Glucose Metabolism and Liver Fat in Early Life

Madelon Liselotte Geurtsen

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Population studies from pregnancy until childhood

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Glucose Metabolism and Liver Fat in Early Life

Population studies from pregnancy until childhood

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Chapter 2.2: **Madelon L. Geurtsen***, Eef E. van Soest*, Ellis Voerman, Eric A.P. Steegers, Vincent W.V. Jaddoe, Romy Gaillard. High maternal early-pregnancy blood glucose concentrations are associated with altered fetal growth and increased risk of adverse birth outcomes. *Diabetologia. 2019 Oct;62(10):1880-1890.* PMID: 31392381. *Authors contributed equally

Chapter 2.3: **Madelon L. Geurtsen**, Vincent W.V. Jaddoe, Romy Gaillard, Janine F. Felix. Associations of maternal early-pregnancy blood glucose and insulin concentrations with DNA methylation in newborns. *Clinical Epigenetics. 2020 Sep 7;12(1):134.* PMID: 32894192.

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1 Introduction

GENERAL INTRODUCTION AND AIMS OF THE THESIS

Healthy early-life development and growth is of lifelong importance. The Developmental Origins of Health and Disease (DOHaD) hypothesis states that adverse exposures during critical periods of growth and development in early life lead to developmental adaption mechanisms. These adverse exposures may have short and long term consequences for growth, body composition and cardio-metabolic health in later life.[1] Maternal gestational diabetes, among other early-life adverse exposures, has been identified as a possible risk factor that might lead to impaired offspring cardio-metabolic health.[2 3] The studies in this thesis are specifically focused on the potential associations of maternal early-pregnancy glucose concentrations on maternal, fetal and childhood outcomes. Previous studies have mainly focused on gestational diabetes or maternal glucose concentrations in mid- and late-pregnancy.[4-9] However, early pregnancy is a critical period for embryonic and placental development.[10 11] Also, I was specifically interested in childhood liver fat development as outcome. The prevalence of childhood non-alcoholic fatty liver disease varies from 3% to 11% in the general population, and is increasing, in line with the global pandemic of childhood obesity.[12-15] An accumulating body of evidence suggests that adverse early-life factors, including disturbed glucose concentrations, contribute to adiposity. However, prospective data on factors in early life in relation with liver fat accumulation are currently lacking.[16-18]

Maternal early-pregnancy glucose concentrations

Gestational diabetes complicates up to 25% of pregnancies, depending on the used diagnostic criteria and the examined population, and this prevalence continues to rise worldwide.[5 19] This rise is partly due to the increasing prevalence of obesity among women of reproductive age and depends on screening tools and diagnostic criteria.[5 20 21] Gestational diabetes is a major risk factor for maternal and fetal perinatal complications, such as miscarriage, fetal structural anomalies, gestational hypertensive disorders, preterm birth, caesarean delivery, large-for-gestational-age infants, as well as for diabetes and obesity in the offspring.[4-7 9] Recent studies suggest that these associations are also present for higher maternal glucose concentrations below the threshold of gestational diabetes.[22 23] A meta-analysis of 25 prospective studies showed that higher maternal glucose concentrations in mid- and late-pregnancy are related to increased risks of perinatal complications.[8] Additionally, the associations are stronger among women who are overweight or obese at the start of pregnancy.[5 20] Current clinical guidelines advise screening for pre-gestational diabetes among women with overweight who also have additional risk-factors.[9 24] In clinical practice, the diagnosis of gestational diabetes is usually made in second half of pregnancy.

High glucose concentrations may already contribute to the risk of adverse effects on maternal, fetal and later offspring health before gestational diabetes and its associated complications, such as fetal macrosomia and polyhydramnios, become apparent.[6] It is likely that women who develop gestational diabetes or hyperglycemia later in pregnancy already have a suboptimal glucose metabolism preconceptionally or in early pregnancy, a critical period for embryonic and placental

development.[10 11] As such, an impaired glucose metabolism may already exert negative effects in that early stage. Fetuses of women with pre-gestational type 1 and type 2 diabetes are at increased risk of macrosomia at birth, but also for delayed growth during early pregnancy. This latter association may be due to poor glucose control already preconceptionally or very early in pregnancy.[25-28] The role of maternal glucose metabolism in early pregnancy in relation to fetal development, pregnancy outcomes and birth outcomes in women without overt diabetes is not clear. Early pregnancy may be an important time window for the effects of suboptimal maternal glucose metabolism on maternal and fetal complications.[6 29] Insight into the influence of maternal blood glucose concentrations from early pregnancy onwards on pregnancy outcomes is important, as maternal blood glucose concentrations might be a major target for potential future intervention strategies.

Epigenetics, more specifically DNA methylation has been suggested as a potential mechanism linking adverse exposures during pregnancy and impaired offspring health.[30 31] Epigenetics refers to changes in DNA structure, without changes in the underlying DNA-sequence, that may affect gene expression. Known forms of epigenetics are the silencing of genes by non-coding ribonucleic acids (RNAs), histone modifications around which the DNA is packed, and DNA methylation.[32] DNA-methylation is the most extensively studied epigenetic process in population studies and it refers to the attachment of a methyl group to the DNA, mainly in places where a cytosine is located next to a guanine nucleotide (Cytosine phosphate Guanine (CpG) sites). This process can change over time through methylation and demethylation, and may be influenced by genetic, stochastic and environmental factors, such as smoking, maternal BMI or air pollution.[33-35] The *in utero* period is a particularly sensitive period for DNA methylation changes. Previous studies using candidate-gene approaches, in which DNA methylation is studied on the promoter of single genes, suggested that maternal gestational diabetes is associated with epigenetic modifications in placenta and cord blood at loci relevant to growth, energy homeostasis, and diabetes mellitus.[30 36-38] Epigenome-wide association studies (EWAS), in which DNA methylation is measured at hundreds of thousands of CpG sites widespread over the genome, looking into gestational diabetes or maternal glucose concentrations showed varying results, with no clear pattern of associations.[19 31 39-45]

Non-alcoholic fatty liver disease in children

Non-alcoholic fatty liver disease is the most common chronic liver disease in children and adolescents worldwide.[15 46 47] Non-alcoholic fatty liver disease in children was first described in 1983 by Moran et al.[48] In the decades since this first report, the prevalence of non-alcoholic fatty liver disease increased in concert with the global pandemic of obesity.[13 47 49] The estimated prevalence in children varies from 3% to 11% in the general population and from 34% to 38% in obese populations, depending on population characteristics and applied diagnostic methods.[12-15] It is difficult to diagnose non-alcoholic fatty liver disease in children, due to the fact that it is a relatively clinically silent disease and because of the difficulty in measuring liver fat.[46] The defini-

tive diagnosis of non-alcoholic fatty liver disease relies on histologic features, with liver biopsy being the gold standard.[13 16 50] In both children and adults non-alcoholic fatty liver disease is histologically defined as macrovesicular steatosis in ≥5.0% of hepatocytes in the absence of other known causes of fatty liver disease.[50 51] Magnetic resonance imaging (MRI) enables noninvasive measurement of liver fat.[52 53] Increased liver fat accumulation and non-alcoholic fatty liver disease reflect a spectrum of severity, ranging from simple liver steatosis to steatohepatitis and fibrosis, which can progress to cirrhosis or hepatocellular carcinoma, and eventually end-stage liver disease.[15 16] Non-alcoholic fatty liver disease is a major risk factor for cardio-metabolic disease.[13 47 54 55] In adults, non-alcoholic fatty liver disease is associated with cardiovascular disease, dyslipidemia, type 2 diabetes, and metabolic syndrome.[16 47 54] As in adults, it is suggested that children with non-alcoholic fatty liver disease have an increased risk for hypertension, dyslipidemia and insulin resistance.[49 56-59] When non-alcoholic fatty liver disease presents earlier in life, affected children may exhibit advanced liver disease earlier in adulthood and have increased comorbidities, such as cardiovascular disease and metabolic syndrome.[29]

Early-life exposures may contribute to the development of non-alcoholic fatty liver disease.[16-18 29] Infancy seems to be a critical period for the development of an altered body composition.[60] In particular dietary patterns in infancy have been shown to track into adulthood.[61] It has been suggested that intake of glucose, fructose and fructose-containing sugars, which are primarily metabolized in the liver, contribute to the development not only of obesity, but also of liver fat accumulation and non-alcoholic fatty liver disease.[61-63] The mechanisms underlying the observed associations of early-life factors with liver fat in children may include changes in DNA methylation.[16 17] The growing global epidemic of obesity suggests that the prevalence of pediatric non-alcoholic fatty liver disease will increase further. Assessing early-life exposures like maternal early-pregnancy glucose concentrations and sugar-containing beverage intake in infancy and their relation with liver fat accumulation broadens the understanding of early-life determinants of non-alcoholic fatty liver disease. Furthermore, identifying and developing a set of basic clinical and biomarker characteristics to establish an accurate prediction tool for children at risk for non-alcoholic fatty liver disease already in early childhood is needed. This may help to develop future preventive strategies aimed at improving body composition and liver health throughout the life course. Therefore, studies included in this thesis were designed to identify early-life exposures, underlying DNA methylation differences and cardio-metabolic consequences of childhood liver fat accumulation.

General aims

In this thesis, I first focused on the associations of maternal early–pregnancy glucose concentrations with pregnancy and offspring outcomes. Second, I studied potential determinants, including maternal early-pregnancy glucose concentrations, and cardio-metabolic consequences of childhood liver fat. I specifically focused on DNA methylation as potential underlying mechanisms for the associations of interest.

General design

The study population in the current thesis are the mothers and children included in the Generation R Study. This is an ongoing prospective population-based cohort study from early pregnancy onwards in Rotterdam, the Netherlands.[64] The Generation R Study is designed to identify early environmental and genetic determinants of growth, development and health in fetal life and childhood. Pregnant women residing in Rotterdam, with an expected delivery date between April 2002 and January 2006 were invited to enrol. In total, 9,778 women were included, of whom 8,880 (91%) were included during pregnancy. Detailed measurements were planned in early pregnancy (<18 weeks of gestation), mid-pregnancy (18 – 25 weeks of gestation) and late pregnancy (>25 weeks of gestation) and included fetal ultrasound measurements, physical examinations, collection of biological samples and self-administered questionnaires. Information on perinatal and maternal pregnancy outcomes, including fetal growth, birth weight, gestational age at birth and complications of pregnancy were available.[64]

DNA methylation was measured in a subsample of children of European ancestry. DNA methylation was measured in these children at birth, 6 and 10 years. In the period from birth to 4 years of age, data collection was performed in all children by questionnaires and visits to the routine child health care centres. At the age of 6, 10 and 13 years, all children were invited to participate in detailed follow-up measurements, including body composition and cardiovascular measurements. The follow-up at 17 years of age is currently ongoing.

The studies presented in this thesis used data from pregnancy, birth, infancy and the follow-up visits at 6 and 10 years of age. Maternal early-pregnancy glucose and insulin concentrations were measured in venous non-fasting blood samples once in early pregnancy. Glucose concentration (mmol/l) is an enzymatic quantity and was measured with c702 module on the Cobas 8000 analyzer (Roche, Almere, the Netherlands). Insulin concentration (pmol/l) was measured with electrochemiluminescence immunoassay on the Cobas e411 analyzer (Roche). Childhood liver fat was assessed using a 3.0 Tesla MRI scanner (Discovery MR750w, GE Healthcare, Milwaukee, Wisconsin, United States).[50 52 53 64]. A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique (IDEAL IQ).[65] The obtained fat fraction maps were analyzed by the Precision Image Analysis (PIA) (Kirkland, Washington, United States) using the sliceOmatic (TomoVision, Magog, Canada) software package. Liver fat fraction was determined by taking four samples of at least 4cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat estimation. Liver fat measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[66 67]

Outline of the thesis

Studies in the first part of this thesis focus on maternal early-pregnancy glucose concentrations. In **Chapter 2.1**, I studied the associations of maternal early-pregnancy glucose concentrations with placental hemodynamics, blood pressure and risks of gestational hypertensive disorders. In

Chapter 2.2, I assessed whether maternal early-pregnancy glucose concentrations are associated with fetal growth and birth outcomes using a longitudinal design. Subsequently, I performed an epigenome-wide association study on maternal early-pregnancy glucose and insulin concentrations with DNA methylation in cord blood, followed by exploratory analyses with previously found maternal early-pregnancy glucose associated outcomes (**Chapter 2.3**). In **Chapter 2.4**, I presented the associations of maternal early-pregnancy glucose concentrations with childhood liver fat accumulation.

In the second part of this thesis I focus on childhood liver fat accumulation. I assessed the associations of environmental exposures in early life with childhood liver fat accumulation. In **Chapter 3.1**, I focused on the associations of infant sugar-containing beverage intake with childhood liver fat accumulation. In **Chapter 3.2**, I extended my approach to assess the association of DNA methylation with childhood liver fat accumulation. Subsequently, I evaluated the consequences of childhood liver fat accumulation by studying the associations of childhood liver fat with cardio-metabolic risk factors at the same age in **Chapter 3.3**. In **Chapter 3.4**, I developed a prediction model with early-life determinants of childhood liver fat accumulation to be able to predict whether children are at risk for developing non-alcoholic fatty liver disease.

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2 Consequences of glucose metabolism in pregnancy

2.1 Associations of maternal early-pregnancy glucose concentrations with placental hemodynamics, blood pressure, and gestational hypertensive disorders

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ABSTRACT

Background: Gestational diabetes is associated with increased risks of gestational hypertension and pre-eclampsia. We hypothesized that high maternal glucose concentrations in early pregnancy are associated with adverse placental adaptations and subsequently altered uteroplacental hemodynamics during pregnancy, predisposing to an increased risk of gestational hypertensive disorders.

Methods: In a population-based prospective cohort study from early pregnancy onwards, among 6,078 pregnant women, maternal early-pregnancy non-fasting glucose concentrations were measured. Mid- and late pregnancy uterine and umbilical artery resistance indices were assessed by Doppler ultrasound. Maternal blood pressure was measured in early, mid-, and late pregnancy and the occurrence of gestational hypertensive disorders was assessed using hospital registries.

Results: Maternal early-pregnancy glucose concentrations were not associated with mid- or late pregnancy placental hemodynamic markers. A 1 mmol/l increase in maternal early-pregnancy glucose concentrations was associated with 0.71 mmHg (95% confidence interval 0.22–1.22) and 0.48 mmHg (95% confidence interval 0.10–0.86) higher systolic and diastolic blood pressure in early pregnancy, respectively, but not with blood pressure in later pregnancy. Also, maternal glucose concentrations were not associated with the risks of gestational hypertension or pre-eclampsia.

Conclusions: Maternal early-pregnancy non-fasting glucose concentrations within the normal range are associated with blood pressure in early pregnancy, but do not seem to affect placental hemodynamics and the risks of gestational hypertensive disorders.

INTRODUCTION

Gestational diabetes complicates up to 17% of all pregnancies and is a strong risk factor for gestational hypertensive disorders.[1 2] In pregnant women with pre-gestational diabetes, hyperglycemia causes a pro-inflammatory environment and cytokine derangements, which act on the endothelium, and lead to placental vascular changes, whereas insulin may have a direct toxic effect on the placenta.[3 4] Also, pregnancies complicated by obesity or gestational diabetes show dysregulation of metabolic, vascular, and inflammatory pathways.5,6 This dysregulation is characterized by increased circulating concentrations of inflammatory molecules and placental overexpression of genes encoding for inflammatory mediators.[5 6] Studies have shown that hyperglycemia during pregnancy is associated with reduced invasiveness of the trophoblast, increased oxidative stress in the maternal and fetal milieu, disrupted vasculogenesis, and macroscopically and histologically altered placentae.[4 7-11] Treatment of gestational diabetes has been shown to reduce the prevalence of pre-eclampsia.[12] It is not known yet to what extent early-pregnancy non-fasting glucose concentrations may influence early placental adaptations, blood pressure, and predispose women to gestational hypertensive disorders.

We hypothesized that high maternal glucose concentrations in early pregnancy are associated with adverse placental adaptations and subsequently altered uteroplacental hemodynamics during pregnancy, predisposing to an increased risk of gestational hypertensive disorders. We examined in a low-risk, multi-ethnic, population-based prospective cohort study among 6,078 pregnant women, the associations of maternal early-pregnancy non-fasting glucose concentrations with placental flow measures, blood pressure throughout pregnancy, and gestational hypertensive disorders.

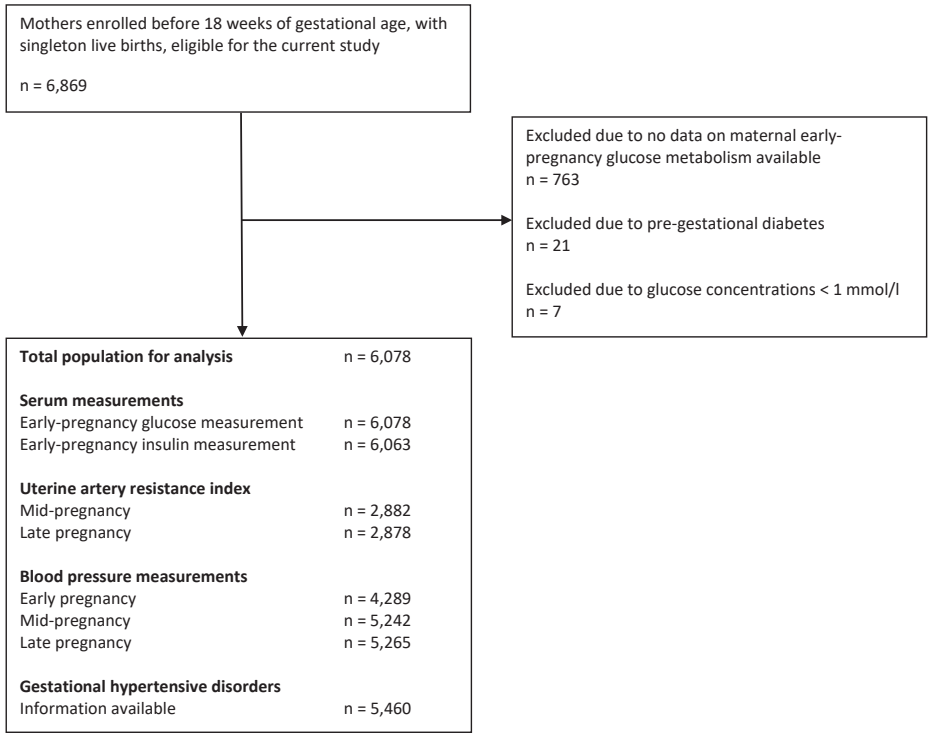
METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards in Rotterdam, The Netherlands. All pregnant woman and their children who were living within the city of Rotterdam at the time of birth were eligible to participate.[13] The study has been approved by the local Medical Ethical Committee (MEC 198.782/2001/31). Written consent was obtained from all participating women. All pregnant women were enrolled between 2001 and 2005. Response rate at birth was 61%.[14] In total, 8,879 women were enrolled during pregnancy. For the current study, 6,869 women were eligible as they enrolled before 18 weeks of gestational age and had singleton live births. Women with no data on maternal early-pregnancy glucose metabolism or with all outcome measures missing were excluded (n = 763). Women with pre-gestational diabetes (n = 21) and women with unreliable glucose concentrations (<1 mmol/l) were excluded (n = 7). The population for analysis comprised

6,078 pregnant women (**Figure 1**). All measurements in pregnancy were performed by trained research assistants who were part of the study team.

Figure 1. Study participants flowchart



Maternal glucose concentrations

Blood samples were collected once in early pregnancy at 13.2 median weeks' gestation (95% range 9.6; 17.6), as described previously.[15] After 30 minutes of fasting, venous blood samples were collected from pregnant women, by specifically trained research nurses who were part of the research team, and temporally stored at room temperature for a maximum of 3 hours. We considered the 30 minutes fasting samples non-fasting samples. This time interval was chosen because of the design of our study, in which it was not possible to obtain fasting samples from all pregnant women. At least every 3 hours, blood samples were transported to a dedicated laboratory facility (Star-MDC, Rotterdam, The Netherlands), for further processing and storage.[16] Glucose (mmol/l) is an enzymatic quantity and was measured with the c702 module on a Cobas 8000 analyzer (Roche, Almere, The Netherlands). Insulin (pmol/l) was measured with electrochemiluminescence immunoassay on a Cobas e411 analyzer (Roche, Almere, The Netherlands). Quality control samples demonstrated intra- and interassay coefficients of variation of 1.30% and 2.50%, respectively. Information on pre-gestational diabetes was obtained from self-reported question-

naires and on gestational diabetes from medical records after delivery. Gestational diabetes was diagnosed by a community midwife or an obstetrician according to Dutch midwifery and obstetric guidelines using the following criteria: either a random glucose concentrations >11.0 mmol/l, a fasting glucose ≥7.0 mmol/l, or a fasting glucose between 6.1 and 6.9 mmol/l with a subsequent abnormal glucose tolerance test.[17]

Placenta hemodynamic characteristics

Ultrasound examinations were carried out in 2 dedicated research centers in the city of Rotterdam in early (median 13.2 weeks gestational age, interquartile range (IQR) 12.2; 14.9), mid- (median 20.4 weeks gestational age, IQR 19.9; 21.1), and late pregnancy (median 30.2 weeks gestational age, IQR 29.9; 30.6). We established gestational age by using data from the first ultrasound examination.[18] Uterine artery resistance index and umbilical artery pulsatility index were derived from flow velocity waveforms in mid- and late pregnancy. Standard deviation scores for uterine artery resistance index and umbilical artery pulsatility index were based on values from the whole study population and represent the equivalent of *z*-scores. Late pregnancy uterine artery notching was diagnosed if a notch was present uni- or bilaterally, as a result from increased blood flow resistance, which is a sign of placental insufficiency.[19]

Blood pressure and gestational hypertensive disorders

Blood pressure was measured at each pregnancy visit (median gestational age 13.2 weeks (IQR 12.2; 14.9); 20.4 weeks (IQR 19.9; 21.1); and 30.2 weeks (IQR 29.9; 30.6)) using an Omron 907 automated digital oscillometer sphygmomanometer (OMRON Healthcare Europe, Hoofddorp, The Netherlands).[20] The mean value of 2 blood pressure readings over a 60-second interval was documented for each participant.[21] Information about hypertensive disorders in pregnancy was obtained from medical records.[16] The occurrence of hypertension and related complications were cross-validated using hospital registries, and defined using criteria of the International Society for the Study of Hypertension in Pregnancy.[22 23] Gestational hypertension was defined as *de novo* hypertension alone (an absolute blood pressure 140/90 mmHg or greater), appearing after 20 weeks gestational age. Pre-eclampsia was defined as *de novo* hypertension (blood pressure ≥140/90 mmHg) after the 20th gestational week with concurrent proteinuria (0.3 g or greater in a 24-hour urine specimen or 2+ or greater (1 g/l) on a voided specimen or 1+ or greater (0.3 g/l) on a catheterized specimen). Any gestational hypertensive disorder was defined as either gestational hypertension or pre-eclampsia.

Covariates

Maternal height (cm) and weight (kg) were measured without shoes and heavy clothing at enrollment and body mass index (BMI, kg/m^2) was calculated. Information about pre-pregnancy weight, ethnicity (European/non-European), and education (higher education yes/no) was obtained by questionnaire.[14] Folic acid supplementation, categorized as use vs. no use, and parity, categorized

as nulliparous or multiparous, were obtained at enrollment by questionnaire.[24] Information about smoking was available from questionnaires, and was classified as "yes" if the woman smoked until pregnancy was known and if she continued to smoke throughout pregnancy.[13]

Statistical analyses

First, we conducted a nonresponse analysis to compare characteristics of women with and without glucose measurements available. Second, we assessed the associations of maternal early-pregnancy non-fasting glucose concentrations continuously with mid- and late pregnancy uterine artery and umbilical artery resistance indices and late pregnancy uterine artery notching, and with blood pressure in early, mid-, and late pregnancy, using linear and logistic regression models. We also analyzed the longitudinal systolic and diastolic blood pressure patterns in women using unbalanced repeated measurement regression models.[25] These models take the correlation between repeated measurements of the same subject into account, and allow for incomplete outcome data. Using fractional polynomials of gestational age, the best-fitting models were constructed. For presentation purposes, we constructed tertiles of maternal glucose concentrations for these analyses. Third, we assessed the associations of maternal early-pregnancy non-fasting glucose concentrations continuously with gestational hypertensive disorders (gestational hypertension and pre-eclampsia), using logistic regression models. For all analyses, we constructed different models to explore whether any association was explained by maternal sociodemographic and lifestyle factors. The basic model was adjusted for gestational age at glucose measurement; the main model was additionally adjusted for gestational age at assessment, maternal ethnicity, age, educational level, smoking, and folic acid supplement use; and the maternal BMI model was additionally adjusted for maternal pre-pregnancy BMI. Included covariates were based on previous studies, strong correlations with exposure and outcomes, and changes in effect estimates of >10%. We further tested but did not observe statistical interactions between maternal pre-pregnancy BMI and maternal early-pregnancy non-fasting glucose concentrations for the associations with uterine and umbilical artery resistance indices and blood pressure. Statistical interaction terms were tested by including the term maternal pre-pregnancy BMI × maternal early-pregnancy non-fasting glucose concentrations in the regression model. We performed 3 sensitivity analyses. First, analyses were repeated using maternal early-pregnancy non-fasting insulin concentrations. Second, to test whether the associations of maternal early-pregnancy non-fasting glucose concentrations with high blood pressure we excluded women with gestational diabetes (n = 66). Third, to test whether a cutoff effect was present, we tested for differences in associations with blood pressure between women in quintiles of glucose concentrations, with the lowest quintile used as the reference group. We used multiple imputation for missing values of covariates according to Markov Chain Monte Carlo method.[26] The percentage of missing data was <10%, except for smoking (15%) and folic acid supplement use (31.2%). Five imputed datasets were created and pooled for analyses. No significant differences in descriptive statistics were found between the original and imputed datasets. The repeated measurement

analysis was performed using the Statistical Analysis System version 9.4 (SAS Institute, Cary, NC), including the Proc Mixed module for unbalanced repeated measurements. All other analyses were performed using the Statistical Package of Social Sciences version 24.0 for Windows (IBM, Armonk, NY).

RESULTS

Population characteristics

Population characteristics are shown in **Table 1**. Mean maternal early-pregnancy glucose concentrations were 4.4 mmol/l. In total, 64 (1.1%) women were diagnosed with gestational diabetes. Late pregnancy uterine artery notching occurred in 312 (10.2%) participants. Gestational hypertension developed in 203 (3.8%) women and pre-eclampsia developed in 131 (2.4%) women. Nonresponse analyses showed that women without glucose measurements were more often parous, had a lower level of educational attainment, used folic acid supplementation more often, were more often of non-European descent, and had a higher mid-pregnancy and a lower late pregnancy uterine artery resistance index (**Table S1**). Histogram for maternal glucose concentrations given in **Figure S1**.

Early-pregnancy glucose concentrations and placental hemodynamics

Maternal early-pregnancy glucose concentrations were not associated with mid- and late pregnancy uterine artery resistance indices, umbilical artery pulsatility indices, and risk of late pregnancy uterine artery notching (**Table 2**).

Early-pregnancy glucose concentrations, blood pressure, and gestational hypertensive disorders

Associations of maternal early-pregnancy glucose concentrations with blood pressure in early, mid-, and late pregnancy are shown in **Table 3**. A 1 mmol/l increase in maternal early-pregnancy glucose concentrations was associated with 0.71 mmHg (95% confidence interval 0.22; 1.22) and 0.48 mmHg (95% confidence interval 0.10; 0.86) higher systolic and diastolic blood pressure in early pregnancy, respectively, but not with blood pressure in later pregnancy. Using repeated measurements analysis (**Figure 2**), we observed that tertiles of maternal early-pregnancy glucose concentrations were not associated with blood pressure over time (*P* value for interaction of early-pregnancy glucose concentrations with gestational age >0.05, Supplementary Table S5 online). Also, maternal early-pregnancy glucose concentrations were not associated with the risks of gestational hypertensive disorders (**Table 4**).

Table 1. Subject characteristics

Characteristics	n = 6,078
Maternal characteristics	
Age, mean (SD), years	29.8 (5.1)
Height, mean (SD), cm	167.5 (7.4)
Weight before pregnancy, mean (SD), kg	66.4 (12.7)
Body Mass Index, median (IQR), kg/m^2	22.6 (20.7 to 25.4)
Parity, No. nulliparous (%)	3,458 (57.4)
Education, No. higher education (%)	2,538 (44.9)
Ethnicity	
Dutch or European, No. (%)	3,558 (61.0)
Surinamese, No. (%)	503 (8.6)
Turkish, No. (%)	472 (8.1)
Moroccan, No. (%)	352 (6.0)
Cape Verdian or Dutch Antilles, No. (%)	410 (7.1)
Smoking	
None, No. (%)	3,712 (72.2)
Early-pregnancy only, No. (%)	452 (8.8)
Continued, No. (%)	974 (19.0)
Folic acid use No. used (%)	2,943 (47.4)
Pre-gestational diabetes mellitus, No. (%)	0 (0)
Blood pressure, mean (SD), mmHg	
Early pregnancy	115 (12.3) /68 (9.6)
Mid-pregnancy	116 (12.0) /67 (9.4)
Late pregnancy	118 (12.0) / 69 (9.4)
Mid-pregnancy uterine artery resistance index, mean (SD)	0.54 (0.09)
Late pregnancy uterine artery resistance index, mean (SD)	0.49 (0.08)
Late pregnancy uterine artery notching, No. (%)	312 (10.2)
Glucose, mean (SD), mmol/l	4.4 (0.84)
Insulin, median (IQR), pmol/l	115.1 (55.4 to 233.4)
Gestational diabetes mellitus, No. (%)	64 (1.1)
Gestational hypertension, No. (%)	203 (3.8)
Pre-eclampsia, No. (%)	131 (2.4)
Birth characteristics	
Males, No.(%)	3,076 (50.6)
Gestational age at delivery, median (IQR), weeks	40.1 (39.1 to 41.0)
Preterm birth, No (%)	310 (5.1)
Birth weight, mean (SD), grams	3,417 (564)
Placenta weight, median (IQR), grams	610 (530 to 720)

Values are observed data and represent means (SD), medians (IQR) or number of subjects (valid %). Abbreviation: IQR: inter quartile range.

Table 2. Associations of maternal early-pregnancy glucose concentrations with mid- and late pregnancy placental flow measures

Maternal early-pregnancy glucose concentrations (mmol/l)	**Uterine artery**		**Umbilical artery**
	Resistance index (95% Confidence Interval)	**Notching** (95% Confidence Interval)	**Pulsatility index** (95% Confidence Interval)
Mid-pregnancy			
Basic model	-0.00 (-0.02 to 0.02)	Not available	0.03 (-0.01 to 0.07)
Main model	-0.00 (-0.05 to 0.04)	Not available	0.03 (-0.01 to 0.07)
BMI model	-0.02 (-0.07 to 0.03)	Not available	0.02 (-0.02 to 0.06)
Late pregnancy			
Basic Model	-0.00 (-0.03 to 0.02)	0.96 (0.84 to 1.09)	-0.02 (-0.06 to 0.01)
Main model	-0.00 (-0.05 to 0.04)	0.95 (0.82 to 1.09)	-0.02 (-0.06 to 0.02)
BMI model	-0.03 (-0.08 to 0.02)	0.92 (0.79 to 1.08)	-0.02 (-0.07 to 0.02)

Values are SDSs (95% CI) from linear regression models, reflecting differences in measures of uterine and umbilical artery flow measures, and OR (95% CI) reflecting difference in risk of late pregnancy uterine artery notching, per 1 mmol/l increase in maternal early-pregnancy glucose concentrations (n = 4,236). Estimates are from multiple imputed data. Basic model: Adjusted for gestational age at glucose measurement. Main model: Gestational age at glucose measurement, gestational age at ultrasound, maternal ethnicity, age, parity, educational level, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI.

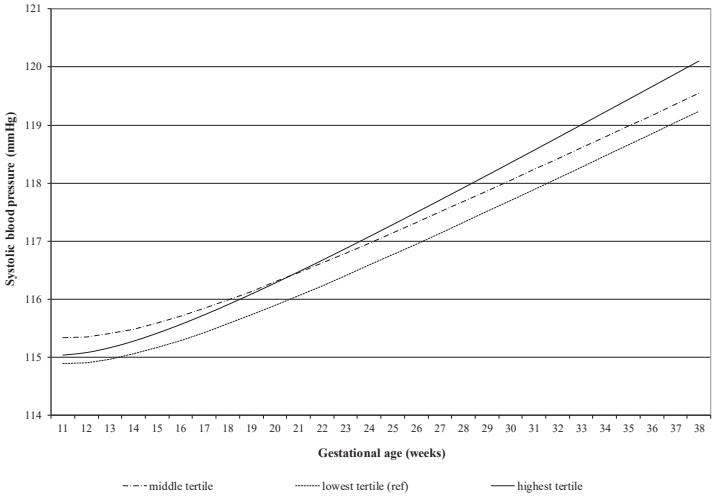
Table 3. Associations of maternal early-pregnancy glucose concentrations with early, mid- and late pregnancy blood pressure

Maternal early-pregnancy glucose concentrations (mmol/l)	**Systolic blood pressure,** mmHg (95% Confidence Interval)	**Diastolic blood pressure,** mmHg (95% Confidence Interval)
Early pregnancy		
Basic model	0.37 (-0.08 to 0.81)	0.40 (0.06 to 0.75)*
Main model	0.47 (0.03 to 0.92)*	0.40 (0.06 to 0.75)*
BMI model	0.71 (0.22 to 1.22)*	0.48 (0.10 to 0.86)*
Mid-pregnancy		
Basic model	0.13 (-0.30 to 0.48)	-0.13 (-0.44 to 0.18)
Main model	0.19 (-0.21 to 0.59)	-0.12 (-0.43 to 0.20)
BMI model	0.36 (-0.09 to 0.80)	-0.02 (-0.37 to 0.33)
Late pregnancy		
Basic model	0.21 (-0.18 to 0.61)	0.19 (-0.12 to 0.50)
Main model	0.25 (-0.15 to 0.65)	0.18 (-0.13 to 0.49)
BMI model	0.36 (-0.08 to 0.80)	0.24 (-0.10 to 0.59)

Values are mmHg (95% CI) from linear regression models, reflecting differences in systolic and diastolic blood pressure, per 1 mmol/l increase in maternal early-pregnancy glucose concentrations (n = 5,265). Estimates are from multiple imputed data. Basic model: Adjusted for gestational age at glucose measurement. Main model: Gestational age at glucose measurement, gestational age at blood pressure measurement, maternal ethnicity, age, parity, educational concentrations, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI. *p value < 0.05

Figure 2. Longitudinal associations between tertiles of maternal early-pregnancy glucose concentrations and blood pressure

a



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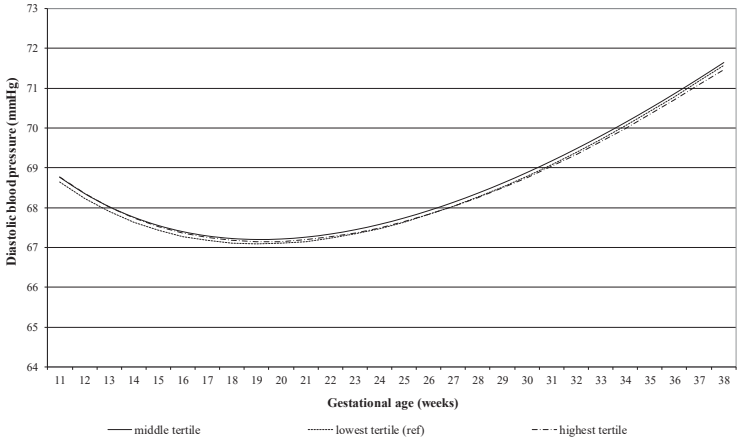

Blood pressure patterns in different maternal early-pregnancy glucose tertiles. (a) Systolic and (b) diastolic blood pressure in different maternal early-pregnancy glucose tertiles (n = 6,078). Results reflect the change in mmHg in mothers with early-pregnancy glucose concentrations in the second (4.0-4.6 mmol/l) and third (4.6-10.3 mmol/l) tertile, compared to those with glucose levels in the first tertile (1.0-4.0 mmol/l). (a) Systolic blood pressure = $\beta_0 + \beta_1 \times$ glucose tertile $+ \beta_2 \times$ gestational age $+ \beta_3 \times$ gestational age$^{-2} + \beta_4 \times$ glucose tertile × gestational age. (b) Diastolic blood pressure = $\beta_0 + \beta_1 \times$ glucose tertile $+ \beta_2 \times$ gestational age $+ \beta_3 \times$ gestational age$^{0.5} + \beta_4 \times$ glucose tertile × gestational age. The models were adjusted for gestational age at intake. The interaction term of maternal early-pregnancy glucose tertile with gestational age in weeks was not significant. Similarly, when glucose was used continuously in the models, no significant interaction of maternal early-pregnancy glucose concentration with gestational age in weeks was observed. Estimates are given in **Table S5**.

Table 4. Associations of maternal early-pregnancy glucose concentrations with the risks of gestational hypertensive disorders

Maternal early-pregnancy glucose concentrations (mmol/l)	**Gestational hypertension** (95% Confidence Interval) n = 203	**Pre-eclampsia** (95% Confidence Interval) n = 131	**Any gestational hypertensive disorder** (95% Confidence Interval) n = 334
Basic model	1.01 (0.86 to 1.20)	0.98 (0.81 to 1.17)	0.95 (0.83 to 1.09)
Main model	1.02 (0.86 to 1.20)	0.87 (0.70 to 1.09)	0.96 (0.84 to 1.10)
BMI model	0.98 (0.82 to 1.18)	0.88 (0.69 to 1.11)	0.94 (0.81 to 1.09)

Values are ORs (95% CI) from logistic regression models, reflecting differences in risk of gestational hypertensive disorders, per 1 mmol/l increase in maternal early-pregnancy glucose concentrations (n = 5,459). Estimates are from multiple imputed data. Basic model: Adjusted for gestational age at glucose measurement. Main model: Gestational age at glucose measurement, maternal ethnicity, age, parity, educational level, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI.

Sensitivity analyses

In mid-pregnancy, higher insulin concentrations were associated with a higher umbilical artery pulsatility index in the basic and main model, but the association attenuated in the BMI model (**Table S2**). In the BMI model, higher early-pregnancy insulin concentrations were associated with a higher early-pregnancy systolic blood pressure (**Table S3**). We found similar results to the main findings when we excluded women with gestational diabetes (data not shown). Finally, no differences in associations with blood pressure between women with non-fasting glucose concentrations in quintiles were observed (data not shown).

DISCUSSION

Our findings suggest that higher maternal early-pregnancy non-fasting glucose concentrations are associated with higher blood pressure in early pregnancy, but no associations were present with blood pressure in mid- or late pregnancy. Also, maternal early-pregnancy non-fasting glucose concentrations were not associated with placental hemodynamics or gestational hypertensive disorders.

Meaning of the current study and findings

Hyperglycemia during pregnancy is associated with miscarriage, fetal structural anomalies, fetal macrosomia, fetal demise, preterm birth, and gestational hypertensive disorders.[27] Limited evidence for early-pregnancy screening for diabetes in the general population exist, although testing can be performed as early as the first prenatal visit if a high degree of suspicion of undiagnosed type 2 diabetes exists.[27] Current clinical guidelines advise screening for pre-gestational diabetes among women with overweight and additional risk factors.[27 28] In clinical practice, the diagnosis of gestational diabetes is usually made in second half of pregnancy. However, high glucose concentrations may already have contributed to risk of gestational hypertensive disorders and

other adverse effects on maternal and fetal health before gestational diabetes and associated complications such as fetal macrosomia and polyhydramnios become apparent.[28] Optimization of glucose regulation in the case of gestational diabetes and pre-gestational diabetes leads to a strong reduction of risk of gestational hypertensive disorders.[29] Therefore, early pregnancy may be a critical period for adverse effects of increased glucose concentrations on fetal and maternal pregnancy outcomes. Previously we reported associations of higher maternal early-pregnancy non-fasting glucose concentrations with decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards, and an increased risk of delivering a large-for-gestational-age infant.[15] Early placenta development may play an important role in these associations. Next to its adverse effects on fetal growth, inadequate placental development may play an important role in the development of gestational hypertensive disorders.

Early pregnancy is a critical period for optimal placenta development. In this period, trophoblast invasion and spiral artery remodeling takes place to ensure adequate blood flow to the placenta, leading to larger vessels with lower resistance and increased end-diastolic flow.[30] Normally, in early pregnancy, cardiac output increases, peripheral vascular resistance is reduced, and blood pressure decreases until mid-pregnancy, returning to baseline at term.[30] If these processes are inadequate, increased blood pressure, abnormal uterine artery Doppler's with higher resistance indices and notching may be observed, and gestational hypertension or pre-eclampsia may develop.

Previous studies have shown that women with prediabetes defined as HbA1c of 5.7–6.4% in early pregnancy represent a high-risk group for development of gestational hypertensive disorders.[31 32] It is unclear how early-pregnancy glucose concentrations across the full range influence placental flow measures, blood pressure, and gestational hypertensive disorders. We hypothesized that higher early-pregnancy non-fasting glucose concentrations negatively influence placental flow measures, blood pressure, and risk of gestational hypertensive disorders. Previous studies report associations of glucose concentrations with placental flow measures.[33 34] In a study among 231 pregnant women with polycystic ovarian syndrome, early-pregnancy and, more strongly, mid-pregnancy fasting glucose concentrations, were positively associated with an increased mid-pregnancy uterine artery pulsatility index.[33] A retrospective study among 155 pre-gestational diabetic women suggested a positive correlation between concentrations of HbA1c and increased vascular resistance in the uterine and umbilical arteries, suggesting that hyperglycemia may influence uterine and placental vessel endothelial function.[34] In the current study in a low-risk healthy population, we did not observe associations of maternal early-pregnancy glucose concentrations with placental flow measures. The difference in results may be explained by our low-risk, non-diabetic population. Also, maternal glucose concentrations in early pregnancy may not influence placental flow measures measured later in pregnancy.

Diabetes and hypertension often occur simultaneously and show a substantial overlap in disease etiology and risk factors, such as genetics, obesity, insulin resistance, and inflammation.[35-37] Due to prolonged exposure to effects of hyperglycemia, we expected to find stronger associations

of maternal early-pregnancy glucose concentrations with blood pressure throughout pregnancy. In the current study, we observed associations of maternal early-pregnancy non-fasting glucose concentrations with early-pregnancy blood pressure, but not later in pregnancy. Possibly, this may be due to the fact that the time between the exposure and the outcome is large, and as the effect estimates are already small and within the normal range in early pregnancy, the effect of maternal early-pregnancy glucose concentrations on blood pressure in mid- or late pregnancy may not be detectable, or no association may present at all. Possibly, a more pronounced effect on cardiovascular outcomes may be observed in the presence of sustained elevated glucose concentrations. It has been shown that gestational diabetes leads to a strongly increased risk of gestational hypertensive disorders.[1 2] Simultaneously, associations with gestational hypertensive disorders have not been found in women diagnosed with prediabetes in early pregnancy although these women are at increased risk of development of gestational diabetes.[32 38] A previous prospective study among 4,589 healthy nulliparous women showed that even within the normal range, the plasma glucose concentration 1 hour after 50-g oral glucose challenge was positively correlated with the likelihood of pre-eclampsia.[39] As parity is a strong risk factor for pre-eclampsia, the baseline risk of gestational hypertensive disorders among this nulliparous population may be higher. In the current study, we did not find associations of maternal early-pregnancy non-fasting glucose concentrations with risk of pre-eclampsia. This difference might be explained by differences in baseline risk and in glucose measurements. Future studies, using early-pregnancy fasting glucose concentrations or glucose concentrations obtained after a standardized oral glucose challenge, are needed to confirm if maternal early-pregnancy glucose concentrations are indeed associated with pre-eclampsia in a low-risk population. We did not observe associations of maternal early-pregnancy glucose concentrations across the full range, with gestational hypertensive disorders. Findings from our study do not support strong effects of non-fasting glucose concentrations in early pregnancy within the normal range on the risks of gestational hypertensive disorders. In clinical practice, testing for pre-gestational diabetes is only recommended among high-risk populations.[27 28 40] As pregnancy physiologically influences the glucose metabolism, future studies focused on pre-pregnancy glucose concentrations may shed an important light on the effects of glucose concentrations on blood pressure, placental flow measures, and risk of gestational hypertensive disorders.

Strengths and limitations

We had a prospective data collection from early pregnancy onwards and a large low-risk sample of 6,078 women with detailed glucose measurements, blood pressure, placental flow measures, and information on gestational hypertensive disorders available. The response rate at baseline was 61%. The nonresponse at baseline might have led to selection of a healthier population. We had a population with a relatively low BMI, a low mean non-fasting glucose concentration, and the sample contained a small number of cases of gestational diabetes, indicating selection toward a nondiabetic population and might affect the generalizability of our findings to higher-risk popu-

lations in which stronger associations are expected. Blood sample collection was performed in a non-fasting state at different time points in the day. The minimum fasting time until blood sample collection was 30 minutes, due to the design of the study. The samples were therefore considered as non-fasting blood samples. Since glucose and insulin concentrations are sensitive toward carbohydrate intake and vary during the day, this may have led to non-differential misclassification and an underestimation of the observed effect estimates. We had no information available on oral glucose tolerance testing in pregnancy. although we included many covariates, there still might be some residual confounding, as in any observational study. Further studies are needed to replicate our findings using more detailed maternal glucose metabolism measurements, including fasting glucose concentrations and detailed postprandial glucose measurements among higher-risk populations.

CONCLUSIONS

Maternal early-pregnancy non-fasting glucose concentrations across the full range are associated with blood pressure in early pregnancy, but not later in pregnancy. Also, maternal early-pregnancy non-fasting glucose concentrations within the normal range are not associated with placental flow measures and gestational hypertensive disorders.

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SUPPLEMENTARY MATERIAL

Table S1. Characteristics of mothers without glucose measurement available

Characteristics	Non-participants n = 763	Participants n = 6,078	*p* value*
Maternal characteristics			
Age, mean (SD), years	29.5 (5.6)	29.8 (5.1)	<0.01
Height, mean (SD), cm	166.7 (7.3)	167.5 (7.4)	0.63
Weight before pregnancy, mean (SD), kg	65.8 (13.6)	66.4 (12.7)	0.48
Body Mass Index, median (IQR), kg/m^2	22.5 (207 to 25.2)	22.6 (20.7 to 25.4)	0.31
Parity, No. nulliparous (%)	414 (55.2)	3,458 (57.4)	0.48
Education, No. higher education (%)	269 (39.4)	2,538 (44.9)	0.02
Ethnicity			<0.01
Dutch or European, No. (%)	373 (52.4)	3,558 (61.0)	
Surinamese, No. (%)	71 (1.0)	503 (8.6)	
Turkish, No. (%)	84 (11.8)	472 (8.1)	
Moroccan, No. (%)	49 (6.9)	352 (6.0)	
Cape Verdian or Dutch Antilles, No. (%)	53 (7.4)	410 (7.1)	
Smoking			0.92
None, No. (%)	471 (72.9)	3,712 (72.2)	
Early-pregnancy only, No. (%)	57 (8.8)	452 (8.8)	
Continued, No. (%)	118 (18.3)	974 (19.0)	
Folic acid use No. used (%)	380 (49.8)	2,943 (47.4)	0.03
Blood pressure, mean (SD) (mmHg)			
Early pregnancy	116 (12.4) / 68 (9.8)	115 (12.3)/68 (9.6)	0.68/0.69
Mid-pregnancy	117 (12.1) / 67 (9.3)	116 (12.0)/67 (9.4)	0.35/0.82
Late pregnancy	118 (12.5) / 69 (9.0)	118 (12.0) / 69 (9.4)	0.30/0.35
Mid-pregnancy uterine artery resistance index, mean (SD)	0.55 (0.08)	0.54 (0.09)	0.04
Late pregnancy uterine artery resistance index, mean (SD)	0.48 (0.07)	0.49 (0.08)	0.04
Third trimester uterine artery notching, No. (%)	40 (10.5)	312 (10.2)	0.81
Glucose, mean (SD), mmol/l	Not available	4.4 (0.84)	
Insulin, median (IQR), pmol/l	Not available	115.1 (55.4 to 233.4)	
Gestational diabetes mellitus, No. (%)	8 (1.1)	64 (1.1)	0.99
Gestational hypertension, No. (%)	28 (4.1)	203 (3.8)	0.63
Pre-eclampsia, No. (%)	15 (2.2)	131 (2.4)	0.92
Birth characteristics			
Males, No. (%)	384 (50.5)	3,076 (50.6)	0.96
Gestational age at delivery, median (IQR), weeks	40.1 (39.0 to 40.9)	40.1 (39.1 to 41.0)	0.44
Preterm birth, No. (%)	36 (4.7)	310 (5.1)	0.68
Birth weight, mean (SD), grams	3,381 (570)	3,417 (564)	0.15
Placenta weight, median (IQR), grams	630 (540 to 710)	610 (530 to 720)	0.71

Values are observed data and represent means (SD), medians (IQR) or number of subjects (valid %). *Differences in subject characteristics between participants with and without glucose measurements available were evaluated using one-way ANOVA tests for continuous variables and chi-square tests for categorical variables. Abbreviations: IQR: inter quartile range; SD: Standard deviation.

Table S2. Associations of maternal early-pregnancy insulin concentrations with mid- and late pregnancy placental flow measures

	Uterine artery			**Umbilical artery**
Maternal early-pregnancy insulin concentrations (SDS)	**Pulsatility index** (95% Confidence Interval)	**Resistance index** (95% Confidence Interval)	**3rd Trimester notching** (95% Confidence Interval)	**Pulsatility index** (95% Confidence Interval)
Mid-pregnancy				
Basic model	0.00 (-0.02 to 0.02)	0.00 (-0.02 to 0.02)	Not available	0.04 (0.01 to 0.07)*
Main model	0.01 (-0.03 to 0.05)	0.01 (-0.03 to 0.05)	Not available	0.03 (0.00 to 0.06)*
BMI model	0.00 (-0.05 to 0.05)	0.01 (-0.04 to 0.05)	Not available	0.02 (-0.02 to 0.06)
Late pregnancy				
Basic model	0.02 (0.00 to 0.04)	0.01 (0.01 to 0.01)	0.97 (0.91 to 1.03)	0.02 (-0.01 to 0.05)
Main model	0.02 (-0.02 to 0.06)	0.00 (-0.03 to 0.04)	0.96 (0.85 to 1.08)	0.01 (-0.02 to 0.04)
BMI model	0.00 (-0.04 to 0.05)	-0.01 (-0.06 to 0.03)	0.94 (0.82 to 1.09)	-0.00 (-0.04 to 0.04)

Values are SDSs (95% CI) from linear regression models, reflecting differences in measures of uterine and umbilical artery flow measures, and OR (95% CI) reflecting difference in risk of 3rd trimester uterine artery notching, per 1 standard deviation increase in maternal early-pregnancy insulin concentrations (n = 4,236). Estimates are from multiple imputed data. SDS: Standard deviation score. Basic model: Adjusted for gestational age at insulin measurement. Main model: Gestational age at insulin measurement, gestational age at ultrasound, maternal ethnicity, age, parity, educational level, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI. *p value <0.05.

Table S3. Associations of maternal early-pregnancy insulin concentrations with early, mid- and late pregnancy blood pressure

Maternal early-pregnancy insulin concentrations (SDS)	**Systolic blood pressure**, mmHg (95% Confidence Interval)	**Diastolic blood pressure**, mmHg (95% Confidence Interval)
Early pregnancy		
Basic model	0.18 (-0.20 to 0.55)	0.11 (-0.18 to 0.40)
Main model	0.28 (-0.10 to 0.66)	0.16 (-0.14 to 0.45)
BMI model	0.49 (0.06 to 0.93)*	0.30 (-0.04 to 0.64)
Mid-pregnancy		
Basic model	0.10 (-0.24 to 0.44)	-0.11 (-0.38 to 0.16)
Main model	0.16 (-0.19 to 0.51)	-0.08 (-0.36 to 0.19)
BMI model	0.24 (-0.15 to 0.64)	-0.04 (-0.34 to 0.27)
Late pregnancy		
Basic model	0.06 (-0.29 to 0.40)	-0.06 (-0.33 to 0.21)
Main model	0.15 (-0.21 to 0.50)	-0.03 (-0.31 to 0.24)
BMI model	0.16 (-0.24 to 0.56)	-0.05 (-0.36 to 0.27)

Values are mmHg (95% CI) from linear regression models, reflecting differences in systolic and diastolic blood pressure, per 1 standard deviation increase in maternal early-pregnancy insulin concentrations (n = 5,265). Estimates are from multiple imputed data. SDS: Standard deviation score. Basic model: Adjusted for gestational age at insulin measurement. Main model: Gestational age at insulin measurement, gestational age at ultrasound, maternal ethnicity, age, parity, educational level, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI. *p value <0.05.

Table S4. Associations of maternal early-pregnancy insulin concentrations with the risks of gestational hypertensive disorders

Maternal early-pregnancy insulin concentrations (SDS)	**Gestational hypertension** (95% Confidence Interval) n = 104	**Pre-eclampsia** (95% Confidence Interval) n = 133	**Any gestational hypertensive disorder** (95% Confidence Interval) n = 334
Basic model	0.87 (0.73 to 1.02)	0.94 (0.77 to 1.13)	0.89 (0.78 to 1.01)
Main model	0.89 (0.75 to 1.06)	0.93 (0.77 to 1.14)	0.91 (0.79 to 1.03)
BMI model	0.86 (0.71 to 1.04)	0.94 (0.75 to 1.18)	0.89 (0.77 to 1.03)

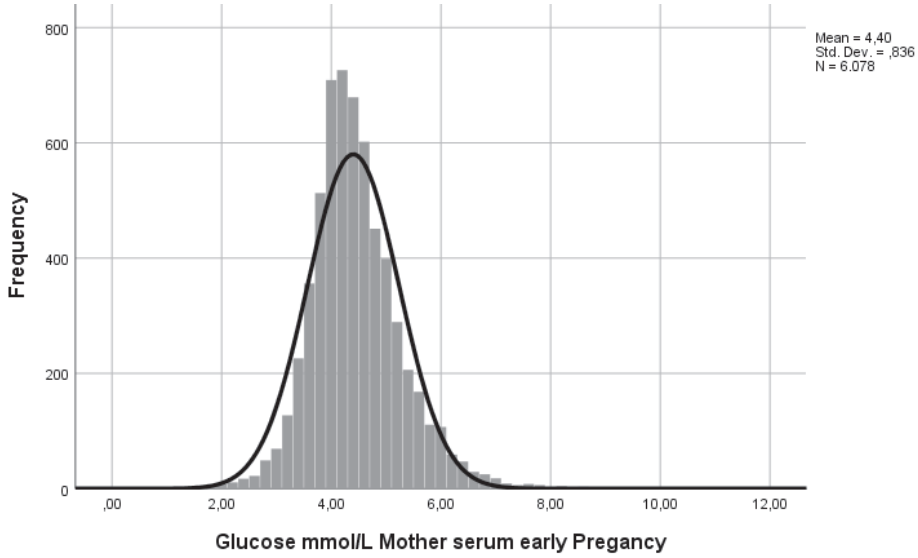
Values are ORs (95% CI) from logistic regression models, reflecting differences in risk of gestational hypertensive disorders, per 1 standard deviation increase in maternal early-pregnancy insulin concentrations (n = 5,427). Estimates are from multiple imputed data. SDS: Standard deviation score. Basic model: Adjusted for gestational age at glucose measurement. Main model: Gestational age at glucose measurement, maternal ethnicity, age, parity, educational level, smoking, and folic acid supplement use. BMI model: Main model additionally adjusted for maternal pre-pregnancy BMI.

Table S5. Associations of tertiles of early pregnancy glucose concentrations with longitudinally measured systolic and diastolic blood pressure

	Difference in systolic blood pressure			
	Intercept (mmHg)	***p* value***	**Slope** (mmHg)	***p* value***
Intercept	111.55	<0.01		
Glucose lowest tertile	*Reference*			
Glucose middle tertile	0.50	0.44		
Glucose highest tertile	-0.14	0.82		
Gestational age in weeks	0.20	<0.01		
Glucose lowest tertile * gestational age (weeks)			*Reference*	
Glucose middle tertile * gestational age (weeks)			-0.01	0.84
Glucose highest tertile * gestational age (weeks)			0.03	0.31
Ga_2	138.19	0.05		
	Difference in diastolic blood pressure			
	Intercept (mmHg)	***p* value***	**Slope** (mmHg)	***p* value***
Intercept	93.74	<0.01		
Glucose lowest tertile	*Reference*			
Glucose middle tertile	0.22	0.67		
Glucose highest tertile	0.14	0.78		
Gestational age in weeks	1.39	<0.01		
Glucose lowest tertile * gestational age (weeks)			*Reference*	
Glucose middle tertile * gestational age (weeks)			-0.01	0.67
Glucose highest tertile * gestational age (weeks)			0.001	0.93
GA05	-12.19	<0.01		

Values are based on repeated non-linear regression models and reflect the change in blood pressure per tertile increase in early-pregnancy glucose concentration (n = 5,265). **p* value reflects the significance level of the estimate.

Figure S1. Maternal early-pregnancy glucose concentrations



2.2 Maternal early-pregnancy blood glucose concentrations are associated with altered fetal growth and increased risk of adverse birth outcomes

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ABSTRACT

Background: The study aimed to assess the associations of maternal early-pregnancy blood glucose concentrations with fetal growth throughout pregnancy and the risks of adverse birth outcomes.

Methods: In a population-based prospective cohort study among 6,116 pregnant women, maternal non-fasting glucose concentrations were measured in blood plasma at a median 13.2 weeks of gestation (95% range 9.6–17.6). We measured fetal growth by ultrasound in each pregnancy period. We obtained information about birth outcomes from medical records and maternal sociodemographic and lifestyle factors from questionnaires.

Results: Higher maternal early-pregnancy non-fasting glucose concentrations were associated with altered fetal growth patterns, characterised by decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards, resulting in an increased length and weight at birth (*p* values ≤0.05). A weaker association of maternal early-pregnancy non-fasting glucose concentrations with fetal head circumference growth rates was present. Higher maternal early-pregnancy non-fasting glucose concentrations were also associated with an increased risk of delivering a large-for-gestational-age infant, but decreased risk of delivering a small-for-gestational-age infant (OR 1.28 [95% CI 1.16, 1.41], OR 0.88 [95% CI 0.79, 0.98] per mmol/l increase in maternal early-pregnancy non-fasting glucose concentrations, respectively). These associations were not explained by maternal sociodemographic factors, lifestyle factors or body mass index. Maternal early-pregnancy non-fasting glucose concentrations were not associated with preterm birth or delivery complications.

Conclusions: Higher maternal early-pregnancy non-fasting glucose concentrations are associated with decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards, and an increased risk of delivering a large-for-gestational-age infant. Future preventive strategies need to focus on screening for an impaired maternal glucose metabolism from preconception and early pregnancy onwards to improve birth outcomes.

INTRODUCTION

Gestational diabetes complicates up to 17% of pregnancies and is a major risk factor for maternal and fetal perinatal complications.[1-3] Recent studies suggest that these associations are also present for higher maternal glucose concentrations below the threshold of gestational diabetes.[4-6] A meta-analysis of 25 prospective studies showed that higher maternal glucose concentrations in mid-pregnancy and late pregnancy are related to increased risks of perinatal complications.[7]

Accumulating evidence suggests that early pregnancy is a critical period for the effects of adverse exposures on embryonic and placental development.[8 9] Little is known, however, about the direct effects of an impaired maternal glucose metabolism from early pregnancy onwards on fetal growth and the risks of adverse birth outcomes in both diabetic and non-diabetic pregnant women.[3] Among women with gestational diabetes, fetal growth may already be abnormal preceding this diagnosis. However, results are inconsistent and difficult to interpret as maternal glucose concentrations before the diagnosis of gestational diabetes are unknown.[10 11] We hypothesised that a maternal glucose metabolism already impaired in early pregnancy affects embryonic and placental development, subsequently leading to altered fetal growth and increased risks of adverse birth outcomes.[2 3 12 13] Insight into the influence of maternal blood glucose concentrations from early pregnancy onwards on fetal development is important, as maternal blood glucose concentrations offer a major target for potential future interventions.

Therefore, in a population-based prospective cohort study among 6,116 pregnant women, we examined whether maternal early-pregnancy non-fasting glucose concentrations across the full range, and not limited to diagnostic thresholds, are associated with fetal growth in each pregnancy period and with the risks of adverse birth outcomes. To obtain further insight into the causality of these associations, we additionally explored whether these associations are explained by maternal sociodemographic factors or lifestyle factors.

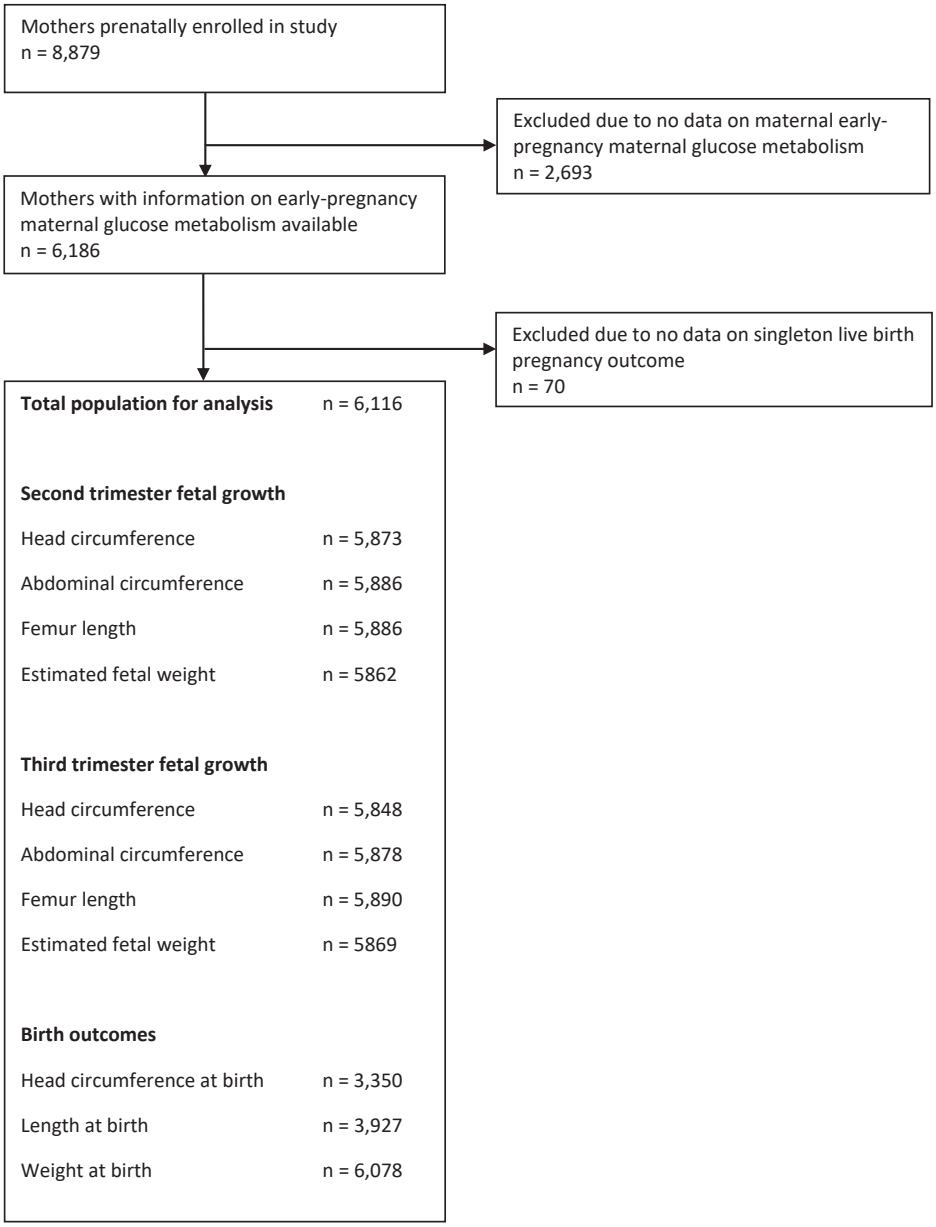
METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort study from early pregnancy onwards in Rotterdam, the Netherlands.[14] The study has been approved by the local Medical Ethical Committee (MEC 198.782/2001/31). Written informed consent was obtained from all participating women. All pregnant women, who were resident in the study area at their delivery date, were enrolled between 2001 and 2005. Translated information packages and questionnaires were available for recruitment of different ethnicities. The enrolment procedure has been described in detail previously.[15] Response rate at birth was 61%.[16] In total, 8,879 women were enrolled during pregnancy, of whom 6,186 had measurements of glucose concentrations available. We excluded pregnancies not leading to singleton live births (n = 70). The population for

analyses comprised 6,116 women (**Figure 1**). Information on pre-gestational diabetes was available, and to the low number (n = 24) women with pre-gestational diabetes were included in the analyses. A sensitivity analysis excluding women with pre-gestational diabetes was performed.

Figure 1. Study participants flowchart



Maternal glucose metabolism

Blood samples were collected once in early pregnancy at 13.2 median weeks' gestation (95% range 9.6–17.6). After 30 minutes of fasting, venous blood samples were collected from pregnant women by research nurses and temporally stored at room temperature. We consider the 30 minutes fasting samples non-fasting samples. This time-interval was chosen because of the design of our study, in which it was not possible to obtain fasting samples from all pregnant women. At least every 3 hours, blood samples were transported to a dedicated laboratory facility of the regional laboratory in Rotterdam, the Netherlands (Star-MDC), for further processing and storage.[17] Glucose (mmol/l) is an enzymatic quantity and was measured with the c702 module on a Cobas 8000 analyser. Insulin (pmol/l) was measured with electrochemiluminescence immunoassay on a Cobas e411 analyser. Quality control samples demonstrated intra- and inter-assay CVs of 1.30% and 2.50%, respectively. We constructed maternal early-pregnancy non-fasting glucose and insulin SD scores (SDSs).

Information on pre-gestational diabetes was obtained from self-reported questionnaires and on gestational diabetes from medical records after delivery. Gestational diabetes was diagnosed by a community midwife or an obstetrician according to Dutch midwifery and obstetric guidelines using the following criteria: either a random glucose concentration >11.0 mmol/l, a fasting glucose ≥7.0 mmol/l or a fasting glucose between 6.1 and 6.9 mmol/l with a subsequent abnormal glucose tolerance test.[18] In clinical practice and for this study sample, an abnormal glucose tolerance test was defined as a glucose concentration greater than 7.8 mmol/l after glucose intake.

Fetal growth patterns and adverse birth outcomes

Fetal ultrasound examinations were carried out in two dedicated research centres in early pregnancy (13.2 median weeks' gestation [95% range 9.6–17.6]), mid-pregnancy (20.5 median weeks' gestation [95% range 18.7–23.1]) and late pregnancy (30.3 median weeks' gestation [95% range 28.5–32.8]). In early pregnancy we used crown–rump length to assess fetal growth only in mothers with a known and reliable first day of the last menstrual period, a regular menstrual cycle of 28 days (range 24–32 days) and who had fetal crown–rump length measured between a gestational age of 10 weeks 0 days and 13 weeks 6 days (n = 1470), as described previously.[19] The first day of the last menstrual period was obtained from the referring letter from the community midwife or hospital. This date was confirmed with the participants at the ultrasound visit and additional information on the regularity and duration of the menstrual cycle was obtained.[19] For mothers without this information, gestational age was established by early-pregnancy fetal ultrasound examination. This strategy was performed because of the large number of mothers who did not know the exact date of their last menstrual period or who had irregular menstrual cycles.[20] Subsequently, in mid-pregnancy and late pregnancy, we measured fetal head circumference, abdominal circumference and femur length to the nearest millimetre using standardised ultrasound procedures. Estimated fetal weight was subsequently calculated using the formula of Hadlock et al.[21] Longitudinal growth curves and gestational-age-adjusted SDSs were constructed

for all fetal biometry measurements.[20] These gestational-age-adjusted SDSs were based on reference growth curves from the whole study population and represent the equivalent of z scores.[20]

Information about offspring sex, gestational age, weight, length and head circumference at birth was obtained from medical records.[14] Since head circumference and length were not routinely measured at birth, fewer measurements were available (n = 3350 for head circumference and n = 3927 for length at birth). Gestational-age-adjusted SDSs for head circumference, length and weight at birth were constructed using North European growth standards as the reference growth curve and represent the equivalent of z scores.[22] Small-for-gestational-age and large-for-gestational-age at birth were defined as the lowest and highest 10 percentiles of gestational age- and sex-adjusted birthweight using North European growth standards.[22] Preterm birth was defined as a gestational age at birth <37 weeks. Information on delivery complications, Caesarean delivery and vacuum extraction, was collected from medical records.

Covariates

Information on maternal age, pre-pregnancy weight, educational level, ethnicity, parity and folic acid supplements use was obtained at enrolment by questionnaires.[14] Height and weight, both without shoes and heavy clothing, were measured at enrolment. Pre-pregnancy body mass index was calculated (self-reported pre-pregnancy weight in kilograms divided by height measured at first study visit in metres squared). Information about smoking and alcohol consumption was assessed by questionnaires. We dichotomised both variables; women were classified as 'yes' when having consumed until pregnancy was known and when they continued to consume throughout pregnancy. Information on total daily energy intake was obtained by a food frequency questionnaire in early pregnancy.[14]

Statistical analysis

First, we conducted a non-response analysis to compare characteristics of women with and without glucose measurements available. Normal distributed data were presented in mean with standard deviation, non-normal distributed data were presented in median with 95% range (i.e. the 2.5th to 97.5th percentile).

Second, we assessed the associations of maternal early-pregnancy non-fasting glucose concentrations with repeatedly measured fetal biometry measurements to assess fetal growth patterns using unbalanced repeated measurement regression models. These models take the correlation between repeated measurements of the same individual into account and allow for incomplete outcome data.[23] We included maternal early-pregnancy non-fasting glucose concentrations in these models as intercept and as interaction term with gestational age to estimate fetal growth rates over time.[23] These analyses were conducted without adjustment for covariates, which most clearly reflects clinical practice.[7]

Third, we examined the associations of maternal early-pregnancy non-fasting glucose concentrations with detailed fetal biometry measurements in gestational-age-adjusted SDS in each

pregnancy period using linear regression models. Analyses were repeated using fetal biometry measurements in absolute values. For these analyses, we constructed different models to explore whether these observed associations were explained by maternal sociodemographic and lifestyle factors: a basic model (adjusted for gestational age at assessment), a maternal ethnicity model (basic model additionally adjusted for ethnicity), a maternal pregnancy-related factors model (maternal ethnicity model additionally adjusted for maternal age, parity, educational level, daily total energy intake, smoking, alcohol consumption and folic acid supplement use) and a maternal BMI model (maternal pregnancy-related factors model additionally adjusted for maternal pre-pregnancy BMI). Included covariates were based on previous studies, strong correlations with maternal glucose concentrations, risk of gestational diabetes and fetal biometry measurements, and changes in effect estimates of >10%.[2 3]

Fourth, we assessed the associations of maternal early-pregnancy non-fasting glucose concentrations with the risks of adverse birth outcomes using multiple logistic regression models using the same adjustment models. We explored whether associations were non-linear by performing quintiles analyses and adding a quadratic term to the original model. However, for all analyses, a linear model had the best fit. Since only seven women had glucose concentrations of >7.8 mmol/l and only 62 women developed gestational diabetes, we were unable to explore the effects of these clinical categories on fetal growth and adverse birth outcomes. We tested but did not observe statistical interactions between maternal ethnicity or pre-pregnancy BMI and maternal early-pregnancy non-fasting glucose concentrations for the associations with fetal biometry measurements and with adverse birth outcomes.[2 3 10 11]

As a sensitivity analysis, analyses were repeated using maternal early-pregnancy non-fasting insulin concentrations. To enable comparison of effect sizes for the associations of different measures of maternal early-pregnancy glucose metabolism with fetal growth and birth outcomes, these sensitivity analyses were performed using maternal early-pregnancy non-fasting glucose and insulin concentrations in SDSs. In addition, we explored whether our observed associations were affected by specific subgroups. We performed five additional sensitivity analyses for the associations of maternal early-pregnancy non-fasting glucose concentrations with fetal biometry measurements in each pregnancy period: (1) excluding women with pre-gestational diabetes (n = 24); (2) excluding women with gestational diabetes (n = 62); (3) among women of Dutch ethnicity only; (4) among women included in early pregnancy only (before 14 weeks' gestation); and (5) among term births only.

Missing data of covariates were imputed using multiple imputation. Five imputed datasets were created and analysed together. Repeated measurement analyses were performed using the Statistical Analysis System version 9.4 (SAS Institute, Cary, NC, USA; Proc Mixed module). All other analyses were performed using the Statistical Package of Social Sciences version 24.0 for Windows (SPSS, Chicago, IL, USA).

Table 1. Subject characteristics

Characteristics	Total group n = 6,116
Maternal characteristics	
Age, years	29.8 ± 5.1
Height, cm	167.5 ± 7.4
Pre-pregnancy weight, kg	64.0 (48.0–99.7)
Pre-pregnancy BMI, kg/m^2	22.6 (18.0–34.7)
Gestational age at intake, weeks	13.2 (9.6–17.6)
Parity (nulliparous)	3474 (57.3)
Ethnicity	
Dutch	3083 (52.2)
European	496 (8.4)
Cape Verdean	245 (4.2)
Moroccan	353 (6.0)
Dutch Antillean	171 (2.9)
Surinamese	506 (8.6)
Turkish	474 (8.1)
Other	545 (9.3)
Education, higher	2550 (44.9)
Total energy intake, kJ	486 (134)
Folic acid use	
No	1183 (25.3)
Start first 10 weeks	1491 (31.9)
Start periconceptional	1997 (42.8)
Smoking during pregnancy, continued	1012 (18.6)
Alcohol use during pregnancy, continued	2095 (39.0)
Gestational hypertensive disorders	
Pre-eclampsia	127 (2.2)
Gestational hypertension	234 (4.1)
Glucose, mmol/l	4.4 ± 0.8
Insulin, pmol/l	114.6 (17.6–716.1)
Impaired glucose tolerance at intake[a]	17 (0.3)
Pre-gestational diabetes	24 (0.5)
Gestational diabetes	62 (1.1)
Birth characteristics	
Male	3100 (50.7)
Gestational age, weeks	40.1 (35.6–42.3)
Preterm birth[b]	310 (5.1)
Small for gestational age[c]	606 (10.0)
Large for gestational age[d]	606 (10.0)
Caesarean delivery	692 (14.5)
Vacuum extraction	774 (15.9)

Values are numbers (%), means ± SD or medians (95% range). [a]Impaired glucose tolerance at intake is defined as >7.8 mmol/l in non-fasting state. [b]Preterm birth is defined as <37 weeks' gestation. [c]Small for gestational age is defined as <10th percentile of age- and sex-adjusted birthweight. [d]Large for gestational age is defined as >90th percentile of age- and sex-adjusted birthweight.

RESULTS

Population characteristics are shown in **Table 1**. Fetal growth characteristics of the study population are shown in **Table 2**. Non-response analyses showed that women without glucose measurements had a higher BMI, had a lower level of educational attainment, were of non-European descent and used folic acid supplements less often (**Table S1**).

Table 2. Fetal growth characteristics of the study population

Fetal growth characteristics	Total group n = 6,116
Mid-pregnancy	
Gestational age, weeks	20.5 (18.7–23.1)
Head circumference, mm	179 ± 13.3
Abdominal circumference, mm	156 ± 13.7
Femur length, mm	33 ± 3.3
Estimated fetal weight, g	377 ± 83.9
Late pregnancy	
Gestational age, weeks	30.3 (28.5–32.8)
Head circumference, mm	285 ± 12.2
Abdominal circumference, mm	264 ± 16.3
Femur length, mm	57 ± 8.7
Estimated fetal weight, g	1611 ± 251.0
Birth	
Gestational age, weeks	40.1 (35.6–42.3)
Birth head circumference, cm	33.8 ± 1.7
Birth length, cm	50.2 ± 2.4
Birthweight, g	3418 ± 563

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

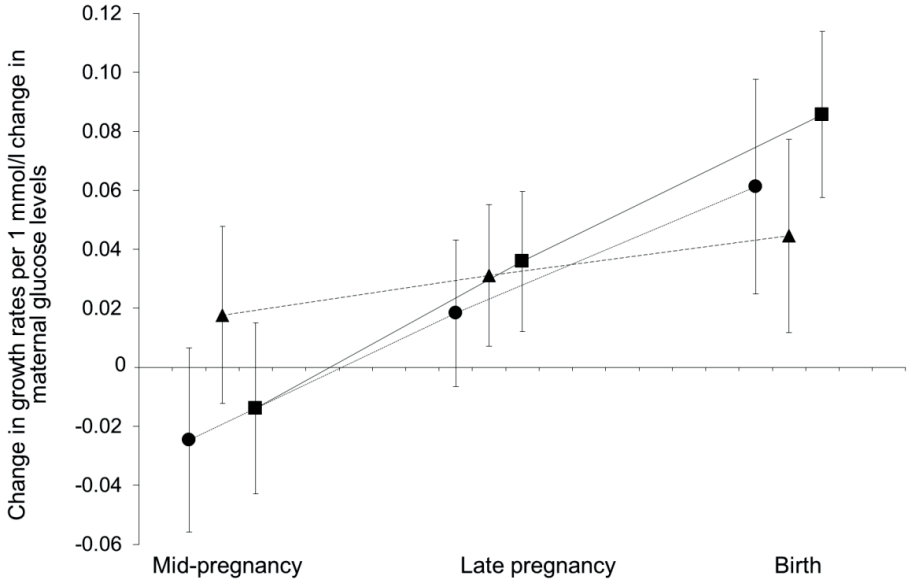
Maternal blood glucose concentrations and fetal growth

Figure 2 shows that higher maternal early-pregnancy non-fasting glucose concentrations were associated with increased rates of fetal length and weight growth from late pregnancy onwards, resulting in increased length and weight at birth (*p* value for interaction with gestational age <0.05). Weaker effect estimates were observed for the associations of maternal early-pregnancy non-fasting glucose concentrations with rate of fetal head circumference growth, but a significant interaction with gestational age was also present (*p* value for interaction with gestational age <0.05).

Figure 3 shows that maternal early-pregnancy non-fasting glucose concentrations were not significantly associated with early-pregnancy fetal crown–rump length. Higher maternal early-pregnancy non-fasting glucose concentrations were associated with decreased mid-pregnancy

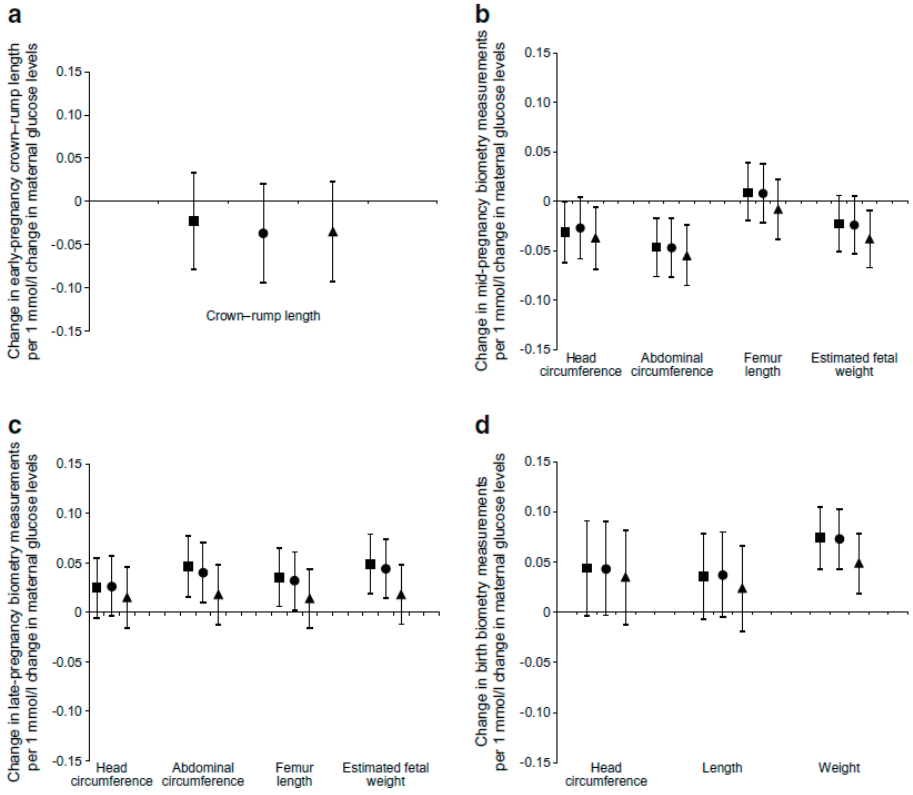
fetal head circumference SDS and abdominal circumference SDS (*p* values ≤0.05). The association of higher maternal early-pregnancy non-fasting glucose concentrations with decreased mid-pregnancy estimated fetal weight SDS did not reach statistical significance (*p* value 0.10). No association with mid-pregnancy femur length was present. However, higher maternal early-pregnancy non-fasting glucose concentrations were associated with increased fetal head circumference SDS, abdominal circumference SDS, femur length SDS and estimated fetal weight SDS in late pregnancy, and head circumference SDS, length SDS and weight SDS at birth (*p* values ≤0.05). These associations were not explained by adjustment for maternal ethnicity, or other maternal pregnancy-related factors, but were partly attenuated after adjustment for maternal pre-pregnancy BMI (maternal ethnicity-adjusted model is given in **Table S2**). The strongest effect estimate was present for birthweight (difference in birthweight in the maternal pregnancy-related model: 0.07 SDS [95% CI 0.04, 0.10] per mmol/l increase in maternal early-pregnancy non-fasting glucose concentrations; *p* value ≤0.05). The associations of maternal early-pregnancy non-fasting glucose concentrations with absolute values of fetal biometry measurements are given in **Table S3** and showed similar findings to the main findings. Per 1 mmol/l increase in maternal early-pregnancy non-fasting glucose concentrations, birthweight increased by 25.4 g (95% CI 9.0, 41.8) in the maternal pregnancy-related model (*p* value ≤0.05).

Figure 2. Differences in fetal growth rates per change in maternal early-pregnancy glucose concentrations



Data are SDS values (95% CI) from repeated measurement regression models that reflect the differences in gestational age-adjusted growth rates in SDS of head circumference (circles), length (triangles) and weight (squares) at mid-pregnancy, late pregnancy and at birth per 1 mmol/l change in maternal early-pregnancy glucose concentrations. As a measure of skeletal length growth from mid-pregnancy onwards, we used fetal femur length SDS in mid-pregnancy and late pregnancy and total body length SDS at birth within the repeated measurements model. All fetal biometry measurements for each pregnancy period were taken at the same time point. The models were adjusted for gestational age at intake. *p* value <0.05 for interaction with gestational age for all models.

Figure 3. Associations of maternal early-pregnancy glucose concentrations in mmol/l with fetal biometry measurements



Data are SDS values (95% CI) from linear regression models that reflect the differences in growth characteristics in SDSs in (**a**) early pregnancy, (**b**) mid-pregnancy, (**c**) late pregnancy and (**d**) at birth, per 1 mmol/l change in maternal early-pregnancy glucose concentrations (n = 6,116). Analyses with crown–rump length were based on subgroup analyses (n = 1,470). Estimates are from multiple imputed data. Squares show basic model: adjusted for gestational age at assessment. Circles show maternal pregnancy-related factors model: basic model additionally adjusted for maternal ethnicity, age, parity, educational level, daily total energy intake, smoking, alcohol consumption and folic acid supplement use. Triangles show BMI model: maternal pregnancy-related factors model additionally adjusted for maternal pre-pregnancy BMI.

Impact of maternal early-pregnancy blood glucose concentrations on adverse birth outcomes

Independent of maternal sociodemographic or lifestyle factors, higher maternal early-pregnancy non-fasting glucose concentrations were associated with an increased risk of delivering a large-for-gestational-age infant, but with a decreased risk of delivering a small-for-gestational-age infant (ORs 1.28 [95% CI 1.16, 1.41] and 0.88 [95% CI 0.79, 0.98] per mmol/l increase in maternal early-pregnancy non-fasting glucose concentrations in the maternal pregnancy-related model (*p* value ≤0.05), respectively) (**Table 3**, and maternal ethnicity-adjusted model is given in **Table S4**). No significant associations were present for maternal early-pregnancy non-fasting glucose concentrations with preterm birth, Caesarean delivery or vacuum extraction.

Table 3. Associations of maternal early-pregnancy glucose concentrations with the risks of adverse birth outcomes

Maternal early-pregnancy glucose concentrations (mmol/l)	Small-for-gestational-age at birth	Large-for-gestational-age at birth	Preterm birth	Caesarean delivery	Vacuum extraction
Basic model	0.89 (0.80, 0.98)*	1.27 (1.16, 1.40)*	1.08 (0.95, 1.24)	1.12 (0.98, 1.27)	0.99 (0.90, 1.09)
Maternal pregnancy-related factors model	0.88 (0.79, 0.98)*	1.28 (1.16, 1.41)*	1.08 (0.94, 1.23)	1.11 (1.00, 1.23)	1.01 (0.91, 1.12)
BMI model	0.91 (0.82, 1.02)	1.21 (1.10, 1.34)*	1.06 (0.92, 1.21)	1.09 (0.99, 1.20)	1.01 (0.90, 1.12)

Values are ORs (95% CI) from logistic regression models that reflect the differences in risks of adverse birth outcomes per 1 mmol/l increase in maternal early-pregnancy glucose concentrations. Estimates are from multiple imputed data. Basic model adjusted for gestational age at assessment. Maternal pregnancy-related factors model: basic model additionally adjusted for maternal ethnicity, age, parity, educational level, daily total energy intake, smoking, alcohol consumption and folic acid supplement use. BMI model: maternal pregnancy-related factors model, additionally adjusted for maternal pre-pregnancy BMI. *p value <0.05.

Sensitivity analyses

The sensitivity analyses using maternal early-pregnancy non-fasting insulin concentrations, instead of maternal early-pregnancy non-fasting glucose concentrations, showed that maternal early-pregnancy non-fasting insulin concentrations were largely similarly associated with fetal growth rates and fetal biometry measurements in each pregnancy period (**Table S5**, **Figure S1** and **S2**). Based on comparison of the effect estimates per SDS increase in maternal early-pregnancy non-fasting glucose and insulin concentrations, the strength of the associations with fetal biometry measurements was also largely the same. Similar results to the main findings were found when we excluded women with pre-gestational diabetes or gestational diabetes and when we restricted our analyses to women of Dutch ethnicity only, women included in early pregnancy only and among term births only (**Table S6**).

DISCUSSION

We observed that maternal early-pregnancy blood glucose concentrations across the full spectrum are associated with altered fetal growth patterns, characterised by decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards, and an increased risk of delivering a large-for-gestational-age infant. These associations were only partly explained by maternal pre-pregnancy BMI, and not by other maternal pregnancy-related factors.

Interpretation of main findings

Maternal gestational diabetes and hyperglycemia diagnosed in the second half of pregnancy are common and major risk factors for adverse birth outcomes. It is likely that women who develop gestational diabetes or hyperglycemia later in pregnancy already have a suboptimal glucose metabolism preconceptionally or in early pregnancy, a critical period for embryonic and placental

development.[10 11] Despite well-known associations of maternal gestational diabetes with adverse birth outcomes, direct effects of a disturbed maternal glucose metabolism from early pregnancy onwards on fetal growth remain unclear. Fetuses of women with pre-gestational type 1 and type 2 diabetes are at increased risk for macrosomia at birth, but also for delayed growth during early pregnancy. This latter association may be due to poor glucose control already preconceptionally or very early in pregnancy.[24-27] The role of maternal glucose metabolism in early pregnancy in relation to fetal development and birth outcomes is not clear in women without overt diabetes.

Among women with gestational diabetes, it has been suggested that fetal growth is already abnormal preceding the diagnosis of gestational diabetes. Results from a cohort study among 533 women showed that, compared with those without gestational diabetes, those with gestational diabetes had smaller fetuses until 24 weeks of gestation, followed by accelerated fetal growth in late pregnancy.[11] A study among 4,069 pregnant women showed that fetuses of women with gestational diabetes had increased abdominal circumference growth rates between 20 and 28 weeks of gestation, with the strongest effects among women with obesity.[10] Similarly, a prospective study among 741 black African women showed increased fetal abdominal circumference growth rates from mid-pregnancy onwards in women with gestational diabetes compared with those without gestational diabetes.[28] As maternal glucose concentrations before the diagnosis of gestational diabetes were not known in these studies, these findings are difficult to interpret in the context of the present study results. We observed that among non-diabetic women, higher maternal early-pregnancy non-fasting glucose concentrations across the full range were associated with decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards resulting in larger size at birth. These associations were independent of maternal ethnicity, a well-known risk factor for gestational diabetes and an important determinant for fetal growth. Also, other maternal pregnancy-related factors, including pre-pregnancy BMI, did not explain the observed associations. When we assessed each pregnancy period separately, we also observed that higher maternal early-pregnancy non-fasting glucose concentrations tended to be associated with smaller fetal biometry measurements in early pregnancy and mid-pregnancy, although for some measurements there was no significant association. Thus, our results suggest that already among non-diabetic women, higher maternal early-pregnancy non-fasting glucose concentrations within the normal range are related to altered fetal growth patterns, characterised by decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards. The presence of associations for all fetal biometry measurements suggests that maternal early-pregnancy non-fasting glucose concentrations affect both fetal fat development and skeletal growth.

Impaired maternal gestational glucose metabolism is a major risk factor for delivering a large-for-gestational-age infant, preterm birth and Caesarean delivery, with even stronger effects among women with overweight and obesity.[2 7 29-32] A retrospective study among more than 6,000 women showed that higher maternal glucose concentrations at 9.5 weeks' gestation were associated with an increased risk of delivering a large-for-gestational-age infant.[3] A case–control study

among 2,050 women with term deliveries observed an association of maternal early-pregnancy glucose concentrations with delivering large-for-gestational-age infants, independent of maternal BMI.[31] A large cohort among 46,000 women showed that higher maternal glucose concentrations between 10 and 24 weeks of gestation were associated with an increased risk of spontaneous preterm birth.[33] We observed that already a small increase in maternal early- pregnancy non-fasting glucose concentrations within the normal range was related to an increased risk of delivering a large-for-gestational-age infant, but a decreased risk of delivering a small-for-gestational-age infant. These associations were independent of maternal pre-pregnancy BMI. BMI is a measure of general adiposity, but does not provide any information on more specific fat compartments, such as visceral fat mass. Alterations in maternal visceral fat mass, which is more metabolically active, might explain part of the observed associations. In addition, our study population is a relatively lean population. The effect of maternal pre-pregnancy BMI on the observed associations might be stronger among more obese populations. We did not observe associations for preterm birth, Caesarean delivery or vacuum extraction. It seems likely that associations of maternal early-pregnancy non-fasting glucose concentrations with delivery complications are partly driven by size at birth as well as other maternal characteristics, such as maternal obesity. Even though we did observe that higher maternal early-pregnancy non-fasting glucose concentrations were associated with an increased risk of a large-for-gestational-age infant, the overall effect on birthweight in the full cohort was relatively small. This may partly explain the lack of associations with the delivery complications. These associations may be more apparent among higher-risk populations. Thus, our findings suggest that in a non-diabetic population, non-fasting glucose concentrations in early pregnancy already partly determine the risk of delivering a large-for-gestational-age infant.

The mechanisms underlying associations of maternal glucose metabolism with reduced fetal growth in the first half of pregnancy and increased fetal growth thereafter are not known. It has been suggested that impaired glucose control during early pregnancy negatively affects placental development, starting with impaired early placentation, which induces placental insufficiency and thereby early fetal growth restriction.[11] In response to the placental insufficiency, it has been suggested that the fetus may induce maternal hyperglycemia to improve nutrient supply and growth during the second half of pregnancy via placental signalling.[11] It has also been hypothesised that hyperglycemia in early pregnancy injures the development of the yolk sac, which is of great importance during the embryonic period, especially in nutrient transport towards the embryo. This may lead to impaired embryonic growth and development. When the yolk sac function is replaced by the placenta at the end of early pregnancy, hyperglycemia together with increased transfer of other nutrients could induce an intrauterine environment that stimulates increased fetal adiposity and growth.[2 34]

Even though the observed effects for the associations of maternal blood glucose concentrations with altered fetal growth patterns and the risk of delivering a large-for-gestational-age infant are relatively small, they are important from an aetiological and preventive perspective. Importantly, we observed the adverse effects of maternal blood glucose concentrations across

the full range of maternal early-pregnancy non-fasting glucose concentrations and not only at the diagnostic thresholds of impaired glucose metabolism. In addition, the observed associations were not explained by maternal sociodemographic factors or lifestyle factors, which suggests that potential intrauterine mechanisms may be involved. Current clinical practice is mainly focused on screening for gestational diabetes based on diagnostic thresholds of maternal glucose concentrations from mid-pregnancy onwards in higher-risk women. However, based on our findings, altered fetal development can already occur among non-diabetic women before mid-pregnancy, which is when screening for gestational diabetes and necessary interventions are currently implemented. Recent RCTs, which are considered the gold standard for studying causality, indicate that treatment of gestational diabetes and maternal hyperglycemia with lifestyle adaptations from mid-pregnancy onwards leads to a decreased risk of adverse birth outcomes compared with no treatment.[35-37] Based on our findings, future RCTs should focus on glucose screening and treatment from preconception and early pregnancy onwards to further improve pregnancy outcomes, among higher-risk populations such as women with overweight and obesity and possibly also among lower-risk populations. These studies should assess the effects of lifestyle interventions that keep an adequate balance between reducing maternal blood glucose concentrations without inducing hypoglycaemia and preventing hyperglycemia. These interventional studies from preconception and early pregnancy onwards will not only provide important novel insights into the effectiveness of these interventions, but also into the causality of the observed associations of maternal early-pregnancy non-fasting glucose concentrations with altered fetal growth and adverse birth outcomes.

Methodological considerations

Major strengths of this study are the population-based prospective design with a large sample size with information on maternal blood glucose concentrations and fetal growth throughout pregnancy. The response rate at baseline was 61%. The non-response at baseline would lead to biased effect estimates if associations were different between those included and not included in the analyses, but this seems unlikely. We had a relatively small number of cases of gestational diabetes, which indicates a selection towards a non-diabetic population and might affect the generalisability of our findings. The observed associations might be stronger among higher-risk populations. Information on gestational diabetes was obtained from medical records after delivery. Accurate diagnosis of gestational diabetes is difficult. A fasting glucose greater than 7.0 mmol/l might also represent pre-existing diabetes and a fasting glucose between 6.1 and 6.9 mmol/l might also represent impaired glucose tolerance, instead of gestational diabetes. Unfortunately, in our study, glucose testing for diagnosis of gestational diabetes was not done for all women for study purposes and no data were available on glucose tolerance before pregnancy. Further studies are needed to replicate our findings among more high-risk populations, including women with impaired glucose tolerance from preconception and early pregnancy onwards and women at risk to develop gestational diabetes. We only measured maternal glucose concentrations

once in early pregnancy. However, it has been suggested that impaired glucose control in early pregnancy persists throughout pregnancy.[38] The fasting time before venous puncture was limited to 30 minutes, due to which we consider our samples non-fasting samples. We were not able to collect blood samples after a longer fasting period due to the design of the study. The blood samples were collected in a non-fasting state at different time-points during the day, depending on time of the study visit. Since glucose concentrations shift very easily during the day and are sensitive towards carbohydrate intake, this may have led to non-differential misclassification of what would be classified as high- or low-glucose concentrations and an underestimation of the observed effect estimates. We also did not have information on 1 hour and 2 hours postprandial glucose concentrations available. However, it has been suggested that maternal fasting glucose concentrations, postprandial glucose concentrations and non-fasting random samples are appropriate measures of maternal glucose metabolism and are related to adverse birth outcomes.[2 7] Non-fasting blood values may better reflect the normal physiological state in pregnant women.[4 31] Further studies are needed to replicate our findings using more detailed maternal glucose measurements, including fasting glucose concentrations and detailed postprandial glucose measurements. Although we included many covariates, there still might be some residual confounding, as in any observational study.

CONCLUSIONS

Maternal early-pregnancy non-fasting blood glucose concentrations are associated with altered fetal growth patterns, characterised by decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards, and an increased risk of delivering a large-for-gestational-age infant. These associations are only partly explained by maternal pre-pregnancy BMI. Instead of targeting maternal glucose metabolism in the second half of pregnancy as in current clinical practice, future preventive strategies need to focus on screening for an impaired maternal glucose metabolism from preconception and early pregnancy onwards to improve fetal growth and birth outcomes.

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SUPPLEMENTARY MATERIAL

Table S1. Non-response analyses

	Participants n = 6,116	**Non-participants** n = 2,763	***p* value***
Maternal characteristics			
Age, years	29.8 ± 5.1	29.4 ± 5.8	0.04
Height, cm	167.5 ± 7.4	166.2 ± 7.4	<0.01
Pre-pregnancy weight, kg	64.0 (48.0–99.7)	63.0 (48.0–100.0)	0.39
Pre-pregnancy BMI, kg/m^2	22.6 (18.0–34.7)	22.8 (17.8–36.0)	0.02
Gestational age at intake, weeks	13.2 (9.6–17.6)	19.6 (10.8–31.4)	<0.01
Parity (nulliparous)	3474 (57.3)	1387 (51.6)	<0.01
Ethnicity			
Dutch	3,083 (52.2)	1,013 (40.4)	<0.01
European	496 (8.4)	175 (7.0)	
Cape Verdean	245 (4.2)	110 (4.4)	
Moroccan	353 (6.0)	227 (9.1)	
Dutch Antillean	171 (2.9)	124 (5.0)	
Surinamese	506 (8.6)	258 (10.3)	
Turkish	474 (8.1)	298 (11.9)	
Other	545 (9.3)	300 (12.0)	
Education, higher	2,550 (44.9)	828 (34.8)	<0.01
Total calorie intake, kJ	486 ± 134	489 (141)	0.49
Folic acid use			
No	1,183 (25.3)	745 (39.4)	<0.01
Start first 10 weeks	1,491 (31.9)	549 (29.1)	
Start periconceptional	1,997 (42.8)	595 (31.5)	
Smoking during pregnancy, continued	1,012 (18.6)	428 (18.6)	0.96
Alcohol use during pregnancy, continued	2,095 (39.0)	691 (30.5)	<0.01
Gestational hypertensive disorders			
Pre-eclampsia	127 (2.2)	60 (2.4)	0.68
Gestational hypertension	234 (4.1)	84 (3.3)	0.11
Pre-gestational diabetes	24 (0.5)	9 (0.4)	0.75
Gestational diabetes	62 (1.1)	29 (1.1)	0.75
Birth Characteristics			
Males	3,100 (50.7)	1,301(49.9)	0.48
Gestational age, weeks	40.1 (35.6–42.3)	40.0 (34.7–42.4)	<0.01
Preterm birth[a]	310 ± 5.1	199 ± 7.6	<0.01
Small-for-gestational-age[b]	606 (10.0)	254 (9.9)	0.89
Large-for-gestational-age[c]	606 (10.0)	234 (8.5)	0.21
Caesarean delivery	692 (14.5)	277 (13.8)	0.45
Vacuum extraction	774 (15.9)	277 (13.8)	0.03

Values are numbers (%), means ± SD or medians (95% range). *Differences in subject characteristics between participants and non-participants were evaluated using one-way ANOVA test for continuous variables and chi-square tests for categorical variables. [a] Preterm birth is defined as <37 weeks' gestation. [b] Small for gestational age is defined as <10th percentile of age-and sex-adjusted birth weight. [c] Large for gestational age is defined as >90th percentile of age-and sex-adjusted birth weight.

Table S2. Associations of maternal early-pregnancy glucose concentrations in mmol/l with fetal biometry measurements

Maternal early-pregnancy glucose concentrations (mmol/l)				
Early pregnancy	**Crown-rump Length**			
Basic model	-0.02 (-0.08, 0.03)			
Ethnicity model	-0.02 (-0.08, 0.03)			
Maternal pregnancy-related factors model	-0.04 (-0.09, 0.02)			
BMI model	-0.04 (-0.09, 0.02)			
Mid-pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Basic model	-0.03 (-0.06, 0.00)*	-0.05 (-0.08, -0.02)*	0.01 (-0.02, 0.04)	-0.02 (-0.05, 0.01)
Ethnicity model	-0.03 (-0.06, 0.00)	-0.04 (-0.07, -0.01)*	0.01 (-0.02, 0.04)	-0.02 (-0.05, 0.01)
Maternal pregnancy-related factors model	-0.03 (-0.06, 0.00)	-0.05 (-0.08, -0.02)*	0.01 (-0.02, 0.04)	-0.02 (-0.05, 0.01)
BMI model	-0.04 (-0.07, -0.01)*	-0.06 (-0.09, -0.02)*	-0.01 (-0.04, 0.02)	-0.04 (-0.07, -0.01)*
Late pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Basic model	0.03 (-0.01, 0.06)	0.05 (0.02, 0.08)*	0.04 (0.01, 0.07)*	0.05 (0.02, 0.08)*
Ethnicity model	0.03 (0.00, 0.06)*	0.05 (0.02, 0.08)*	0.04 (0.01, 0.07)*	0.05 (0.02, 0.08)*
Maternal pregnancy-related factors model	0.03 (0.00, 0.06)	0.04 (0.01, 0.07)*	0.03 (0.00, 0.06)*	0.04 (0.01, 0.07)*
BMI model	0.02 (-0.02, 0.05)	0.02 (-0.01, 0.05)	0.01 (-0.02, 0.04)	0.02 (-0.01, 0.05)
Birth	**Head circumference**	**Abdominal circumference**	**Length**	**Weight**
Basic model	0.04 (0.00, 0,09)	NA	0.04 (-0.01, 0.08)	0.07 (0.04, 0.11)*
Ethnicity model	0.05 (0.01, 0,10)*	NA	0.04 (0.00, 0.09)*	0.08 (0.05, 0.11)*
Maternal pregnancy-related factors model	0.04 (0.00, 0,09)	NA	0.04 (-0.01, 0.08)	0.07 (0.04, 0.10)*
BMI model	0.04 (-0.01, 0.08)	NA	0.02 (-0.02, 0.07)	0.05 (0.02, 0.08)*

Data are SDS values (95% CI) from linear regression models that reflect the differences in growth characteristics per 1 mmol/l in maternal early-pregnancy glucose concentrations. Analyses with crown-rump length were based on subgroup analyses (n = 1,470). Estimates are from multiple imputed data. Basic model adjusted for gestational age at assessment. Ethnicity model: basic model additionally adjusted for maternal ethnicity. Maternal pregnancy-related factors model: ethnicity model additionally adjusted for maternal age, parity, educational level, daily total calorie intake, smoking, alcohol consumption and folic acid supplement use. BMI model: maternal pregnancy-related factors model additionally adjusted for maternal prepregnancy BMI. *p value <0.05. Abbreviations: NA, not available.

Table S3. Associations of maternal early-pregnancy glucose concentrations in mmol/l with absolute fetal biometry measurements

Maternal early-pregnancy glucose concentrations (mmol/l)	**Head circumference** (mm/ at birth cm)	**Abdominal circumference** (mm/ at birth NA)	**Femur length** (mm/ length at birth cm)	**Estimated fetal weight** (gram/ weight at birth gram)
Mid-pregnancy	-0.32 (-0.72, 0.08)	-0.51 (-0.92, -0.10)*	-0.02 (-0.11, 0.08)	-1.95 (-4.48, 0.57)
Late pregnancy	0.26 (-0.12, 0.63)	0.55 (0.06, 1.05)*	0.08 (-0.01, 0.17)	8.46 (0.86, 16.06)*
Birth	0.04 (-0.03, 0.11)	NA	0.07 (-0.02, 0.15)	25.38 (8.98, 41.79)*

Data are exact values (95% CI) from linear regression models that reflect the differences in growth characteristics per 1 mmol/l in maternal early-pregnancy glucose concentrations. Estimates are from multiple imputed data. Models are adjusted according maternal pregnancy-related factors model adjusted for gestational age at assessment, maternal ethnicity, age, parity, educatoinal level, daily total calorie intake, smoking, alcohol consumption and folic acid supplement use. *p value <0.05. Abbreviations: NA, not available.

Table S4. Associations of maternal early-pregnancy glucose concentrations in mmol/l with the risks of adverse birth outcomes

Maternal early-pregnancy glucose concentrations (mmol/l)	**Small size for gestational age at birth**	**Large size for gestational age at birth**	**Preterm birth**	**Caesarean delivery**	**Vacuum extraction**
Basic model	0.89 (0.80, 0.98)*	1.27 (1.16, 1.40)*	1.08 (0.95, 1.24)	1.12 (0.98, 1.27)	0.99 (0.90, 1.09)
Ethnicity model	0.87 (0.79, 0.97)*	1.29 (1.18, 1.42)*	1.08 (0.94, 1.23)	1.12 (0.99, 1.27)	1.00 (0.91, 1.11)
Maternal pregnancy-related factors model	0.88 (0.79, 0.98)*	1.28 (1.16, 1.41)*	1.08 (0.94, 1.23)	1.11 (1.00, 1.23)	1.01 (0.91, 1.12)
BMI model	0.91 (0.82, 1.02)	1.21 (1.10, 1.34)*	1.06 (0.92, 1.21)	1.09 (0.99, 1.20)	1.01 (0.90, 1.12)

Values are ORs (95% CI) from logistic regression models that reflect the differences in risks of adverse birth outcomes per 1 mmol/l in maternal early-pregnancy glucose concentrations. Estimates are from multiple imputed data. Basic model adjusted for gestational age at assesment. Ethnicity model: basic model additionally adjusted for maternal ethnicity. Maternal pregnancy-related factors model: ethnicity model additionally adjusted for maternal age, parity, educational level, daily total calorie intake, smoking, alcohol consumption and folic acid supplement use. BMI model: maternal pregnancy-related factors model additionally adjusted for maternal prepregnancy BMI. *p value <0.05.

Table S5. Associations of maternal early-pregnancy glucose and insulin concentrations in standard deviation scores with fetal biometry measurements

Early pregnancy	**Crown-rump Length**			
Glucose (SD)	-0.02 (-0.07, 0.03)			
Insulin (SD)	-0.02 (-0.07, 0.04)			
Mid-pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Glucose (SD)	-0.02 (-0.05, 0.00)	-0.04 (-0.07, -0.02)*	0.01 (-0.02, 0.03)	-0.02 (-0.05, 0.00)
Insulin (SD)	-0.01 (-0.04, 0.02)	-0.02 (-0.04, 0.01)	0.04 (0.01, 0.06)*	0.01 (-0.02, 0.03)
Late pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Glucose (SD)	0.02 (0.00, 0.05)	0.03 (0.01, 0.06)*	0.03 (0.00, 0.05)*	0.04 (0.01, 0.06)*
Insulin (SD)	0.00 (-0.03, 0.03)	0.04 (0.01, 0.06)*	0.06 (0.04, 0.09)*	0.05 (0.03, 0.08)*
Birth	**Head circumference**	**Abdominal circumference**	**Length**	**Weight**
Glucose (SD)	0.04 (0.00, 0.08)	NA	0.03 (0.00, 0.07)	0.06 (0.04, 0.09)*
Insulin (SD)	0.06 (0.01, 0.09)*	NA	0.08 (0.04, 0.12)*	0.06 (0.03, 0.08)*

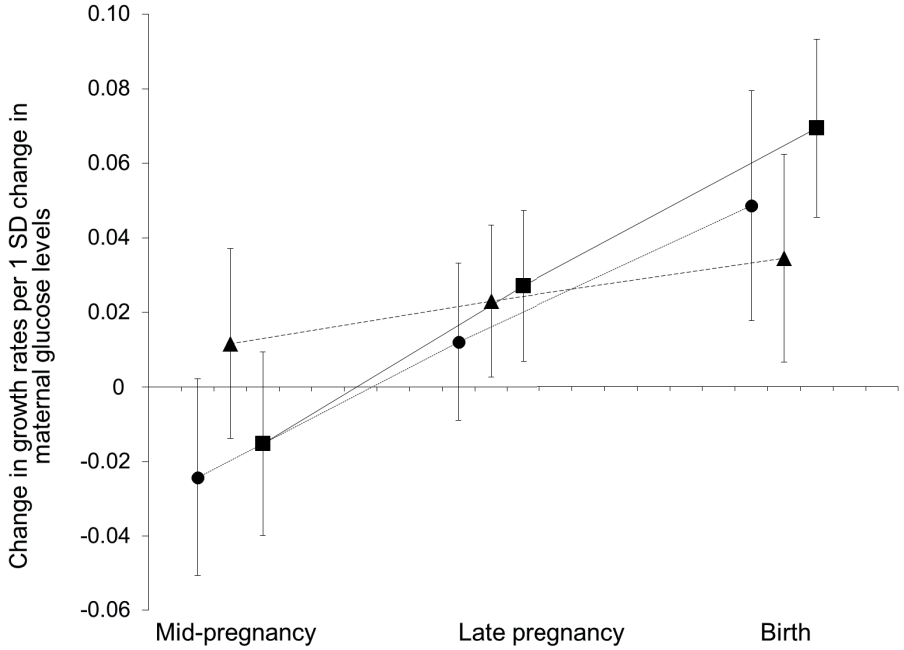
Data are SDS values (95% CI) from linear regression models that reflect the differences in growth characteristics per 1 SD change in maternal early-pregnancy glucose and insulin concentrations. Insulin was log-transformed before the construction of SD score. Analyses with crown-rump length were based on subgroup analyses (n = 1,470). Estimates are from multiple imputed data. Models are adjusted according maternal pregnancy-related factors model adjusted for gestational age at assessment, maternal ethnicity, age, parity, educational level, daily total calorie intake, smoking, alcohol consumption and folic acid supplement use. *p value <0.05. Abbreviations: NA, not available.

Table S6. Sensitivity analyses of fetal biometry measurements

Early pregnancy	**Crown-rump Length**			
Main model	-0.04 (-0.09, 0.02)			
No pre-gestational diabetes mellitus	-0.05 (-0.10, 0.01)			
No gestational diabetes mellitus	-0.05 (-0.11, 0.01)			
Dutch ethnicity	-0.04 (-0.11, 0.03)			
Included <14 weeks gestation	-0.04 (-0.10, 0.02)			
No preterm	-0.05 (-0.11, 0.01)			
Mid-pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Main model	-0.03 (-0.06, 0.00)	-0.05 (-0.08, -0.02)*	0.01 (-0.02, 0.04)	-0.02 (-0.05, 0.01)
No pre-gestational diabetes mellitus	-0.02 (-0.06, 0.01)	-0.05 (-0.08, -0.02)*	0.01 (-0.03, 0.04)	-0.03 (-0.06, 0.00)
No gestational diabetes mellitus	-0.03 (-0.06, 0.00)	-0.05 (-0.08, -0.02)*	0.01 (-0.02, 0.04)	-0.03 (-0.06, 0.00)
Dutch ethnicity	-0.04 (-0.09, 0.00)	-0.07 (-0.12, -0.03)*	0.01 (-0.03, 0.05)	-0.04 (-0.08, 0.00)
Included <14 weeks gestation	0.00 (-0.02, 0.02)	-0.04 (-0.07, -0.01)*	0.03 (-0.01, 0.06)	-0.01 (-0.04, 0.03)
No preterm	-0.03 (-0.06, 0.01)	-0.05 (-0.08, -0.02)*	0.00 (-0.03, 0.03)	-0.03 (-0.06, 0.00)
Late pregnancy	**Head circumference**	**Abdominal circumference**	**Femur length**	**Estimated fetal weight**
Main model	0.03 (0.00, 0.06)	0.04 (0.01, 0.07)*	0.03 (0.00, 0.06)*	0.04 (0.01, 0.07)*
No pre-gestational diabetes mellitus	0.03 (0.00, 0.06)	0.03 (0.00, 0.06)	0.02 (-0.01, 0.06)	0.03 (0.00, 0.07)*
No gestational diabetes mellitus	0.02 (-0.01, 0.05)	0.03 (0.00, 0.06)*	0.03 (0.00, 0.06)*	0.04 (0.01, 0.07)*
Dutch ethnicity	0.00 (-0.05, 0.04)	0.02 (-0.02, 0.07)	0.03 (-0.01, 0.07)	0.03 (-0.01, 0.07)
Included <14 weeks gestation	0.04 (0.00, 0.08)*	0.04 (0.00, 0.08)*	0.06 (0.02, 0.10)*	0.06 (0.02, 0.09)*
No preterm	0.03 (-0.01, 0.06)	0.03 (0.00, 0.07)*	0.03 (0.00, 0.06)*	0.04 (0.01, 0.07)*
Birth	**Head circumference**	**Abdominal circumference**	**Length**	**Weight**
Main model	0.04 (0.00, 0,09)	NA	0.04 (-0.01, 0.08)	0.07 (0.04, 0.10)*
No pre-gestational diabetes mellitus	0.03 (-0.02, 0,08)	NA	0.04 (0.00, 0.09)	0.07 (0.04, 0.10)*
No gestational diabetes mellitus	0.04 (-0.01, 0.09)	NA	0.04 (-0.01, 0.08)	0.06 (0.04, 0.10)*
Dutch ethnicity	0.02 (-0.04, 0.08)	NA	0.02 (-0.03, 0.08)	0.06 (0.01, 0.10)*
Included <14 weeks gestation	0.02 (-0.04, 0.08)	NA	0.03 (-0.02, 0.08)	0.09 (0.05, 0.12)*
No preterm	0.05 (0.00, 0,10)*	NA	0.04 (-0.01, 0.08)	0.07 (0.04, 0.10)*

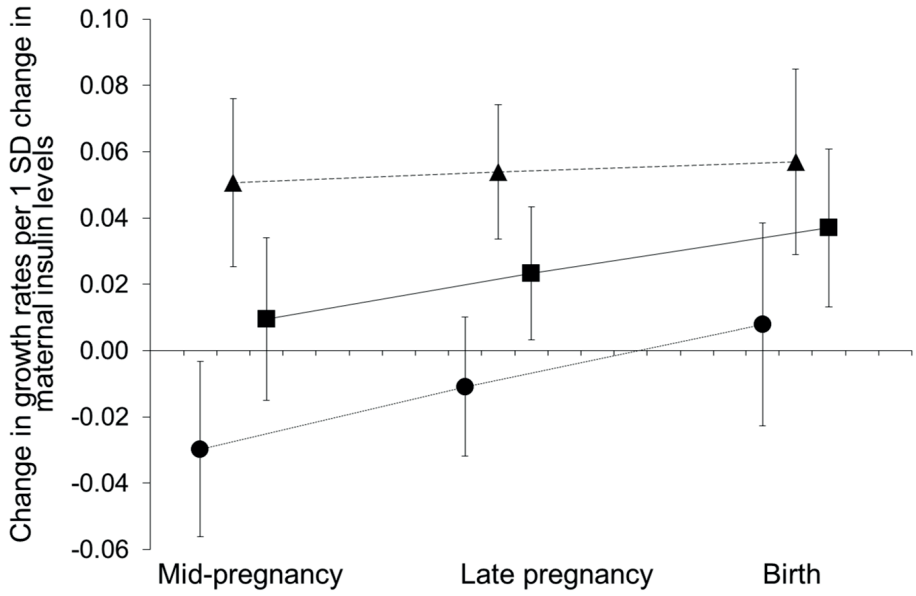
Data are SDS values (95% CI) from linear regression models that reflect the differences in growth characteristics per 1 mmol/l in maternal early-pregnancy glucose concentrations. Analyses with crown-rump length were based on subgroup analyses (n = 1,470). Estimates are from multiple imputed data. Models are adjusted according maternal pregnancy-related factors model adjusted for gestational age at assessment, maternal ethnicity, age, parity, educational level, daily total calorie intake, smoking, alcohol consumption and folic acid supplement use. *p value <0.05. Abbreviations: NA, not available.

Figure S1. Maternal early-pregnancy glucose concentrations in standard deviation scores and longitudinal fetal growth rates



Data are SDS values (95% CI) from repeated measurement regression models that reflect the differences in gestational age-adjusted growth rates in SDS of head circumference (circles), length (triangles), and weight (squares) at mid-pregnancy, late pregnancy and at birth per 1 SD change in maternal early-pregnancy glucose concentrations. As a measure of skeletal length growth from mid-pregnancy onwards, we used fetal femur length SDS in mid-pregnancy and late pregnancy and total body length SDS at birth within the repeated measurements model. All fetal biometry measurements for each pregnancy period were taken at the same time point. The models were adjusted for gestational age at intake. *p* value for interaction with gestational age for all models <0.05.

Figure S2. Maternal early-pregnancy insulin concentrations in standard deviation scores and longitudinal fetal growth rates



Data are SDS values (95% CI) from repeated measurement regression models that reflect the differences in gestational age-adjusted growth rates in SDS of head circumference (circles), length (triangles), and weight (squares) at mid-pregnancy, late pregnancy and at birth per 1 SD change in maternal early-pregnancy insulin concentrations. As a measure of skeletal length growth from mid-pregnancy onwards, we used fetal femur length SDS in mid-pregnancy and late pregnancy and total body length SDS at birth within the repeated measurements model. All fetal biometry measurements for each pregnancy period were taken at the same time point. The models were adjusted for gestational age at intake. *p* value for interaction with gestational age for all models <0.05.

2.3 Associations of maternal early-pregnancy blood glucose and insulin concentrations with DNA methylation in newborns

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ABSTRACT

Background: Intrauterine exposure to a disturbed maternal glucose metabolism is associated with adverse offspring outcomes. DNA methylation is a potential mechanism underlying these associations. We examined whether maternal early pregnancy glucose and insulin concentrations are associated with newborn DNA methylation.

Methods: In a population-based prospective cohort study among 935 pregnant women, maternal plasma concentrations of non-fasting glucose and insulin were measured at a median of 13.1 weeks of gestation (95% range 9.4 – 17.4). DNA methylation was measured using the Infinium HumanMethylation450 BeadChip (Illumina). We analyzed associations of maternal early-pregnancy glucose and insulin concentrations with single-CpG DNA methylation using robust linear regression models. Differentially methylated regions were analyzed using the dmrff package in R. We stratified the analyses on women with normal weight versus women with overweight or obesity. We also performed a look-up of CpGs and differently methylated regions from previous studies to be associated with maternal gestational diabetes, hyperglycemia or hyperinsulinemia, or with type 2 diabetes in adults.

Results: Maternal early-pregnancy glucose and insulin concentrations were not associated with DNA methylation at single CpGs nor with differentially methylated regions in the total group. In analyses stratified on maternal BMI, maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG (cg03617420, *XKR6*) among women with normal weight and at another (cg12081946, *IL17D*) among women with overweight or obesity. No stratum-specific associations were found for maternal early-pregnancy insulin concentrations. The two CpGs were not associated with birth weight or childhood glycemic measures (*p* values > 0.1). Maternal early-pregnancy insulin concentrations were associated with one CpG known to be related to adult type 2 diabetes. Enrichment among nominally significant findings in our maternal early-pregnancy glucose concentrations was found for CpGs identified in a previous study on adult type 2 diabetes.

Conclusions: Maternal early-pregnancy glucose concentrations, but not insulin concentrations, were associated with DNA methylation at one CpG each in the subgroups of women with normal weight, and of women with overweight or obesity. No associations were present in the full group. The role of these CpGs in mechanisms underlying offspring health outcomes needs further study. Future studies should replicate our results in larger samples with early-pregnancy information on maternal fasting glucose metabolism.

INTRODUCTION

The prevalence of gestational diabetes is rising worldwide and has been reported to complicate up to 25% of all pregnancies.[1 2] This rise is partly due to the increasing prevalence of obesity among women of reproductive age and depends on screening tools and diagnostic criteria.[2-4] Intrauterine exposure to maternal gestational diabetes or impaired glucose tolerance measured in mid-pregnancy and late pregnancy is associated with increased risks of adverse maternal and fetal perinatal outcomes, and of diabetes and obesity in the offspring.[5-8] These associations of increased risks on perinatal outcomes are already present for higher maternal glucose concentrations below the threshold of gestational diabetes.[9 10] Additionally, the associations are stronger among women who are overweight or obese at the start of their pregnancy.[2 3] Women who develop hyperglycemia and gestational diabetes may already have suboptimal glucose metabolism earlier in pregnancy. The first trimester of pregnancy is a critical period for embryonic and placental growth and development.[11] As such, impaired maternal glucose metabolism may already exert negative effects in that early stage. Early-pregnancy glucose metabolism has been shown to be associated with altered fetal growth, adverse birth outcomes and childhood glucose metabolism, but not with other childhood cardio-metabolic outcomes after adjusting for maternal pre-pregnancy BMI.[9 12 13] Thus, early pregnancy may be an important time window for the effects of suboptimal maternal glycemic measures and as such an influential period for future interventions.

The mechanisms underlying these associations are unknown. DNA methylation has been suggested as a potential mechanism linking adverse exposures during pregnancy and impaired offspring health.[14 15] Previous studies using candidate-gene approaches suggested that maternal gestational diabetes is associated with epigenetic modifications in placenta and cord blood at loci relevant to growth, energy homeostasis, and diabetes.[14 16-18] Epigenome-wide association studies (EWAS) of gestational diabetes or maternal glucose concentrations showed varying results, with no clear pattern of associations.[1 15 19-25] The inconsistent results of candidate-gene studies and EWAS may be due to differences in study design. The studies varied in their exposure definition: gestational diabetes as binary exposure or glucose concentrations after an oral challenge test, in the tissues in which DNA methylation was measured: placenta or blood, and in the extent of adjustment for covariates, with most not adjusting for cell type heterogeneity. Also, the majority had limited sample sizes.[15 19 21-25] It is not known whether maternal glucose and insulin concentrations across the full range in early pregnancy are associated with cord blood DNA methylation and whether these associations differ between women with normal weight versus women with overweight or obesity. Insight into these associations and their underlying mechanisms is important, as maternal blood glucose metabolism can be a target for preventive interventions to improve child health outcomes.

We hypothesized that maternal early-pregnancy glucose and insulin concentrations are associated with offspring DNA methylation at birth. Therefore, we conducted an epigenome-wide association analysis in a population-based prospective cohort study, with maternal glucose and insulin concentrations measured at a median of 13.1 weeks of gestation (95% range 9.4 – 17.4).

As a secondary analysis, we stratified on maternal body mass index (BMI) categories to observe if maternal BMI modifies the studied associations. We also examined whether differential DNA methylation at any CpGs found to be associated with maternal glucose or insulin concentrations in cord blood persisted in peripheral blood of 10-year-old children. To obtain further insight into the potential significance of the observed DNA methylation changes, we conducted exploratory analyses on the associations of identified CpGs with offspring health outcomes. We also performed a look-up in our results of CpGs identified to be associated with maternal glucose metabolism during pregnancy or with type 2 diabetes in adults in previous literature.

RESULTS

Subject characteristics

The population characteristics for the total group and stratified on maternal BMI in women with normal weight versus women with overweight or obesity are shown in **Table 1.** The mean maternal early-pregnancy glucose concentration was 4.4 mmol/l (standard deviation 0.8) and the median maternal early-pregnancy insulin concentration was 126.3 pmol/l (95% range 19.9 – 774.6). Gestational diabetes was diagnosed in 12 (1.3%) women and 59 (6.3%) women were obese. Non-response analyses showed that mothers without data on early-pregnancy glucose and insulin measurements delivered more often female children (**Table S1**).

Associations of maternal early-pregnancy glucose and insulin concentrations with DNA methylation at birth

After Bonferroni (*p* value cutoff < 1.0×10^{-7}) or false-discovery rate (FDR) correction, we did not observe associations of maternal early-pregnancy glucose or insulin concentrations with offspring DNA methylation in cord blood. These models were adjusted for gestational age at glucose/insulin measurement, maternal age, educational level, parity, smoking, pre-pregnancy BMI, child sex, cell type proportions and batch. The results of both analyses are presented in **Figure S1a** and **Figure S1b**. The CpGs with *p* values < 1.0×10^{-4} for both analyses are shown in **Table S2** and **Table S3**. A model without adjustment for maternal pre-pregnancy BMI showed largely similar results **Table S4** and **Table S5**.

The analyses stratified on maternal BMI showed that among women with normal weight maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG (cg03617420 in *XKR6;* effect estimate = 7.3×10^{-3} (standard error (SE) 1.3×10^{-3}), *p* value = 7.4×10^{-9}) (**Figure 1a**). DNA methylation at this CpG was not significantly associated with glucose concentrations in women with overweight or obesity (effect estimate = -2.4×10^{-3} (SE 3.2×10^{-3}), *p* value = 0.46). Among women with overweight or obesity maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG (cg12081946 in *IL17D*; effect estimate = -3.4×10^{-2} (SE 5.6×10^{-3}), *p* value = 8.9×10^{-10}) (**Figure 1b**). DNA methylation at this CpG

was not significantly associated with glucose concentrations in women with normal weight (effect estimate = 3.1 x 10^{-3} (SE 4.1 x 10^{-3}), *p* value = 0.45). Maternal early-pregnancy insulin concentrations were not associated with DNA methylation in cord blood in women with normal weight and in women with overweight or obesity (**Figure S2a** and **Figure S2b**).

Neither maternal early-pregnancy glucose nor insulin concentrations were associated with differentially methylated regions in cord blood, analysed using the dmrff package in R.[26] The differentially methylated regions with *p* values < 1.0 x 10^{-4} are shown in **Table S6** and **Table S7**.

Maternal glucose concentrations were not associated with DNA methylation levels at the two identified CpGs in peripheral blood of 10-year-old children (*p* values > 0.1) (**Table S8**). In exploratory analyses, the two identified CpGs were not associated with birth weight or childhood glucose concentrations, which both were previously found to be associated with maternal early-pregnancy glucose concentrations in our data (*p* values > 0.1).[9 13]

Table 1. Maternal and birth characteristics of the study population

Characteristics	**Total group** n = 935	**Maternal normal weight** n = 667	**Maternal overweight/obesity** n = 234	***p* value**
Maternal characteristics				
Age, years	31.7 ± 4.2	31.8 ± 4.2	31.7 ± 4.1	0.69
Height, cm	170.8 ± 6.2	171.1 ± 6.3	170.4 ± 5.8	0.25
Pre-pregnancy body mass index, kg/m^2	23.3 ± 3.8	21.8 ± 1.6	28.5 ± 3.4	<0.01
Women with				
Underweight	34 (3.6)	-	-	
Normal weight	667 (71.3)	667	-	
Overweight	175 (18.7)	-	175	
Obesity	59 (6.3)	-	59	
Gestational age at glucose/ insulin measurement, weeks	13.1 (9.4 – 17.4)	13.4 (8.3 – 17.4)	12.9 (9.5 – 17.5)	0.19
Parity, nulliparous	563 (60.3)	409 (61.3)	130 (55.6)	0.23
Education, higher education	596 (64.9)	405 (71.6)	97 (50.0)	<0.01
Smoking during pregnancy, continued	173 (20.8)	101 (19.6)	40 (22.6)	0.40
Glucose, mmol/l	4.4 ± 0.8	4.4 ± 0.8	4.5 ± 0.8	0.08
Insulin, pmol/l	126.3 (19.9 – 774.6)	119.6 (19.6 – 764.5)	153.6 (19.0 – 847.4)	0.09
Gestational diabetes	10 (1.3)	8 (1.7)	1 (0.5)	0.31
Child characteristics				
Male	491 (52.5)	311 (54.4)	96 (49.0)	0.19
Gestational age at birth, weeks	40.3 (36.4 – 42.3)	40.4 (36.5 – 42.3)	40.4 (36.3 – 42.3)	0.60
Birth weight, grams	3552 ± 514	3552 ± 493	3599 ± 571	0.31

Values are means ± SD, medians (95% range) or numbers of subjects (valid %) shown for the total group and stratified for maternal pre-pregnancy body mass index. The stratified groups are women with normal weight versus women with overweight or obesity (data for women with underweight are not separately shown n = 34). Differences were tested using Student's t-tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively, and χ2-test were used for dichotomous variables.

Figure 1a. Epigenome-wide association study results of maternal early-pregnancy glucose concentrations and DNA methylation in cord blood in women with normal weight

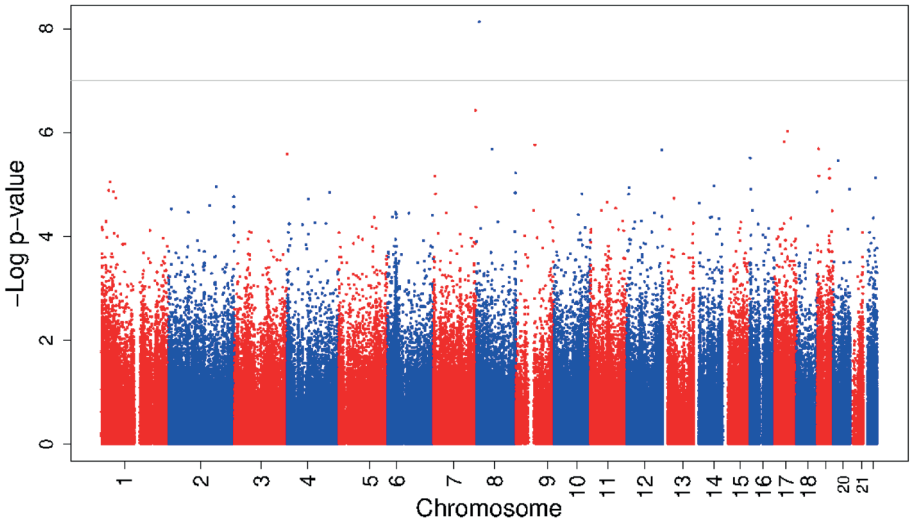
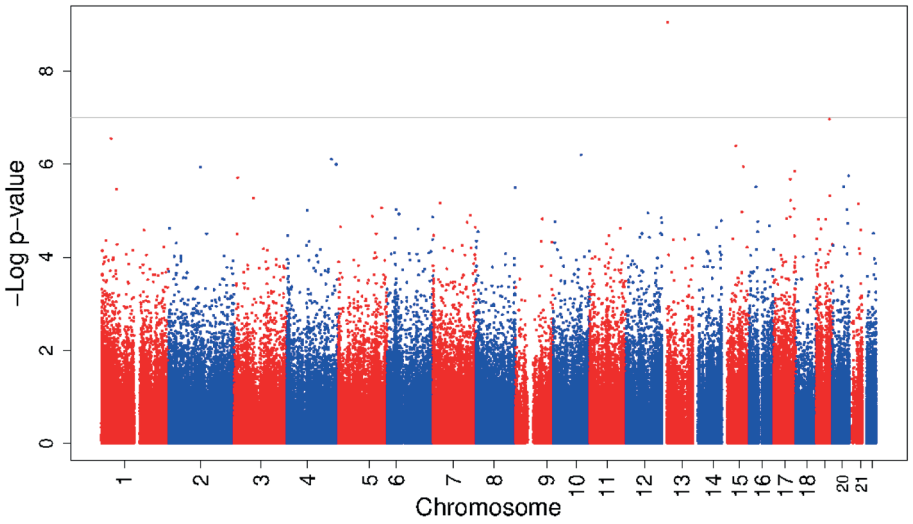
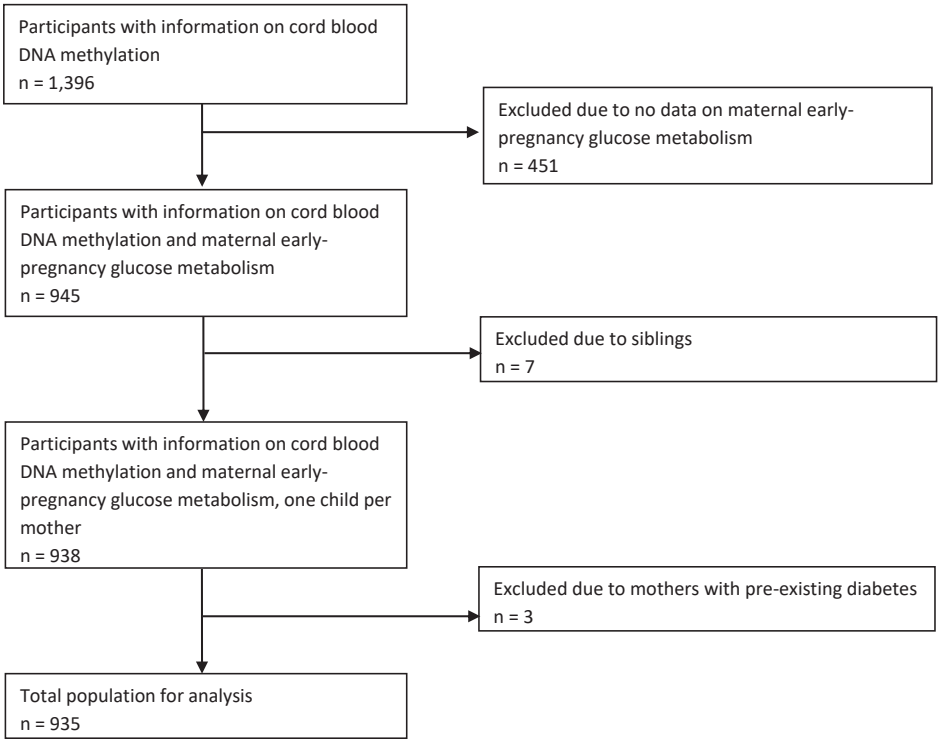


Figure 1b. Epigenome-wide association study results of maternal early-pregnancy glucose concentrations and DNA methylation in cord blood in women with overweight or obesity



In 1a. the Manhattan plot shows the results of the epigenome-wide association study of maternal early-pregnancy glucose concentrations and DNA methylation in cord blood in women with normal weight. In 1b Manhattan plot of the results of the epigenome-wide association study of maternal early-pregnancy glucose concentrations and DNA methylation in cord blood in women with overweight or obesity. In both figures the x-axis represents the autosomal (1 – 22) chromosomes and the y-axis shows the $-\log_{10}$ (*p* value). The models were adjusted for gestational age at assessment, maternal age at intake, educational level, parity, smoking, child sex, cell type proportions and batch.

Figure 2. Study participants flowchart



Look-up of maternal glucose metabolism and adult type 2 diabetes-associated CpGs

In a look-up in our results of CpGs and DMRs identified in previous studies to be associated with maternal glucose metabolism or with adult type 2 diabetes, we found that one CpG, cg1680945 at *MDN1*, known to be related to adult type 2 diabetes was also significantly associated with maternal early-pregnancy insulin concentrations (effect estimate = -3.3×10^{-3} (SE 1.1×10^{-3}), p value = 2.2×10^{-3}).[27] The look-up of other previously described CpGs and DMRs in the maternal early-pregnancy glucose and insulin EWAS results showed no associations (**Table S9**; **Table S10**; **Table S11**; **Table S12**).[1 14 17 18 22 23 25 27-42] We found enrichment for findings from one previous study on adult type 2 diabetes among the 24,935 nominally significant CpGs from the maternal early-pregnancy glucose EWAS results (Fisher combined probability p value = 0.04).[40] No evidence for enrichment of the CpGs from other previous studies among the 24,935 nominally significant CpGs from the maternal early-pregnancy glucose cord blood analysis, nor among the 19,418 nominally significant CpGs from the maternal early-pregnancy insulin cord blood analysis was found (lowest Fisher combined probability p value = 0.15 in maternal early-pregnancy glucose EWAS results and p value = 0.12 in insulin EWAS results).[1 14 17 18 22 23 25 27-42]

DISCUSSION

In this population-based EWAS we did not observe associations of maternal early-pregnancy glucose and insulin concentrations across the full spectrum with offspring cord blood DNA methylation in the full group. However, after stratification on maternal BMI, maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG each among women with normal weight and among women with overweight or obesity. Associations of DNA methylation at these CpG sites did not persist in 10-year-old children. Also, we did not find associations with offspring health outcomes. Maternal early-pregnancy insulin was associated with one CpG known from a previous adult type 2 diabetes-associated study. Also, we found enrichment of CpGs identified in a previous EWAS on adult type 2 diabetes among our maternal early-pregnancy glucose EWAS results. Overall, our results constitute a first step towards a better understanding of a potential role of DNA methylation underlying the associations of maternal glycemic traits in early pregnancy with offspring health outcomes.

Interpretation of main findings

Gestational diabetes or impaired glucose tolerance diagnosed in the second half of pregnancy increases the risks of adverse birth outcomes, of obesity and diabetes in the offspring.[5 6 8] It has been suggested that women who develop gestational diabetes or hyperglycemia later in pregnancy already have suboptimal glucose metabolism before or in early pregnancy.[43 44] Maternal glycemic measures in early pregnancy have been described to be associated with altered fetal growth and glucose metabolism in childhood, but not with child adiposity, lipid levels and blood pressure after adjustment for maternal pre-pregnancy BMI.[9 12 13] Thus, early pregnancy may already be a critical period for the effects of maternal glucose concentrations on offspring birth outcomes and glycemic health in childhood. The associations of maternal glucose metabolism with offspring outcomes may be explained by differential DNA methylation. Therefore, we hypothesized that maternal early-pregnancy glucose and insulin concentrations are associated with offspring DNA methylation levels at birth, and that these associations may be different for women with normal weight and women with overweight or obesity.

Results from a recent meta-analysis from seven pregnancy cohorts among 3,677 mother-newborn pairs showed that gestational diabetes was not associated with differential methylation at single CpG level, but it was associated with lower cord blood methylation levels within two specific regions.[1] In the current population-based EWASs, we did not find any associations in the full group, but maternal early-pregnancy glucose concentrations were associated with DNA methylation at cg03617420 (*XKR6*) among women with normal weight, and at cg12081946 (*IL17D*) among women with overweight or obesity. The effect estimates of both CpGs were in opposite directions for women with normal weight and women with overweight or obesity, which could imply a modifying effect of maternal BMI, as we hypothesized.

XKR6, XK related 6 gene, is located on chromosome 8 and is classified as a member of the Kell blood group complex subunit-related family. Genetic variants in this gene have previously been associated with type 2 diabetes, lipid concentrations, systolic blood pressure and kidney function, among others.[45-48] *XKR6* is expressed in many tissues, most strongly in testis and lymphocytes, but also in the cerebellum and pancreas, among others. *IL17D*, interleukin 17D, is part of the cytokine family and located on chromosome 13, and has been previously associated with autoimmune and inflammatory diseases.[49] Autoimmune processes are part of the pathogenesis of type 1 diabetes.[50] Genetic variants close to *IL17D* have been associated with PR segment on electrocardiogram.[51] *IL17D* is most strongly expressed in brain and skeletal muscle. Based on Roadmap Epigenomics Data Complete Collection extracted from the UCSC Genome Browser, both cg03617420 and cg12081946 coincide with DNAseI hypersensitivity clusters and transcription factor binding regions, indicating a location in potential regulatory elements.

DNA methylation levels at these CpGs have not been previously described in relation to maternal early-pregnancy glucose concentrations and our results need replication in larger groups. We found that DNA methylation at one CpG, cg1680945 (*MDN1*), which was associated with adult type 2 diabetes in a previous study, was also associated with maternal early-pregnancy insulin concentrations.[27] We also found enrichment for CpGs identified in a previous EWAS on adult type 2 diabetes among the nominally significant CpGs from the maternal early-pregnancy glucose EWAS.[40] Maternal early-pregnancy glucose and insulin concentrations were not associated with any of the other previously reported maternal gestational diabetes, hyperglycemia or hyperinsulinemia, or adult type 2 diabetes-associated CpGs or differently methylated regions.[1 14 17 18 22 23 25 28-39 41 42]

The lack of identified associations in our total study group may have multiple reasons. Our study population is relatively healthy with on average lean women, with limited variability in maternal early-pregnancy glucose and insulin concentrations and with a low percentage of women who developed gestational diabetes. Associations of maternal early-pregnancy glucose and insulin concentrations with DNA methylation may be more apparent when using fasting glucose and insulin concentrations or among high-risk populations, as observed in studies in women with gestational diabetes.[22 31 34] Besides this, the moderate sample size of this study also limits the power to detect smaller differences. Another possibility is that associations of maternal early-pregnancy glucose and insulin concentrations with DNA methylation in offspring are more apparent in other tissues than cord blood, such as placental tissue, body fat, skeletal muscle, liver or pancreas. As shown in the analysis stratified on BMI, for some CpGs, associations may not be apparent in the full group as the directions of effect may be opposite between certain subgroups in the study population.

Maternal glucose concentrations were not associated with DNA methylation in child peripheral blood at age 10 years at the two CpGs identified in cord blood. This could imply a temporary effect of maternal glucose metabolism on offspring DNA methylation or it may be due to the relatively small sample size at age 10 years. Further studies should repeat these analyses among different

age-groups in children to replicate our findings at birth and explore persistence of differential DNA methylation at the two identified CpGs. Also, the two identified CpGs were not associated with birth weight or childhood glucose concentrations in exploratory analyses. This may be due to the relatively small sample size or it may indicate, that DNA methylation at these sites does not represent a biological pathway linking maternal glucose levels to birth weight or childhood glucose concentrations. Further studies, including studies among high-risk populations are needed to examine the exact pathways involved in the associations of maternal glycemic measures during pregnancy with adverse birth outcomes and with offspring health outcomes, such as diabetes at later ages, in more detail.

This study suggests that maternal glycemic traits are associated with DNA methylation, and that these associations may differ between mothers with overweight/obesity and those without. The role of the identified differential DNA methylation in pathways to offspring health needs further study. This is a first step towards discovering the underlying biological pathways and, if confirmed, it emphasizes the first trimester being a potentially important window of pregnancy for intervention studies to improve child health outcomes. Further, larger studies, with maternal early-pregnancy fasting blood samples, are needed to replicate our results.

Methodological considerations

Major strengths of this study are the population-based prospective design and the fact that we have information on maternal plasma glucose and insulin concentrations in early pregnancy in combination with cord blood DNA methylation. In addition to single-CpG analyses, differential methylated regions were also evaluated. We were able to adjust for a large number of potential confounders and for estimated cell type proportions. The relatively small number of mothers with gestational diabetes (1.3% versus 2-5% in the general Dutch population[52]) may be due to the fact that information on gestational diabetes was taken from medical records and there was no structural testing of all pregnant women. However, the low number of mothers with gestational diabetes and obesity included in the sample may also indicate a selection towards a healthy, non-diabetic and lean population that might influence the generalizability of our findings and may have limited our statistical power to detect significant associations. Glucose and insulin concentrations were measured once during early pregnancy. Future studies are needed to measure maternal glucose and insulin concentrations at multiple time points during pregnancy to observe whether normal glucose and insulin concentrations in early pregnancy will worsen or maintain normal during pregnancy and whether patterns of glucose and insulin concentrations during pregnancy may be more informative than single measurements. The blood samples in the study are non-fasting. They were collected after a fasting time of at least 30 minutes. Since glucose and insulin concentrations vary during the day and are sensitive to carbohydrate intake, this may have led to non-differential misclassification of glucose and insulin concentrations. However, it has been suggested that maternal non-fasting glucose concentrations may better reflect the normal physiological state in pregnancy.[10 53] Ideally, data on oral glucose tolerance tests would have been

included, but these are not available in the Generation R Study. Blood samples were collected and processed in a standardized way, but time from sampling to freezing could be up to four hours. This may have affected the measured glucose concentrations. DNA methylation was measured in blood, which may differ from methylation patterns in other tissues. The study participants are of European ancestry and therefore, the findings might not be generalizable to other populations.

CONCLUSIONS

Maternal early-pregnancy blood glucose and insulin concentrations were not associated with differential DNA methylation at birth in the full group. However, maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG in *XKR6* among women with normal weight and at another CpG in *IL17D* among women with overweight or obesity. Their role in mechanisms underlying offspring health outcomes needs further study. These results await confirmation by future studies in larger samples with early-pregnancy information on maternal fasting glucose metabolism and exploring potential tissue-specific methylation effects in the offspring.

METHODS

Study design

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[54] The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained for all participants.[54] In total, 8,879 women were enrolled during pregnancy (response rate at baseline: 61%), of whom 6,186 had measurements of glucose and insulin concentrations available. DNA methylation was measured in cord blood of a randomly selected European-ancestry subset of n = 1,396 mothers. Out of these mothers n = 945 had measurements on early-pregnancy glucose metabolism available. We excluded women with pre-existing diabetes (n = 3), twin pregnancies and in case of multiple (non-twin) children per mother, we excluded one of each sibling pair, based on data completeness or, if equal, randomly (n = 7). The population for analysis of this study comprised 935 mother-newborn pairs (**Figure 2**).

Maternal glucose and insulin concentrations assessment

Blood samples were collected once in early pregnancy at 13.1 median weeks' gestation (95% range 9.4 – 17.4), as described previously. After at least 30 minutes of fasting, venous blood samples were collected from pregnant women by research nurses and temporally stored at room

temperature. Samples were minimally 30 minutes fasting. As we did not have information on the exact fasting duration, we consider all samples random. The time-interval of 30 minutes was chosen because of the design of the study, in which it was not possible to obtain fasting samples from all pregnant women. At least every 3 hours, blood samples were transported to a dedicated laboratory facility of the regional laboratory in Rotterdam, the Netherlands (Star-MDC), for further processing and storage.[55] Glucose (mmol/l) was measured with the c702 module on a Cobas 8000 analyser. Insulin (pmol/l) was measured with electrochemiluminescence immunoassay on a Cobas e411 analyser. Quality control samples demonstrated intra- and inter-assay CVs of 0.9% and 1.2% for glucose concentrations and of 1.3% and 2.5% for insulin concentrations, respectively.

Information on pre-existing diabetes was obtained from self-reported questionnaires and on gestational diabetes from medical records after delivery. Gestational diabetes was diagnosed by a community midwife or an obstetrician according to Dutch midwifery and obstetric guidelines at the time of inclusion into the study, using the following criteria: either a random glucose concentration > 11.0 mmol/l, a fasting glucose ≥ 7.0 mmol/l or a fasting glucose between 6.1 and 6.9 mmol/l with a subsequent abnormal glucose tolerance test.[56]

DNA methylation

DNA was extracted from cord blood using the salting-out method. Five hundred nanograms of DNA per sample underwent bisulfite conversion using the EZ-96 DNA Methylation kit (Shallow) (Zymo Research Corporation, Irvine, CA, USA). Samples were plated randomly onto 96-well plates. Samples were processed with the Illumina Infinium HumanMethylation450 (450k) BeadChip (Illumina Inc., San Diego, CA, USA). Quality control of analyzed samples was performed using standardized criteria. Quality control and normalization of the array data was performed according to the Control Probe Adjustment and reduction of global CORrelation (CPACOR) workflow using R.[57 58] Probes that had a detection *p* value ≥ 1E-16 were set to missing per array. Next, the intensity values were quantile normalized for each of the six probe type categories separately: type II red/green, type I methylated red/green and type I unmethylated red/green. Beta values were calculated as proportion of methylated intensity value to the sum of (methylated and unmethylated intensities plus 100). Arrays with observed technical problems such as failed bisulfite conversion, hybridization or extension, as well as arrays with a sex mismatch were removed from subsequent analyses. Additionally, only arrays with a call rate > 95% per sample were processed further. Probes on the X and Y chromosomes were excluded from the analyses. Outlying methylation beta values were excluded using the following method: values < (25th percentile – 3*interquartile range (3IQR)) and values > (75th percentile + 3IQR) were removed.[59] For each analysis, we excluded plates with fewer than 3 samples because of convergence issues. This did not lead to exclusions in the analyses of the full group, but led to exclusion of 4 and 14 participants, in the normal weight and overweight/obese stratum, respectively. For all CpGs and differentially methylated regions, the official gene name of the nearest gene was noted using

Illumina's annotation information and we enhanced the annotation provided by Illumina with the UCSC Genome Browser build hg19 using the CpG location.[60 61]

Covariates

Information on maternal age, pre-pregnancy weight, educational level and parity was obtained from questionnaires at enrolment.[62] Maternal smoking during pregnancy was assessed by questionnaires in pregnancy. We measured maternal height at enrolment without shoes and heavy clothing. Pre-pregnancy BMI was calculated (self-reported pre-pregnancy weight in kilograms divided by height measured at enrolment in meters, squared). Information on gestational age at birth, child sex and birth weight was obtained from medical records. To adjust for batch effects, plate number was included as a covariate in the analyses. We estimated leukocyte subtypes using a cord blood-specific reference.[63] This method estimates the relative proportions of six white blood cell subtypes (CD4+ T-lymphocytes, CD8+ T-lymphocytes, natural killer cells, B-lymphocytes, monocytes and granulocytes) and nucleated red blood cells.

Statistical analysis

First, non-response analysis was conducted among participants with singleton children and information available on cord blood DNA methylation, comparing participants with to those without data on maternal early-pregnancy glucose metabolism available, using Student's t-tests, Mann-Whitney tests and Chi-square tests. Second, we used robust linear regression models in an EWAS framework to assess the associations of maternal early-pregnancy glucose and insulin concentrations with single-CpG DNA methylation in cord blood.[58] Maternal early-pregnancy insulin had a skewed distribution and was natural log-transformed for the analyses. The analyses were performed in two models: first model; adjusted for gestational age at glucose/insulin measurement, maternal age at intake, educational level, parity, smoking, child sex, cell type proportions and batch, and a secondary (main) model additionally adjusted for pre-pregnancy BMI. Since maternal obesity enhances the effect of higher glucose and insulin concentrations on adverse offspring outcomes, the effect on DNA methylation could be modified by maternal BMI. Therefore, as a secondary analysis we stratified women into two strata of women with normal weight, and women with overweight or obesity and repeated the main linear regression models in these strata. Included covariates were based on previous studies and factors known to be strongly associated with DNA methylation.[22 64-66] Multiple testing was accounted for using Bonferroni correction, with CpGs with a p value < 1.0×10^{-7} considered significant. Additionally, we planned *a priori* to also report results using FDR correction for multiple testing, using the method by Benjamini and Hochberg.[67] Third, we identified differentially methylated regions using the dmrff package (https://github.com/perishky/dmrff), which identifies differentially methylated regions by combining EWAS summary statistics from nearby CpGs.[26] Significant differentially methylated regions were defined as regions spanning a set of CpG sites with at most 500 bp between consecutive sites with nominal EWAS p values <0.05 and effect estimates with the same direction. Fourth, we

examined whether associations of any CpGs identified in cord blood persisted in peripheral blood of 10-year-old children, using the main model additionally adjusted for child age at measurement. Missing covariate data were multiple-imputed using the Markov chain Monte Carlo method. All analyses were performed using R version 3.4.3.[58]

Associations of identified CpGs with offspring health outcomes

Exploratory analyses were performed in the relevant strata of maternal BMI to examine associations of identified CpGs with offspring birth weight and childhood glucose concentrations measured at age 10 years. We chose these outcomes, because we have previously found them to be associated with maternal early-pregnancy glucose concentrations.[9 13] We ran linear regression models using gestational age and sex adjusted birth weight standard deviation scores (SDS) and childhood glucose concentrations as outcomes. Birth weight SDS were calculated based on the Niklasson reference charts, using Growth Analyzer (version 3.5; Dutch Growth Research Foundation, Rotterdam, the Netherlands).[68] Models were adjusted for maternal age, educational level, parity and smoking, as well as for plate number and the seven cell types from the cord blood reference.[63] Childhood glucose models were additionally adjusted for child sex and age at glucose measurement.

Look-up of previously identified CpGs

We performed a look-up in our maternal early-pregnancy glucose and insulin results of previously described maternal glucose metabolism-associated CpGs and DMRs and of previously described adult type 2 diabetes-associated CpGs in adults. The PubMed search terms are described in Note S1. We took those studies into account that: 1) included more than 50 participants in total, 2) measured DNA methylation in cord blood or peripheral blood, 3) were epigenome-wide studies or candidate-gene studies, and 4) reported *p* values for single CpGs or differential methylated regions. Significance was determined based on a Bonferroni corrected p value < 0.05/number of tested CpGs per reference study. We also evaluated enrichment of these CpGs among CpGs with a p value < 0.05 in our EWAS results using a hypergeometric test.

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SUPPLEMENTARY MATERIAL

Further detailed online resources can be found in the published article online: https://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-020-00924-3#Sec18

2.4 Maternal early-pregnancy glucose concentrations and liver fat among school age children

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ABSTRACT

Background: Gestational diabetes appears to be associated with offspring non-alcoholic fatty liver disease. We hypothesized that maternal glucose concentrations across the full range may have persistent effects on offspring liver fat accumulation.

Methods: In a multi-ethnic population-based prospective cohort study among 2,168 women and their offspring, maternal early-pregnancy glucose concentrations were measured at a median of 13.1 weeks' gestation (95% range 9.6-17.2 weeks). Liver fat fraction was measured at 10 years by magnetic resonance imaging. Non-alcoholic fatty liver disease was defined as liver fat fraction ≥5.0%. We performed analyses among all mothers with different ethnic backgrounds and those of European ancestry only.

Results: The multi-ethnic group had a median maternal early-pregnancy glucose concentration of 4.3 mmol/l (IQR 3.9-4.9) and a 2.8% (n = 60) prevalence of non-alcoholic fatty liver disease. The models adjusted for child age and sex only showed that in the multi-ethnic group higher maternal early-pregnancy glucose concentrations were associated with higher liver fat accumulation and higher odds of non-alcoholic fatty liver disease, but these associations attenuated into non-significance after adjustment for potential confounders. Among mothers of European ancestry only, maternal early-pregnancy glucose concentrations were associated with increased odds of non-alcoholic fatty liver disease (odds ratio 1.93 (95% CI: 1.30; 2.86 after adjustment for confounders) per 1 mmol/l increase in maternal early-pregnancy glucose concentration). This association was not explained by maternal pre-pregnancy and childhood BMI, organ fat and metabolic markers.

Conclusions: In this study, maternal early-pregnancy glucose concentrations were among mothers of European ancestry associated with offspring non-alcoholic fatty liver disease. The associations of higher maternal early-pregnancy glucose concentrations with offspring non-alcoholic fatty liver disease may differ between ethnic groups.

INTRODUCTION

Pre-existing diabetes and gestational diabetes are complicating up to 25% of pregnancies.[1-3] Recent studies suggest that gestational diabetes leads to impaired offspring cardiovascular and metabolic health in childhood and adulthood.[4-7] The observed associations seem not to be restricted to the clinical diagnosis of gestational diabetes but are also present across the full range of maternal glucose concentrations.[8-10] Previous studies suggest that gestational diabetes is also associated with offspring markers of liver pathology.[11-16] Results from animal studies suggest that offspring of maternal pregnancy hyperglycemia are predisposed to develop liver steatosis.[13-16] In humans, a case-control study among 25 mothers showed that intrahepatocellular lipid content, as measured by magnetic resonance spectroscopy, was increased in neonates of mothers with both obesity and gestational diabetes compared to neonates of mothers with both normal weight and without gestational diabetes.[12] Another study among 1,215 mother-child pairs reported that maternal pregnancy diabetes or glycosuria was associated with an increased risk for ultrasound-diagnosed non-alcoholic fatty liver disease at 17.8 years of age, independent of maternal pre-pregnancy BMI.[11] We have previously shown that maternal early-pregnancy glucose metabolism is associated with childhood glucose metabolism, but not with other childhood cardio-metabolic outcomes after adjustment for maternal pre-pregnancy BMI.[9] Also liver fat accumulation is related to risk factors for cardio-metabolic disease, independent of total body fat.[17 18] We hypothesized that higher maternal glucose concentrations across the full range in early pregnancy are associated with liver fat accumulation in the offspring and that these associations may differ between ethnic groups.[40] Such associations may predispose individuals to liver and cardio-metabolic disease in later life.

We assessed the associations of maternal early-pregnancy glucose concentrations with offspring liver fat accumulation and non-alcoholic fatty liver disease with magnetic resonance imaging (MRI) at 10 years of age in a multi-ethnic population-based prospective cohort among 2,168 mothers and their children. Because both glucose concentrations, liver fat and the associations between them may differ between ethnic groups,[40] we performed analyses in the full multi-ethnic group and in the group of European ancestry only.

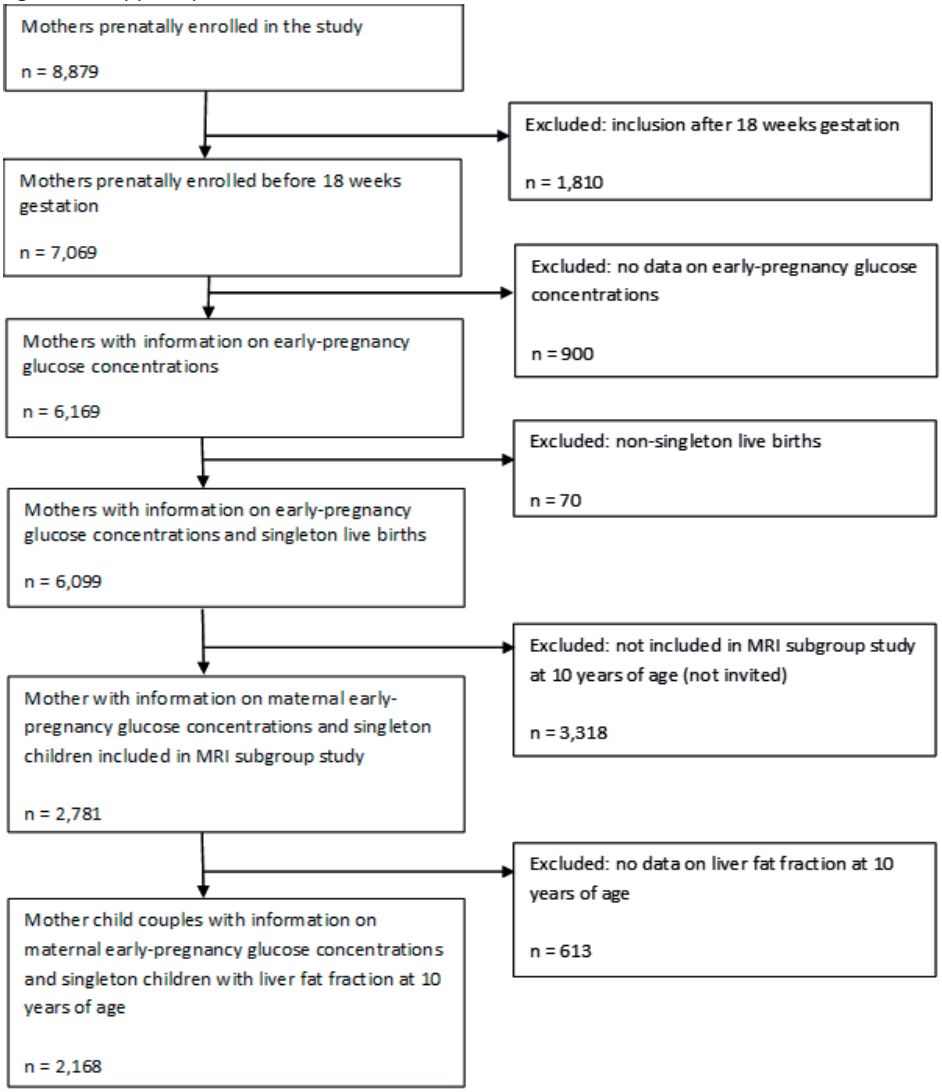
METHODS

Study population

This study was embedded in the Generation R Study. This is a multi-ethnic population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[19] The study has been approved by the Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained for all participants.[20] All pregnant women were enrolled between 2001 and 2005. The enrollment procedure

has been described in detail previously.[21] In total, 8,879 women were enrolled during pregnancy, of whom 6,099 were enrolled in early pregnancy, had measurements of glucose concentrations available and had singleton pregnancies. MRI-based liver fat measurements at 10 years of age were available in a subgroup of 2,168 of their children (**Figure 1**). None of these children had a history of jaundice, medication use, alcohol use, smoking, or drug use, based on information from questionnaires at 10 years of age. Missing measurements were mainly due to whether or not the child attended the MRI subgroup study at 10 years of age, lost to follow-up, no data on liver fat or MRI artifacts.[20]

Figure 1. Study participants flowchart



Maternal early-pregnancy glucose and insulin concentrations

Non-fasting blood samples were collected once in early pregnancy at 13.1 median weeks' gestation (95% range, 9.6-17.2), as previously described.[8 22] Briefly, venous blood samples were collected from pregnant women. Although samples were minimally 30 minutes fasting, we did not have information on the exact fasting duration and consider all samples random. Glucose concentration (mmol/l) is an enzymatic quantity and was measured with c702 module on the Cobas 8000 analyzer (Roche, Almere, the Netherlands). Insulin concentration (pmol/l) was measured with electrochemiluminescence immunoassay on the Cobas e411 analyzer (Roche). Quality control samples demonstrated intra- and inter-assay CVs of 0.9% and 1.2% for glucose concentrations and of 1.3% and 2.5% for insulin concentrations, respectively. Information on pre-existing diabetes was obtained from self-reported questionnaires and on gestational diabetes from medical records after delivery.[20] Gestational diabetes was diagnosed by a community midwife or an obstetrician according to Dutch midwifery and obstetric guidelines.[20 23]

Liver fat at 10 years

We measured liver fat using a 3.0 Tesla MRI scanner (Discovery MR750w, GE Healthcare, Milwaukee, Wisconsin, United States) as described previously.[20 24-26] A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique (IDEAL IQ) for generating a precise liver fat fraction image.[27] The IDEAL IQ scan is based on a carefully tuned 6-echo echo planar imaging acquisition. The obtained fat fraction maps were analyzed by the Precision Image Analysis (PIA) (Kirkland, Washington, United States) using the sliceOmatic (TomoVision, Magog, Canada) software package. All extraneous structures and any image artifacts were removed manually.[28] Liver fat fraction was determined by taking four samples of at least 4cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat estimation. Liver fat measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[29 30] Non-alcoholic fatty liver disease was defined as liver fat ≥5.0%.[24 30 31] We studied liver fat fraction across the full range and dichotomized in low, <5.0%, and high, ≥5.0%, based on the clinical cutoff for non-alcoholic fatty liver disease.[32] As a sensitivity analysis, we dichotomized liver fat into low, ≤2.0%, and high, >2.0%, based on the median liver fat fraction in our population and on previous work from our group describing that liver fat accumulation above 2.0% is already associated with an increased cardio-metabolic risk profile in children.[17]

Covariates

Information was obtained by questionnaires on maternal age, parity, ethnicity, education level, smoking, alcohol consumption, folic acid supplement use, pre-pregnancy weight, and total daily energy intake during pregnancy.[19] We categorized ethnicity into European (Dutch n = 1,258 (58.8%) and other European n = 168 (7.7%)) versus Non-European (Cape Verdean n = 98 (4.6%), other African n = 21 (1.0%), Dutch Antillean n = 42 (2.0%), Surinamese n = 172 (8.0%), American

n = 43 (2.0%), Asian n = 48 (2.1%), Indonesian n = 75 (3.5%), Turkish n = 117 (5.5%), Moroccan n = 95 (4.4%), Oceanian n = 4 (0.2%)). We measured maternal height without shoes at intake and calculated pre-pregnancy BMI. Non-fasting venous blood samples were obtained in early pregnancy, total cholesterol (mmol/l), triglyceride (mmol/l) and high-density lipoprotein (HDL) cholesterol (mmol/l) concentrations were analyzed. Low-density lipoprotein (LDL) cholesterol (mmol/l) concentrations were calculated using the Friedewald equation. Maternal dyslipidemia was defined as having three or more out of the following four adverse factors: total cholesterol above the seventy-fifth percentile; triglycerides above the seventy-fifth percentile; HDL cholesterol below the twenty-fifth percentile, and LDL cholesterol above the seventy-fifth percentile of our study population.Information on child gestational age at birth, sex, and birth weight was obtained from medical records.[20] We obtained information on breastfeeding in infancy by questionnaire.[20] Non-fasting blood samples were collected to determine concentrations of insulin, total cholesterol, triglycerides, HDL cholesterol and LDL cholesterol at 6 years of age. At the 10 years of age follow-up visit, we measured childhood height and weight, both without shoes and heavy clothing, and calculated BMI and sex- and age-adjusted childhood BMI standard deviation score (SDS) based on Dutch reference growth charts (Growth Analyzer 4.0, Dutch Growth Research Foundation).[33] Visceral fat mass was obtained by MRI scans, as described previously.[20] Non-fasting venous blood samples were obtained and we measured glucose and insulin concentrations. Physical activity and screen time were assessed with questionnaires at 10 years of age.[34]

Statistical analysis

We conducted a non-response analysis to compare characteristics of mothers and children with and without liver MRI scan measurements with Student's t-tests, Mann-Whitney tests and Chi-square tests. Second, we used linear and logistic regression models to assess associations of maternal early-pregnancy glucose concentrations across the full range with liver fat accumulation and with the odds of non-alcoholic fatty liver disease. Potential covariates were first selected based on previous literature, their association with both the exposure and the outcome or a change in the effect estimates of >10% in the basic model as shown with the Directed Acyclic Graph, subsequently we performed a backward model selection analysis (**Figure S1**).[11 35] The basic model was adjusted for gestational age at assessment, child sex and age at follow-up measurements. The main confounder model was additionally adjusted for maternal ethnicity, education, smoking, alcohol consumption and folic acid supplement use during pregnancy, and child physical activity and screen time. We further adjusted any significant association in the main model for maternal pre-pregnancy BMI, dyslipidemia, and child metabolic markers at 6 years, BMI at 10 years, visceral fat mass at 10 years, and glucose concentrations at 10 years to explore whether any significant association was explained by these covariates.[3 9 36]

Because both glucose concentrations, liver fat and the associations between them may differ between ethnic groups, we performed analyses in the full multi-ethnic group and in the European ancestry only groups (**Table S1**).[37] Unfortunately, the other ethnic subgroups were too small to

perform ethnic specific analyses. As sensitivity analysis, first, we repeated all analyses using maternal early-pregnancy insulin concentrations as exposure as another marker of maternal glucose metabolism in early pregnancy. Maternal early-pregnancy insulin concentrations were natural log-transformed before the SDS construction due to the skewed distribution. Second, to assess the associations of maternal early-pregnancy glucose concentrations with a potentially clinically relevant liver fat cutoff, we repeated the analyses using liver fat dichotomized in low, ≤2.0%, and high, >2.0%, liver fat. Third, we explored whether our observed associations were affected by specific subgroups in our study population. We first excluded women with the pre-existing diabetes or gestational diabetes (total n = 28) to focus specifically on a non-diabetic population. Second, we excluded women with glucose concentrations sampled at >14 weeks' gestation to assess the associations of first trimester maternal glucose concentrations with liver fat accumulation at school age (n = 702); The distribution of liver fat was skewed and natural log-transformed values were used in all linear regression analyses. Missing data in the covariates were multiple-imputed using Markov chain Monte Carlo approach. Five imputed datasets were created and analyzed together. All statistical analyses were performed using the Statistical Product and Service Solutions (SPSS) Statistics version 25.0 for Windows (IBM, Chicago, Illinois, United States).

RESULTS

Subject characteristics

The median maternal early-pregnancy glucose concentration was 4.3 mmol/l (95% range, 3.0-6.4, interquartile range (IQR) 3.9-4.9). The median liver fat fraction was 2.0% (95% range, 1.2-5.2%, IQR 1.7-2.5) and the prevalence of non-alcoholic fatty liver disease was 2.8% (n = 60) in children at 10 years of age (**Table 1**). Mothers of children with non-alcoholic fatty liver disease had a higher BMI, were less often from European ancestry, had slightly higher level of educational attainment and those children had higher BMI and visceral fat mass compared to children without non-alcoholic fatty liver disease in the full multi-ethnic group (**Table 1**). In the European ancestry only group, mothers of children with non-alcoholic fatty liver disease had higher glucose concentrations in early pregnancy, and those children were less active compared to children without non-alcoholic fatty liver disease (**Table S2**). Mothers of the European ancestry only group had similar glucose concentrations and had slightly higher level of educational attainment compared to the full multi-ethnic group (**Table S3**). The correlation coefficient for the correlation between maternal early-pregnancy glucose and maternal pre-pregnancy BMI was 0.15 (**Table S4**). Non-response analyses showed that participants without outcome measurements had mothers with a slightly lower level of educational attainment (**Table S5**).

Table 1. Subject characteristics - full multi-ethnic group

	Total group n = 2,168	NAFLD no n = 2,108	NAFLD yes n = 60	*p* value
Maternal characteristics				
Age at enrollment, years	30.8 ± 4.6	30.9 ± 4.6	30.1 ± 6.0	0.36
Gestational age at glucose/insulin measurement, weeks	13.1 (9.6, 17.2)	13.1 (9.6, 17.2)	13.1 (11.2, 17.9)	0.20
Pre-pregnancy body mass index, kg/m^2	22.5 (18.1, 35.2)	22.4 (18.1, 34.9)	24.9 (18.3, 42.8)	<0.01
Parity, nulliparous	1,317 (61.0)	1,284 (61.2)	33 (55.0)	0.33
Ethnicity, European	1,426 (66.6)	1,401 (67.3)	25 (42.4)	<0.01
Education, higher	1,115 (53.6)	1,099 (54.2)	16 (29.1)	<0.01
Smoking during pregnancy, continued	334 (18.7)	329 (19.0)	5 (10.4)	0.14
Alcohol consumption, during pregnancy	622 (37.2)	609 (37.5)	13 (28.3)	0.20
Folic acid supplement use, yes	1,024 (71.4)	994 (71.5)	30 (68.2)	0.64
Daily energy intake, kcal/day	2,060 ± 572	2,061 ± 571	2,053 ± 610	0.93
Dyslipidemia	233 (10.7)	226 (10.7)	7 (11.7)	0.82
Glucose, mmol/l	4.4 ± 0.8	4.4 (± 0.8)	4.6 ± 1.0	0.12
Insulin, pmol/l	113.1 (19.8, 669.6)	112.8 (19.7, 673.2)	171.0 (22.8, 672.6)	0.09
Pre-existing Diabetes	6 (0.3)	5 (0.3)	1 (1.9)	0.04
Gestational Diabetes	22 (1.1)	22 (1.1)	0 (0)	0.43
Child characteristics				
Sex, female	1,113 (51.3)	1,082 (51.3)	31 (51.7)	0.96
Birth weight, grams	3,447 ± 548	3,475 ± 549	3,347 ± 535	0.15
Gestational age at birth, weeks	40.3 (36.0, 42.4)	40.3 (36.0, 42.4)	39.9 (34.5, 42.8)	0.08
Ever breastfed, yes	1,761 (93.0)	1,721 (93.1)	40 (87.0)	0.11
Insulin at 6 years, pmol/l	113.5 (18.1, 409.9)	113.1 (17.7, 409.8)	130.7 (34.1, 412.5)	0.42
Total cholesterol at 6 years, mmol/l	4.2 ± 0.6	4.2 ± 0.6	4.4 ± 0.7	0.09
LDL cholesterol at 6 years, mmol/l	2.4 ± 0.6	2.4 ± 0.6	2.4 ± 0.6	0.61
HDL cholesterol at 6 years, mmol/l	1.3 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	0.49
Triglycerides at 6 years, mmol/l	1.0 (0.4, 2.4)	1.0 (0.4, 2.4)	1.1 (0.4, 3.1)	0.10
Age 10 years at outcome follow-up measurements, years	9.8 ± 0.4	9.8 ± 0.3	9.9 ± 0.5	0.34
Playing sports at 10 years, hours/day	1.3 (0.3, 3.5)	1.3 (0.3, 3.5)	1.1 (0.1, 3.5)	0.15
Screen time at 10 years, ≥2 hours/day	852 (51.5)	824 (51.2)	28 (62.2)	0.15
Body mass index at 10 years, kg/m^2	16.9 (14.0, 24.3)	16.9 (14.0, 23.9)	21.9 (15.5, 31.0)	<0.01
Visceral fat mass at 10 years, grams	369.0 (164, 1,005)	364.1 (163, 948)	804.4 (242, 1,849)	<0.01
Glucose at 10 years, mmol/l	5.2 ± 0.9	5.3 ± 0.9	5.1 ± 0.7	0.34
Insulin at 10 years, pmol/l	180.8 (37.1, 625.7)	180,0 (36.8, 610,5)	208.8 (41.7, 830.5)	0.09
Liver fat fraction at 10 years, %	2.0 (1.2, 5.2)	2.0 (1.2, 4.0)	6.5 (5.1, 20.4)	<0.01
Liver fat dichotomized, high ≥2.0%	1,086 (50.1)	1,026 (48.7)	60 (100)	<0.01
Non-alcoholic fatty liver disease	60 (2.8)	-	-	-

Values are observed and represent numbers (valid %), means ± SD, or medians (95% range).

Maternal early-pregnancy glucose concentrations and childhood liver fat

In the full group, results from the basic models showed that higher maternal early-pregnancy glucose concentrations were associated with higher liver fat accumulation (difference 0.04 (95% Confidence Interval (CI): 0.02; 0.07) SDS per 1 mmol/l increase in maternal early-pregnancy glucose concentration) and with increased odds of non-alcoholic fatty liver disease (odds ratio (OR) 1.27 (95% CI: 1.10; 1.46) per 1 mmol/l increase in maternal early-pregnancy glucose concentration) (**Table 2**). These associations attenuated into non-significance in the main confounder model. In mother-child pairs of European ancestry only, higher maternal early-pregnancy glucose concentrations were associated with increased odds of non-alcoholic fatty liver disease (OR 1.93 (95% CI: 1.30; 2.86) per 1 mmol/l increase in maternal early-pregnancy glucose concentration in the main confounder model). These associations were not explained by maternal pre-pregnancy BMI, and dyslipidemia. Also, childhood metabolic markers at 6 years, BMI and visceral fat mass child glucose concentrations at 10 years of age, did not explain the observed associations (**Table 3**). Maternal glucose concentrations were not associated with liver fat accumulation among mother-child pairs of European ancestry only (**Table 3**).

Table 2. Associations between maternal early-pregnancy glucose concentrations with childhood liver fat fraction and non-alcoholic fatty liver disease in the full multi-ethnic group

			Liver Fat at School Age n = 2,168	
Maternal early-pregnancy glucose mmol/l	**Difference liver fat fraction SDS (95% Confidence Interval)**	***p* value**	**Odds ratio NAFLD yes/no (95% Confidence Interval)**	***p* value**
Basic model	0.04 (0.01; 0.07)	0.12	1.26 (1.09; 1.45)	0.11
Main confounder model	0.03 (-0.02; 0.08)	0.27	1.20 (0.90; 1.59)	0.21
Maternal body mass index model	0.01 (-0.04; 0.05)	0.84	1.18 (0.87, 1.59)	0.30
Maternal dyslipidemia model	0.03 (-0.02; 0.08)	0.29	1.25 (0.93, 1.67)	0.14
Child metabolic markers at 6 years model	0.03 (-0.02, 0.08)	0.27	1.24 (0.93, 1.66)	0.15
Child body mass index at 10 years model	0.01 (-0.04, 0.06)	0.68	1.13 (0.84, 1.53)	0.42
Child visceral fat mass at 10 years model	0.02 (-0.02, 0.06)	0.47	1.30 (0.95, 1.79)	0.11
Child glucose concentrations at 10 years model	0.03 (-0.02, 0.08)	0.30	1.26 (0.94; 1.69)	0.12

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per maternal early-pregnancy glucose concentrations in mmol/l. Values are ORs (95% CIs) that reflect the risk of NAFLD per maternal early-pregnancy glucose concentrations in mmol/l. Basic model: adjusted for child sex and age 10 years at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal ethnicity, education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; SDS, standard deviation score.

Table 3. Associations between maternal early-pregnancy glucose concentrations with childhood liver fat fraction and non-alcoholic fatty liver disease in group of European ancestry

	Liver Fat at School Age n = 1,426			
Maternal early-pregnancy glucose mmol/l	**Difference liver fat fraction SDS (95% Confidence Interval)**	***p* value**	**Odds ratio NAFLD yes/no (95% Confidence Interval)**	***p* value**
Basic model	0.03 (-0.03; 0.08)	0.38	1.93 (1.31; 2.84)	<0.01
Main confounder model	0.02 (-0.04; 0.08)	0.49	1.95 (1.32; 2.88)	<0.01
Maternal body mass index model	0.00 (-0.06; 0.06)	0.90	1.86 (1.24; 2.78)	<0.01
Maternal dyslipidemia model	0.02 (-0.04; 0.08)	0.49	1.92 (1.30; 2.86)	<0.01
Child metabolic markers at 6 years model	0.02 (-0.04; 0.08)	0.50	1.96 (1.31; 2.95)	<0.01
Child body mass index model	0.01 (-0.05; 0.06)	0.78	1.66 (1.04; 2.64)	0.03
Child visceral fat mass at 10 years model	0.00 (-0.05; 0.06)	0.89	1.82 (1.19; 2.79)	<0.01
Child glucose concentrations model	0.02 (-0.04; 0.08)	0.50	1.95 (1.32; 2.88)	<0.01

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per maternal early-pregnancy glucose concentrations in mmol/l in mother-child pairs of European ancestry only. Values are ORs (95% CIs) that reflect the risk of NAFLD per maternal early-pregnancy glucose concentrations in mmol/l. Basic model: adjusted for child sex and age at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; SDS, standard deviation score.

Sensitivity analyses

When we repeated the main analyses by using insulin concentrations we observed largely the same patterns and tendencies as for glucose concentrations (**Table S6**). When we repeated the analyses with childhood liver fat accumulation categorized into ≤2.0% versus >2% we observed odds in similar direction but smaller as for maternal early-pregnancy glucose concentrations with non-alcoholic fatty liver disease (**Table S7**). No differences in findings were present when mothers with pre-existing diabetes or gestational diabetes or mothers with glucose measurements after 14 weeks gestation were excluded from the analyses in both the full multi-ethnic group and the European ancestry only group (**Table S8 and Table S9**).

DISCUSSION

In this prospective cohort study, we observed that maternal early-pregnancy glucose concentrations were only among mothers of European ancestry associated with offspring non-alcoholic fatty liver disease. These associations were not explained by maternal pre-pregnancy BMI, and dyslipidemia. Also, childhood metabolic markers at 6 years, or BMI, visceral fat mass and glucose

concentrations at 10 years, did not explain the observed associations. No associations were observed in the full group.

Interpretation of main findings

Non-alcoholic fatty liver disease ranges from liver steatosis, to fibrosis, cirrhosis, and eventually end-stage liver disease.[38] In adults, non-alcoholic fatty liver disease is associated with type 2 diabetes, cardiovascular disease, dyslipidemia, and metabolic syndrome.[17 32 38 39] We previously reported that elevated liver fat is associated with an adverse cardio-metabolic risk profile in children.[17] Gestational diabetes and hyperglycemia diagnosed in second half of pregnancy are associated with an altered offspring body fat composition, cardiovascular and metabolic health.[4-6 36 40] Studies in women with gestational diabetes showed an association with offspring markers of liver pathology.[11 12] These findings, together with observations from animal studies, suggest that maternal gestational hyperglycemia might be related to offspring liver fat development.[13-15] More specifically, early pregnancy might be a critical period for effects of intrauterine maternal glucose exposure on liver health, because the embryonic development of the metabolic systems and of the placenta already occurs in the first weeks after conception.[41] Therefore, we hypothesized that higher maternal glucose concentrations across the full range in early pregnancy are associated with liver fat accumulation in offspring.

In this study, in children 10 years of age we did not observe that maternal early-pregnancy glucose concentrations were associated with childhood liver fat accumulation and with risk of non-alcoholic fatty liver disease. Because both glucose concentrations, liver fat and the associations between ethnic subgroups strongly differ, we performed analyses in the full multi-ethnic group and in the group of European ancestry only. In the European ancestry only group, the largest ethnic subgroup, we observed an almost 2-fold increase in odds of non-alcoholic fatty liver disease, independent of maternal pre-pregnancy BMI and dyslipidemia, childhood metabolic markers at 6 years, or BMI, visceral fat mass and of glucose concentrations at 10 years. This may suggest that there is also an intrauterine effect of maternal early-pregnancy glucose concentrations on childhood liver fat accumulation through other pathways than through maternal pre-pregnancy or child BMI, or child glucose concentrations in this subgroup. Due to smaller sample sizes for the other individual ethnic subgroups, we could not test these associations in each ethnic subgroup separately. We did not observe associations of maternal early-pregnancy glucose concentrations with liver fat across the full range in the total study sample and in the largest ethnic subgroup. The lack of association in the total group might be due to a modifying effect of ethnicity with per ethnic subgroup opposite directions of effect estimates. The lack of association in the largest ethnic subgroup could be due to the moderate sample size, together with the relatively small variability in liver fat accumulation in this population of children. Further studies are needed to explore these associations among high-risk populations and evaluating liver fat accumulation in older offspring.

The underlying pathogenic mechanisms behind the abnormal metabolic risk profile in offspring of mothers with gestational diabetes are largely unknown. Animal studies have suggested that *in utero* exposure to high glucose concentrations may induce ectopic fat storage.[13-15] For instance, mouse models of maternal insulin resistance have shown impairment of gene expression involved in fatty acid oxidative capacity and lipogenesis in offspring liver.[15 16 42] The accelerated hepatic fat storage in mouse offspring appear to persist into adulthood, suggesting a lasting impact of the maternal intrauterine environment on pathways of hepatic lipid metabolism.[16 42] Another speculation is that the higher insulin resistance in the offspring of mothers with gestational diabetes is associated with higher liver fat accumulation, although the direction of effect is not yet defined.[8 17] In mothers with gestational diabetes a higher risk for non-alcoholic liver fat disease after pregnancy is observed, supporting the hypothesis of a link between insulin resistance and liver fat accumulation.[43]

Given the high prevalence of both obesity and impaired glucose metabolism in preconceptional women, these may represent pivotal targets if proven causal for public health in preventing offspring obesity and metabolic disease, like non-alcoholic fatty liver disease.[1-3] Our findings emphasize the importance of developing preventive strategies before and in early pregnancy to improve liver and metabolic health outcomes in children. Further studies should characterize the maternal metabolic environment in early pregnancy to provide insights into the causality of early-life determinants of non-alcoholic fatty liver disease taking into account ethnic background.

Methodological considerations

The population-based prospective longitudinal design of this study together with the large sample size with data collection from early pregnancy onwards and the availability of MRI-measured liver fat fraction at 10 years of age are major strengths of this study. The children who underwent MRI measurements at 10 years of age constitute a subgroup of the full Generation R Study population. This may have led to biased effect estimates if associations were different between those included and not included in the analyses, which seems unlikely since the non-response analysis showed hardly any differences. The prevalence of gestational diabetes in our sample was lower than expected (1.1% versus 2-5% in the general Dutch population[44]) likely due to the use of medical records after delivery to obtain information on the diagnosis of gestational diabetes and to lack of universal screening, which may have led to misclassification. The small number of children with non-alcoholic fatty liver disease is likely explained by the fact that we measured liver fat in a relatively healthy study population at a young age, which could have limited our statistical power to detect significant associations and may affect the generalizability of our findings. The main analyses focused on non-alcoholic fatty liver disease were based on only 60 in the full and 25 children in the Europeans ancestry only group with MRI-diagnosed non-alcoholic fatty liver disease. Therefore, these results need to be interpreted carefully and need further replication. The blood samples in the study were random maternal glucose concentrations obtained once during pregnancy at non-fixed times throughout the day, which may have led to misclassification

of glucose concentrations. However, previous studies showed that random maternal glucose concentrations in pregnancy are related to increased risk of adverse outcomes in mothers and children.[8 9 22 45] These associations were in similar directions as those for maternal fasting and postprandial glucose concentrations.[35 46] Information on many covariates was available, yet some residual confounding may have influenced the results.

CONCLUSIONS

Maternal early-pregnancy glucose concentrations were among mothers of European ancestry associated with offspring non-alcoholic fatty liver disease. These associations were independent of maternal pre-pregnancy and childhood BMI, organ fat and metabolic markers. No associations were observed in the full multi-ethnic group. Further studies are needed to explore the causality of the observed associations. Optimizing maternal pre-pregnancy BMI and glucose concentrations could be starting points for prevention strategies to improve liver health among future generations.

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SUPPLEMENTARY MATERIAL

Table S1. Differences between groups of various ethnic backgrounds

Maternal ethnicity	Glucose, mmol/l	Liver fat fraction, %	Non-alcoholic fatty liver disease
Total group (n = 2,141)	4.44 ± 0.8	2.00 (1.2, 5.2)	60 (2.8)
European			
Dutch (n = 1,258)	4.42 ± 0.8	1.97 (1.2, 4.5)	23 (1.8)
Other European (n = 168)	4.29 ± 0.7	2.07 (1.2, 4.7)	2 (1.2)
Non-European			
Cape Verdean (n = 98)	4.51 ± 0.9	2.22 (1.2, 8.4)	6 (6.1)
Other African (n = 21)	4.40 ± 0.7	2.11 (1.3, 4.8)	0 (0.0)
Dutch Antillean (n = 42)	4.46 ± 1.1	1.81 (1.3, 10.9)	1 (2.4)
Surinamese (n = 172)	4.48 ± 0.8	2.00 (1.2, 5.5)	6 (3.5)
American (n = 43)	4.30 ± 0.7	2.06 (1.3, 5.6)	1 (2.3)
Asian (n = 48)	4.85 ± 1.1	1.87 (1.3, 4.4)	0 (0.0)
Indonesian (n = 75)	4.44 ± 0.9	1.99 (1.2, 7.0)	4 (5.3)
Turkish (n = 117)	4.51 ± 0.8	2.45 (1.2, 13.8)	13 (11.1)
Moroccan (n = 95)	4.58 ± 0.9	2.03 (1.3, 8.9)	3 (3.2)
Oceanian (n = 4)	4.28 ± 0.8	1.78 (1.5, 3.2)	0 (0.0)

Values are observed and represent numbers (valid %), means ± SD, or medians (95% range).

Table S2. Subject characteristics - European only group

	Europeans only n = 1,426	NAFLD no n = 1,401	NAFLD yes n = 25	*p* value
Maternal characteristics				
Age at enrollment, years	31.7 ± 4.0	31.7 ± 4.0	31.1 ± 5.0	0.49
Gestational age at glucose/insulin measurement, weeks	12.8 (9.6, 17.0)	12.9 (9.6, 16.8)	12.4 (10.9, 17.0)	0.62
Prepregnancy body mass index, kg/m^2	22.2 (18.1, 34.3)	22.2 (18.1, 31.3)	24.3 (18.1, 34.2)	0.05
Parity, nulliparous	901 (63.3)	513 (36.7)	10 (40.0)	0.73
Education, higher	923 (65.4)	914 (65.9)	9 (36.0)	<0.01
Smoking during pregnancy, continued	217 (18.7)	215 (18.9)	2 (9.5)	0.28
Alcohol consumption, during pregnancy	414 (38.2)	407 (38.3)	7 (33.3)	0.64
Folic acid supplement use, yes	662 (70.6)	648 (70.4)	14 (77.8)	0.50
Daily energy intake, kcal/day	2,053 ± 587	2,055 ± 586	1,966 ± 651	0.51
Dyslipidemia	141 (9.9)	140 (10.0)	1 (4.0)	0.32
Glucose, mmol/l	4.4 ± 0.8	4.4 ± 0.8	5.0 ± 1.2	<0.01
Insulin, pmol/l	102.1 (19.2, 518.6)	102.1 (19.1, 440.7)	103.9 (19.8, 846.0)	0.15
Pre-existing Diabetes	2 (0.2)	2 (0.2)	0 (0)	0.85
Gestational Diabetes	15 (1.1)	15 (1.1)	0 (0)	0.61
Child characteristics				
Sex, female	722 (50.6)	708 (50.5)	14 (56.0)	0.59
Birth weight, grams	3,500 ± 540	3,500 ± 540	3,447 ± 521	0.62
Gestational age at birth, weeks	40.3 (36.0, 42.4)	40.3 (36.0, 42.1)	40.0 (37.0, 42.6)	0.62
Ever breastfed, yes	1,195 (92.1)	1,177 (92.0)	18 (94.7)	0.66
Insulin at 6 years, pmol/l	115.1 (18.5, 394.3)	114.1 (18.3, 394.5)	155.4 (60.3, 398.2)	0.06
Total cholesterol at 6 years, mmol/l	4.2 ± 0.6	4.2 ± 0.6	4.4 ± 0.8	0.15
LDL cholesterol at 6 years, mmol/l	2.3 ± 0.6	2.3 ± 0.6	2.5 ± 0.7	0.45
HDL cholesterol at 6 years, mmol/l	1.3 ± 0.3	1.3 ± 0.3	1.4 ± 0.3	0.34
Triglycerides at 6 years, mmol/l	1.0 (0.4, 2.3)	1.0 (0.4, 2.3)	0.9 (0.4, 2.4)	0.60
Age 10 years at outcome follow-up measurements, years	9.8 ± 0.3	9.8 ± 0.3	9.8 ± 0.3	0.82
Playing sports at 10 years, hours/day	1.4 (0.4, 3.5)	1.4 (0.4, 3.5)	1.2 (0.1, 2.5)	<0.01
Screen time at 10 years, ≥2 hours/day	552 (45.9)	538 (45.6)	14 (63.6)	0.09
Body mass index at 10 years, kg/m^2	16.6 (14.0, 22.6)	16.6 (14.0, 22.0)	21.3 (16.2, 28.6)	<0.01
Visceral fat mass at 10 years, grams	371.7 (168, 981)	369.9 (168, 920)	782,1 (301, 1,360)	<0.01
Glucose at 10 years, mmol/l	5.3 ± 1.0	5.3 ± 1.0	5.2 ± 0.8	0.76
Insulin at 10 years, pmol/l	172.1 (34.9, 577,9)	171.2 (34.8, 573.7)	212.8 (40.8, 826.0)	0.15
Liver fat fraction at 10 years, %	2.0 (1.2, 4.5)	2.0 (1.2, 4.0)	6.2 (5.1, 14.0)	<0.01
Liver fat dichotomized, high ≥2.0%	687 (48.2)	662 (47.3)	25 (100.0)	<0.01
Non-alcoholic fatty liver disease	25 (1.8)	-	-	-

Values are observed and represent numbers (valid %), means ± SD, or medians (95% range).

Table S3. Subject characteristics - full multi-ethnic group versus Europeans only group

	Total group n = 2,168	**Europeans only n = 1,426**
Maternal characteristics		
Age at enrollment, years	30.8 ± 4.6	31.7 ± 4.0
Gestational age at glucose/insulin measurement, weeks	13.1 (9.6, 17.2)	12.8 (9.6, 17.0)
Pre-pregnancy body mass index, kg/m^2	22.5 (18.1, 35.2)	22.2 (18.1, 34.3)
Parity, nulliparous	1,317 (61.0)	901 (63.3)
Ethnicity, European	1,426 (66.6)	-
Education, higher	1,115 (53.6)	923 (65.4)
Smoking during pregnancy, continued	334 (18.7)	217 (18.7)
Alcohol consumption, during pregnancy	854 (51.1)	554 (51.1)
Folic acid supplement use, yes	1,024 (71.4)	662 (70.6)
Daily energy intake, kcal/day	2,060 ± 572	2053 ± 587
Glucose, mmol/l	4.4 ± 0.8	4.4 ± 0.8
Insulin, pmol/l	113.1 (19.8, 669.6)	102.1 (19.2, 518.6)
Pre-existing Diabetes	6 (0.3)	2 (0.2)
Gestational Diabetes	22 (1.1)	15 (1.1)
Child characteristics		
Sex, female	1,113 (51.3)	722 (50.6)
Birth weight, grams	3,447 ± 548	3,500 ± 540
Gestational age at birth, weeks	40.3 (36.0, 42.4)	40.3 (36.0, 42.4)
Ever breastfed, yes	1,761 (93.0)	1,195 (92.1)
Age at outcome follow-up measurements, years	9.8 ± 0.4	9.8 ± 0.3
Playing sports, hours/day	1.3 (0.3, 3.5)	1.4 (0.4, 3.5)
Screen time, ≥2 hours/day	852 (51.5)	552 (45.9)
Body mass index, kg/m^2	16.9 (14.0, 24.3)	16.6 (14.0, 22.6)
Glucose, mmol/l	5.2 ± 0.9	5.3 ± 1.0
Insulin, pmol/l	180.8 (37.1, 625.7)	172.1 (34.9, 577,9)
Liver fat fraction, %	2.0 (1.2, 5.2)	2.0 (1.2, 4.5)
Liver fat dichotomized, high ≥2.0%	1,086 (50.1)	687 (48.2)
Non-alcoholic fatty liver disease	60 (2.8)	25 (1.8)

Values are observed and represent numbers (valid %), means ± SD, or medians (95% range). Number of missings per covariate: maternal ethnicity, n = 27 (1.2%); maternal educational level, n = 86 (4.0%); smoking during pregnancy, n = 384 (17.7%); alcohol consumption, n = 497 (22.9%); folic acid supplement use during pregnancy, n = 733 (33.8%); pre-existing diabetes n = 254 (11.7%); gestational diabetes, n = 133 (6.1%); ever breastfed, n = 274 (12.6%); screen time, n = 514 (23.7%).

Table S4. Correlation coefficients between maternal glucose and insulin concentrations, childhood liver fat fraction and non-alcoholic fatty liver disease, and body mass index of mother and child

	Maternal glucose	Maternal insulin	Maternal pre-pregnancy BMI	Child liver fat	Child NAFLD	Child BMI
Maternal glucose	1	0.53*	0.15*	0.01	0.02	0.03
Maternal insulin	0.53*	1	0.23*	0.05**	0.04	0.07*
Maternal pre-pregnancy BMI	0.15*	0.23*	1	0.18*	0.09*	0.35*
Child liver fat	0.01	0.05**	0.18*	1	0.28*	0.37*
Child NAFLD	0.02	0.04	0.09*	0.28*	1	0.19*
Child BMI	0.03	0.07*	0.35*	0.37*	0.19*	1

Values are Spearman correlation coefficients.*P value <0.01, **P value <0.05.

Table S5. Comparison of characteristics between mothers and children with and without outcome measurements

	Participants	**Non-participants**	
	n = 2,168	n = 613	***p* value**
Maternal characteristics			
Age at enrollment, years	30.8 ± 4.6	30.5 ± 5.0	0.15
Gestational age at glucose/insulin measurement, weeks	13.1 (9.6, 17.2)	13.2 (9.8, 17.6)	0.06
Pre-pregnancy body mass index, kg/m^2	22.5 (18.1, 35.2)	22.6 (18.1, 33.6)	0.83
Parity, nulliparous	1,317 (61.0)	367 (60.5)	0.80
Ethnicity, European	1,426 (66.6)	379 (62.6)	0.07
Education, higher	1,115 (53.6)	266 (46.0)	<0.01
Smoking during pregnancy, continued	334 (18.7)	92 (17.8)	0.63
Alcohol consumption, during pregnancy	854 (51.1)	226 (47.0)	0.11
Folic acid supplement use, yes	1,024 (71.4)	300 (70.8)	0.81
Daily energy intake, kcal/day	2,060 ± 572	2,015 ± 550	0.16
Glucose, mmol/l	4.4 ± 0.8	4.4 ± 0.8	0.74
Insulin, pmol/l	113.1 (19.8, 669.6)	116.7 (19.7, 575.6)	0.75
Pre-existing Diabetes	6 (0.3)	0 (0.0)	0.19
Gestational Diabetes	22 (1.1)	7 (1.2)	0.78
Child characteristics			
Sex, female	1,113 (51.3)	294 (48.0)	0.14
Birth weight, grams	3,447 ± 548	3,421 ± 530	0.29
Gestational age at birth, weeks	40.3 (36.0, 42.4)	40.3 (36.3, 42.3)	0.90
Ever breastfed, yes	1,761 (93.0)	462 (93.0)	0.99

Values are observed and represent numbers (valid %). means ± SD, or medians (95% range). Differences were tested using Student *t* tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively, and $\chi 2$ test was used for dichotomous variables. Number of missings per covariate in participants: maternal ethnicity, n = 27 (1.2%); maternal educational level, n = 86 (4.0%); smoking during pregnancy, n = 384 (17.7%); alcohol consumption, n = 497 (22.9%); folic acid supplement use during pregnancy, n = 733 (33.8%); pre-existing diabetes n = 254 (11.7%); gestational diabetes, n = 133 (6.1%); ever breastfed, n = 274 (12.6%); screen time, n = 514 (23.7%).

Table S6. Associations between maternal early-pregnancy glucose and insulin concentrations SDS with childhood liver fat fraction and non-alcoholic fatty liver disease

	Liver Fat at School Age n = 2,168			
Maternal early-pregnancy glucose SDS	**Difference liver fat fraction SDS (95% Confidence Interval)**	***p* value**	**Odds ratio NAFLD yes/no (95% Confidence Interval)**	***p* value**
Basic model	0.03 (0.01; 0.06)	0.12	1.22 (1.08; 1.37)	0.11
Main confounder model	0.02 (-0.02; 0.07)	0.27	1.17 (0.92; 1.48)	0.21
Maternal body mass index model	0.00 (-0.04; 0.05)	0.84	1.15 (0.89; 1.48)	0.30
Maternal dyslipidemia model	0.02 (-0.02; 0.07)	0.29	1.20 (0.94; 1.54)	0.14
Child metabolic markers at 6 years model	0.02 (-0.02; 0.07)	0.27	1.20 (0.94; 1.54)	0.15
Child body mass index at 10 years model	0.01 (-0.03; 0.05)	0.68	1.11 (0.86; 1.44)	0.42
Child visceral fat mass at 10 years model	0.01 (-0.02; 0.05)	0.47	1.25 (0.95; 1.64)	0.11
Child glucose concentrations at 10 years model	0.02 (-0.02; 0.07)	0.30	1.22 (0.95; 1.56)	0.12
Maternal early-pregnancy insulin SDS				
Basic model	0.06 (0.02; 0.10)	<0.01	1.23 (0.95; 1.59)	0.11
Main confounder model	0.03 (-0.01; 0.08)	0.12	1.09 (0.84; 1.41)	0.51
Maternal body mass index model	0.00 (-0.04; 0.05)	0.85	1.01 (0.78; 1.31)	0.96
Maternal dyslipidemia model	0.02 (-0.02; 0.07)	0.30	1.06 (0.81; 1.38)	0.69
Child metabolic markers at 6 years model	0.03 (-0.01; 0.08)	0.15	1.06 (0.81; 1.39)	0.65
Child body mass index at 10 years model	0.01 (-0.03; 0.05)	0.52	0.95 (0.73; 1.25)	0.73
Child visceral fat mass at 10 years model	0.02 (-0.02; 0.05)	0.45	1.07 (0.80; 1.43)	0.64
Child glucose concentrations at 10 years model	0.03 (-0.01; 0.07)	0.16	1.06 (0.81; 1.39)	0.66

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per maternal early-pregnancy glucose or insulin concentrations in SDS. Values are ORs (95% CIs) that reflect the risk of NAFLD at 10 years of age per maternal early-pregnancy glucose or insulin concentrations in SDS. Basic model: adjusted for child sex and age 10 years at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal ethnicity, education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total-cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; SDS, standard deviation score.

Table S7. Associations between maternal early-pregnancy glucose with childhood liver fat fraction below or above 2% liver fat

	Liver Fat at School Age n = 2,168	
Maternal early-pregnancy glucose mmol/l	**Odds ratio Liver fat >2% yes/no (95% Confidence Interval)**	***p* value**
Basic model	1.05 (1.00; 1.11)	0.34
Main confounder model	1.04 (0.94; 1.15)	0.49
Maternal body mass index model	1.00 (0.90; 1.11)	0.95
Maternal dyslipidemia model	1.04 (0.94; 1.14)	0.50
Child metabolic markers at 6 years model	1.04 (0.94; 1.15)	0.48
Child body mass index model	1.01 (0.91; 1.12)	0.89
Child visceral fat mass at 10 years model	1.02 (0.92; 1.14)	0.69
Child glucose concentrations model	1.03 (0.92; 1.15)	0.66

Values are ORs (95% CIs) that reflect the risk of more than 2% liver fat at 10 years of age per maternal early-pregnancy glucose concentrations in mmol/l. Basic model: adjusted for child sex and age 10 years at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal ethnicity, education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total-cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. Liver fat >2% was defined as "yes" when liver fat >2.0% and as "no" when liver fat ≤2.0%. Abbreviations: BMI, Body Mass Index; SDS, standard deviation score.

Table S8. Sensitivity analyses on the associations between maternal early-pregnancy glucose with childhood liver fat fraction and non-alcoholic fatty liver disease among the full multi-ethnic group

			Liver Fat at School Age	
Maternal early-pregnancy glucose mmol/l	**Difference liver fat fraction SDS (95% Confidence Interval)**	***p* value**	**Odds ratio NAFLD yes/no (95% Confidence Interval)**	***p* value**
Women without pre-existing or gestational diabetes (n = 1,771)				
Basic model	0.04 (0.00; 0.08)	0.15	1.20 (1.02; 1.41)	0.27
Main confounder model	0.03 (-0.03; 0.08)	0.38	1.15 (0.83; 1.60)	0.39
Maternal body mass index model	0.00 (-0.05; 0.06)	0.91	1.09 (0.77; 1.53)	0.63
Maternal dyslipidemia model	0.02 (-0.03; 0.08)	0.40	1.15 (0.83; 1.60)	0.39
Child metabolic markers at 6 years model	0.03 (-0.03; 0.08)	0.36	1.16 (0.83; 1.62)	0.38
Child body mass index model	0.01 (-0.04; 0.06)	0.68	1.08 (0.77; 1.50)	0.66
Child visceral fat mass at 10 years model	0.02 (-0.03; 0.07)	0.37	1.21 (0.85; 1.74)	0.29
Child glucose concentrations model	0.02 (-0.03; 0.08)	0.40	1.18 (0.85; 1.64)	0.34
Women included before 14 weeks gestation (n = 1,466)				
Basic model	0.03 (0.00; 0.06)	0.40	1.20 (1.00; 1.45)	0.32
Main confounder model	0.02 (-0.04; 0.08)	0.48	1.23 (0.85; 1.78)	0.27
Maternal body mass index model	0.00 (-0.06; 0.06)	0.95	1.15 (0.78; 1.69)	0.48
Maternal dyslipidemia model	0.02 (-0.04; 0.08)	0.53	1.22 (0.85; 1.77)	0.29
Child metabolic markers at 6 years model	0.02 (-0.04; 0.08)	0.48	1.25 (0.86; 1.81)	0.25
Child body mass index model	0.01 (-0.05; 0.06)	0.74	1.12 (0.78; 1.63)	0.54
Child visceral fat mass at 10 years model	0.01 (-0.05; 0.06)	0.78	1.22 (0.82; 1.81)	0.34
Child glucose concentrations model	0.02 (-0.04; 0.08)	0.50	1.25 (0.86; 1.81)	0.24

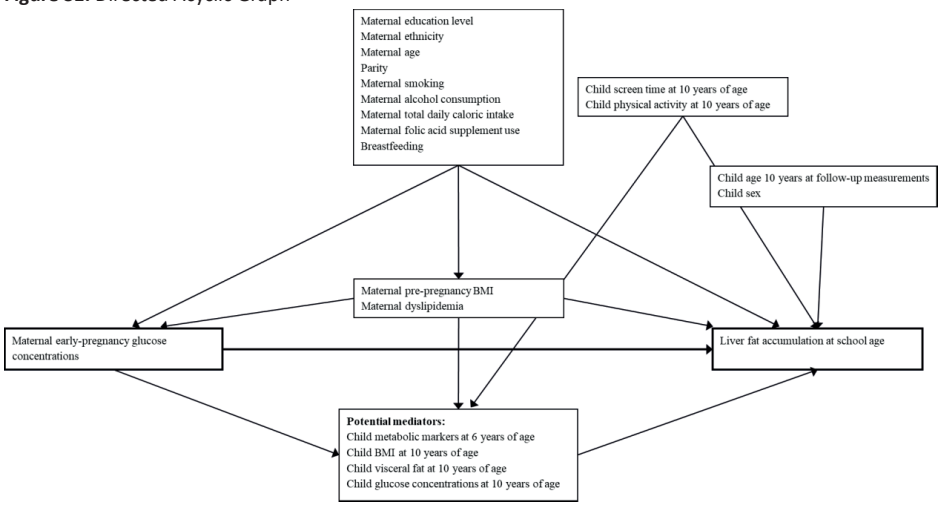
Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per maternal early-pregnancy glucose concentrations in mmol/l. Values are ORs (95% CIs) that reflect the risk of NAFLD at 10 years of age per maternal early-pregnancy glucose in mmol/l. Basic model: adjusted for child sex and age 10 years at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal ethnicity, education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total-cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; SDS, standard deviation score.

Table S9. Sensitivity analyses on the associations between maternal early-pregnancy glucose with childhood liver fat fraction and non-alcoholic fatty liver disease among the European ancestry only group

			Liver Fat at School Age	
Maternal early-pregnancy glucose mmol/l	**Difference liver fat fraction SDS (95% Confidence Interval)**	***p* value**	**Odds ratio NAFLD yes/no (95% Confidence Interval)**	***p* value**
Women without pre-existing or gestational diabetes (n = 1,184)				
Basic model	0.03 (-0.03; 0.10)	0.29	1.95 (1.29; 2.97)	<0.01
Main confounder model	0.03 (-0.03; 0.09)	0.31	1.99 (1.30; 3.05)	<0.01
Maternal body mass index model	0.01 (-0.05; 0.07)	0.69	1.96 (1.27; 3.01)	<0.01
Maternal dyslipidemia model	0.03 (-0.03; 0.09)	0.36	1.98 (1.29; 3.03)	<0.01
Child metabolic markers at 6 years model	0.03 (-0.03; 0.09)	0.34	2.02 (1.30; 3.12)	<0.01
Child body mass index model	0.02 (-0.04; 0.07)	0.62	1.67 (1.02; 2.72)	0.04
Child visceral fat mass at 10 years model	0.02 (-0.04; 0.08)	0.51	1.91 (1.21; 3.01)	<0.01
Child glucose concentrations model	0.03 (-0.03; 0.09)	0.36	2.00 (1.30; 3.07)	<0.01
Women included before 14 weeks gestation (n = 1,037)				
Basic model	0.01 (-0.06; 0.08)	0.76	1.81 (1.18; 2.77)	<0.01
Main confounder model	0.01 (-0.06; 0.07)	0.82	1.80 (1.17; 2.76)	<0.01
Maternal body mass index model	-0.01 (-0.08; 0.05)	0.69	1.72 (1.10; 2.69)	0.02
Maternal dyslipidemia model	0.01 (-0.06; 0.07)	0.85	1.86 (1.20; 2.89)	<0.01
Child metabolic markers at 6 years model	0.01 (-0.06; 0.07)	0.86	1.88 (1.20; 2.95)	<0.01
Child body mass index model	-0.01 (-0.07; 0.06)	0.87	1.58 (0.95; 2.62)	0.08
Child visceral fat mass at 10 years model	-0.01 (-0.07; 0.05)	0.71	1.70 (1.07; 2.68)	0.03
Child glucose concentrations model	0.01 (-0.06; 0.07)	0.86	1.82 (1.18; 2.81)	<0.01

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per maternal early-pregnancy glucose concentrations in mmol/l in mother-child pairs of European ancestry only. Values are ORs (95% CIs) that reflect the risk of NAFLD per maternal early-pregnancy glucose concentrations in mmol/l. Basic model: adjusted for child sex and age at outcome follow-up measurements. Main model: basic model additionally adjusted for maternal education, child physical activity. Maternal BMI model: main model additionally adjusted for maternal pre-pregnancy BMI. Maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia in early pregnancy. Child metabolic markers at 6 years model: main model additionally adjusted for child insulin, total-cholesterol, LDL and HDL-cholesterol and triglycerides concentrations at 6 years of age. Child BMI model: main model additionally adjusted for child BMI at 10 years of age. Child visceral fat mass model: main model additionally adjusted for child MRI-measured visceral fat mass at 10 years of age. Child glucose concentrations model: main model additionally adjusted for child glucose concentrations at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: BMI, Body Mass Index; NAFLD, non-alcoholic fatty liver disease; SDS, standard deviation score.

Figure S1. Directed Acyclic Graph



Covariate selection was primarily based on the Directed Acyclic Graph and subsequent on backward model selection analysis. The final model included child sex and age 10 years at follow-up measurements, maternal ethnicity, education, and child physical activity. We selected maternal ethnicity, education, smoking, alcohol consumption, folic acid supplement use, and child physical activity and screen time in the model, based on previous literature, their association with both the exposure and the outcome or change in effect estimates of >10% in the basic model(1, 2). Thereafter, we selected variables for the main model using backward selection and stopped when all *p* values <0.20(3). Maternal age, parity, and total daily caloric intake, and breastfeeding were not included in the main model as they did not affect the observed associations. Maternal smoking, alcohol consumption, folic acid supplement and child screen time were removed with backward selection from the main model having a *p* value >0.20(3). To observe the added confounding effect of maternal pre-pregnancy BMI in the observed associations we created an extra model, the maternal BMI model additionally adjusted for maternal pre-pregnancy BMI. Previous studies have suggested that maternal pre-pregnancy BMI largely explains the associations between gestational diabetes and offspring outcomes(4, 5). Next to this, we assessed the possible confounding effect of maternal dyslipidemia in an extra model, the maternal dyslipidemia model: main model additionally adjusted for maternal dyslipidemia. As we showed in our Directed Acyclic Graph, child metabolic markers at 6 years of age, BMI at 10 years, visceral fat mass at 10 years of age or child glucose concentrations at 10 years of age may mediate potential associations between maternal glucose concentrations and liver fat accumulation at school age. To explore the mediating role of child metabolic markers at 6 years of age, BMI at 10 years, visceral fat mass at 10 years As we showed in our Directed Acyclic Graph, child BMI at 10 years of age or child glucose concentrations at 10 years of age may mediate potential associations between maternal glucose concentrations and liver fat accumulation at school age. To explore the mediating role of child BMI at 10 years of age and child glucose concentrations at 10 years of age, we additionally corrected for these characteristics in separate models.

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3 | Determinants and outcomes of liver fat in childhood

3.1 Sugar-containing beverage intake in infancy and liver fat accumulation at school age

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ABSTRACT

Background: Sugar-containing beverage intake is a major risk for obesity in both children and adults and seems to be associated with non-alcoholic fatty liver disease in adults. To examine the associations of sugar-containing beverage intake at 1 year with liver fat accumulation and non-alcoholic fatty liver disease among school age children.

Methods: In a population-based prospective cohort study, we assessed sugar-containing beverage intake at 1 year with a validated Food Frequency Questionnaire among 1,940 infants. Liver fat fraction and non-alcoholic fatty liver disease (liver fat fraction ≥5.0%) were assessed with Magnetic Resonance Imaging.

Results: Higher sugar-containing beverage intake at 1 year was not associated with higher liver fat accumulation at 10 years (*p* value for trend 0.38). However, as compared to infants with <1.0 serving/day, those with >2.0 servings/day had the highest odds of non-alcoholic fatty liver disease (Odds Ratio 3.02 (95% Confidence Interval 1.34, 6.83)). These associations were largely independent by sugar-containing beverage intake and body mass index at school age. Stratified analyses suggested stronger associations of sugar-containing beverage intake at 1 year with non-alcoholic fatty liver disease at 10 years among children of mothers with low educational attainment and among overweight or obese children.

Conclusions: Higher sugar-containing beverage intake in infancy was associated with non-alcoholic fatty liver disease in school age children, independent of sugar-containing beverage intake and body mass index at school age. Limiting the intake of sugar-containing beverages already in infancy may be helpful in preventing liver steatosis at school age.

INTRODUCTION

High intake of sugar-containing beverage is a strong risk factor for obesity across the life course.[1-3] Results from prospective studies show that sugar-containing beverage intake already in infancy is related to body mass index (BMI) in adulthood.[3-5] Also, findings from randomized controlled trials suggest that higher consumption of sugar-containing beverages increases adiposity in children.[6-8] Recent studies in adults observed that higher intake of sugar-containing beverages is not only associated with general adiposity, but also with increased liver fat accumulation.[9 10] Increased liver fat accumulation and non-alcoholic fatty liver disease reflect a heterogeneous spectrum, ranging from liver steatosis, to steatohepatitis, fibrosis, cirrhosis, and eventually end-stage liver disease.[11] We have recently reported associations of liver fat across the full spectrum with risk factors for cardio-metabolic disease already at school age.[12] Early life exposures may contribute to the development not only of obesity, but also of liver fat accumulation and non-alcoholic fatty liver disease.[11 13 14] We hypothesized that intake of sugar-containing beverages in infancy is associated with liver fat accumulation at school age.

We examined in a population-based prospective cohort study among 1,940 children the associations of sugar-containing beverages intake at age 1 year with liver fat accumulation and non-alcoholic fatty liver disease assessed with magnetic resonance imaging (MRI) at 10 years. We additionally explored whether any association was explained by socio-demographic, lifestyle factors, or sugar-containing beverage intake and BMI at school age.

METHODS

Study population

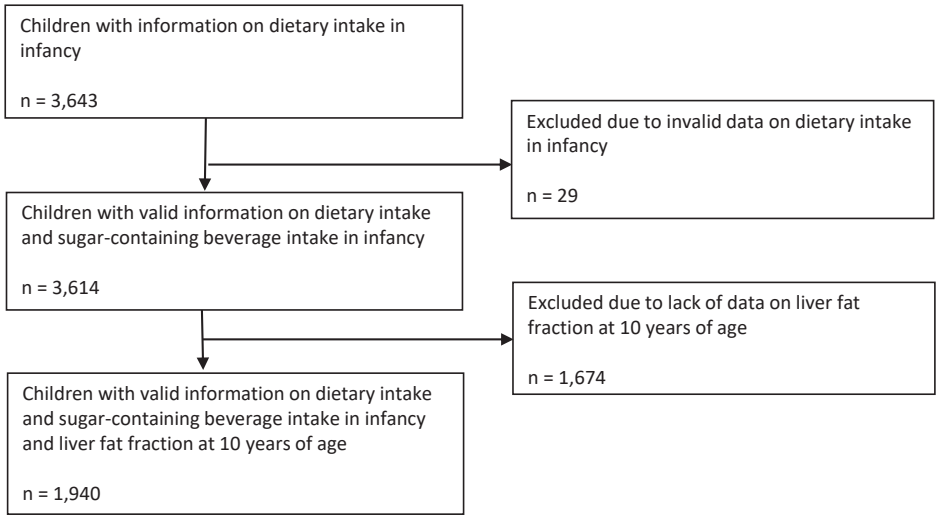
This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[15] The study has been approved by the Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from all parents.[15] All children were born between April 2002 and January 2006. The infant food-frequency questionnaire (FFQ) was implemented at a later stage during the study, therefore this study was performed in a subgroup of the total population.[16] Out of the 5,088 mothers who received the FFQ, 3,643 completed it. In total, 3,614 infants had valid information on dietary intake assessed by the FFQ at the age of 1 year. A subgroup of these children were invited for MRI measurements at age 10 years. The population for analysis comprised 1,940 children (**Figure 1**).

Intake of sugar-containing beverages

Infant sugar-containing beverage intake was assessed at a mean age of 13.6 months (standard deviation (SD) 1.8). Parents of the children completed the FFQ, using the last month as reference

period. The 211-item semi-quantitative FFQ was designed in cooperation with the division of Human Nutrition of Wageningen University, the Netherlands, and based on an existing validated FFQ.[17] The FFQ was modified to foods frequently consumed during early life, according to a National Dutch food consumption survey among 941 Dutch children aged 9 – 18 months.[16] The intraclass correlation coefficient for sugar-containing beverage intake estimated from 3-day recalls and the FFQ was calculated in a validation study to be 0.76.[3] Total sugar-containing beverage intake included intake of fruit juices, fruit concentrates, soft drinks and lemonades. As previously defined, we converted the intake of sugar-containing beverages consumption into the number of servings per day, with 1 serving equaling 150 g (NEVO-2011).[18] The consumption of sugar-containing beverages was assessed continuously and categorized into three categories: low <1.0 serving/day, medium 1.0 – 2.0 servings/day, and high >2.0 servings/day. The reference group was <1.0 serving/day based on the cut-off in the diet quality score for preschool children.[19]

Figure 1. Study participants flowchart



Liver fat at 10 years

As previously described, we measured liver fat using a 3.0 Tesla MRI scanner (Discovery MR750w, GE Healthcare, Milwaukee, Wisconsin, United States).[15 20-22] The children wore light clothing without metal objects while undergoing the body scan. A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique (IDEAL IQ) for generating a precise liver fat fraction image.[23] The IDEAL IQ scan is based on a carefully tuned 6-echo echo planar imaging acquisition. The obtained fat-fraction maps were analyzed by the Precision Image Analysis (PIA, Kirkland, Washington, United States) using the sliceOmatic (TomoVision, Magog, Canada) software package. All extraneous structures and any image artifacts were removed manually.[24] Liver fat fraction was determined by taking four samples of at least

4cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat estimation. Liver fat measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[25 26] As previously described, non-alcoholic fatty liver disease was defined as liver fat ≥5.0%.[20 26 27]

Covariates

At enrolment in the study, we obtained information on maternal age, parity, education level, smoking, net household income and pre-pregnancy weight by questionnaires. We measured maternal height and calculated pre-pregnancy BMI. Information on child age, sex and birth weight was obtained from medical records. Child´s ethnicity was obtained by questionnaires and was defined based on country of birth of the parents.[28] We categorized ethnicity into European (Dutch and other European) versus non-European (African (Cape Verdean, other African, Dutch Antillean, and Surinamese-Creole), American, Asian (Indonesian, other Asian, Surinamese-Hindu and Surinamese-unspecified), Turkish, Moroccan, Oceanian). At the 10-year follow-up visit, we measured childhood height and weight, both without shoes and heavy clothing, calculated BMI and further calculated sex- and age-adjusted childhood BMI standard deviation scores (SDS) based on Dutch reference growth charts (Growth Analyzer 4.0).[29] Childhood BMI was categorized into normal weight versus overweight or obesity.[30] Physical activity and screen time were assessed with questionnaires at school age.[31] The child diet quality score for preschool children was used, previously calculated with information from the FFQ at 1 year of age.[19] Sugar-containing beverage intake at the age of 8 years was assessed with the validated 71-item semi-quantitative FFQ.[32 33] The Dutch 2015 Guidelines for a Healthy Diet were used to calculate energy and nutrient intake at the age of 8 years.[34]

Statistical analysis

First, we conducted a non-response analysis among infants with a valid FFQ, comparing children with and those without liver MRI scans with Student's t-tests, Mann-Whitney tests and Chi-square tests. Second, we examined the associations of sugar-containing beverage intake with liver fat accumulation using linear regression models. Third, we used logistic regression models to assess the associations of sugar-containing beverage intake with the odds of non-alcoholic fatty liver disease. Analyses were performed using sugar-containing beverage intake as a continuous measure and categorized (low <1.0 serving/day (reference); medium 1.0 – 2.0 servings/day; and high >2.0 servings/day). The basic model was adjusted for age at the MRI visit, sex and total energy intake at 1 year; the confounder model was additionally adjusted for maternal pre-pregnancy BMI, education and net household income, child ethnicity, physical activity and screen time; and the mediator model was additionally adjusted for sugar-containing beverage intake at 8 years and BMI at 10 years of age. Included covariates were based on previous studies, strong correlations with consumption of sugar-containing beverages and with liver fat accumulation, changes in effect estimates of >10% and based on the Directed Acyclic Graph we constructed with

these covariates (Supporting Fig. S1).[10 35 36] As secondary analysis, we examined the associations of sugar-containing beverage intake at 8 years with liver fat accumulation at 10 years using similar models, with adjustment for total energy intake at 8 years. The distribution of liver fat was skewed and natural log-transformed values were used in all linear regression analyses. To assess whether the associations differed by type of sugar-containing beverage intake, we repeated the analyses separately for intake of fruit juice and for intake of soft drinks and lemonade. Based on previous findings, we hypothesized that the association of sugar-containing beverage intake with liver fat accumulation might differ by maternal educational level.[36] Since we observed a statistically significant interactions between sugar-containing beverage intake at 1 year with maternal education and with childhood BMI, we performed additional stratified analyses.[12 36] We did not observe a statistically significant interaction between sugar-containing beverage intake at 1 year with child sex.[3 35] As sensitivity analyses, we first repeated the confounder model with sugar-containing beverage intake standardized for total daily energy intake using the residual method and without adjustment for total daily energy intake as a confounder, since energy standardization could possibly reduce the measurement error.[37] Second, we examined the associations of sugar-containing beverage intake at 1 year with liver fat accumulation among children of Dutch ethnicity only, and among singleton children only. Missing data in the covariates were multiple-imputed using Markov chain Monte Carlo approach. Five imputed datasets were created and analyzed together. All statistical analyses were performed using the Statistical Package of Social Sciences version 25.0 for Windows (SPSS IBM, Chicago, Illinois, United States).

RESULTS

Subject characteristics

Table 1 shows that the median sugar-containing beverage intake at 1 year was 0.9 serving per day (95% range 0.0 – 3.7). The median liver fat fraction was 2.0% (95% range 1.2 – 4.6), 1.9% (95% range 1.2 – 4.3) and 2.0% (95% range 1.2 – 6.1) in the groups with low, medium and high sugar-containing beverage intake at 1 year, respectively. **Table S1** shows that children without liver fat measurement were less often European and had higher total daily energy intake at 1 year. Although total sugar-containing beverage intake at 1 year and at 8 years was similar between study participants and non-participants, fruit juice intake at 1 year was slightly higher in non-participating infants.

Sugar-containing beverage intake in infancy and liver fat accumulation

Figure 2 shows that the distribution of liver fat fraction at 10 years differed per infant sugar-containing beverage intake category. The percentage of children with liver fat fraction of ≥5.0% liver fat increased from 1.4% (n = 14/1,015) in the low intake group to 4.0% (n = 14/353) in the high intake group. After adjusting for confounders, sugar-containing beverage intake at 1 year

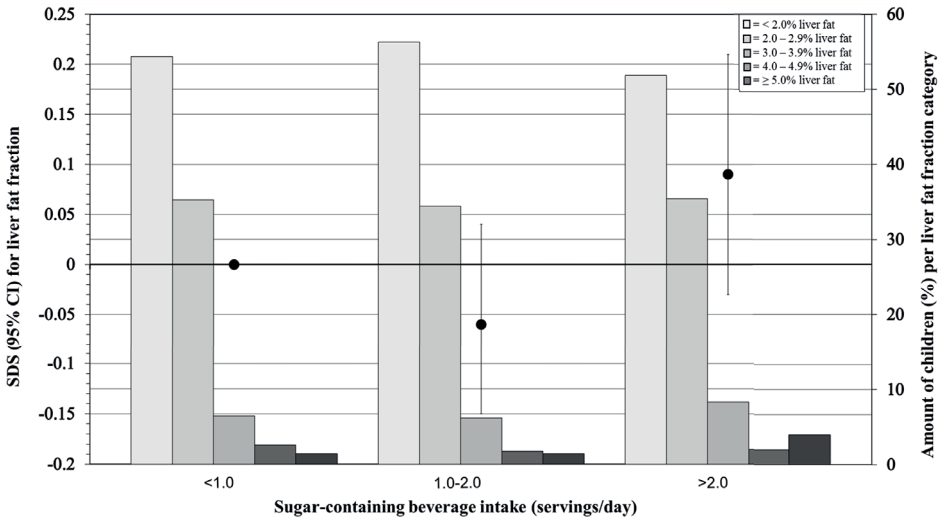
Table 1. Subject characteristics

	Total group	Sugar-containing beverage intake in infancy		
		Low <1.0 serving/day	Medium 1.0-2.0 servings/day	High >2.0 servings/day
	n = 1,940	n = 1,015	n = 572	n = 353
Maternal characteristics				
Age at enrollment, years	31.8 ± 4.3	32.1 ± 4.3	31.5 ± 4.2	31.4 ± 4.7
Pre-pregnancy BMI, kg/m^2	23.4 ± 4.0	23.2 ± 3.8	23.6 ± 4.3	23.5 ± 3.9
Parity, nulliparous	1,154 (61.1)	604 (61.2)	343 (61.5)	207 (60.3)
Education, higher	1,181 (62.4)	643 (65.3)	341 (60.9)	197 (56.8)
Smoking during pregnancy, continued	188 (10.8)	84 (9.1)	51 (10.0)	53 (16.8)
Net household income, ≥2,200 euros/month	1,176 (70.4)	615 (70.9)	356 (71.9)	205 (66.3)
Child characteristics				
Sex, male	937 (48.3)	490 (48.3)	275 (48.1)	172 (48.7)
Ethnicity, European	1,496 (77.4)	767 (75.8)	459 (80.5)	270 (76.9)
Birth weight, grams	3,452 ± 574	3,427 ± 584	3,481 ± 559	3,477 ± 568
Age at 1-year FFQ, months	13.6 ± 1.8	13.4 ± 1.6	13.7 ± 1.9	13.9 ± 2.1
Total energy intake at 1 year, kcal/day	1,306 ± 385	1,219 ± 357	1,323 ± 350	1,531 ± 421
Diet quality score at 1 year, 0-10	4.3 ± 1.4	4.4 ± 1.4	4.2 ± 1.3	4.2 ± 1.4
Sugar-containing beverages at 1 year, servings/day				
Total	0.9 (0.0, 3.7)	0.5 (0.0, 1.0)	1.6 (1.0, 1.9)	2.8 (2.0, 5.6)
Fruit juice	0.1 (0.0, 1.9)	0.0 (0.0, 0.9)	0.1 (0.0, 1.9)	0.4 (0.0, 3.7)
Soft drinks and lemonade	0.7 (0.0, 2.8)	0.3 (0.0, 0.9)	1.1 (0.0, 1.9)	1.9 (0.0, 4.6)
Age at 8-year FFQ, years	8.1 ± 0.1	8.1 ± 0.2	8.1 ± 0.1	8.1 ± 0.2
Total energy intake at 8 years, kcal/day	1,469 ± 341	1,445 ± 342	1,481 ± 326	1,517 ± 356
Diet quality score at 8 years, 0-10	4.6 ± 1.2	4.6 ± 1.2	4.6 ± 1.2	4.5 ± 1.3
Sugar-containing beverages at 8 years, servings/day				
Total	2.2 (0.1, 8.9)	2.0 (0.0, 7.8)	2.4 (0.1, 7.9)	2.6 (0.1, 10.4)
Fruit juice	0.4 (0.0, 3.2)	0.4 (0.0, 3.1)	0.4 (0.0, 3.6)	0.4 (0.0, 3.7)
Soft drinks and lemonade	1.5 (0.0, 7.8)	1.2 (0.0, 6.9)	1.5 (0.0, 7.4)	1.7 (0.0, 9.2)
Age at 10-year visit, years	9.8 ± 0.3	9.8 ± 0.2	9.8 ± 0.3	9.8 ± 0.3
Physical activity, hours/day	1.4 (0.3, 3.5)	1.3 (0.3, 3.3)	1.4 (0.3, 3.5)	1.5 (0.4, 3.8)
Screen time, ≥2 hours/day	796 (49.5)	384 (46.5)	239 (49.7)	173 (57.5)
BMI, kg/m^2	17.2 ± 2.4	17.1 ± 2.5	17.1 ± 2.3	17.6 ± 2.5
Liver fat fraction, %	2.0 (1.2, 4.7)	2.0 (1.2, 4.6)	1.9 (1.2, 4.3)	2.0 (1.2; 6.1)
NAFLD	36 (1.9)	14 (1.4)	8 (1.4)	14 (4.0)

Values, but not imputed data, are observed and represent numbers (valid %), means ± SD, or medians (95% range) shown for the total group and stratified for sugar-containing beverage intake at 1 year. Maternal characteristics were obtained when they were enrolled in the study, mostly in early pregnancy. Child's ethnicity based on the parents country of birth was categorized into European (Dutch and other European) and non-European (African (Cape Verdean, other African, Dutch Antillean, and Surinamese-Creole), American, Asian (Indonesian, other Asian, Surinamese-Hindu and Surinamese-unspecified), Turkish, Moroccan, Oceanian). Abbreviations: BMI, body mass index; FFQ, Food Frequency Questionnaire; NAFLD, nonalcoholic fatty liver disease.

was neither continuously nor categorically associated with liver fat fraction across the full range. There were no substantial differences in results between the basic, confounder or mediator models (**Table 2, Figure S2** and **Figure S3**).

Figure 2. Associations of sugar-containing beverage intake at 1 year with liver fat fraction in children of school age



Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake category as compared to the reference group (children with <1.0 serving per day intake of sugar-containing beverage). Associations are adjusted for child age at 10 years, sex, total energy intake, maternal pre-pregnancy BMI, education, net household income, child ethnicity, physical activity and screen time. The bars are presenting the liver fat fraction categories (<2.0; 2.0 - 2.9; 3.0 - 3.9; 4.0 - 4.9; ≥5.0 % liver fat) per sugar-containing beverage intake. BMI, body mass index; CI, Confidence Interval.

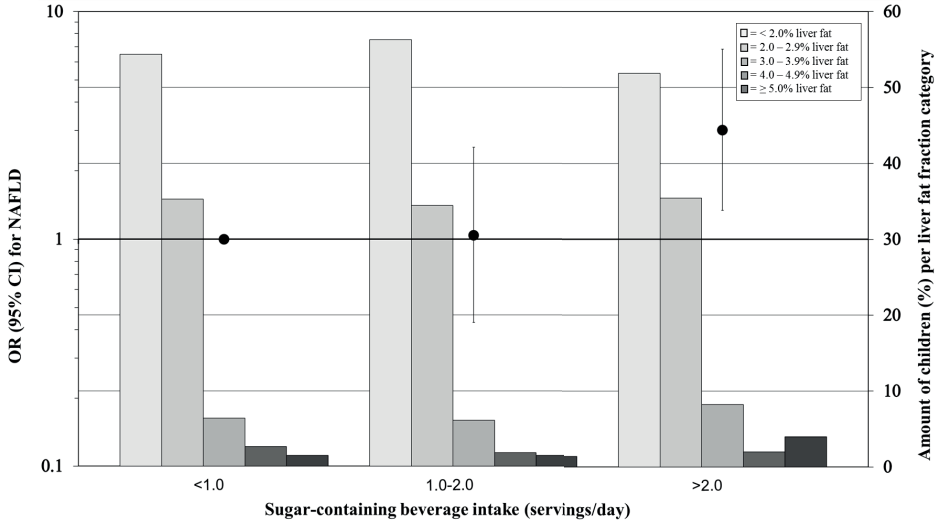
Table 2. Associations between sugar-containing beverage intake in infancy and liver fat fraction and non-alcoholic fatty liver disease in children at school age

	MRI measured liver fat at school age n = 1,940	
Sugar-containing beverages (servings/day) at 1 year	**Liver fat fraction SDS**	**NAFLD yes/no**
Basic model	0.037 (0.02, 0.06)	1.44 (1.27, 1.64)*
Confounder model	0.026 (−0.02, 0.07)	1.34 (1.06, 1.69)**
Mediator model	−0.004 (−0.05, 0.04)	1.34 (0.97, 1.83)

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake per day at 1 year. Values are ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake per day at 1 year. *P value <0.01, **P value <0.05. Basic model: adjusted for child at 10 years of age, sex, and total energy intake. Confounder model: basic model additionally adjusted for maternal pre-pregnancy BMI, education, net household income, child ethnicity, physical activity and screen time. Mediator model: confounder model additionally adjusted for sugar-containing beverage intake at 8 years of age and BMI at 10 years of age. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations: CI, confidential interval; MRI, magnetic resonance imaging; OR, odds ratio; SDS, standard deviation score.

Higher sugar-containing beverage intake at 1 year was associated with higher odds of non-alcoholic fatty liver disease (*p* value for trend <0.05) (**Figure 3**). As compared to infants with <1.0 serving/day, infants with >2.0 servings/day had the highest odds of non-alcoholic fatty liver disease (Odds Ratio (OR) 3.02 (95% CI 1.34, 6.83)). There were no differences in results between the basic and confounder models (**Table 2**). Also, the effect estimates were only slightly affected and of borderline significance after additional adjustment for sugar-containing beverage intake at 8 years and BMI at 10 years (**Table 2** and **Figure S4** and **Figure S5**).

Figure 3. Associations of sugar-containing beverage intake at 1 year with odds of non-alcoholic fatty liver disease in children of school age



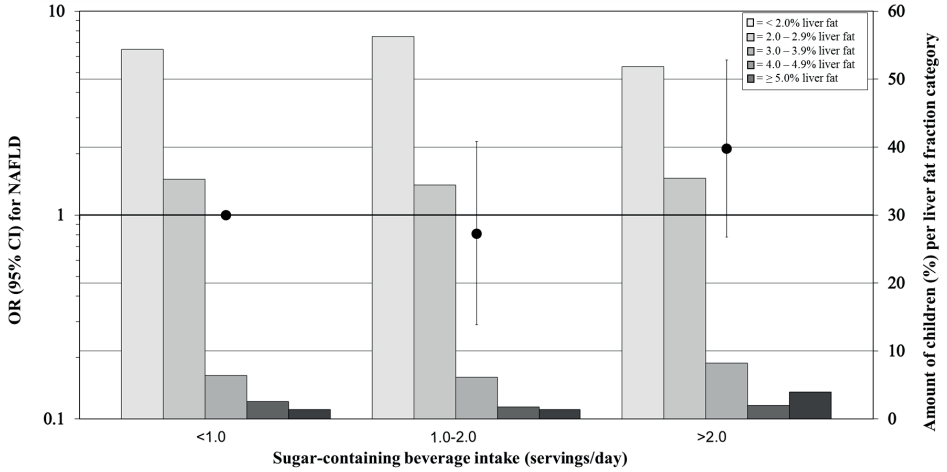
Values are Odds Ratios (95% Confidence Intervals) that reflect the risk of non-alcoholic fatty liver disease per sugar-containing beverage intake category at 1 year as compared to the reference group (children with <1.0 serving per day intake of sugar-containing beverage). Associations are adjusted for child age at 10 years, sex, total energy intake, maternal pre-pregnancy BMI, education, net household income, child ethnicity, physical activity and screen time. The bars are presenting the liver fat fraction categories (<2.0; 2.0 - 2.9; 3.0 - 3.9; 4.0 - 4.9; ≥5.0 % liver fat) per sugar-containing beverage intake. BMI, body mass index; CI, Confidence Interval.

Analyses stratified for maternal education level suggested that among children from mothers with lower or medium level of educational attainment, higher sugar-containing beverage intake at 1 year was associated with increased liver fat fraction, whereas in mothers with higher education no association was observed (**Table S2**). Stratified analyses in sugar-containing beverage intake at 1 year with non-alcoholic fatty liver disease, suggested that the odds of non-alcoholic fatty liver disease are stronger among children of mothers with lower or medium level of educational attainment compared to children of mothers with higher level of educational attainment and among overweight or obese children compared to normal weight children (**Table S2**).

Sugar-containing beverage intake at 1 year categorized in either fruit juice or soft drinks and lemonade was not associated with liver fat fraction or with non-alcoholic fatty liver disease at

school age (**Table S3**). Sugar-containing beverage intake at 8 years was not associated with liver fat fraction or with non-alcoholic fatty liver disease at school age (**Table S4**).

Figure 4. Associations between sugar-containing beverage intake in infancy and odds of NAFLD in children of school age - mediator model



The black circles represent ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake category in infancy as compared to the reference group (children with <1.0 serving/day intake of sugar-containing beverage) as scaled on the left y-axis. These associations are adjusted for child at 10 years of age, sex, total energy intake, maternal pre-pregnancy BMI, education, net household income, child ethnicity, physical activity, and screen time, sugar-containing beverage intake at 8 years of age and BMI at 10 years of age. The bars present the amount of children (in %) per liver fat fraction categories (<2.0%, 2.0%-2.9%, 3.0%-3.9%, 4.0%-4.9%, ≥5.0% liver fat) per sugar-containing beverage intake as scaled on the right y-axis. Trend: OR, 1.41 (95% CI, 0.83-2.40) per 1 serving/day increase in sugar-containing beverage intake.

Sensitivity analyses

When we used sugar-containing beverage intake standardized for total daily energy intake instead of sugar-containing beverage intake unstandardized, the effect estimates were largely similar for the associations with non-alcoholic fatty liver disease (**Table S5**). Also, we observed similar results to the main findings when we restricted our analyses to children of Dutch ethnicity only or among singleton children only (**Table S5**).

DISCUSSION

We observed that higher sugar-containing beverage intake during infancy is associated with an increased risk of non-alcoholic fatty liver disease in children of school age. The associations seems to be independent of sugar-containing beverage intake at 8 years and BMI at 10 years, and tended to be stronger among children of mothers with lower educational attainment and among children with overweight or obesity.

Interpretation of main findings

Sugar-containing beverage consumption is the main source of added sugar intake in the total daily energy intake of children and adults nowadays.[6 8 38] In adults, sugar-containing beverage intake is strongly associated with the development of non-alcoholic fatty liver disease.[10 39] Increased liver fat accumulation and non-alcoholic fatty liver disease reflect a heterogeneous spectrum, ranging from liver steatosis, to steatohepatitis, fibrosis, cirrhosis, and eventually end-stage liver disease.[11] Non-alcoholic fatty liver disease is associated with an increased risk of cardiovascular disease, dyslipidemia and type 2 diabetes in adults.[40] Using data from the same cohort as the current study, we recently reported associations of liver fat across the full spectrum with risk factors for cardio-metabolic disease already at 10 years.[12] Dietary patterns in infancy have been shown to track into adulthood.[39] Early lifestyle exposures are suggested to contribute to the development not only of obesity, but also of liver fat accumulation and non-alcoholic fatty liver disease.[11 13 14] We hypothesized that intake of sugar-containing beverages at 1 year is associated with liver fat accumulation in children of school age.

Two large cross-sectional studies among middle-aged adults observed that sugar-containing beverage consumption was, independently of BMI, associated with increased liver fat accumulation.[9 10] A recent randomized controlled trial among 40 adolescent boys diagnosed with non-alcoholic fatty liver disease, demonstrated that restricting sugar intake reduces liver fat accumulation.[35] In the current study, we observed that infants who consume more than two sugar-containing beverage servings per day had the highest odds of non-alcoholic fatty liver disease at age 10 years. We also observed that the association of sugar-containing beverage intake at 1 year with non-alcoholic fatty liver disease at school age was largely independent of sugar-containing beverage intake at 8 years, which could be a possible mediator in the association. Next to this, childhood BMI, a known risk factor for non-alcoholic fatty liver disease, did not seem to explain the observed associations. Stratified analyses showed stronger associations for sugar-containing beverage intake with both liver fat accumulation and non-alcoholic fatty liver disease among children from mothers with a lower level of educational attainment. The combination of lower maternal education, seen as proxy for family socio-economic status, and higher sugar-containing beverage intake at 1 year, might track from infancy into childhood and exacerbate liver fat accumulation. Stratified analyses on BMI at 10 years showed also stronger associations for sugar-containing beverage intake with non-alcoholic fatty liver disease among children with overweight or obesity. Thus, our findings suggest that sugar-containing beverage intake in infancy seems to be associated with the development of non-alcoholic fatty liver disease at age 10 years, and that these associations are stronger among overweight or obese children. We did not observe associations between sugar-containing beverage intake at 1 year and liver fat across the full range. It seems likely that due to the relatively large group of infants with low sugar-containing beverage intake at 1 year, together with the limited and still healthy spectrum of liver fat across the full range at school age, the differences in sugar-containing beverage intake at 1 year are too small to observe an effect on liver fat fraction across the full range at 10 years.

The absence of association of sugar-containing beverage intake at 8 years with liver fat might be explained by reverse causality since parents of children who are overweight or obese might reduce or underreport total energy intake and sugar-containing beverage intake.

Multiple mechanisms underlying the associations of sugar-containing beverage intake and liver fat accumulation have been proposed. It has been suggested that glucose, and especially fructose and fructose-containing sugars, all primarily metabolized in the liver increase hepatic *de novo* lipogenesis.[1 35 41] Next to this, consumption of sugar-containing beverages induces peaks in blood glucose, insulin and triglyceride concentrations, which may lead to insulin resistance and subsequently to liver fat accumulation.[1 9 10 39] Also, intake of liquid food leads to less satiety, more postprandial hunger and therefore to increased total daily energy intake.[9 42] Based on our findings, future studies should explore lifestyle interventions from infancy onwards to reduce sugar-containing beverage intake and keep an adequate healthy total daily energy intake. Intervention studies from early life onwards will both provide important new insights into the effectiveness of these interventions and into the causality of the observed associations of sugar-containing beverage intake in infancy and non-alcoholic fatty liver disease in later life.

Methodological considerations

Major strengths of this study are the population-based prospective longitudinal design with a large sample size, with information on sugar-containing beverage intake during infancy and on liver fat fraction measured with MRI at age 10 years. A subgroup of the study population were invited for MRI measurements at age 10 years (54% (n = 1,940), the non-response at the outcome measurement could lead to biased effect estimates if associations were different between those included and not included in the analyses, but this seems unlikely. To assess the average sugar-containing beverage intake at 1 year the 211 semi-quantitative FFQ was used, which may be subject to underreporting. The study population contained a relatively small number of children with overweight or obesity, which indicates a selection towards a lean population that might affect the generalizability of our findings. The healthy and young study population might also explain the small number of cases with non-alcoholic fatty liver disease, which could have limited our statistical power to detect significant associations. However, our findings are novel since prospective data on sugar-containing beverage intake during infancy and its relation with liver fat accumulation are lacking and lifestyle exposures in early life are suggested track into adulthood. Since we had a young study population, our results are not likely biased by alcohol use, known history of jaundice, hepatitis, smoking or medication use. We did not include breastfeeding as a covariate in our analyses, since previously we did not observe an association of breastfeeding with liver fat fraction[43]. Finally, although many covariates were included, there still might be some residual confounding, as in any observational study.

CONCLUSIONS

Higher sugar-containing beverage intake in infancy was associated with non-alcoholic fatty liver disease in school age children, independently of sugar-containing beverage intake and of BMI at school age. These associations tended to be stronger among children of mothers with a lower level of educational attainment and among children with overweight or obesity. Future preventive strategies should focus on the intake of sugar-containing beverage already from infancy onwards to reduce the risk of non-alcoholic fatty liver disease in later life.

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SUPPLEMENTARY MATERIAL

Table S1. Comparison of characteristics between participating and non-participating children

	Participants n = 1,940	**Non-participants** n = 1,674	***p* value**
Maternal characteristics			
Age at enrollment, years	31.8 ± 4.3	30.9 ± 4.8	<0.01
Pre-pregnancy BMI, kg/m^2	23.4 ± 4.0	23.4 ± 4.0	0.72
Parity, nulliparous	1,154 (61.1)	936 (57.5)	0.03
Education, higher	1,181 (62.4)	849 (53.5)	<0.01
Smoking during pregnancy, continued	188 (10.8)	210 (14.2)	<0.01
Net household income, ≥2,200 euros/month	1,176 (70.4)	887 (65.0)	<0.01
Child characteristics			
Sex, male	937 (48.3)	833 (49.9)	0.38
Ethnicity, European	1,496 (77.4)	1,212 (73.6)	<0.01
Birth weight, grams	3,452 ± 574	3,433 ± 584	0.31
Age at 1-year FFQ, months	13.6 ± 1.8	13.7 ± 1.9	0.05
Total energy intake at 1 year, kcal/day	1,306 ± 385	1,349 ± 439	<0.01
Diet quality score at 1 year, 0-10	4.3 ± 1.4	4.2 ± 1.4	0.06
Sugar-containing beverages at 1 year, servings/day			
Total	0.9 (0.0, 3.7)	1.0 (0.0, 4.6)	0.37
Fruit juice	0.1 (0.0, 1.9)	0.1 (0.0, 2.8)	0.37
Soft drinks and lemonade	0.7 (0.0, 2.8)	0.6 (0.0, 2.8)	0.57
Age at 8-year FFQ, servings/day	8.1 ± 0.1	8.1 ± 0.1	0.87
Total energy intake at 8 years, kcal/day	1,469 ± 341	1,483 ± 378	0.34
Diet quality score at 8 years, 0-10	4.6 ± 1.2	4.6 ± 1.2	0.99
Sugar-containing beverages at 8 years, servings/day			
Total	2.2 (0.1, 8.9)	2.2 (0.0, 8.9)	0.76
Fruit juice	0.4 (0.0, 3.2)	0.5 (0.0, 3.7)	0.28
Soft drinks and lemonade	1.5 (0.0, 7.8)	1.5 (0.0, 7.8)	0.77
Age at 10-year visit, years	9.8 ± 0.3	9.7 ± 0.3	0.09
BMI, kg/m^2	17.2 ± 2.4	17.2 ± 2.6	0.91
Physical activity, hours/day	1.4 (0.3, 3.5)	1.4 (0.3, 3.7)	0.94
Screen time, ≥2 hours/day	796 (49.5)	353 (49.3)	0.93
BMI, kg/m^2	17.2 ± 2.4	17.2 ± 2.6	0.91

Values are observed and represent numbers (valid %). means ± SD, or medians (95% range). Differences were tested using Student *t* tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively, and $\chi 2$ test was used for dichotomous variables. Maternal characteristics were obtained when they were enrolled in the study, mostly in early pregnancy. Child's ethnicity based on the parents country of birth was categorized into European (Dutch and other European) and non-European (African (Cape Verdean, other African, Dutch Antillean, and Surinamese-Creole), American, Asian (Indonesian, other Asian, Surinamese-Hindu and Surinamese-unspecified), Turkish, Moroccan, Oceanian). Abbreviations: BMI, body mass index; FFQ, Food Frequency Questionnaire; SD, standard deviation.

Table S2. Sugar-containing beverage intake at 1 year with liver fat fraction and non-alcoholic fatty liver disease in school-aged children, confounder model stratified

	MRI measured liver fat at school age n = 1,940	
Sugar-containing beverages (servings/day) at 1 year	**Liver fat fraction SDS**	**NAFLD yes/no**
Lower/medium level of educational attainment n = 711	0.09 (0.03, 0.16)*	1.48 (1.12 1.97)*
Higher level of educational attainment n = 1,181	−0.04 (−0.09, 0.03)	1.04 (0.59, 1.81)
Normal weight n = 1,509	0.03 (−0.01, 0.07)	1.24 (0.78, 1.97)
Overweight or obese n = 285	0.05 (−0.09, 0.20)	1.47 (1.05, 2.07)**

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake per day at 1 year. Values are ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake per day at 1 year. *P value <0.01, **P value <0.05. Confounder model adjusted for child at 10 years of age, sex, total energy intake, ethnicity, physical activity, screen time, maternal pre-pregnancy BMI, education, and net household income. The analyses were stratified for a lower/medium level of maternal educational attainment versus a higher level of maternal educational attainment and for children with normal weight versus children with overweight or obesity. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%. Abbreviations; CI, confidence interval; MRI, magnetic resonance imaging; NAFLD, nonalcoholic fatty liver disease; OR, odds ratio; SDS, standard deviation score.

Table S3. Sugar-containing beverage intake at 1 year with liver fat fraction and non-alcoholic fatty liver disease in children of school age, confounder model for sugar-containing beverage intake subtype

	MRI measured liver fat at school age n = 1,940	
Sugar-containing beverages (servings/day) at 1 year	**Liver fat fraction SDS**	**NAFLD yes/no**
Fruit juice	0.05 (−0.02, 0.12)	1.35 (0.96, 1.89)
Soft drinks and lemonade	0.01 (−0.04, 0.06)	1.33 (0.97, 1.84)

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake subcategory per day at 1 year. Values are ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake subcategory per day at 1 year. Confounder model adjusted for child at 10 years of age, sex, total energy intake, ethnicity, physical activity, and screen time, maternal pre-pregnancy BMI, education, and net household income. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%.

Table S4. Sugar-containing beverage intake at 8 years with liver fat fraction and non-alcoholic fatty liver disease in children of school age

	MRI measured liver fat at school age n = 2,352	
Sugar-containing beverages (servings/day) at 8 years	**Liver fat fraction SDS**	**NAFLD yes/no**
Basic model	−0.016 (−0.02, 0.01)	0.95 (0.83, 1.08)
Confounder model	−0.003 (−0.02, 0.01)	0.95 (0.84, 1.08)
Mediator model	−0.002 (−0.02, 0.01)	0.96 (0.84, 1.08)
Basic model		
<1.0 serving/day	Reference	Reference
1.0-2.0 servings/day	−0.034 (−0.14, 0.08)	0.75 (0.52, 1.09)
>2.0 servings/day	−0.057 (−0.16, 0.05)	0.69 (0.36, 1.33)
p value for trend	0.285	0.292
Confounder model		
<1.0 serving/day	Reference	Reference
1.0-2.0 servings/day	0.000 (−0.11, 0.11)	0.82 (0.39, 1.71)
>2.0 servings/day	−0.009 (−0.11, 0.10)	0.81 (0.42, 1.59)
p value for trend	0.851	0.573
Mediator model		
<1.0 serving/day	Reference	Reference
1.0-2.0 servings/day	0.022 (−0.08, 0.13)	0.74 (0.34, 1.60)
>2.0 servings/day	0.025 (−0.07, 0.12)	0.78 (0.39, 1.57)
p value for trend	0.637	0.541

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake per day at 8 years. Values are ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake per day at 8 years. Basic model: adjusted for child at 10 years of age, sex, total energy intake. Confounder model: basic model additionally adjusted for child ethnicity, physical activity, screen time, maternal pre-pregnancy BMI, education, and net household income. Mediator model: confounder model additionally adjusted for sugar-containing beverage intake at 8 years and BMI at 10 years. NAFLD was defined as "yes" when liver fat ≥5.0% and as "no" when liver fat <5.0%.

Table S5. Sensitivity analyses of sugar-containing beverage intake at 1 year with liver fat fraction and non-alcoholic fatty liver disease in children of school age

	MRI measured liver fat at school age n = 1,940	
Sugar-containing beverages (servings/day) at 1 year	**Liver fat fraction SDS**	**NAFLD yes/no**
Residual method adjusted model n = 1,940	0.022 (−0.10, 0.14)	1.14 (0.49, 2.61)
Dutch-only model n = 1,371	0.019 (−0.03, 0.07)	1.44 (0.95, 2.18)
Residual method adjusted model		
<1.0 serving/day	*Reference*	*Reference*
1.0-2.0 servings/day	−0.076 (−0.19, 0.04)	0.92 (0.36, 2.38)
>2.0 servings/day	0.042 (−0.15, 0.24)	2.22 (0.68, 7.26)
p value for trend	0.95	0.26
Dutch-only model		
<1.0 serving/day	*Reference*	*Reference*
1.0-2.0 servings/day	0.003 (−0.10, 0.11)	1.23 (0.36, 4.15)
>2.0 servings/day	0.071 (−0.06, 0.20)	2.51 (0.74, 8.50)
p value for trend	0.35	0.16

Values are regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake per day at 1 year. Values are ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake per day at 1 year. Models are adjusted according to the confounder model: child at 10 years of age, sex, total energy intake, ethnicity, physical activity, and screen time, maternal pre-pregnancy BMI, education, and net household income. In residual method adjusted model, sugar-containing beverage intake was standardized for energy using the residual method. NAFLD was defined as “yes” when liver fat ≥5.0% and as “no” when liver fat <5.0%.

Figure S1. Directed acyclic graph of associations between sugar-containing beverage intake in infancy and liver fat accumulation at school age with potential covariates

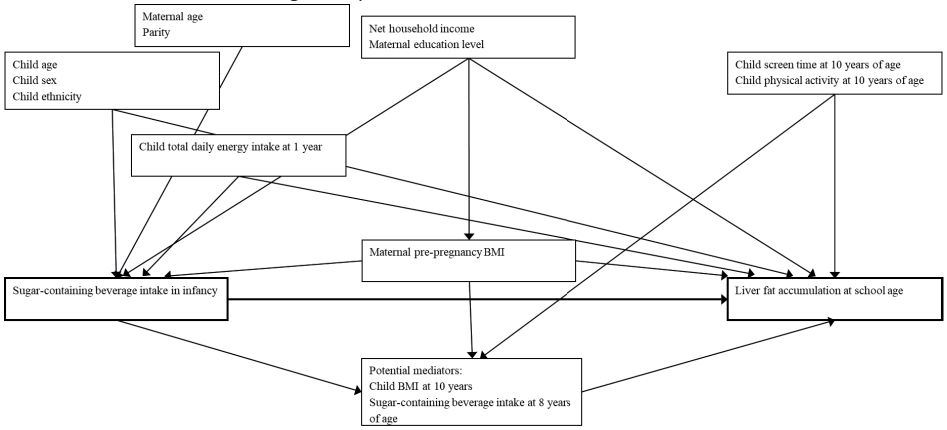
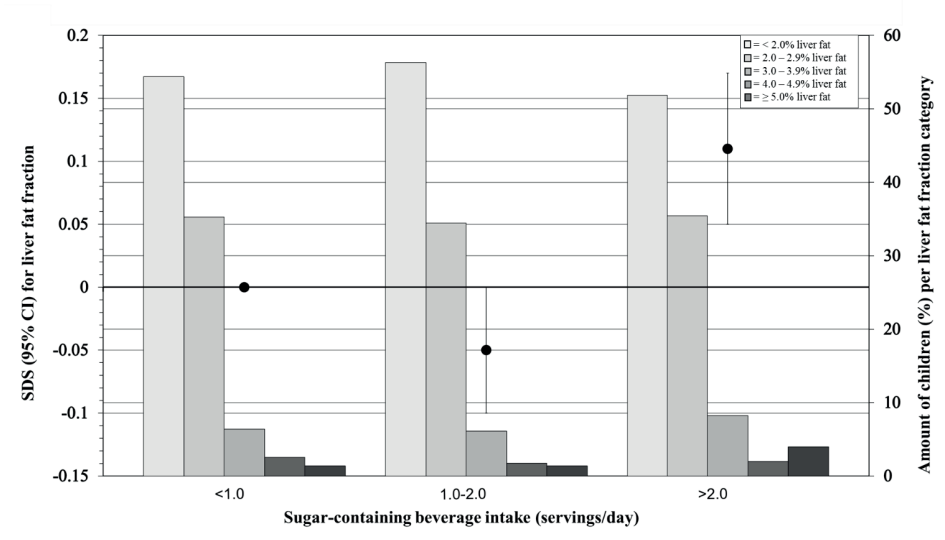
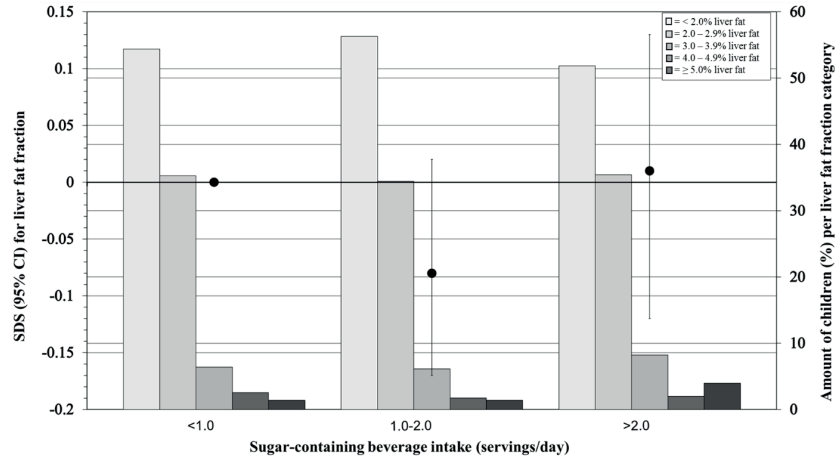


Figure S2. Associations between sugar-containing beverage intake in infancy and liver fat fraction in children of school age - basic model



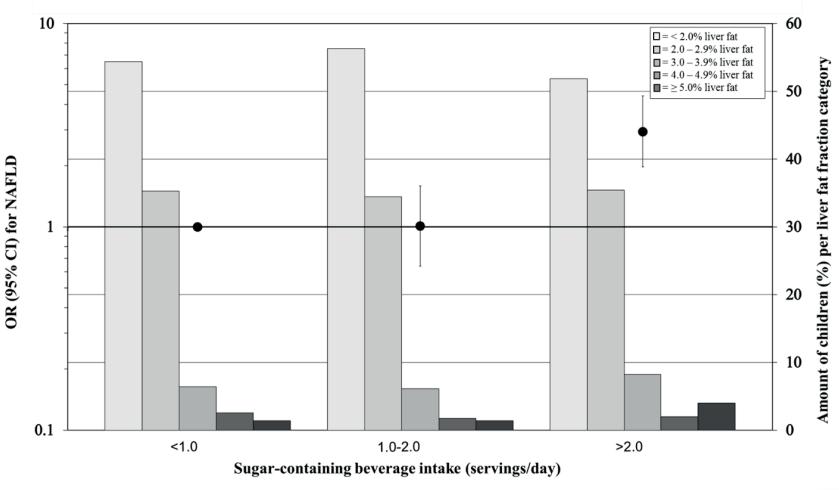
The black circles represent regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake category as compared to the reference group (children with <1.0 serving/day intake of sugar-containing beverage) as scaled on the left y-axis. These associations are adjusted for child at 10 years of age, sex, and total energy intake. The bars present the amount of children (in %) per the liver fat fraction categories (<2.0%, 2.0%-2.9%, 3.0%-3.9%, 4.0%-4.9%, ≥5.0% liver fat) per sugar-containing beverage intake as scaled on the right y-axis. Trend: 0.04 SDS (95% CI, 0.01-0.06) per 1 serving/day increase in sugar-containing beverage intake.

Figure S3. Associations between sugar-containing beverage intake in infancy and liver fat fraction in children of school age - mediator model



The black circles represent regression coefficients (95% CIs) from linear regression models that reflect differences in liver fat fraction in SDS per sugar-containing beverage intake category as compared to the reference group (children with <1.0 serving/day intake of sugar-containing beverage) as scaled on the left y-axis. These associations are adjusted for child at 10 years of age, sex, total energy intake, maternal pre-pregnancy BMI, education, net household income, child ethnicity, physical activity, and screen time, sugar-containing beverage intake at 8 years of age and BMI at 10 years of age. The bars present the amount of children (in %) per liver fat fraction categories (<2.0%, 2.0%-2.9%, 3.0%-3.9%, 4.0%-4.9%, ≥5.0% liver fat) per sugar-containing beverage intake as scaled on the right y-axis. Trend: -0.01 SDS (95% CI, -0.07-0.05) per 1 serving/day increase in sugar-containing beverage intake.

Figure S4. Associations between sugar-containing beverage intake in infancy and odds of NAFLD in children of school age - basic model



The black circles represent ORs (95% CIs) that reflect the risk of NAFLD per sugar-containing beverage intake category in infancy as compared to the reference group (children with <1.0 serving/day intake of sugar-containing beverage) as scaled on the left y-axis. These associations are adjusted for child at 10 years of age, sex, total energy intake. The bars present the amount of children (in %) per liver fat fraction categories (<2.0%, 2.0%-2.9%, 3.0%-3.9%, 4.0%-4.9%, ≥5.0% liver fat) per sugar-containing beverage intake as scaled on the right y-axis. Trend: OR, 1.70 (95% CI, 1.36-2.14) per 1 serving/day increase in sugar-containing beverage intake.

3.2 Newborn and childhood differential DNA methylation and liver Fat in school age children

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ABSTRACT

Background: Non-alcoholic fatty liver disease is the most common chronic liver disease in children in western countries. Adverse early-life exposures are associated with higher liver fat percentages in children. Differential DNA methylation may underlie these associations. We aimed to identify differential DNA methylation in newborns and children associated with liver fat accumulation in childhood. We also examined whether DNA methylation at 22 Cytosine-phosphate-Guanine sites (CpGs) associated with adult non-alcoholic fatty liver disease are associated with liver fat in children.

Methods: In a population-based prospective cohort study, we analyzed epigenome-wide DNA methylation data of 785 newborns and 344 10-year-old children in relation to liver fat fraction at 10 years. DNA methylation was measured using the Infinium HumanMethylation450 BeadChip (Illumina). We measured liver fat fraction by Magnetic Resonance Imaging. Associations of single CpG DNA methylation at the two time points with liver fat accumulation were analyzed using robust linear regression models. We also analyzed differentially methylation regions using the dmrff package. We looked-up associations of 22 known adult CpGs at both ages with liver fat at 10 years.

Results: The median liver fat fraction was 2.0% (95% range: 1.3, 5.1). No single CpGs and no differentially methylated regions were associated with liver fat accumulation. None of the 22 known adult CpGs were associated with liver fat in children.

Conclusions: DNA methylation at birth and in childhood was not associated with liver fat accumulation in 10-year-old children in this study. This may be due to modest sample sizes or DNA methylation changes being a consequence rather than a determinant of liver fat.

INTRODUCTION

Non-alcoholic fatty liver disease is a pathologic excess of ≥5% fat in hepatic cells, not caused by alcohol consumption, genetic or metabolic disorders, medication, or viral infections.[1] Due to the high prevalence of obesity, non-alcoholic fatty liver disease has become the most common chronic liver disease in both children and adults in western countries.[2-5] Non-alcoholic fatty liver disease is associated with an adverse cardio-metabolic risk profile in children.[3] In adults it is associated with cardio-metabolic diseases and hepatocellular carcinoma, and it is a leading indication for liver transplantation.[4 6] An accumulating body of evidence suggests that adverse exposures in early life contribute to the development of obesity and non-alcoholic fatty liver disease.[5 7]

The mechanisms underlying the observed associations of early-life factors with liver fat in children and adults may include changes in DNA methylation.[5 7] DNA methylation is an epigenetic mechanism that is highly dynamic in early life and affects accessibility of DNA for transcription and thereby gene expression.[8] Various adverse early-life factors have been associated with differential DNA methylation.[9-12] Recent studies using liver biopsy samples of adults with non-alcoholic fatty liver disease suggest differential DNA methylation is cross-sectionally associated with non-alcoholic fatty liver disease.[8 13-15] A meta-analysis of population-based cohorts in adults identified 22 Cytosine-phosphate-Guanine sites (CpGs) in peripheral blood at which DNA methylation was associated with non-alcoholic fatty liver disease.[6]

We hypothesized that differential DNA methylation at birth and in childhood is associated with liver fat accumulation in children. We performed an epigenome-wide association study (EWAS) to assess whether DNA methylation at birth and at age 10 years is associated with liver fat accumulation measured with Magnetic Resonance Imaging (MRI) in 10-year-old children participating in a population-based prospective cohort study. Analyses were focused on both single CpGs and differentially DNA methylated regions (DMRs). As a secondary analysis, we examined if DNA methylation at birth and at age 10 years is associated with higher (>2%) versus lower (≤2%) liver fat accumulation. We also examined whether DNA methylation at the 22 CpGs known to be associated with non-alcoholic fatty liver disease in adults, is also associated with liver fat in children.[6]

RESULTS

Subject characteristics

The median liver fat fraction was 2.0% for both groups (newborns 95% range: 1.3, 4.6; 10-year-old children 95% range: 1.3, 5.1)). The prevalence of non-alcoholic fatty liver disease at age 10 years was 2.2% (n = 17/785) in the group with DNA methylation data at birth and 2.6% (n = 9/344) in the group with DNA methylation data at age 10 years. The baseline characteristics of the study population are presented in **Table 1**. Non-response analyses comparing singleton children with DNA methylation data, with and without information on liver fat fraction available, showed that

participants in the newborn group were slightly more often female and more often overweight, had somewhat older and higher educated mothers, who more often stopped smoking during pregnancy compared to non-participants in the newborn group. In the childhood group, non-response analyses showed that participants were slightly older compared to the non-participants (**Table 2**).

Table 1. Subject characteristics

	Newborns (n = 785)	**Childhood** (n = 344)
Maternal characteristics		
Age, mean (SD), years	32.1 ± 4.0	32.1 ± 4.0
Prepregnancy body mass index, mean (SD), kg/m^2	23.2 ± 3.9	23.4 ± 4.0
Parity, n (%), nulliparous	477 (60.8%)	205 (59.6%)
Education, n (%), higher education	535 (68.2%)	232 (67.4%)
Smoking during pregnancy, n (%), continued	94 (12.0%)	43 (12.5%)
Child characteristics		
Gestational age at birth, median (95%), weeks	40.4 (37.0 – 42.3)	40.3 (36.9 – 42.4)
Age, mean (SD), years	0	9.8 ± 0.3
Males, n (%)	378 (48.2%)	170 (49.4%)
Birth weight, mean (SD), g	3556 ± 505	3578 ± 515
Body mass index at 10 years, mean (SD), kg/m^2	17.0 ± 2.1	17.1 ± 2.0
Children with		
underweight, n (%)	62 (7.9)	19 (5.5)
normal weight, n (%)	637 (81.1)	287 (83.4)
overweight, n (%)	79 (10.1)	37 (10.8)
obesity, n (%)	7 (0.9)	1 (0.3)
Liver fat fraction, median (95% range), %	2.0 (1.3 – 4.6)	2.0 (1.3 – 5.1)
Prevalence non-alcoholic fatty liver disease, n (%)	17 (2.2%)	9 (2.6%)

Values are observed data and represent means ± SD, medians (95% range) or numbers of subjects (valid %).

Epigenome-wide association study of childhood liver fat accumulation

We assessed associations of DNA methylation in cord blood and in whole peripheral blood at 10 years with liver fat as a continuous measure in 10-year-old children. In the main models, adjusted for maternal age, education level, early-pregnancy BMI and smoking, gestational age at birth (cord blood analyses) or child age (childhood analyses), child sex, cell type proportions and batch, we did not observe any CpGs at birth or at 10 years to be associated with liver fat accumulation at 10 years after Bonferroni (p value <1.0 x 10^{-7}) or false-discovery rate (FDR) correction. The Manhattan plots of both EWAS analysis of liver fat accumulation are presented in **Figure S1a** and **Figure S1b**. **Table S1** and **S2** show the CpGs with p values <1.0 x 10^{-4} for newborns and for 10-year-old children, respectively. We did not identify significantly associated differentially methylated regions associated with liver fat accumulation, nor did we find associations of individual CpG

Table 2. Comparison of child characteristics between children included and not included in the analyses

	Newborns Participants (n = 785)	Non-participants (n = 604)	*p* value	**Children** Participants (n = 344)	Non-participants (n = 120)	*p* value
Maternal characteristics						
Age, mean (SD), years	32.1 ± 4.0	31.3 ± 4.5	<0.01	32.1 ± 4.0	32.5 ± 4.1	0.41
Prepregnancy body mass index, mean (SD), kg/m^2	23.2 ± 3.9	23.2 ± 3.8	0.95	23.4 ± 4.0	22.7 ± 3.2	0.12
Parity, n (%), nulliparous	477 (60.8%)	365 (60.6%)	0.96	205 (59.6%)	73 (61.3%)	0.74
Education, n (%), higher education	535 (68.2%)	357 (61.1%)	<0.01	232 (67.4%)	75 (65.2%)	0.86
Smoking during pregnancy, n (%), continued	94 (12.0%)	88 (18.0%)	<0.01	43 (12.5%)	8 (9.3%)	0.70
Child characteristics						
Gestational age at birth, median (95%), weeks	40.4 (37.0 – 42.3)	40.3 (36.3 – 42.4)	0.45	40.3 (36.9 – 42.4)	40.4 (37.6 – 42.4)	0.63
Age, mean (SD), years	NA	NA	NA	9.8 ± 0.3	9.7 ± 0.3	<0.01
Males, n (%)	378 (48.2%)	325 (53.8%)	0.04	170 (49.4%)	61 (50.8%)	0.79
Birth weight, mean (SD), g	3556 ± 505	3528 ± 518	0.31	3578 ± 515	3547 ± 498	0.57
Body mass index, mean (SD), kg/m^2	17.0 ± 2.1	17.0 ± 2.1	0.77	17.1 ± 2.0	17.2 ± 2.2	0.62
Children with						
underweight, n (%)	62 (7.9)	30 (8.1)	0.12	19 (5.5)	8 (6.7)	0.21
normal weight, n (%)	637 (81.1)	299 (80.6)	0.05	287 (83.4)	97 (80.8)	0.42
overweight, n (%)	79 (10.1)	38 (6.3)	<0.01	37 (10.8)	13 (10.8)	0.52
obesity, n (%)	7 (0.9)	4 (0.7)	0.09	1 (0.3)	2 (1.7)	0.11

Values are observed data and represent means (SD), medians (95% range) or numbers of subjects (valid %). Differences were tested using Student's t-tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively and using $\chi 2$-test for dichotomous variables.

sites with higher versus lower liver fat accumulation. **Table S3** and **S4** show the differentially methylated regions with *p* values <1.0 x 10^{-4} for newborns and 10-year-old children, respectively. **Table S5** and **S6** show the CpGs with *p* values <1.0 x 10^{-4} for newborns and for 10-year-old children for higher versus lower liver fat, respectively. Results of the basic model and of the model additionally adjusted for childhood body mass index (BMI) were not substantially different from the results in the main model. The mean percent differences in effect estimates between the main model and the basic model, and between the main model and the childhood BMI model in cord blood were 2.5% and 10.9%, respectively. In the child peripheral blood analyses at 10 years, the mean percent differences were 1.6% and 3.9%, respectively. In **Table S7** and **Table S8** we show the results of the basic and childhood BMI models for the CpGs probes with *p* values <1.0 x 10^{-4} identified in the main model.

Table 3. Associations of 22 adult non-alcoholic fatty liver disease-associated CpGs with liver fat fraction in children*

				Newborns			Children		
CpG	Chr	Position	Gene	Effect*	SE*	*p* value	Effect*	SE*	*p* value
cg09469355	1	2161886	*SKI*	0.002	0.03	0.96	-0.002	0.07	0.98
cg17901584	1	55353706	*DHCR24*	-0.005	0.02	0.74	-0.069	0.04	0.08
cg03725309	1	109757585	*SARS*	-0.012	0.02	0.45	0.027	0.05	0.56
cg14476101	1	120255992	*PHGDH*	-0.003	0.02	0.99	0.011	0.04	0.78
cg19693031	1	145441552	*TXNIP*	-0.011	0.03	0.72	-0.031	0.04	0.45
cg06690548	4	139162808	*SLC7A11*	-0.086	0.05	0.08	-0.027	0.06	0.67
cg05119988	4	166251189	*SC4MOL*	0.003	0.02	0.88	0.003	0.03	0.92
cg03957124	6	37016869	*COX6A1P2***	0.011	0.02	0.59	0.027	0.05	0.58
cg18120259	6	43894639	*LOC100132354***	0.024	0.02	0.31	-0.124	0.05	0.02
cg17501210	6	166970252	*RPS6KA2*	0.086	0.07	0.21	-0.137	0.08	0.10
cg21429551	7	30635762	*GARS*	0.015	0.02	0.42	0.017	0.03	0.52
cg11376147	11	57261198	*SLC43A1*	-0.004	0.03	0.89	0.107	0.07	0.11
cg00574958	11	68607622	*CPT1A*	0.028	0.04	0.43	-0.019	0.08	0.79
cg26894079	11	122954435	*ASAM*	0.004	0.03	0.88	-0.020	0.04	0.63
cg11024682	17	17730094	*SREBF1*	-0.023	0.04	0.54	-0.005	0.07	0.93
cg14020176	17	72764985	*SLC9A3R1*	0.006	0.03	0.84	-0.007	0.06	0.90
cg19016694	17	80821826	*TBCD*	0.016	0.03	0.55	-0.062	0.06	0.30
cg15860624	19	3811194	*ZFR2*	0.011	0.02	0.61	0.002	0.05	0.97
cg02711608	19	47287964	*SLC1A5*	-0.025	0.03	0.44	0.004	0.06	0.95
cg08309687	21	35320596	*LINC00649***	-0.004	0.03	0.88	-0.008	0.04	0.84
cg27243685	21	43642366	*ABCG1*	0.042	0.04	0.32	-0.023	0.09	0.81
cg06500161	21	43656587	*ABCG1*	0.018	0.03	0.57	0.023	0.05	0.66

*Effect estimates represent the change in liver fat fraction (%) per 10% difference in DNA methylation beta and standard error. Associations are adjusted for maternal age, education level, early-pregnancy BMI and smoking, age at birth or child age at measurement, child sex, cell type proportions and batch. *Gene names added using information from the UCSC Genome Browser build hg19. Other gene names from original paper by Ma *et al*, 2019. BMI, Body Mass Index; Chr, chromosome; n, number; SE, standard error.

Look-up of CpGs associated with adult liver fat

None of the 22 CpGs differentially methylated regions known for their associations with non-alcoholic fatty liver disease in adults, were associated with liver fat in children (Bonferroni corrected *p* value cutoff <0.05/22 = 2.3 x 10^{-3}, **Table 3**). We found no evidence for enrichment of the 22 CpGs among the 18,848 nominally significant CpGs from the cord blood analysis and among the 23,173 nominally significant CpGs from the 10-year-old analysis (Fisher combined probability *p* value = 1.00 in newborns and *p* value = 0.68 in 10-year-old children).

Candidate genes analysis associated with liver fat

We examined if there was enrichment of CpGs located in regions within a 4 Mb window (+/- 2 Mb) surrounding the 9 single-nucleotide polymorphisms (SNPs) identified to be associated with non-alcoholic fatty liver disease in adults, among all nominally significant CpGs in our analyses.[16 17] A total of 7,225 CpGs were present in these regions in the newborn dataset and 7,244 CpGs in the 10-year-old dataset. In newborns, 299 of these CpGs were nominally significant (*p* value <0.05). In 10-year-old children, this was the case for 347 CpGs. There was no enrichment for CpGs associated with liver fat accumulation at either age (Fisher combined probability *p* value = 0.47 in newborns and *p* value = 0.86 in 10-year-old children).

Top CpG probes functions and related biological processes

In an explorative analysis, significantly enriched gene ontology (GO) terms based on the annotated genes of the 32 CpG probes with *p* values <1.0 x 10^{-4} in cord blood pointed towards processes related to triglyceride, acylglycerol and lipid metabolic processes, digestive tract development, digestive system development and digestive tract morphogenesis, among others (**Table S9**). The same analysis using the 76 CpG probes with *p* values <1.0 x 10^{-4} in child peripheral blood revealed processes related to cell cycle functions, organ morphogenesis and development, among others (**Table S10**). We did not observe functional enrichment of Kyoto Encyclopedia of Genes and Genomes (KEGG) terms ((FDR <0.05). Next to this, we did not observe significant enrichment of DNAse hypersensitivity sites among the CpG <1.0 x 10^{-4} (smallest *p* value in cord blood analyses 0.09 and in childhood analyses 0.25).

DISCUSSION

In the first epigenome-wide association study on liver fat accumulation in children we did not observe differential DNA methylation in newborns or 10-year-old children related to liver fat accumulation analyzed as a continuous measure or related to higher versus lower liver fat accumulation measured by MRI at age 10 years. Also, DNA methylation at 22 CpGs known to be associated with non-alcoholic fatty liver disease in adults, was not associated with liver fat in children.

Interpretation of main findings

Non-alcoholic fatty liver disease has an increasing prevalence in both children and adults.[5 18] It is a major risk factor for adverse cardio-metabolic health in children and for cardio-metabolic diseases and liver diseases in adults.[3 4 6] Adverse early-life factors have been described to be associated with liver fat development.[5 7] These associations may be explained by DNA methylation changes in response to these early-life exposures that lead to liver fat development.[5 19]

Among adults it has been demonstrated that differential DNA methylation is present in liver biopsy samples of adults with non-alcoholic fatty liver disease.[8 13-15 20] All these studies used liver histology, the current gold standard for diagnosing non-alcoholic fatty liver disease.[2 5] As a consequence, these studies are limited by small sample sizes, histologically heterogeneous groups varying in severity of non-alcoholic fatty liver disease, older study populations, wide BMI ranges and having only few or no healthy controls. None of these reports controlled for cell heterogeneity in their analyses. A recent meta-analysis of four multiethnic population-based cohort studies in adults showed that DNA methylation at 22 CpGs in peripheral blood was associated with non-alcoholic fatty liver disease diagnosed with either computed tomography or ultrasound imaging (FDR <0.05).[6] In our study we did not observe differential DNA methylation at single CpGs or differentially methylated regions in cord blood or child peripheral blood associated with liver fat accumulation assessed by MRI in 10-year-old children. Also, DNA methylation at the 22 CpGs known from adults studies was not associated with liver fat in children.[6] It is possible that small, but potentially biologically important, DNA methylation differences may be associated with liver fat accumulation in children. These differences would be difficult to detect in the moderate sample size of the current study. Besides this, the variability in liver fat accumulation in this population of children was relatively small, which may also partly explain the lack of identified associations. In addition, our study population is a relatively lean population. Associations of DNA methylation with liver fat accumulation may be more apparent among higher risk populations, as observed in adult studies.[8 13-15 20] Another possibility is that DNA methylation truly is not associated with liver fat accumulation in children. As has been suggested for phenotypes such as obesity, differential DNA methylation may be mostly a consequence rather than a cause of liver fat accumulation. If that is indeed the case, then the duration of exposure to increased liver fat in this population of 10-year-old children may not have been sufficient to induce differential DNA methylation.[21]

The present population-based study is the first to examine the association of differential DNA methylation with liver fat fraction measured with MRI in children. Although the hypothesis of early-life factors contributing to the development of liver fat accumulation through DNA methylation cannot be completely discarded based on this study, we found no evidence to support associations of differential DNA methylation in newborns or children with liver fat accumulation at 10 years. Future studies should investigate in large longitudinal studies the associations of differential DNA methylation with liver fat accumulation in children.

Methodological considerations

Strengths of this study are the prospective and cross-sectional analyses with information on DNA methylation at two ages. We used a sensitive imaging-based method to enable non-invasive measurement of liver fat.[22 23] Although our sample size is relatively large for epigenome-wide analyses, it might still be too small to detect more minor effect sizes.[8 13-15] However, to the best of our knowledge, similar data on DNA methylation and MRI-measured liver fat accumulation in children are not currently available elsewhere. DNA methylation was measured in blood, which may differ from DNA methylation in liver cells. The relatively small number of children with obesity in the included sample indicates a selection towards a lean population that may affect the generalizability of our findings.

CONCLUSIONS

DNA methylation at birth and in childhood was not associated with liver fat accumulation in 10-year-old children in this study. This may be due to modest sample sizes or DNA methylation changes being a consequence rather than a determinant of liver fat. Future studies should investigate in large longitudinal studies the associations and timing of differential DNA methylation with liver phenotypes in children.

METHODS

Study design

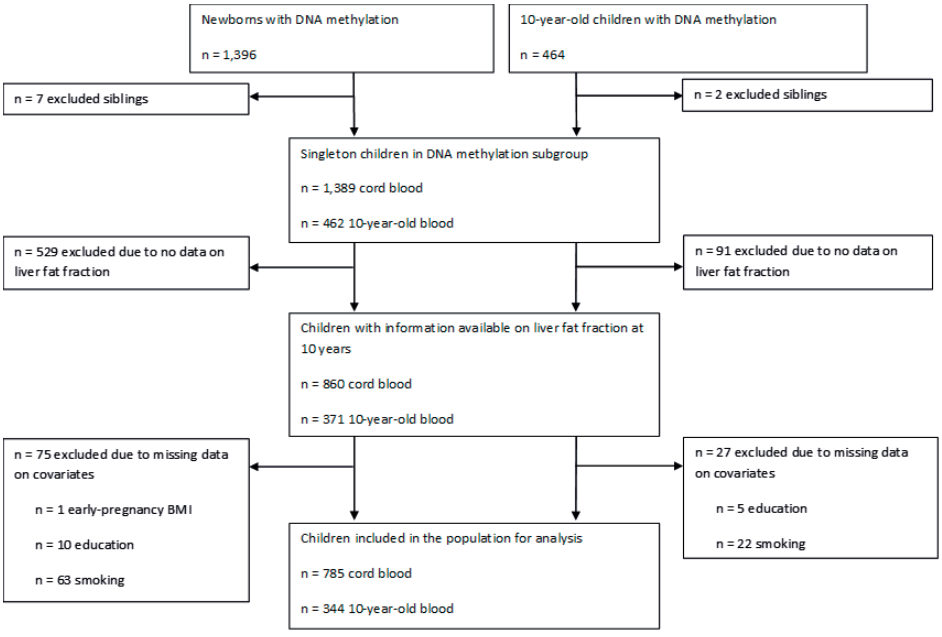
This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[24] The study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained for all participants.[24] All 9,778 participating live-born children were born between April 2002 and January 2006. DNA methylation was measured in a randomly selected European-ancestry subset of 1,396 newborns and 464 10-year-old children. The liver fat MRI measurements were performed in a subgroup of children at age 10 years. We excluded children without complete data on liver fat fraction and covariates. The population for analysis of this study comprised 785 newborns and 344 10-year-old children (**Figure 1**).

DNA methylation

DNA was extracted from cord blood and whole peripheral blood at 10 years using the salting-out method. Five hundred nanograms of DNA per sample underwent bisulfite conversion using the EZ-96 DNA Methylation kit (Shallow) (Zymo Research Corporation, Irvine, CA, USA). Samples

were plated randomly onto 96-well plates. Samples were processed with the Illumina Infinium HumanMethylation450 (450k) BeadChip (Illumina Inc., San Diego, CA, USA). Quality control of analyzed samples was performed using standardized criteria. Quality control and normalization of the HumanMethylation450 BeadChip array data was performed according to the Control Probe Adjustment and reduction of global CORrelation (CPACOR) workflow using R.[25 26] Probes that had a detection *p* value ≥1E-16 were set to missing per array. Next, the intensity values were quantile normalized for each of the six probe type categories separately: type II red/green, type I methylated red/green and type I unmethylated red/green. Beta values were calculated as proportion of methylated intensity value to the sum of methylated and unmethylated intensities plus 100. Arrays with observed technical problems such as failed bisulfite conversion, hybridization or extension, as well as arrays with a sex mismatch were removed from subsequent analyses. Additionally, only arrays with a call rate >95% per sample were processed further. Probes on the X and Y chromosomes were excluded from the analyses. The final datasets contained 457,774 probes in the newborn dataset and 458,563 probes in the 10-year-old dataset. For all CpGs and differentially methylated regions, the official gene name of the nearest gene was noted using Illumina's annotation information and we enhanced the annotation provided by Illumina with the UCSC Genome Browser build hg19.[27 28]

Figure 1. Study participants flowchart



Liver fat fraction at 10 years

We measured liver fat using a 3.0 Tesla MRI (Discovery MR750w, GE Healthcare, Milwaukee, WI, USA).[1 22-24] The children wore light clothing without metal objects while undergoing the body scan. A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique (IDEAL IQ) for generating a precise liver fat fraction image.[29] The IDEAL IQ scan is based on a carefully tuned 6-echo echo planar imaging acquisition. The obtained fat-fraction maps were subsequently analyzed by the Precision Image Analysis (PIA, Kirkland, WA, USA) using the sliceOmatic (TomoVision, Magog, QC, CAN) software package. All extraneous structures and any image artifacts were removed manually.[30] Liver fat fraction was measured independent of any outcome, determined by taking four samples of at least 4 cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat fraction estimation. Liver fat fraction measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[31 32] As previously described, non-alcoholic fatty liver disease was defined as liver fat fraction ≥5.0%.[1 32 33] We studied liver fat accumulation across the full spectrum as our primary objective. As the secondary objective we dichotomized liver fat into low, ≤2.0%, and high, >2.0%, liver fat accumulation. This cutoff was based on the median in our population and on previous work from our group describing that liver fat accumulation above 2.0% is already associated with an increased cardio-metabolic risk profile in children.[34] Due to lower numbers of cases, we could not dichotomize liver fat accumulation based on the clinical cut-off of ≥5.0%.

Covariates

At enrolment in the study information on maternal age and educational level was obtained by questionnaires. Maternal smoking during pregnancy was assessed by questionnaires in pregnancy. We measured maternal height and weight at enrolment to calculate early-pregnancy BMI.[35] Information on gestational age at birth, child sex and age at 10 years visit was obtained from medical records. We measured height and weight in the children, without shoes and heavy clothing. Childhood BMI was calculated and sex- and age-adjusted childhood BMI standard deviation scores were calculated (Growth Analyzer 4.0, Dutch Growth Research Foundation).[36]

Look-up study of adult CpGs associated with liver fat

We examined in our data the associations of the 22 CpGs known from previous literature to be associated with liver fat accumulation in in adults with liver fat accumulation in children.[6] A Bonferroni corrected p value $<0.05/22 = 2.3 \times 10^{-3}$ was used to define significance. We also evaluated whether the 22 CpGs were enriched among CpGs with a $p<0.05$ in our results using a hypergeometric test.

Genes previously associated with liver fat

We assessed the number of nominally significant single CpGs from our analyses that were located within a 4 Mb window (+/- 2 Mb) surrounding the 9 SNPs identified in two previous genome-wide association studies (GWAS) of liver fat accumulation in adolescents and adults of European descent.[16 17] With a hypergeometric test, we calculated enrichment of the CpGs surrounding the 9 SNPs among CpGs with a p<0.05 in our results.

Pathway analysis

To identify biological processes associated with the genes annotated to the CpG probes with *p* values <1.0 x 10^{-4} identified in cord blood and in child peripheral blood at 10 years associated with liver fat accumulation, we used the DAVID bioinformatics resource to test for enrichment in GO biological processes and KEGG pathways.[37] The online program epigenetic Functional element Overlap analysis of the Results of Genome Wide Association Study Experiments (eFORGE) was used to examine enrichment for DNAse hypersensitivity site enrichment among the most significantly associated CpGs in both cord blood and in child peripheral blood at 10 years.[38]

Statistical analysis

First, non-response analysis was conducted among singleton children with DNA methylation data, and with or without complete data on liver fat and covariates available, using Student's t-tests, Mann-Whitney tests and Chi-square tests. Second, we used robust linear regression models to assess the associations of DNA methylation in cord blood and in whole peripheral blood at 10 years with liver fat fraction as a continuous measure in 10-year-old children.[26] The analyses were performed in three models, namely a basic model (adjusted for gestational age at birth, child sex, cell type proportions and batch), a main model (additionally adjusted for maternal age, education level, early-pregnancy BMI and smoking), and a childhood BMI model (additionally adjusted for childhood BMI at 10 years). The statistical models for DNA methylation measured in 10-year-old children were the same, with the only difference that they were adjusted for child age at the time of measurement instead of gestational age at birth. We adjusted for leukocyte subtypes using the cord blood-specific Gervin reference for the cord blood analyses and the Reinius reference set for the analyses at 10 years using the minfi Bioconductor package in R.[39-42] Included covariates were based on previous studies and strong correlations with DNA methylation and liver fat.[2 6] Since the outcome liver fat had a skewed distribution, it was natural log-transformed. Multiple testing was accounted for using Bonferroni correction, with CpGs with a *p* value <1.0 x 10^{-7} considered significant. Additionally, we planned to report results using FDR correction for multiple testing, using the method by Benjamini and Hochberg.[43] Third, we identified differentially methylated regions using the dmrff package (https://github.com/perishky/dmrff), which identifies differentially methylated regions by combining EWAS summary statistics from nearby CpGs.[44] Significant differentially methylated regions were defined based on the following criteria: 1. Within one differentially methylated region, the distance between two neighboring probes can be at most

500 base pairs; 2. the regions have nominal EWAS *p* values <0.05 and 3. EWAS effect estimates for the individual CpGs in a differentially methylated regions have the same direction. All analyses were performed using R version 3.4.3.[26]

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SUPPLEMENTARY MATERIAL

Further detailed online resources can be found in the published article online: https://clinicalepigeneticsjournal.biomedcentral.com/articles/10.1186/s13148-019-0799-6#Sec21.

3.3 Liver fat and cardio-metabolic risk factors among school age children

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ABSTRACT

Background: Non-alcoholic fatty liver disease is a major risk factor for cardio-metabolic disease in adults. The burden of liver fat and associated cardio-metabolic risk factors in healthy children is unknown.

Methods: In a population-based prospective cohort study among 3,170 10-year-old children, we assessed whether both liver fat accumulation across the full range and non-alcoholic fatty liver disease are associated with cardio-metabolic risk factors already in childhood. Liver fat fraction was measured by Magnetic Resonance Imaging and non-alcoholic fatty liver disease was defined as liver fat fraction ≥5.0%. We measured body mass index, blood pressure, and insulin, glucose, lipids and C-reactive protein concentrations. Cardio-metabolic clustering was defined as having three or more risk factors out of high visceral fat mass, high blood pressure, low high-density-lipoprotein cholesterol or high triglycerides, and high insulin concentrations.

Results: Non-alcoholic fatty liver disease prevalences were 1.0%, 9.1% and 25.0% among normal weight, overweight and obese children, respectively. Both higher liver fat within the normal range (<5.0% liver fat) and non-alcoholic fatty liver disease were associated with higher blood pressure, insulin resistance, total-cholesterol, triglycerides and C-reactive protein concentrations (*p* values <0.05). As compared to children with <2.0% liver fat, children with ≥5.0% liver fat had the highest odds of cardio-metabolic clustering (Odds Ratio 24.43 (95% Confidence Interval 12.25, 48.60)). The associations remained similar after adjustment for body mass index and tended to be stronger in overweight and obese children.

Conclusions: Higher liver fat is, across the full range and independently of body mass index, associated with an adverse cardio-metabolic risk profile already in childhood. Future preventive strategies focused on improving cardio-metabolic outcomes in later life may need to target liver fat development in childhood.

INTRODUCTION

Non-alcoholic fatty liver disease is a major risk factor for cardio-metabolic disease, end-stage liver disease and subsequent need for liver transplantation.[1-4] In adults, non-alcoholic fatty liver disease is associated with cardiovascular disease, dyslipidemia, type 2 diabetes mellitus and metabolic syndrome.[1 3 5 6] Due to high rates of childhood overweight and obesity, non-alcoholic fatty liver disease has become the most common chronic liver disease in children in western countries.[3 7] The estimated prevalence in children varies from 3% to 11%, depending on population characteristics and diagnostic methods.[2 8 9] Studies on the cardio-metabolic consequences of non-alcoholic fatty liver disease in children are scarce. Previous studies in small population-based samples, among older or only obese children, suggested that non-alcoholic fatty liver disease is associated with increased risks of insulin resistance, hypertension and dyslipidemia.[5 7 10-14] It is not known whether liver fat also influences cardio-metabolic risk factors in children without obesity or non-alcoholic fatty liver disease. The limited number of studies focused on liver fat in children is partly due to the difficulty in measuring liver fat. Liver biopsy is the gold standard for diagnosing non-alcoholic fatty liver disease, but is not possible to perform in population-based samples.[2 6] Magnetic Resonance Imaging (MRI) enables non-invasive measurement of liver fat.[15 16]

We performed a cross-sectional analysis among 3,170 10-year-old children participating in a population-based prospective cohort study to examine whether liver fat accumulation across the full range and non-alcoholic fatty liver disease assessed with MRI are associated with cardio-metabolic risk factors.

METHODS

Study population

This study was embedded in the Generation R Study, a population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[17] The study has been approved by the Medical Ethical Committee of the Erasmus University Medical Center in Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained from parents for all participants.[17] All children were born between April 2002 and January 2006. In total, 4,245 children attended the MRI subgroup study at 10 years. None of these children had a history of jaundice, medication use, alcohol use, smoking and drugs, based on information from questionnaires at 10 years. We included children with at least one cardio-metabolic outcome available. The population for analysis of this subgroup study comprised 3,170 children (**Figure S1**). Missing measurements were mainly due to no data on liver fat, MRI-artefacts or blood sampling.

Liver fat at 10 years

We measured liver fat using a 3.0 Tesla MRI (Discovery MR750w, GE Healthcare, Milwaukee, Wisconsin, United States).[15-18] The children wore light clothing without metal objects while undergoing the body scan. A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique (IDEAL IQ) for generating a precise liver fat fraction image.[19] The IDEAL IQ scan is based on a carefully tuned 6-echo echo planar imaging acquisition. The obtained fat-fraction maps were analyzed by the Precision Image Analysis (PIA, Kirkland, Washington, United States) using the sliceOmatic (TomoVision, Magog, Canada) software package. All extraneous structures and any image artifacts were removed manually.[20] Liver fat fraction was determined by taking four samples of at least 4cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat estimation. Liver fat measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[21 22] As previously described, non-alcoholic fatty liver disease was defined as liver fat ≥5.0%.[7 18 22] To study the associations across the full spectrum, liver fat was first categorized into six categories (0.0 – 0.9, 1.0 – 1.9, 2.0 – 2.9, 3.0 – 3.9, 4.0 – 4.9, and >5.0%). Since only 5 children were in the 0.0 – 0.9 group, we combined them with the 1.0 – 1.9 group. In total 5 categories were used; <2.0% (n = 1,590), 2.0 to 2.9% (n = 1,160), 3.0 to 3.9% (n = 250), 4.0 to 4.9% (n = 80) and ≥5.0% (n = 90). The reference group was <2.0%, since it is the largest group and contains the median of the sample. Due to lower numbers, no further subcategories were possible for >5.0% liver fat.

Cardio-metabolic risk factors at 10 years

We measured blood pressure at the right brachial artery four times with one minute intervals, using the validated automatic sphygmanometer Datascope Accutor Plus (Paramus, New Jersey, United States).[23] We calculated the mean value for systolic and diastolic blood pressure using the last three blood pressure measurements of each participant. Thirty minute fasting venous blood samples were collected to measure glucose, insulin, total-cholesterol, HDL-cholesterol, triglycerides and C-reactive protein concentrations.[17] We consider the 30 minutes fasting samples non-fasting samples. This time-interval was chosen because of the design of our study, in which it was not possible to obtain fasting samples from all children. Glucose, total cholesterol, HDL-cholesterol, C-reactive protein and triglycerides concentrations were measured using the c702 module on the Cobas 8000 analyzer. Insulin was measured with electrochemiluminescence immunoassay (ECLIA) on the E411 module (Roche, Almere, the Netherlands). Concentrations of LDL-cholesterol were calculated according to the Friedewald formula.[24] Insulin resistance was estimated with the homeostatic model assessment of insulin resistance (HOMA-IR) using the formula: insulin resistance = (insulin (μU/L) x glucose (mmol/l)) / 22.5.[25] Visceral fat mass was obtained by MRI scans, as previously described.[17 26] We defined children with clustering of cardio-metabolic risk factors being at risk for metabolic syndrome phenotype, in line with previous studies.[27 28] Clustering of cardio-metabolic risk factors was defined as having three or more

out of the following four adverse risk factors: visceral fat mass above the 75th percentile; systolic or diastolic blood pressure above the 75th percentile; HDL-cholesterol below the 25th percentile or triglycerides above the 75th percentile; and insulin above the 75th percentile of our study population.

Covariates

At enrolment in the study, we obtained maternal education level and pre-pregnancy weight by questionnaires, measured maternal height and calculated pre-pregnancy BMI. Information on child age and sex was obtained from medical records, and on ethnicity from questionnaires. We measured childhood height and weight, both without shoes and heavy clothing, calculated BMI at 10 years, and further calculated sex- and age- adjusted childhood BMI standard deviation scores (SDS) (Growth Analyzer 4.0, Dutch Growth Research Foundation).[29] Childhood BMI was categorized into underweight, normal weight, overweight and obesity, using the International Obesity Task Force cutoffs.[30]

Statistical analysis

First, we examined differences in subject characteristics between childhood BMI groups with ANOVA tests for continuous variables and Chi-square tests for categorical variables. We used similar methods to assess the differences for cardio-metabolic risk factors between children with and without non-alcoholic fatty liver disease in normal weight, overweight and obese children. For non-response-analyses, we compared participants and non-participants with Student's t-tests, Mann-Whitney tests and Chi-square tests.

Second, we used linear regression models to assess the associations of liver fat across the full range and non-alcoholic fatty liver disease, both compared to the reference group, with cardio-metabolic risk factors at 10 years. Analyses were performed for the total group and also separately for normal weight and overweight or obese children, to which we further refer as overweight children.

Third, we used logistic regression models to assess the associations of liver fat in categories with the odds of adverse levels of single and clustered cardio-metabolic risk factors at 10 years. Only cases with complete data on cardio-metabolic outcomes were used for the analyses with clustered cardio-metabolic risk factors. For all analyses, we presented a basic model, adjusted for child age, sex and ethnicity; and a confounder model, which was additionally adjusted for maternal pre-pregnancy BMI and education. Since we were interested in the associations of liver fat with cardio-metabolic risk factors independently of BMI, we analyzed an extra model, which was additionally adjusted for child BMI at 10 years (BMI model). We adjusted for child BMI in a separate model to observe the additional confounding effect of BMI in our associations. Covariates were included in the models based on previous studies, strong correlations with liver fat, risk of non-alcoholic fatty liver disease and with cardio-metabolic risk factors, and if they changed the effect estimates >10%.[2 8] Since insulin, HOMA-IR, triglycerides and C-reactive protein concentra-

tions were skewed, we used their natural logged values in all linear regression analyses. Due to a violation of the normality of the residuals assumption in the linear regression models, caused by a skewed distribution of liver fat, we also log-transformed liver fat when used continuously. To enable comparison of effect sizes of different measures, we constructed SDS ((observed value – mean) / SD) for all variables. We found a statistically significant interaction between liver fat and BMI for systolic blood pressure, HOMA-IR, triglycerides and C-reactive protein. No statistical interactions between liver fat and sex or between liver fat and ethnicity were observed in the associations with cardio-metabolic risk factors. As sensitivity analyses, we repeated the analyses with adjustment for visceral fat mass instead of BMI, to explore whether any association was affected by visceral fat. Missing data of covariates were multiple-imputed using Markov chain Monte Carlo approach. Five imputed datasets were created and analyzed together. All statistical analyses were performed using the Statistical Package of Social Sciences version 25.0 for Windows (SPSS IBM, Chicago, Illinois, United States).

RESULTS

Subject characteristics

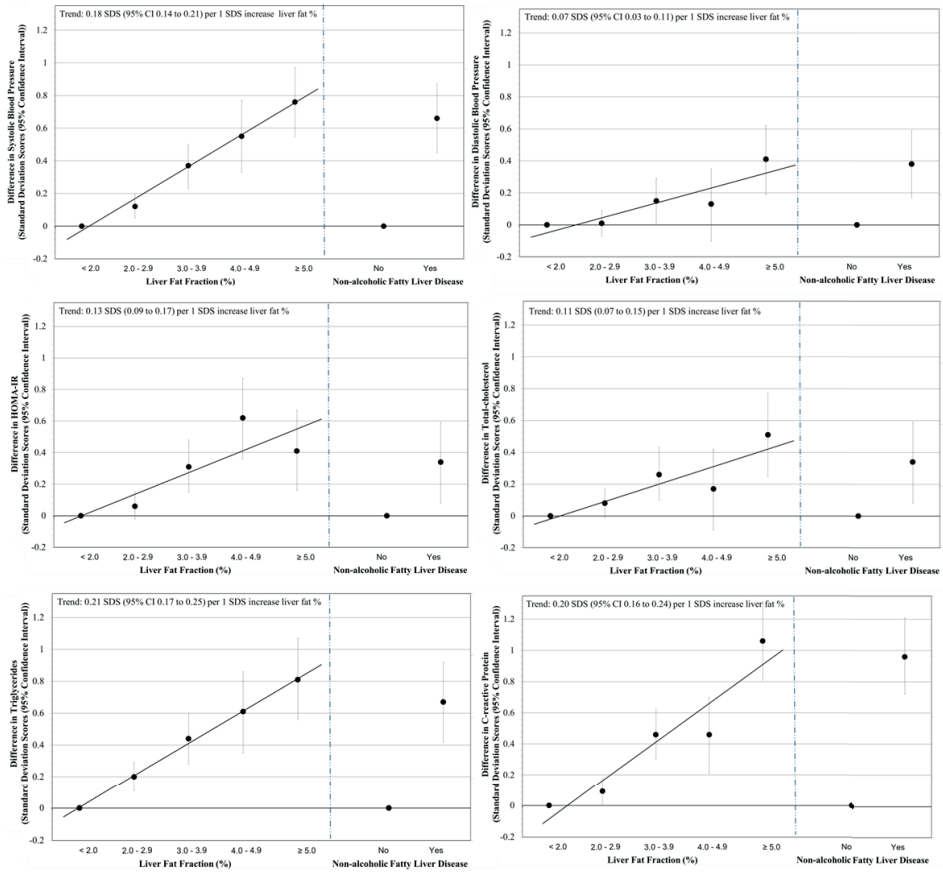
The median liver fat fraction was 1.8% (95% range: 1.1, 3.1), 2.0% (95% range: 1.2, 4.1), 2.5% (95% range: (1.4, 8.7) and 3.1% (95% range: 1.7, 17.9) in underweight, normal weight, overweight and obese children, respectively (**Table 1**). Prevalences of non-alcoholic fatty liver disease were 2.8% (n = 90) in the total group and 1.0% (n = 26), 9.1% (n = 41) and 25.0% (n = 23) in children with normal weight, overweight and obesity, respectively. We observed in all BMI groups higher levels of adverse cardio-metabolic risk factors in children with non-alcoholic fatty liver disease, compared to those without non-alcoholic fatty liver disease (**Table 2**). Non-response analyses showed that participants were slightly more often European and had lower BMI compared to non-participants (**Table S1**).

Liver fat and cardio-metabolic risk factors

Higher liver fat and non-alcoholic fatty liver disease were associated with higher systolic and diastolic blood pressure, HOMA-IR, and total-cholesterol, triglycerides and C-reactive protein concentrations (*p* values <0.05)(**Figure 1**). As compared to the reference group of children with <2.0% of liver fat, children with ≥5.0% of liver fat tended to have the strongest associations with the cardio-metabolic risk factors (differences for systolic blood pressure (0.76 (95% CI 0.55, 0.97) SDS), diastolic blood pressure (0.41 (95% CI 0.19, 0.62) SDS), HOMA-IR (0.41 (95% CI 0.16, 0.67) SDS, total-cholesterol (0.51 (95% CI 1.24, 3.67) SDS), triglycerides (0.81 (95% CI 0.56, 1.07) SDS), and C-reactive protein (1.06 (95% CI 0.81, 1.31) SDS). **Figure S2** shows similar results for the basic models. These associations of liver fat and non-alcoholic fatty liver disease with cardio-metabolic risk factors were also present after additional adjustment for childhood BMI (**Figure S3**). Liver fat

and non-alcoholic fatty liver disease were positively associated with insulin and LDL-cholesterol, negatively associated with HDL-cholesterol and no associations were observed with glucose (**Table S2**). Stratified analyses showed that the associations of liver fat and non-alcoholic fatty liver disease with cardio-metabolic outcomes were present among both normal weight and overweight children, with a tendency for stronger effect estimates among overweight children (**Table S3**). The sensitivity analyses using visceral fat instead of BMI, showed no consistent differences in associations of liver fat and non-alcoholic fatty liver disease with cardio-metabolic risk factors (**Table S4**).

Figure 1. Associations of liver fat fraction and non-alcoholic fatty liver disease with cardio-metabolic risk factors at school age



Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in childhood cardio-metabolic risk factors in SDS per SDS change in childhood liver fat fraction as compared to the reference group (children with <2.0% of liver fat; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5.0% of liver fat; right side of each figure). Associations are adjusted for child's age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. BMI, body mass index; CI, Confidence Interval; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

Table 1. Subject characteristics

	Total (n = 3,170)	**Underweight** (n = 212)	**Normal weight** (n = 2,410)	**Overweight** (n = 456)	**Obesity** (n = 92)	***p* value**
Maternal characteristics						
Age, mean (SD), years	31.1 (4.9)	31.3 (4.7)	31.4 (4.7)	30.0 (5.2)	29.0 (5.9)	<0.01
Prepregnancy body mass index, mean (SD), kg/m^2	23.5 (4.2)	21.8 (3.2)	23.1 (3.8)	25.4 (4.6)	28.8 (6.1)	<0.01
Parity, n (%), nulliparous	1769 (55.8)	132 (62.3)	1325 (55.0)	257 (56.4)	55 (59.8)	0. 18
Education, n (%), higher education	1540 (52.7)	109 (54.5)	1273 (57.2)	150 (36.1)	8 (10.0)	<0.01
Child characteristics						
Age, mean (SD), years	9.8 (0.3)	9.8 (0.4)	9.8 (0.3)	9.9 (0.4)	9.8 (0.3)	0.03
Boys, n (%)	1563 (49.3)	112 (52.8)	1221 (50.7)	191 (41.9)	39 (42.4)	<0.01
Ethnicity, n (%), European	2118 (68.2)	150 (72.1)	1706 (72.2)	229 (51.3)	33 (37.5)	<0.01
Birth weight, mean (SD), g	3445 (557)	3264 (575)	3458 (549)	3483 (553)	3376 (679)	<0.01
Body mass index, mean (SD), kg/m^2	17.5 (2.7)	14.1 (0.5)	16.8 (1.3)	21.3 (1.2)	23.5 (2.1)	<0.01
Visceral fat mass, median (95% range), g	358 (161; 982)	245 (135; 478)	338 (162; 709)	602 (268; 1216)	853 (362; 1862)	<0.01
Liver fat fraction, median (95% range), %	2.0 (1.2; 5.3)	1.8 (1.1; 3.1)	2.0 (1.2; 4.1)	2.5 (1.4; 8.7)	3.1 (1.7; 17.9)	<0.01
Prevalence non-alcoholic fatty liver disease, n (%)	90 (2.8)	1 (0.5)	26 (1.0)	41 (9.1)	23 (25.0)	<0.01
Systolic blood pressure, mean (SD), mmHg	103.3 (8.0)	99.3 (7.6)	102.4 (7.5)	107.3 (7.8)	113.0 (8.8)	<0.01
Diastolic blood pressure, mean (SD), mmHg	58.6 (6.4)	57.6 (6.3)	58.4 (6.4)	59.5 (6.5)	61.8 (7.6)	<0.01
Insulin, median (95% range), pmol/l	182 (35.2; 629.1)	144 (27.9; 471.8)	172 (34.5; 569.2)	242 (48.0; 798.2)	339 (45.2; 1178.0)	<0.01
Glucose, mean (SD), mmol/l	5.3 (0.9)	5.3 (1.0)	5.3 (1.0)	5.2 (0.8)	5.3 (0.7)	0.11
HOMA-IR, median (95% range)	7.0 (1.1; 28.8)	5.7 (0.9; 22.8)	6.6 (1.1; 26.9)	9.3 (1.6; 32.1)	12.4 (1.5; 50.5)	<0.01
Total – cholesterol, mean (SD), mmol/l	4.3 (0.7)	4.3 (0.6)	4.3 (0.6)	4.4 (0.7)	4.5 (0.7)	<0.01
HDL – cholesterol, mean (SD), mmol/l	1.5 (0.3)	1.6 (0.4)	1.5 (0.3)	1.4 (0.3)	1.2 (0.2)	<0.01
LDL – cholesterol, mean (SD), mmol/l	2.3 (0.6)	2.3 (0.5)	2.3 (0.6)	2.5 (0.6)	2.6 (0.7)	<0.01
Triglycerides, median (95% range), mmol/l	1.0 (0.4; 2.6)	0.87 (0.4; 2.2)	0.9 (0.4; 2.5)	1.1 (0.5; 2.9)	1.5 (0.5; 3.8)	<0.01

Table 1. Subject characteristics (continued)

	Total (n = 3,170)	**Underweight** (n = 212)	**Normal weight** (n = 2,410)	**Overweight** (n = 456)	**Obesity** (n = 92)	***p* value**
C-reactive protein, median (95% range), mg/l	0.3 (0.3; 5.7)	0.3 (0.3; 6.1)	0.3 (0.3; 4.4)	0.9 (0.3; 10.2)	1.5 (0.3; 14.2)	<0.01
Prevalence cardio-metabolic clustering, n (%)	254 (13.3)	2 (1.8)	106 (7.2)	114 (42.1)	32 (72.7)	<0.01

Values are observed, but not imputed data and represent means (SD), medians (95% range) or numbers of subjects (valid %). Differences between BMI categories were tested using one-way ANOVA tests for continuous variables and χ^2-test for categorical variables. HOMA-IR was calculated using the formula: insulin resistance = (insulin (μU/L) x glucose (mmol/l)) / 22.5. LDL-cholesterol is calculated according to the Friedewald formula. Cardio-metabolic clustering was defined as having three or more risk factors (high (>75th percentile) visceral fat mass, high (>75th percentile) systolic or diastolic blood pressure, low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides, and high (>75th percentile) insulin. The prevalence of cardio-metabolic clustering was calculated in a subgroup of complete cases (n = 1,906). HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n, number; SD, standard deviation.

Table 2. Children with and without non-alcoholic fatty liver disease among different body mass index groups with cardio-metabolic risk factors

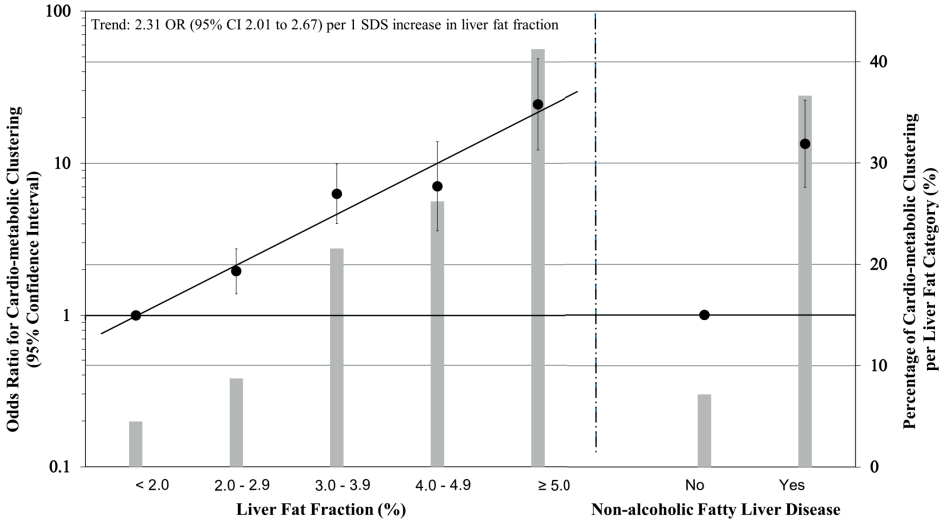
	Systolic blood pressure (mmHg) mean (SD)	**Diastolic blood pressure** (mmHg) mean (SD)	**HOMA-IR** median (95% range)	**Total – cholesterol** (mmol/l) mean (SD)	**Triglycerides** (mmol/l) median (95% range)	**C-reactive protein** (mg/l) median (95% range)
Children with normal weight ***Non-alcoholic Fatty Liver Disease,*** *n (%)*						
No; 2,384 (99.0)	102.4 (7.5)	58.4 (6.4)	6.6 (1.8;19.6)	4.3 (0.6)	0.9 (0.4;2.5)	0.3 (0.3;4.3)
Yes; 26 (1.0)	104.7 (7.9)	61.7 (5.9)	6.5 (1.5;21.4)	4.4 (0.8)	1.39 (0.3;3.5)	0.3 (0.3;34.0)
Children with overweight ***Non-alcoholic Fatty Liver Disease,*** *n (%)*						
No; 415 (90.9)	107.1 (7.6)	59.3 (6.4)	8.8 (2.5;23.3)	4.4 (0.7)	1.1 (0.5;2.9)	0.7 (0.3;8.7)
Yes; 41(9.1)	109.2 (9.8)	60.9 (7.0)	10.6 (2.5;37.6)	4.9 (0.8)	1.4 (0.5;3.0)	1.8 (0.3;18.1)
Children with obesity ***Non-alcoholic Fatty Liver Disease,*** *n (%)*						
No; 69(75.0)	112.6 (8.7)	61.4 (7.8)	13.7 (2.3;45.5)	4.6 (0.7)	1.4 (0.4;3.8)	1.4 (0.3;9.6)
Yes; 23(25.0)	114.0 (9.2)	62.7 (6.9)	11.4 (4.5;40.7)	4.3 (0.7)	1.6 (0.6;3.0)	1.9 (0.3;18.0)

Values are observed, but not imputed data and represent means (SD), medians (95% range) or numbers of subjects (valid %). HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n, number; SD, standard deviation.

Liver fat and clustering of cardio-metabolic risk factors

In children with non-alcoholic fatty liver disease the prevalence of cardio-metabolic clustering was 66.7% (n = 30) compared to a prevalence of 12.0% (n = 224) in children without non-alcoholic fatty liver disease. **Figure S4** and **Figure S5** show liver fat continuously with cardio-metabolic clustering present and not present, respectively. Higher liver fat was associated with higher odds of cardio-metabolic clustering, already from a liver fat fraction of ≥2.0% onwards (*p* values <0.05) (**Figure 2**). As compared to the reference group of children with <2.0% of liver fat, children with ≥5.0% of liver fat had the highest odds of cardio-metabolic clustering (Odds Ratio (OR) 24.43 (95% CI 12.25, 48.60)). The strongest association for liver fat was observed with high visceral fat mass, with an OR 27.80 (95% CI 14.50, 53.30) (**Figure 7** in Supplemental Material). **Figure 6** in Supplemental Material shows similar results for the basic models and the associations were not materially affected after further adjustment for childhood BMI (**Figure 8** in Supplemental Material). Due to the moderate correlation between liver fat and visceral fat, we also performed the analyses for the cardio-metabolic clustering excluding visceral fat, these showed slightly smaller but still statistically significant odds ratios (**Table 5** in Supplemental Material).

Figure 2. Associations of liver fat fraction and non-alcoholic fatty liver disease with odds of clustering of cardio-metabolic risk factors at school age



Values are odds ratios (95% Confidence Intervals) analyzed in a subgroup of cases with complete data for all cardio-metabolic variables (n = 1,906) that reflect the risk of cardio-metabolic clustering per increase in liver fat fraction as compared to the reference group (<2.0%; left side of the figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5.0% of liver fat; right side of the figure). Bars represent the percentage of cardio-metabolic clustering per liver fat fraction group. Cardio-metabolic clustering was defined as having three or more risk factors (high (>75th percentile) visceral fat mass, high (>75th percentile) systolic or diastolic blood pressure, low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides, and high (>75th percentile) insulin. Associations are adjusted for child age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. SDS, standard deviation scores. Trend lines is given only when *p* value for linear trend <0.05.

DISCUSSION

We observed that not only non-alcoholic fatty liver disease but also a higher liver fat across the full range is associated with an adverse cardio-metabolic profile in school age children. Adverse cardio-metabolic clustering was already observed from a liver fat fraction of ≥2.0% onwards. The associations were independent of BMI and tended to be stronger in overweight and obese children than in normal weight children.

Interpretation of main findings

Non-alcoholic fatty liver disease has a prevalence of up to 30% in the general adult population.[6 31] Due to the high rates of childhood overweight and obesity, non-alcoholic fatty liver disease has also become the most common chronic liver disease in children in the developed world.[3 18] Previous studies in selected populations estimated childhood prevalences of non-alcoholic fatty liver disease between 3% and 11%. The differences in prevalences were mainly due to heterogeneity in sample selection and diagnostic methods.[2 8 9] In a population-based sample, using a sensitive imaging-based method for liver fat assessment, we observed a prevalence of 2.8% for non-alcoholic fatty liver disease in all children with the highest prevalence up to 25.0% among obese children. Non-alcoholic fatty liver disease was not only present among obese children, but also among normal weight children. This high prevalence of non-alcoholic fatty liver disease in 10-year-old children is an important population health problem.

Non-alcoholic fatty liver disease is strongly associated with cardiovascular disease, dyslipidemia and type 2 diabetes mellitus in adults.[1 3 5 18] A cross-sectional study in 571 obese children aged 8 – 18 years showed that, as compared to children without non-alcoholic fatty liver disease, those with non-alcoholic fatty liver disease had a higher BMI, insulin resistance and triglycerides concentrations.[5] Three case-control studies reported that children with non-alcoholic fatty liver disease had a more adverse cardio-metabolic profile.[11 12 14] In line with these previous studies, we observed that non-alcoholic fatty liver disease was associated with higher blood pressure, insulin resistance, adverse lipids profile and increased C-reactive protein concentrations at 10 years.

To the best of our knowledge, no previous studies assessed the associations of liver fat accumulation across the full range. The cut-off point for defining non-alcoholic fatty liver disease in children and adults is originally derived from adult studies.[22] We observed that children with liver fat of ≥5.0% had the highest odds of cardio-metabolic risk factor clustering. However, we also observed that even small increases in liver fat from ≥2.0% onwards were associated with adverse cardio-metabolic risk factors. Our results suggest that in children the cut-off for increased risk of an adverse cardio-metabolic risk profile is already between 2.0% and 3.0% liver fat, instead of the current cut-off of ≥5%. We could not test a lower cut-off because in our study group, only 5 children had liver fat lower than 1.0%. These findings suggest that diagnosing non-alcoholic fatty liver disease in children might need a lower threshold than 5.0% liver fat. Current conventional ultrasounds cannot measure this low liver fat percentage, but future improvements in resolution

of ultrasound techniques may enable detection of lower fat percentages. We also observed that the associations of liver fat with cardio-metabolic risk factors in childhood were independent of BMI, and present among both normal weight and overweight children, with stronger effect estimates among overweight children. The combination of a higher liver fat and a higher BMI might exacerbate the adverse cardio-metabolic health profile. Next to BMI, visceral fat is also known to correlate with liver fat.[26] However, our results suggest that the associations of liver fat with cardo-metabolic risk factors were independent of visceral fat. Thus, not only non-alcoholic fatty liver disease, but also small increases in liver fat accumulation within the normal range are, independent of BMI and visceral fat, related to an adverse cardio-metabolic risk profile already in childhood.

The directions of the associations of liver fat with cardio-metabolic risk factors cannot be concluded from a cross-sectional analysis. Future prospective follow-up studies should explore prospectively whether liver fat in childhood leads to increased risks of cardiovascular disease. In our study, we will perform follow-up studies in cardiovascular risk factors at age 18 years. Several mechanisms have been described linking liver fat with cardio-metabolic risk factors.[4] Increased visceral fat mass may alter lipid metabolism and trigger insulin resistance, that may subsequently lead to non-alcoholic fatty liver disease and cardiovascular disease.[4 32 33] On the other hand, liver fat can be the source of systemic release of inflammatory cytokines and pro-atherogenic factors leading to cardio-metabolic diseases, including hypertension.[3 4 26 33] Findings from previous studies suggest a strong association of non-alcoholic fatty liver disease with the metabolic syndrome.[4 33] Also, studies in both adults and children showed associations of non-alcoholic fatty liver disease with hypertension as part of the metabolic syndrome.[34-36] Adults with non-alcoholic fatty liver disease had increased carotid-artery intima-media thickness and increased prevalence of carotid atherosclerotic plaques.[35] Possible underlying mechanisms may include chronic inflammation leading to pro-atherogenic factors leading to arterial damage and hypertension.[33] The strong associations of both higher liver fat with systolic blood pressure and with C-reactive protein in our study supports this hypothesis. Prospective analyses or mendelian randomization approaches may help to elucidate the directions of the observed associations. Our study suggests that increased levels of liver fat are common and associated with harmful cardio-metabolic consequences in childhood, predisposing children to cardiovascular disease later in life. Future studies should focus on specific lifestyle related factors influencing liver fat from early childhood onwards.

Methodological considerations

Major strengths of this study are the cross-sectional analysis performed in an ongoing prospective cohort study with a large sample size, with information on liver fat fraction measured with MRI and on cardio-metabolic outcomes in children at a young age. The non-response at MRI visit would lead to biased effect estimates if associations were different between those included and not included in the analyses, but this seems unlikely. We had a relatively small number of children with obesity, which indicates a selection towards a lean population that might affect the

generalizability of our findings. The healthy and young study population possibly also explains the small number of children with liver fat fraction above the clinical cut-off of 5.0%. This might have limited our statistical power to detect significant associations. However, these findings are novel since little data is available on liver fat in healthy children and its relation with cardio-metabolic risk factors. The fasting time before blood sampling was limited to 30 minutes, and thus we consider our samples non-fasting samples.[17] The blood samples were collected at different time-points during the day, depending on time of the study visit. Since glucose and insulin levels shift very easily during the day and are sensitive towards carbohydrate intake, this may have led to non-differential misclassification of children with high- or low glucose and insulin levels and thus underestimation of the observed effect estimates. On the other hand, for lipid levels it has been shown that non-fastening blood sampling is superior to fasting in accurately predicting cardio-metabolic events for adults in later life.[37] Therefore, we believe our findings for triglycerides and cholesterol are less likely influenced by the non-fasting state. Overall, these results need to be carefully interpreted and further studies are needed to replicate our findings with fasting blood samples in children. Since we had a young study population, our results are not likely biased by alcohol use, known history of jaundice, hepatitis, smoking, drugs and medication use. We had no data available on Tanner stages. The pubertal increase of sex hormones may be important in predisposition for non-alcoholic fatty liver disease.[38] In our population we did not observe sex differences, possibly due to the young age. Although many covariates were included, there still might be some residual confounding, as in any observational study.

CONCLUSIONS

Liver fat across the full range is associated with an adverse cardio-metabolic risk profile already in children of school age. The associations were independent of BMI and tended to be stronger in overweight and obese children. Future preventive strategies focused on improving cardio-metabolic outcomes in later life may need to target liver fat metabolism already in young childhood.

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SUPPLEMENTARY MATERIAL

Table S1. Comparison of child characteristics between children included and not included in the analyses

Characteristics	Participants (n = 3,170)	Non-participants (n = 965)	*p* value
Age, mean (SD), years	9.8 (0.3)	9.8 (0.4)	0.10
Boys, n (%)	1563 (49.3)	505 (52.3)	0.10
Ethnicity, n (%), European	2118 (68.2)	576 (61.2)	<0.01
Birth weight, mean (SD), g	3446 (558)	3409 (531)	0.33
Body mass index, mean (SD), kg/m^2	17.5 (2.7)	17.8 (3.0)	0.01
Systolic blood pressure, mean (SD), mmHg	103.3 (8.0)	103.3 (8.1)	0.49
Diastolic blood pressure, mean (SD), mmHg	58.6 (6.4)	58.8 (6.6)	0.61
Insulin, median (95% range), pmol/l	182.3 (35.2, 629.1)	174.3 (35.8, 740.4)	0.61
Glucose, mean (SD), mmol/l	5.3 (0.9)	5.2 (1.0)	0.04
HOMA-IR, median (95% range)	7.0 (1.1, 28.8)	6.7 (1.1, 32.4)	0.34
Total – cholesterol, mean (SD), mmol/l	4.3 (0.7)	4.3 (0.6)	0.38
HDL – cholesterol, mean (SD), mmol/l	1.5 (0.3)	1.5 (0.3)	0.52
LDL – cholesterol, mean (SD), mmol/l	2.3 (0.6)	2.3 (0.6)	0.42
Triglycerides, median (95% range), mmol/l	1.0 (0.4, 2.6)	1.0 (0.4, 2.4)	0.42
C-reactive protein, median (95% range), mg/l	0.3 (0.3, 5.7)	0.3 (0.3, 5.1)	0.28

Values are observed data and represent means (SD), medians (95% range) or numbers of subjects (valid %). Differences were tested using Student's t-tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively and using χ^2-test for dichotomous variables. HOMA-IR was calculated using the formula: insulin resistance = (insulin (μU/L) x glucose (mmol/L)) / 22.5. LDL-cholesterol is calculated according to the Friedewald formula. HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n, number; SD, standard deviation.

Table S2. Associations of liver fat fraction and non-alcoholic fatty liver disease with insulin, glucose, HDL-cholesterol and LDL-cholesterol

	Insulin, Glucose, HDL-cholesterol and LDL-cholesterol at 10 years in Standard Deviation Scores Difference (95% Confidence Interval)			
	Insulin (n = 2,246)	**Glucose** (n = 2,252)	**HDL-cholesterol** (n = 2,253)	**LDL-cholesterol** (n = 2,242)
Liver fat fraction				
Basic model	0.14 (0.10;0.18)*	0.02 (-0.02;0.07)	-0.13 (-0.17;-0.09)*	0.09 (0.04;0.13)*
Confounder model	0.14 (0.09;0.18)*	0.03 (-0.01;0.07)	-0.11 (-0.15;-0.07)*	0.09 (0.05;0.13)*
BMI model	0.06 (0.02;0.11)*	0.05 (0.01;0.10)†	-0.05 (-0.10;-0.01)†	0.05 (0.00;0.09)†
Non-alcoholic Fatty Liver Disease				
Basic model	0.41 (0.16;0.66)*	-0.04 (-0.29;0.21)	-0.37 (-0.61;-0.12)*	0.30 (0.17;0.43)†
Confounder model	0.38 (0.13;0.64)*	-0.03 (-0.29;0.23)	-0.31 (-0.56;-0.06)†	0.31 (0.05;0.56)†
BMI model	0.13 (-0.12;0.38)	0.02 (-0.24;0.28)	-0.11 (-0.36;0.14)	0.16 (-0.09;0.42)

Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in insulin and glucose in SDS per SDS change in childhood liver fat fraction. $^*p$ value <0.01, $^\dagger p$ value <0.05. Associations are adjusted for child age, sex, ethnicity in the basic models, further adjusted for maternal pre-pregnancy BMI and maternal education in the confounder models and additionally adjusted for childhood BMI at ten years of age in the BMI model. N, number; SDS, standard deviation scores.

Table S3. Associations of liver fat fraction and non-alcoholic fatty liver disease with cardio-metabolic risk factors at school age among children with normal weight and with overweight/obesity

	Cardio-metabolic risk factors at 10 years in Standard Deviation Scores Difference (95% Confidence Interval)					
	Systolic blood pressure	**Diastolic blood pressure**	**HOMA-IR**	**Total – cholesterol**	**Triglycerides**	**C-reactive protein**
Liver fat fraction						
Normal weight group	n = 2,323 0.09 (0.04;0.13)*	n = 2,324 0.06 (0.01;0.11)†	n = 1,729 0.07 (0.01;0.13)†	n = 1,729 0.07 (0.01;0.12)†	n = 1,727 0.15 (0.10;0.21)*	n = 1,730 0.07 (0.02;0.12)*
Overweight group	n = 536 0.13 (0.07;0.19)*	n = 536 0.08 (0.02;0.15)†	n = 377 0.14 (0.07;0.22)*	n = 382 0.13 (0.05;0.21)*	n = 380 0.20 (0.12;0.27)*	n = 383 0.20 (0.11;0.29)*
Non-alcoholic Fatty Liver Disease						
Normal weight group	n = 2,323 0.24 (-0.15;0.62)	n = 2,324 0.45 (0.05;0.85)†	n = 1,729 -0.11 (-0.55;0.32)	n = 1,729 0.17 (-0.27;0.60)	n = 1,727 0.47 (0.03;0.91)†	n = 1,730 0.28 (-0.11;0.67)
Overweight group	n = 536 0.37 (0.10;0.63)*	n = 536 0.31 (0.04;0.58)†	n = 377 0.32 (0.00;0.63)	n = 382 0.45 (0.11;0.80)*	n = 380 0.49 (0.17;0.81)*	n = 383 0.76 (0.37;1.14)*

Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in childhood cardio-metabolic risk factors in SDS per SDS change in childhood liver fat fraction or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat). *p value <0.01, †p value <0.05. Associations are adjusted for child's age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n, number; SDS, standard deviation scores.

Table S4. Associations of liver fat fraction and non-alcoholic fatty liver disease with cardio-metabolic risk factors at school age

	Cardio-metabolic risk factors at 10 years in Standard Deviation Scores Difference (95% Confidence Interval) (n = 3,170)					
	Systolic blood pressure	**Diastolic blood pressure**	**HOMA-IR**	**Total – cholesterol**	**Triglycerides**	**C-reactive protein**
Liver fat fraction						
Confounder model	0.17 (0.14;0.21)*	0.07 (0.03;0.11)*	0.13 (0.09;0.17)*	0.11 (0.07;0.15)*	0.21 (0.17;0.25)*	0.20 (0.16;0.24)*
BMI model	0.07 (0.03;0.10)*	0.05 (0.01;0.09)*	0.07 (0.02;0.11)*	0.09 (0.04;0.13)*	0.16 (0.12;0.21)*	0.12 (0.08;0.16)*
Visceral fat model	0.07 (0.03;0.11)*	0.05 (0.00;0.09)*	0.11 (0.06;0.17)*	0.08 (0.03;0.13)*	0.13 (0.08;0.18)*	0.10 (0.05;0.15)*
Non-alcoholic Fatty Liver Disease						
Confounder model	0.66 (0.45;0.87)*	0.38 (0.17;0.59)*	0.34 (0.08;0.59)*	0.45 (0.20;0.70)*	0.67 (0.42;0.93)*	0.96 (0.71;1.21)*
BMI model	0.29 (0.09;0.49)*	0.31 (0.10;0.53)*	0.11 (-0.14;0.37)	0.36 (0.10;0.61)*	0.48 (0.23;0.74)*	0.67 (0.42;0.91)*
Visceral fat model	0.20 (-0.03;0.44)	0.25 (0.00;0.49)*	0.20 (-0.10;0.49)	0.32 (0.03;0.62)*	0.18 (-0.11;0.47)	0.59 (0.31;0.87)*

Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in childhood cardio-metabolic risk factors in SDS per SDS change in childhood liver fat fraction or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat). *p value <0.01, †p value <0.05. Confounder model adjusted for child's age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. BMI model: confounder model additionally adjusted for child BMI at 10 years. Visceral fat model: confounder model additionally adjusted for visceral fat mass at 10 years. HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; n, number; SDS, standard deviation scores.

Table S5. Associations of liver fat fraction with odds of clustering of cardio-metabolic risk factors without visceral fat mass – confounder models

Liver fat fraction (%)	**Clustering of cardio-metabolic risk factors without taking into account visceral fat mass** (n = 3,170)
< 2.0	*Reference group*
2.0 – 2.9	1.36 (1.08;1.71)*
3.0 – 3.9	2.85 (1.94;4.20)*
4.0 – 4.9	2.78 (1.50;5.14)*
≥ 5.0	6.68 (3.48;12,81)*

Values are odds ratios (95% Confidence Intervals) analyzed in a subgroup of complete cases (n = 1,906) that reflect the odds of cardio-metabolic clustering without taking into account visceral fat mass, defined as having two or more out of high (>75th percentile) systolic or diastolic blood pressure, low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides, and high (>75th percentile) insulin for children with increasing liver fat fraction compared to the reference group (children with <2% of liver fat). *p value <0.01. Associations are adjusted for child age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. N, number.

Figure S1. Study participants flowchart

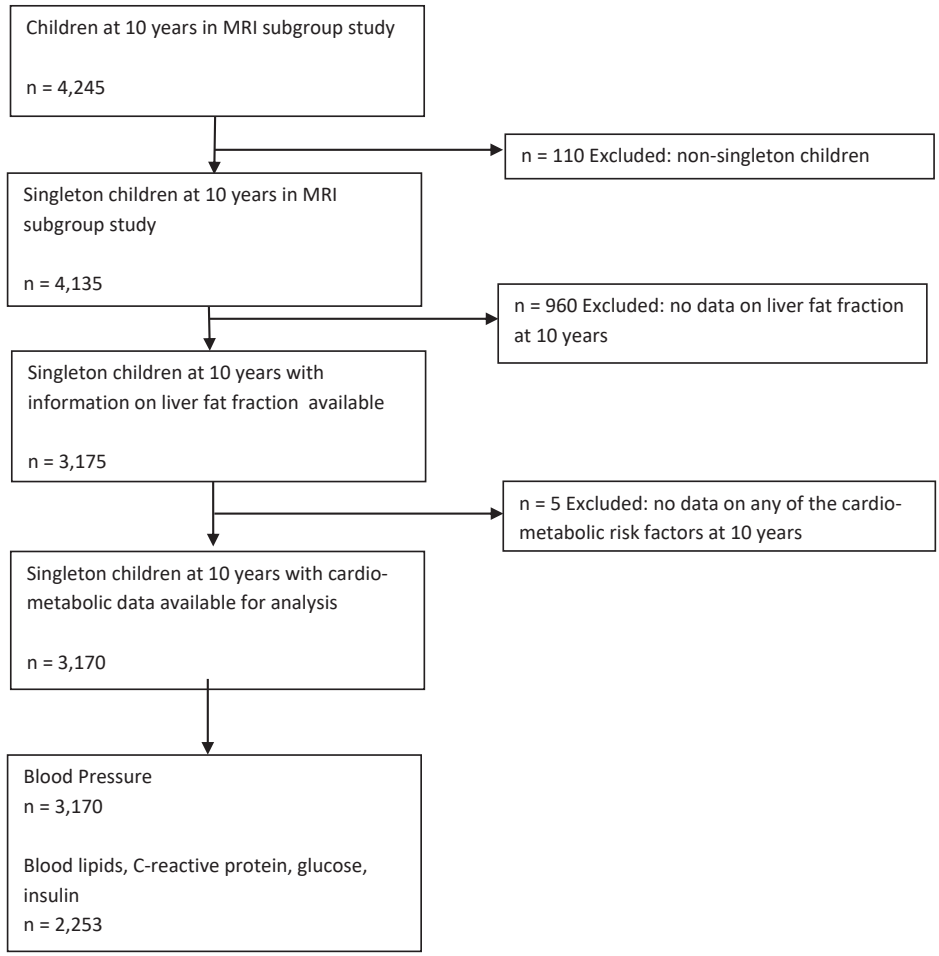
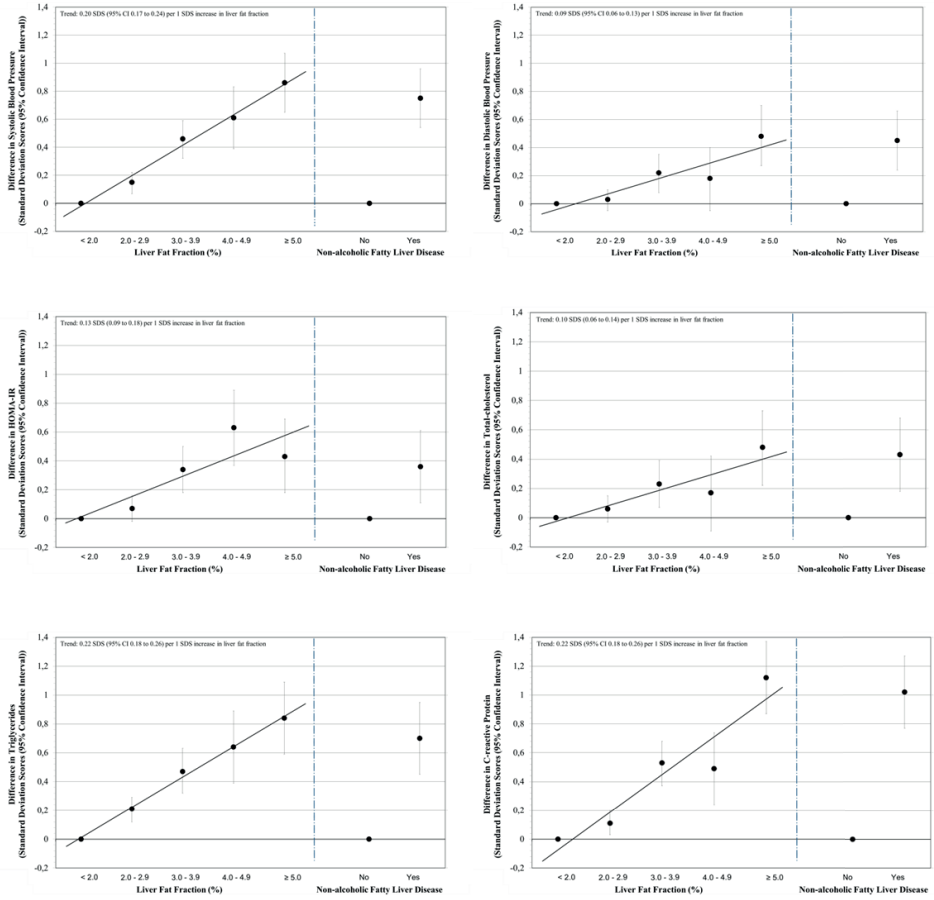
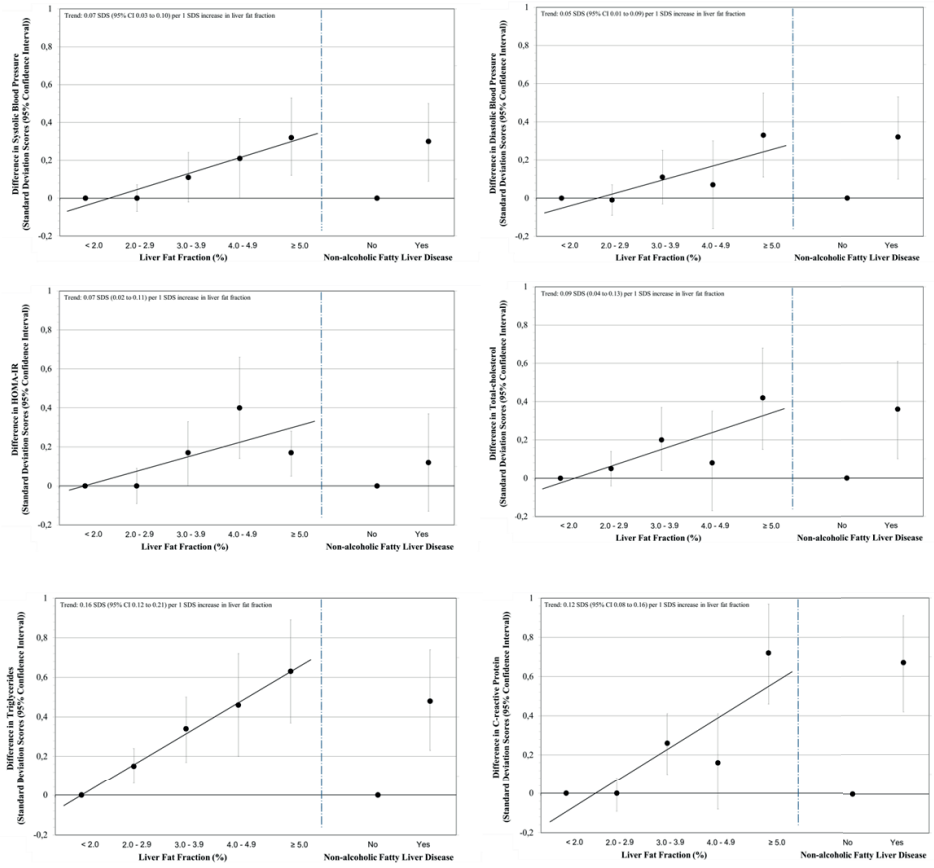


Figure S2. Associations of liver fat fraction and non-alcoholic fatty liver disease with cardio-metabolic risk factors at school age – basic models



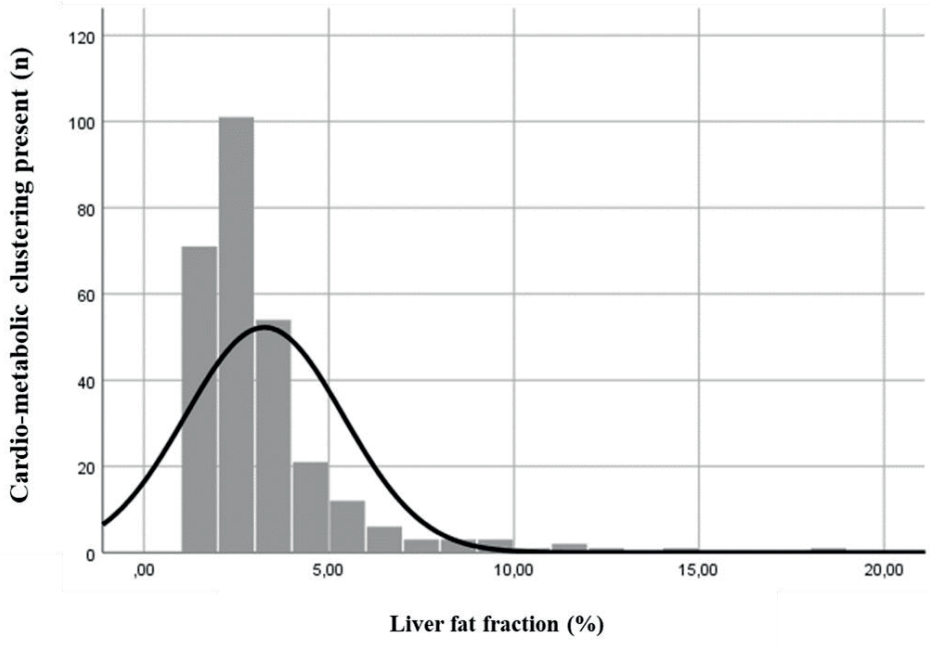
Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in childhood cardio-metabolic risk factors in SDS per SDS change in childhood liver fat fraction as compared to the reference group (children with <2.0% of liver fat; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat; right side of each figure). Associations are adjusted for child age, sex, ethnicity. HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

Figure S3. Associations of liver fat fraction and non-alcoholic fatty liver disease with cardio-metabolic risk factors at school age – body mass index models



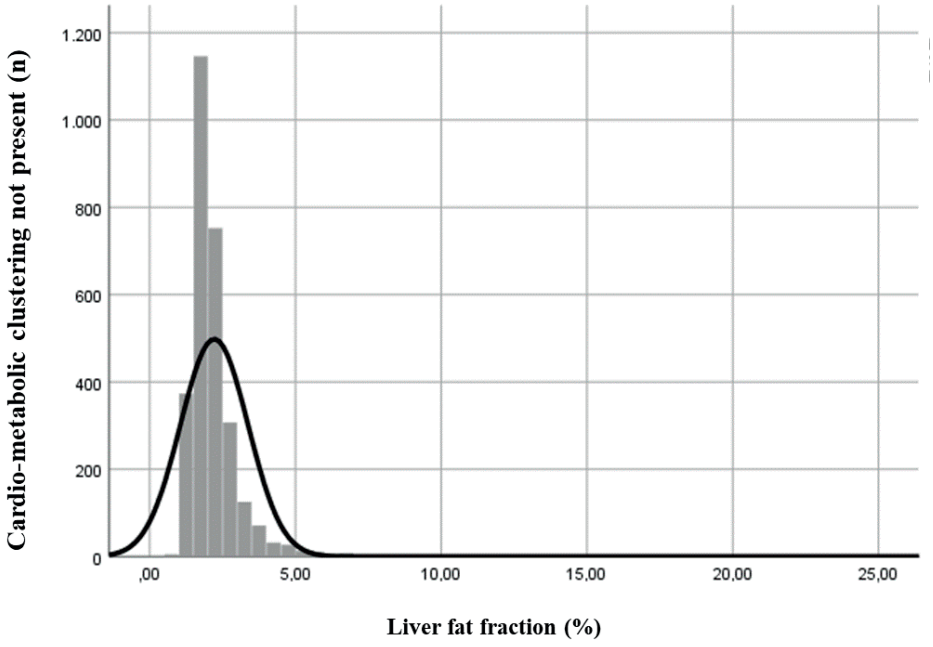
Values are regression coefficients (95% Confidence Intervals) from linear regression models that reflect differences in childhood cardio-metabolic risk factors in SDS per SDS change in childhood liver fat fraction as compared to the reference group (children with <2.0% of liver fat; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat; right side of each figure). Associations are adjusted for child age, sex, ethnicity, maternal pre-pregnancy BMI, maternal education and childhood BMI at ten years of age. HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

Figure S4. Histogram of liver fat continuously of children with cardio-metabolic clustering



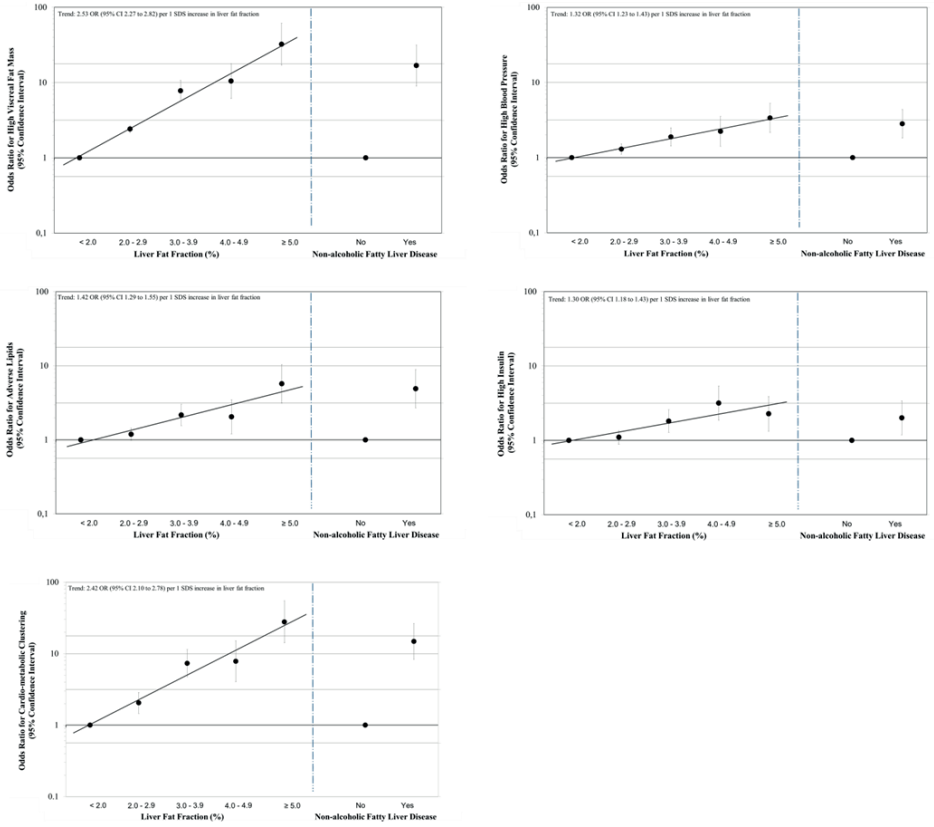
Histogram of liver fat continuously (%) for children with cardio-metabolic clustering present.

Figure S5. Histogram of liver fat continuously of children without cardio-metabolic clustering



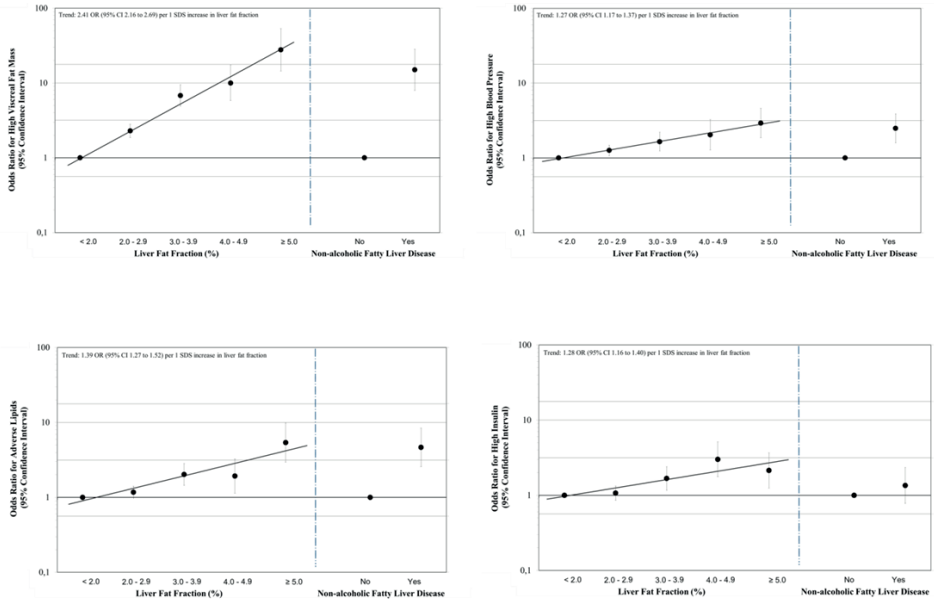
Histogram of liver fat continuously (%) for children without cardio-metabolic clustering present.

Figure S6. Associations of liver fat fraction and non-alcoholic fatty liver disease with odds of adverse levels of single and clustered cardio-metabolic risk factors at school age – basic models



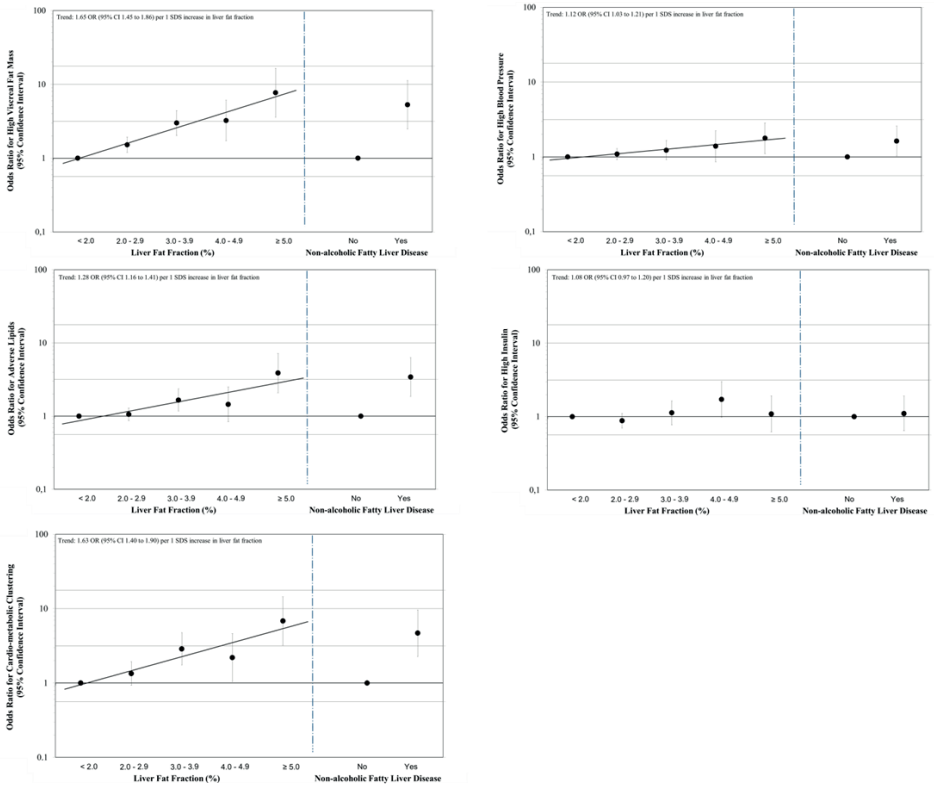
Values are odds ratios (95% Confidence Intervals) that reflect the risk of high (>75th percentile) visceral fat mass, high (>75th percentile) systolic or diastolic blood pressure, low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides, and high (>75th percentile) insulin and of cardio-metabolic clustering per SDS increase in liver fat fraction as compared to the reference group (<2.0%; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat; right side of each figure). Cardio-metabolic clustering was defined as having three or more of these risk factors and was analyzed in a subgroup of cases with complete data for all cardio-metabolic variables (n = 1,906). Associations are adjusted for child age, sex, ethnicity. OR, Odds Ratio; SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

Figure S7. Associations of liver fat fraction and non-alcoholic fatty liver disease with odds of cardio-metabolic risk factors at school age – confounder models



Values are odds ratios (95% Confidence Intervals) that reflect the risk of high (>75th percentile) visceral fat mass, high (>75th percentile) systolic or diastolic blood pressure (shown as high blood pressure), low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides (shown as adverse lipids), and high (>75th percentile) insulin per increase in liver fat fraction as compared to the reference group (<2.0%; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat; right side of each figure). Associations are adjusted for child age, sex, ethnicity, maternal pre-pregnancy BMI and maternal education. SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

Figure S8. Associations of liver fat fraction and non-alcoholic fatty liver disease with odds of adverse levels of single and clustered cardio-metabolic risk factors at school age – body mass index models



Values are odds ratios (95% Confidence Intervals) that reflect the risk of high (>75th percentile) visceral fat mass, high (>75th percentile) systolic or diastolic blood pressure (shown as high blood pressure), low (<25th percentile) HDL-cholesterol or high (>75th percentile) triglycerides (shown as adverse lipids), and high (>75th percentile) insulin and of cardio-metabolic clustering per increase in liver fat fraction as compared to the reference group (<2.0%; left side of each figure), or for children with non-alcoholic fatty liver disease as compared to the reference group (children with <5% of liver fat; right side of the figure). Cardio-metabolic clustering was defined as having three or more of these risk factors and was analyzed in a subgroup of cases with complete data for all cardio-metabolic variables (n = 1,906). Associations are adjusted for child age, sex, ethnicity in the basic models, further adjusted for maternal pre-pregnancy BMI and maternal education in the confounder models and additionally adjusted for childhood BMI at ten years of age in the BMI model. OR, Odds Ratio; SDS, standard deviation scores. Trend lines are given only when *p* value for linear trend <0.05.

3.4 Early-life prediction of childhood non-alcoholic fatty liver disease

Manuscript in progress.

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ABSTRACT

Background: Non-alcoholic fatty liver disease may develop from childhood onwards. Early-life seems to be an important period for the development of obesity and also of non-alcoholic fatty liver disease. We developed an early-life prediction model for childhood non-alcoholic fatty liver disease.

Methods: In a multi-ethnic population-based prospective cohort study among 3,175 children, we measured liver fat fraction at 10 years of age by magnetic resonance imaging. Non-alcoholic fatty liver disease was defined as liver fat fraction ≥5.0%. The area under the receiver operating characteristics curve (AUC) were obtained with multiple logistic regression analyses from eight predefined prediction models.

Results: The main prediction model included child sex, ethnicity, age and body mass index (BMI) at 6 years of age. The AUC of this model was 0.70 (95% Confidence Interval (CI): 0.64; 0.75). The model performance increased with addition of maternal pregnancy factors including: maternal age, education, pre-pregnancy BMI, pregnancy smoking, pregnancy folic acid supplement use, (AUC 0.73 (95% CI: 0.68; 0.78)). In models additionally including infant factors, childhood blood pressure, childhood liver enzyme, and childhood metabolic factors did non-significantly increase the AUC effect estimate. All prediction characteristics combined in the full model yielded the best model performance with an AUC of 0.77 (95% CI: 0.73; 0.82), a sensitivity of 47% at 90% specificity.

Conclusions: We showed that easily obtainable childhood characteristics enable prediction of non-alcoholic fatty liver disease at 10 years of age in a population-based cohort. The discriminative value was further improved by adding early-life and child cardio-metabolic characteristics. If externally validated, these models might serve as a prediction tool in clinical practice for identification of children in mid-childhood at risk for non-alcoholic fatty liver disease in later childhood.

INTRODUCTION

Non-alcoholic fatty liver disease is the most common chronic liver disease in children in industrialized countries, affecting 3% to 11% of the general pediatric population.[1 2] The prevalence of childhood non-alcoholic fatty liver disease is increasing because of the global rise in children with obesity, which is the major risk factor for non-alcoholic fatty liver disease.[2 3] Non-alcoholic fatty liver disease exhibits a spectrum of severity, ranging from simple liver steatosis and fibrosis, to cirrhosis or hepatocellular carcinoma and eventually end-stage liver disease.[4] Children with non-alcoholic fatty liver disease have an increased risk for hypertension, dyslipidemia and insulin resistance.[5 6] Also, children with non-alcoholic fatty liver disease may exhibit advanced liver and cardiovascular disease in young adulthood.[2 7] Diagnosis of non-alcoholic fatty liver disease in children is difficult, due to lack of specific symptoms and of easy methods to measure liver fat.[2] Liver biopsy is the gold standard for diagnosing non-alcoholic fatty liver disease.[1 8] Advanced imaging methods are not feasible with respect to high healthcare costs on population-based level.[1 4 8] Recent North American Society for Pediatric Gastroenterology, Hepatology and Nutrition (NASPGHAN) guidelines for non-alcoholic fatty liver disease screening in children propose the use of alanine aminotransferase (ALT) concentrations.[9] Unfortunately, since ALT cut-off points in children are not clearly defined, ALT screening remains controversial. In addition, measurement of ALT requires blood sampling, which may not be available in all settings. In adults, algorithms have been developed to predict magnetic resonance imaging (MRI)-derived non-alcoholic fatty liver disease based on anthropometric indices and routine blood biomarkers such as ALT, aspartate transaminase (AST), triglycerides and insulin concentrations.[10-14] These adult prediction models have poor performance for prediction of non-alcoholic fatty liver disease in children.[15-17] Thus, currently no accurate validated prediction tools for non-alcoholic fatty liver disease in childhood exist.[17-19] Previously reported associations of maternal pregnancy, infant and childhood factors with childhood liver fat, suggest that early life might be a critical period for liver fat development and non-alcoholic fatty liver disease.[4 20-24] The purpose of this study was to explore whether we can identify a combination of maternal, infant and childhood factors that can predict non-alcoholic fatty liver disease in children.

In a population-based prospective cohort study, we aimed to develop a prognostic prediction model for MRI-diagnosed non-alcoholic fatty liver disease in children 10 years of age based on maternal, infant and childhood characteristics.

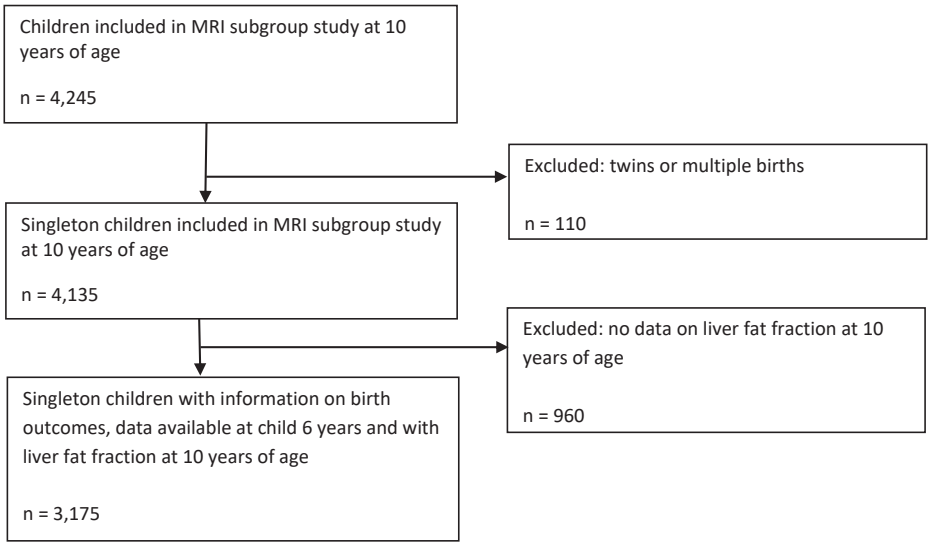
METHODS

Study population

This study was embedded in the Generation R Study. This is a multi-ethnic population-based prospective cohort from early fetal life onwards, based in Rotterdam, the Netherlands.[25] The

study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam (MEC 198.782/2001/31). Written informed consent was obtained for all participants.[26] All pregnant women were enrolled between 2001 and 2005. The enrollment procedure has been described in detail previously.[27] At the follow-up visit at 10 years of age a subgroup of 4,245 children were included for the MRI measurements. For the current study, we included singleton children with MRI-based liver fat measurements at 10 years of age and at least one characteristic in early life or mid-childhood available. After applying exclusions, the population for analyses was 3,175 children (**Figure 1**). None of these children had a history of jaundice, medication use, alcohol use, smoking, or drug use, based on information from questionnaires at 10 years of age completed by parents. Missing measurements were mainly due to no data on liver

Figure 1. Study participants flowchart



fat or MRI artifacts.[26]

Early-life cardio-metabolic characteristics

We selected maternal preconception and pregnancy characteristics, as well as infant characteristics as early-life predictors. In mid-childhood cardio-metabolic characteristics were selected as predictors. We selected the predictors based on their associations with non-alcoholic fatty liver disease in literature in children or adults.[1 4 5 20 21 24 28] The predictors were clustered according to the clinical practice availability and time of assessment within the cohort. In the following paragraphs we describe the way in which the maternal and child characteristics were obtained per cluster of predictors.

Main characteristics: The mid-childhood characteristics in the main model were chosen based on both easy clinical accessibility and known important determinants of non-alcoholic fatty liver disease.[21] Information on child sex was obtained from medical records.[26] Child ethnicity was based on parental countries of birth obtained through questionnaire. A child was considered of Dutch background if both parents were born in the Netherlands, and of non-Dutch origin if one or both of the parents were born abroad. If the parents were born in different countries, the country of birth of the mother determined the ethnic background of the child. We categorized child ethnicity into European n = 2,119 (68.1%), Surinamese n = 229 (7.4%), Turkish n = 165 (5.3%), Moroccan n = 153 (4.9%), Cape Verdean or Dutch Antillean n = 189 (6.0%), and other n = 256 (8.2%). At the follow-up visit around 6 years of age, we measured childhood height and weight, both without shoes and heavy clothing, and calculated body mass index (BMI) as weight (in kg) divided by height (in m) squared and sex- and age-adjusted childhood BMI standard deviation scores based on Dutch reference growth charts (Growth Analyzer 4.0, Dutch Growth Research Foundation).[29] Child BMI categories were obtained using the International Obesity Task Force cutoffs.[30]

Maternal pregnancy characteristics: Information was obtained by questionnaires on maternal age, education level (highest completed education), smoking, folic acid supplement use, and pre-pregnancy weight.[25] Maternal height was measured at study enrolment and pre-pregnancy BMI was calculated and categorized in clinical categories.

Infant characteristics: Information about gestational age at birth was obtained from medical records and on breastfeeding in infancy by questionnaire.[26] Infant sugar-containing beverage intake was assessed with the food-frequency questionnaire at a mean age of 13.6 months (standard deviation (SD) 1.8).[31] As previously defined, we converted the intake of sugar-containing beverages consumption into the number of servings per day, with 1 serving equaling 150 grams (NEVO-2011).[32]

Childhood blood pressure: Blood pressure was measured at the right brachial artery four times with one-minute intervals, using the validated automatic sphygmomanometer Datascope Accutor Plus (Paramus, New Jersey).[33] We calculated the mean value for systolic and diastolic blood pressure using the last three blood pressure measurements of each participant.

Childhood metabolic markers: Non-fasting blood samples were collected to determine serum concentrations of ALT, total cholesterol, triglycerides, and C-Reactive Protein with the c702 module of the Cobas 8000 analyzer, and for insulin with the electrochemiluminescence immunoassay on the E411 module of the Cobas 8000 analyzer (Roche, Almere, The Netherlands). Quality control samples demonstrated intra- and interassay coefficients of variation ranging from 1.34% to 1.98%, 0.77 % to 1.39%, and 0.87 to 2.40%, respectively.

Liver fat at 10 years

We measured liver fat using a 3.0 Tesla MRI scanner (Discovery MR750w, GE Healthcare, Milwaukee, Wisconsin, United States) as described previously.[8 9 26 34] A liver fat scan was performed using a single-breath-hold, 3D volume and a special 3-point proton density weighted Dixon technique

(IDEAL IQ) for generating a precise liver fat fraction image.[35] The IDEAL IQ scan is based on a carefully tuned 6-echo echo planar imaging acquisition. The obtained fat fraction maps were analyzed by the Precision Image Analysis (PIA) (Kirkland, Washington, United States) using the sliceOmatic (TomoVision, Magog, Canada) software package. All extraneous structures and any image artifacts were removed manually.[36] Liver fat fraction was determined by taking four samples of at least 4cm^2 from the central portion of the hepatic volume. Subsequently, the mean signal intensities were averaged to generate an overall mean liver fat estimation. Liver fat measured with IDEAL IQ using MRI is reproducible, highly precise and validated in adults.[37 38] Non-alcoholic fatty liver disease was defined as liver fat ≥5.0%.[9 38 39] We studied liver fat fraction dichotomized in low, <5.0%, and high, ≥5.0%, based on the clinical cutoff for non-alcoholic fatty liver disease.[40]

Statistical analysis

A non-response analysis was conducted to compare characteristics of mothers and children with and without liver MRI scan measurements with Student's t-tests, Mann-Whitney tests and Chi-square tests. All variables were categorized in clinical categories and a missing category was added to allow for missing values when using the final risk score. We used different predefined multivariable logistic regression models to assess the discriminative value for non-alcoholic fatty liver disease at 10 years of age. Eight models were constructed with clustering of variables based on literature, timing of assessment and ease of clinical accessibility, starting with a model with mid-childhood characteristics, then in addition to mid-childhood characteristics we assessed prediction clusters separately and prediction clusters combined.[1 5 20 21 28] The eight predefined models were (1) main model including child sex, ethnicity, age and BMI at 6 years; thereafter the main model combined with (2) maternal pregnancy model including maternal age, education, pre-pregnancy BMI, smoking, and folic acid supplement use; (3) infant model including gestational age at birth, ever breastfed, and sugar-containing beverage intake at 1 year of age; (4) childhood blood pressure model including systolic blood pressure and diastolic blood pressure at 6 years of age; (5) childhood liver enzyme model including ALT at 6 years of age; (6) childhood metabolic model including total cholesterol, triglycerides, C-Reactive Protein, and insulin concentrations at 6 years of age. Last, two models combing previous predictors: (7) easily obtainable characteristics including the main model combined with the variables included in the maternal pregnancy, infant and child blood pressure models; (8) full model including the main model combined with all other models. The predicted values from these regression models were obtained. The model performance on discriminative ability was assessed by calculation of the Area Under the Receiver Operating Characteristic curves (AUC), along with the sensitivity at different specificity levels. We compared the AUCs of the main model with the other models using the DeLong tests.[41] Positive and negative predictive values, and positive and negative likelihood ratios were calculated. The AUC analyses were repeated for each cluster without the main model characteristics. We assessed in secondary analyses the risk prediction for non-alcoholic fatty liver disease at 10 years of age for three children with different combinations of risk factors using the main model and

the model with the best AUC. As a sensitivity analysis, we explored whether the observed AUCs were affected by missing not at random in the blood samples, we repeated the analyses for the liver enzyme and childhood metabolic models in a subgroup of cases with complete data for all cardio-metabolic variables (n = 1,976). The statistical analyses were performed using the Statistical Product and Service Solutions (SPSS) Statistics version 25.0 for Windows (IBM, Chicago, Illinois, United States), and R version 3.6.3 (R Foundation for Statistical Computing) (R Core Team 2020).

RESULTS

Subject characteristics

Table 1 shows that 90 (2.8%) children of the included 3,175 children had MRI-diagnosed non-alcoholic fatty liver disease at 10 years of age. Non-response analyses showed that children without MRI-measured liver fat assessment were more likely to have mothers with lower educational attainment, the children were less often of European and more often of Turkish ethnic background and had a slightly higher BMI at 6 years of age (**Table S1**).

Prediction of child non-alcoholic fatty liver disease

The discriminative performance for prediction of non-alcoholic fatty liver disease of the main model was moderate (AUC 0.70 (95% Confidence Interval (CI): 0.64; 0.75), with a sensitivity of 32% at 90% specificity) (**Figure 2** and **Table 2**). The model performance increased significantly with addition of the separate cluster of maternal pregnancy model (AUC 0.73 (95% CI: 0.68; 0.78)). Also, the combined cluster models improved the main model performance significantly (*p* values for model comparison to the main model all <0.05) (**Table 2**). The infant, childhood blood pressure and childhood metabolic biomarkers models slightly improved the AUC, but the differences with the main model were not significant. Also, adding ALT did not improve the model performance. Performance of the full model was fair (AUC 0.77 (95% CI: 0.73; 0.82), with a sensitivity of 47% at 90% specificity, a positive predictive value of 12.1% and negative predictive value of 98.3%, a positive likelihood ratio of 4.7 and negative likelihood ratio of 0.59) (**Table 2**). The odds ratios for the individual predictors of non-alcoholic fatty liver disease at 10 years of age in the final model are given in **Table 3**. **Table S2** shows that without the mid-childhood characteristics of the main model considerably lower model performances were observed for all models of the early-life and child cardio-metabolic characteristics.

We calculated the non-alcoholic fatty liver disease risk prediction profiles for three children with different combinations of risk factors using the main and full prediction models (**Figure 3,** using the risk calculator **Excel sheet 1**)). A child with a healthy risk profile in mid-childhood had a risk of 0.7% and 1.4% for non-alcoholic fatty liver disease at 10 years of age according to the main and full model, respectively. For a child with an unhealthy risk profile in mid-childhood the risk

Table 1. Study population characteristics

	Total group	**No NAFLD (liver fat <5.0)** n = 3,085 (97.2%)	**Yes NAFLD (liver fat ≥5.0)** n = 90 (2.8%)	***p* value**
Maternal characteristics				
Age at enrollment, years	31.1 ± 4.9	31.1 ± 4.8	30.6 ± 5.5	0.38
Pre-pregnancy BMI, kg/m^2	22.6 (18.0, 34.9)	22.5 (18.0, 34.7)	24.6 (18.7, 40.6)	**<0.01**
BMI at child 6 years, kg/m^2	24.8 (19.1, 39.5)	24.8 (19.1, 39.5)	24.9 (19.5, 39.7)	0.90
Education, higher	1,542 (52.7)	1,514 (53.2)	28 (35.4)	**<0.01**
Smoking during pregnancy, stopped or no smoking	2,127 (82.3)	2,064 (82.2)	63 (85.1)	0.51
Folic acid supplement use, yes	1,444 (71.3)	1,402 (71.5)	42 (65.6)	0.30
Birth characteristics				
Sex, female	1,606 (50.6)	1,560 (50.6)	46 (51.1)	0.92
Gestational age at birth, weeks	40.1 (35.8, 42.3)	40.1 (35.8, 42.3)	39.7 (33.7, 42.5)	**<0.01**
Child ethnicity				
European	2,119 (68.1)	2,072 (68.5)	47 (43.4)	**<0.01**
Surinamese	229 (7.4)	221 (7.3)	8 (9.1)	0.53
Turkish	165 (5.3)	150 (5.0)	15 (17.0)	**<0.01**
Moroccan	153 (4.9)	150 (5.0)	3 (3.4)	0.50
Cape Verdean or Dutch Antilles	189 (6.1)	178 (5.9)	11 (12.5)	**0.01**
Other	256 (8.2)	252 (8.3)	4 (4.5)	0.20
Early-childhood characteristics				
Ever breastfed, yes	2,493 (92.9)	2,430 (93.0)	63 (87.5)	0.07
Sugar-containing beverages, servings/day at 1 year	1.0 (0.0, 3.7)	1.0 (0.0, 3.7)	1.3 (0.0, 5.1)	0.19
Mid-childhood characteristics				
Age at 6 years, years	6.0 ± 0.4	6.0 ± 0.4	6.1 ± 0.5	0.13
BMI at 6 years, kg/m^2	15.8 (13.6, 20.3)	15.7 (13.6, 20.0)	17.3 (14.0, 23.8)	**<0.01**
Systolic blood pressure, mmHg	102.1 ± 7.8	102.0 ± 7.8	105.1 ± 8.6	**<0.01**
Diastolic blood pressure, mmHg	60.3 ± 6.5	60.2 ± 6.5	62.5 ± 8.0	**0.02**
ALT u/l	20.0 ± 5.3	20.0 ± 5.4	19.9 ± 3.9	0.88
Total cholesterol, mmol/l	4.2 ± 0.6	4.2 ± 0.6	4.4 ± 0.6	0.07
Triglycerides, mmol/l	1.0 (0.4, 2.4)	1.0 (0.4, 2.4)	1.1 (0.4, 3.0)	0.17
C-Reactive Protein, mg/l	0.3 (0.1, 9.3)	0.3 (0.1, 9.3)	0.7 (0.0, 20.4)	**<0.01**
Insulin, pmol/l	110.4 (17.0, 393.9)	110.2 (16.9, 394.2)	121.2 (23.8, 406.2)	0.70
Late-childhood characteristics				
Age at 10 years, years	9.8 ± 0.3	9.8 ± 0.3	9.8 ± 0.4	0.60
BMI at 10 years, kg/m^2	16.9 (14.0, 24.2)	16.9 (14.0, 23.8)	21.4 (14.7, 29.8)	**<0.01**
Liver fat fraction, %	2.0 (1.2, 5.3)	2.0 (1.2, 4.1)	6.5 (5.1, 19.5)	**<0.01**

Values are observed and represent numbers (valid %), means ± SD, or medians (95% range). Abbreviations: BMI, body mass index; NAFLD, non-alcoholic fatty liver disease.

Table 2. Model performance metrics of child non-alcoholic fatty liver disease prediction models

Non-alcoholic fatty liver disease at 10 years of age									
Models	**AUC** (95% Confidence Interval)	***p* value between AUCs***	**Sensitivity at specificity of**			**Positive predicted value**	**Negative predicted value**	**Positive likelihood ratio**	**Negative likelihood ratio**
			70%	80%	90%				
Main	0.70 (0.64; 0.75)	*Reference*	57	48	32	8.5%	97.8%	3.2	0.76
Early-life characteristics									
Maternal	0.73 (0.68; 0.78)	0.04	59	46	37	9.7%	98.0%	3.7	0.70
Infant	0.72 (0.67; 0.78)	0.11	62	46	36	9.5%	98.0%	3.6	0.71
Child cardio-metabolic characteristics									
Blood pressure	0.72 (0.66; 0.77)	0.24	60	51	32	8.5%	97.8%	3.2	0.76
Liver enzyme	0.70 (0.66; 0.77)	0.37	60	44	32	8.5%	97.8%	3.2	0.76
Metabolic biomarkers	0.72 (0.67; 0.78)	0.20	59	54	34	9.0%	97.9%	3.4	0.73
Combined characteristics									
Easily obtainable	0.76 (0.71; 0.81)	<0.01	71	52	42	10.9%	98.2%	4.2	0.64
Full	0.77 (0.73; 0.82)	<0.01	72	58	47	12.1%	98.3%	4.7	0.59

The Area Under the Receiver Operating Characteristic Curves (AUC) are shown for the prediction of non-alcoholic fatty liver disease at 10 years of age. **p* values are obtained using DeLong's test for comparison of the AUC of the main model with the AUC of the other models. The prevalence of non-alcoholic fatty liver disease was 2.8% (n = 90) in our study population. The sensitivity is given for different specificities derived of the corresponding AUCs. The positive and negative predicted value, and positive and negative likelihood ratio of the predictions models are calculated for a specificity of 90% with corresponding sensitivity. Main model: child sex, ethnicity, age and BMI at 6 years. Maternal model: main model with maternal age, education, smoking during pregnancy, folic acid supplement use during pregnancy, and pre-pregnancy BMI. Infant model: main model with gestational age at birth, ever breastfed, and sugar-containing beverage intake at 1 year of age. Blood pressure model: main model with systolic blood pressure and diastolic blood pressure at 6 years of age. Liver enzyme model: main model with ALT at 6 years of age. Cardio-metabolic biomarkers: main model with total cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age. Easy obtainable model: main combined with early-life characteristics and with blood pressure at 6 years of age. Full model: main model combined with early-life and child cardio-metabolic characteristics. Abbreviations: AUC, Area Under the Receiver Operating Characteristic Curves; NAFLD, Non-alcoholic fatty liver disease.

was 28.1% and 93.1% for non-alcoholic fatty liver disease at 10 years of age according to the main and full model, respectively.

Sensitivity analyses

When we repeated the analyses in the subgroup with complete blood sample information, we observed similar AUC effect estimates and no significant differences (**Table S3**).

Table 3. Odds ratios of the full model for non-alcoholic fatty liver disease at 10 years of age

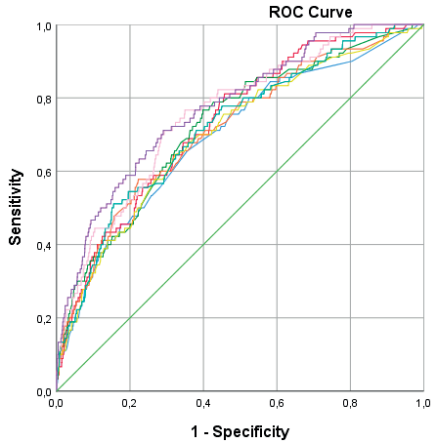
		Non-alcoholic fatty liver disease
Variable	**Categories**	**Odds Ratio** (95% Confidence Interval)
Intercept		0.00
Child sex	Girls (n = 1,606)	*Reference*
	Boys (n = 1,569)	1.08 (0.70; 1.69)
Child ethnicity	European (n = 2,119)	*Reference*
	Surinamese (n = 229)	1.18 (0.52; 2.68)
	Turkish (n = 165)	2.71 (1.35; 5.45)
	Moroccan (n = 153)	0.46 (0.13; 1.62)
	Cape Verdean or Dutch Antilles (n = 189)	1.79 (0.83; 3.87)
	Other (n = 256)	0.64 (0.22; 1.85)
	Missing (n = 64)	0.69 (0.13; 3.70)
Child age at 6 years	<6 years (n = 1,734)	*Reference*
	6 - 7 years (n = 1,116)	1.74 (1.06; 2.87)
	≥7 years (n = 114)	1.07 (0.34; 3.41)
	Missing (n = 211)	1.97 (0.69; 2.81)
Child BMI at 6 years	Underweight (n = 358)	1.44 (0.70; 2.99)
	Normal weight (n = 2,003)	*Reference*
	Overweight (n = 199)	3.39 (1.76; 6.53)
	Obesity (n = 40)	2.53 (0.68; 9.41)
	Missing (n = 575)	1.07 (0.53; 2.13)
Maternal age	<25 years (n = 364)	0.89 (0.46; 1.69)
	25-35 years (n = 2,177)	*Reference*
	≥35 years (n = 634)	1.20 (0.68; 2.12)
Educational level	High (n = 1,542)	*Reference*
	Low (n = 1,384)	1.37 (0.79; 2.36)
	Missing (n = 249)	1.45 (0.58; 3.65)
Maternal smoking	No (n = 2,127)	*Reference*
	Yes (n = 458)	0.83 (0.42; 1.64)
	Missing (n = 590)	1.10 (0.61; 1.99)
Folic acid supplement use	Yes (n = 817)	*Reference*
	No (n = 1,207)	0.82 (0.49; 1.39)
	Missing (n = 1,151)	0.66 (0.37; 1.18)
Pre-pregnancy BMI	<25 kg/m^2 (n = 1,713)	*Reference*
	25.0-30.0 kg/m^2 (n = 446)	1.86 (1.01; 3.39)*
	≥30.0 kg/m^2 (n = 164)	1.86 (0.83; 4.14)
	Missing (n = 852)	1.26 (0.70; 2.29)

Table 3. Odds ratios of the full model for non-alcoholic fatty liver disease at 10 years of age (continued)

		Non-alcoholic fatty liver disease
Variable	**Categories**	**Odds Ratio** (95% Confidence Interval)
Gestational age at birth	Preterm (n = 173)	1.61 (0.72; 3.60)
	Aterm (n = 2,829)	*reference*
	Missing (n = 133)	0.91 (0.09; 8.93)
Ever breastfed	Yes (n = 2,493)	*Reference*
	No (n = 191)	1.48 (0.68; 3.20)
	Missing (n = 491)	0.50 (0.19; 1.34)
Sugar-containing beverages intake at 1 year of age	<2 servings per day (n = 1,540)	*Reference*
	≥2 servings per day (n = 353)	2.55 (1.25; 5.19)
	Missing (n = 1,282)	0.83 (0.42; 1.66)
Systolic blood pressure at 6 years of age	<100 mmHg (n = 1,156)	*Reference*
	100 - 110 mmHg (n = 1,166)	1.71 (0.94; 3.12)
	≥110 mmHg (n = 450)	1.70 (0.76; 3.81)
Diastolic blood pressure at 6 years of age	<60 mmHg (n = 1,365)	*Reference*
	60 - 70 mmHg (n = 1,209)	0.62 (0.36; 1.08)
	≥70 mmHg (n = 198)	1.66 (0.74; 3.71)
Blood pressure	*Missing (n = 403)*	0.58 (0.16; 2.05)
ALT concentrations	<20 u/l (n = 1,079)	*Reference*
	20 - 25 u/l (n = 653)	1.15 (0.62; 2.12)
	≥25 u/l (n = 280)	0.57 (0.21; 1.54)
Total cholesterol concentrations	<4.0 mmol/l (n = 719)	*Reference*
	4.0 - 4.5 mmol/l (n = 637)	1.65 (0.78; 3.49)
	4.5 - 5.0 mmol/l (n = 440)	1.56 (0.70; 3.49)
	≥5.0 mmol/l (n = 184)	1.66 (0.64; 4.36)
Triglycerides concentrations	<1.0 mmol/l (n = 1,069)	*Reference*
	1.0 - 1.5 mmol/l (n = 623)	1.24 (0.64; 2.41)
	≥1.5 mmol/l (n = 324)	1.66 (0.76; 3.61)
C-Reactive Protein concentrations	<1.0 mg/l (n = 1,552)	*Reference*
	1.0 - 5.0 mg/l (n = 356)	1.61 (0.83; 3.12)
	≥5.0 mg/l (n = 118)	2.79 (1.08; 7.23)
Insulin concentrations	<50 pmol/l (n = 381)	*Reference*
	50 - 100 pmol/l) (n = 530)	1.48 (0.57; 3.80)
	100 - 150 pmol/l) (n = 407)	1.83 (0.69; 4.86)
	150 - 200 pmol/l (n = 267)	0.74 (0.20; 2.74)
	≥200 pmol/l (n = 417)	1.51 (0.56; 4.05)
Blood sample	*Missing* (n = 1,173)	3.25 (1.14; 9.25)

Values are odds ratios for the predicted risk of child non-alcoholic fatty liver disease at 10 years of age assessed with the full model. Full model: child sex, ethnicity, age and BMI at 6 years, maternal age, education, smoking during pregnancy, folic acid supplement use during pregnancy, pre-pregnancy BMI, gestational age at birth, ever breastfed, sugar-containing beverage intake at 1 year of age, systolic blood pressure and diastolic blood pressure at 6 years of age, ALT, total cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age.

Figure 2. Screening performance for child non-alcoholic fatty liver disease



Models	AUC (95% Confidence Interval)
Main (light blue line)	0.70 (0.64; 0.75)
Early-life characteristics	
Maternal pregnancy (red line)	0.73 (0.68; 0.78)
Infant (dark green line)	0.72 (0.67; 0.78)
Child cardio-metabolic characteristics	
Chilhood blood pressure (orange line)	0.72 (0.66; 0.77)
Childhood liver enzyme (yellow line)	0.70 (0.66; 0.77)
Childhood metabolic (turquoise line)	0.72 (0.67; 0.78)
Combined characteristics	
Easy obtainable (pink line)	0.76 (0.71; 0.81)
Full (purple line)	0.77 (0.73; 0.82)

Eight models were used to calculate the Area Under the Receiver Operating Characteristic Curves (AUC) for the prediction of non-alcoholic fatty liver disease at 10 years of age. The prevalence of non-alcoholic fatty liver disease was 2.8% (n = 90) in our study population. Main model: child sex, ethnicity, age and BMI at 6 years of age. Maternal pregnancy model: main model with maternal age, education, maternal smoking during pregnancy, folic acid supplement use during pregnancy, and pre-pregnancy BMI. Infant model: main model with gestational age at birth, ever breastfed, and sugar-containing beverage intake at 1 year. Childhood blood pressure model: main model with systolic blood pressure and diastolic blood pressure at 6 years of age. Childhood liver enzyme model: main model with ALT at 6 years of age. Childhood metabolic model: main model with total-cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age. Easy obtainable model: main combined with maternal pregnancy, infant and childhood blood pressure models. Full model: main model combined with early-life and child cardio-metabolic characteristics.

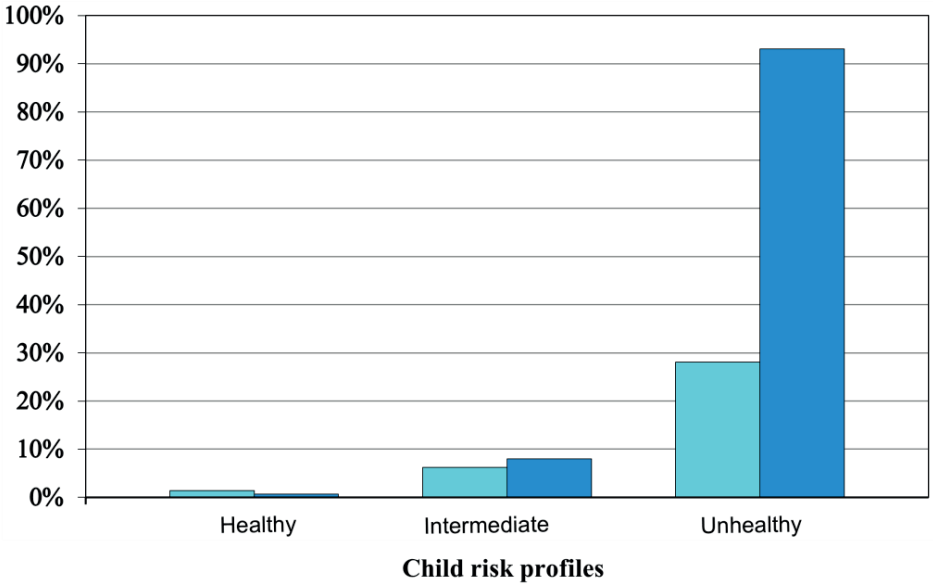
DISCUSSION

In this population-based prospective cohort study, we observed that easily obtainable child characteristics can moderately identify children at risk for developing non-alcoholic fatty liver disease in later childhood. The discriminative value of this prediction model was further improved with addition of maternal pregnancy, infant and child cardio-metabolic characteristics. The full model had a fair performance for the prediction of child non-alcoholic fatty liver disease in later childhood in a multi-ethnic low-risk population. After external validation, these models might serve as a prediction tool in clinical practice for identification of children in mid-childhood at risk for non-alcoholic fatty liver disease in later childhood.

Interpretation of main findings

The increasing prevalence of non-alcoholic fatty liver disease among children emphasizes the need for adequate early identification of children who will most likely benefit from preventive strategies to prevent liver fat accumulation development. Non-alcoholic fatty liver disease in children is a difficult disease to diagnose, as it is mostly a clinically silent disease and it is not easy to accurately measure liver fat.[2] The gold standard for diagnosing non-alcoholic fatty liver disease

Figure 3. Risk predictions for child non-alcoholic fatty liver disease for different health



Values are percentages for the predicted risk of child non-alcoholic fatty liver disease at 10 years of age for 3 children with different example health profiles in mid-childhood. The risk predictions were calculated with both the main and full model. Main model: child sex, ethnicity, age and BMI at 6 years. Full model: main model with maternal age, education, smoking during pregnancy, folic acid supplement use during pregnancy, pre-pregnancy BMI, gestational age at birth, ever breastfed, sugar-containing beverage intake at 1 year of age, systolic blood pressure and diastolic blood pressure at 6 years of age, ALT, total-cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age.

Healthy: risk profile represents a girl of 5 years of age with Dutch ethnic background and with normal weight. She has a mother who was 32 years of age during her pregnancy, with a high level of educational attainment, who did not smoke during pregnancy, who took folic acid supplements during pregnancy, and who had a pre-pregnancy BMI of 23.5 kg/m^2. The girl was born a term, and she was breastfed. Her intake of sugar-containing beverages around 1 year of age were below 1 serving per day on average. At 5 years of age she has a blood pressure of 90 / 55 mmHg, her blood concentrations are: ALT 19.5 u/l, total-cholesterol 3.2 mmol/l, triglycerides 1.1 mmol/l, C-Reactive Protein 0.8 mg/l, and insulin 75 pmol/l.

Intermediate: risk profile represents a boy of 7 years of age with Dutch Antillean ethnic background and with underweight. He has a mother who was 23 years of age during his pregnancy, with a low level of educational attainment, no information about smoking during pregnancy, who took folic acid supplements during pregnancy, and who a pre-pregnancy BMI of 21.0 kg/m^2. The boy was born a term, he was breastfed. His intake of sugar-containing beverages around 1 year of age were less than 2 servings per day on average. At 7 years of age he has a blood pressure of 105 / 65 mmHg, his blood concentrations are: ALT 20.5 u/l, total-cholesterol 4.1 mmol/l, triglycerides 1.6 mmol/l, C-Reactive Protein 1.2 mg/l, and insulin 120 pmol/l.

Unhealthy: risk profile of a boy of 6.5 years of age with Turkish ethnic background and with overweight. He has a mother who was 36 years of age during his pregnancy, with a low level of educational attainment, who smoked during pregnancy, who took no folic acid supplements during pregnancy, and a who had a pre-pregnancy BMI of 29.0 kg/m^2. The boy was born preterm, he was not breastfed. His intake of sugar-containing beverages around 1 year of age were more than 2 servings per day on average. At 6.5 years of age he has a blood pressure of 105 / 75 mmHg, his blood concentrations are: ALT 24.5 u/l, total-cholesterol 4.4 mmol/l, triglycerides 2.1 mmol/l, C-Reactive Protein 5.5 mg/l, and insulin 140 pmol/l.

is liver biopsy, or with imaging methods, both are not feasible for routine screening with respect to health care expenditures and biopsy-related risks.[1 4 8] Hence, there is a considerable interest in developing non-invasive, accurate and cost-effective prediction models. Several non-alcoholic fatty liver disease prediction models have been developed in adults to address this issue, but none of these models have sufficiently high predictive ability in children.[15-17] This may partly be

due to differences in risk factors and the unique histological features of pediatric non-alcoholic fatty liver disease compared to adults, with a portal pattern of fibrosis in children rather than a lobar involvement in adults.[2 42] In children only a few diagnostic algorithms have been developed, all of which without using early-life characteristics, and in selected populations, such as among hospitalized children or children with obesity. The diagnostic prediction models consisted of either clinical and routine laboratory tests or of specialized tests, for instance of cytokeratin-18.[43-45] In a study of 203 children admitted to a tertiary care liver unit at a mean age of 12 years, of which 141 (69%) had biopsy-proven non-alcoholic fatty liver disease, the diagnostic Pediatric NAFLD fibrosis Index (PNFI) was developed, which is based on child age, waist circumference and triglyceride concentrations (AUC 0.85 (95% CI: 0.80; 0.90)).[43] The high prevalence rate of non-alcoholic fatty liver disease in this pediatric population makes it difficult to generalize to a population-based pediatric population. Validation of the PNFI in a retrospective chart review study using clinical and laboratory data of 146 children with a mean age of 14 years with obesity and confirmed non-alcoholic fatty liver disease showed inadequate sensitivity in accurately predicting fibrosis.[17] Another study in 56 outpatient children with obesity and a mean age of 10 years developed a diagnostic predictive model for MRI-diagnosed non-alcoholic fatty liver disease using waist-to-height ratio, insulin resistance, adiponectin and ALT (AUC 0.94 (95% CI: 0.89; 0.99); without adiponectin AUC 0.88 (95% CI: 0.79; 0.97)).[18] The population prevalence of non-alcoholic fatty liver disease in that population was 46% (n = 26).[18] This model has not yet been externally validated. To date there are no accurate validated screening tools for child non-alcoholic fatty liver disease in the general population.[17-19] Previously reported associations of maternal pregnancy, infant and childhood factors with childhood liver fat, suggest that early life might be a critical period for liver fat development and non-alcoholic fatty liver disease.[4 20-24] No previous study in children has considered early-life environment for predicting later childhood non-alcoholic fatty liver disease. Development of non-invasive child prediction tools is needed to accurate identify children at risk for non-alcoholic fatty liver disease already on a population level.

We developed different prediction models for prognostic prediction in mid-childhood of non-alcoholic fatty liver disease in later childhood. The models were developed in a population-based cohort study using easy obtainable maternal, infant and childhood characteristics. We predefined the predictors based on their associations with non-alcoholic fatty liver disease in literature.[1 4 5 20 21 24 28] The predictors were clustered *a priori* according to their availability in clinical practice and time of assessment within our cohort. A moderate model performance of our main model was already observed using only four mid-childhood characteristics. These predictors can easily and routinely be obtained in daily clinical practice. The addition of maternal preconception and pregnancy characteristics significantly improved the prediction model. We observed the best model performance for maternal pregnancy, infant, childhood cardio-metabolic characteristics together with the main childhood characteristics to predict child non-alcoholic fatty liver disease in later childhood.

The limited additional predictive value of the cluster with ALT concentrations at 6 years of age is in contrast with the current NASPGHAN guidelines for non-alcoholic fatty liver disease screening in children, which recommends the use of ALT concentrations.[9] Our observations could be due to the relatively healthy population, with ALT concentrations reflecting a relatively healthy range. Possibly, the predictive value of ALT concentrations would be stronger in high-risk populations. On the other hand, this finding is in agreement with studies performed in adults, showing that liver enzymes are not reliably associated with non-alcoholic fatty liver disease prediction as compared to liver biopsy.[46] We found that the prediction models without the main childhood characteristics had an inferior model performance compared to models taking the main childhood characteristics into account as well. These observations underline the importance of the predictors in the main childhood model for assessing the risk of later childhood non-alcoholic fatty liver disease.

After external validation, the proposed predictions models could provide a screening tool to estimate the child non-alcoholic fatty liver disease risk profile in mid-childhood. This could be applied on a population level to identify children who will most likely benefit from strategies to prevent liver fat accumulation development and subsequent associated comorbidities. Lifestyle modifications concerning diet and physical exercise are currently the mainstay of recommendations.[2 47] A recent randomized controlled trial in 40 adolescent boys with non-alcoholic fatty liver disease showed that dietary sugar restriction was associated with significant improvements in liver steatosis, providing strong evidence for the potential benefits of sugar reduction in child-onset non-alcoholic fatty liver disease.[48] Currently, there is a lack of evidence for optimal lifestyle guidelines in children with non-alcoholic fatty liver disease. Further studies should develop lifestyle strategies to prevent child non-alcoholic fatty liver disease.

Methodological considerations

Major strengths of this study are the prospective data collection in an ongoing population-based cohort study with a large sample size and liver fat fraction measured with MRI. The non-response at the MRI visit might have led to selection of a healthier population, which might affect the generalizability of results to high-risk populations. We also had a relatively small number of cases with childhood non-alcoholic fatty liver disease. Therefore, we lacked statistical power to perform stepwise model estimation to select candidate predictors. As an alternative, we predefined predictors based on easy clinical applicability and their associations with non-alcoholic fatty liver disease in the literature.[1 5 20 21 28] External validation of the prediction models is needed to assess generalizability to other pediatric populations. High quality data were obtained for a large number of maternal, infant and child characteristics through hands-on measurements, blood sampling and questionnaires. The fasting time before blood sampling was limited to 30 minutes, and thus we consider our samples non-fasting samples.[26] The blood samples were collected at different time-points during the day, depending on time of the study visit. Since insulin concentrations vary during the day and are sensitive to carbohydrate intake, this may have led to non-differential misclassification of insulin concentrations.and thus underestimation of the observed effect esti-

mates. However, semi-fasted insulin resistance is moderately correlated with fasting values.[49] For lipid concentrations it seems that non-fasting blood sampling is superior to fasting in accurately predicting cardio-metabolic events for adults in later life.[50] Therefore, we believe our findings for total cholesterol and triglycerides are less likely influenced by the non-fasting state. Although we used validated questionnaires to assess lifestyle and socio-demographic characteristics, measurement error, recall bias or reporting bias may still have affected the study and this could have resulted in non-differential misclassification and may have attenuated the studied model performance.

CONCLUSIONS

In mid-childhood, easily obtainable child characteristics can moderately predict which children are at risk for developing non-alcoholic fatty liver disease in later childhood. The discriminative value of this prediction model was further improved with addition of early-life maternal, infant and child cardio-metabolic characteristics. The full model had a fair performance for the prediction of child non-alcoholic fatty liver disease in later childhood in a multi-ethnic low-risk population-based cohort. After external validation, these models might serve as a prediction tool in clinical practice for identification of children in mid-childhood at risk for non-alcoholic fatty liver disease in later childhood.

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SUPPLEMENTARY TABLES CHAPTER 3.4

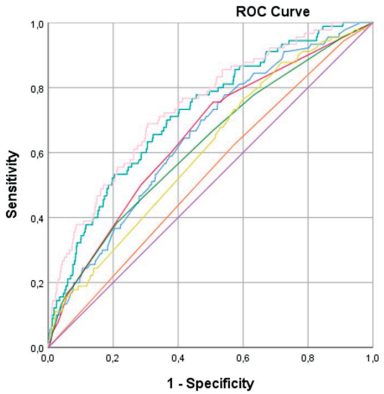
Table S1. Comparison of characteristics between mothers and children with and without outcome measurements

	Participants	**Nonparticipants**	
	n = 3,175	n = 960	***p* value**
Maternal characteristics			
Age at enrollment, years	31.1 ± 4.9	30.9 ± 5.2	0.36
Pre-pregnancy BMI, kg/m^2	22.6 (18.0, 34.9)	22.6 (18.0, 34.0)	0.90
BMI at child 6 years, kg/m^2	24.8 (19.1, 39.5)	24.8 (19.2, 39.6)	0.89
Education, higher	1,542 (52.7)	385 (44.7)	**<0.01**
Smoking during pregnancy, stopped or no smoking	2,127 (82.3)	654 (82.5)	0.90
Folic acid supplement use, yes	1,444 (71.3)	455 (71.8)	0.84
Birth characteristics			
Sex, female	1,606 (50.6)	460 (47.9)	0.15
Gestational age at birth, weeks	40.1 (35.8, 42.3)	40.1 (36.0, 42.3)	0.78
Child ethnicity			
European	2,119 (68.1)	575 (61.4)	**<0.01**
Surinamese	229 (7.4)	61 (6.5)	0.36
Turkish	165 (5.3)	91 (9.7)	**<0.01**
Moroccan	153 (4.9)	58 (6.2)	0.13
Cape Verdean or Dutch Antilles	189 (6.1)	58 (6.2)	0.92
Other	256 (8.2)	93 (9.9)	0.11
Early-childhood characteristics			
Ever breastfed, yes	2,493 (92.9)	702 (93.1)	0.84
Sugar-containing beverages, servings/day at 1 year	1.0 (0.0, 3.7)	1.0 (0.0, 3.8)	0.11
Mid-childhood characteristics			
Age at 6 years, years	6.0 ± 0.4	6.1 ± 0.5	**<0.01**
BMI at 6 years, kg/m^2	15.8 (13.6, 20.3)	15.9 (13.8, 20.8)	**0.02**
Systolic blood pressure, mmHg	102.1 ± 7.8	102.6 ± 8.4	0.08
Diastolic blood pressure, mmHg	60.3 ± 6.5	60.5 ± 7.0	0.46
ALT u/l	20.0 ± 5.3	19.6 ± 5.1	0.14
Total cholesterol, mmol/l	4.2 ± 0.6	4.3 ± 0.6	0.09
Triglycerides, mmol/l	1.0 (0.4, 2.4)	1.0 (0.4, 2.3)	0.38
C-Reactive Protein, mg/l	0.3 (0.1, 9.3)	0.3 (0.1, 13.4)	0.13
Insulin, pmol/l	110.4 (17.0, 393.9)	115.5 (17.0, 392,6)	0.23

Values are observed and represent numbers (valid %). means ± SD, or medians (95% range). Differences were tested using Student *t* tests and Mann-Whitney tests for normally and non-normally distributed variables, respectively, and $\chi 2$ test was used for dichotomous variables.

Table S2. Screening performance for non-alcoholic fatty liver disease of each cluster separately without the main child characteristics

Screening performance in children 6 years of age for non-alcoholic fatty liver disease at 10 years of age

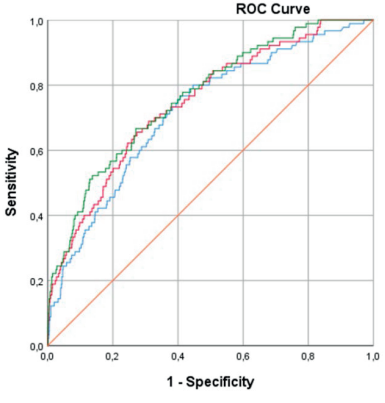


Models	AUC (95% Confidence Interval)	*p* value between AUCs*
Early-life characteristics		
Maternal (light blue line)	0.65 (0.60; 0.70)	0.03
Infant (red line)	0.65 (0.59; 0.71)	0.07
Childcardiometabolic characteristics		
Blood pressure (dark green line)	0.62 (0.56; 0.68)	0.03
Liver enzyme (orange line)	0.53 (0.47; 0.59)	<0.01
Metabolic biomarkers (yellow line)	0.61 (0.55; 0.66)	<0.01
Combined characteristics		
Easy obtainable (turquoise line)	0.72 (0.67; 0.77)	0.30
Full (pink line)	0.74 (0.69; 0.79)	0.36

Seven models were used to calculate the Area Under the Receiver Operating Characteristic Curves (AUC) for the prediction of non-alcoholic fatty liver disease at 10 years of age. The prevalence of non-alcoholic fatty liver disease was 2.8% (n = 90) in the study population. **p* values are obtained using DeLong's test for comparison of the AUC of the models without the main child characteristics with the AUC of the models with the main child characteristics as shown in Table 2 in the manuscript. Maternal model: maternal age, education, maternal smoking during pregnancy, folic acid supplement use during pregnancy, and pre-pregnancy BMI. Infant model: gestational age at birth, ever breastfed, and sugar-containing beverage intake at 1 year. Blood pressure model: systolic blood pressure and diastolic blood pressure at 6 years of age. Liver enzyme model: ALT at 6 years of age. Metabolic biomarkers: total cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age. Easy obtainable model: early-life characteristics combined with blood pressure at 6 years of age. Full model: early-life and child cardiometabolic characteristics combined.

Table S3. Screening performance for non-alcoholic fatty liver disease with complete blood sample information

Screening performance in children 6 years of age for non-alcoholic fatty liver disease at 10 years of age



Models	AUC (95% Confidence Interval)	*p* value between AUCs*
Main (light blue line)	0.69 (0.62; 0.76)	0.92
Early-life characteristics		
Maternal (red line)	0.75 (0.69; 0.81)	0.75
Infant (dark green line)	0.72 (0.66; 0.79)	0.99
Child cardiometabolic characteristics		
Blood pressure (orange line)	0.72 (0.65; 0.78)	0.98
Liver enzyme (yellow line)	0.70 (0.63; 0.77)	0.98
Metabolic biomarkers (turquoise line)	0.72 (0.66; 0.79)	0.95
Combined characteristics		
Easily obtainable (pink line)	0.78 (0.72; 0.84)	0.63
Full (purple line)	0.81 (0.75; 0.86)	0.39

Eight models were used to calculate with Area Under the Receiver Operating Characteristic Curves (AUC) for the prediction of non-alcoholic fatty liver disease at 10 years of age in participants with complete information on blood sampling (n = 1,976). The prevalence of non-alcoholic fatty liver disease was 2.6% (n = 51) in this sub-sample population. **p* values are obtained using DeLong's test for comparison of the AUC of the model in the sub-sample population with the AUC of the full population as shown in Table 2 in the manuscript. Main model: child sex, ethnicity, age and BMI at 6 years of age. Maternal model: main model with maternal age, education, maternal smoking during pregnancy, folic acid supplement use during pregnancy, and pre-pregnancy BMI. Infant model: main model with gestational age at birth, ever breastfed, and sugar-containing beverage intake at 1 year. Blood pressure model: main model with systolic blood pressure and diastolic blood pressure at 6 years of age. Liver enzyme model: main model with ALT at 6 years of age. Metabolic biomarkers: main model with total cholesterol, triglycerides, C-Reactive Protein, and insulin at 6 years of age. Easy obtainable model: main combined with early-life characteristics and with blood pressure at 6 years of age. Full model: main model combined with early-life and child cardiometabolic characteristics.

4 General discussion and future perspectives

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

Healthy early-life development and growth is of lifelong importance. Barker et al. proposed the Fetal Origins of Health and Disease hypothesis in the early 1990s.[1] This hypothesis states that adverse exposures during critical periods of growth and development in early life lead to developmental adaption mechanisms. These adverse exposures may have short and long term consequences for growth, body composition and cardio-metabolic health in later life. This concept has now been generally accepted as the Developmental Origins of Health and Disease (DOHaD) hypothesis and suggests an enormous potential for early-life prevention strategies.[2] Currently, we are in the pandemic time window of the SARS-CoV-2 virus. Disproportionate burdens of infections, hospitalizations, and deaths from COVID-19 are observed in minority groups with higher prevalence of obesity and associated cardio-metabolic diseases.[3-5] Before this pandemic, preventive strategies and public health care lacked appreciation, possibly since the gain on personal level is less clear compared to curative health care. One of the most essential lessons we can already learn from the COVID-19 health crisis is the importance of preventive strategies and healthy lifestyle in general, and especially to strive to recognize and reduce social disparities. The future challenge is to hold on to these insights and implement preventive strategies to support society in a healthy lifestyle and thus to reduce the prevalence of lifestyle-related diseases from the earliest phases of life.

The overall objective of this thesis was to identify early-life factors which play an important role in the development of adverse fetal, pregnancy, birth and child cardio-metabolic outcomes in a low-risk, multi-ethnic population. Previous literature has suggested a putative role of maternal gestational diabetes in the health of the mother herself and in offspring early-life development and health.[6-11] In this thesis, I assessed as a first objective the associations of maternal early–pregnancy glucose concentrations across the full range with fetal, pregnancy, birth and child outcomes. As a second objective, I studied potential determinants, including maternal early-pregnancy glucose concentrations and cardio-metabolic consequences of childhood liver fat. I specifically focused on DNA methylation as potential underlying mechanism for the associations of interest.

Maternal early-pregnancy glucose concentrations

Throughout the first part of this thesis, I focused on maternal early-pregnancy glucose concentrations using a population-based approach. I identified several associations of maternal early-pregnancy glucose concentrations with fetal growth, maternal early-pregnancy blood pressure, size at birth, offspring DNA methylation and childhood liver fat accumulation.

In clinical practice, the diagnosis of gestational diabetes is usually made in second half of pregnancy. However, high glucose concentrations may already contribute to the risk of adverse effects on fetal, maternal and later offspring health before gestational diabetes and its associated complications, such as fetal macrosomia and polyhydramnios, become apparent.[8 12] Addition-

ally, these associations are stronger among women who are overweight or obese at the start of pregnancy.[7 13] The role of maternal glucose metabolism in early pregnancy in relation to fetal development, pregnancy, birth and child outcomes in women without overt diabetes is not clear. Early pregnancy may be an important time window for the effects of suboptimal maternal glucose metabolism on fetal and maternal complications.[8] Early placental development could play an essential role in these associations, although the exact mechanisms through which maternal early-pregnancy hyperglycemia affects placentation and placental function is unknown.[14] Previously, it has been shown that women with HbA1c-defined prediabetes or with gestational diabetes are at increased risk for gestational hypertensive disorders.[12 13 15] Treatment of gestational diabetes has shown to reduce the prevalence of pre-eclampsia.[16 17] In **Chapter 2.1**, I hypothesized that higher maternal early-pregnancy glucose concentrations would be associated with suboptimal placental flow, higher blood pressure and increased risks of gestational hypertensive disorders. I expected to find stronger associations of maternal early-pregnancy glucose concentrations with blood pressure throughout pregnancy, due to prolonged exposure time to a hyperglycaemic environment. Instead, I observed associations of maternal early-pregnancy glucose concentrations with maternal blood pressure in early pregnancy, but not in later pregnancy. I observed no associations of maternal early-pregnancy glucose concentrations with blood pressure in mid- or late pregnancy, placental hemodynamics or gestational hypertensive disorders. The difference in results with previous studies may be explained by my low-risk population. Also, maternal glucose concentrations in early pregnancy may not influence placental flow measures assessed later in pregnancy. My findings may be explained by the fact that an association of maternal early-pregnancy glucose concentrations with blood pressure in mid- or late pregnancy may be too small to detect, as the effect estimates observed for early-pregnancy blood pressure with values within the normal range were already small. Also, there might be no association between maternal early-pregnancy glucose concentrations and blood pressure. It might be that stronger associations of maternal early-pregnancy glucose concentrations with adverse pregnancy outcomes are present in high-risk populations.

Next to pregnancy outcomes, the role of maternal glucose metabolism in early pregnancy in relation to fetal development and birth outcomes is important. Therefore, in **Chapter 2.2** , I studied whether maternal glucose concentrations measured in early pregnancy are associated with fetal growth throughout pregnancy and with risks of adverse birth outcomes. Previously, higher maternal glucose concentrations below the threshold of gestational diabetes measured in mid and late pregnancy were shown to be associated with increased risks of perinatal complications.[10] I observed that among non-diabetic women, higher maternal early-pregnancy glucose concentrations were associated with decreased fetal growth rates in mid-pregnancy and increased fetal growth rates from late pregnancy onwards resulting in larger size at birth. These associations were independent of maternal ethnicity, a well-known risk factor for gestational diabetes and an important determinant of fetal growth. Also, other maternal pregnancy-related factors, including pre-pregnancy body mass index, did not explain the observed associations. The presence of as-

sociations for all fetal biometry measurements suggests that maternal early-pregnancy glucose concentrations affect both fetal fat development and skeletal growth. I also observed that a small increase in maternal early-pregnancy glucose concentrations within the normal range was related to an increased risk of delivering a large-for-gestational-age infant, but a decreased risk of delivering a small-for-gestational-age infant. These associations were independent of maternal pre-pregnancy body mass index.

Epigenetics, more specifically DNA methylation, has been suggested as a potential mechanism linking adverse exposures during pregnancy and impaired offspring health.[18 19] In **Chapter 2.3**, I studied DNA methylation as a potential mechanism linking maternal glucose and insulin concentrations in early pregnancy with impaired offspring health.[18 19] Previous studies using candidate-gene approaches suggested that maternal gestational diabetes is associated with epigenetic modifications in placenta and cord blood at loci relevant to growth, energy homeostasis, and diabetes mellitus.[18 20-22] Epigenome-wide association studies (EWAS) of gestational diabetes or maternal glucose concentrations showed varying results, with no clear pattern of associations.[6 19 23-29] The inconsistent results of candidate-gene studies and EWAS may be due to differences in study design. The studies varied in their exposure definition: gestational diabetes as binary exposure or glucose concentrations after an oral challenge test. Also, the studies varied in tissues in which DNA methylation was measured: placenta or blood. Next to this, the studies were different in the extent of adjustment for covariates, with most studies not adjusting for cell type heterogeneity. Also, the majority had limited sample sizes with numbers ranging from 44 to 313 for cord blood samples.[19 23 25-29] I observed that maternal early-pregnancy glucose and insulin concentrations were not associated with differential DNA methylation at birth in the full group. However, after stratification on maternal body mass index, maternal early-pregnancy glucose concentrations were associated with DNA methylation at one Cytosine phosphate Guanine (CpG) site located in *XKR6* among women with normal weight and at another CpG site in *IL17D* among women with overweight or obesity. Associations of DNA methylation at these CpG sites did not persist in children 10 years of age. Also, I did not observe associations with offspring health outcomes, more specifically with birth weight and child glucose concentrations. The effect estimates of both CpGs were in opposite directions for women with normal weight and for women with overweight or obesity, which could imply a modifying effect of maternal body mass index in these associations. These results constitute a first step towards a better understanding of a potential role of DNA methylation underlying the associations of maternal glycaemic traits in early pregnancy with offspring health outcomes. In addition, they imply that different mechanisms may be involved in different subgroups.

Next to possible underlying mechanisms outcomes, the role of maternal glucose metabolism in early pregnancy in relation to adverse child outcomes is important. I assessed in **Chapter 2.4** the associations of maternal early-pregnancy glucose concentrations with offspring liver fat accumulation and non-alcoholic fatty liver disease measured using magnetic resonance imaging (MRI) at 10 years of age. Previous studies suggest that gestational diabetes is associated with

offspring markers of liver pathology.[30-36] In this study in children 10 years of age, maternal early-pregnancy glucose concentrations were not associated with childhood liver fat accumulation or with risk of non-alcoholic fatty liver disease. Because both glucose concentrations, liver fat and the associations between them may differ between ethnic subgroups, I performed analyses in the full multi-ethnic group and in the group of European ancestry only, as the largest ethnic subgroup. In the European ancestry group, I observed that a 1 mmol/l increase in maternal early-pregnancy glucose concentration was associated with an almost twofold increase in odds of non-alcoholic fatty liver disease, independent of maternal pre-pregnancy and child body mass index and of child glucose concentrations. This may suggest that there is also an intrauterine effect of maternal early-pregnancy glucose concentrations on childhood non-alcoholic fatty liver disease through other pathways in this subgroup. I did not observe associations of maternal early-pregnancy glucose concentrations with liver fat across the full range in the total study sample and in the European ancestry group. The lack of association in the total group might be due to a modifying effect of ethnicity, with directions of effect estimates differing per ethnic subgroup. The lack of association in European ancestry group could be due to the moderate sample size, combined with the relatively small variability in liver fat accumulation in this population of children. Further studies are needed to explore these associations among higher-risk populations and to evaluate liver fat accumulation in older offspring. Methodological considerations of these studies will be discussed in following paragraphs.

Main findings

- Sugar-containing beverage intake at 1 year is associated with increased odds of non-alcoholic fatty liver disease at 10 years of age, but not with liver fat accumulation across the full range.
- DNA methylation at birth and in childhood is not associated with liver fat accumulation in children 10 years of age. This may be due to modest sample sizes or DNA methylation changes being a consequence rather than a determinant of liver fat accumulation.
- Liver fat accumulation across the full range is associated with an adverse cardio-metabolic risk profile already in children 10 years of age. The associations were independent of child body mass index and tended to be stronger in children with overweight or obesity.
- Easily accessible clinical characteristics can be used to aid in prediction of which children are at risk for developing non-alcoholic fatty liver disease.

Underlying mechanisms

The cellular and molecular mechanisms underlying the observed associations of maternal glucose metabolism with fetal, pregnancy, birth and child cardio-metabolic outcomes are largely unknown. A change in nutrient supply to the fetus is a plausible explanation, with glucose being the principal energy substrate and the primary stimulus for fetal secretion of the growth-promoting

hormone insulin.[37 38] Early pregnancy is a critical period for optimal placental development.[39] In this period, trophoblast invasion and spiral artery remodelling take place to ensure adequate blood flow to the developing placenta, leading to larger vessels with lower resistance and increased end-diastolic flow.[37 40] In pregnant women with pre-gestational diabetes, hyperglycemia is hypothesized to cause a pro-inflammatory environment and cytokine derangements that act on the endothelium and lead to placental vascular changes, placental insufficiency and as a result early fetal growth restriction.[41 42] Studies have shown that hyperglycemia during pregnancy is associated with reduced invasiveness of the trophoblast, increased oxidative stress in the maternal and fetal milieu, disrupted vasculogenesis, and macroscopically and histologically altered placentae.[37 41 43] Maternal insulin concentrations do not cross the placenta, but do affect maternal metabolism and the development of the placenta, leading to alterations in fetal-placental blood flow patterns.[44] It has been suggested that the fetus may induce maternal hyperglycemia In response to placental insufficiency, to improve nutrient supply and growth during the second half of pregnancy via placental signaling.[37 45] It has also been hypothesized that maternal hyperglycemia in early pregnancy affects the development of the yolk sac, which is of great importance during the embryonic period, especially in nutrient transport towards the embryo. This may lead to impaired embryonic growth and development. When the yolk sac function is replaced by the placenta at the end of early pregnancy, maternal hyperglycemia together with increased transfer of other nutrients could induce an intrauterine environment which stimulates increased fetal adiposity and growth.[13 37 46] Animal studies have suggested that *in utero* exposure to high glucose concentrations may induce ectopic fat storage.[32-34 36] For instance, mouse models of maternal insulin resistance have shown impairment of gene expression involved in fatty acid oxidative capacity and lipogenesis in offspring liver.[34 35 47] The accelerated hepatic fat storage in mouse offspring appear to persist into adulthood, suggesting a long-lasting impact of the maternal intrauterine environment on pathways of hepatic lipid metabolism.[35 47] Another speculation is that higher insulin resistance in the offspring of mothers with gestational diabetes is associated with higher liver fat accumulation, although the direction of effect is not yet defined.[48 49] In mothers with gestational diabetes a higher risk for non-alcoholic fatty liver disease after pregnancy is observed, supporting the hypothesis of a link between insulin resistance and liver fat accumulation.[50]

Various adverse early-life factors have been associated with differential DNA methylation.[51-54] I observed that maternal early-pregnancy glucose concentrations were associated with DNA methylation at one CpG in *XKR6* among women with normal weight and at another CpG in *IL17D* among women with overweight or obesity. The effect estimates of both CpGs were in opposite directions for women with normal weight and for women with overweight or obesity, which could imply a modifying effect of maternal body mass index in these associations. Their role in mechanisms underlying offspring health outcomes needs further study, especially whether these differently methylated CpGs have an effect on gene expression as a functional consequence. These results await confirmation by future studies in larger samples with early-pregnancy information

on maternal fasting glucose metabolism and exploring potential functional consequences of the differential methylation.

Non-alcoholic fatty liver disease in children

Throughout the second part of this thesis, I focused on childhood liver fat accumulation measured with MRI at 10 years of age. I identified potential early-life determinants and possible cardiometabolic consequences of childhood liver fat accumulation, but no associated DNA methylation patterns. Subsequently, I combined the newly observed associations with existing literature and designed a prediction tool to select children early in childhood at risk for non-alcoholic fatty liver disease development at 10 years of age.

In children, non-alcoholic fatty liver disease is related to the current obesity epidemic and is now the most common cause of chronic liver disease worldwide.[55] Early-life exposures may contribute to the development of not only obesity but also of liver fat accumulation and non-alcoholic fatty liver disease.[56-58] Sugar-containing beverage consumption is the main source of added sugar intake in the total daily energy intake of children and adults.[59-61] Recent studies in adults showed that higher intake of sugar-containing beverages is associated with increased liver fat accumulation in addition to the observed associations with general adiposity.[62 63] In **Chapter 3.1**, I hypothesized that intake of sugar-containing beverages at 1 year of age is associated with liver fat accumulation at 10 years of age. I observed that as compared to infants with less than 1 sugar-containing beverage serving per day, those who consumed more than two sugar-containing beverage servings per day had the highest odds of non-alcoholic fatty liver disease at 10 years of age. I also observed that the association of sugar-containing beverage intake at 1 year of age with non-alcoholic fatty liver disease at school age was largely independent of sugar-containing beverage intake at 8 years of age. Furthermore, childhood body mass index, a known risk factor for non-alcoholic fatty liver disease, did not explain the observed associations. Stratified analyses showed stronger associations between sugar-containing beverage intake and both liver fat accumulation and non-alcoholic fatty liver disease among children from mothers with a lower level of educational attainment. The combination of lower maternal education, used here as proxy for family socio-economic status, and higher sugar-containing beverage intake in infancy might track from infancy into childhood and exacerbate liver fat accumulation. Stratified analyses on body mass index at 10 years of age also showed stronger associations between sugar-containing beverage intake and non-alcoholic fatty liver disease among children with overweight or obesity. I did not observe associations between sugar-containing beverage intake at 1 year and liver fat accumulation across the full range. It appears likely that, due to the relatively large group of infants with low sugar-containing beverage intake in infancy, together with the limited variability and still healthy spectrum of liver fat accumulation across the full range at school age, the differences in sugar-containing beverage intake in infancy are too small to observe an effect on liver fat accumulation across the full range at 10 years of age. The differences observed for early-life intake of sugar-containing beverages with the risk of non-alcoholic fatty liver disease at 10 years

of age were not present for the sugar-containing beverage intake at 8 years of age. The absence of association between sugar-containing beverage intake at 8 years of age and liver fat accumulation might indicate that only early life is a critical period for the influence of sugar-containing beverage intake on the development of liver fat accumulation. However, it might also be explained by information bias, because parents of children who are overweight or obese might reduce or selectively underreport total energy intake and sugar-containing beverage intake. Alternatively, sugar-containing beverage intake in infancy may not track strongly into childhood or tracking might be affected by selective loss-to-follow-up in the infants with high sugar-containing beverage intake.

The mechanisms underlying adverse exposures in early life associated with liver fat accumulation in children and adults may include changes in DNA methylation.[56 57] In **Chapter 3.2**, I hypothesized that differential DNA methylation assessed with EWAS at birth and in childhood is associated with liver fat accumulation at 10 years of age. Recent studies in adults with non-alcoholic fatty liver disease suggest differential DNA methylation is cross-sectionally associated with non-alcoholic fatty liver disease.[64-67] All these studies used liver biopsy to histologically confirm non-alcoholic fatty liver disease, the current gold standard for diagnosing non-alcoholic fatty liver disease.[56 68] As a consequence, these studies are limited by small sample sizes, histologically heterogeneous groups varying in the severity of non-alcoholic fatty liver disease, older study populations, wide body mass index ranges, and having only few or no healthy controls. None of these reports controlled for cell type heterogeneity in their analyses. A recent meta-analysis of four multi-ethnic population-based cohort studies in adults showed that DNA methylation at 22 CpGs in peripheral blood was associated with non-alcoholic fatty liver disease diagnosed with either computed tomography or ultrasound imaging.[69] I did not observe differential DNA methylation at single CpGs or in differentially methylated regions in cord blood or child peripheral blood in association with MRI-diagnosed liver fat accumulation in children at 10 years of age. The associations of the 22 CpGs identified in adults could also not be replicated in children.[69] It is possible that small, but potentially biologically important, DNA methylation differences may be associated with liver fat accumulation in children. These differences would be difficult to detect in the moderate sample size of the current study. Besides this, the variability in liver fat accumulation in this population of children was relatively small, which may also partly explain the lack of identified associations. In addition, my study population is a relatively lean population. Associations of DNA methylation with liver fat accumulation may be more apparent among higher risk populations, as observed in adult studies.[64-67 70] Another potential explanation for the fact that I did not find associations is that DNA methylation might not be associated with liver fat accumulation in children. As has been suggested for phenotypes such as obesity, differential DNA methylation may be mostly a consequence rather than a cause of liver fat accumulation. If this is the case, the duration of exposure to increased liver fat in this population of children 10 years of age may not have been sufficient to induce differential DNA methylation.[71 72]

In adults, non-alcoholic fatty liver disease is associated with cardiovascular disease, dyslipidemia, type 2 diabetes mellitus, and metabolic syndrome.[56 73-75] Studies on the cardio-metabolic consequences of non-alcoholic fatty liver disease in children are scarce. Previous studies in small population-based samples, among children who were older or only among children with obesity suggested that non-alcoholic fatty liver disease is associated with increased risks of insulin resistance, hypertension and dyslipidemia.[73 76-81] It is not known whether liver fat accumulation is also associated with cardio-metabolic risk factors in children without obesity or non-alcoholic fatty liver disease. In **Chapter 3.3**, I observed in a population-based sample a prevalence in all children of 2.8% for non-alcoholic fatty liver disease using a sensitive imaging-based method for liver fat assessment with the highest prevalence up to 25% among children with obesity. Non-alcoholic fatty liver disease was not only present among children with obesity, but also among children with normal weight. This high prevalence of non-alcoholic fatty liver disease in children of 10 years of age is an important population health problem. I observed that both liver fat accumulation across the full range and non-alcoholic fatty liver disease were associated with higher blood pressure, insulin resistance, adverse lipid profile, and increased C-reactive protein concentrations at 10 years of age. These associations were independent of child body mass index and present both in children who were normal weight and who were overweight or obese with stronger effect estimates in the latter group.

The diagnosis of non-alcoholic fatty liver disease in children is difficult, partly due to the relatively clinically silent disease and because of the difficulty in measuring liver fat.[82] Routine detection based on liver biopsy, which is the gold standard for diagnosing non-alcoholic fatty liver disease, or with imaging methods is not feasible with respect to health care expenditures and biopsy-related risks. In adults, algorithms have been developed to predict non-alcoholic fatty liver disease based on anthropometric indices and routine blood biomarkers such as transaminases, triglycerides and insulin.[83-87] In children, there are no accurate prediction tools for non-alcoholic fatty liver disease.[88 89] No previous study in children has considered early-life adverse lifestyle and physical characteristics in predicting childhood non-alcoholic fatty liver disease. When non-alcoholic fatty liver disease presents earlier in life, affected children may exhibit advanced liver disease earlier in adulthood and have increased comorbidities, such as cardiovascular disease and metabolic syndrome. Thus, early detection may be key to the prevention of liver disease and its complications in the population. Therefore, I aimed to identify a set of basic clinical and biomarker characteristics to establish an accurate prediction tool for children at risk of non-alcoholic fatty liver disease in early childhood. In **Chapter 3.4**, I compared different predictor clusters and provide an early-life prediction model to aid in the clinical prediction of which children are at risk for developing non-alcoholic fatty liver disease. The early risk prediction for non-alcoholic fatty liver disease may help to develop future preventive strategies aimed at improving body composition and liver health throughout the life course. My childhood non-alcoholic fatty liver disease prediction models need to be externally validated to assess generalizability to other populations.

Methodological considerations of these studies will be discussed in following paragraphs.

Main findings

- Maternal early-pregnancy non-fasting glucose concentrations are associated with blood pressure in early pregnancy, but not with mid- and late-pregnancy blood pressure, and not with placental hemodynamics or gestational hypertensive disorders.
- Maternal early-pregnancy non-fasting glucose concentrations are associated with decreased fetal growth rates in mid-pregnancy, and with increased fetal growth rates from late pregnancy onwards and with an increased risk of delivering a large-for-gestational-age infant.
- Maternal early-pregnancy non-fasting glucose concentrations, but not insulin concentrations, are associated with offspring DNA methylation at one CpG each in the subgroups of women with normal weight and women with overweight or obesity. No associations of maternal early-pregnancy non-fasting glucose concentrations with offspring DNA methylation are present in the full group.
- Maternal early-pregnancy non-fasting glucose concentrations are associated with offspring non-alcoholic fatty liver disease only among mothers of European ancestry. No associations are observed in the full multi-ethnic group.

Underlying mechanisms

Several mechanisms underlying the observed associations of sugar-containing beverage intake and liver fat accumulation, and of liver fat accumulation with cardio-metabolic risk factors have been proposed.

Glucose, and especially fructose and fructose-containing sugars, all primarily metabolized in the liver, have been suggested to increase hepatic *de novo* lipogenesis.[90-92] Next to this, consumption of sugar-containing beverages induces peaks in blood glucose, insulin and triglyceride concentrations, which may lead to insulin resistance and subsequently to liver fat accumulation.[62 63 91 93] Also, intake of liquid food leads to less satiety, more postprandial hunger and therefore to an increased total daily energy intake.[62 94]

The directions of the associations of child liver fat accumulation with cardio-metabolic risk factors cannot be concluded from my cross-sectional analyses. The link between liver fat accumulation and cardio-metabolic risk factors has been explained by several mechanisms.[95] When visceral fat mass is increased, this may alter lipid metabolism and trigger insulin resistance, that may subsequently lead to non-alcoholic fatty liver disease and cardiovascular disease development.[95-97] On the other hand, liver fat can be the source of systemic release of inflammatory cytokines and pro-atherogenic factors leading to cardio-metabolic diseases, including hypertension.[75 96-98] Findings from previous studies suggest a strong association of non-alcoholic fatty liver disease with the metabolic syndrome.[96 97] Also, studies in both adults and children showed associations of non-alcoholic fatty liver disease with hypertension.[99-101] Adults with non-alcoholic fatty liver

disease had increased carotid-artery intima-media thickness and increased prevalence of carotid atherosclerotic plaques[100]. Possible underlying mechanisms may include chronic inflammation leading to pro-atherogenic factors leading to arterial damage and hypertension.[97] The strong associations of both higher liver fat accumulation with systolic blood pressure and with C-reactive protein in my study support this hypothesis. Prospective longitudinal analyses or causal inference approaches such as Mendelian randomization may help to elucidate the directions of the observed associations.

Although the hypothesis of early-life adverse outcomes contributing to the development of liver fat accumulation through DNA methylation was not supported by my findings, it cannot be completely discarded based on my analyses. Future studies should investigate the associations of differential DNA methylation with liver fat accumulation in children in large longitudinal studies.

Methodological considerations

Strengths and limitations for each study are described in **Chapter 2** and **Chapter 3** of this thesis. In the following paragraphs general methodological considerations regarding selection bias, information bias, confounding, causality, and specific issues in epigenetic studies, that may have played a role in my studies are discussed.

Selection bias

Selection bias may occur due to selective non-response at baseline or selective loss to follow-up. Selection bias at baseline can arise from the procedures used to select study participants or factors that influence the study participation, leading to a difference in the estimated association between the exposure and the outcome of interest for those who participate in the study and those who were eligible for the study. In the Generation R Study the participation rate at birth was 61%.[102] The non-response appears not to be at random. As compared to the general population in Rotterdam, women from the Generation R Study were less likely to be from ethnic minority groups, had a higher socioeconomic status, and less often had adverse perinatal outcomes.[103] This selective non-response at baseline suggests a selection towards a more affluent and healthy study population, which may have led to lower prevalence rates of gestational diabetes, childhood liver fat accumulation, and associated risk factors, and subsequently reduced statistical power. Also, it may affect the generalizability of my findings to less affluent and healthy populations. Selection towards a relatively healthy population may have biased the observed effect estimates, although it is difficult to quantify the extent. Multiple imputation was applied in my studies to limit the risk of selection bias due to random missing values in covariates.[104 105]

Selective loss to follow-up can arise when the association between the exposure and outcome of interest is different between those participating in the studies described in this thesis and those lost to follow-up. I used data from the follow-up at 6 and 10 years of age.[102] Compared to the total follow-up group a lower percentage of children participated in the MRI measurements of liver and organ fat at 10 year of age, since a subgroup was invited for MRI measurements and

due to non-consent. Compared to the baseline characteristics, mothers and children who did visit the research centre in the follow-up evaluations were more frequently of Dutch ethnic origin, higher educated and generally had healthier lifestyle habits than mothers and children who did not participate in follow-up visits. I also used data from the subgroup of Dutch mothers and their children participating in additional EWAS assessments, in which the loss of follow-up at 6 and 10 years of age was low. This selective loss to follow-up suggests a selection towards a healthier population. Although it is difficult to speculate if this might have biased my effect estimates, this seems unlikely since participants did not differ from non-participants regarding the exposures of interest, but it may affect the generalizability of my findings.

Information bias

Information bias may arise from measurement error or misclassification of an exposure or outcome. In this thesis, I relied on anthropometric measurements, such as body mass index and blood pressure, which might have greater measurement error and be less accurate, but on the other hand are easier and cheaper to obtain in large epidemiological studies as compared to imaging techniques of body composition. High accuracy and reproducibility have been reported for MRI, which was used in this thesis.[106 107] Misclassification can be non-differential or differential. These terms refer to the mechanism for misclassification. Non-differential misclassification is a random error and occurs unrelated to the exposure or outcome status, and generally leads to an underestimation of the effect estimates than the actual effect. Differential misclassification is a non-random error, which occurs when the exposure is misclassified and this is related to the outcome status, and generally leads to biased results, which can be either overestimated or underestimated. Similarly, misclassification of the outcome is non-differential when it is unrelated to the exposure, otherwise it is differential.

In this thesis, information on exposures and outcomes was obtained prospectively by physical and ultrasound examinations, blood samples, MRI data and parental questionnaires. Differential misclassification is unlikely, since exposure data used in my studies were collected before assessment of the outcomes, the data collectors were blinded to the exposure status when assessing the outcomes and parents as well as data collectors were unaware of the specific research questions under study. However, non-differential misclassification might have occurred. If I had non-differential misclassification in my studies, the effect-estimates reported are conservative compared to the assumed true effect. We obtained high quality data for a large number of maternal and child characteristics through hands-on measurements, blood withdrawal and questionnaires. Although I used validated questionnaires to assess socio-demographic and lifestyle characteristics, these measurements may still have been affected by measurement error, recall bias and reporting bias.

In the studies included in this thesis, the fasting time before blood sampling was limited to 30 minutes, and thus I consider my samples non-fasting samples.[102] The blood samples were collected at different time-points during the day, depending on time of the study visit. Since glucose and insulin concentrations vary during the day and are sensitive to carbohydrate intake,

this may have led to non-differential misclassification of maternal glucose and insulin concentrations. However, it has been suggested that maternal non-fasting glucose concentrations may better reflect the normal physiological state in pregnancy.[108 109] In children these different blood sampling time points could have led to non-differential misclassification of glucose and insulin concentrations and thus underestimation of the observed effect estimates. However, semi-fasted insulin resistance is moderately correlated with fasting values.[110] For lipid concentrations it seems that non-fasting blood sampling is superior to fasting in accurately predicting cardio-metabolic events for adults in later life.[111] Therefore, I believe my findings for triglycerides and cholesterol are less likely influenced by the non-fasting state. Blood samples were collected, stored on ice and processed in a standardized way, but time from sampling to freezing could be up to four hours. This may have affected the measured glucose concentration, since glucose concentrations may decline in serum tubes, again possibly leading to non-differential misclassification and an underestimation of my associations.

Self-reported lifestyle habits, such as sugar-containing beverage intake studied as a potential determinant of child liver fat accumulation, may have been selectively underreported by parents due to for example prior knowledge. Parents can be aware of potential negative effects of the lifestyle behaviours collected in the study and this could have resulted in non-differential misclassification and may have attenuated the studied associations.

Confounding

Confounding occurs when all or some of the observed effect between an exposure and an outcome is in fact explained by other variables that affect the outcome but are not themselves affected by the exposure. If a confounding factor is not taken into account, results might be biased and the true exposure effect is obscured. To account for confounding, I adjusted all analyses in this thesis for multiple potential confounding. I selected covariates based on their associations with the exposures and outcomes of interest as identified by literature or in my previous studies, or when a change in effect estimates of more than 10% was observed when adding the confounder to the basic model. As in any observational study, residual confounding might still be present due to unknown or unmeasured confounding variables.

Methodological issues in DNA methylation measurements

In my DNA methylation studies I used a hypothesis-free epigenome-wide approach, which gives the opportunity of finding new associations. The effect estimates in this type of research are generally small. In both EWAS, I used a Bonferroni-correction to adjust for multiple testing. This correction assumes that the tests performed are independent. However, DNA methylation is correlated between multiple CpG sites. Thus, the Bonferroni-correction may be too strict, potentially leading to false negative results. One way of resolving this issue is by increasing power. Generation R has one of the largest datasets of childhood DNA methylation in the world, but the number of tests performed require even larger populations, which can only be achieved through

collaboration.[112] Unfortunately, due to differences in assessment of the phenotypical variables it was not possible to perform meta-analyses with other cohorts. Another way to deal with multiple testing correction, is to reduce the number of tests performed. Besides single CpG sites, I also studied clusters of CpG sites using the dmrff package (https://github.com/perishky/dmrff), which identifies differentially methylated regions by combining EWAS summary statistics from nearby CpGs.[113] Next to these methodological perspective, it is biologically plausible that DNA methylation in a region of correlated CpG sites explains more variance in the phenotype of interest than DNA methylation at a single CpG site alone.[114] EWAS studies are subject to confounding by environmental, genetic, and technical factors. For instance, DNA methylation differences in blood samples are strongly influenced by cellular heterogeneity. Consequently, EWAS studies need to adjust for cell type composition, as was performed in my DNA methylation studies. A further challenge in EWAS is the question to what extent the DNA methylation levels in one tissue are informative of those in another, potentially more relevant tissue. In my studies, I had access to cord blood and peripheral blood. For instance, in the setting of the study of DNA methylation with liver fat accumulation, liver tissue would ideally be the tissue of interest, but this is not available in population studies. One study assessed the concordance between single CpG sites in peripheral blood and liver tissue in 27 adults and found that only 6% of the variable CpGs showed moderate correlations and 4% strong correlations.[115] Even if there is no correlation between cord blood or peripheral blood DNA methylation with DNA methylation in liver tissue, methylation in blood may still be a possible biomarker of future or current non-alcoholic fatty liver disease.

Causality

The observational nature of the prospective cohort design in which the studies included in this thesis were embedded, does not allow to conclude *causality* in the associations. To imply in prospective cohort studies whether there is evidence for a *causal* relationship between an exposure and outcome the Bradford Hill criteria can be used.[116] These criteria include examination of the biological gradients, the consistency with previous literature, the temporality of the associations, the strength, specificity, and plausibility of the effect estimates and whether there is a dose-response relationship. Most of my findings are in line with previous literature, and since the majority of the studies in this thesis had a longitudinal study design, this supports the temporality between exposures and outcomes. In the cross-sectional study on child liver fat accumulation with cardio-metabolic risk factors at the same age, I am unable to state the direction of effects because it is uncertain whether the exposure occurred before the outcomes. I observed a possible biological gradient for the association of higher maternal early-pregnancy glucose concentrations with fetal growth, birth weight and child liver fat accumulation at 10 years of age. I reported plausible underlying mechanisms of my results and reported on coherence with experimental and animal studies. For my EWAS studies, causality is even more complex, since DNA methylation can be an exposure as well as an outcome, or a biomarker for the actual outcome of interest.[117] Mendelian Randomization could be used as an approach to further examine causality.[118] Mende-

lian Randomization studies use known genetic variants associated with the exposure of interest as unconfounded instrumental variables not affected by confounding, to examine whether the exposure is causally related to the outcome.[118]

FUTURE RESEARCH

Current clinical practice is mainly focused on screening for gestational diabetes based on diagnostic thresholds of maternal glucose concentrations from mid-pregnancy onwards in higher risk women. However, based on findings of the studies in this thesis, altered fetal development can already occur among non-diabetic women before mid-pregnancy when screening for gestational diabetes and necessary interventions are currently implemented. Recent randomized controlled trials, which are considered the golden standard for studying causality, indicate that treatment of gestational diabetes and maternal hyperglycemia with lifestyle adaptations from mid-pregnancy onwards leads to a decreased risk of adverse birth outcomes compared to no treatment.[17 119 120] Based on my findings, future randomized controlled trials should already focus on glucose screening and treatment from preconception and early pregnancy onwards to further improve pregnancy outcomes, among both higher risk populations such as women with overweight or obesity and possibly also among lower risk populations. These studies should assess the effect of lifestyle interventions, which keep an adequate balance between reducing maternal blood glucose concentrations to prevent hyperglycemia, but without inducing hypoglycemia. These intervention studies from preconception and early pregnancy onwards will not only provide important novel insight into the effectiveness of these interventions, but also into the causality of the observed associations of maternal early-pregnancy glucose concentrations with altered fetal growth and adverse birth outcomes.

As mentioned previously, maternal glucose and insulin concentrations were measured once during early pregnancy. Future studies including maternal glucose and insulin concentrations measured at multiple time points during pregnancy, preferable also preconceptionally, are needed to observe whether normal glucose and insulin concentrations before or in early pregnancy will worsen or maintain normal during pregnancy and whether patterns of glucose and insulin concentrations during pregnancy may be more informative than single measurements. Also, potential misclassification of maternal early-pregnancy glucose and insulin concentrations might be present in the studies in this thesis. Ideally, data on oral glucose tolerance tests should be included in glucose metabolism related follow up studies.

Overall, there are still many questions with regard to childhood non-alcoholic fatty liver disease, as most studies to date have been conducted in adults. Understanding the natural history and pathogenesis of non-alcoholic fatty liver disease from early life to adulthood is essential, in order to better prevent and improve future care of the disease. Focus of further studies should be on identifying early-life factors, like nutrients, epigenetics, genetics, and environmental factors,

which influence the development of non-alcoholic fatty liver disease during childhood and should take ethnic background into account. The microbiome may be explored as possible key driver in early life of child non-alcoholic fatty liver disease, since it can influence nutrient utilization, immune function and host gene expression.[121] Next to this, the field of metabolomics can also lead to advancements in understanding the systemic metabolic signatures of processes involved in non-alcoholic fatty liver disease in children. The relevance of screening for non-alcoholic fatty liver disease in children is emphasized with the growing evidence for the increasing prevalence of the disease, and beside the hepatic also the extra-hepatic complications of non-alcoholic fatty liver disease. Future studies should validate my current prediction model results. Risk stratification could help to determine which children would benefit most from preventive strategies, intensive multidisciplinary care and treatment. Preventive strategies remain the cornerstone of preventing non-alcoholic fatty liver disease. Methods to prevent non-alcoholic fatty liver disease in children have not yet been studied. Based on my findings, future studies should explore new strategies for education, particularly on lifestyle interventions from infancy onwards, especially to reduce sugar-containing beverage intake. Intervention studies from early life onwards will provide important new insights into the effectiveness of these interventions as well as the causality of the observed associations between sugar-containing beverage intake in infancy and non-alcoholic fatty liver disease in later life.

In order to better understand how maternal pregnancy glucose concentrations and offspring DNA methylation are related during fetal and later child development, I need to further address issues of power, measurement error and cell-type specificity of tissue. Sample size and reproducibility of my results within the field of epidemiology would benefit from more collaboration between different cohorts. In the last decade the number of CpG sites measured on profiling platforms increased, from 485,000 on the Illumina 450K array to the HumanMethylationEPIC Beadchip (EPIC) array, which measures 850,000 CpGs. The EPIC platform includes more CpG sites located at regulatory elements, like enhancer regions.[122 123] Peripheral blood derived DNA was used in my studies. The cell-type specificity of DNA methylation makes it difficult to extrapolate the findings towards other, potentially pathophysiologically more relevant tissues, such as liver tissue.[124] Also, future studies should investigate in large longitudinal studies the associations of differential DNA methylation with liver fat accumulation in children to assess whether DNA methylation is one of the underlying mechanisms in the association of early-life adverse outcomes with liver fat accumulation and to separate cause and effect. In this thesis, I have assessed DNA methylation, which is only one component of epigenomics. Data on DNA methylation is often studied solely, since it is relatively easy to obtain and to measure in larger populations. Future challenges include gaining a better understanding of how epigenetics, genetics and environment together may affect child health, as well as the integration of all types of epigenetic mechanisms and their functional consequences such as differences in gene expression.

Implications for prevention and policy development

Early-life exposure to high maternal glucose concentrations from early pregnancy onwards might affect maternal and offspring health in the short and long term. The effect estimates for the observed associations were small. However, these small effects might contribute to the total burden of cardio-metabolic disease in women and their offspring. They might induce intergenerational effects by influencing fetal growth and development. Given the high prevalence of both obesity and impaired glucose metabolism in preconceptional women and if proved causal, these may represent pivotal targets for public health in preventing offspring obesity and metabolic disease, such as non-alcoholic fatty liver disease.

Governmental policies currently focus more on secondary prevention than on primary prevention of lifestyle medicine in fertile women and men. As the current COVID-19 pandemic also underlines, primary prevention is the most important health strategy. Therefore, especially during preconception and early pregnancy, preventive strategies should educate and motivate both women and men of all social strata to pursue a healthy lifestyle. Health professionals should address lifestyle habits during regular appointments, since preconception consultations are not yet standard procedure in the Netherlands. Also, clinicians who provide preventive health care in the Netherlands should be better supported by the government. Promotion of preconception consultations and governmental campaigns might increase awareness among women and men with a wish to start a family.

The studies in this thesis provides the insight that sugar-containing beverage intake in infancy possibly affects child liver fat accumulation. These effects are even stronger among children of mothers with lower educational attainment and among children with overweight or obesity. These findings may imply that it is of great importance to invest in prevention of sugar-containing beverage intake already from infancy onwards. One of the main predictors of childhood liver fat accumulation is a higher child body mass index. The COVID-19 pandemic has forced countries worldwide to implement strict social distancing and sanitary regimes. These regimes include among others the lockdown, children being homeschooled by their remotely working parents. These events have further increased and probably are currently still increasing the prevalence of overweight and obesity among children due to more screen time, less physical activity, and less social activities while maintaining the same or a higher daily energy intake.[125] This may lead to a further increase in the prevalence of child obesity and related diseases, such as non-alcoholic fatty liver disease. This alarming knowledge should again emphasize the importance of preventive strategies and healthy lifestyle in general. A recent intervention study in the Netherlands assessed whether a school environment promoting healthy behaviours in children with a daily healthy lunch and structured physical activity sessions each day could lower child body mass index.[126] The 'Healthy Primary School of the Future' was shown effective after 1 and 2 years of follow-up. Although, the long term effects on child body mass index still need to be explored, this intervention study can be seen as an example that aiding in healthy lifestyle can contribute to reduce child health inequalities and improve the long-term health perspectives of children.

Clinical relevance

The observations from my studies may have important relevance in current clinical practice to improve maternal and offspring health in early life and to prevent long-term cardio-metabolic complications:

§ I show that maternal non-fasting glucose concentrations in early pregnancy are associated with decreased fetal growth rates in mid-pregnancy, with increased fetal growth rates from late pregnancy onwards, and with an increased risk of delivering a large-for-gestational-age infant. This is in line with previous literature showing that higher maternal glucose concentrations below the threshold of gestational diabetes measured in mid and late pregnancy were associated with increased risks of perinatal complications.[10] In current clinical practice, gestational diabetes is usually diagnosed in the second half of pregnancy. However, high glucose concentrations may already contribute to the risk of adverse effects on fetal, maternal and later offspring health before gestational diabetes and its associated complications become apparent.[8 12] **Therefore, I suggest that intervention studies should assess whether glucose screening before or early in pregnancy supports early identification and prevention of maternal glucose related health problems during pregnancy and in later life of the offspring.**

§ I observed that high intake of sugar-containing beverages at 1 year of age is associated with increased odds of non-alcoholic fatty liver disease at 10 years of age. Since prevention is the major target in treating childhood non-alcoholic fatty liver disease, new strategies for education, particularly on early-life nutrition and lifestyle modification are required. **My results suggest that limiting the intake of sugar-containing beverages from infancy onwards may help to prevent non-alcoholic fatty liver disease at school age, which should be a target in future prevention strategies and education programs.**

§ My study on liver fat accumulation across the full range with adverse cardio-metabolic risk profiles in children of 10 years of age highlights the importance of assessing not only liver fat accumulation but also the cardio-metabolic profile in children with non-alcoholic fatty liver disease both in children of normal weight and in those with overweight or obesity. **These results suggest that non-alcoholic fatty liver disease is already present in school age children and related with impaired cardio-metabolic health.**

§ The prognostic prediction model for child non-alcoholic fatty liver disease developed with easily accessible clinical characteristics may be used after external validation, to aid in prediction of which children in mid-childhood are at risk for developing non-alcoholic fatty liver disease in later childhood. **The early risk prediction for non-alcoholic fatty liver disease may help to develop future preventive strategies aimed at improving body composition, liver health and associated cardio-metabolic disease throughout the life course.**

CONCLUSIONS

Findings presented in this thesis suggest that early-life exposures, such as maternal early-pregnancy glucose concentrations, are associated with maternal and child health outcomes in the short and long term. In my studies, I identified associations between maternal early-pregnancy glucose concentrations and fetal growth, maternal early-pregnancy blood pressure, and childhood liver fat accumulation. I identified some evidence that maternal early-pregnancy glucose concentrations are associated with offspring DNA methylation stratified for maternal body mass index. Also, I observed that sugar-containing beverage intake in infancy was associated with childhood non-alcoholic fatty liver disease. I showed no evidence for associations between DNA methylation and child liver fat accumulation. Liver fat accumulation at 10 years of age was already associated with increased cardio-metabolic risk clustering. Early-life exposures can be used in mid-childhood for prediction of child non-alcoholic fatty liver disease in later childhood. Further studies should validate this prediction model in a low-risk multi-ethnic population. In the long run, this will lead to development of new preventive strategies and interventions before and in early-pregnancy, and for young children, aiming at a healthy development and growth from early-life onward.

Ultimately, this thesis may be like planting a few scientific seeds which will grow and blossom into beautiful and healthy green.

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5 Summary
Samenvatting

SUMMARY

Optimal development in early life is of great importance for lifelong health. The Developmental Origins of Health and Disease (DOHaD) hypothesis suggests that adverse exposures during critical periods of growth and development in early life lead to developmental adaptations. These fetal adaptations initially lead to short-term survival, but may lead to an increased susceptibility to cardio-metabolic diseases later in life. Several decades ago, the basis for this hypothesis was laid by large-scale epidemiological studies reporting that low birth weight, preterm birth and adverse maternal lifestyle factors are associated with an adverse cardiovascular health profile in the offspring. These findings emphasize the importance of early-life prevention strategies.

The first objective of this thesis was to examine the hypothesis that maternal early-pregnancy glucose concentrations are associated with adverse fetal, pregnancy, birth and child cardio-metabolic outcomes. Also, as second objective, we studied potential determinants of a specific outcome, childhood liver fat, as well as the cardio-metabolic consequences of childhood liver fat. We focused on DNA methylation as potential underlying mechanisms for the associations of interest. All studies described in this thesis were conducted in the Generation R Study, a population-based cohort study in Rotterdam, the Netherlands. Below I present a brief overview of the individual studies and their results from this thesis.

Chapter 1 provides the background, hypothesis, aims and design for the studies presented in this thesis.

In **Chapter 2**, studies focusing on maternal early-pregnancy glucose concentrations are described. In **Chapter 2.1**, we tested our hypothesis that maternal early-pregnancy glucose concentrations are associated with suboptimal placental flow, higher blood pressure and increased risks of gestational hypertensive disorders. We observed that maternal early-pregnancy glucose concentrations were associated with higher maternal blood pressure in early pregnancy, but not in later pregnancy. Maternal early-pregnancy glucose concentrations were not associated with placental hemodynamics or gestational hypertensive disorders. The findings of our study do not support strong effects of maternal glucose concentrations in early pregnancy within the normal range on the risks of gestational hypertensive disorders. It might be that stronger associations of maternal early-pregnancy glucose concentrations with adverse pregnancy outcomes are present in higher-risk populations.

In **Chapter 2.2**, we assessed the associations of maternal early-pregnancy glucose concentrations with fetal growth throughout pregnancy and the risks of adverse birth outcomes. We observed that higher maternal early-pregnancy glucose concentrations were associated with altered fetal growth patterns, resulting in increased length and weight at birth. Maternal early-pregnancy glucose concentrations were also associated with an increased risk for delivering a large-for-gestational-age infant, but a decreased risk for delivering a small-for-gestational-age infant. These associations were not explained by maternal sociodemographic or lifestyle factors, or by pre-pregnancy body mass index. Maternal early-pregnancy glucose concentrations were

not associated with preterm birth or delivery complications. If proven causal, maternal glucose metabolism may represent a pivotal target for public health in preventing offspring adverse health outcomes.

In **Chapter 2.3**, we examined whether maternal early-pregnancy glucose and insulin concentrations were associated with newborn DNA methylation. Maternal early-pregnancy glucose and insulin concentrations were not associated with DNA methylation at single CpG sites, nor with differentially methylated regions in the total population. In analyses stratified on maternal body mass index, maternal early-pregnancy glucose concentrations were associated with DNA methylation at a CpG site in *XKR6* among women with normal weight and at another CpG site in *IL17D* among women with overweight or obesity. No stratum-specific associations were found for maternal early-pregnancy insulin concentrations. These results constitute a first step towards a better understanding of a potential role of DNA methylation in the associations of maternal glycaemic traits in early pregnancy with offspring health outcomes, since it implies a modifying effect of maternal body mass index in the observed associations. In addition, they imply that different mechanisms may be involved in different subgroups.

Gestational diabetes seems to be associated with offspring non-alcoholic fatty liver disease. In **Chapter 2.4**, we assessed the associations of maternal early-pregnancy glucose concentrations with offspring fat accumulation and non-alcoholic fatty liver disease with magnetic resonance imaging (MRI) at 10 years of age. We observed that maternal early-pregnancy glucose concentrations were associated with offspring non-alcoholic fatty liver disease, but only among mothers of European ancestry and not among the multi-ethnic population. These associations were independent of maternal pre-pregnancy and child body mass index, and of child glucose concentrations. No associations were observed in the full multi-ethnic group, this might be due to a modifying effect of ethnicity, with directions of effect estimates differing per ethnic subgroup. These results await confirmation by future studies in larger samples with early-pregnancy information on maternal fasting glucose metabolism and exploring potential functional consequences of the differential methylation.

In **Chapter 3**, we describe studies focused on child liver fat accumulation. Sugar-containing beverage intake is a major risk factor for obesity in both children and adults and appears to be associated with non-alcoholic fatty liver disease in adults. The purpose of **Chapter 3.1** was to examine the associations between sugar-containing beverage intake in infancy and child liver fat accumulation at 10 years of age. We observed that higher sugar-containing beverage intake in infancy is associated with an increased risk of non-alcoholic fatty liver disease in children of 10 years of age. The associations were stronger among children of mothers with lower educational attainment as compared to those with higher educational attainment and among children with overweight or obesity as compared to children with normal weight. Our results suggest that limiting the intake of sugar-containing beverages from infancy onwards may help to prevent liver steatosis at school age, which could be a target in future prevention strategies and education programs.

In **Chapter 3.2**, we aimed to identify whether DNA methylation in newborns and children was associated with liver fat accumulation at 10 years of age. We also examined if DNA methylation at CpG sites associated with adult non-alcoholic fatty liver disease is associated with liver fat in children. DNA methylation at birth and in childhood was not associated with liver fat accumulation in children 10 years of age in this study. Also, DNA methylation at 22 CpGs known to be associated with non-alcoholic fatty liver disease in adults was not associated with liver fat in children. This may be due to modest sample sizes or to DNA methylation changes being a consequence rather than a determinant of liver fat. Although the hypothesis of early-life adverse outcomes contributing to the development of liver fat accumulation through DNA methylation was not supported by our findings, it cannot be completely discarded based on our analyses. Future studies should investigate the associations of differential DNA methylation with liver fat accumulation in children in larger longitudinal studies.

The burden of liver fat and associated cardio-metabolic risk factors in healthy children is unknown. We performed a cross-sectional analysis in children at 10 years of age in **Chapter 3.3**, to examine whether liver fat accumulation and non-alcoholic fatty liver disease are associated with cardio-metabolic risk factors. We observed that not only non-alcoholic fatty liver disease, but also a higher liver fat fraction across the full range is associated with an adverse cardio-metabolic profile in children. Clustering of cardio-metabolic risk factors was already observed from a liver fat fraction of ≥2.0% onward. These associations were independent of child body mass index and tended to be stronger in children who were overweight and obese than in children who had a normal weight. These results suggest that non-alcoholic fatty liver disease is already present in children with both normal weight and overweight or obesity at school age and that it is related with impaired cardio-metabolic health.

In **Chapter 3.4**, we compared different child non-alcoholic fatty liver disease predictor clusters, and we provided an early-life prediction model to aid in the clinical prediction of which children are at risk for developing non-alcoholic fatty liver disease. Our childhood non-alcoholic fatty liver disease prediction models need to be externally validated to assess generalizability to other populations.

Finally, in **Chapter 4**, we discuss the findings and interpretations of the individual studies in the broader context of the existing literature. We conclude with several recommendations for future research and present implications for clinical practice and policy.

In conclusion, the studies presented in this thesis suggest that early-life exposures, such as maternal early-pregnancy glucose concentrations, are associated in the short- and long-term with maternal and child health outcomes. Next to this, we identified early-life predictors of child non-alcoholic fatty liver disease. Ultimately, we expect that this will lead to the development of new preventive strategies and interventions before and in early pregnancy, and for young children, aiming at a healthy development and growth from early-life onwards.

SAMENVATTING

In de beginfase van het leven optimaal groeien en ontwikkelen is van groot belang voor een levenslange gezondheid. Volgens de 'Developmental Origins of Health and Disease (DOHaD)' hypothese leidt blootstelling aan ongunstige omstandigheden tijdens essentiële perioden in de beginfase van het leven van het kind tot aanpassingen in de foetale ontwikkeling. Op de korte termijn dragen deze foetale aanpassingen bij aan een hogere kans op overleving, maar op de lange termijn leiden ze tot een verhoogd risico op hart- en metabole ziekten. Grote epidemiologische studies hebben beschreven dat er associaties zijn tussen enerzijds een laag geboortegewicht, vroeggeboorte en ongezonde maternale leefstijlfactoren tijdens de zwangerschap, en anderzijds een verhoogd risico op hart- en vaatziekten. Het identificeren van factoren die van invloed zijn op de hart- en metabole gezondheid van zwangere vrouwen en hun kinderen, zou kunnen bijdragen aan het ontwikkelen van preventiestrategieën om de optimale groei en ontwikkeling van jonge kinderen gedurende het hele leven én in toekomstige generaties te verbeteren.

Het doel van dit proefschrift is ten eerste te onderzoeken of maternale glucosewaarden vroeg in de zwangerschap van invloed zijn op nadelige hart- en metabole gezondheidsuitkomsten bij de foetus, op zwangerschapsuitkomsten, geboortecomplicaties en op de lange termijn ontwikkeling van het kind. Ten tweede is het doel te onderzoeken wat vroege determinanten van leververvetting bij kinderen zijn, wat de hart- en metabole consequenties hiervan zijn en welke parameters men zou kunnen gebruiken om te voorspellen welke kinderen een verhoogd risico hebben op leververvetting. Daarnaast hebben we voor beide hoofdvragen DNA-methylering bestudeerd als mogelijk onderliggend mechanisme voor de gevonden associaties. Alle onderzoeken beschreven in dit proefschrift zijn uitgevoerd binnen het Generation R Onderzoek. Dit is een prospectieve cohort studie waarin kinderen, geboren in Rotterdam, gevolgd worden in hun ontwikkeling, gestart vanaf het foetale leven tot in de jongvolwassenheid. Het Generation R Onderzoek heeft tot doel om factoren die van invloed zijn op de groei, de ontwikkeling en de gezondheid in het foetale leven en in de kindertijd te identificeren.

Hoofdstuk 1 beschrijft de achtergrond voor de hypotheses die de basis vormen van de studies in dit proefschrift.

In **Hoofdstuk 2** worden de studies beschreven waarin we de invloed van maternale glucosewaarden vroeg in de zwangerschap op uitkomsten, bij zowel moeder als kind, hebben bestudeerd.

In **Hoofdstuk 2.1** hebben we de hypothese getest of maternale glucosewaarden vroeg in de zwangerschap geassocieerd zijn met een suboptimale placentadoorbloeding, een hogere maternale bloeddruk en een verhoogd risico op hypertensieve zwangerschapscomplicaties. Uit onze studie blijkt dat maternale glucosewaarden vroeg in de zwangerschap geassocieerd zijn met een hogere maternale bloeddruk vroeg in de zwangerschap, maar niet later in de zwangerschap. Maternale glucosewaarden vroeg in de zwangerschap zijn niet geassocieerd met placentadoorbloeding of met het risico op hypertensieve zwangerschapscomplicaties. Deze bevindingen laten geen sterke effecten van maternale glucosewaarden vroeg in de zwangerschap zien op het

optreden van zwangerschapsgerelateerde hypertensieve aandoeningen. Mogelijk zijn er sterkere associaties aanwezig in hogere risico populaties.

In **Hoofdstuk 2.2** hebben we de associaties van maternale glucosewaarden in de beginfase van de zwangerschap met de foetale groei tijdens de zwangerschap en met de risico's van ongunstige geboorte-uitkomsten onderzocht. We hebben gezien dat hogere maternale glucosewaarden vroeg in de zwangerschap geassocieerd zijn met verandering in foetale groeipatronen, die resulteren in een toegenomen lengte en gewicht bij de geboorte. Daarnaast zijn hogere maternale glucosewaarden vroeg in de zwangerschap geassocieerd met een hoger risico op een te hoog geboortegewicht voor de zwangerschapsduur en met een lager risico op een te laag geboortegewicht voor de zwangerschapsduur. Onze bevindingen worden niet verklaard door maternale sociaal-demografische en leefstijlfactoren, of door maternale body mass index voor de zwangerschap. Maternale glucosewaarden vroeg in de zwangerschap zijn niet geassocieerd met vroeggeboorte of ongunstige geboorte-uitkomsten. Het maternaal glucosemetabolisme zou een belangrijke factor kunnen zijn bij het ontwikkelen van nieuwe preventiestrategieën ter verbetering van de gezondheid van moeder en kind.

In **Hoofdstuk 2.3** hebben we gekeken of maternale glucose- en insulinewaarden in de beginfase van de zwangerschap geassocieerd zijn met DNA-methylering bij de geboorte. In onze totale onderzoekspopulatie zien we dat maternale glucose- en insulinewaarden vroeg in de zwangerschap niet geassocieerd zijn met DNA-methylering op CpG niveau en niet met differentiële methylering op regio niveau. Toen we de totale groep splitsten op basis van maternale body mass index, hebben we gezien dat maternale glucosewaarden vroeg in de zwangerschap geassocieerd zijn met DNA-methylering op een CpG in *XKR6* bij vrouwen met een normaal gewicht en met DNA-methylering op een CpG in *IL17D* bij vrouwen met overgewicht of obesitas. Er zijn geen maternaal body mass index-specifieke associaties gevonden voor maternale insulinewaarden vroeg in de zwangerschap. De resultaten van deze studie zijn een startpunt in het beter begrijpen van de mogelijke rol van DNA-methylering in de associaties van maternaal glucosemetabolisme vroeg in de zwangerschap met gezondheidsuitkomsten van het kind. Bovendien impliceren deze bevindingen dat er verschillende DNA-methylering mechanismen betrokken zouden kunnen zijn bij verschillende subgroepen.

In **Hoofdstuk 2.4** hebben we de associaties van maternale glucosewaarden vroeg in de zwangerschap onderzocht met de hoeveelheid leververvetting bij 10-jarige kinderen. Het percentage leververvetting is gemeten met een MRI scan. We hebben aangetoond dat hogere maternale glucosewaarden vroeg in de zwangerschap geassocieerd zijn met het vaker optreden van niet-alcoholische leververvetting bij kinderen. Deze associatie zien we alleen bij moeders van Europese afkomst. De gevonden associaties zijn onafhankelijk van de maternale body mass index voor de zwangerschap en onafhankelijk van de body mass index en glucosewaarden bij kinderen. In de totale, multi-etnische onderzoekspopulatie hebben we geen associaties gevonden. Toekomstige studies zouden ter bevestiging van onze resultaten het onderzoek moeten herhalen in grotere groepen met informatie over nuchter gemeten maternaal glucose in de vroege zwangerschap.

In **Hoofdstuk 3** worden de door ons uitgevoerde studies beschreven ten aanzien van leververvetting bij het kind.

De inname van suikerhoudende dranken is bij zowel kinderen als volwassenen een belangrijke risicofactor voor het ontwikkelen van obesitas. Een hogere inname van suikerhoudende dranken lijkt verband te houden met niet-alcoholische leververvetting bij volwassenen. Het doel van **Hoofdstuk 3.1** is om de associatie te onderzoeken tussen de inname van suikerhoudende dranken op de leeftijd van 1 jaar en de hoeveelheid leververvetting bij kinderen op 10-jarige leeftijd. We hebben vastgesteld dat een hogere inname van suikerhoudende dranken op de leeftijd van 1 jaar geassocieerd is met een verhoogd risico op niet-alcoholische leververvetting bij kinderen op 10-jarige leeftijd. Deze associatie is sterker bij kinderen van moeders met een lager opleidingsniveau, dan bij kinderen van moeders met een hoger opleidingsniveau. Ook is deze associatie sterker bij kinderen met overgewicht of obesitas ten opzichte van kinderen met een normaal gewicht. Onze resultaten suggereren dat het beperken van de inname van suikerhoudende dranken vanaf de peutertijd kan helpen om leververvetting op de schoolleeftijd te voorkomen. Dit zou een speerpunt kunnen worden in toekomstige preventiestrategieën.

In **Hoofdstuk 3.2** hebben we de hypothese getest dat DNA-methylering bij de geboorte en op de schoolleeftijd geassocieerd is met de mate van leververvetting op 10-jarige leeftijd. We hebben ook onderzocht of DNA-methylering op bekende CpGs geassocieerd met niet-alcoholische leververvetting bij volwassenen, ook geassocieerd is met leververvetting bij kinderen. DNA-methylering bij de geboorte en op schoolleeftijd is in onze studie niet geassocieerd met de mate van leververvetting op 10-jarige leeftijd. Ook is de DNA-methylering waarvan bekend is dat het verband houdt met niet-alcoholische leververvetting bij volwassenen, niet geassocieerd met leververvetting bij kinderen. Dit kan mogelijk komen door een te kleine studiepopulatie of doordat veranderingen in DNA-methylering eerder een gevolg, dan een determinant van leververvetting zijn. Hoewel onze bevindingen de hypothese dat ongunstige omstandigheden in het vroege leven bijdragen aan de ontwikkeling van leververvetting via DNA-methylering niet ondersteunen, kan deze hypothese op basis van onze analyses nog niet volledig worden verworpen. Toekomstige studies zouden DNA-methylering met de hoeveelheid leververvetting bij kinderen in grotere longitudinale studies moeten onderzoeken.

De impact van leververvetting en de bijbehorende hart- en metabole risicofactoren bij gezonde kinderen is onbekend. We hebben 10-jarige kinderen onderzocht in **Hoofdstuk 3.3**, waarbij we hebben gekeken of de mate van leververvetting en het optreden van niet-alcoholische leververvetting geassocieerd is met hart- en metabole risicofactoren. We zien dat een hoger percentage leververvetting en het optreden van niet-alcoholische leververvetting geassocieerd zijn met een ongunstig hart- en metabool risicoprofiel bij 10-jarige kinderen. Clustering van hart- en metabole risicofactoren zijn al waargenomen vanaf een levervet-percentage van 2.0%. Deze associaties zijn onafhankelijk van de body mass index van kinderen en zijn sterker bij kinderen met overgewicht en obesitas dan bij kinderen met een normaal gewicht. Deze resultaten suggereren dat niet-alcoholische leververvetting al aanwezig is bij kinderen met zowel een normaal gewicht, als

met overgewicht of obesitas op de schoolleeftijd en dat leververvetting verband houdt met een verminderde hart- en metabole gezondheid.

In **Hoofdstuk 3.4** hebben we verschillende clusters van voorspellende factoren voor niet-alcoholische leververvetting bij kinderen vergeleken. We hebben laten zien dat gemakkelijk te verkrijgen karakteristieken van het kind een redelijk goede voorspelling kunnen genereren om kinderen met een verhoogd risico op het ontwikkelen van niet-alcoholische leververvetting te identificeren. De onderscheidende waarde van dit voorspellingsmodel is verder verbeterd door het toevoegen van maternale kenmerken en hart- en metabole kenmerken van het kind. Onze voorspellingsmodellen voor niet-alcoholische leververvetting bij kinderen moeten eerst extern worden gevalideerd om de bruikbaarheid in andere populaties te beoordelen. Hierna zouden ze in de klinische praktijk kunnen worden toegepast ter identificatie van kinderen die een verhoogd risico lopen op niet-alcoholische leververvetting.

In **Hoofdstuk 4** sluiten we het proefschrift af met het bespreken van de belangrijkste bevindingen uit de beschreven studies en plaatsen we de bevindingen in een bredere context. Daarnaast geven we aanbevelingen voor toekomstig onderzoek en beschrijven we implicaties voor de klinische praktijk en beleidsvorming.

Concluderend suggereren de studies in dit proefschrift dat blootstellingen in de beginfase van het leven, zoals maternale glucosewaarden tijdens de vroege zwangerschap, op korte en langere termijn geassocieerd zijn met de gezondheidsuitkomsten voor moeder en kind. Daarnaast hebben we vroege voorspellende factoren voor niet-alcoholische leververvetting bij kinderen geïdentificeerd. Onze bevindingen zullen bijdragen aan nieuwe preventiestrategieën gericht op het verbeteren van de gezondheid voor en tijdens de vroege zwangerschap en in de vroege kindertijd, om een gezonde ontwikkeling en groei al vanaf het vroege leven na te streven.

A

List of publications
PhD portfolio
About the author
Words of gratitude &
Dankwoord

LIST OF PUBLICATIONS

Madelon L. Geurtsen, Charlie C. Obihara. Een zuigeling met een zwarte, harige tong [An infant with a black hairy tongue]. *Nederlands Tijdschrift voor Geneeskunde. 2016;160:D130. Dutch.* PMID: 27229695.

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PHD PORTFOLIO

Name PhD candidate:	Madelon Liselotte Geurtsen
Erasmus MC Department:	The Generation R Study
	Pediatrics, Erasmus MC, Rotterdam
Medical School:	VU University Amsterdam, 2007 - 2013
Research School:	Netherlands Institute for Health Sciences (NIHES), Rotterdam, 2017 - 2019
PhD period:	May 2017 - January 2021
Promotor:	Prof. dr. V.W.V. Jaddoe
Copromotor:	Dr. J.F. Felix

	Year	Workload (ETCS)
PhD training		
Master of Science in Clinical Epidemiology, NIHES, Rotterdam, the Netherlands	2017 - 2019	70.0
Common core		
Principles of Research in Medicine and epidemiology	2017	
Methods of Public Health Research	2017	
Clinical Trials	2017	
Health Economics	2017	
The Practice of Epidemiologic Analysis	2017	
Fundamentals of Medical Decision Making	2017	
Study Design	2018	
Biostatistical Methods I: Basic Principles	2018	
Biostatistical Methods II: Classical Regression Models	2018	
Introduction to Medical Writing	2019	
Clinical Translation of Epidemiology	2017	
Clinical Epidemiology	2018	
Principles in Causal Inference	2018	
Elective courses		
Review of Mathematics and Introduction to Statistics	2018	
Intermediate Course in R	2018	
Genomics in Molecular Medicine	2018	
Logistic Regression	2019	
Genome Wide Association Studies	2018	
Human Epigenomics	2018	
Principles of Epidemiologic Data-analysis	2017	
Repeated Measurements in Clinical Studies	2019	
General Academic Courses		
Scientific Integrity for PhD students, Erasmus MC, Rotterdam	2018	0.3
Seminars and workshops		
Research meetings Generation R Study	2017 - 2021	1.0

Maternal and Child Health meetings	2017 - 2021	1.0
Molecular Epidemiology meeting	2017 - 2021	1.0
LifeCycle Project - Consortium meetings (Rotterdam, Oulu, Bristol)	2017 - 2019	1.0
Systematic Literature Search (part I and II), Medical Library	2017	0.4
Endnote Workshop	2017	0.2
Jonge Onderzoekers Dag, Tulips for Child Health, Utrecht	2019, 2020	1.4
DataSHIELD, Newcastle University, England	2019	0.7
Conferences		
Developmental Origins of Health and Disease (DOHaD), Rotterdam *Poster presentation*	2017	0.7
Sophia Research Day, Rotterdam *Oral presentation*	2018	0.7
3rd Paula Rantakallio Symposium on Birth Cohorts and Longitudinal Studies, Oulu, Finland *Poster presentation*	2018	0.7
Sophia Research Day, Rotterdam *Oral presentation*	2019	1.4
PRE-cis-E annual meeting, oral presentation, Rotterdam *Oral presentation*	2020	0.7
European Academy of Paediatric societies, virtual congress *Oral presentation*	2020	0.7
Teaching activities		
Eef E.L. van Soest, Master Student	2017 -2018	3.0
Jazmin Taubert, PhD Student	December 2020 - June 2021	
Jasmin de Groot, Master student	Januari - June 2021	
Irene C. Marques, PhD student	February 2021 - June 2021	
Peer review		
Clinical Epigenetics, European Journal of Epidemiology, Pediatric Allergy and Immunology, Journal of Gerontology Medical Science	2018 - 2021	1.0
Scholarship		
Vereniging Trustfonds Erasmus MC University Medical Center, Rotterdam	2018	

*1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours.

ABOUT THE AUTHOR

Madelon Geurtsen was born on 15th of December 1987 in Loenen, the Netherlands. As a little child she climbed the trees of a forest near Hilvarenbeek, where she spent the first half of primary school. Thereafter, she explored the neighbourhood of Wijk en Aalburg, where she finished the second half of primary school. From this village she cycled to her secondary school the Stedelijk Gymnasium in 's Hertogenbosch and graduated in 2006. Her journey continued by train to Zwolle, to study Health and Life Sciences there for one year at the dependence of the VU University of Amsterdam and she obtained her propedeutics. In 2007 she moved from Zwolle to Amsterdam, to start with medical school at the VU University of Amsterdam. After obtaining her medical degree in 2013, she started working as a resident in pediatrics (ANIOS) in the Elisabeth-TweeSteden Hospital in Tilburg. In 2015 she started her residency to become a pediatrician. In 2017, she interrupted her residency to start with her PhD project focused on maternal glucose metabolism and child liver fat in early life under supervision of Prof. dr. Vincent Jaddoe and dr. Janine Felix at the department of Generation R at the Erasmus MC Rotterdam. During her project, she obtained her Master of Science degree in Clinical Epidemiology at the Netherlands Institute of Health Sciences (NIHES) in 2019. She will continue her pediatric residency from September 2021 onwards.

Madelon lives in Rotterdam with her beloved husband Martijn and son Sem, together with three cats Benji, Balou and Beau and a lot of plants.

WORDS OF GRATITUDE & DANKWOORD

"Wisdom is like a baobab tree, no one individually can embrace it" – African proverb

Onderzoek is samenwerken, daarom een groot dankjewel aan iedereen die op welk vlak dan ook geholpen heeft. Ergens op de weg van het begin tot het einde, of tijdens mijn gehele promotietraject. Graag bedank ik een aantal van jullie in het bijzonder.

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“An end has a start” – Editors

"Wisdom is like a baobab tree
no one individual can embrace it"
- African proverb