Perinatal Determinants of Child Brain Development

A Population-Based Neuroimaging Study

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The work presented in this thesis was conducted at the Department of Child and Adolescent Psychiatry/Psychology and the Generation R Study Group, Erasmus Medical Center, Rotterdam, the Netherlands. The Generation R Study is conducted by the Erasmus Medical Center in close collaboration with the Faculty of Social Sciences of the Erasmus University Rotterdam, the Municipal Health Service Rotterdam area, the Rotterdam Homecare Foundation, and the Stichting Trombosedienst and Artsenlaboratorium Rijnmond (STAR-MDC), Rotterdam, the Netherlands.

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Perinatal Determinants of Child Brain Development

A Population-Based Neuroimaging Study

Perinatale determinanten van hersenontwikkeling in de kindertijd

Een epidemiologische neuroimaging studie

Thesis

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Chapter 1

General Introduction

INTRODUCTION

"Neuroscience is by far the most exciting branch of science because the brain is the most fascinating object in the universe. Every human brain is different - the brain makes each human unique and defines who he or she is."

—— *Stanley B. Prusiner*

Compared to the objects we can see and touch around us, it has always been more challenging to get to know ourselves. Dating back almost 5000 years, ancient Egyptians believed that the heart was the center of emotions and intelligence. Likewise, the first and the most classic traditional Chinese medicine bible *Hunangdi Neijing* (literally *The Yellow Emperor's Classic of Internal Medicine*) argues: 'The heart is the organ similar to the monarch and is responsible for spirit and mental activity'. Hippocrates (460-379 B.C.E.), the father of Western medicine, was one of the first to argue that the brain is the seat of thought, sensation, emotion and cognition. However, this view was not universally accepted. For example, the famous philosopher Aristotle (384-322 B.C.E.) clung to the belief that the heart was the center of intellect, and considered the function of the brain was to 'cool the passions of the heart'. During the Roman empire, physician Galen (130-200 C.E.) embraced the Hippocratic view of brain via careful animal dissections. From the Renaissance to modern centuries, human's knowledge on the brain has been substantially expanded.[1] Nowadays, the human brain, an organ comprising only 2% of total body weight, attracts continuous and increasing attention from the scientific world.

About the brain — an overview

The brain consists of three main parts: the cerebrum, the cerebellum, and the brain stem. In this thesis, I focus on the cerebrum, and only briefly mention the cerebellum.

With two hemispheres bridged by corpus callosum, the cerebrum is the largest part of the brain. The surface of the cerebrum is called the cerebral cortex, which has a folded appearance with hills ('gyri') and valleys ('sulci'). Enabling large cortical surface in a limited skull size, cortical folding first occurred in the ancestors of mammals and is an important milestone along evolution. Characterized by the gyrification index, the human brain is the largest and most intensively folded primate brain.[2] The cortex consists of gray matter, which contains neuronal cell bodies, neuropil, glial cells, synapses, and capillaries. The cerebral cortex is responsible for multiple functions, depending on the specific lobes (i.e., the frontal lobes, the temporal lobes, the parietal lobes, and the occipital lobes). Beneath the cerebral gray matter are long-range axons covered with a sheath of myelin, termed white matter, that connect different brain areas to each other. Myelination greatly

increases the speed of neurotransmitter signaling.[3] In addition to the cortical gray and white matter, subcortical gray matter including the diencephalon, pituitary gland, limbic structures and the basal ganglia are present deeper in the cerebrum. These structures are involved in complex activities such as memory and emotion (e.g., hippocampus and amygdala), and hormone production (e.g., hypothalamus and pituitary gland).

As the second-largest part of the brain, the cerebellum locates underneath the occipital lobe and contains more than half the brain's neurons. Similar to the cerebrum, the greatly folded cerebellum is divided into two hemispheres, with gray matter in the out layer and white matter deep inside. Although the most remarkable function of the cerebellum is coordinating body movements and motor skills learning, the cerebellum's role in cognitive processes has attracted increasing research interest.[4]

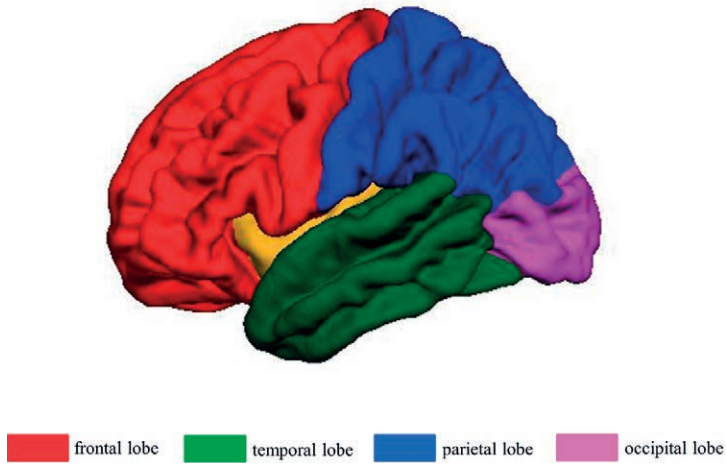

Figure 1.1. Lobes of the brain

Brain growth across gestation and childhood

The human brain development begins in the embryonic period (i.e., from conception to 8 weeks of gestation), when the neural stem cells (also called neural progenitor cells) appear, and the first brain structure—the neural tube—is formed.[5] The fetal period (i.e., from the ninth gestational week through the end of gestation) is a critical phase characterized by enormous subsequent neuron production, migration, and differentiation, and both the cerebellum and the cerebral cortex experience exponential volumetric increase during the second half of gestation.[6] The human brain also gradually develops the characteristic mature pattern of gyral and sulcal folding by the end of gestation.

During the postnatal period and childhood brain growth continues but volume reductions add a level of complexity to the exponential growth in the fetal period. For example, there are continuous age-related increases in cerebral white matter volume

during childhood and early adulthood, but the volumes of frontal cortex and subcortical structures such as the thalamus and nucleus accumbens show a decrease trend with age.[7] Further, cortical thickness of most of the lateral frontal, lateral temporal, parietal and occipital isocortex show cubic trajectories, with a period of initial increase in childhood, followed by adolescent decline and stabilization in adulthood.[8] Human myelination is also a largely postnatal process that peaks in early childhood.[9]

Neurodevelopment with the DOHaD framework

The attempt to discover the causes of abnormal development or non-communicable diseases has been long, and possibly it will remain an endless journey. Although genetic variability may be an answer, it is far from capturing the whole picture.[10] In 1990s, David Barker and his colleagues reported associations between low birth weight and adverse health outcomes in childhood and adulthood, such as high systolic blood pressure, elevated risk of mortality of cardiovascular disease, impaired glucose tolerance, and type II diabetes.[11,12] Based on these findings, they developed the concept that early life, mostly prenatal, events can cause permanent changes in physiology that may later predispose people to disease, which was originally termed Barker Hypothesis, and is now known as the Developmental Origins of Health and Disease (DOHaD).[10,13] This hypothesis has been supported by cumulative evidence, including studies among men and women who were born around the Dutch famine of 1944–1945, suggesting that prenatal exposure to undernutrition is predictive of not only cardio-metabolic consequences, but also neuropsychiatric disorders such as schizophrenia in adult life.[14] In addition, the effects of undernutrition are dependent of its timing. Much evidence from animal models and observational human studies shows that the period from conception to early childhood, namely the period when organogenesis and rapid body growth take place, is critical to the immediate and future health of the offspring. The underlying mechanisms mediating the programming effects of environmental adversities are less clear. Candidate explanations include excessive exposure to maternal glucocorticoids, dysregulation in the development of the hypothalamic-pituitary-adrenal (HPA) axis, irreversible changes in organ structure, and alteration in gene expression.[15]

In addition to cardiovascular and metabolic diseases that were the initial conditions of interest of DOHaD and remain as the dominant focus, neurodevelopmental diversities as consequences of early life exposure have gained attention in the recent decades. A largely consistent body of evidence related environmental chemical or other toxic substance exposure to differences in neurodevelopment. For example, maternal gestational lead levels were inversely related to mental development in infants and toddlers;[16,17] higher exposure to polycyclic aromatic hydrocarbons (PAHs) in fetal life was associated with IQ deficit and behavior problems at age 5-7 years;[18,19] and prenatal exposure to tobacco predicted irritability, attention and behavioral problems, and cognitive impairment in childhood.[20,21]

The association of other perinatal (i.e., prenatal and early postnatal) determinants, such as maternal mental health and nutrients with neurodevelopment of the offspring has been investigated to a lesser extent.

Maternal mental health

Pregnancy can be both exciting and tough for the mother, physically as well as mentally. Mood swings during or following gestation are not rare, but symptoms beyond the normal range can have substantial clinical implications. In high-income countries, 10% - 13% women experience some type of mental disorders during the perinatal phase.[22] These numbers can reach up to 15% - 20% in the settings of low- and middle-income countries.[23] As the most common mental health issue among the pregnant women, perinatal depression not only affects the wellbeing of the mother, but also predicts adverse developmental outcomes in the offspring, including suboptimal neurodevelopment. For example, children born to women with major depressive disorder during pregnancy had lower motor development scores and lower language scores in infancy.[24] More postpartum maternal depressive symptoms predicted a higher risk of autism spectrum disorders (ASD), lower intelligence, and more behavior problems of the child.[25,26] However, investigations on the relation between maternal depression and child brain development, which may be the biological mechanism underlying these cognitive or neuropsychiatric outcomes, remain limited. A few previous studies suggested that more maternal depressive symptoms during the perinatal phase were related to a larger amygdala of the offspring in childhood, and a sex difference was reported.[27,28] Associations between antenatal maternal depressive symptoms and right hippocampal volume in neonates were also found.[29] However, there is limited research of the long-term effect of prenatal exposure to depressive symptoms on brain morphological development. In addition, since depression is a chronic condition with notable recurrence,[30] it is important is take into account the persistency of exposure to depressive symptoms over time.

Maternal nutrients

Maternal nutrients during pregnancy are critical for intrauterine growth and development of the child, because many nutrients cannot be synthesized by the fetus and must be obtained from the mother and transported through the placenta. Depending on the biochemical and physiological features, deficiency of different nutrients during pregnancy may have distinct consequences on short- and long-term neurodevelopment of the child. Below, I discuss three important micronutrients: vitamin D, folate and fatty acids.

Vitamin D is a fat-soluble vitamin with the main source of natural sunlight. Since the major biological function of vitamin D involves maintaining normal blood levels of calcium and phosphorus, maternal vitamin D status during pregnancy has been mostly linked to bone health of the offspring.[31-33] However, vitamin D is also an important neurosteroid that is involved in proliferation and differentiation of neurons,[34] and vitamin D receptors

can be found in widespread brain tissue. Emerging evidence has related maternal vitamin D levels to child neurodevelopment. However, despite a few animal studies suggesting lower cortical volumes and greater lateral ventricle volume in rats born to vitamin D deficient mothers,[35,36] it remains largely unknown whether maternal gestational vitamin D status predicts variation in brain morphology of the offspring in humans.

Folate also belongs to the vitamin family and involves in deoxythymidylate synthesis, purine synthesis, and various methylation reactions as an important coenzyme. Folate deficiency of the mother during the periconceptional period is best known to increase the risk of neural tube defects (NTDs) of the fetus, which leads to miscarriage or severe birth defects.[37] In addition, emerging evidence shows that exposure to low folate levels during pregnancy is also associated with long-term neuropsychiatric outcomes such as more behavioral problems and autistic traits in childhood.[38,39]. Embedded in the current cohort, smaller HC in utero and less total brain volume at age 6-8 years have been found in children exposed to low folate levels in pregnancy.[40,41] With brain imaging data of children aged 9-11 years, investigating prenatal folate status in relation to brain developmental trajectories across fetal life and childhood would provide insights on the long-term effects of intrauterine folate exposure, as well as the age-dependent brain plasticity.[42]

Fatty acids and their associated derivatives are the primary components of lipids. Most fatty acids can be endogenously synthesized by human, but linoleic acid (LA) and α-linolenic acid (ALA) are essential fatty acids that must be obtained from food and serve as the precursors of other polyunsaturated fatty acids (PUFAs) from the omega-3 (ω-3) and omega-6 (ω-6) families. Long chain PUFAs (LC-PUFAs) such as docosahexaenoic acid (DHA) and arachidonic acid (ARA) are also termed conditionally essential fatty acids because of the inefficient endogenous production. PUFAs are primarily involved in lipid metabolism, and therefore have been mostly related to cardiovascular and metabolic outcomes. To date, investigations on the impact of maternal status of fatty acids, including trans fatty acids (TFAs) that are primarily found in industrial oil, on child brain development remain scarce, despite the evidence suggesting roles of specific fatty acids (e.g., ω-3 PUFAs) in the process of neurogenesis and neuroplasticity.[43-45]

Gestational duration

Gestational age at birth (GAB) is an important indicator of obstetric outcome. Both preterm (i.e., GAB<37 weeks) and post-term (i.e., GAB>42 weeks) birth have been associated with elevated mortality in neonates and infants.[46,47] Long-term neurodevelopmental outcomes related to GAB are frequently reported. Further, a few previous studies have shown that preterm birth is associated with differences in brain morphology, such as less gray and white matter volumes across infancy and adulthood.[48-50] However, there has been no study examining the relation between GAB as a continuum

and long-term brain development, which has important clinical implication given the worldwide prevalence of elective cesarean deliveries often between 37-39 weeks.[51]

Unveiling determinants of brain development with epidemiology and neuroimaging

Randomized controlled trials (RCTs) are the gold standard for establishing cause-effect relations, but they cannot always be easily implemented in reality and frequently encounter ethic issues. Stemming from the investigations into the causes of the 19th-century cholera epidemics by John Snow, modern epidemiology has become the study of 'the distribution and determinants of health-related events in specified populations, and the application of this study to the control of health problems'.[52] Applying advanced epidemiological methods enables investigators to identify determinants of health or developmental conditions while minimizing biases in observational studies.

With the rapid growth of modern neuroimaging modalities, cerebral tissue and function can now be assessed quantitatively, thus the potential for reproducible evaluation is unlocked.[53] Without radiative hazards, magnetic resonance imaging (MRI) is a non-invasive modality to obtain brain data with high quality. Brain morphological outcomes such as volumetric measures can be then estimated after data reconstruction using software such as FreeSurfer or FSL. In addition, diffusion tensor imaging (DTI) is a unique MRI technique that measures the extent of diffusion in all directions in the three-dimensional space and hence maps the anisotropy of water diffusion in the brain, thus reflecting the organization and architecture of white matter fibers.

A major concern on current neuroimaging studies is limited sample sizes,[54] which potentially brings the issue of inadequate statistical power especially when a number

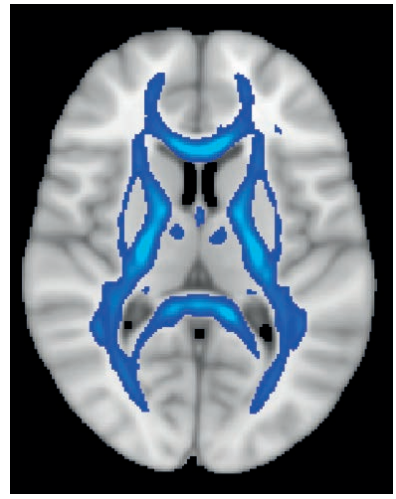

Figure 1.2. White matter microstructure of the brain

of confounding factors should be taken into account. With a large sample size, this population-based neuroimaging study enabled the application of modern epidemiological methods to minimize various biases and obtain valid results. In addition, the prospective design warranted the temporal relations between the determinants and outcomes of interest, and the application of repeated measures contributed to a more comprehensive understanding from a longitudinal perspective.

THIS THESIS

General aim

Using data from one of the largest pediatric neuroimaging studies worldwide, the aim of this thesis was to use epidemiological methods to investigate prospective associations of several modifiable perinatal determinants (i.e., maternal depressive symptoms, maternal nutrients, and gestational duration) with brain morphology and white matter microstructure in children aged 9-11 years old. I also aimed to study whether any observed brain differences are in turn related to neurodevelopmental outcomes of the child.

Setting

All studies in this thesis were embedded in the ongoing Generation R Study, a population-based prospective cohort from fetal life onwards in Rotterdam, the Netherlands. Pregnant women with an expected delivery date between April 2002 and January 2006 were eligible for participation in the study. Data collection started upon recruitment in pregnancy and participants and their children were followed up in the preschool period (0-4 years), early childhood (6 years), mid-childhood (10 years), and early adolescence (13 years).[55] Maternal and paternal psychopathology was assessed repeatedly using validated questionnaires from mid-gestation onwards. Biological samples such as maternal blood collected in pregnancy were assayed to determine the status of several nutrients. Ultrasound assessment was performed at each trimester to collect information on anthropometry including head circumference (HC) of the fetus, as well as to assist pregnancy dating (ultrasound in the first trimester).[56] In addition, children were invited to participate in a brain imaging session that included high-resolution structural, diffusion tensor, and resting-state functional MRI sequences at age 6-8 years and 9-11 years.[57,58]

Outline

In chapter 2, the association of maternal depressive symptoms across the perinatal period and childhood with offspring brain morphology and white matter microstructure at age 6-8 years (section 2.1) and 9-11 years (section 2.2) was examined. Chapter 3

focused on the association of maternal nutrients during pregnancy, including vitamin D (section 3.1), folate (section 3.2), PUFAs (section 3.3), and TFAs (section 3.4) with child brain morphology at age 9-11 years. In addition, earlier brain measures such as HC in fetal life and total brain volume at age 6-8 years were examined in section 3.2 and section 3.4. In chapter 4, the relation between gestational duration and brain morphometry at age 9-11 years was examined. The main findings of these studies are presented, and practical implications with future directions are discussed in chapter 5. Chapter 6 gives a brief summary of this thesis.

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Chapter 2

Parental Mental Health and Brain Development in Childhood

2.1

Prenatal exposure to maternal and paternal depressive symptoms and white matter microstructure in children

El Marroun H., Zou R., Muetzel R.L., Jaddoe V.W., Verhulst F.C., White T., & Tiemeier H. (2018)

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ABSTRACT

Background: Prenatal maternal depression has been associated with multiple problems in offspring involving affect, cognition and neuroendocrine functioning. This suggests that prenatal depression influences neurodevelopment. However, the underlying neurodevelopmental mechanism remains unclear. We prospectively assessed whether maternal depressive symptoms during pregnancy and at the child's age 3 years are related to white matter microstructure in 690 children. The association of paternal depressive symptoms with childhood white matter microstructure was assessed to evaluate genetic or familial confounding.

Methods: Parental depressive symptoms were measured using the Brief Symptom Inventory. In children aged 6-9 years, we used diffusion tensor imaging to assess white matter microstructure characteristics including fractional anisotropy (FA) and mean diffusivity (MD).

Results: Exposure to maternal depressive symptoms during pregnancy was associated with higher MD in the uncinate fasciculus and to lower FA and higher MD in the cingulum bundle. No associations of maternal depressive symptoms at the child's age of 3 years with white matter characteristics were observed. Paternal depressive symptoms also showed a trend towards significance for a lower FA in the cingulum bundle.

Conclusions: Prenatal maternal depressive symptoms were associated with higher MD in the uncinate fasciculus and the cingulum bundle. These structures are part of the limbic system, which is involved in motivation, emotion, learning, and memory. As paternal depressive symptoms were also related to lower FA in the cingulum, the observed effect may partly reflect a genetic predisposition and shared environmental family factors and to a lesser extent a specific intrauterine effect.

INTRODUCTION

Stress, anxiety or depression during pregnancy may affect fetal development and thus negatively impact offspring (reviewed in Dunkel Schetter, 2011; Goodman, 2007; Mulder et al., 2002). Prenatal depression has been related to low birth weight and preterm birth, affective problems, poor cognitive functioning, and impaired neuroendocrine functioning. Previously, we demonstrated that prenatal maternal depressive symptoms were related to decreased fetal (head) growth (El Marroun et al., 2012; Henrichs et al., 2010), behavioral and emotional problems (El Marroun et al., 2014), and executive functioning problems in childhood (El Marroun et al., 2017). These findings suggest that prenatal maternal depression influences neurodevelopment through underlying mechanisms that have yet to be elucidated.

Neuroimaging can be used to better understand the underlying neurobiological effects of prenatal maternal depression on offspring. Recently, Sandman and colleagues showed that exposure to maternal depressive symptoms was associated with cortical thinning in 6-to-9 year old children, particularly in the prefrontal cortex (Sandman, Buss, Head, & Davis, 2015). Similarly, our own research group showed a thinner superior frontal cortex in children exposed to maternal depressive symptoms (El Marroun et al., 2016). However, brain regions do not function in isolation as they are interconnected through a vast network of myelinated axons. Thus, it is important to study the structural connectivity of the brain as well.

Very limited information is available on the associations of prenatal maternal depression with the structural connectivity in offspring. To our knowledge only three studies from two cohorts have been published on this specific topic. A recent study in neonates (6-14 days of age) from the Growing Up in Singapore Towards Healthy Outcomes (GUSTO) study demonstrated that prenatal maternal depression was associated with lower fractional anisotropy (FA) and axial diffusivity (but not volume) in the right amygdala (Rifkin-Graboi et al., 2013). At follow-up around 4.5 years of age, prenatal depressive symptoms were associated with larger volumes of the right amygdala, while postnatal depressive symptoms were related to higher FA in the right amygdala, particularly in girls (Wen et al., 2017). Further, in a subgroup of the Alberta Pregnancy Outcomes and Nutrition (APrON) cohort maternal depressive symptoms were related to diffusivity measures of white matter tracts emanating from the inferior frontal and middle temporal region, which included the uncinate, the inferior-fronto-occipital and arcuate fasciculi (Lebel et al., 2015).

In the current study, we will investigate the relation between prenatal exposure to maternal depressive symptoms and white matter microstructure assessed using diffusion tensor imaging (DTI) in childhood (6-9 years). A challenge in investigating this research question is to disentangle whether an association reflects an actual intrauter-

ine effect or whether it is confounded by genetic and environmental factors. To address this, we used a prospective design taking into account maternal depressive symptoms during pregnancy, maternal depressive symptoms at the child's age 3 years as well as paternal depressive symptoms during pregnancy. Using maternal depressive symptoms at 3 years will provide information on whether specific timing of the exposure to depressive symptoms is important. Furthermore, prenatal exposure to paternal depressive symptoms will provide insight as to whether genetic factors or confounding by shared environmental family factors play a role (G. D. Smith, 2008b).

Based on our prior findings that maternal depressive symptoms were related to decreased cortical thickness in the frontal brain region (El Marroun et al., 2016), and on prior literature showing emotional problems in children prenatally exposed to maternal depressive symptoms (Dunkel Schetter, 2011; El Marroun et al., 2014; Goodman, 2007; Goodman et al., 2011), we hypothesize that children exposed to prenatal depressive symptoms will have atypical white matter microstructure, particularly in the limbic system. The limbic system is involved in motivation, emotion, learning, and memory. Like the approach used in the study of Lebel et al. 2015 in which white matter tracts were selected based on the findings in cortical thickness, we focused on five different white matter tracts that are either in the frontal brain area (forceps minor), connect the frontal brain area with other parts of the brain (superior and inferior longitudinal fasciculus), or are part of the limbic system (cingulate and uncinate fasciculus).

MATERIALS AND METHODS

Study population

This study reports findings from the Generation R Study; a population-based cohort in Rotterdam (the Netherlands) designed to identify environmental and genetic determinants of maternal and child development, including physical and mental health, from fetal life onwards (Jaddoe et al., 2012). The Medical Ethics Committee of the Erasmus Medical Centre approved the protocol for this neuroimaging study.

In total, 1070 children aged 6-to-9 years were scanned in a neuroimaging study component (White et al., 2013). Subjects with missing data (n=37, 3.5%), poor data quality (n=255, 23.8%), or major cerebral abnormalities (n=2, 0.2%) were excluded from the analyses. Children of mothers who used antidepressants were excluded (n=36, 3.3%). Another 50 (4.7%) children were excluded because of missing information on prenatal maternal depressive symptoms. Further, children with pervasive developmental problems or autism were also excluded (n=54) as they may have different neurodevelopmental trajectories (Courchesne et al., 2007). The final study population consisted of 636 children . Children with psychiatric problems such as attention deficit

hyperactivity disorder (ADHD) and aggressive problems (n=78) were oversampled, thus a sensitivity analysis was performed excluding these children.

Prenatal exposure to maternal and paternal depressive symptoms

Depressive symptoms were assessed with the Brief Symptom Inventory (BSI) at, on average, 20.6 weeks of gestation. The BSI is a validated self-report questionnaire with 53 items (Derogatis & Melisaratos, 1983), which define a spectrum of psychiatric symptoms; in the current study we used the six-item depression scale. Internal consistency for the depression scale was α=0.80. At the child's age of 3, the BSI was assessed again to determine maternal depressive symptoms. Paternal depressive symptoms during pregnancy were assessed using the same instrument (BSI) at the same time in a separate questionnaire for fathers. Parents with a score higher than 0.75 have clinically relevant depressive symptoms according to the Dutch norm data in the manual (de Beurs, 2004).

Magnetic Resonance Imaging

Prior to neuroimaging, all children were first familiarized with MRI scanning during a mock scanning session. All images were acquired using the same sequence on the same scanner (3 Tesla GE 750 Discovery). DTI data were acquired using a single-shot, echo-planar sequence with the following parameters: TR=11.000 ms, TE=83 ms, flip angle=90, matrix=128×128, FOV=256×256 mm, slice thickness=2 mm, number of slices=77, acquisition time=7 min 40 s. In total, 35 volumes with diffusion weighting (b=1000 s/mm^2) and 3 volumes without diffusion weighting (b=0 s/mm^2) were acquired.

Image preprocessing and probabilistic fiber tractography

The technical details of the image preprocessing and fiber tractography in our cohort have been extensively described previously (Muetzel et al., 2015). Data were processed using the functional MRI of the Brain's Software Library (FMRIB, FSL) and the Camino Diffusion MRI Toolkit (Cook et al., 2006). Image processing tools were performed in Python v2.7 through the Neuroimaging in Python Pipelines and Interfaces package v0.92) (Gorgolewski et al., 2011). First, using the FSL "eddy_correct" tool (Jenkinson & Smith, 2001) images were adjusted for eddy-current and motion induced artifacts (Haselgrove & Moore, 1996). Using the FSL Brain Extraction Tool (S. M. Smith, 2002) non-brain tissue was removed. The diffusion tensor was fit using the RESTORE method implemented in Camino (Chang, Jones, & Pierpaoli, 2005). Common scalar maps, that is, fractional anisotropy (FA) and mean diffusivity (MD) were computed. Fully automated probabilistic fiber tractography was performed using the FSL plugin, "AutoPtx" (http://fsl.fmrib.ox.ac.uk/fsl/fslwiki/AutoPtx) (de Groot et al., 2015). The tracts used in the current study are shown in Figure 1.

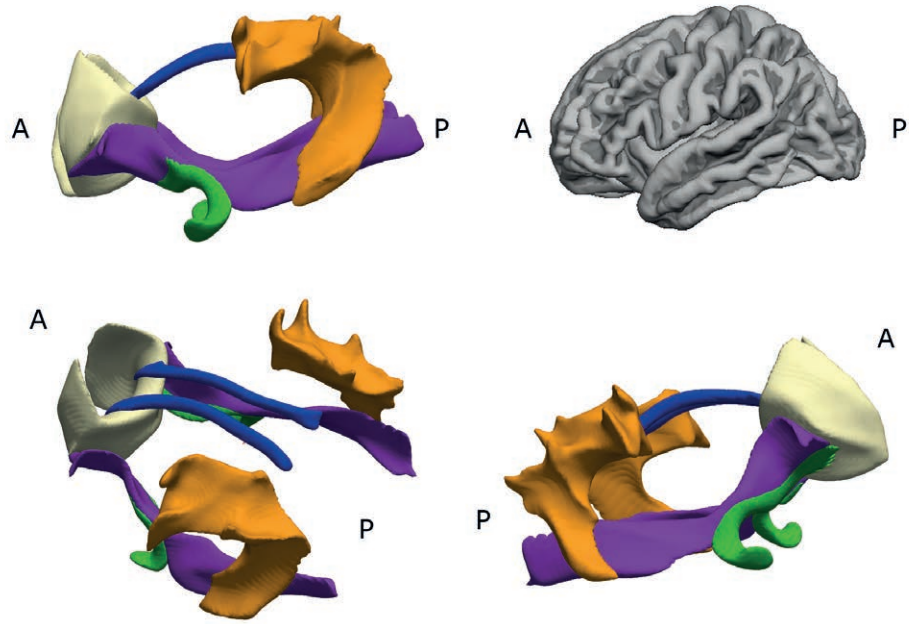

Figure 1. White matter tracts of interest. The five tracts used in the analyses: blue = cingulum bundle, green= uncinate fasciculus, orange = superior longitudinal fasciculus, cream= forceps minor, and purple = inferior fronto-occipital fasciculus. A = Anterior, P = Posterior.

Additional measures

The following demographic information was collected: maternal age at intake, educational level, ethnicity, prenatal smoking and alcohol use, maternal diet, age of the child at MRI and IQ assessment, and gender of the child. Educational level was categorized into three levels: primary (no or primary education), secondary (lower and intermediate vocational training), and higher (higher vocational education and university) education. Maternal ethnicity was defined according to the classification of Statistics Netherlands and was categorized in Dutch, non-Dutch Western and non-Dutch non-Western. Information on maternal smoking and alcohol use during pregnancy was collected with questionnaires during each trimester. Maternal smoking was categorized into: 'never smoked in pregnancy', 'smoked until pregnancy was known' and 'continued to smoke in pregnancy'. Maternal alcohol use was categorized in: 'never drank in pregnancy', 'drank until pregnancy was known', 'continued to drink in pregnancy occasionally' and 'continued to drink in pregnancy frequently'. We assessed maternal diet during pregnancy using a food frequency questionnaire, and computed a diet quality score (Nguyen et al., 2017). Non-verbal IQ in children was assessed using two subtests of a Dutch IQ test: Snijders-Oomen Niet-verbale intelligentie Test–Revisie (SON-R 2½-7)

(Tellegen, Winkel, B., & Laros, 2005). Information on child emotional and behavioral problems was assessed at 6 years using the well-validated Child Behavior Check List (CBCL) (Achenbach & Rescorla, 2000).

Statistical analyses

The associations of maternal and paternal depressive symptoms during pregnancy with FA and MD were analyzed using linear regression analyses. Maternal or paternal depressive symptoms were used as determinants and childhood white matter characteristics were used as outcomes. Because there were no a priori hypotheses about specific hemispheric effects and the correlation between the left and right white matter tracts are high (Spearman's $r > 0.70$), we initially averaged the data of the left and right hemisphere. When trends of associations between depressive symptoms and the outcome were observed (two-tailed $p<0.10$), we ran additional models for the left and right white matter tracts separately. Next, the method of Julious was used to compare the effect estimates of the left and the right hemisphere (Julious, 2004). Based on the change-in-estimate method (Mickey & Greenland, 1989) of 5%, maternal drinking habits and maternal diet were not used as covariates as these variables did not affect the association. Likewise, gestational age at birth did not confound the association of interest if birth weight was also controlled for. All models were corrected for maternal age at intake, educational level, smoking during pregnancy and maternal depressive symptoms at 3 years, child gender, age at scanning, birth weight, and emotional and behavioral problems in childhood. A false discovery rate (FDR) correction for multiple testing was applied to adjust for multiple testing. Spearman correlation coefficients were calculated of maternal and paternal depressive symptoms. On average, 6.5% of data across the covariates was missing. To avoid the bias of complete case analysis, we accounted for missing information on the confounders by using multiple imputation methods; 10 imputed datasets were generated using a fully conditional specified model to handle missing values and were based on the relations between all variables (Greenland & Finkle, 1995). Only the pooled estimates of the analyses of these 10 imputed datasets are reported.

RESULTS

Table 1 shows the demographic information of the study group (n=636). Children (50.6% boys) were born at a mean gestational age of 39.9 weeks with a mean birth weight of 3443 grams, and the average IQ was 102.8. These demographics characteristics were very similar when we excluded children with psychiatric problems (ADHD and Aggressive problems) that were oversampled (Supplemental Table 1). Maternal

Table 1. Descriptive statistics of study population (total sample)

	Sample N=636
Maternal characteristics	
Age at intake	30.8 ± 4.8
Depressive symptoms during pregnancy	.27 ± .54
Clinically relevant depressive symptoms (%)	13.0
Depressive symptoms at 3 years	.12 ± .30
Clinically relevant depressive symptoms (%)	4.9
Educational level (%)	
Primary	6.4
Secondary	44.4
Higher	49.2
Ethnicity	
Dutch	67.6
Non-Dutch Western	6.4
Non-Dutch Non-Western	26.0
Alcohol use (%) #	
Never drank in pregnancy	35.7
Drank until pregnancy was known	13.5
Continued to drink in pregnancy occasionally	40.0
Continued to drink in pregnancy frequently	10.8
Smoking habits (%)	
Never smoked in pregnancy	76.5
Smoked until pregnancy was known	6.9
Continued to smoke in pregnancy	16.6
Paternal characteristics	
Age at intake	32.9 ± 5.3
Prenatal depressive symptoms *	.10 ± .28
Clinically relevant depressive symptoms (%)	2.8
Child Characteristics	
Gender (% boys)	50.6
Gestational age at birth (weeks)	39.9 ± 1.9
Birth weight (grams)	3444 ± 562
Non-verbal IQ at age 5 years	102.8 ± 14.1
Child emotional and behavioral problems at 6 years	21.5 ± 15.2
Age at MRI assessment (years)	8.0 ± 1.0

Notes: Frequent continued alcohol use is defined as '1 or more glasses of alcohol per week in at least two trimesters'.

* Information about prenatal depressive symptoms of the father was available in n=461 children.

and paternal depressive symptoms during pregnancy showed a low correlation (Spearman's r=0.21). Maternal depressive symptoms during pregnancy had a low correlation with maternal depressive symptoms at 3 years (Spearman's r= 0.34).

Fractional anisotropy

No associations were observed between prenatal maternal depressive symptoms and FA in the forceps minor, superior longitudinal fasciculus, and inferior fronto-occipital fasciculus (Table 2). A trend was present for the association of prenatal depressive symptoms with a lower FA in the cingulum bundle (B=-.008, 95% Confidence Interval

Table 2. The association between prenatal maternal depressive symptoms and white matter tracts in children (total sample)

	Prenatal maternal depressive symptoms	N	Fractional anisotropy (FA)		Mean diffusivity (MD)	
			B (95% CI)	p-value #	B (95% CI)	p-value #
White matter tracts of interest	Forceps minor	636	-.004 (-.004 to .006)	.202	.001 (-.006 to .008)	.767
	Superior longitudinal fasciculus	636	-.002 (-.007 to .002)	.353	.004 (-.001 to .008)	.096
	Left superior longitudinal fasciculus				.004 (-.001 to .008)	.121
	Right superior longitudinal fasciculus				.004 (-.001 to .008)	.112
	Uncinate fasciculus	636	-.001 (-.005 to .004)	.790	.004 (-.000 to .008)	.054
	Left uncinate fasciculus				.005 (.000 to .009)	.040
	Right uncinate fasciculus				.003 (-.001 to .007)	.146
	Cingulum bundle	604	-.008 (-.017 to .000)	.059	.007 (.002 to .012)	.012
	Left cingulum bundle	607	-.006 (-.023 to .007)	.215	.006 (.000 to .012)	.047
	Right cingulum bundle	622	-.010 (-.019 to -.002)	.020	.008 (.002 to .013)	.006
	Inferior fronto-occipital fasciculus	636	-.002 (-.006 to .003)	.449	.002 (-.002 to .006)	.299
	Left inferior fronto-occipital fasciculus					
	Right inferior fronto-occipital fasciculus					

Notes: Linear regression analyses were used. B represents the association of prenatal maternal depressive symptoms with fractional anisotropy and MD in children. The regression models presented were adjusted for maternal age, education and smoking, maternal depressive symptoms at 3 years and age and gender of the child, birth weight and emotional and behavioral problems at 5 years.

The p-values of the 5 average white tracts of interest were corrected for multiple testing, but none of the 5 p-values survived the FDR correction for multiple testing.

(CI) -.017 to .000, p=.059), and prenatal maternal depressive symptoms were related to FA in the right cingulum bundle (B=-.010, 95%CI -.019 to -.002, p=.020).

Table 3. The association between maternal depressive symptoms at 3 years, paternal depressive symptoms and white matter tracts in children (total sample)

		N	Fractional anisotropy		Mean diffusivity (MD)	
			B (95% CI)	p-value	B (95% CI)	p-value
	Maternal depressive symptoms at 3 years					
White matter tracts	Forceps minor	636	.000 (-.009 to .009)	.970	.001 (-.010 to .013)	.804
	Superior longitudinal fasciculus	636	.005 (-.003 to .013)	.198	-.005 (-.012 to .001)	.121
	Uncinate fasciculus	636	.000 (-.008 to .008)	.942	.000 (-.006 to .006)	.905
	Cingulum bundle	604	-.003 (-.018 to .011)	.647	-.001 (-.010 to .008)	.904
	Inferior fronto-occipital fasciculus	636	.003 (-.004 to .011)	.343	-.002 (-.009 to .004)	.467
	Paternal depressive symptoms during pregnancy					
White matter tracts	Forceps minor	461	-.006 (-.017 to .004)	.239	.002 (-.010 to .014)	.752
	Superior longitudinal fasciculus	461	-.002 (-.010 to .007)	.728	-.002 (-.010 to .006)	.629
	Uncinate fasciculus	461	-.003 (-.012 to .006)	.473	.002 (-.005 to .009)	.604
	Cingulum bundle	438	-.015 (-.031 to .001)	.059	.003 (-.006 to .012)	.536
	Left cingulum bundle	441	-.017 (-.033 to .002)	.077		
	Right cingulum bundle	451	-.015 (-.032 to .001)	.067		
	Inferior fronto-occipital fasciculus	461	.004 (-.004 to .012)	.298	-.004 (-.011 to .004)	.309

Notes: Linear regression analyses were used. B represents the association of prenatal maternal depressive symptoms with fractional anisotropy and MD in children. The regression models presented were adjusted for maternal age, education and smoking, maternal depressive symptoms at 3 years and age and gender of the child, birth weight and emotional and behavioral problems at 5 years.

Diffusivity

No relations were observed between prenatal maternal depressive symptoms and MD of the forceps minor, superior longitudinal fasciculus or inferior fronto-occipital fasciculus (Table 2). Prenatal depressive symptoms were related to higher MD in the uncinate fasciculus (trend) (B=.004, 95%CI: -.000 to .008, p=.054) and in the cingulum bundle (B=.007, 95% CI: .002 to .012, p=.012). Subsequently, the left and right white matter tracts were separately analyzed. Maternal depressive symptoms during pregnancy were related to higher MD in the left uncinate fasciculus (B=.005, 95%CI: .000 to .009, p=.040), but not in the right uncinate fasciculus.

Maternal depressive symptoms at 3 years

Maternal depressive symptoms at 3 years were not related to FA or MD in the different white matter tracts as shown in Table 3.

Paternal depressive symptoms during pregnancy

Paternal depressive symptoms showed a trend towards significance for a lower FA in the cingulum bundle (Bcingulum bundle=-.015, 95% CI: -.031 to .001, p=.059), and this was seen in the left and right cingulum bundle (Bleft =-.017, 95%CI: -.033 to .002, p=.077 and (Bright =-.015, 95%CI: -.032 to .001, p=.067). Paternal depressive symptoms were not associated with FA in any of the other white matter tracts nor were paternal depressive symptoms during pregnancy associated with MD of the white matter tracts in childhood (Table 3).

Left and right hemisphere

Using the method of Julious, we found no differences between the estimates of the right hemisphere compared to those of the left hemisphere.

Sensitivity analyses

Children with psychiatric problems (ADHD and aggressive problems) were excluded. The associations of maternal depressive symptoms during pregnancy with MD in the cingulum and uncinate remained comparable (n=558, see Supplemental Table 2). Associations of maternal and paternal depressive symptoms with white matter tracts in this subgroup (n=78 for maternal depressive symptoms and n=54 for paternal depressive symptoms) were in mostly same direction but were not statistically significant possibly due to a lack of sufficient power.

DISCUSSION

Main findings

In this prospective study, we examined the association between maternal depressive symptoms during pregnancy and white matter tract characteristics in childhood. Our results suggest that exposure to prenatal maternal depressive symptoms during pregnancy was associated with higher levels of diffusivity in the cingulum bundle. No relation of maternal depressive symptoms at 3 years with childhood white matter microstructure was observed. However, we did find that exposure to paternal depressive symptoms during pregnancy was associated with slightly lower FA of the cingulum bundle.

Existing literature

The existing literature concerning the potential consequences of maternal depressive symptoms during pregnancy for child brain development is sparse. Prenatal maternal depression has been associated with lower FA and axial diffusivity (but not in volume) in the right amygdala in young infants (Rifkin-Graboi et al., 2013), which is partly in line with the current findings. Using functional MRI, prenatal maternal depression has been linked to altered functional connectivity in the amygdala in early postnatal life (Qiu et al., 2015). Additionally, in children exposed to early socio-emotional deprivation (in Romanian orphanages) factional anisotropy values were significantly decreased in the uncinate fasciculus (Eluvathingal et al., 2006). Thus, although our study group was not a clinical sample, the results seem to be in accordance with the existing literature.

Explanations

First, as our results show an association of maternal depressive symptoms during pregnancy with white matter tract characteristics, but no associations of maternal depressive symptoms at the child's age of 3 years with white matter microstructure. A potential mechanism may involve prenatal stress and distress in women with elevated depressive symptoms during pregnancy, which may lead to elevated hypothalamus–pituitary–adrenal (HPA) axis activity with the release of glucocorticoids (e.g. cortisol). Cortisol during pregnancy may then negatively influence offspring neurodevelopment in this vulnerable period, e.g., elevated maternal cortisol levels during pregnancy have been related to lower intelligence at age 7 years (LeWinn et al., 2009). Fetal exposure to synthetic glucocorticoids in mid-gestation (betamethasone treatment for fetal lung maturation in mothers at risk for preterm delivery) has been related to differences in the cingulate cortex and frontal brain regions (Davis, Sandman, Buss, Wing, & Head, 2013). As exposure to depressive symptoms at 3 years was not related to white mat-

ter microstructure, this may suggest that the brain is more susceptible to depressive symptoms in pregnancy.

Another potential explanation for this association could be parenting practices. Maternal depression, and even life-time maternal depression, has been related to poor parenting skills (Lovejoy, Graczyk, O'Hare, & Neuman, 2000). Depressed mothers have been found to be less responsive to child behavior, communicate less effectively, and have fewer positive interactions with their children. Further, mothers who are depressed are more likely to use corporal punishment (Chung, McCollum, Elo, Lee, & Culhane, 2004) and maternal depression has been linked to physical and verbal child abuse. Decreased white matter density in adolescents and young adults exposed to parental verbal abuse and physical neglect during childhood has been shown (Choi, Jeong, Rohan, Polcari, & Teicher, 2009; Huang, Gundapuneedi, & Rao, 2012). The white matter abnormalities were observed in areas such as the cingulum bundle and the fornix. However, the explanation of poor parenting practices is less likely as our results did not show a relation between maternal depressive symptoms at 3 years and childhood white matter microstructure.

A third potential mechanism to explain the relation between maternal depressive symptoms during pregnancy and childhood white matter microstructure is the presence of epiphenomena of depressive symptoms, e.g., smoking during pregnancy, unhealthy nutrition or low socioeconomic status. We attempted to adjust for these epiphenomena, but unmeasured residual confounding (e.g. poor prenatal care, or other lifestyle factors) might nonetheless be present.

Finally, a potential mechanism for our findings could be an underlying biological vulnerability (genetic predisposition) associated with both maternal depressive symptoms as well as white matter development. Depression is likely to be caused by a combination of genetic vulnerability and environmental factors. The heritability of perinatal depression was estimated at 54% in twins and 44% in siblings (Viktorin et al., 2015). Measures of white matter microstructure, such as FA, are also heritable (Kochunov et al., 2010). Interestingly, the genes encoding for brain-derived neurotrophic factor (BDNF) and catechol-O-methyl transferase (COMT) have been identified to be potentially involved in depression (Nestler et al., 2002; Opmeer, Kortekaas, & Aleman, 2010) and exert small effects on white matter development (Chiang et al., 2011; Thomason et al., 2010). Further, shared environmental family factors (e.g., poverty) may also explain the association of depressive symptoms with structural connectivity. Next to this, genomic imprinting, which is a unique form of epigenetic regulation in mammals leading to the preferential expression of the maternal or paternal allele of certain genes with wide spread implications for the development and function of the brain, could play a role (Perez, Rubinstein, & Dulac, 2016). This potential explanation is likely as our results demonstrate that both maternal and paternal depressive symptoms were similarly

related to childhood FA in the cingulum suggesting that a heritable vulnerability or shared environmental family factors or the combination of both (epigenetic processes) may play a role in this relation. Further, if the effects are due to an intrauterine exposure, then maternal exposure during pregnancy would have a clearly greater influence than paternal exposure on the child's white matter microstructure (G. D. Smith, 2008a). In the current study, the confidence intervals of the estimates in the prenatal maternal models and paternal models are overlapping and thus the results suggest that this is not the case.

Strengths and weaknesses

The strengths of the study are the prospective design, the relatively large group of children that underwent neuroimaging, and, particularly, the use of paternal depressive symptoms as a contrast. However, we acknowledge that this study has its limitations. We measured prenatal depressive symptoms of the mother and father only once during pregnancy. Therefore, we do not know whether depressive symptoms varied in intensity or were persistent throughout pregnancy. Although we used depressive symptoms of the father as a contrast in the current sample, paternal depressive symptoms were lower than maternal depressive symptoms, resulting in less power and precision. Furthermore, we only measured white matter microstructure parameters once, and therefore it is impossible to infer conclusions about neurodevelopmental trajectories. Moreover, we performed many statistical tests, and the FA and MD values of the different tracts were somewhat correlated. We used an FDR to correct for multiple testing, but none of the p-values survived this correction and therefore the results should be interpreted with caution. Finally, we were not able to investigate other important tracts, which are part of the limbic system including the fornix and terminal stria. These tracts are smaller and curved and thus more difficult to obtain via tractography.

CONCLUSION

Overall, our findings suggest that prenatal exposure to maternal depressive symptoms is associated with differences in the microstructure of white matter tracts in young children. As paternal depressive symptoms were also related to decreased FA, the observed effect likely reflects a genetic predisposition or shared environmental family factors and to a lesser extent a specific intrauterine effect. It is thus important to take into account both fathers and mothers psychopathology in future studies. Longitudinal neuroimaging studies are needed to explore structural and functional neurodevelopmental effects of prenatal exposure to depression.

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SUPPLEMENTAL MATERIALS

Table S1. Descriptive statistics of study population (sample without psychiatric disorders for sensitivity analyses)

	Sample N=558
Maternal characteristics	
Age at intake	30.8 ± 4.8
Depressive symptoms during pregnancy	.26 ± .53
Clinically relevant depressive symptoms (%)	12.4
Depressive symptoms at 3 years	.15 ± .39
Clinically relevant depressive symptoms (%)	4.8
Educational level (%)	
Primary	6.7
Secondary	45.2
Higher	48.1
Ethnicity	
Dutch	67.2
Non-Dutch Western	6.6
Non-Dutch Non-Western	26.2
Alcohol use (%) #	
Never drank in pregnancy	35.8
Drank until pregnancy was known	13.2
Continued to drink in pregnancy occasionally	39.5
Continued to drink in pregnancy frequently	11.5
Smoking habits (%)	
Never smoked in pregnancy	76.6
Smoked until pregnancy was known	6.9
Continued to smoke in pregnancy	16.5
Paternal characteristics	
Age at intake	33.2 ± 5.5
Prenatal depressive symptoms *	.10 ± .29
Clinically relevant depressive symptoms (%)	2.2
Child Characteristics	
Gender (% boys)	49.3
Gestational age at birth (weeks)	39.9 ± 1.9
Birth weight (grams)	3443 ± 570
Non-verbal IQ at age 5 years	102.9 ± 14.2
Child emotional and behavioral problems at 6 years	18.8 ± 13.4
Age at MRI assessment (years)	7.9 ± 1.0

Frequent continued alcohol use is defined as '1 or more glasses of alcohol per week in at least two trimesters'.
* Information about prenatal depressive symptoms of the father was available in n=407 children.

Table S2. The association between prenatal maternal depressive symptoms and white matter tracts in children (sample without psychiatric disorders for sensitivity analyses)

Prenatal maternal depressive symptoms	N	**Fractional anisotropy (FA)**		**Mean diffusivity (MD)**	
		B (95% CI)	p-value #	B (95% CI)	p-value #
Forceps minor	558	-.003 (-.009 to .003)	.298	-.001 (-.008 to .006)	.881
Superior longitudinal fasciculus	558	-.002 (-.007 to .004)	.446	.003 (-.001 to .008)	.125
Uncinate fasciculus	558	-.001 (-.005 to .004)	.784	.004 (.000 to .008)	.053
Left uncinate fasciculus	558			.005 (.000 to .010)	.033
Right uncinate fasciculus	558			.003 (-.001 to .007)	.165
Cingulum bundle	527	-.007 (-.016 to .002)	.152	.006 (.000 to .012)	.033
Left cingulum bundle	530			.006 (-.001 to .012)	.072
Right cingulum bundle	544			.007 (.001 to .013)	.023
Inferior fronto-occipital fasciculus	558	.000 (-.005 to .004)	.963	.002 (-.003 to .006)	.399

Notes: Linear regression analyses were used. B represents the association of prenatal maternal depressive symptoms with fractional anisotropy and MD in children. The regression models presented were adjusted for maternal age, education and smoking, maternal depressive symptoms at 3 years and age and gender of the child, birth weight and emotional and behavioral problems at 5 years.

The p-values of the 5 tracts were corrected for multiple testing, but none p-values survived the FDR correction for multiple testing.

2.2

Exposure to maternal depressive symptoms in fetal life or childhood and offspring brain development: A population-based imaging study

Zou R., Tiemeier H., van der Ende J., Verhulst F.C., Muetzel R.L., White T., Hillegers M., & El Marroun H. (2019)

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ABSTRACT

Objective: The authors examined associations of exposure to maternal depressive symptoms at different developmental stages from fetal life to preadolescence with child brain development, including volumetrics and white matter microstructure.

Methods: This study was embedded in a longitudinal birth cohort in Rotterdam, the Netherlands. Participants were 3,469 mother-child pairs with data on maternal depressive symptoms and child neuroimaging at age 10. The authors also measured child emotional and behavioral problems at the time of neuroimaging. The association of maternal depressive symptoms with child brain development at each assessment was examined. Maternal depressive symptom trajectories were modeled across fetal life and childhood to determine the association of maternal depressive symptom patterns over time with child brain development.

Results: The single-time-point analyses showed that maternal depressive symptoms at child age 2 months were associated with smaller total gray matter volume and lower global fractional anisotropy (FA), whereas maternal depressive symptoms assessed prenatally or in childhood were not. The trajectory analyses suggested in particular that children exposed to persistently high levels of maternal depressive symptoms across the perinatal period had smaller gray and white matter volumes as well as alterations (i.e., lower FA) in white matter microstructure compared with non-exposed children. Furthermore, the gray matter volume differences mediated the association between postnatal maternal depressive symptoms and child attention problems.

Conclusions: Perinatal maternal depressive symptoms were consistently associated with child brain development assessed 10 years later. These results suggest that the postnatal period is a window of vulnerability for adversities such as maternal depressive symptoms.

INTRODUCTION

Maternal depression is closely linked to the wellbeing of mothers and their offspring (1). There is clear evidence that perinatal (i.e., prenatal and postnatal) maternal depressive symptoms are associated with various adverse neonatal outcomes, such as low birth weight (2). Further, the long-term consequences of maternal depressive symptoms on offspring cognition, emotion and behavior are well documented. Children exposed to maternal depression in the postpartum period are more likely to develop internalizing and externalizing problems, and attention-deficit/hyperactivity disorder (ADHD) as young children and adolescents (3, 4).

To date, however, little is known about the neurobiology underlying the associations of maternal depressive symptoms with cognitive, emotional, and behavioral problems in offspring. Previous neuroimaging studies on prenatal or postnatal depression primarily focused on offspring limbic system development, in particular the structure and function of the amygdala and the hippocampus (5-10). A few other studies have adopted an exploratory whole-brain approach and suggested that prenatal and postnatal maternal depressive symptoms predict offspring cortical thinning in childhood, particularly in the frontal lobe (11, 12). There are also recent studies suggesting associations between perinatal maternal depression and child brain microstructure in various developmental phases (13). However, these findings are not consistent, and several important gaps remain. First, the critical period during which offspring brain development is most vulnerable to maternal depressive symptoms remains unclear. Second, although persistently high depressive symptoms in mothers are associated with offspring internalizing and externalizing problems (14, 15), few studies have addressed whether persistence of depressive symptoms in mothers affects offspring brain development.

Our aim in this study was to explore whether maternal depressive symptoms repeatedly assessed from the perinatal period until child age ten years are associated with offspring brain development. We also examined the associations between empirically defined groups of maternal depressive symptom trajectories and child brain development. And, finally, we investigated whether any observed brain differences mediated the association of maternal depressive symptoms with child problems.

METHODS

Setting

This study was embedded in the Generation R Study, a population-based cohort from fetal life onwards in Rotterdam, the Netherlands (16). Women with an expected delivery date between April 2002 and January 2006 were eligible. The study was approved

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

Study population

The children participating in Generation R were invited for a neuroimaging session at age ten years. In total, 4245 children visited the research center. Of these, 3992 children underwent the neuroimaging assessment. We excluded 134 children without data on maternal depressive symptoms. Further, 389 children were excluded due to insufficient quality of neuroimaging data, leaving 3469 mother-child pairs for the final analyses (see Figure S1 in the supplement).

Maternal depressive symptoms

Information on maternal psychopathology was obtained during pregnancy (on average at 20.6 weeks of gestation), postnatal period (child age two months), early childhood (age three years), and preadolescence (age ten years) with the Brief Symptoms Inventory (BSI) (17), a validated self-reported questionnaire containing 53 items. A weighted score for the depressive symptoms scale (6 items, each ranging from 0 to 4 points) was calculated by summing the item scores and dividing by the number of completed items. In line with the existing literature on the chronicity of maternal depressive symptoms (18), the depressive symptoms scores across the assessments were moderately correlated (Spearman correlation coefficients ranging from 0.26 to 0.44), with the strongest correlation between depressive symptoms scores at 20 weeks of gestation and those at child age 2 months. The Cronbach's α of the BSI depressive symptoms scale at the four assessments ranged from 0.82 to 0.85. Mothers also completed the Edinburg Postnatal Depression Scale (EPDS) at child age 2 months. Using the validated BSI cut-off of 0.75, we compared the dichotomized score with the EPDS (validated cut-off of 13) and found a sensitivity of 0.62 and a specificity of 0.97 (19), demonstrating moderate to good quality of the BSI's measurement of clinical depression.

Scores on 'interpersonal sensitivity', 'anxiety', and 'hostility', also measured repeatedlywith the BSI, were used to test the specificity of any observed association.

Neuroimaging

All children were familiarized with Magnetic Resonance Imaging (MRI) scanning during a mock scanning session. All images were acquired using the same sequence on the same scanner (3 Tesla GE 750w Discovery). Following a three-plane localizer scan, a high-resolution T1-weighted inversion recovery fast spoiled gradient recalled sequence was acquired with the parameters: T_R=8.77 ms, T_E=3.4 ms, T_I=600 ms, flip angle=10°, field of view (FOV)=220 mm×220 mm, Acquisition Matrix=220×220, slice thickness=1mm, number of slices=230. Diffusion tensor imaging (DTI) data were acquired using an

echo-planar sequence with three b=0 scans and 35 diffusion weighted images (b=1000 s/mm^2) with the following parameters: T_R=12500 ms, T_E=72.8 ms, FOV=240 mm×240 mm, Acquisition Matrix=120×120, slice thickness=2 mm, number of slices=65.

Cortical reconstruction and volumetric segmentation were performed with FreeSurfer v.6.0.0 (http://surfer.nmr.mgh.harvard.edu/). FreeSurfer procedures have shown good test-retest reliability across manufacturers and field strengths (20). The quality of FreeSurfer output was visually inspected, after which data with insufficient quality were eliminated. Metrics of volume, including total gray matter volume and total white matter volume, and volumes of the specific limbic structures were extracted. DTI data were processed using the functional MRI of the Brain's Software Library (FMRIB,FSL) and the Camino Diffusion MRI Toolkit (21, 22). The diffusion tensor was fit using the RESTORE method implemented in Camino. Common scalar maps, including fractional anisotropy (FA) and mean diffusivity (MD), were computed. FA and MD are common measures to quantify white matter microstructure. FA describes the degree of anisotropic diffusion, while MD describes the average diffusion in all directions. Fully automated probabilistic fiber tractography was performed using the FSL plugin AutoPtx (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/AutoPtx)(23). Diffusion image quality was assured by a combination of manual and automated checks, including the examination of slice-wise variation in the diffusion signal, the sum-of-square error of the tensor calculation, and inter-subject registration accuracy.

Covariates

The following demographic variables were selected as potential confounders based on the literature (10, 14, 24): maternal ethnicity, marital status, educational level, monthly household income, number of individuals living in the household, maternal smoking, alcohol use, age at intake, child age at scan, sex, birth weight, and presence of siblings. In addition, information on maternal lifetime history of depression was collected during pregnancy, and seeking professional help for depression postnatally was assessed by questionnaire. These variables were not considered as confounders but aided interpretation of the results. Maternal prenatal antidepressants use was assessed by self-report and pharmacy records and was explored in sensitivity analyses. Emotional and behavioral problems in children at age ten years were measured with the validated Brief Problem Monitor (BPM) for mediation analyses (25).

Statistical analysis

A hierarchical approach was used to examine the association of maternal depressive symptoms with offspring brain outcomes. In primary analyses, we examined global volumetric measures including total gray matter and total white matter volumes, and overall white matter microstructure measures including global FA and MD. If an asso-

ciation with a global metric was observed, we performed post-hoc analyses of the cortical thickness of specific brain lobes and the microstructure of predefined tracts: forceps major, forceps minor, uncinate fasciculus, inferior longitudinal fasciculus, superior longitudinal fasciculus, and cingulum bundle. Based on previous studies, volumes of specific limbic structures, including the thalamus, amygdala, and hippocampus were examined independently of global findings in supplementary analyses. Additionally, we tested for the presence of indirect effects as suggested by Hayes et al. (26) to examine whether the potential differences in brain measures mediated the association of maternal depressive symptoms with child emotional and behavioral problems.

The association between maternal depressive symptoms at four specific time points (further referred to as single-time-point analyses) and offspring brain neuroimaging measures was examined separately with linear regression analyses to avoid inflated standard errors and unstable estimations due to collinearity of the repeated measures (27). Likewise, the models with total gray matter and white matter volume as the outcome were not adjusted for intracranial volume given the high correlation ($r>0.89$). We present initial unadjusted analyses and analyses adjusted for covariates selected using a change-in-estimate criterion of 5%.

Because heterogeneity in the timing of the onset and persistence of maternal depression during perinatal period has been described (28), we aimed to identify between-group difference rather than within-group variability. Hence, a latent class growth analysis (LCGA) was used to account for the interrelation of repeatedly measured maternal depressive symptoms. Trajectories of depressive symptoms were modeled in mothers for whom data were available from two or more time points ($n=3071$). Because the distribution of maternal depressive symptoms was skewed, the developmental trajectories were modeled with censored normal distribution. The full-information maximum likelihood algorithm was used to handle missing data. Based on previous experience (14), models with two to five trajectories were estimated and compared. Model selection was further based on the bootstrap likelihood ratio test (BLRT), the Lo-Mendell-Rubin (LMR) test, and the Bayesian Information Criterion (BIC) (29). As a supplementary reference, a low Akaike's Information Criterion (AIC) and high entropy were used to define good model fit. Interpretability of the groups was taken into account to decide on the number of groups. Models were not adopted if an additional group failed to provide substantive information. Subsequently, the associations between maternal depression trajectory membership and offspring brain neuroimaging measures were assessed with linear regression analyses. Individual's posterior probability of trajectory membership was corrected in regression models to account for the uncertainty of the classification.

We additionally explored interaction by child sex in the above analyses. In sensitivity analyses we excluded 86 mothers who reported prenatal antidepressants use, as

exposure to maternal antidepressant has been associated with altered neonatal brain outcomes (30).

On average 5.8% of data on the covariates were missing (missing at random indicated by Little's test) and were accounted for by multiple imputation. Determinants and outcomes were not imputed; 10 imputed datasets were generated.

In this exploratory study, a false discovery rate (FDR) correction was applied to the primary volumetric and white matter microstructure outcomes in the adjusted models to minimize false positive findings due to multiple comparisons. The number of tests was 8 for single-time-point analyses and 6 for trajectory analyses. Statistical significance was set as $\alpha < 0.05$ (2-sided). Mplus v.7.1.0 was employed to perform LCGA; SPSS v.24.0 (including the PROCESS macro v.3.1 for mediation analysis) was used for all other analyses.

Non-response analyses

The characteristics of the 3469 participants were compared with those of the 523 participants for whom data were not available on maternal depressive symptoms or child neuroimaging. Analysis of variance or the Kruskal-Wallis test was used for continuous variables and the chi-square test for categorical variables. The results showed that mothers with missing data were younger on average (30.6±5.4 years for non-responders vs. 31.2±4.8 years for responders), more often non-Dutch (42.9% vs. 58.8%), more often single (17.6% vs. 11.3%), and less highly educated (38.9% vs. 51.7%). Excluded children had a lower birth weight on average (3359±572 grams vs. 3423±573 grams) than participating children.

RESULTS

Descriptive statistics

Table 1 summarizes the demographic and exposure information of the study population. The mean age of the children at scanning was 10.1 years. Half (50.5%) of the children were girls, and 58.7% had a Dutch national origin. The mean scores for maternal depressive symptoms at four time points ranged from 0.12 to 0.20.

Maternal depressive symptoms at specific time points and child brain development

Table 2 shows that higher maternal depressive symptom scores at all four time points were associated with smaller total gray matter volume in unadjusted models. After adjusting for covariates, only exposure to maternal depressive symptoms at child age two months was associated with smaller total gray matter volumes in offspring at age

Table 1. Demographic and exposure characteristics of the study population in a study of maternal depressive symptoms and offspring brain development[a]

Characteristics		
	N	%
Maternal ethnicity		
Dutch	2039	58.8
Non-Dutch Western	289	8.3
Non-Dutch Non-Western	1141	32.9
Marital status , with partner	3078	88.7
Educational level		
Primary or lower	234	6.7
Secondary	1440	41.5
Higher	1795	51.7
Household income , €/month		
< 1200	234	6.7
1200 – 2000	541	15.6
2000 – 4000	1462	42.1
> 4000	1232	35.5
Smoking		
Never smoked in pregnancy	2700	77.8
Smoked until pregnancy was known	309	8.9
Continued to smoke in pregnancy	460	13.5
Alcohol use		
Never drank in pregnancy	1267	36.5
Drank until pregnancy was known	626	18.0
Continued to drink in pregnancy occasionally	1247	35.9
Continued to drink in pregnancy frequently	329	9.5
Child sex, male	1717	49.5
Siblings (Yes)	580	16.7
	Mean	SD
Maternal age at intake (years)	31.2	4.8
Maternal depressive symptoms scores		
20 weeks of gestation (n=2628)	0.2	0.4
Child age 2 months (n=2330)	0.2	0.4
Child age 3 years (n=2491)	0.1	0.3
Child age 10 years (n=3006)	0.2	0.4
Child age at MRI scan (years)	10.1	0.6
Birth weight (grams)	3422.6	573.0
Number of individuals living in the household	2.2	0.8

a. Imputed statistics are shown (except for maternal depressive symptom scores).

Table 2. Maternal depressive symptoms at single time points and child brain development

Maternal depressive symptoms	global volumetric measures (cm^3)						global white matter microstructure measures					
	Total Gray Matter			**Total White Matter**			**Fractional Anisotropy (FA)**			**Mean Diffusivity (MD)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
20 weeks of gestation (n=2348)							(n=2243)					
Model 1[a]	-19.14	-25.32, -12.97	<0.001	-10.17	-15.02, -5.32	<0.001	-0.28	-0.46, -0.10	0.002	0.02	-0.01, 0.04	0.15
Model 2[b]	-2.58	-8.02, 2.85	0.35	-2.32	-6.79, 2.16	0.31	-0.08	-0.28, 0.11	0.41	0.02	-0.01, 0.04	0.19
Child age 2 months (n=2083)							(n=2037)					
Model 1	-12.21	-18.43, -6.00	<0.001	-4.98	-9.92, -0.05	0.05	-0.29	-0.47, -0.12	0.001	0.01	-0.01, 0.03	0.17
Model 2	-7.29	-12.51, -2.06	0.006[c]	-3.05	-7.41, 1.32	0.17	-0.22	-0.40, -0.03	0.02	0.01	-0.01, 0.03	0.41
Child age 3 years (n=2207)							(n=2183)					
Model 1	-9.40	-17.99, -0.80	0.03	-6.09	-12.95, 0.76	0.08	-0.17	-0.41, 0.08	0.19	0.02	-0.01, 0.05	0.22
Model 2	0.89	-6.19, 7.98	0.81	-1.06	-6.96, 4.85	0.73	-0.04	-0.29, 0.22	0.78	0.02	-0.01, 0.05	0.27
Child age 10 years (n=2676)							(n=2577)					
Model 1	-11.18	-17.27, -5.10	<0.001	-6.27	-11.08, -1.46	0.01	-0.18	-0.35, -0.01	0.04	0.01	-0.02, 0.03	0.67
Model 2	-1.98	-7.03, 3.06	0.44	-1.90	-6.10, 2.30	0.37	-0.08	-0.25, 0.10	0.40	0.003	-0.02, 0.03	0.81

a. Model 1 was adjusted for no covariates.

b. Model 2 was adjusted for child age at scan, child sex, maternal ethnicity, maternal age at intake, maternal educational level, marital status, household income, child birth weight, maternal smoking and alcohol use.

c. These results survived multiple comparison correction. An FDR correction was performed for volumetric measures and white matter microstructure measures separately in model 2.

ten. Specifically, a 1-point increase on the BSI depressive symptoms scale corresponded to a 7.29cm^3 (0.96%) reduction in total gray matter; thus children exposed to the highest BSI score had up to 29.16cm^3 (3.8%) less gray matter. The Cohen's f^2 measure was 0.004, indicating a small effect size. Post-hoc analyses showed that maternal depressive symptoms at two months were associated with thinner cortices in the left and right frontal lobe, and the left temporal lobe (Figure 1). No association was found with total white matter volume. Maternal depressive symptoms at other time points were not associated with total gray matter or white matter volume after taking into account the covariates.

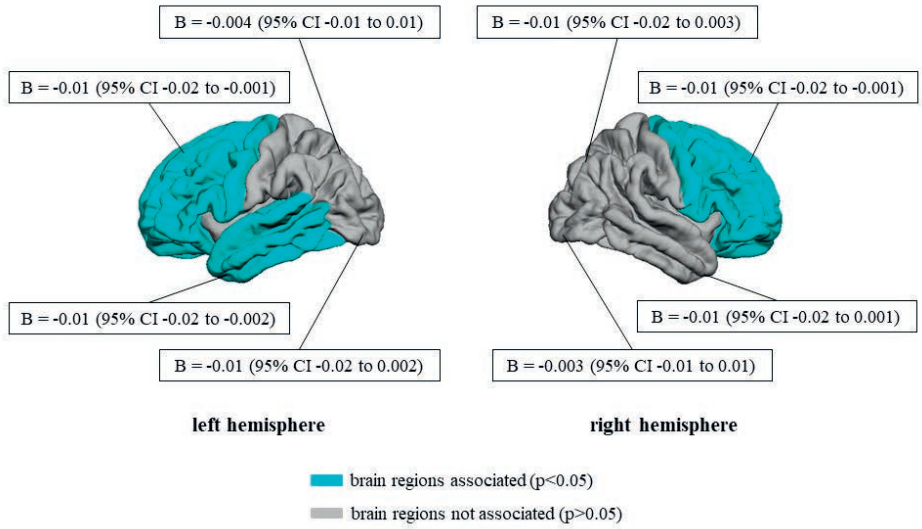

Figure 1. Maternal depressive symptoms at child age 2 months and child cortical thickness. Models were adjusted for child age at the time of MRI scanning (the mean age at scanning was 10.1 years [SD=0.6]), child sex, maternal ethnicity, maternal age at intake, maternal education level, marital status, household income, child birth weight, maternal smoking, and maternal alcohol use. B represents the difference in thickness, in millimeters.

In the supplementary analyses, no association was found between maternal depressive symptoms at any time point and volumes of the thalamus, amygdala, or hippocampus (see Table S1 in the supplement).

Table 2 also shows that maternal depressive symptoms at child age two months predicted lower offspring white matter FA, but the association did not survive multiple comparison correction. The Cohen's f^2 was 0.003, indicating a small effect size. Post-hoc analyses of specific tracts demonstrated that maternal depressive symptoms at two months were related to lower FA in the forceps minor (b=-0.01, 95% CI=-0.01 to -0.001, p=0.008) after correcting for the covariates. Again, maternal depressive symptoms at other time points were not associated with white matter microstructure.

No associations were observed between maternal symptoms of interpersonal sensitivity, anxiety, or hostility and child brain outcomes at any time point. No interaction by child sex was observed in any association tested.

We found evidence suggesting that the child's total gray matter volume mediated the association of maternal depressive symptoms at child age two months with child attention problems at ten years (covariates adjusted unstandardized indirect effect=0.04, bootstrapped 95% CI=0.01 to 0.07; direct effect=-0.12, 95% CI=-0.17 to 0.40. Figure S2). This mediating association was not driven by cortical thickness of any specific brain lobe. The association of maternal depressive symptoms with internalizing or externalizing problems was not mediated by child brain morphology.

Maternal depressive symptoms trajectories and child brain development

The four-class model with quadratic and cubic terms was found to be the optimal model, with the lowest BIC and p-values < 0.05 for BLRT and LMR (Table S2). The average posterior probabilities of the trajectories ranged from 0.79 to 0.89, which were satisfactory. Figure 2 shows the trajectory groups; the first and largest class (n=2120, 69.0%), termed 'no symptoms', consisted of mothers reporting no or very few depressive symptoms at all time points. The second class (n=845, 27.5%), termed 'low symptoms', included mothers with a very modest and stable level of depressive symptoms. In the smallest group (n=43, 1.4%), maternal depressive symptoms sharply increased from moderate levels prenatally and at three years to high levels at age ten, thus this group was labeled 'increasing symptoms'. Finally, there was a group of mothers (n=63, 2.1%) with already high symptom levels in pregnancy that peaked in the early postnatal period. In these mothers, depressive symptoms were lower but remained above the clinical cutoff score at child ages three and ten years. This group was labeled 'perinatal high symptoms'. Interestingly, a larger proportion of mothers with 'perinatal high symptoms' had a history of depression (71.2% vs. 20.6% for mothers with 'no symptoms', p<0.001), and more sought professional help for depression postnatally (41.9% vs. 0.4%, p<0.001).

Table 3 lists the adjusted association of the trajectory groups with child brain development. Children whose mothers were in the perinatal high symptoms group had smaller total gray matter and total white matter volumes than the children whose mothers were in the no symptoms group. Specifically, children of mothers with perinatal high symptoms had a 21.09 cm^3 (2.7%) less total gray matter volume and 15.57 cm^3 (3.4%) less total white matter volume than those in the reference groups. No differences in cortical thickness between groups were found. Children whose mothers were in the low symptoms or increasing symptoms group did not differ in gray matter or white matter volumes from the reference group.

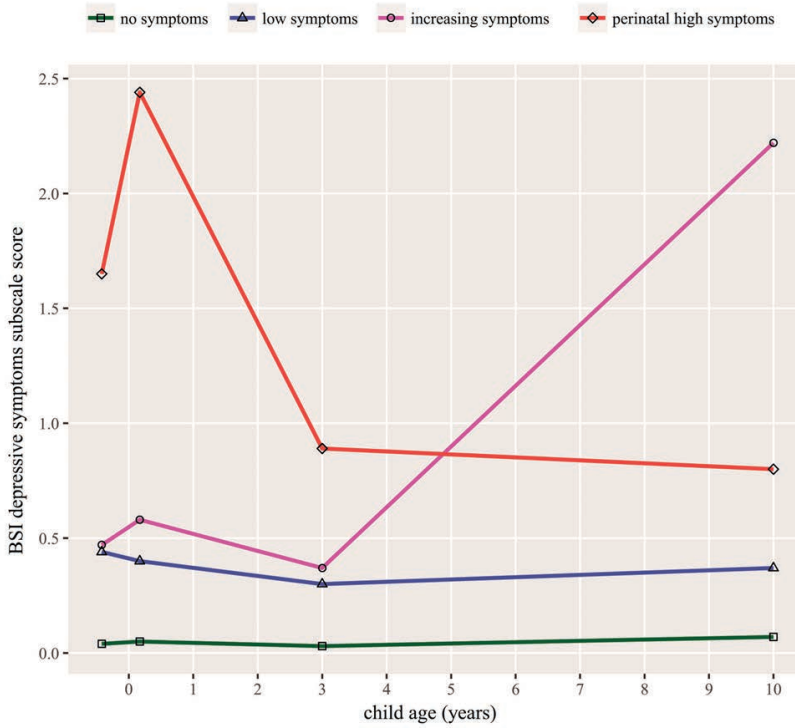

Figure 2. Maternal depressive symptoms trajectories across fetal life and childhood. Maternal symptoms were assessed during pregnancy (on average at 20.6 weeks of gestation) and at child ages 2 months, 3 years, and 10 years.

There were no differences in volumes of the thalamus, amygdala, and hippocampus between the maternal depressive symptoms trajectory groups (Table S1).

Moreover, children whose mothers were in the perinatal high symptoms group showed lower white matter FA than the reference group. Post-hoc analyses further indicated that exposure to perinatal high symptoms was associated with lower FA in the forceps major (b=-0.01, 95% CI=-0.02 to -0.0003, p=0.04), the forceps minor (b=-0,01, 95% CI=-0.02 to -0.001, p=0.03), and the uncinate fasciculus (b=-0.01, 95% CI=-0.02 to -0.002, p=0.02). No associations were found between maternal depressive symptoms trajectories and MD. Again, no interaction by child sex was detected in any of these analyses.

Results obtained in the sensitivity analyses excluding mothers who reported prenatal antidepressants use were similar to the main analyses (Table S3).

Table 3. Maternal depressive symptoms trajectories and child brain development

Trajectory group	global volumetric measures (cm^3)						global white matter microstructure measures					
	Total Gray Matter			**Total White Matter**			**Fractional Anisotropy (FA)**			**Mean Diffusivity (MD)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
n		2735			2735			2647			2647	
Model 1[a]												
No	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Low	-7.95	-13.44, -2.47	0.004	-2.94	-7.30, 1.42	0.19	-0.23	-0.39, -0.07	0.005	0.02	-4×10^{-4}, 0.04	0.06
Increasing	-35.35	-56.77, -13.93	0.001	-24.35	-41.40, -7.31	0.005	0.02	-0.57, 0.61	0.95	-0.05	-0.12, 0.03	0.23
Perinatal high	-40.78	-58.17, -23.38	<0.001	-24.65	-38.50, -10.81	<0.001	-0.80	-1.28, -0.32	0.001	0.05	-0.01, 0.11	0.08
Model 2[b]												
No	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Low	-2.91	-7.46, 1.64	0.21	-1.58	-5.36, 2.20	0.41	-0.14	-0.31, 0.02	0.08	0.02	-0.003, 0.04	0.10
Increasing	-10.72	-28.10, 6.66	0.23	-9.93	-24.38, 4.52	0.18	0.22	-0.36, 0.81	0.45	-0.04	-0.11, 0.03	0.27
Perinatal high	-21.09	-35.46, -6.71	0.004[c]	-15.57	-27.52, -3.62	0.01[c]	-0.53	-1.02, -0.04	0.04	0.04	-0.02, 0.10	0.17

a. Model 1 was adjusted for posterior probability of maternal trajectory membership.

b. Model 2 was additionally adjusted for child age at scan, sex, maternal ethnicity, maternal age at intake, maternal educational level, marital status, household income, child birth weight, maternal smoking and alcohol use.

c. These results survived multiple comparison correction. An FDR correction was performed for volumetric measures and white matter microstructure measures separately in model 2.

DISCUSSION

In this population-based study, maternal depressive symptoms in the postnatal period were associated with smaller gray matter in offspring. Children exposed to persistently high maternal depressive symptoms across the perinatal period had smaller gray and white matter volumes. Consistently, alterations (i.e., lower FA) in white matter microstructure were observed, particularly in the forceps minor. Notably, the gray matter volume differences mediated the association of postnatal maternal depressive symptoms with child attention problems.

Very few studies have focused on the associations of maternal depressive symptoms with child brain development, and results have been inconsistent. In line with previous literature (7, 10, 11, 30), we found no associations of prenatal maternal depressive symptoms with volumes of gray and white matter, amygdala, or hippocampus in the offspring, although some studies observed associations between prenatal maternal depression and amygdala or hippocampus volume in children at younger ages (10, 31). Similarly, no relation was reported between postnatal maternal depression and amygdala volumes in offspring (10). In contrast, a longitudinal study suggested that persistent maternal depressive symptoms from birth until late childhood were related to larger amygdala in ten-year-old children (6). However, these findings should be interpreted with caution given the small sample size combined with limited adjustment for relevant confounding factors. Recent studies have reported associations between prenatal maternal depression and white matter structure in 1-month-old infants or 8-year-old children (5, 13). In addition, Lebel et al. (12) reported that postnatal maternal depressive symptoms were associated with white matter microstructure in preschool children. Although our results also suggested associations between perinatal maternal depressive symptoms and child global FA in white matter, these findings should be interpreted cautiously because they did not survive multiple comparison correction. Additionally, unlike a few previous studies that suggested sex-dependent effects of perinatal depression on amygdala volume or brain microstructure (10, 13), we observed no sex-specific associations. Possibly, differences in age and confounder adjustment explain these discrepancies. Finally, our post-hoc analysis showing that postnatal maternal depressive symptoms may be associated with thinner cortices in the frontal lobes is consistent with findings from a previous study (12).

Our study extends the existing literature of maternal depression and child brain development with trajectory analyses, which provide information beyond the single-time-point analyses and account for the patterns of depressive symptoms over time. Maternal depressive symptoms in the perinatal period, in particular the postnatal period, are more likely to affect offspring brain development, which suggests a critical period of sensitivity. Furthermore, our study suggests that gray matter volume may

be involved in the neurobiological mechanism underlying the association of maternal depression with child attention problems, which has rarely been reported, highlighting the possibility that interventions reducing maternal depression may have lasting effects on child development.

The finding that maternal depressive symptoms at child age two months were associated with offspring brain development in childhood is very interesting, because much of postnatal brain development is experience-dependent (32). Previous studies have suggested the postpartum months as a critical period for child behavioral outcomes, such as internalizing problems and responsiveness disturbance in those exposed to maternal depression (33, 34). Our results suggest this is also true for brain development. In the postnatal period, the brain undergoes rapid growth in gray matter, mainly as a result of synaptogenesis and dendritic arborization (35). The postnatal period is also when the primary myelination process in white matter occurs (36). White matter volume, on the other hand, increases steadily in both prenatal and postnatal periods (37). This may explain why smaller white matter volumes were observed only in children exposed to persistently high maternal depressive symptoms across the perinatal period.

Several potential mechanisms underlying our results deserve discussion. First, impaired mother-infant interaction may have a mediating role. For the infant, neural architecture must be formed within the embedding context of parents' support (38). There is evidence showing that postpartum maternal depression predicts poor mother-infant attachment and parenting (1, 39, 40), which have both been related to smaller gray matter volumes and abnormal white matter microstructure development (41, 42). Second, early postnatal exposure to maternal depression predicted elevated cortisol levels in offspring (43), possibly as a result of the cortisol synchrony in mother-infant dyads through breastfeeding or physical interactions (44), which have been associated with altered brain structure, such as smaller limbic regions (45). Third and perhaps foremost, mothers in the perinatal high symptoms group suffered more from lifetime depression, and were more likely to have sought professional help for depression, suggesting an inherent vulnerability that increases the risk of developing depression particularly in the perinatal period (46). Thus, the genetic susceptibility to depression, which in turn may be related to offspring brain differences (47), could underlie the association observed in the perinatal period.

Most probably, more chronic and severe maternal depressive symptoms exert cumulative effects on offspring brain development across the perinatal period or childhood. Indeed, the depressive symptoms scores were correlated from fetal life to childhood, in particular between depressive symptoms scores in the perinatal period. Women in the perinatal high symptoms group had the most chronic and severe depressive symptoms from their pregnancy onwards even though these symptoms decreased over time. Thus, we must be careful when interpreting the timing of effects, because depression is a chronic and recurrent disorder. The correlation of maternal depressive symptoms over

time makes it difficult to attribute any effect to a particular time point in development. Also, we cannot infer causality from this study; we can only speculate that the perinatal environment and genetic susceptibility together mediate the association of maternal depressive symptoms with child brain development.

The strengths of this study are the relatively large sample size, repeated assessments of depressive symptoms, and the combination of single-time-point analyses and trajectory analyses. Moreover, the population-based nature of the study enhances generalizability of the results. A few limitations must also be discussed. First, only cross-sectional neuroimaging data were used, so we cannot determine whether the effects are permanent or transient. Second, although we adjusted for a number of potential confounding factors, unmeasured residual confounding by extraneous factors such as breastfeeding, diet, and maternal externalizing symptoms cannot be ruled out. Third, since the prevalence of depression was low in this study population, the power to detect an association may have been more limited than suggested by the sample size, and the results must be generalized carefully to more vulnerable populations.

In conclusion, this study shows that exposure to perinatal maternal depressive symptoms, but not to maternal depressive symptoms in childhood, is associated with brain morphological differences in the offspring assessed at age ten years. These findings suggest that the perinatal period, particularly the postnatal period, may be critical for prevention of maternal depressive symptoms in view of the long-term association with child brain development.

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SUPPLEMENTAL MATERIALS

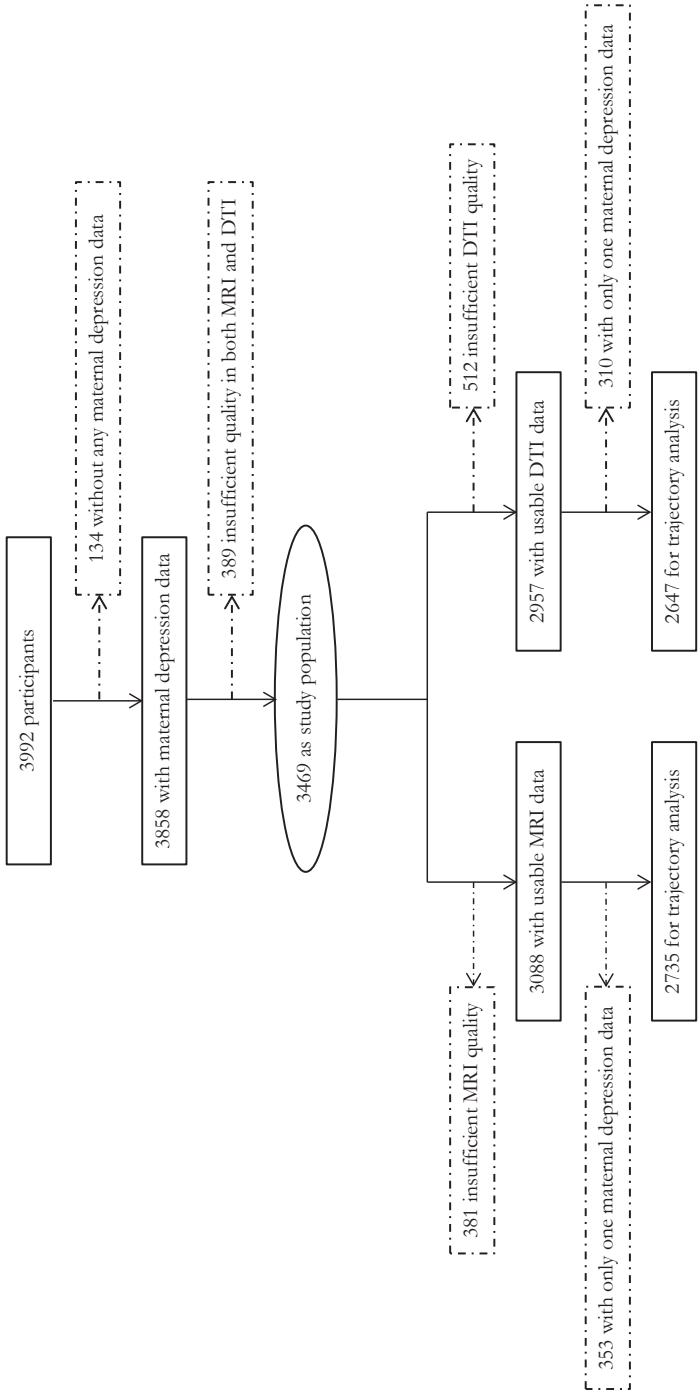

Figure S1. Flow chart of the study population

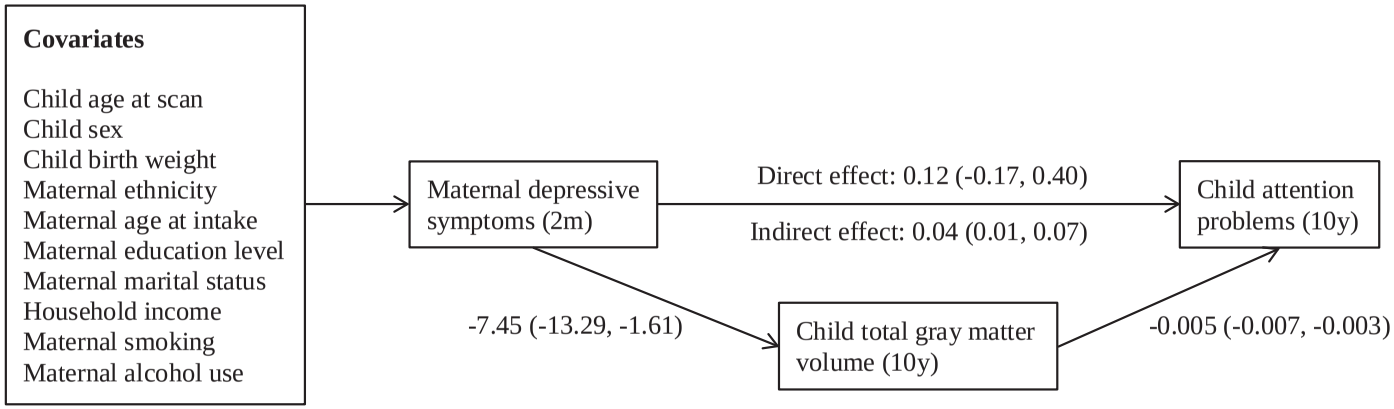

Figure S2. Mediation models between maternal depressive symptoms, child total gray matter volume, and child attention problems (n=1760)

Table S1. Maternal depressive symptoms and volumes of specific limbic structures in children

Maternal depressive symptoms	**Thalamus** (cm^3)			**Amygdala** (cm^3)			**Hippocampus** (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
20 weeks of gestation (n=2348)									
Model 1[a]	0.02	-0.06, 0.11	0.58	0.03	-0.003, 0.06	0.08	0.05	-0.001, 0.11	0.05
Model 2[b]	0.04	-0.06, 0.13	0.44	0.02	-0.01, 0.05	0.20	0.06	-0.01, 0.12	0.07
Child age 2 months (n=2083)									
Model 1	0.02	-0.07, 0.10	0.74	-0.01	-0.04, 0.02	0.51	-0.03	-0.08, 0.03	0.38
Model 2	0.02	-0.07, 0.11	0.71	-0.02	-0.05, 0.01	0.21	-0.03	-0.09, 0.03	0.32
Child age 3 years (n=2207)									
Model 1	-0.02	-0.14, 0.10	0.74	0.02	-0.03, 0.06	0.46	-0.04	-0.12, 0.03	0.27
Model 2	-0.01	-0.13, 0.11	0.88	0.01	-0.03, 0.05	0.61	-0.05	-0.13, 0.03	0.24
Child age 10 years (n=2676)									
Model 1	0.04	-0.05, 0.12	0.41	0.01	-0.02, 0.04	0.66	0.01	-0.04, 0.07	0.62
Model 2	0.03	-0.06, 0.12	0.48	0.01	-0.02, 0.04	0.71	0.01	-0.05, 0.07	0.76
Trajectory groups (n=2735)	**Thalamus** (cm^3)			**Amygdala** (cm^3)			**Hippocampus** (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1[c]									
No	ref	-	-	ref	-	-	ref	-	-
Low	0.02	-0.06, 0.10	0.60	0.01	-0.01, 0.04	0.29	0.02	-0.04, 0.06	0.57
Increasing	-0.16	-0.46, 0.14	0.29	-0.04	-0.14, 0.06	0.41	-0.11	-0.30, 0.08	0.27
Perinatal high	0.04	-0.20, 0.28	0.73	-0.01	-0.09, 0.08	0.86	0.03	-0.13, 0.19	0.73
Model 2[c]									
No	ref	-	-	ref	-	-	ref	-	-
Low	0.02	-0.06, 0.10	0.66	0.01	-0.02, 0.03	0.62	0.01	-0.04, 0.06	0.71
Increasing	-0.16	-0.46, 0.14	0.30	-0.05	-0.15, 0.05	0.35	-0.12	-0.32, 0.07	0.22
Perinatal high	0.03	-0.22, 0.28	0.81	-0.01	-0.10, 0.07	0.80	0.02	-0.14, 0.19	0.77

a. Model 1 was adjusted for intracranial volume.

b. Model 2 was additionally adjusted for child age at scan, child sex, maternal ethnicity, maternal age at intake, maternal educational level, marital status, household income, child birth weight, maternal smoking and alcohol use.

c. Models were additionally adjusted for posterior probability of trajectory membership.

Table S2. Comparison of LCGA-based trajectories model fit indices[a]

Number of class	Growth factors[b]	Fit Indices				
		Entropy	AIC[c]	BIC[d]	LMR[e]	BLRT[f]
2	i, s	0.744	14525.824	14580.092	P<0.001	P<0.001
	i, s, q	0.890	16239.757	16306.084	P<0.001	P<0.001
	i, s, q, c	0.924	16523.886	16602.273	P<0.001	P<0.001
3	i, s	0.665	14287.055	14359.412	P=0.006	P<0.001
	i, s, q	0.635	14220.958	14311.404	P=0.010	P<0.001
	i, s, q, c	0.686	14191.399	14299.935	P<0.001	P<0.001
4	i, s	0.734	14168.920	14259.366	P=0.004	P<0.001
	i, s, q	0.703	14070.867	14185.433	P=0.080	P<0.001
	i, s, q, c	0.738	14040.140	14178.825	P=0.004	P<0.001
5	i, s	0.677	14131.408	14239.944	P=0.124	P<0.001
	i, s, q	0.749	13964.574	14103.259	P=0.252	P<0.001
	i, s, q, c	0.761	13937.861	14106.694	P=0.567	P<0.001

[a] LCGA=Latent Class Growth Analysis

[b] i=intercept, s=slope, q=quadratic, c=cubic, all with significance in the models

[c] AIC=Akaike's Information Criterion

[d] BIC=Bayesian Information Criterion

[e] LMR=Lo-Mendell-Rubin test

[f] BLRT=Bootstrap Likelihood Ratio Test

Table S3. Sensitivity analyses of maternal depressive symptoms and child brain development

Maternal depressive symptoms	global volumetric measures (cm^3)						global white matter microstructure measures					
	Total Gray Matter			**Total White Matter**			**Fractional Anisotropy (FA)**			**Mean Diffusivity (MD)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
20 weeks of gestation (n=2348)							(n=2243)					
Model 1[a]	-19.14	-25.32, -12.97	<0.001	-10.17	-15.02, -5.32	<0.001	-0.28	-0.46, -0.10	0.002	0.02	-0.01, 0.04	0.15
Model 2[b]	-2.58	-8.02, 2.85	0.35	-2.32	-6.79, 2.16	0.31	-0.08	-0.28, 0.11	0.41	0.02	-0.01, 0.04	0.19
Child age 2 months (n=2028)							(n=1985)					
Model 1	-11.34	-17.99, -4.70	0.001	-3.60	-8.84, 1.64	0.18	-0.29	-0.48, -0.10	0.002	0.02	-0.004, 0.04	0.12
Model 2	-7.53	-13.11, -1.94	0.008	-2.73	-7.37, 1.92	0.25	-0.22	-0.41, -0.02	0.03	0.01	-0.01, 0.03	0.34
Child age 3 years (n=2148)							(n=2127)					
Model 1	-9.22	-18.29, -0.16	0.05	-5.55	-12.72, 1.63	0.13	-0.23	-0.49, 0.03	0.08	0.03	-0.01, 0.06	0.14
Model 2	-0.44	-7.91, 7.04	0.91	-1.64	-7.83, 4.54	0.60	-0.11	-0.37, 0.16	0.44	0.02	-0.01, 0.06	0.18
Child age 10 years (n=2603)							(n=2511)					
Model 1	-11.00	-17.31, -4.70	0.001	-6.02	-10.97, -1.07	0.02	-0.21	-0.39, -0.03	0.02	0.01	-0.01, 0.03	0.45
Model 2	-2.32	-7.55, 2.91	0.38	-2.20	-6.54, 2.13	0.32	-0.10	-0.29, 0.08	0.27	0.01	-0.02, 0.03	0.59

Table S3. Sensitivity analyses of maternal depressive symptoms and child brain development (*continued*)

Trajectory group	global volumetric measures (cm^3)						global white matter microstructure measures					
	Total Gray Matter			**Total White Matter**			**Fractional Anisotropy (FA)**			**Mean Diffusivity (MD)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
n		2671			2671			2586			2586	
Model 1[c]												
No	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Low	-6.97	-12.55, -1.39	0.01	-2.16	-6.58, 2.26	0.34	-0.22	-0.38, -0.05	0.01	0.02	0.001, 0.04	0.04
Increasing	-37.14	-59.58, -14.69	0.001	-24.92	-42.67, -7.17	0.006	-0.09	-0.70, 0.52	0.77	-0.04	-0.11, 0.04	0.34
Perinatal high	-39.67	-58.37, -20.98	<0.001	-22.25	-37.03, -7.47	0.003	-0.87	-1.38, -0.35	0.001	0.07	0.002, 0.13	0.04
Model 2[c]												
No	ref	-	-	ref	-	-	ref	-	-	ref	-	-
Low	-2.20	-6.84, 2.45	0.35	-1.12	-4.96, 2.72	0.57	-0.13	-0.30, 0.04	0.13	0.02	-0.002, 0.04	0.07
Increasing	-14.71	-32.94, 3.52	0.11	-12.46	-27.54, 2.63	0.11	0.12	-0.49, 0.72	0.71	-0.03	-0.11, 0.04	0.38
Perinatal high	-20.74	-36.18, -5.29	0.008	-14.56	-27.34, -1.77	0.03	-0.58	-1.10, -0.05	0.03	0.05	-0.01, 0.12	0.12

a. Model 1 was adjusted for no covariates.

b. Model 2 was adjusted for child age at scan, child sex, maternal ethnicity, maternal age at intake, maternal educational level, marital status, household income, child birth weight, maternal smoking and alcohol use.

c. Models were additionally adjusted for posterior probability of trajectory membership.

Chapter 3

Prenatal Nutrients and Brain Development in Childhood

3.1

A prospective population-based study of gestational vitamin D status and brain morphology in preadolescents

Zou R., El Marroun H., McGrath J.J., Muetzel R.L., Hillegers M., White T., & Tiemeier H. (2020)

Neuroimage, 209:116514.

ABSTRACT

Low vitamin D level during pregnancy has been associated with adverse neurodevelopmental outcomes such as autism spectrum disorders (ASD) in children. However, the underlying neurobiological mechanism remains largely unknown. This study investigated the association between gestational 25-hydroxyvitamin D [25(OH)D] concentration and brain morphology in 2597 children at the age of 10 years in the population-based Generation R Study. We studied both 25(OH)D in maternal venous blood in mid-gestation and in umbilical cord blood at delivery, in relation to brain volumetric measures and surface-based cortical metrics including cortical thickness, surface area, and gyrification using linear regression. We found exposure to higher maternal 25(OH) D concentrations in mid-gestation was associated with a larger cerebellar volume in children (b=0.02, 95%CI 0.001 to 0.04), however this association did not remain after correction for multiple comparisons. In addition, children exposed to persistently deficient (i.e., <25 nmol/L) 25(OH)D concentration from mid-gestation to delivery showed less cerebral gray matter and white matter volumes, as well as smaller surface area and less gyrification at age 10 years than those with persistently sufficient (i.e., ≥ 50 nmol/L) 25(OH)D concentration. These results suggest temporal relationships between gestational vitamin D concentration and brain morphological development in children.

INTRODUCTION

Vitamin D is an essential micronutrient that is mainly synthesized in the skin by exposure to sunlight (Bendik et al., 2014). In fetal life, vitamin D is mainly transported from mother to fetus through the placenta in the form of 25-hydroxyvitamin D [25(OH)D] (McAree et al., 2013). Maternal serum 25(OH)D concentration and that of the fetus measured in cord blood are highly correlated (Glorieux et al., 1981; Kimball et al., 2008), suggesting maternal vitamin D level is a reliable indicator of fetal vitamin D status.

Vitamin D deficiency is prevalent worldwide, with people living in Europe, the Middle East, and Asia at particular risk (Lips, 2007). It is also known that women, especially those in pregnancy, are more likely to be vitamin D deficient (Gellert et al., 2017; Vinkhuyzen et al., 2016). Maternal vitamin D deficiency has repeatedly been associated with adverse birth outcomes such as fetal growth restriction and preterm birth (Bodnar et al., 2015; Leffelaar et al., 2010). In recent years, emerging evidence also associates low maternal vitamin D level during pregnancy with long-term cognitive and neuropsychiatric outcomes of the offspring. For instance, Keim et al. (Keim et al., 2014) found maternal vitamin D concentration in mid and late gestation was positively associated with child IQ at age 7. Two neonatal studies reported an association of vitamin D deficiency with increased risk of schizophrenia (Eyles et al., 2018; McGrath et al., 2010). In addition, animal studies using rodents and epidemiological studies in humans showed that gestational vitamin deficiency was associated with an increased risk of autism spectrum disorders (ASD) or more autism-related phenotypes in offspring (Ali et al., 2019; Chen et al., 2016; Magnusson et al., 2016; Vuillermot et al., 2017), which has also been supported by the evidence from our present cohort (Vinkhuyzen et al., 2018; Vinkhuyzen et al., 2017). However, these relationships remain inconclusive due to some inconsistent findings. For example, maternal vitamin D levels during pregnancy were not related to ASD symptoms in children from 5 to 18 years old in a Spanish birth cohort (Lopez-Vicente et al., 2019), and a case-control study in Southern California, Unite States reported no association between neonatal vitamin D levels and ASD in childhood (Windham et al., 2019).

Using neuroimaging techniques, clear associations have been established between cognitive, emotional, and behavioral phenotypes and brain morphology. For instance, reduced brain volumes and less gyrification are frequently reported in children with poor cognitive outcomes or ASD (Arhan et al., 2017; Blanken et al., 2015; Duret et al., 2018; Libero et al., 2014; Pangelinan et al., 2011). To date, however, the evidence linking early life exposure to low vitamin D and brain development remains scarce. There are a few animal studies showing that rats exposed to vitamin D deficiency in gestation had a smaller brain volume and larger cerebral ventricles (Eyles et al., 2003;

Feron et al., 2005). Similarly, in studies in older adults using a cross-sectional design, a lower vitamin D concentration was associated with smaller brain volume and larger cerebral ventricles (Annweiler et al., 2013; Hooshmand et al., 2014). However, we know of no study exploring maternal gestational vitamin D status and brain morphology in children. Such studies may further our understanding of the biological mechanism underlying the established association between gestational vitamin D and child neurodevelopmental outcomes, and justify interventions such as vitamin D supplementation during pregnancy. Therefore, we investigated the association of maternal vitamin D concentration during pregnancy with offspring brain morphology at age 10 years. Based on the existing literature, we hypothesized that low gestational vitamin D level was associated with global alterations in brain morphology in children.

MATERIAL AND METHODS

Study design

This study was embedded in the Generation R Study (Kooijman et al., 2016), a population-based prospective cohort in Rotterdam, the Netherlands. Pregnant women living in the study area with an expected delivery date between April 2002 to January 2006 were recruited. In total 8879 mothers were enrolled in the study prenatally, who gave birth to 8976 live-born children. The study has been approved by the Medical Ethics Committee of Erasmus Medical Center, Rotterdam. Written informed consent was obtained from all participants.

Participants

Of the 8976 mother-child dyads, we excluded 966 without any information on gestational vitamin D concentration. This left 8010 children, of which 6156 visited the research center at age 9-11 years and were invited for a magnetic resonance imaging (MRI) assessment of the brain (White et al., 2018); among the 3363 children that underwent brain MRI assessment, 2715 children had usable brain morphological data after quality inspection. We also randomly excluded 118 siblings to rule out potential clustered data (i.e., children born to the same mother and thus exposed to shared genetic or environmental factors shaping their brain development), leaving 2597 children as the study population. Of these children, 2427 had vitamin D concentration information in mid-gestation, 1706 had vitamin D concentration information at delivery, and 1536 had information on both assessments (see Supplemental Figure S1 for the flow diagram).

Vitamin D concentration

Maternal venous blood samples were collected during mid-pregnancy at a median gestational age of 20.4 (range 18.1-24.9) weeks. Cord blood from the umbilical vein was collected at delivery, at a median gestational age of 40.3 (range 27.6-43.4) weeks. Samples were analyzed using isotope dilution liquid chromatography-tandem mass spectrometry (LC-MS/MS) at the Eyles Laboratory of the Queensland Brain Institute, University of Queensland, Australia. Vitamin D status was assessed by measuring 25(OH)D, defined as the sum of 25-hydroxyvitamin D_2 [$25(OH)D_2$] and 25-hydroxyvitamin D_3 [$25(OH)D_3$] in serum (Eyles et al., 2009). Assay accuracy was assessed using certified reference materials purchased from the Australian National Institute of Standards and Technology (NIST SRM 972a Levels 1-4). Further details of the assay methodology have been described elsewhere (Vinkhuyzen et al., 2016).

Structural neuroimaging

Prior to neuroimaging, all children were familiarized with MRI scanning during a mock scanning session. All images were acquired using the same sequence on the same scanner (3 Tesla GE MR 750w Discovery). Following a three-plane localizer scan, a high-resolution T1-weighted inversion recovery fast spoiled gradient recalled sequence was acquired. Detailed information on the sequence and imaging procedure can be found elsewhere (White et al., 2018).

Volumetric segmentation and cortical reconstruction were performed with FreeSurfer v.6.0.0 (http://surfer.nmr.mgh.harvard.edu/). The standard reconstruction stream was applied, and surface-based models of white matter and gray matter were generated. Thickness maps for each subject were smoothed with a 10 mm full-width half-maximum Gaussian kernel. Local gyrification index (LGI) maps were smoothed using a 5 mm full-width half-maximum Gaussian kernel. The quality of surface reconstruction was visually inspected, after which data with insufficient quality were eliminated.

Covariates

Possible confounders were chosen based on prior literature (Morales et al., 2015; Vinkhuyzen et al., 2018; Whitehouse et al., 2012) and directed acyclic graphs (Shrier and Platt, 2008). Information on maternal age at intake, ethnicity, marital status, education, household income, smoking and alcohol use in pregnancy, and vitamin supplement use in pregnancy was collected at enrollment of the study with questionnaire. Maternal ethnicity was determined from the country of birth of the parents according to the largest ethnic groups in our study population and used to define broad categories based on similarities in skin color and/or cultural background (Eilers et al., 2013; Voorburg/Heerlen, 2004a; Voortman et al., 2015). The categories were Dutch, Non-Dutch

Western (European, North American, and Oceanian); Turkish and Moroccan; African (Cape Verdean, other African, Surinamese-Creole, and Dutch Antillean); and Other (Asian, Surinamese-Hindu, Surinamese-unspecified, and South and Central American). Educational level was categorized into primary or low, secondary, and higher (Voorburg/Heerlen, 2004b). Household income in pregnancy was categorized into less than €1200, €1200 to €2000, and more than €2000 per month. Maternal smoking and alcohol use in pregnancy were assessed in each trimester of pregnancy. Maternal smoking was categorized into 'never smoked in pregnancy', 'smoked until pregnancy was known', and 'continued to smoke in pregnancy' (Roza et al., 2007). Maternal alcohol use was categorized into 'never drank in pregnancy', 'drank until pregnancy was known', 'continued to drink in pregnancy occasionally', and 'continued to drink in pregnancy frequently (defined as one or more glass/week for at least two trimesters)'. Child date of birth and sex were obtained from medical record at birth. Season of blood sampling was recorded at the moment of blood sampling of 25(OH)D.

Statistical analysis

Information on maternal or child characteristics was presented as mean (standard error) or median (95% range) for continuous variables and number (percentage) for categorical variables. First, 25(OH)D concentration was studied as a continuous variable. Second, 25(OH)D concentration was categorized to 'deficient', 'insufficient' and 'sufficient' groups using the cut-off of 25 nmol/L and 50 nmol/L (Garcia et al., 2017; Osteoporosis, 2003; Vinkhuyzen et al., 2018); the 'sufficient' group was set as the reference. For a region of interest (ROI) approach, we used multiple linear regression to examine the association of 25(OH)D status in mid-gestation and at delivery with child brain volumetric measures, including total brain volume, cerebral gray matter volume, cerebral white matter volume, and cerebellar volume. Additionally, we associated 25(OH)D status across the two assessments with these measures to investigate whether exposure to consistently low 25(OH)D levels from mid-gestation to delivery was related to brain volumes in children. In a supplementary analysis, we also examined whether children exposed to low 25(OH)D at one assessment and sufficient 25(OH)D at the other assessment had different brain volumes than those with sufficient 25(OH)D levels as assessed in maternal blood in mid-gestation and in umbilical cord blood. We also investigated 25(OH)D concentration in relation to volumes of subcortical structures (i.e., the thalamus, amygdala, hippocampus, putamen, pallidum, caudate, and accumbens) and the lateral ventricles in secondary analyses. For an exploratory surface-based brain analysis we used linear regression run in a custom-in-house package ('QdecR', http://github.com/slamballais/QDECR) at each cortical vertex to examine the association of gestational 25(OH)D concentration with cortical thickness, surface area, and gyrification. In the surface-based models for single vitamin D assessment,

25(OH)D concentration was introduced as a continuous variable only. Regression analyses were run in two models. The first analyses (Model 1) were adjusted for age at the neuroimaging assessment and sex of the child. In a second step, we further adjusted for other potential confounders (Model 2). In particular, we adjusted for season of blood sampling in mid-gestation and season of blood sampling at delivery simultaneously in the fully adjusted model (Model 2) when investigating 25(OH)D levels across the two assessments in relation to brain morphology.

We performed two sensitivity analyses to test the robustness of the primary analyses. First, information on 25(OH)D concentration and covariates between the participants and non-participants was compared to apply inverse probability weighting (IPW). This approach addresses selection bias and helps obtain results more representative for the initial population (Forns et al., 2018; Nohr and Liew, 2018). Inverse probability weights were calculated with logistic regression. Second, using information from genomic components (Medina-Gomez et al., 2015), we re-ran analyses including only the 1092 children of European ancestry to eliminate effect modification by ethnicity due to genetic or dietary variations.

Missing covariate data (proportions ranging from 1.5% to 15.1%) were accounted for by multiple imputation with the 'Mice' package (missing at random indicated by Little's test) (van Buuren and Groothuis-Oudshoorn, 2011). A total of 10 imputed datasets were generated with 10 iterations. Only pooled results are reported. Statistical significance was set as $\alpha < 0.05$ (2-sided). Furthermore, a false discovery rate (FDR) correction was applied to the two primary and two secondary analyses separately to minimize false positive findings due to multiple testing (Benjamini and Hochberg, 1995). For the surface-based brain analyses, correction for multiple testing was performed using built-in Gaussian Monte Carlo Simulations (Hagler et al., 2006). Cluster-wise p-values were Bonferroni-corrected for two hemispheres ($p<0.025$), and a cluster forming threshold (CFT) of $p=0.001$ was selected for significance testing because it has shown high correspondence with actual permutation testing at the smoothing kernels used (Greve and Fischl, 2018; Muetzel et al., 2019). All analyses were run using the R statistical software (version 3.5.1).

RESULTS

Descriptive statistics

Table 1 shows the demographic information of the study population. Children (49.5% boys) were scanned at an average age of 10.1 years. Over half of them (57.3%) were of Dutch national origin. The median 25(OH)D concentration in mid-pregnancy was 53.8 nmol/L, and the median 25(OH)D concentration at delivery was 31.0 nmol/L. 25(OH)

D concentration at delivery was significantly correlated with 25(OH)D concentration in mid-pregnancy (r=0.56).

Table 1. Demographics of participants (n=2597)

Maternal characteristics	
Ethnicity, N (%)	
Dutch	1489 (57.3)
Non-Dutch Western	208 (8.0)
Turkish and Moroccan	307 (11.8)
African	278 (10.7)
Other	315 (12.1)
Age at intake, mean (SD), years	30.8 (4.8)
Marital status (with partner), N (%)	2292 (88.3)
Education level, N (%)	
Primary or low	189 (7.3)
Secondary	1084 (41.7)
Higher	1324 (51.0)
Household income per month, N (%)	
< €1200	429 (16.5)
€1200-2000	455 (17.5)
> €2000	1713 (66.0)
Smoking, N (%)	
Never smoked in pregnancy	1983 (76.4)
Smoked until pregnancy was known	243 (9.4)
Continued to smoke in pregnancy	371 (14.3)
Alcohol use, N (%)	
Never drank in pregnancy	1046 (40.3)
Drank until pregnancy was known	366 (14.1)
Continued to drink in pregnancy occasionally	958 (36.9)
Continued to drink in pregnancy frequently	227 (8.7)
Vitamin supplement use in pregnancy (Yes), N (%)	883 (34.0)
25(OH)D concentration (n=2427), median (95% range), nmol/L	53.8 (11.2, 110.7)
Season of blood sampling (n=2427), N (%)	
Spring	669 (27.6)
Summer	468 (19.3)
Autumn	621 (25.6)
Winter	669 (27.6)
Child characteristics	
Age at the neuroimaging assessment, mean (SD), years	10.1 (0.6)
Gender (boys), N (%)	1286 (49.5)
25(OH)D concentration in cold blood (n=1706), median (95% range), nmol/L	31.0 (7.3, 72.5)
Season of cord blood sampling (n=1706), N (%)	
Spring	500 (29.3)
Summer	496 (29.1)
Autumn	317 (18.6)
Winter	393 (23.0)

Imputed data were shown (except for 25(OH)D concentration and season of blood sample collection).

Gestational 25(OH)D concentration and child brain volumes

25(OH)D concentration in mid-gestation was positively associated with child total brain volume at age 10 years [b=0.49, representing 0.49 cm^3 difference (larger) in total brain volume per 1 nmol/L increase of 25(OH)D concentration, 95% CI 0.37 to 0.61, p<0.001] in Model 1. However, after adjusting for the additional covariates, this association did not remain (b=0.06, 95% CI -0.08 to 0.21, p=0.39). Higher 25(OH)D concentration in mid-pregnancy was also associated with larger volumes of cerebral gray matter, cerebral white matter, and the cerebellum in children when correcting for child age at the neuroimaging assessment and sex, but only the association with cerebellar volume remained after full adjustment, as shown in Table 2. However, this association did not survive after correcting for multiple testing. Likewise, 25(OH)D concentration at delivery was associated with child total brain volumes (b=0.73, 95% CI 0.52 to 0.94, p<0.001) in Model 1, but not in Model 2 (b=0.06, 95% CI -0.20 to 0.32, p=0.65). A similar pattern was observed for the associations of 25(OH)D concentration at delivery with volumes of cerebral gray and white matter, and cerebellar volume.

Table 2. Gestational 25(OH)D concentration and child brain volume at age 10 years

25(OH)D concentration in mid-gestation (N=2427)	**Cerebral Gray Matter (cm^3)**			**Cerebral White Matter (cm^3)**			**Cerebellar Volume (cm^3)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	0.27	0.20, 0.33	<0.001	0.16	0.10, 0.21	<0.001	0.06	0.05, 0.08	<0.001
Categorical									
Sufficient (n=1316)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=630)	-9.99	-14.66, -5.31	<0.001	-6.08	-10.11, -2.06	0.003	-2.64	-3.77, -1.51	<0.001
Deficient (n=481)	-23.58	-28.71, -18.45	<0.001	-15.67	-20.09, -11.26	<0.001	-5.07	-6.31, -3.82	<0.001
Model 2									
Continuous	0.02	-0.06, 0.10	0.65	0.03	-0.04, 0.10	0.45	0.02	0.001, 0.04	0.04
Categorical									
Sufficient (n=1316)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=630)	-1.35	-6.17, 3.48	0.58	-1.97	-6.22, 2.28	0.36	-1.11	-2.31, 0.08	0.07
Deficient (n=481)	-3.24	-9.81, 3.34	0.33	-5.98	-11.72, -0.23	0.04	-1.34	-2.95, 0.27	0.10

Table 2. Gestational 25(OH)D concentration and child brain volume at age 10 years (*continued*)

25(OH)D concentration at delivery (N=1706)	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	0.39	0.28, 0.50	<0.001	0.24	0.14, 0.33	<0.001	0.10	0.08, 0.13	<0.001
Categorical									
Sufficient (n=376)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=656)	-9.88	-16.15, -3.60	0.002	-8.98	-14.33, -3.63	0.001	-2.10	-3.59, -0.61	0.006
Deficient (n=674)	-22.78	-29.03, -16.54	<0.001	-14.98	-20.31, -9.66	<0.001	-5.64	-7.12, -4.16	<0.001
Model 2									
Continuous	0.01	-0.12, 0.15	0.83	0.02	-0.10, 0.14	0.79	0.03	-0.004, 0.06	0.08
Categorical									
Sufficient (n=376)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=656)	-3.95	-10.27, 2.38	0.22	-5.35	-10.87, 0.17	0.06	-0.96	-2.49, 0.56	0.22
Deficient (n=674)	-3.24	-10.88, 4.39	0.40	-3.65	-10.31, 3.00	0.28	-1.75	-3.59, 0.09	0.06

Model 1 was adjusted for child age at time of the neuroimaging assessment and sex; Model 2 was additionally adjusted for maternal ethnicity, marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, and season of blood sampling. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.

Next we tested the associations of categories of 25(OH)D levels with brain volumetric measures. There was no evidence suggesting brain volume differences in children exposed to 'deficient' or 'insufficient' 25(OH)D concentration in gestation compared to those exposed to 'sufficient' 25(OH)D concentration after taking into account covariates and multiple testing.

Of the 1536 children with 25(OH)D concentration data at both assessments, using the same cut-offs as above, 230 were defined as exposed to 'consistently deficient' 25(OH)D concentration. Likewise, 168 were determined as 'consistently insufficient' and 291 were determined as 'consistently sufficient'. Table 3 shows the adjusted and unadjusted results, here we focus on the adjusted results only. Children exposed in utero to 'consistently insufficient' 25(OH)D concentration had a smaller total brain volume (b=-18.20, 95% CI -35.81 to -0.59, p=0.04) and a smaller cerebral white matter volume than the reference group [i.e., the 291 children exposed to 'consistently sufficient' 25(OH)D concentration from mid-gestation to delivery]. Also, children exposed to 'consistently deficient' 25(OH)D concentration showed a smaller total brain volume (b=-36.47, 95% CI -62.83 to -10.12, p=0.007), and smaller cerebral gray and white matter volumes at 10 years of age than the reference group. After FDR correction most associations

remained, only the difference in total brain volume between the 'consistently insufficient' group and the reference group disappeared. No association was found between 25(OH)D status from mid-gestation to delivery and cerebellar volume. In addition, no brain volumetric differences were observed between children exposed to low 25(OH)D at one assessment only (n=606) and those with 'consistently sufficient' 25(OH)D concentration (data not shown).

Table 3. 25(OH)D status from mid-gestation to delivery in relation to child brain volume at age 10 years (n=689)

25(OH)D status from mid-gestation to delivery	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-10.73	-19.69, -1.76	0.02	-10.47	-18.25, -2.68	0.008	-2.17	-4.32, -0.02	0.05
Consistently deficient	-29.48	-37.64, -21.32	<0.001	-18.53	-25.61, -11.44	<0.001	-7.24	-9.20, -5.28	<0.001
Model 2									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-7.02	-16.42, 2.38	0.14	-10.09	-18.43, -1.75	0.02[a]	-1.09	-3.38, 1.21	0.35
Consistently deficient	-18.77	-32.90, -4.64	0.009[a]	-14.77	-27.23, -2.32	0.02[a]	-2.84	-6.25, 0.56	0.10

Model 1 was adjusted for child age at time of the neuroimaging assessment and sex; Model 2 was additionally adjusted for maternal ethnicity, marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, season of blood sampling in mid-gestation and season of blood sampling at delivery; n =230 for 'consistently deficient' group; n=168 for 'consistently insufficient' group; and n=291 for 'consistently sufficient' group. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.
a These p-values survived FDR correction for multiple testing.

Figure 1 shows the results of the subcortical structures. After adjusting for covariates, although a marginal positive association between 25(OH)D concentration in mid-gestation and the volume of the pallidum was observed, gestational 25(OH)D concentration was not associated with the volume of any subcortical structures in children after correcting for multiple testing. We found no association between gestational 25(OH)D concentration and lateral ventricle volume. 25(OH)D status from mid-gestation to delivery was not associated with the volume of the subcortical structures or the lateral ventricle (data not shown).

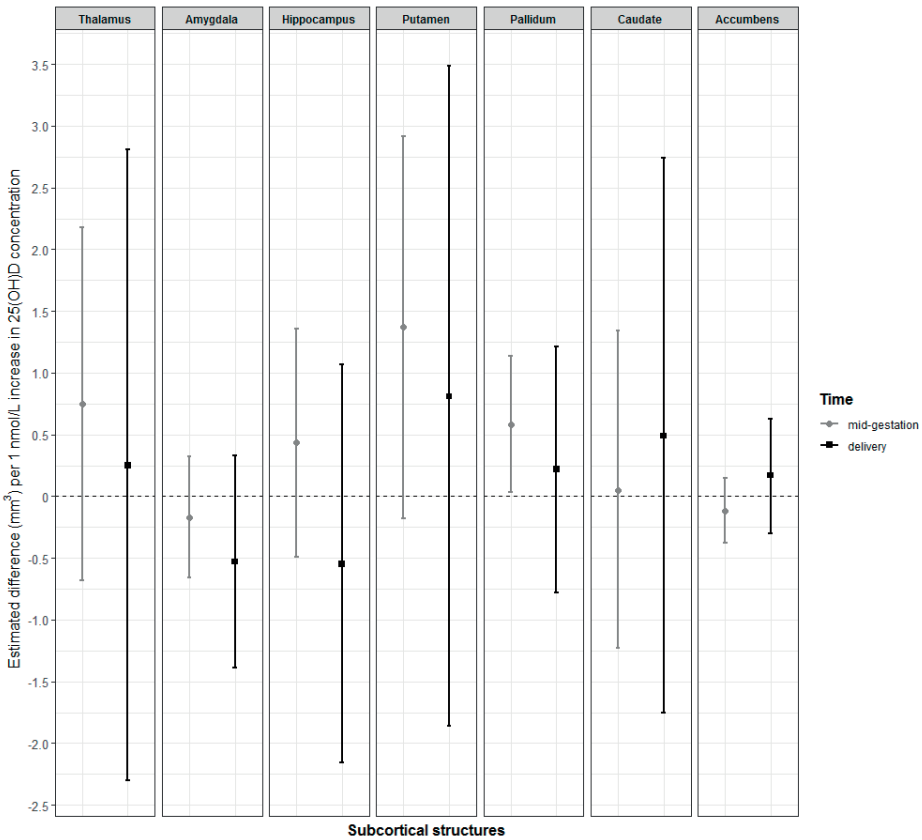

Figure 1. Gestational 25(OH)D concentration and volumes of subcortical structures in children at age 10 years. Models were adjusted for child age at time of the neuroimaging assessment and sex, maternal ethnicity, age at intake, marital status, education, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, household income, season of blood sampling, and child intracranial volume. n=2427 for 25(OH)D concentration in mid-gestation, and n=1706 for 25(OH)D concentration at delivery.

Gestational 25(OH)D concentration and child surface-based brain morphometry

In the analyses only adjusted for child age at the neuroimaging assessment and sex, 25(OH)D concentration in mid-gestation and at delivery was associated with widespread differences in cortical thickness, surface area, and gyrification in both hemispheres. However, after full adjustment for confounding variables, no association remained. Compared to children exposed to 'consistently sufficient' 25 (OH)D concentration from mid-gestation to delivery, those exposed to 'consistently insufficient' 25 (OH)D concentration showed smaller surface area in the temporal region in the right hemisphere, and those exposed to 'consistently deficient' 25(OH)D concentration showed smaller surface area in the frontal and occipital region in the right hemisphere,

as well as less gyrification in the temporal region in the left hemisphere after adjustment for all covariates (Figure 2).

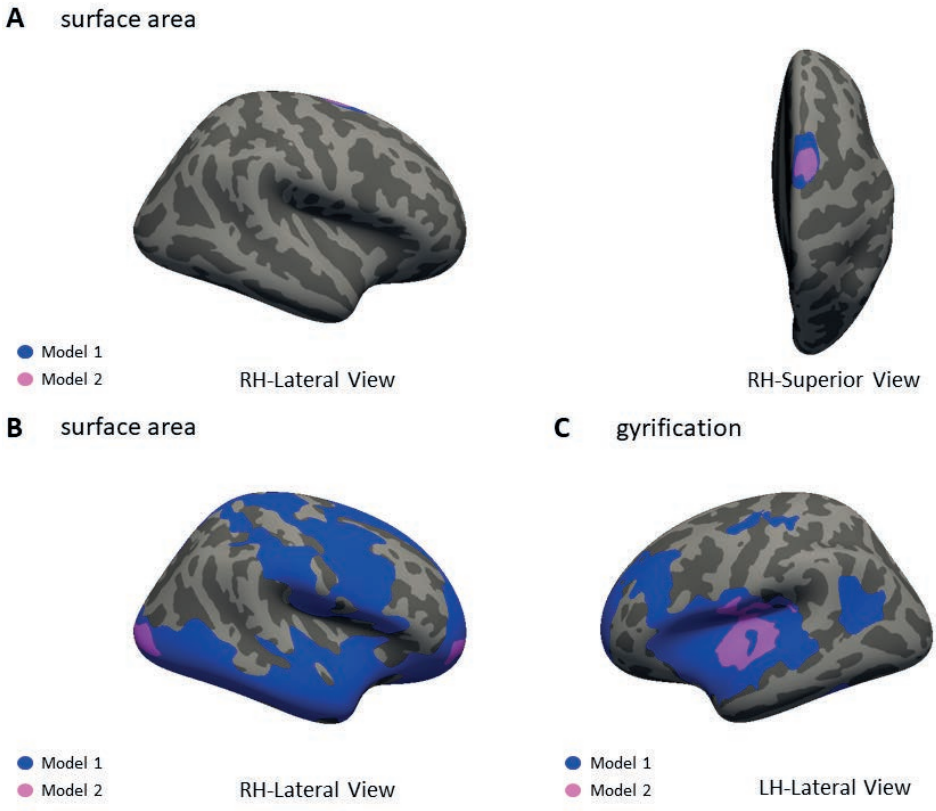

Figure 2. 25(OH)D status from mid-gestation to delivery in relation to surface-based cortical metrics in children at age 10 years. Compared to children with 'consistently sufficient' 25(OH)D concentration (as assessed in maternal blood in mid-gestation and in umbilical cord blood, n=288), those exposed to 'consistently insufficient' 25 (OH)D concentration (n=165) had significantly smaller surface area in the colored regions (section A); those exposed to 'consistently deficient' 25(OH)D concentration (n=229) showed significantly smaller surface area and less gyrification in the colored regions (section B, C). These associations remained if corrected for multiple comparisons. Model 1 was adjusted for child age at time of the neuroimaging assessment and sex; Model 2 was additionally adjusted for maternal ethnicity, marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, season of blood sampling in mid-gestation and season of blood sampling at delivery. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L. LH=left hemisphere; RH=right hemisphere.

Sensitivity analyses

As shown in Table S1 and Table S2, results from inverse probability weighted regression were generally consistent with the main analyses. Table S3 and Table S4 demonstrate

that there were no associations between gestational 25(OH)D concentration and brain volumes at 10 years in children of European ancestry. However, the sample sizes of these analyses were considerably smaller.

DISCUSSION

In this population-based study, we found that exposure to persistently low vitamin D levels was associated with a smaller brain (specially less cerebral gray and white matter volumes) and differed surface-based cortical metrics such as surface area and gyrification in children, using repeated assessment of 25(OH)D concentration from mid-gestation to delivery. Also, there was a positive association between mid-gestational 25(OH)D concentration and offspring cerebellar volume, but this did not survive multiple comparison correction.

Research on gestational vitamin D status and brain morphology is scarce. In animal studies, rats born to vitamin D_3-deficient mothers showed smaller cortical volumes and larger lateral ventricle volumes than controls (Eyles et al., 2003; Feron et al., 2005). Similarly, in cross-sectional human studies, low vitamin D concentrations have been associated with a smaller total brain volume and a larger lateral ventricle volume in adults and the elderly, albeit these findings are inconsistent (Annweiler et al., 2012; Annweiler et al., 2013; Zivadinov et al., 2013). In our study, we found an association between gestational 25(OH)D concentration and the offspring total brain only if we studied persistent vitamin insufficiency. In another study no association between 25 (OH)D concentration and bilateral amygdala or hippocampus volume was found (Annweiler et al., 2010), which is in line with the current study. However, these studies are not comparable in terms of study design and subjects. Further, one study indicated that mothers with lower 25(OH)D concentration during pregnancy had offspring with smaller head circumference (as a marker for brain development) from the second trimester until birth (Miliku et al., 2016), while in a recent study no association was found between gestational 25(OH)D concentration and infant head circumference at the age of 6 or 12 months (Hauta-Alus et al., 2019). Several explanations for the discrepancy with previous findings must be discussed.

First, as an important neurosteriod, vitamin D has important functions in the proliferation and differentiation of neurons, calcium signaling within the brain, neurotrophic and neuroprotective actions, and may alter neurotransmission and synaptic plasticity (Cui et al., 2017; Groves et al., 2014). An emerging concept suggests that vitamin deficiency may weaken the integrity of perineuronal nets (PNNs), thereby neural-circuit function is disturbed and cognitive processes such as learning and memory are impeded (Mayne and Burne, 2019). These mechanisms may function at such a micro

level that brain morphological measures are not modalities with adequate sensitivity to capture any arising differences. Interestingly, we observed an association between maternal vitamin D concentration in mid-gestation and child cerebellar volume. This association remained when accounting for selection bias in the sensitivity analysis. Recent longitudinal studies suggested that deficient maternal vitamin D status in pregnancy is associated with adverse motor and social development in children in early childhood (Darling et al., 2017; Dhamayanti et al., 2019), which may be explained by the reduction in cerebellar volume because smaller cerebellum has been associated with worse motor and cognitive performance (D'Ambrosio et al., 2017). Moreover, emerging evidence shows that cerebellum involves in the complex neural underpinnings of ASD (Becker and Stoodley, 2013), suggesting a potential mediating role of cerebellum in the association of gestational vitamin D status with child autistic traits (Vinkhuyzen et al., 2018). Further studies exploring such a mediation pathway are warranted.

Second, it is feasible that more prolonged exposure to vitamin D in gestation may exert a cumulative effect on child brain development, which is not evident with more transient prenatal exposures. Exposure to persistent vitamin D deficiency from mid-gestation to delivery has been associated with more severe autism-related traits in our previous study (Vinkhuyzen et al., 2018). Our analysis suggests that exposure to persistently deficient or insufficient 25(OH)D concentration from mid-gestation onwards is also associated with smaller brain volumes, which may be accounted for by reduced regional surface area rather than cortical thickness. Difference in brain volumes were reported in children with and without ASD at age 10 years (Lange et al., 2015), but such unspecific neurological findings can also be indicative of a higher risk for other child problems such as early onset schizophrenia (Arango et al., 2012). In contrast to our finding from the mid-gestational assessment, these results suggest that the cerebrum and not the cerebellum is most sensitive to vitamin D, and that multiple assessments are needed to reliably identify vitamin deficiency. In addition, these findings were not found when we analyzed only European children. This could possibly be explained by a reduced ability to detect small differences in far fewer subjects (in particular the 'consistently deficient' group), but also an effect modification by ethnicity. Possibly, more pigmented children in the Netherlands are affected more by persistently low gestational vitamin D status (partly this could simply reflect less misclassification). Besides, interestingly, children exposed to persistent vitamin D deficiency and those with more autistic traits both showed decreased gyrification in the frontal, superior temporal and inferior parietal cortices in the left hemisphere when the same covariates were adjusted for (Blanken et al., 2015), suggesting that gyrification in these regions may play a role in the relationship between gestational vitamin D deficiency and autistic traits in childhood.

Third, gestational vitamin D concentration at both assessments was significantly associated with brain volumes and cortical metrics when only child age at the neuroimaging assessment and sex were adjusted for, while no significances remained when adjusting for all covariates. It has been suggested that education and income are both important indicators of family socioeconomic status (SES) that relates to child development (Ahmadi Doulabi et al., 2017). Moreover, SES has been suggested as a determinant of smoking, alcohol use and vitamin supplement use in pregnant women (Najman et al., 1998; Skagerström et al., 2011; Sullivan et al., 2009). Therefore, it is possible that less rigorous control for confounding explains some of the previous findings.

The strengths of our study were the longitudinal study design to examine the temporal association of gestational vitamin D exposure with brain morphology, the inclusion of large sample size enabling us to detect small effects, and the implementation of IPW in the sensitivity analyses to reduce selection bias. Several limitations, however, should also be mentioned. First, vitamin D concentration in blood fluctuates with diet and sun exposure, and the half-life of vitamin D is relatively short, thus the assessed values at a specific time point may not be substantially representative for the average level over the target period. Additionally, as previously reported (Wegienka et al., 2016), vitamin D concentration measured in child cord blood was significantly lower than that of the mother in mid-gestation and no specific cut-offs have been established. Second, we measured 25(OH)D concentration only in mid-gestation and at delivery. The period of early pregnancy, which can be critical in terms of brain development, could not be investigated. Also, the possible influence of child 25(OH)D level at age 10 years cannot be ruled out. Third, child brain morphology was only assessed once in preadolescence, thus whether any observed association is transient or lasting, and whether gestational vitamin D concentration is associated with brain morphology in other developmental phases cannot be determined.

To the best of our knowledge, this is the first longitudinal study to investigate the association between gestational vitamin D status and brain morphological development in children. We found limited evidence for associations between gestational vitamin D level at single assessments and child brain morphology at age 10 years, but observed differed brain volumes and cortical morphometry in children exposed to persistently low vitamin D levels from mid-gestation to delivery. Further studies are needed to ascertain the possible alterations in cerebellar volume and the more generalized gray and white matter changes, and explore how these findings are related to child neurodevelopment.

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SUPPLEMENTAL MATERIALS

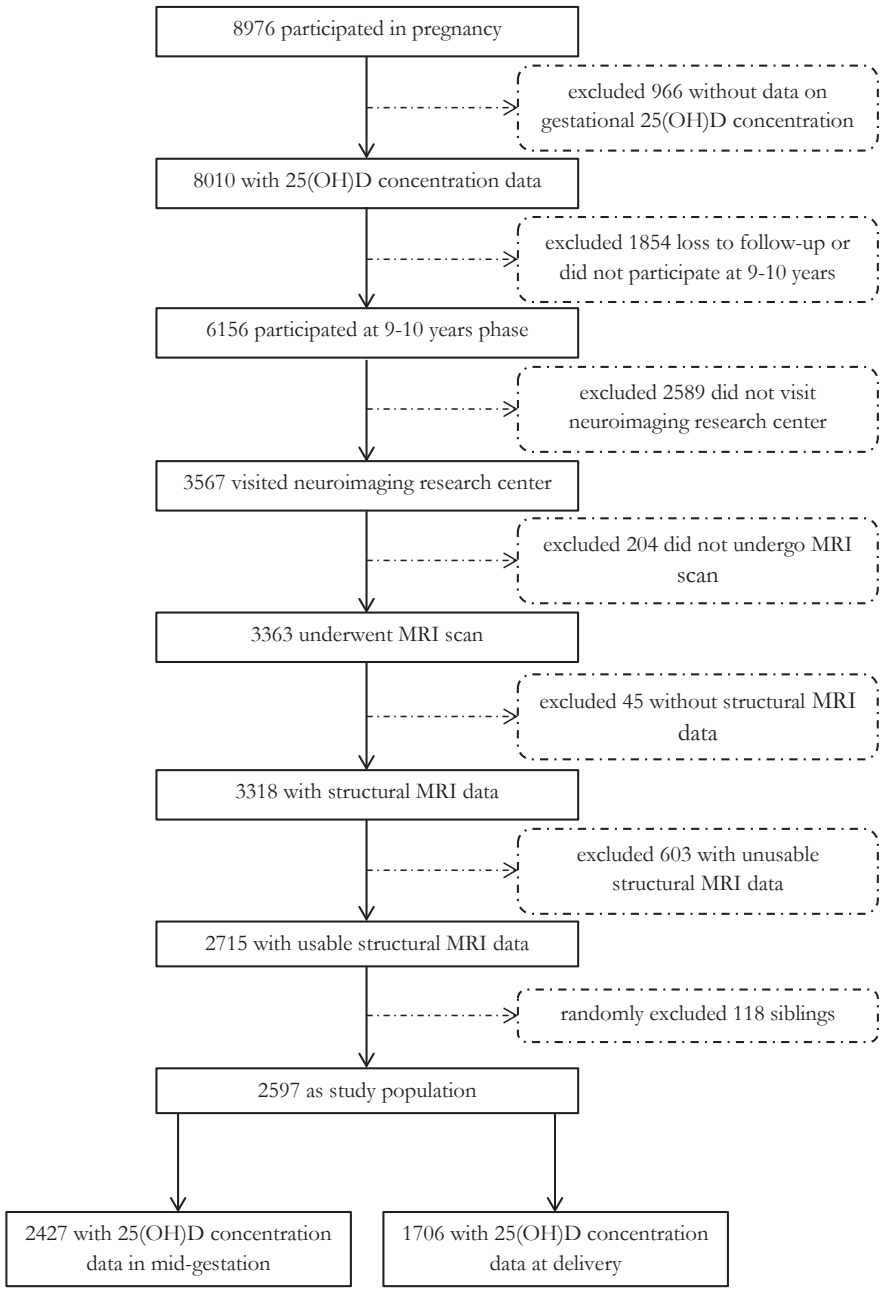

Figure S1. Flow diagram of study population selection

Table S1. Gestational 25(OH)D concentration and child brain volume at age 10 years, inverse probability weighted

25(OH)D concentration in mid-gestation (N=2427)	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	0.29	0.22, 0.35	<0.001	0.17	0.11, 0.22	<0.001	0.07	0.05, 0.08	<0.001
Categorical									
Sufficient (n=1316)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=630)	-11.82	-16.78, -6.87	<0.001	-6.56	-10.82, -2.29	0.003	-3.15	-4.41, -1.88	<0.001
Deficient (n=481)	-22.15	-27.66, -16.65	<0.001	-14.48	-19.24, -9.71	<0.001	-5.06	-6.33, -3.79	<0.001
Model 2									
Continuous	0.03	-0.05, 0.10	0.53	0.03	-0.04, 0.10	0.39	0.02	0.004, 0.04	0.02
Categorical									
Sufficient (n=1316)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=630)	-1.86	-6.96, 3.23	0.47	-1.80	-6.41, 2.82	0.45	-1.40	-2.71, -0.08	0.04
Deficient (n=481)	-2.56	-9.00, 3.88	0.44	-5.37	-11.10, 0.36	0.07	-1.59	-3.27, 0.09	0.06
25(OH)D concentration at delivery (N=1706)	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	0.43	0.31, 0.54	<0.001	0.26	0.16, 0.37	<0.001	0.11	0.08, 0.14	<0.001
Categorical									
Sufficient (n=376)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=656)	-11.59	-17.90, -5.29	<0.001	-9.53	-15.23, -3.84	0.001	-2.26	-3.84, -0.68	0.005
Deficient (n=674)	-25.20	-31.50, -18.90	<0.001	-16.75	-22.46, -11.05	<0.001	-5.96	-7.54, -4.38	<0.001
Model 2									
Continuous	0.02	-0.12, 0.16	0.77	0.04	-0.09, 0.17	0.57	0.03	-0.002, 0.07	0.07
Categorical									
Sufficient (n=376)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=656)	-4.24	-10.55, 2.06	0.19	-5.21	-11.04, 0.62	0.08	-0.92	-2.54, 0.70	0.27
Deficient (n=674)	-4.55	-12.35, 3.25	0.25	-5.32	-12.63, 1.99	0.15	-1.89	-3.87, 0.10	0.06

Model 1 was adjusted for child age at time of the neuroimaging assessment and sex; Model 2 was additionally adjusted for maternal ethnicity, marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, and season of blood sampling. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.

Table S2. 25(OH)D status from mid-gestation to delivery in relation to child brain volume at age 10 years (n=689), inverse probability weighted

25(OH)D status from mid-gestation to delivery	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-13.90	-23.06, -4.74	0.003	-10.80	-18.74, -2.86	0.008	-2.68	-4.95, -0.41	0.02
Consistently deficient	-27.85	-36.62, -19.07	<0.001	-17.46	-25.39, -9.54	<0.001	-6.68	-8.77, -4.59	<0.001
Model 2									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-9.58	-19.03, -0.13	0.05	-10.82	-19.30, -2.34	0.01	-1.35	-3.81, 1.10	0.28
Consistently deficient	-20.47	-34.39, -6.54	0.004	-18.02	-31.17, -4.87	0.007	-2.78	-6.72, 1.15	0.17

Model 1 was adjusted for child age at time of the neuroimaging assessment and sex; Model 2 was additionally adjusted for maternal ethnicity, marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, season of blood sampling in mid-gestation and season of blood sampling at delivery; n =230 for 'consistently deficient' group; n=168 for 'consistently insufficient' group; and n=291 for 'consistently sufficient' group. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.

Table S3. Gestational 25(OH)D concentration and child brain volume at age 10 years, children of European ancestry only (n=1092)

25(OH)D concentration in mid-gestation (N=1017)	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	-0.01	-0.12, 0.10	0.91	-0.03	-0.13, 0.07	0.59	0.01	-0.02, 0.03	0.68
Categorical									
Sufficient (n=707)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=264)	1.02	-5.84, 7.89	0.77	-0.59	-6.66, 5.48	0.85	0.36	-1.25, 1.98	0.66
Deficient (n=46)	-4.75	-19.22, 9.71	0.52	-2.91	-15.70, 9.89	0.66	1.46	-1.95, 4.86	0.40
Model 2									
Continuous	-0.07	-0.19, 0.05	0.25	-0.05	-0.16, 0.06	0.34	0.01	-0.02, 0.04	0.36
Categorical									
Sufficient (n=707)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=264)	3.78	-3.47, 11.03	0.31	0.04	-6.42, 6.49	0.99	0.08	-1.64, 1.79	0.93
Deficient (n=46)	-0.46	-15.32, 14.40	0.95	-1.27	-14.50, 11.97	0.85	1.31	-2.20, 4.83	0.46
25(OH)D concentration at delivery (N=909)	**Cerebral Gray Matter (cm^3)**			**Cerebral White Matter (cm^3)**			**Cerebellar Volume (cm^3)**		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Continuous	0.01	-0.16, 0.17	0.95	-0.02	-0.16, 0.12	0.74	0.03	-0.01, 0.07	0.14
Categorical									
Sufficient (n=294)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=424)	-4.13	-11.46, 3.20	0.27	-7.72	-14.00, -1.44	0.02	-1.70	-3.38, -0.03	0.05
Deficient (n=191)	-2.43	-11.42, 6.55	0.60	0.25	-7.45, 7.95	0.95	-1.65	-3.70, 0.40	0.11
Model 2									
Continuous	-0.13	-0.32, 0.05	0.16	-0.13	-0.30, 0.03	0.11	0.02	-0.02, 0.06	0.33
Categorical									
Sufficient (n=294)	reference	-	-	reference	-	-	reference	-	-
Insufficient (n=424)	-0.87	-8.64, 6.90	0.83	-5.21	-11.91, 1.50	0.13	-1.51	-3.29, 0.27	0.10
Deficient (n=191)	4.17	-6.14, 14.47	0.43	5.14	-3.75, 14.03	0.26	-1.40	-3.76, 0.96	0.25

Model 1 was adjusted for child age at time of the neuroimaging assessment, sex, and principal components of ancestry; Model 2 was additionally adjusted for maternal marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, and season of blood sampling. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.

Table S4. 25(OH)D status from mid-gestation to delivery in relation to child brain volume at age 10 years, children of European ancestry only (n=333)

25(OH) D status from mid-gestation to delivery	Cerebral Gray Matter (cm^3)			Cerebral White Matter (cm^3)			Cerebellar Volume (cm^3)		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-1.27	-12.41, 9.87	0.82	-7.15	-16.97, 2.66	0.15	-0.27	-2.97, 2.43	0.85
Consistently deficient	-14.27	-38.72, 10.19	0.25	7.49	-14.06, 29.03	0.49	-3.40	-9.33, 2.54	0.26
Model 2									
Consistently sufficient	reference	-	-	reference	-	-	reference	-	-
Consistently insufficient	-1.78	-13.76, 10.20	0.77	-8.89	-19.47, 1.69	0.10	-0.63	-3.56, 2.30	0.67
Consistently deficient	-6.89	-33.56, 19.78	0.61	11.03	-12.57, 34.63	0.36	-2.83	-9.45, 3.79	0.40

Model 1 was adjusted for child age at time of the neuroimaging assessment, sex, and principal components of ancestry; Model 2 was additionally adjusted for maternal marital status, education, age at intake, household income, smoking and alcohol use in pregnancy, vitamin supplement use in pregnancy, season of blood sampling in mid-gestation and season of blood sampling at delivery; n =15 for 'consistently deficient' group; n=98 for 'consistently insufficient' group; and n=220 for 'consistently sufficient' group. Deficient is 25(OH)D concentration < 25 nmol/L; insufficient is 25(OH)D concentration 25 to < 50 nmol/L; sufficient is 25(OH)D concentration ≥ 50 nmol/L.

3.2

Maternal folate levels during pregnancy and offspring brain development in late childhood

Zou R., El Marroun H., Cecil C., Jaddoe V.W.V., Hillegers M., Tiemeier H., & White T. (2020)

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ABSTRACT

Background: Cumulative evidence shows that low maternal folate levels during pregnancy are associated with offspring neuropsychiatric disorders even in the absence of neural tube defects. However, the relationship between prenatal exposure to folate and brain development in late childhood has been rarely investigated.

Methods: In 2095 children from a prospective population-based cohort in Rotterdam, the Netherlands, we examined the association of maternal folate levels during pregnancy with downstream brain development in offspring. Maternal folate concentrations were measured from venous blood in early gestation. Child structural neuroimaging data were measured at age 9-11 years. In addition, measures of child head circumference using fetal ultrasound in the third trimester and total brain volume using magnetic resonance imaging at age 6-8 years were used for analyses with repeated assessments of brain development.

Results: Maternal folate deficiency (i.e., <7 nmol/L) during pregnancy was associated with smaller total brain volume (B=-18.7 cm^3, 95% CI -37.2 to -0.2) and smaller cerebral white matter (B=-7.2 cm^3, 95% CI -11.8 to -2.6) in children aged 9-11 years. No differences in cortical thickness or surface area were observed. Analysis of the repeated brain assessments showed that children exposed to deficient folate concentrations in utero had persistently smaller brains compared to controls from the third trimester to childhood (β=-0.4, 95% CI -0.6 to -0.1).

Conclusions: Low maternal folate levels during pregnancy are associated with altered offspring brain development in childhood, suggesting the importance of essential folate concentrations in early pregnancy.

INTRODUCTION

Folate is an essential water-soluble vitamin that can only be obtained from the diet. In adults, low folate levels have been related to an increased risk of cardiovascular disease mortality, cognitive dysfunction and many cancers [1-3]. Moreover, offspring of mothers with low folate status during pregnancy has increased risks of neural tube defects (NTDs) and congenital heart defects [4]. Exposure to low folate levels in gestation has also been associated with other adverse outcomes such as low birthweight, childhood brain and spinal cord tumors, and metabolic consequences in children [5-7].

Recent research has also shown associations between maternal folate status during pregnancy and various long-term neuropsychiatric outcomes in offspring. For example, higher maternal folate concentrations and greater folate intake in early pregnancy were predictive of better cognitive and psychomotor development and less emotional problems in early childhood [8-10]. In addition, exposure to low folate status in gestation was associated with an increased risk of schizophrenia and more hyperactivity and peer problems in childhood [11, 12], and several studies suggested that higher maternal plasma folate levels and folic acid supplementation during pregnancy, notably when supplementation was initiated during the periconceptional period, were related to less autistic traits and lower risk of autism spectrum disorder (ASD) in the offspring [13].

Emerging evidence has suggested relationships between maternal folate status during pregnancy and offspring brain development, which likely underlie the observed cognitive and behavioral outcomes. Previous work in our research group showed that, even in the absence of NTDs, lower maternal plasma folate concentrations in early pregnancy were associated with a smaller offspring head circumference (HC), an indicator of global brain volume for fetus or neonates, at 30 weeks of gestation [14-16]. Embedded in the same cohort, we also found Dutch children exposed to low folate levels in utero had less total brain volume (TBV) at age 6 to 8 years [17]. A recent study based on three observational cohorts in the United States observed thicker cortices in children and adolescents with greater folic acid in gestation, which was associated with a lower risk of psychosis spectrum symptoms [18]. Given the complex growth trajectories of the human cerebral cortex and the inherent plasticity of the developing brain [19-21], studies describing the brain developmental trajectories related to prenatal folate exposure are needed.

Building on our previous work, our aim was to first investigate prospective associations between maternal folate levels during pregnancy and child structural brain morphology at age 9-11 years. Second, we used repeated brain measures to examine prenatal exposure to folate in relation to the long-term trajectories of brain development from fetal life to childhood. We expected that children exposed to low folate levels during pregnancy would continue to show decreased brain volumes.

METHODS

Study design

This study was embedded in the Generation R Study, a prospective cohort study in which pregnant women with an expected delivery date between April 2002 and January 2006 were recruited [22]. The study was approved by the Medical Ethical Committee of the Erasmus Medical Center, and written informed consent was obtained prior to participation.

Participants

In total 8976 children were born to 8879 mothers participating during pregnancy. Of the 97 twin births, one child from each twin pair was randomly excluded. Further, 2848 children were excluded due to the lack of information on maternal plasma folate concentration during pregnancy. Of the remaining 6031 children, 3766 visited our research center at age 9-11 years, and 2595 underwent a brain magnetic resonance imaging (MRI) with parental consent. After excluding those without a complete T_1-weighted MRI (n=15), assessed using heterogeneous scanning parameters (n=18), with major incidental findings (n=15) [23], with braces (n=56), and with data of insufficient quality (n=396), 2095 children were determined as the study population (see Supplemental Figure S1 for the flow chart).

Maternal folate concentration

Venous blood samples were collected from the mothers in early pregnancy (mean=13.3 weeks, SD=1.9 weeks), after which they were transported to a regional laboratory within 3 hours and centrifuged and stored at -80 °C [24]. Folate concentrations were analyzed using an immune electrochemiluminence assay on the Architect System (Abbott Diagnostics B.V.). The between-run coefficients of variation ranged between 1.5 and 8.9%. The analytic range for plasma folate concentration was 1.8-45.3 nmol/L (0.8-20.0 ng/mL). A plasma folate concentration of less than 7 nmol/L (3.1 ng/mL) was considered 'folate-deficient' in our primary analysis [10, 14].

Neuroimaging

Prior to the MRI scanning, children underwent a mock scanning session to become familiar with the neuroimaging assessment. MR images were acquired on a 3-Tesla GE Discovery MR750w MRI System (General Electric, Milwaukee, WI, USA) scanner using an 8-channel head coil. T_1-weighted sequences were obtained using a 3D coronal inversion recovery fast spoiled gradient recalled sequence with the following parameters: repetition time=8.77 ms, echo time=3.4 ms, inversion time=600 ms, flip angle=10°, field of view=220 mm×220 mm, acquisition matrix=220×220, slice thickness=1mm,

number of slices=230, and arc acceleration=2. Volumetric segmentation and cortical reconstruction were performed with FreeSurfer v.6.0.0 (http://surfer.nmr.mgh.harvard.edu/). The quality of the FreeSurfer output for each individual was visually inspected and rated on a three-point Likert-scale (insufficient/sufficient/excellent), after which data with insufficient quality were eliminated. In addition, utilizing an automated quality assessment (Q/A) program that provides a valid continuous measure of the quality of structural MRI images [25], we found that the automated Q/A metric was not correlated with maternal folate concentration (r=-0.02, p=0.31), suggesting that results were not due to poor quality data.

Covariates

Based on prior literature and directed acyclic graphs [8, 14, 17, 26], we selected the following potential confounding variables: child sex and age at neuroimaging, maternal age at enrollment, ethnicity, marital status, educational level, pre-pregnancy body mass index (BMI), parity, smoking and alcohol use during pregnancy, and family income. Information on child sex and date of birth (to calculate age at neuroimaging) was obtained from medical records. Maternal information on age at enrollment, ethnicity, marital status, educational level, parity, smoking and alcohol use during pregnancy, and family income was collected with questionnaires during pregnancy. Maternal pre-pregnancy BMI was calculated by self-reported pre-pregnancy height and weight.

Additional measures

For the analysis with repeated assessments we used data on child HC measured by ultrasound in the third trimester (mean=30.4 weeks, SD=0.9 week) to estimate fetal brain volume using a validated formula: $TBV=(1/2)\times(\pi/6)\times(HC/\pi)^3$ [27]. We also used TBV measured by MRI at child age 6-8 years (mean=7.7 years, SD=0.9 year) [28]. Child age at these assessments and gestational age at birth were obtained [29].

For supplementary analyses, self-report information on periconceptional maternal folic acid or vitamin supplement use was collected in 1720 mothers and assessed in relation to child brain morphology. In addition, information on mother-report Child Behavior Checklist (CBCL) at child age 10 years (mean=9.7, SD=0.3) was available in 1803 children of the present study sample [30]. We used scores of the internalizing and externalizing subscales to examine maternal folate levels in relation to child emotional and behavioral problems that might be concomitant with brain morphological differences.

Statistical analyses

For descriptive statistics, continuous variables were analyzed using t-tests or Wilcoxon tests; categorical variables were analyzed using Chi-square tests. In a non-response

analysis, we compared maternal and child characteristics between participating children ('respondents') and those with information on maternal folate concentration during pregnancy but were not included in the study population ('non-respondents').

We used multiple linear regression with folate status as a dichotomized independent variable to investigate whether children exposed to deficient maternal folate concentrations during pregnancy showed differences in global or regional brain volumes (i.e., TBV, cortical gray matter volume, cerebral white matter volume, and subcortical gray matter volume) compared to those with normal concentrations (the reference group). We also tested possible linear relationships by introducing maternal folate concentration as a continuous trait. Twelve children with a maternal folate concentration of 45.3 nmol/L (i.e., the upper detection limit) were excluded from this analysis to rule out possible invalid values, and a quadratic term was introduced to test for non-linear relationships. For exploratory surface-based brain analyses we used linear regression run in a custom-in-house package ('QdecR', http://github.com/slamballais/QDECR) at each cortical vertex to examine the cortical metrics including cortical thickness and surface area [31].

In a follow-up analysis, we re-ran the analysis excluding children with preterm birth (i.e., gestation age at birth < 37 weeks), small for gestational age (SGA, defined as a SD score ≤ −2.3 and based on SD curves derived from the total Generation R cohort) [32], and/or born to mothers with gestational complications (i.e., preeclampsia, diabetes, and/or pregnancy-induced hypertension) to examine whether gestational and obstetrical complications explained any observed association.

Second, we used linear mixed models to investigate the association of maternal folate levels with repeatedly assessed TBV in a sub-sample of 354 children with brain measurements at all three time points (i.e., the third trimester in gestation, 6-8 years, and 9-11 years). Prior to analyses, TBV was standardized using Z-transforms to gain comparability across measurements. Both intercept and slope were included as random effects in the model, and the restricted maximum likelihood (REML) estimation was used. Interactions of maternal folate levels with child age at assessments and sex were examined.

All regression analyses described above were performed using multi-stage models. The initial model (Model 1) was only adjusted for child sex and age at neuroimaging. Using a change-in-estimate criterion (i.e., candidate covariates were only included if their inclusion changed the effect estimate of maternal folate status more than 5%) [33], maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income were additionally adjusted for in Model 2. Correction for child intracranial volume was performed for models with regional brain volumes (Model 3) to investigate whether any observed regional differences were independent of global effects.

Since the established folate status cutoff (i.e., 7 nmol/L) is primarily based on hematologic indicators or risk of NTDs [34], we ran a secondary analysis using less rigorous folate concentration cutoffs (i.e., 8, 9, 10, 11, and 12 nmol/L) to empirically explore a maternal folate level that might be specifically relevant for child brain development in the long term. The level of 8 nmol/L (3.5 ng/mL) was an earlier cutoff of the Erasmus MC laboratory prior to the publication of an international standard.

In supplementary analyses, we examined the relation between maternal folic acid supplementation in the periconceptional period and child brain volumes. We also investigated the association of maternal folate levels during pregnancy with child emotional and behavioral outcomes at age 10 years using linear regression. The CBCL internalizing and externalizing scores were square root transformed prior to analysis due to skewed distribution of the raw values.

In addition, we performed a sensitivity analysis using targeted maximum likelihood estimation (TMLE) because it outperforms naïve regressions and propensity-score methods in minimizing the impact of model misspecification and near-violation of the practical positivity assumption (i.e., when children with certain combinations of confounders were rarely exposed to maternal folate deficiency during pregnancy) [35, 36].

On average, 5.2% of the covariates were missing, which was accounted for using multiple imputation (missing at random indicated by Little's test) [37]. A total of 20 imputed datasets were generated and we report the pooled results (for the sensitivity analysis we report the results from the first imputed dataset). Statistical significance was set as $\alpha<0.05$ (two-sided) and false discovery rate (FDR) was used to correct for multiple testing for each of the three regional brain volumes [38]. For the surface-based analyses, correction for multiple testing was performed using built-in Gaussian Monte Carlo Simulations [39]. Cluster-wise p-values were Bonferroni corrected for the two hemispheres ($p<0.025$), and a cluster forming threshold (CFT) of $p=0.001$ was selected for significance testing because it has shown high correspondence with actual permutation testing at the smoothing kernels used [31, 40]. All statistical analyses were performed using R version 3.6.2 (The R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Descriptive statistics

Table 1 shows descriptive statistics on maternal and child characteristics between folate-deficient and folate-normal groups. Children underwent neuroimaging at ap-

proximately 10 years-of-age. Folate-deficient mothers were younger, lower educated, had less family income, and were more often single parents than folate-normal mothers.

Table 1. Descriptive statistics

	Folate-deficient (n=103)	**Folate-normal** (n=1992)	**p-value**
Maternal characteristics			
Age at enrollment, years	27.8 (5.9)	30.9 (4.5)	<0.001
Ethnicity			
Dutch	33 (32.0)	1216 (61.0)	
Non-Dutch Western	8 (7.8)	246 (12.3)	<0.001
Non-Dutch non-Western	62 (60.2)	530 (26.6)	
Marital status (with partner)	74 (71.8)	1793 (90.0)	<0.001
Highest education completed			
Primary or secondary	85 (82.5)	896 (45.0)	<0.001
Higher	18 (17.5)	1096 (55.0)	
Pre-pregnancy BMI	24.1 (4.8)	23.4 (4.0)	0.18
Parity (≥1)	48 (46.6)	756 (38.0)	0.10
Smoking during pregnancy			
Never	74 (71.8)	1519 (76.3)	0.37
Yes	29 (28.2)	473 (23.7)	
Alcohol use during pregnancy			
Never drank in pregnancy	50 (48.5)	721 (36.2)	
Drank until pregnancy was known	23 (22.3)	299 (15.0)	<0.001
Continued drinking throughout pregnancy	30 (29.1)	972 (48.8)	
Family income, €/months			
≤ 2000	73 (70.9)	584 (29.3)	<0.001
> 2000	30 (29.1)	1408 (70.7)	
Breastfeeding duration, months	3.5 (3.7)	4.5 (3.7)	0.005
Plasma folate concentration, nmol/L	5.9 (0.8)	20.1 (8.7)	<0.001
Child characteristics			
Age at neuroimaging, years	10.1 (0.5)	10.1 (0.6)	0.39
Sex (male)	51 (49.5)	983 (49.3)	0.97
Gestational age at birth, weeks	39.5 (2.4)	40.0 (1.7)	0.05

Imputed statistics were displayed. Continuous data were presented in mean (SD) and compared using t-test or Wilcoxon test; categorical data were presented in number (%) and compared using Chi-square test.

As shown in the non-response analysis (Supplemental Table S1), there were no significant differences in child sex or age at neuroimaging between respondents and non-respondents. However, participating mothers were older, more often Dutch, higher educated, less often single parents, had higher family income, and were less

often smokers but more often alcohol consumers during pregnancy. In particular, respondents had higher folate concentrations than non-respondents (19.4 nmol/L vs. 16.6 nmol/L, p<0.001).

Mothers who used folic acid or vitamin supplement in the periconceptional period were less likely folate deficient during pregnancy than those who did not (1.0% vs. 25.2%, p<0.001).

Maternal folate status and child brain volumes

Table 2 shows the relationships between maternal folate status and child brain volumes. In the fully adjusted model 2, children exposed to deficient maternal folate concentrations during pregnancy showed a smaller TBV (B=-18.7, 95% CI -37.2 to -0.2, in cm^3) compared to the reference group. Children exposed to low folate during pregnancy also showed smaller cerebral white and subcortical gray matter volumes, where cerebral white matter volume remained significant after additional correction for intracranial volume (Model 3). In addition, analyzing maternal folate concentration along a continuum was positively associated with TBV (B=0.5, 95% CI 0.04 to 1.0) and cerebral white matter volume (B=0.2, 95% CI 0.02 to 0.5) of the child after full adjustment for covariates. Differences in cerebral white matter volume disappeared when child intracranial volume was additionally corrected for, suggesting a global effect (Supplemental Table S2). We found no evidence for a quadratic relationship between maternal folate concentration and either TBV or cerebral white matter volume.

Table 2. Maternal folate status during pregnancy and child brain volumes at age 10 years

Folate status	Model	Total brain volume			Cortical gray matter			Cerebral white matter			Subcortical gray matter		
		B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Normal	-	ref	-	-	ref	-	-	ref	-	-	ref	-	-
	1	-46.8	-65.4, -28.1	<0.001	-18.6	-27.9, -9.2	<0.001	-21.9	-30.4, -13.5	<0.001	-1.9	-2.7, -1.1	<0.001
Deficient	2	-18.7	-37.2, -0.2	0.05	-3.8	-13.0, 5.4	0.41	-12.7	-21.2, -4.2	0.003[a]	-0.9	-1.7, -0.1	0.03[a]
	3	-	-	-	2.0	-3.2, 7.2	0.44	-7.2	-11.8, -2.6	0.002[a]	-0.5	-1.0, 0.1	0.10

B's represent the volumetric difference (in cm^3) of children exposed to deficient maternal folate concentrations (<7 nmol/L, n=103) compared to those with normal maternal folate concentrations (≥7 nmol/L, n=1992).

Model 1 was adjusted for child sex and age at neuroimaging.

Model 2 was additionally adjusted for maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income.

Model 3 was additionally adjusted for child intracranial volume.

[a] <0.05 after a false discovery rate (FDR) correction (for p-values of regional measures in Model 2 and Model 3 separately).

After excluding children with preterm birth, SGA, or exposed to maternal complications (n=239), maternal folate deficiency (i.e., <7 nmol/L) was still associated with a smaller white matter volume (B=-7.2, 95% CI -12.0 to -2.3) that was independent of TBV. In addition, the positive relation between maternal folate concentration as a continuum and child TBV remained (B=0.5, 95 CI% 0.04 to 1.0).

When using 8, 9, 10, 11, or 12 nmol/L as the cutoff, children exposed to folate deficiency in utero still showed less TBV, smaller cerebral white matter, and smaller subcortical gray matter than the reference group. However, the volumetric differences in both cerebral white matter and subcortical gray matter disappeared after additional correction for intracranial volume (see Figure 1).

Maternal folate status and child cortical metrics

Of the 2095 children, 2084 had available data for surface-based analyses, of which 102 were exposed to folate deficiency during pregnancy. After adjusting for child sex and age at neuroimaging, children exposed to deficient folate concentrations in gestation displayed thicker cortices in the frontal lobe in the left hemisphere, and smaller surface area in the frontal and temporal lobes bilaterally. After full adjustment for covariates, however, no differences in cortical thickness or surface area remained.

Maternal folate status and child TBV trajectories

Figure 2 shows cross-sectional differences in TBV between the folate-deficient group and the folate-normal group at each of the three time points, suggesting that children exposed to deficient maternal folate concentrations in pregnancy had consistently less TBV over time. Results from the linear mixed model showed that children exposed to deficient folate concentrations during pregnancy had persistently smaller brain than the reference group from the third trimester to childhood (see Table 3). We observed no relationships between maternal folate concentration as a continuum and child TBV trajectories. We found no evidence for interactions between maternal folate levels and child age or sex.

In addition, no differences in child TBV trajectories were found when <8, 9, 10, 11, or 12 nmol/L was used to define folate deficiency.

Maternal supplement use and child brain volumes

Maternal folic acid supplementation in the periconceptional period predicted higher TBV (B=36.1, 95% CI 48.3 to 24.0), cortical gray matter volume (B=17.4, 95% CI 23.5 to 11.3), cerebral white matter (B=11.8, 95% CI 17.3 to 6.2), and subcortical gray matter volume (B=1.6, 95% CI 2.1 to 1.0) in children after adjusting for child sex and age at neuroimaging. However, no differences were present when correcting for all covariates.

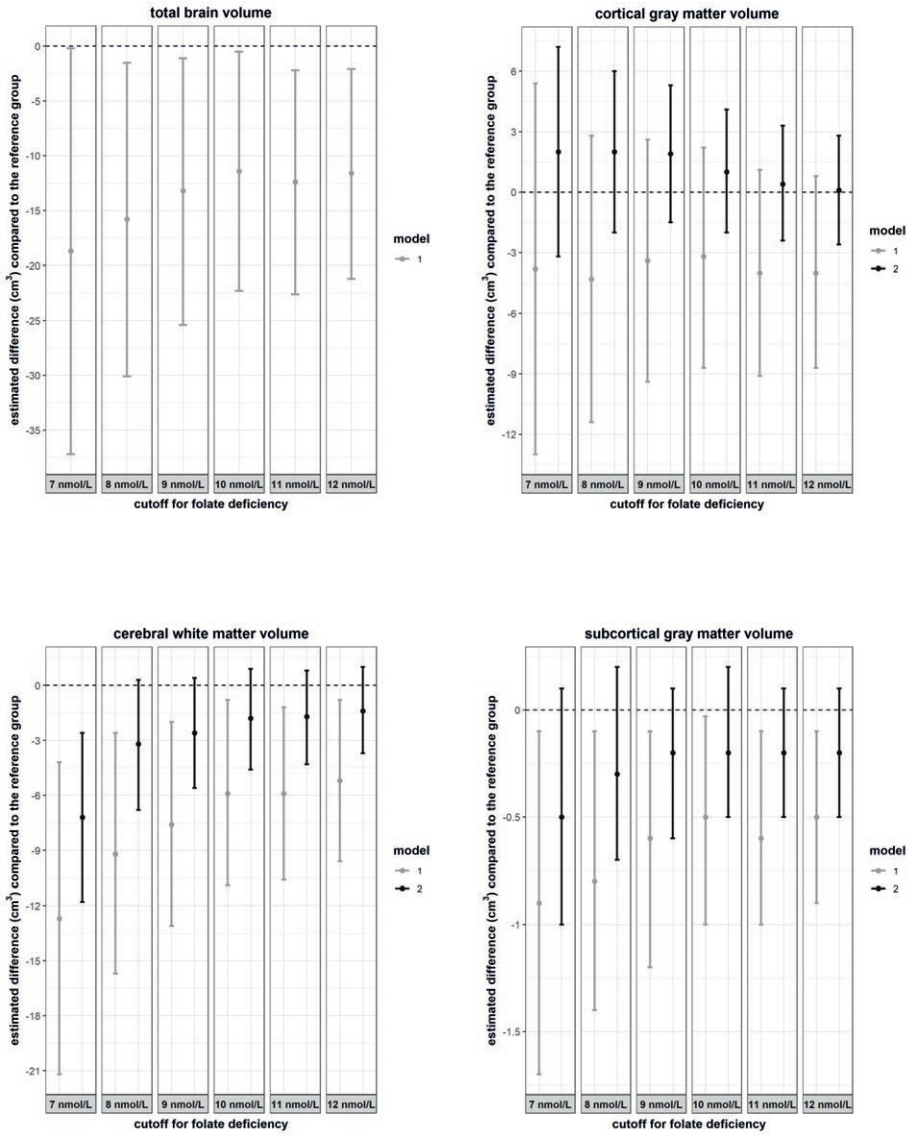

Figure 1. Maternal folate deficiency and child brain volumes at age 10 years, a comparison of using different cutoffs. Estimated brain volumetric differences (with 95% CI) of children exposed to maternal folate deficiency (determined using cutoff values of 7 to 12) during pregnancy compared to the reference group. The sample sizes for the deficient group and reference group were n=1992 vs. n=103 using cutoff value of 7; n=1913 vs. n=182 using cutoff value of 8; n=1817 vs. n=278 using cutoff value of 9; n=1525 vs. n=370 using cutoff value of 10; n=1644 vs. n=451 using cutoff value of 11; and n=1547 vs. n=548 using cutoff value of 12, respectively. Model 1 was adjusted for child sex, age at neuroimaging, maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income. Model 2 was additionally adjusted for child intracranial volume, and was only used for the volumes of regional brain structures (i.e., cortical gray matter, cerebral white matter, and subcortical gray matter) to investigate whether any observed regional differences were independent of global effects.

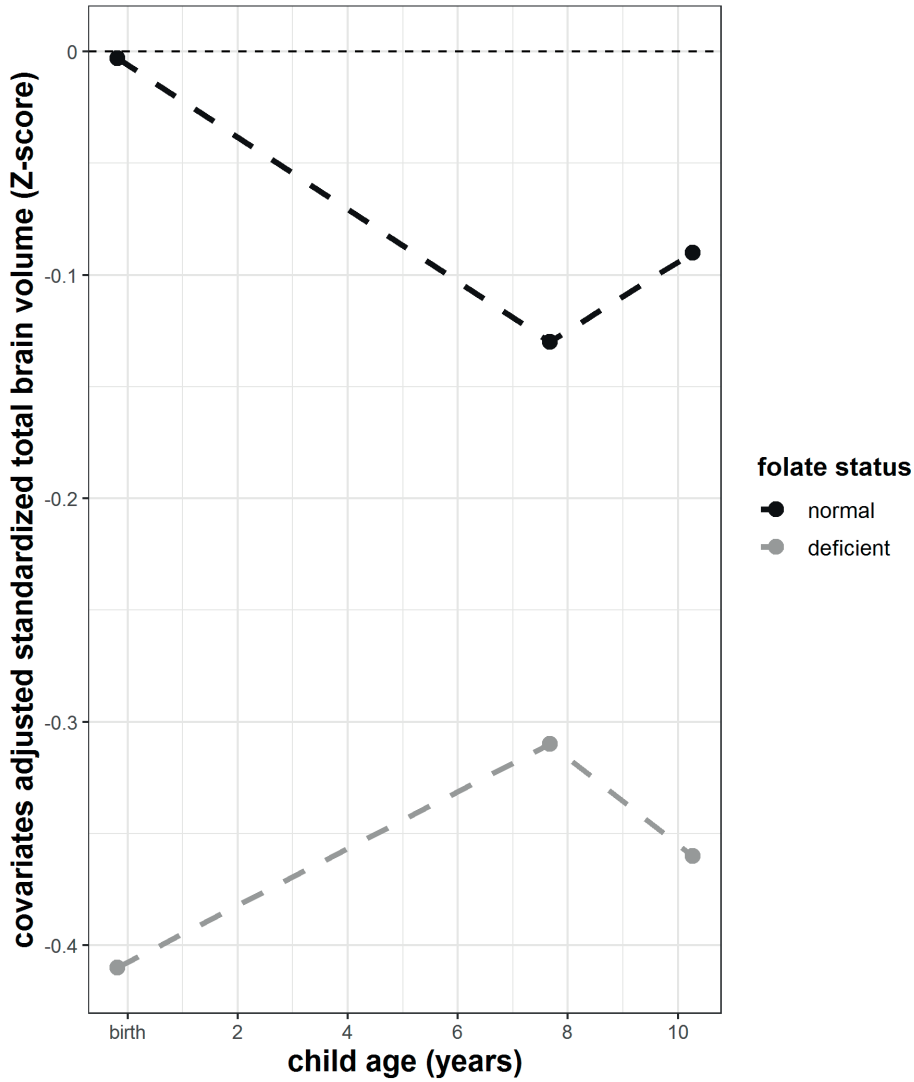

Figure 2. Maternal folate status and child brain developmental trajectories. The sample size for the deficient group (i.e., <7 nmol/L) and normal group was n=29 and n=325, respectively. Brain assessment was performed in the third trimester (mean=30.4 weeks of gestation), middle childhood (mean=7.7 years), and late childhood (mean=10.1 years). Standardized total brain volume was adjusted for child sex and age at assessment, maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income.

Maternal folate status and child emotional and behavioral outcomes

Supplemental Table S3 shows the results of maternal folate levels during pregnancy in relation to child emotional and behavioral problems at age 10 years. When only child sex and age at the CBCL assessment were adjusted for, children born to folate-deficient

Table 3. Maternal folate status during pregnancy and child brain development from the third trimester to childhood

Maternal folate status	N	Model	Standardized total brain volume (Z-score)		
			β	95% CI	p
Normal	325	-	ref	-	-
Deficient	29	1	-0.5	-0.8, -0.2	0.001
		2	-0.4	-0.6, -0.1	0.02

β's represent the difference in Z-scores of children exposed to deficient maternal folate concentrations (<7 nmol/L, 87 observations corresponding to 29 subjects) compared to those with normal maternal folate concentrations (≥7 nmol/L, 975 observations corresponding to 325 subjects). One score stands for one SD.
Model 1 was adjusted for child sex and age at neuroimaging.
Model 2 was additionally adjusted for maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income.

mothers were 0.3 (95% CI 0.1 to 0.6) points higher in the square root transformed CBCL internalizing score at age 10 years compared to those born to folate-normal mothers. These findings did not remain after adjustment for all covariates. Maternal folate deficiency during pregnancy was not related to child behavioral problems in either model. Further, there was no evidence suggesting any association of maternal folate level as a continuum with child emotional or behavioral problems.

Sensitivity analysis

Analyses using TMLE showed consistent results with the main analyses: compared to the reference group, folate-deficient children had lower TBV (B=-23.7, 95% CI -42.3 to -5.1) and cerebral white matter volume (B=-15.1, 95% CI -22.8 to -7.5) at 10 years-of-age, including longitudinal brain volumetric differences (β=-0.5, 95% CI -0.7 to -0.3) from the third trimester to late childhood.

DISCUSSION

We found evidence of reduced brain volumes in children exposed to low folate levels in gestation using a prospective population-based study. The analysis of the repeatedly measured TBV strengthened this finding and revealed that the lower volumes likely originated from fetal life and persisted across childhood. Thus, in spite of the inherent plasticity of the developing brain, global decreases in brain volume may persist even nine-to-eleven years after exposure to deficient folate status during pregnancy, suggesting long-term subtle consequences of exposure to low folate levels in utero [21].

Despite a few studies on folate and brain morphology in geriatric populations [41, 42], investigations on maternal folate status during pregnancy and offspring brain

development are limited. Embedded in the present cohort, low maternal folate concentrations in gestation were associated with a smaller fetal HC in the third trimester [14]. This is supported by Scholtz et al. [12], who reported a positive association between maternal folate in early pregnancy and offspring HC at birth. Findings from the current study extend our previous work showing less TBV in school-age children exposed to low folate levels in gestation [17]. Interestingly, we also observed smaller white matter volume that was independent of TBV reduction in offspring exposed to deficient maternal folate concentrations (i.e., <7 nmol/L), which has not been reported before. Another recent study reported an association between prenatal exposure to folic acid fortification and thicker cortices in the frontal and temporal regions in American youth [18]. We did not find similar differences in cortical thickness; however, variations in age range, race and ethnicity, and neuroimaging techniques, or our more stringent cluster-wise p-value threshold may explain this discrepancy.

Our study showed that the relation between prenatal folate levels and brain volume in childhood was not likely explained by gestational or obstetrical complications, implying that exposure to low folate in utero may have specific impact on brain development. Several mechanisms are possible. In animal studies, folate deficiency during pregnancy results in brain tissue loss in offspring via the selective upregulation of folate receptors and heterogeneous nuclear ribonucleoprotein-E1, which are in turn associated with increased cell loss and premature differentiation of fetal tissues [43]. An experimental study with rats suggests that maternal folate deficiency increases neuronal apoptosis in the cortex of offspring [44]. Also, it is known that prenatal folate deficiency increases the risk of NTDs in offspring, and defects in the skull are commonly observed in NTDs [45, 46]. Thus, even in the absence of NTDs, prenatal folate deficiency could exert a subtle adverse impact on the normal skull development of fetus, potentially by disrupting the proliferation of neuro crest stem cells that generate a diverse array of cell types (including bone cells) during development [47, 48]. This could lead to a reduced intracranial volume that restricts the physical space for brain growth. Moreover, as an important coenzyme, folate involves in various biological processes such as DNA and purine synthesis and placental amino acid transportation that are critical for normal fetal growth [49, 50]. The disturbance of these processes due to low folate levels likely has nonspecific influences on multiple brain structures, resulting in global alterations in brain development.

DNA methylation may be another mechanism underlying the association between prenatal folate exposure and brain development. Maternal folate depletion has been associated with DNA methylation changes that can lead to increased oxidative DNA damage in the brains of adult mice [51]. Other animal studies also show that maternal folic acid supplementation increases DNA methylation and DNA methyltransferase expression and activity in the brain of offspring [51, 52]. An epigenome-wide meta-

analysis found that maternal plasma folate during gestation was associated with DNA methylation levels of genes implicated in neurodevelopment, such as *ACP2* and *GRM8* [53]. *APC2* is essential for axonal projections and may underlie the observed differences in white matter volume [54]. *GRM8* encodes a glutamate receptor interacting with neurotransmitters in the central nervous system and thus is involved in synaptic signaling [55]. A recent randomized trial also suggests that continued folic acid supplementation (400 μg/d) throughout the second and third trimesters of pregnancy results in significantly lower DNA methylation levels at several brain development related genes in newborns, including *LINE-1*, *IFG2*, and *BDNF* [56].

Interestingly, the difference between the TBV trajectory curves of the two groups appears to become less over time, especially from birth to middle childhood (Figure 2), although not statistically significant. In humans, brain plasticity allows for considerable adaptation and enhances the efficiency of the developing brain in response to experiences, which is crucial for optimal neurodevelopment [57, 58]. In the Dutch Hunger Winter study, reduced TBV in the elderly was not observed in all subjects exposed to prenatal famine [59], implying that prenatal adverse effects on brain development may, over a lifetime, be compensated by postnatal factors or ageing. The normalization of neurodevelopment may occur in certain, yet unknown phases after birth. However, we found no evidence of an interaction with time (child age) in the analysis of repeated measured TBV to support this hypothesis. Follow-up study waves including large brain neuroimaging datasets from adolescence into adulthood are needed for future investigations into the long-term effects of maternal folate status on offspring brain developmental trajectories. It is also worth noting that, Figure 1 suggests a dose effect of maternal folate concentrations during pregnancy on TBV as well as the volumes of cerebral white matter and subcortical gray matter of the child, which plateaus after 10 nmol/L. Although most regional volumetric differences disappeared after correction for intracranial volume, we cautiously speculate that a maternal plasma folate concentration of 10 nmol/L or above, which is higher than that for preventing NTDs, predicts optimal brain development of the offspring. Further investigations are needed to confirm this threshold.

We found no associations between maternal folic acid supplementation in the periconceptional period and child brain volumes when adjusting for all covariates (i.e., child sex and age at neuroimaging, maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income). It could be that compared to plasma folate levels, self-reported supplement use was more subjected to information bias, and more highly correlated with socioeconomic indicators that were simultaneously adjusted for in the model, including educational level and income. In addition, maternal folate intake from non-supplement sources is important for child brain development, and these were not systematically collected in our study.

Embedded in the same cohort, Steenweg de Graaff et al. [8] reported more emotional problems in toddlers exposed to prenatal folate deficiency. However, the current study showed no relation between maternal folate levels and child emotional or behavioral problems at age 10 years after full adjustment for covariates. These findings are in line with our previous findings at 6-8 years [17], suggesting an absence of relation between maternal folate levels during pregnancy and offspring emotional or behavioral problems across mid- and late childhood. However, since these emotional and behavioral outcomes were collected at the same age (i.e., 10 years) as the MRI measures, the lack of an association may not necessarily exclude the possibility that clinically relevant emotional or behavioral problems could emerge later in development. Major psychiatric illnesses, such as mood and psychotic disorders tend to emerge during late adolescence and early adulthood and are thought to be associated with developmental brain trajectories in late childhood and adolescence [60, 61]. Thus, the brain differences we show may be associated with an elevated risk of general psychopathology at later stages of development. In addition, the regional volumetric reduction in white matter we observed in children born to folate-deficient women is very interesting. White matter tracts are known to mediate the essential connectivity and enable human cognitive capacities [62], and previous studies have shown that larger white matter volume is associated with favorable cognitive outcomes such as higher intelligence quotient (IQ) and information proceeding speed in children and adolescents [63, 64]. Since prenatal exposure to folate deficiency has been associated with poorer cognitive performance in childhood [17], further studies examining the role of white matter in this relation are warranted.

This study has several strengths. Our longitudinal design allows for the detection of temporal relationships between prenatal folate exposure and brain development assessed 10 years later. Further, the analysis of repeated brain measures provides valuable insights into gestational folate status in relation to brain morphology from a developmental perspective. Moreover, in the sensitivity analysis we supported our primary findings by applying TMLE to minimize bias due to unbalanced exposure distribution. Some limitations of this study should also be acknowledged. First, maternal folate concentrations were only measured in early pregnancy, so we cannot determine whether exposure to folate in the other phases in gestation is associated with brain development in children. Second, we used ultrasound to estimate fetal TBV. While such measures are less accurate than measured by MRI, direct comparisons show correlations above 0.9 [15, 27]. In addition, scanner differences between the two MRI assessments might not be entirely eliminated by standardization. Third, due to being an observational design, residual confounding variables such as genetic predisposition or effects caused by other nutrients cannot be ruled out, thus no causal conclusion can be drawn. Finally, the non-response analysis showed that respondents were exposed

to higher folate levels in gestation, and were from families with higher socioeconomic status than non-respondents, indicating possible attrition.

To conclude, in line with the evidence on the neuroprotective effects of prenatal folic acid supplementation [65], low maternal folate levels during pregnancy are prospectively associated with reduced offspring brain volumes that persist into late childhood. The global volumetric differences originate from fetal life and persist throughout childhood. Therefore, the importance of adequate folate levels (e.g., via periconceptional folic acid supplementation) in pregnancy on child brain development is a vital public health message, which could be particularly true in low and middle-income countries where the prevalence of folate deficiency among women of reproductive age is still not optimal, or in higher-income countries, including much of Europe, where folic acid fortification is not mandatory [66-68]. Further investigations are needed to track the observed brain differences throughout the lifespan and explore their potential neuropsychiatric consequences.

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SUPPLEMENTAL MATERIALS

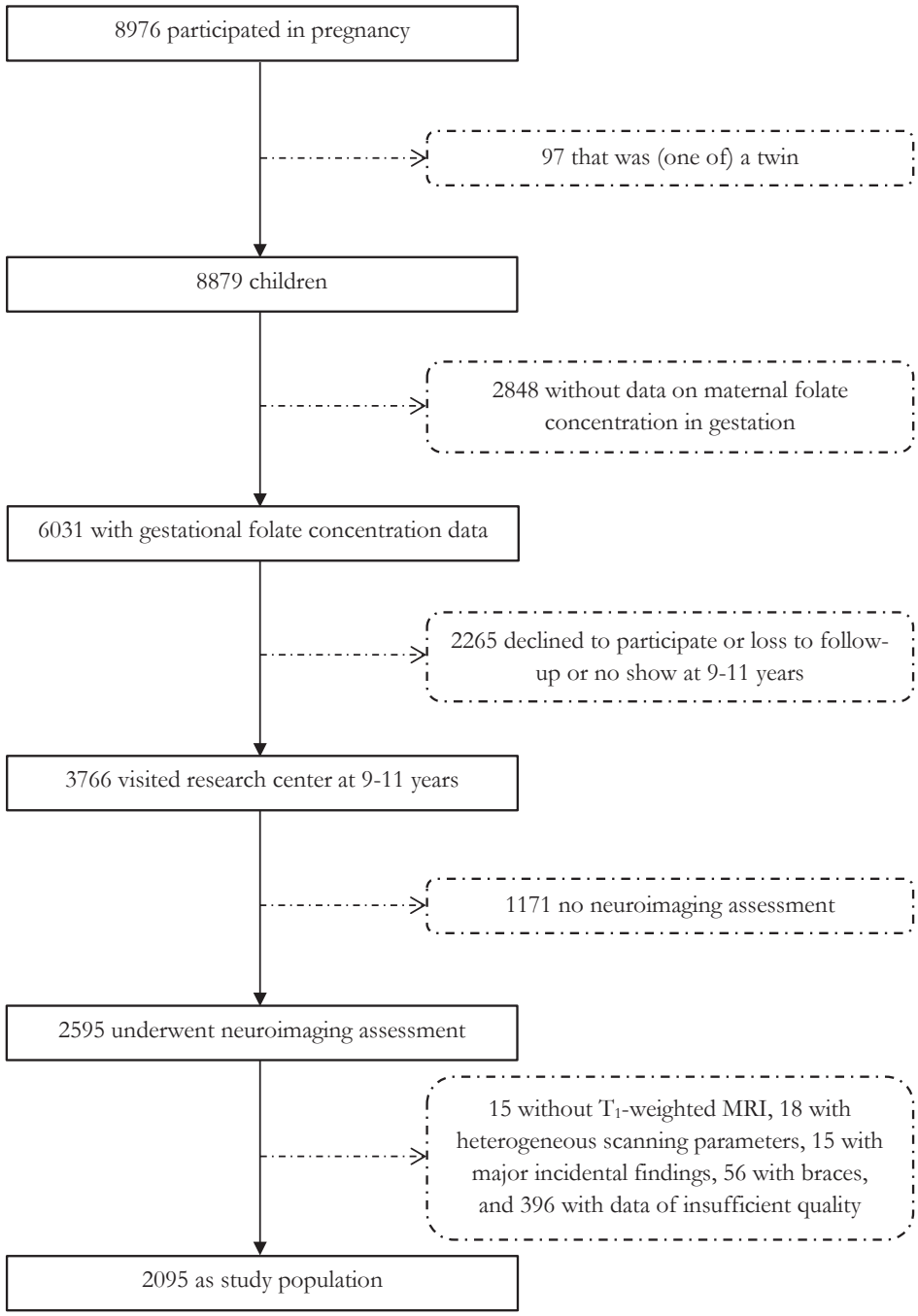

Figure S1. Flow chart

Table S1. Non-response analysis

Characteristics	Respondents (n=2095)	Non-respondents (n=3936)	p-value
Maternal age at enrollment, years	30.8 (4.7)	29.3 (5.2)	<0.001
Maternal ethnicity			
Dutch	1249 (59.6)	1827 (49.1)	
Non-Dutch Western	254 (12.1)	446 (12.0)	<0.001
Non-Dutch non-Western	592 (28.3)	1450 (38.9)	
Maternal marital status (with partner)	1867 (89.1)	3063 (85.3)	<0.001
Maternal highest education completed			
Primary or secondary	981 (46.8)	2173 (60.4)	<0.001
Higher	1114 (53.2)	1422 (39.6)	
Maternal pre-pregnancy BMI	23.4 (4.1)	23.6 (4.3)	0.24
Parity (≥1)	804 (38.4)	1760 (45.2)	<0.001
Maternal smoking during pregnancy			
Never	1593 (76.0)	2419 (69.6)	<0.001
Yes	502 (24.0)	1059 (30.4)	
Maternal alcohol use during pregnancy			
Never drank in pregnancy	771 (36.8)	1708 (48.2)	
Drank until pregnancy was known	322 (15.4)	498 (14.1)	<0.001
Continued drinking in pregnancy	1002 (47.8)	1336 (37.7)	
Family income, €/months			
≤ 2000	657 (31.4)	1227 (41.7)	<0.001
> 2000	1438 (68.6)	1715 (58.3)	
Breastfeeding duration, months	4.5 (3.7)	4.6 (3.8)	0.36
Maternal plasma folate concentration, nmol/L	19.4 (9.1)	16.6 (8.9)	<0.001
Child age at neuroimaging, years	10.1 (0.6)	10.2 (0.8)	0.25
Child sex (male)	1034 (49.4)	2015 (51.2)	0.17
Child gestational age at birth, weeks	40.0 (1.8)	39.8 (2.0)	<0.001

Imputed data were used for respondents. Continuous data were presented in mean (SD) and compared using t-test or Wilcoxon test; categorical data were presented in number (%) and compared using Chi-square test.

Table S2. Maternal folate concentration during pregnancy and child brain volumes at age 10 years

Model	Total brain volume			Cortical gray matter			Cerebral white matter			Subcortical gray matter		
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
1	1.5	1.0, 2.0	<0.001	0.7	0.5, 0.9	<0.001	0.6	0.4, 0.8	<0.001	0.1	0.03, 0.1	<0.001
2	0.5	0.04, 1.0	0.03	0.2	-0.1, 0.4	0.19	0.2	0.02, 0.5	0.03	0.02	-0.0002, 0.04	0.05
3	-	-	-	-0.1	-0.2, 0.1	0.36	0.04	-0.1, 0.2	0.55	0.003	-0.01, 0.02	0.64

B's represent the volumetric difference (in cm^3) of children (n=2083) per 1 nmol/L increase in maternal folate concentration during pregnancy.
Model 1 was adjusted for child sex and age at neuroimaging.
Model 2 was additionally adjusted for maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income.
Model 3 was additionally adjusted for child intracranial volume.

Table S3. Maternal folate status during pregnancy in relation to child emotional and behavioral problems at age 10 years

Maternal folate levels during pregnancy	Model	CBCL emotional problems			CBCL behavioral problems		
		B	95% CI	p-value	B	95% CI	p-value
Normal	-	ref	-	-	ref	-	-
Deficient	1	0.3	0.1, 0.6	0.01	0.1	-0.1, 0.4	0.34
	2	0.2	-0.1, 0.5	0.14	-0.01	-0.3, 0.3	0.97
Continuum	1	-0.01	-0.01, 0.002	0.06	-0.004	-0.01, 0.002	0.20
	2	-0.002	-0.01, 0.004	0.54	0.0001	-0.01, 0.01	0.97

B's represent the difference in the square root transformed child CBCL internalizing or externalizing score of the folate-deficient (i.e., <7 nmol/L) group compared to the reference group, or per 1 nmol/L increase in maternal folate concentration. The sample size was 1802 (1724 normal vs. 78 deficient) for the analysis of emotional problems, and was 1801 (1723 normal vs. 78 deficient) for the analysis of behavioral problems. When using folate concentration as a continuum, 10 children exposed to maternal folate concentration of 45.3 nmol/L (the upper detect limit) were excluded in both analyses.
Model 1 was adjusted for child sex and age at CBCL assessment.
Model 2 was additionally adjusted for maternal age at enrollment, ethnicity, marital status, educational level, alcohol use during pregnancy, and family income.
CBCL - Child Behavior Checklist.

3.3

Maternal polyunsaturated fatty acids during pregnancy and offspring brain development in childhood

Zou R., El Marroun H., Voortman T., Hillegers M., White T., & Tiemeier H. (2021)

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ABSTRACT

Background: Emerging evidence suggests an association of maternal polyunsaturated fatty acids (PUFAs) concentrations during pregnancy with child cognitive and neuropsychiatric outcomes such as intelligence and autistic traits. However, little is known about prenatal maternal PUFAs in relation to child brain development, which may underlie these associations.

Objective: We aimed to investigate the association of maternal PUFA status during pregnancy with child brain morphology, including volumetric and white matter microstructure measures.

Design: This study was embedded in a prospective population-based study. In total, 1553 mother-child dyads of Dutch origin were included. Maternal plasma glycerophospholipid PUFAs were assessed in mid-pregnancy. Child brain morphological outcomes including total gray and white matter volumes, and white matter microstructure quantified by global fractional anisotropy and mean diffusivity were measured using magnetic resonance imaging (including diffusion tensor imaging) at age 9-11 years.

Results: Maternal ω-3 long-chain PUFAs (LC-PUFAs) concentrations during pregnancy had an inverted U-shaped relation with child total gray volume (linear term: β=16.7, 95% CI 2.0, 31.5; quadratic term: β=-1.1, 95% CI -2.1, -0.07) and total white matter volume (linear term: β=15.7, 95% CI 3.6, 27.8; quadratic term: β=-1.0, 95% CI -1.8, -0.16). Maternal gestational ω-6 LC-PUFAs levels did not predict brain volumetric differences in children, albeit the linolenic acid concentration was inversely associated with child total white matter volume. Maternal PUFA status during pregnancy was not related to child white matter microstructure.

Conclusions: Sufficient maternal ω-3 PUFAs during pregnancy may be related to more optimal child brain development in the long-term. In particular, exposure to lower ω-3 PUFAs concentrations in fetal life was associated with less brain volume in childhood. Maternal ω-6 LC-PUFAs were not related to child brain morphology.

INTRODUCTION

Fatty acids play vital physiological roles as components of complex lipids involved in metabolism, gene transcription, and cell signaling (1, 2). Humans are able to synthesize most fatty acids, but polyunsaturated fatty acids (PUFAs) from the omega-3 (ω-3) family and the omega-6 (ω-6) family must be obtained from diet such as fatty fish and plant-based oils. Moreover, the ability of PUFA biosynthesis is substantially determined by rate-limiting enzymes for fatty acid metabolism, which are encoded by genetic variants that are robustly related to ethnicity (3).

The embryo and fetus are dependent on the placenta for its supply of PUFAs as essential nutrients to maintain proper physiological functioning (4). Emerging evidence has linked maternal PUFA levels during pregnancy to fetal growth, neonatal respiratory distress syndrome, and childhood metabolic outcomes such as adiposity (5-7). More recent studies also suggested associations between prenatal PUFA status and neurodevelopment in children. For example, previous work in our research group showed that a lower maternal gestational plasma PUFA ω-3 to ω-6 ratio, manifested by lower ω-3 PUFAs and higher ω-6 PUFAs, predicted more emotional problems and autistic traits in the offspring at age 6 years (8, 9). Also, a lower PUFA ω-3 to ω-6 ratio in cord blood was related to more subclinical attention deficit and hyperactivity disorder (ADHD) symptoms in 7-year-old Spanish children (10). In addition, ω-3 PUFAs during pregnancy were positively related to childhood cognitive function such as sequential processing and intelligence quotient (IQ) (11, 12), and lower gestational ω-6 PUFAs were associated with less externalizing behaviors in children at age 6-7 years (9, 13).

The biological basis underlying the neurodevelopmental outcomes related to PUFA status in fetal life remains largely unknown. A general hypothesis posits that specific PUFAs such as eicosapentaenoic acid (EPA, C20:5ω-3), docosahexaenoic acid (DHA, C22:5ω-3), arachidonic acid (ARA, C20:4ω-6), and adrenic acid (C22:4ω-6) are critical components for the development of the central nervous system given their role in neuronal membranes, neurogenesis, and myelination (14-16). However, we know of no studies relating prenatal exposure to PUFAs to brain morphology in childhood (17).

In this population-based study, we aimed to investigate the prospective associations between maternal PUFA status during pregnancy and child brain development. Due to a lack of prior knowledge, an exploratory approach was used to primarily investigate global measures. Based on the studies described above, we hypothesized that higher ω-3 PUFAs, in particular higher DHA, and lower ω-6 PUFAs during pregnancy were related to larger brain volumes and more optimal white matter microstructure in childhood.

SUBJECTS AND METHODS

Setting

This study was embedded in the Generation R Study, an ongoing prospective cohort in Rotterdam, the Netherlands (18). Pregnant women living in Rotterdam with an expected delivery date between April 2002 and January 2006 were invited to participate. The Generation R Study has been approved by the Medical Ethical Committee of the Erasmus Medical Center. Written informed consent was obtained from all participants or their caregivers.

Participants

We included mother-child dyads with a Dutch origin (determined by the country of birth of the mother's parents) given the ethnic differences in PUFA intake and metabolism, and related confounder patterns (19, 20). In total, 4153 children were born to 4096 Dutch mothers enrolled during pregnancy; of these, 57 children were randomly excluded as one of each twin pair to eliminate clustered data (21). Further, 748 children were excluded due to missing information on maternal fatty acids concentrations during pregnancy. Of the remaining 3348 children, 1693 visited the research center and underwent neuroimaging assessment at age 9-11 years. After quality control, 1553 children with usable neuroimaging data were included as the study population (see Supplemental Figure 1 for the flow chart).

Maternal fatty acid assessment

As previously described (9), maternal non-fasting venous samples were drawn in mid-pregnancy and stored at room temperature before being transported to the regional laboratory for processing and storage for future studies. The samples were centrifuged and thereafter stored at -80 °C. For the fatty acid analysis, plasma samples were transported to the Division of Metabolic Diseases and Nutritional Medicine, University of Munich Medical Center, in 2010. After being thawed, plasma glycerophospholipid fatty acids were analyzed and the composition was determined by a sensitive and precise high-throughput method (see Supplemental Method for more details) (22). The average coefficient of variation was 15.7%. The concentrations of individual fatty acids are expressed as weight percentage (%, wt:wt) of all glycerophospholipid fatty acids detected with a chain length between 14 and 22 carbon atoms. The plasma total fatty acid concentration was measured in mg/L. Since bio-magnification of PUFAs with 20 and 22 carbons and bio-reduction of PUFAs with 18 carbons in the placenta have been shown (23), we summed concentrations of long-chain PUFAs (LC-PUFAs) with 20 and 22 carbon atoms from the ω-3 and ω-6 families separately (referred to as ω-3 LC-PUFAs and ω-6 LC-PUFAs hereafter) in the primary analyses. Alpha-linolenic acid

(ALA) and linolenic acid (LA), the two precursor PUFAs with 18 carbon atoms, as well as specific individual PUFAs including EPA, DHA, ARA, and adrenic acid were examined in the secondary analyses. In addition, the concentrations of all PUFAs (i.e., those with 18 to 22 carbon atoms) from the ω-3 and ω-6 families (referred to as ω-3 and ω-6 PUFAs) were reported in a supplementary analysis to allow comparison with prior studies of fatty acids. The full profile for each fatty acid used in this study, including both the relative (%, wt:wt) and absolute (mg/L) concentrations, can be found in Supplemental Table 1.

Neuroimaging

Prior to the magnetic resonance imaging (MRI) scanning, children underwent a mock scanning session to become familiar with the neuroimaging assessment. All images were acquired on a 3-Tesla GE Discovery MR750w MRI System (General Electric, Milwaukee, WI, USA) scanner using an 8-channel head coil. High-resolution T_1-weighted sequences were obtained using a 3D coronal inversion recovery fast spoiled gradient recalled (IR-FSPGR, BRAVO) sequence. Diffusion tensor imaging (DTI) data were acquired using an axial spin echo, echo planar imaging sequence with three b = 0 scans and 35 diffusion-weighted images. T_1-weighted images were rated for imaging quality both during and after the MRI acquisition. Further information on the neuroimaging procedure and quality control is described elsewhere (24).

Volumetric segmentation and cortical reconstruction were performed with FreeSurfer v.6.0.0 (http://surfer.nmr.mgh.harvard.edu/), and the standard reconstruction stream was applied. The quality of FreeSurfer output was visually inspected, and data with insufficient quality were eliminated. DTI data were processed using the Functional MRI of the Brain Software Library and the Camino Diffusion MRI Toolkit (25, 26). The diffusion tensor was fitted using the RESTORE method implemented in Camino. The quality of the diffusion images was assessed using a combination of manual and automated checks. Common scalar maps including global fractional anisotropy (FA) and mean diffusivity (MD) were computed to quantify whole-brain white matter microstructure. FA is a scalar that provides a rotationally invariant measure of hydrogen diffusion within a voxel, and MD describes the average diffusion in all directions (27).

Covariates

Based on prior literature investigating childhood health outcomes related to prenatal PUFAs exposure (8, 9, 28), we selected the following covariates: child sex and age at the time of neuroimaging, and maternal age at enrollment, educational level (categorized as 'primary or below', 'secondary', and 'higher'), marital status ('single' or 'with partner'), pre-pregnancy body mass index (BMI), psychopathology, smoking ('never smoked', 'smoked until pregnancy was known', or 'continued smoking throughout pregnancy'),

alcohol use ('never drank', 'drank until pregnancy was known', 'continued drinking occasionally', or 'continued drinking frequently' defined as '1 or more glasses of alcohol per week in at least two trimesters'), vitamin supplement use ('yes' or 'no'), total energy intake and diet quality during pregnancy, gestational age at fatty acid assessment, and family income ('<1200€', '1200-2000€', or '>2000€' per month). Child sex and date of birth (to calculate age at neuroimaging) were obtained from medical records. Maternal pre-pregnancy BMI was calculated by self-reported pre-pregnancy height and weight. Information on all other covariates was collected with questionnaires during pregnancy. Maternal dietary intake was assessed with a 289-item food frequency questionnaire, from which diet quality was calculated as adherence to Dutch dietary guidelines (29).

Additional measures

Information on child academic performance at age 12 years, indexed by the CITO [Centraal Instituut voor Test Ontwikkeling (English: Central Institute for Test Development)] score (https://www.cito.nl/), was available in 736 children in the present sample. The CITO test assesses language and mathematic skills at the end of primary education in the Netherlands. We standardized the CITO raw score ranging between 500 and 550 using Z-transformation, with a higher score indicating higher academic achievement. Child age at the CITO test was also assessed.

Statistical analyses

For descriptive statistics, continuous variables were presented as mean (standard deviation, SD) and categorical variables were presented as number (%). In a non-response analysis, we compared maternal and child characteristics between participants with complete data on the predictor and the outcome ('respondents') and those with information on the predictor but without data on the outcome ('non-respondents'). Continuous variables were compared with t-test or Wilcoxon tests; categorical variables were compared with Chi-square tests. Based on non-response analysis, inverse probability weighting was applied to all analyses to account for attrition (30).

In the primary analyses, we related maternal ω-3 and ω-6 LC-PUFAs concentrations during pregnancy to child total gray and white matter volumes and white matter microstructure at age 9-11 years using multiple linear regression. As suggested by studies on PUFAs and cardiovascular outcomes (31, 32), non-linearity was tested by introducing quadratic terms to the linear model, and we compared the model fit with the likelihood ratio test (33). In a next step, non-linear associations were depicted with natural cubic splines. Potential sex differences were tested by adding an interaction term to the model. In post-hoc analyses, we associated maternal LC-PUFAs levels with the volumes of individual brain lobes (i.e., the frontal lobe, temporal lobe, parietal lobe, and occipital lobe) to investigate which brain lobes accounted for the global volumetric differences.

We also examined ALA, LA, EPA, DHA, ARA, and adrenic acid in secondary analyses. In a supplementary analysis, we examined maternal ω-3 and ω-6 PUFAs to allow comparison with previous studies of maternal PUFA status and child neurodevelopment.

In a follow-up analysis, we related maternal LC-PUFAs levels during pregnancy and child brain morphology at age 10 years to child CITO score at age 12 years. Further, we performed a mediation analysis to investigate whether any observed brain differences mediated the association between prenatal exposure to LC-PUFAs and academic performance of the child.

All regression analyses described were performed using multi-stage models. The initial model (Model 1) was only adjusted for child sex and age at neuroimaging (age at the CITO test when the CITO scores were the outcome measures). In Model 2, we additionally adjusted for maternal age at enrollment, educational level, smoking and alcohol use, diet quality during pregnancy, and family income. In Model 3, maternal plasma total fatty acid concentration was additionally adjusted for to investigate whether any observed differences were independent of absolute fatty acid concentration (34).

To rule out that fatty acid transfer abnormalities from mother to the fetus in the third trimester due to shortened gestational duration and/or placenta dysfunction (5, 35) drive results, we re-ran the primary analyses after excluding children with preterm birth (defined as a gestational age at birth <37 weeks) and/or small for gestational age (SGA, defined as a SD score ≤ −2.3 and based on SD curves derived from the total Generation R cohort) in sensitivity analyses (36, 37).

We used multiple imputation to account for missing data on covariates (missing at random indicated by Little's test) (38, 39). A total of 20 imputed datasets was generated with 20 iterations, and only pooled results are reported. Statistical significance was set as $\alpha<0.05$ (two-sided). To minimize false positive findings, a multiple comparison correction using the Benjamini-Hochberg method was performed for primary analyses, and only the p-values of the linear terms were corrected for non-linear model. All statistical analyses were performed using R version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Descriptive information

Table 1 shows descriptive statistics of the maternal and child characteristics of the study population. Children underwent neuroimaging assessment at age 10.1 (range: 8.9 to 12.0) years, and there were slightly more girls (51.4%) than boys. Maternal plasma ω-6 LC-PUFAs concentrations during pregnancy were higher than ω-3 LC-PUFAs concentrations (14.7% vs. 6.5 %, wt:wt). Non-response analysis showed that compared to par-

Table 1. Descriptive information (N=1553)[1]

	Statistics
Maternal characteristics	
Age at enrollment, year, mean (SD)	31.9 (4.0)
Educational level, n (%)	
Primary or below	25 (1.6)
Secondary	527 (33.9)
Higher	1001 (64.5)
Marital status (with partner), n (%)	1462 (94.1)
Pre-pregnancy BMI, mean (SD)	23.1 (3.9)
Psychopathology during pregnancy (GSI)[2], mean (SD)	0.2 (0.2)
Smoking during pregnancy, n (%)	
Never	1192 (76.8)
Smoked until pregnancy was known	153 (9.9)
Continued smoking throughout pregnancy	208 (13.4)
Alcohol drinking during pregnancy, n (%)	
Never	451 (29.0)
Drank until pregnancy was known	244 (15.7)
Continued drinking occasionally	660 (42.5)
Continued drinking frequently[3]	198 (12.7)
Family income, €/month, n (%)	
< 1200	63 (4.1)
1200-2000	206 (13.3)
> 2000	1284 (82.7)
Vitamin supplementation use (yes), n (%)	620 (39.9)
Total energy intake, kcal, mean (SD)	2154.9 (483.7)
Diet quality, score, mean (SD)	8.1 (1.5)
Gestational age at fatty acid assessment, week, mean (SD)	20.6 (1.1)
Plasma total fatty acid concentration, mg/L, mean (SD)	1651.9 (216.5)
Plasma ω-3 LC-PUFAs[4] concentration, %, wt:wt[5], mean (SD)	6.5 (1.4)
Plasma ω-6 LC-PUFAs[4] concentration, %, wt:wt[5], mean (SD)	14.7 (1.6)
Child characteristics	
Sex (male), n (%)	771 (49.6)
Age at neuroimaging assessment, year, mean (SD)	10.1 (0.6)

1. Statistics of the first imputed dataset were reported.
2. GSI-global severity index.
3. Defined as '1 or more glasses of alcohol per week in at least two trimesters'.
4. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 to 22 carbon atoms in this study.
5. Weight percentage of all glycerophospholipid fatty acids.

ticipating mothers, non-participating mothers were younger (30.9 vs. 31.9 years), lower educated (with higher education: 52.7% vs. 64.5%), had less family income (>2000 €/month: 76.7% vs. 82.7%), and had a lower plasma total fatty acid concentration (1620.9 vs. 1651.9 mg/L) and a lower ω-3 LC-PUFA level (6.2% vs. 6.5%, wt:wt, Supplemental Table 2).

Maternal PUFAs and child brain volumes

Table 2 demonstrates the association of maternal LC-PUFA levels during pregnancy with child total gray and white matter volumes at age 10 years. First, we examined the linear model. In the minimally adjusted model (Model 1), higher maternal ω-3 PUFAs

Table 2. Maternal LC-PUFAs concentrations during pregnancy in relation to child brain volumes at age 10 years[1]

LC-PUFAs concentrations[2]	Total gray matter volume (cm^3)			Total white matter volume (cm^3)		
	β	95% CI	p-value	β	95% CI	p-value
linear model						
ω-3						
Model 1	4.3	1.7, 6.8	<0.001	3.0	1.0, 5.1	0.003
Model 2	1.9	-0.33, 4.1	0.10	2.1	0.27, 3.9	0.02
Model 3	1.8[3]	-0.40, 4.0	0.11	2.0[3]	0.16, 3.9	0.03
ω-6						
Model 1	-1.0	-2.9, 0.83	0.28	0.46	-1.0, 2.0	0.55
Model 2	0.48	-1.5, 2.5	0.64	1.2	-0.32, 2.8	0.12
Model 3	0.50	-1.5, 2.5	0.62	1.3	-0.28, 2.8	0.11
non-linear model (results from Model 3 only)						
ω-3	16.7	2.0, 31.5	0.03[4]	15.7	3.6, 27.8	0.01[4]
(ω-3)^2	-1.1	-2.1, -0.07	0.04	-1.0	-1.8, -0.16	0.02
ω-6	1.7	-21.4, 24.7	0.89	15.6	-4.7, 35.9	0.13
(ω-6)^2	-0.04	-0.82, 0.74	0.92	-0.49	-1.2, 0.20	0.17

1. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 and 22 carbon atoms in this study.
2. Linear regression was used to examine maternal LC-PUFAs concentrations in relation to child brain volumes. β's represent volumetric differences per 1 %, wt:wt increase in ω-3 or ω-6 LC-PUFAs concentrations. Model 1 was adjusted for child sex and age at neuroimaging. Model 2 was additionally adjusted for maternal age at enrollment, educational level, smoking and alcohol use in pregnancy, diet quality in pregnancy, and family income. Model 3 was additionally adjusted for maternal plasma total fatty acids concentration (mg/L). All models were weighted by inverse probability to count for attrition. N=1397.
3. Using likelihood-ratio test, non-linear model shows better model fit than the linear model.
4. Adjusted p-value ≤ 0.05 after multiple comparison correction using the Benjamini-Hochberg method. Only p-values for the linear terms in the non-linear model were corrected for multiple comparison.

levels during pregnancy were associated with both higher total gray matter volume (β=4.3, 95% CI 1.7, 6.8, cm^3) and higher total white matter volume (β=3.0, 95%CI 1.0, 5.1, cm^3) in children. In the model fully adjusted for covariates (Model 2), only the association of maternal ω-3 LC-PUFAs levels with total white matter volume remained (β=2.1, 95% CI 0.27, 3.9, cm^3). This association was independent of plasma total fatty acid concentration of the mother (Model 3). Maternal ω-6 LC-PUFAs concentrations during pregnancy were not related to child total gray or white matter volume in any model.

Next, we tested non-linear associations of maternal ω-3 LC-PUFAs concentrations with child total gray and white matter volumes. We found evidence that a quadratic term improved the model fit compared to the linear model. After adjusting for all covariates, both the slope and the quadratic terms of the independent variable (i.e., maternal ω-3 LC-PUFAs concentrations) were associated with the dependent variables (i.e., child total gray and white matter volumes, see Table 2). Figure 1 visualizes these inverted U-shaped relations, suggesting that both low and high maternal ω-3 LC-PUFA levels during pregnancy were associated with lower child total gray and white mat-

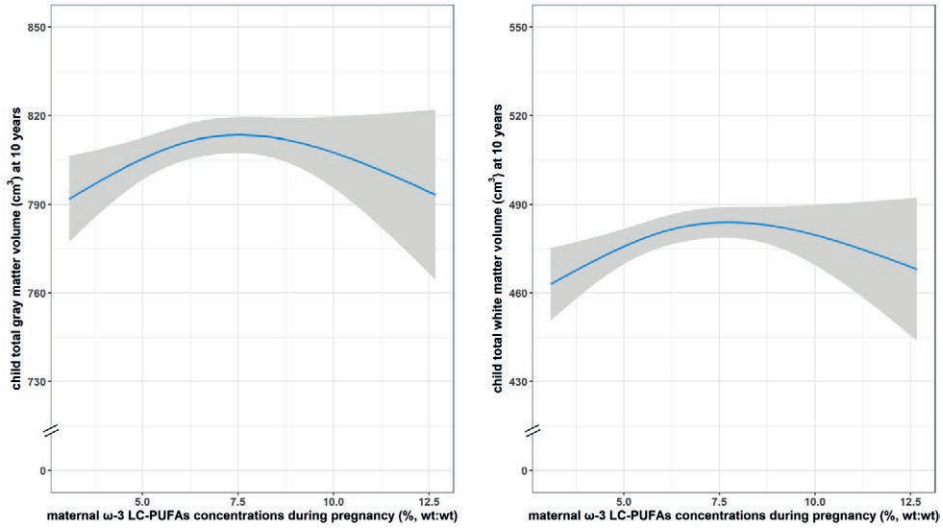

Figure 1. Maternal ω-3 LC-PUFAs concentrations during pregnancy in relation to child brain volumes at age 10 years. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 and 22 carbon atoms in this study. Linear regression was used to examine maternal ω-3 LC-PUFAs concentrations in relation to child brain volumes. The curve and shadow represent mean values and 95% CIs of child total gray or white matter volume predicted by maternal plasma ω-3 LC-PUFA concentrations (%, wt/wt) during pregnancy. Non-linear relations were depicted with natural cubic splines, with one knot placed at the median (6.4). Model was adjusted for child sex and age at neuroimaging assessment, maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, family income, and maternal plasma total fatty acids concentration (mg/L), and was weighted by inverse probability to count for attrition. N=1397.

ter volumes. However, the confidence intervals suggest that the lower end of the ω-3 LC-PUFAs concentrations (e.g., <7%, wt:wt) drives the non-linear association with both gray and white matter volumes more than the higher end (e.g., >8%, wt:wt). In the post-hoc analyses, we found that maternal ω-3 LC-PUFAs concentrations during pregnancy had a similar inverted U-shaped association with the volume of the frontal lobe (Supplemental Figure 2), which likely accounted for the findings with global gray and white matter. We found no evidence for non-linear relations between maternal ω-6 LC-PUFAs levels and child total gray or white matter volume. No evidence for sex differences was found in any model (Supplemental Table 3 shows the results stratified by sex).

Table 3 shows the results of the analysis of the individual PUFAs. After adjustment for all covariates, we observed an inverse linear relation between maternal LA concentra-

Table 3. Maternal plasma concentrations of individual PUFAs during pregnancy in relation to child brain volumes at age 10 years[1]

Individual PUFAs concentrations[2,3]	Total gray matter volume (cm^3)			Total white matter volume (cm^3)		
	β	95% CI	p-value	β	95% CI	p-value
Linear model						
ALA	4.9	-25.3, 35.1	0.75	-7.3	-32.8, 18.3	0.58
LA	-1.0	-2.3, 0.30	0.13	-1.3	-2.3, -0.29	0.01
EPA	-1.5	-9.7, 6.6	0.71	1.4	-5.6, 8.5	0.69
DHA	2.2	-0.70, 5.1	0.14	2.6[4]	0.23, 5.0	0.03
ARA	0.12	-2.1, 2.3	0.91	0.89	-0.82, 2.6	0.31
Adrenic acid	17.4	-10.9, 45.8	0.23	9.4	-15.8, 34.6	0.46
Non-linear model[5]						
ALA	-	-	-	-	-	-
LA	-	-	-	-	-	-
EPA	-	-	-	-	-	-
DHA	-	-	-	19.6	4.5, 34.7	0.01
(DHA^2)	-	-	-	-1.6	-3.0, -0.3	0.02
ARA	-	-	-	-	-	-
Adrenic acid	-	-	-	-	-	-

1. PUFAs - polyunsaturated fatty acids.
2. ALA - α-linolenic acid; LA - linolenic acid; EPA - eicosapentaenoic acid; DHA - docosahexaenoic acid; ARA - arachidonic acid.
3. Linear regression was used to examine maternal individual PUFAs levels in relation to child brain volumes. β's represent volumetric differences per 1 %, wt:wt increase in individual PUFAs concentrations. Model was adjusted for child sex and age at neuroimaging assessment, maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, family income, and maternal plasma total fatty acids concentration (mg/L), and was weighted by inverse probability to count for attrition. N=1397.
4. Using likelihood-ratio test, non-linear model shows better model fit than the linear model.
5. Results are only shown if there is evidence for non-linear relations.

tions and total white matter volume in children. In addition, there was evidence for an inverted U-shaped association between maternal DHA concentrations and child total white matter volume (Supplemental Figure 3). We found no evidence suggesting linear or non-linear relations between maternal ALA, EPA, ARA, or adrenic acid concentrations and child total gray or white matter volume.

Higher maternal ω-3 LC-PUFAs concentrations during pregnancy predicted higher child CITO scores at age 12 years (β=0.06, 95% CI 0.01, 0.10) in models fully adjusted for covariates. Also, both total gray and total white matter volumes at age 10 years were positively related to child CITO scores (β=0.002, 95% CI 0.001, 0.003; and β=0.002, 95% CI 0.001, 0.004, respectively). In a subgroup of 484 children exposed to maternal ω-3 LC-PUFA concentrations of 7%, wt:wt or below (cut-off based on inflection point in the relation between maternal ω-3 LC-PUFAs concentrations and child total gray and white matter volumes, see Figure 1), the mediation analysis showed a significant indirect effect of total gray matter volume (β=0.02, bootstrapped 95% CI 0.0003, 0.04) and of total white matter volume (β=0.01, bootstrapped 95% CI 0.0003, 0.03) at age 10 years in the relation between maternal ω-3 LC-PUFAs concentrations during pregnancy and child CITO scores at age 12 years in unadjusted analyses. There was no evidence of an indirect effect after adjusting for all covariates.

The analyses on maternal ω-3 and ω-6 PUFAs concentrations in relation to child total gray and white matter volumes yielded similar results to the primary analyses (Supplemental Table 4), except for an inverted U-shaped relation between maternal ω-6 PUFAs and child total white matter volume, suggesting that high ω-6 PUFAs concentrations (e.g., >36%, wt:wt) were associated with reduced total white matter volume in children (Supplemental Figure 4). Since this relation was not observed when analyzing ω-6 LC-PUFAs, this finding was likely driven by the inclusion of LA given its inverse association with total white matter volume of the child.

Maternal PUFAs and child white matter microstructure

As shown in Table 4, maternal ω-3 or ω-6 LC-PUFAs concentrations during pregnancy were not related to child global FA or MD at age 10 years in any model, and no sex differences were found. No association was found between the individual PUFAs concentrations of the mother and white matter microstructure of the child either (data not shown). In addition, there were no relations between maternal ω-3 or ω-6 PUFAs concentrations and child white matter microstructure (Supplemental Table 5).

Sensitivity analyses

After excluding 94 children who were born preterm or SGA, results were very consistent with the findings in the total study population.

Table 4. Maternal LC-PUFAs concentrations during pregnancy and child white matter microstructure at age 10 years[1]

LC-PUFAs concentrations[2]	Global Fractional Anisotrophy			Global Mean Diffusivity		
	β	95% CI	p-value	β	95% CI	p-value
ω-3						
Model 1	0.06	-0.02, 0.14	0.15	-0.0001	-0.01, 0.01	0.99
Model 2	0.01	-0.06, 0.09	0.74	-0.001	-0.01, 0.01	0.78
Model 3	0.01	-0.06, 0.09	0.72	-0.002	-0.01, 0.01	0.74
ω-6						
Model 1	0.002	-0.07, 0.07	0.95	0.001	-0.01, 0.01	0.88
Model 2	0.03	-0.04, 0.10	0.45	0.002	-0.01, 0.01	0.63
Model 3	0.03	-0.04, 0.10	0.47	0.002	-0.01, 0.01	0.58

1. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 and 22 carbon atoms in this study.
2. Linear regression was used to examine maternal LC-PUFAs concentrations in relation to child white matter microstructure. β's represent differences in the indicators of white matter microstructure per 1 %, wt:wt increase in ω-3 or ω-6 LC-PUFAs concentrations. Model 1 was adjusted for child sex and age at neuroimaging assessment. Model 2 was additionally adjusted for maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, and family income. Model 3 was additionally adjusted for maternal plasma total fatty acids concentration (mg/L). All models were weighted by inverse probability to count for attrition. N=1348.

DISCUSSION

In a prospective population-based study, we found a non-linear association of maternal ω-3 PUFAs concentrations during pregnancy with total gray and white matter volumes in the offspring 10 years later. In contrast, maternal gestational ω-6 LC-PUFAs concentrations were not related to volumetric differences in total gray or white matter of the child, only LA concentration was inversely associated with child total white matter volume. Maternal PUFAs during pregnancy were not related to child white matter microstructure.

Most of the existing studies relating PUFAs to human brain morphology were performed in adults and elderly persons, and suggested larger brain volumes and superior white matter microstructure in participants with higher DHA levels or fish oil supplementation, which is rich in ω-3 PUFAs (40, 41). Investigations on maternal PUFA status during pregnancy and offspring brain morphology remain sparse. In a randomized controlled trial (RCT), Ogundipe et al. (42) observed larger total gray matter volumes in 24 male infants born to mothers with DHA and ARA supplementation in pregnancy than in the placebo group. Our study showed lower total white matter volume in children born to women with low DHA during pregnancy, but maternal

DHA or ARA levels were not related to child total gray matter volume at age 10 years. No evidence for sex differences was found. Different timing of child brain assessment may explain the heterogeneous findings. In addition, an animal study reported thinner rostral (i.e., frontal) neocortex in mouse offspring of mothers fed with a deficient ω-3 PUFA diet during pregnancy (43). Our results correspond to this finding, because a thinner cortex corresponds to less gray matter volume, and we could attribute the volumetric differences to the frontal lobe.

Our results suggest not only that children exposed to lower ω-3 PUFAs during pregnancy may have less total gray and white matter volumes, but the inverted U-shaped curve suggests an inverse association of ω-3 PUFAs above a certain level (e.g., 8%, wt:wt) during pregnancy with global brain volumes in childhood. This, however, is a less robust explanation given the upper boundary of the estimated association with brain volumes at higher ω-3 PUFAs concentrations. Moreover, this finding is not supported by prior animal or human studies. Therefore, we cautiously speculate that this represents a ceiling effect, i.e., there is no further benefit of prenatal ω-3 PUFAs on long-term brain development at levels above 8%.

Several explanations for the reduced global brain volumes in children exposed to low maternal ω-3 PUFA levels are possible. First, although the basis for the proliferation, migration, and molecular specification is the embryonic period, the human brain experiences accelerated growth in the second half of gestation, manifested by substantial neurogenesis, axonal growth, dendritic differentiation, and synaptogenesis (44, 45). ω-3 PUFAs serve as elements of the cell membrane and, as suggested by animal studies, may enhance neurogenesis across fetal life and adulthood via modulating membrane proteins, cytokines and/or neurotrophins, and increasing levels of signaling factors involved in synaptic plasticity (46-48). In particular, DHA, which is highly enriched in neuronal and synaptic membranes (49, 50), may play a critical role in the regulation of synapse formation and cell proliferation through its effects on cellular phosphatides such as phosphatidylserine (51, 52). Suboptimal neurogenesis and synaptogenesis due to low ω-3 PUFAs (and DHA) may potentially lead to reduced brain volumes of the fetus persisting into childhood. Second, low maternal ω-3 PUFA levels during pregnancy have been related to decreased expression of certain genes such as brain-derived neurotrophic factor (BDNF) and leukemia inhibitory factor (LIF) in the offspring as a result of altered DNA methylation (53, 54). Higher BDNF has been associated with larger gray matter volumes in humans (55), and a smaller cerebral cortex was observed in fetal mice with reduced LIF levels due to decreased neurogenesis (56). Third, exposure to lower ω-3 PUFAs in utero has been related to metabolic outcomes such as increased risk of obesity and lower high-density lipoprotein (HDL) in childhood (5, 57). Obese children aged 8-10 years had less regional gray matter than those with a normal weight (58). Therefore, low ω-3 PUFAs in fetal life may exert an indirect impact

on the long-term brain development. Since LA competes with ω-3 PUFAs for the desaturation enzymes, the reduced total white matter volume in children exposed to high maternal LA concentrations during pregnancy might be attributed to the concurrent low ω-3 PUFAs levels.

Although both maternal LC-PUFAs concentrations during pregnancy and child total gray and white matter volumes at age 10 years were positively related to child academic performance at age 12 years, brain morphology did not mediate this effect. However, it is important to note that, given the limited sample size, our mediation analysis was likely underpowered to detect moderate mediation effects (59). Studies with larger sample sizes are needed to investigate whether global brain morphological differences underlie the associations between prenatal PUFAs exposure and cognitive development in childhood.

ω-3 PUFAs involve in myelin and have been related to improved white matter integrity characterized by increased FA in adults (60, 61). However, we observed no associations between maternal gestational PUFA status and child white matter microstructure. Human myelination is largely a postnatal process that continues into early adulthood, and postpartum factors such as optimal early life nutrition may contribute to alleviating or eliminating the adverse impact of exposure to low ω-3 PUFAs during pregnancy on myelination (62, 63). In addition, compared to fatty acids with 24 carbons such as nervonic acid (C24:1ω-9) and lignoceric acid (C24:0), ω-3 and ω-6 PUFAs are possibly less sensitive markers for myelination (64).

The strengths of this study include the prospective design, the relatively large sample size, and the application of inverse probability weighting to account for selection bias due to loss to follow-up. Several limitations also need to be acknowledged. First, maternal PUFAs were only measured in mid-pregnancy. We could thus not capture PUFAs fluctuations in the periconceptional period that can be critical for embryonic neurodevelopment. However, mid-gestational PUFAs can serve as a proxy of the average level because the absolute concentrations of most PUFAs are stable across pregnancy (65). Further studies incorporating measures of PUFA status of the child could elucidate the cumulative and/or interaction effects of PUFAs levels across fetal life and childhood on long-term brain development. Second, we only examined child brain morphological measures at age 10 years. Future studies using other neuroimaging modalities such as functional MRI with repeated assessments are warranted to better understand the role of prenatal PUFAs exposure in the brain developmental trajectory. Third, due to the population-based nature, we were not able to assess gestational PUFAs exposure in relation to brain development in disadvantaged children, such as those with SGA and/or low birth weight with sufficient power. Finally, since this is an observational study, although we adjusted for several variables including maternal diet and lifestyle, which

were likely related to both maternal PUFA status and child brain development, genetic or residual environmental confounding factors impede drawing causal conclusions.

To conclude, a low maternal ω-3 PUFAs level during pregnancy was associated with reduced total gray and white matter volumes in the offspring in late childhood. Further studies are warranted to ascertain the causal relationship to justify ω-3 PUFAs supplementation in pregnant women with low ω-3 PUFAs levels for child brain development in the long term.

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SUPPLEMENTAL METHOD. FATTY ACIDS ASSAY

A total of 100 µl of plasma, 100 µl of internal standard B, and 0.6 ml methanol (pre-cooled to 5°C) were mixed in glass tubes. The precipitated proteins were separated from the methanolic phase by centrifugation at 900 g for 5 min, and 25 µl sodium methoxide solution was added to the methanolic supernatant containing mainly polar lipids. Following selective synthesis of methyl esters from glycerophospholipid fatty acids at room temperature for 3 min, fatty acid methyl esters (FAMEs) were extracted by adding 300 µl hexane and shaking the tubes for 30 s. The upper hexane phase, which contains the extracted glycerophospholipid FAMEs, was transferred into a 2 ml vial. The extraction was repeated and combined extracts were dried under nitrogen flow at room temperature. The dry residue was taken up in 50 µl hexane (containing 2 g/l BHT) for gas chromatography analysis.

To evaluate lipid compositions in the methanolic supernatant after plasma protein precipitation and to compare the recovery of plasma phospholipids (PhLs) in the methanolic supernatant with the recovery of PhLs in Folch extracts, the supernatant was deposited on a Thin-layer chromatography (TLC) plate. Lipid classes were separated by TLC and fatty acids bound in the different lipids were converted to FAMEs by acid catalyzed transesterification.

To optimize base catalyzed transesterification and FAME extraction, a model sample containing 100 µl water (representing plasma), 100 µl internal standard B, and 100 µl octadecane standard (not participating in the reactions) was applied. The ratio of the peak areas of methyl pentadecanoate to octadecane was used as indicator for transesterification as well as for extraction efficiency.

SUPPLEMENTAL MATERIALS

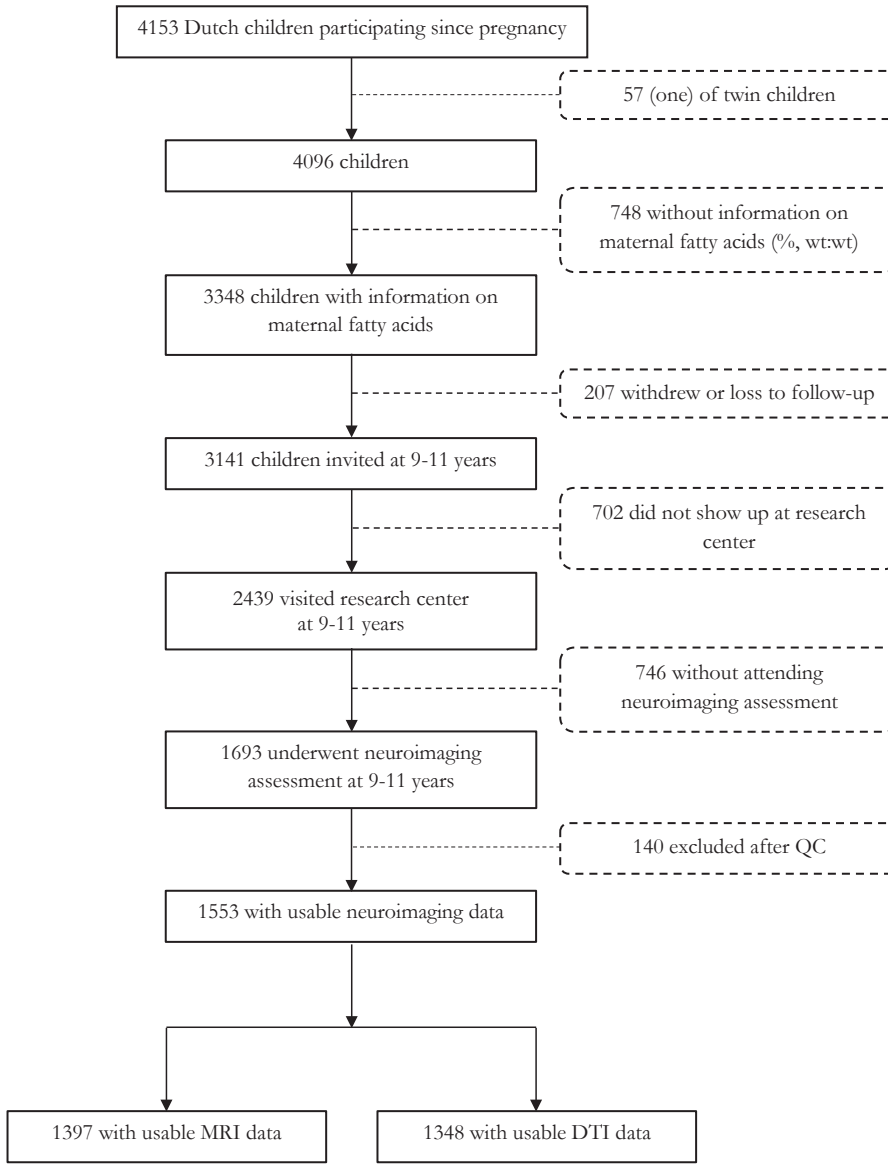

Figure S1. Flow diagram of study population

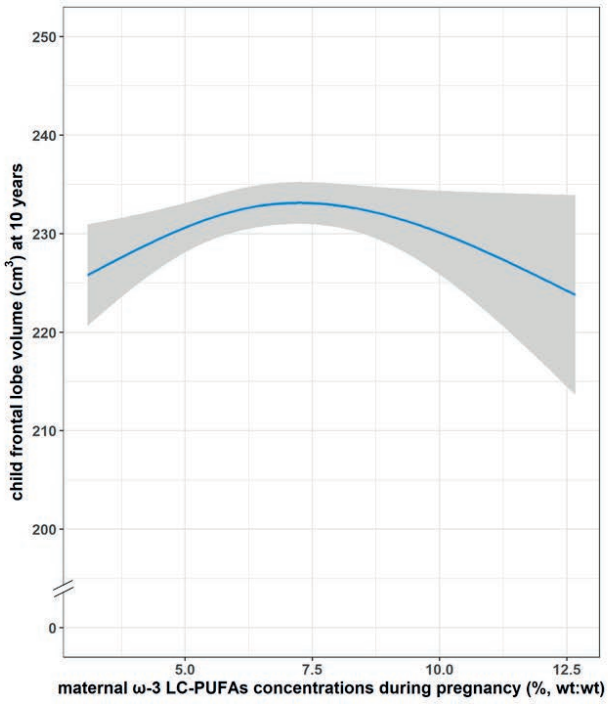

Figure S2. Maternal ω-3 LC-PUFAs concentrations during pregnancy in relation to child frontal lobe volume at age 10 years. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 and 22 carbon atoms in this study. Linear regression was used to examine maternal ω-3 LC-PUFAs concentrations in relation to child frontal lobe volume. The curve and shadow represent mean values and 95% CIs of child frontal lobe volume predicted by maternal plasma ω-3 LC-PUFA concentrations (%, wt/wt) during pregnancy. Non-linear relations were depicted with natural cubic splines, with one knot placed at the median (6.4). Model was adjusted for child sex and age at neuroimaging assessment, maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, family income, and maternal plasma total fatty acids concentration (mg/L), and was weighted by inverse probability to count for attrition. N=1397.

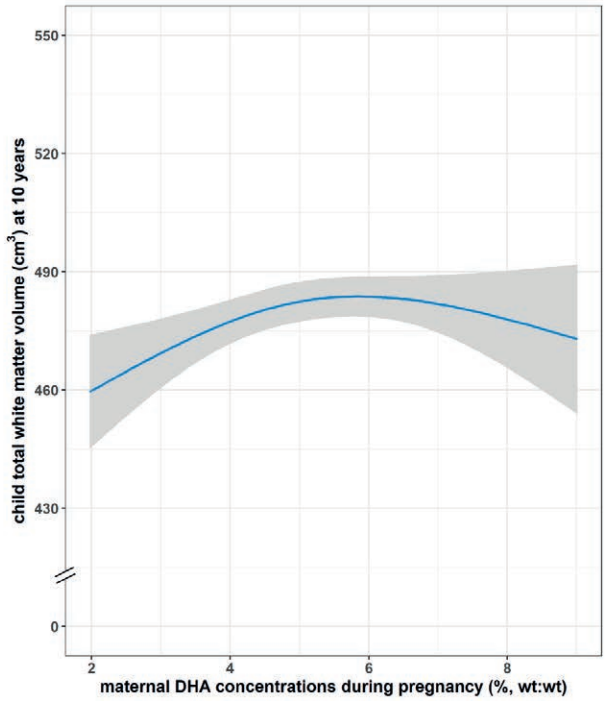

Figure S3. Maternal DHA concentrations during pregnancy and child total white matter volume at age 10 years. DHA - docosahexaenoic acid. Linear regression was used to examine maternal DHA concentrations in relation to child white matter volume. The curve and shadow represent mean values and 95% CIs of child total white matter volume predicted by maternal plasma DHA concentrations (%, wt/wt) during pregnancy. Non-linear relations were depicted with natural cubic splines, with one knot placed at the median (4.9). Model was adjusted for child sex and age at neuroimaging assessment, maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, family income, and maternal plasma total fatty acids concentration (mg/L), and was weighted by inverse probability to count for attrition. N=1397.

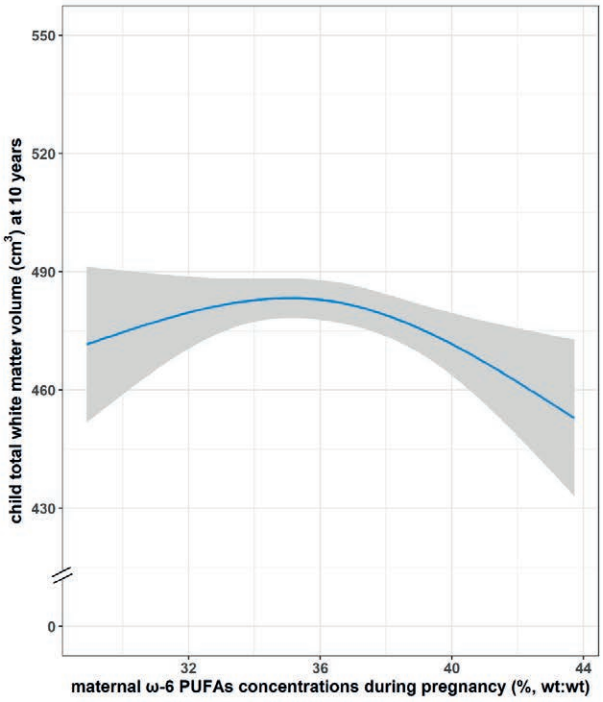

Figure S4. Maternal ω-6 PUFAs concentrations and child total white matter volume at age 10 years. PUFAs - polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 18 to 22 carbon atoms in this study. Linear regression was used to examine maternal ω-6 PUFAs concentrations in relation to child white matter volume. The curve and shadow represent mean values and 95% CIs of child total white matter volume predicted by maternal plasma ω-6 PUFA concentrations (%, wt/wt) during pregnancy. Non-linear relations were depicted with natural cubic splines, with one knot placed at the median (36.3). Model was adjusted for child sex and age at neuroimaging assessment, maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, family income, and maternal plasma total fatty acids concentration (mg/L), and was weighted by inverse probability to count for attrition. N=1397.

Table S1. Full profile of maternal plasma fatty acids

Fatty acids[1]	Relative concentration [mean (SD), %, wt:wt]	Absolute concentration [mean (SD), mg/L]
Saturated fatty acids		
Mytistic acid (C14:0)	0.68 (0.18)	11.2 (3.4)
Palmitic acid (C16:0)	30.7 (1.1)	507.3 (71.5)
Margaric acid (C17:0)	0.35 (0.05)	5.8 (0.98)
Stearic acid (C18:0)	11.3 (0.74)	186.5 (27.3)
Monounsaturated fatty acids (cis)		
Pentadecanoic acid (C15:1ω-5)	0.07 (0.02)	1.1 (0.3)
Palmitoleic acid (C16:1ω-7)	0.79 (0.28)	13.2 (5.4)
Vaccenic acid (C18:1ω-7)	1.5 (0.2)	25.1 (4.2)
Oleic acid (C18:1ω-9)	10.8 (1.1)	177.8 (30.0)
Eicosenoic acid (C20:1ω-9)	0.2 (0.04)	3.3 (0.65)
Polyunsaturated fatty acids		
α-Linolenic acid (C18:3ω-3)	0.34 (0.1)	5.6 (1.8)
Eicosatrienoic acid (C20:3ω-3)	0.1 (0.02)	1.6 (0.39)
Eicosapentaenoic acid (C20:5ω-3)	0.62 (0.33)	10.2 (5.5)
Docosapentaenoic acid (C22:5ω-3)	0.8 (0.22)	13.2 (4.0)
Docosahexaenoic acid (C22:6ω-3)	5 (1.1)	82.3 (19.6)
Linoleic acid (C18:2ω-6)	21.5 (2.5)	354.9 (58.9)
γ-linolenic acid (C18:3ω-6)	0.09 (0.04)	1.5 (0.66)
Eicosadienoic acid (C20:2ω-6)	0.51 (0.09)	8.4 (1.7)
Dihomo-γ-linolenic acid (C20:3ω-6)	3.8 (0.68)	63.8 (15.4)
Arachidonic acid (C20:4ω-6)	9.5 (1.4)	156.2 (30.8)
Adrenic acid (C22:4ω-6)	0.41 (0.1)	6.9 (2.0)
Osbond acid (C22:5ω-6)	0.47 (0.16)	7.8 (2.9)
Mead acid (C20:3ω-9)	0.15 (0.06)	2.5 (1.1)
Trans fatty acids		
C16:1t	0.06 (0.02)	0.93 (0.26)
C18:1t	0.23 (0.1)	3.8 (1.7)
C18:2tt	0.07 (0.02)	1.2 (0.39)
Sum	100	1651.9 (216.5)

[1.] Fatty acids with a chain length between 14 and 22 carbon atoms were assessed.

Table S2. Non-response analysis[1]

Characteristics	Respondents (n=1553)	Non-respondents (n=1795)	p-value
Maternal			
Age at enrollment, year	31.9 (4.0)	30.9 (4.7)	<0.001
Educational level			
Primary or below	25 (1.6)	96 (5.4)	
Secondary	527 (33.9)	742 (41.9)	<0.001
Higher	1001 (64.5)	933 (52.7)	
Marital status (with partner)	1462 (94.1)	1600 (91.3)	0.002
Pre-pregnancy BMI	23.1 (3.9)	23.4 (4.1)	0.07
Psychopathology during pregnancy (GSI)[2]	0.2 (0.2)	0.2 (0.3)	0.002
Smoking during pregnancy			
Never	1192 (76.8)	1164 (69.7)	
Smoked until pregnancy was known	153 (9.9)	146 (8.7)	<0.001
Continued smoking throughout pregnancy	208 (13.4)	361 (21.6)	
Alcohol drinking during pregnancy			
Never	451 (29.0)	592 (34.5)	
Drank until pregnancy was known	244 (15.7)	282 (16.4)	0.002
Continued drinking occasionally	660 (42.5)	665 (38.8)	
Continued drinking frequently[3]	198 (12.7)	176 (10.3)	
Family income, €/month			
< 1200	63 (4.1)	135 (8.7)	
1200-2000	206 (13.3)	227 (14.6)	<0.001
> 2000	1284 (82.7)	1190 (76.7)	
Vitamin supplementation use (yes)	620 (39.9)	534 (33.6)	<0.001
Total energy intake, kcal	2154.9 (483.7)	2141.5 (528.1)	0.47
Diet quality, score	8.1 (1.5)	7.8 (1.6)	<0.001
Gestational age at fatty acid assessment, week	20.6 (1.1)	20.7 (1.2)	0.004
Plasma total fatty acid concentration, mg/L	1651.9 (216.5)	1620.9 (214.2)	<0.001
Plasma ω-3 LC-PUFAs[4] concentration, %, wt:wt[5]	6.5 (1.4)	6.2 (1.4)	<0.001
Plasma ω-6 LC-PUFAs[4] concentration, %, wt:wt[5]	14.7 (1.6)	14.6 (1.7)	0.08
Child			
Sex (male), n (%)	771 (49.6)	923 (51.4)	0.32
Age at neuroimaging assessment, year, n (%)	10.1 (0.6)	10.3 (0.9)	0.17

1. Continuous variables are presented as mean (SD) and compared using t-test or Wilcoxon test; categorical variables were presented as number (%) and compared using Chi-square test. Statistics of the first imputed dataset were reported for respondents.
2. GSI - global severity index.
3. Defined as '1 or more glasses of alcohol per week in at least two trimesters'.
4. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 to 22 carbon atoms in this study.
5. Weight percentage of all glycerophospholipid fatty acids.

Table S3. Maternal LC-PUFAs concentrations during pregnancy in relation to child brain volumes at age 10 years, stratified by sex[1]

Sex	LC-PUFAs concentrations[2]	Total gray matter volume (cm^3)			Total white matter volume (cm^3)		
		β	95% CI	p-value	β	95% CI	p-value
Male (n=699)	*linear model*						
	ω-3	2.0	-1.4, 5.4	0.26	2.5	-0.47, 5.5	0.10
	ω-6	0.10	-3.0, 3.2	0.95	1.3	-1.1, 3.7	0.30
	non-linear model						
	ω-3	22.8	-1.8, 47.4	0.07	19.2	-1.1, 39.5	0.06
	(ω-3)^2	-1.5	-3.3, 0.20	0.08	-1.2	-2.7, 0.22	0.10
	ω-6	-13.6	-47.8, 20.7	0.44	4.3	-26.4, 35.1	0.78
	(ω-6)^2	0.46	-0.70, 1.6	0.44	-0.10	-1.2, 0.95	0.85
Female (n=698)	*linear model*						
	ω-3	1.7	-1.1, 4.5	0.23	1.5	-0.75, 3.7	0.19
	ω-6	0.78	-1.6, 3.2	0.52	1.2	-0.72, 3.1	0.22
	non-linear model						
	ω-3	13.5	-4.5, 31.6	0.14	13.4	-0.40, 27.2	0.06
	(ω-3)^2	-0.85	-2.1, 0.38	0.18	-0.86	-1.8, 0.08	0.07
	ω-6	20.0	-11.0, 51.1	0.21	27.1	0.41, 53.8	0.05
	(ω-6)^2	-0.66	-1.7, 0.38	0.22	-0.88	-1.8, 0.02	0.06

1. LC-PUFAs - long-chain polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 20 to 22 carbon atoms in this study.
2. Linear regression was used to examine maternal LC-PUFAs concentrations in relation to child brain volumes. β's represent differences in child brain volumes per 1 %, wt:wt increase in ω-3 or ω-6 LC-PUFAs concentrations. All models were adjusted for child age at neuroimaging, maternal age at enrollment, educational level, smoking and alcohol use in pregnancy, diet quality in pregnancy, family income, and maternal plasma total fatty acids concentration (mg/L), and were weighted by inverse probability to count for attrition. N=1397.

Table S4. Maternal PUFAs concentrations during pregnancy in relation to child brain volumes at age 10 years[1,2]

PUFAs concentrations	Total gray matter volume (cm^3)			Total white matter volume (cm^3)		
	β	95% CI	p-value	β	95% CI	p-value
linear model						
ω-3						
Model 1	4.3	1.8, 6.9	<0.001	3.0	0.97, 5.1	0.004
Model 2	1.9	-0.30, 4.1	0.09	2.1	0.23, 3.9	0.03
Model 3	1.9[3]	-0.36, 4.1	0.10	2.0[3]	0.12, 3.9	0.04
ω-6						
Model 1	-2.1	-3.8, -0.52	0.01	-1.5	-2.7, -0.28	0.02
Model 2	-1.3	-2.8, 0.27	0.11	-1.2	-2.4, 0.04	0.06
Model 3	-1.3	-2.8, 0.25	0.10	-1.2[3]	-2.5, 0.005	0.05
non-linear model (results from Model 3 only)						
ω-3	17.6	2.0, 33.2	0.03	16.4	3.5, 29.4	0.01
(ω-3)^2	-1.1	-2.1, -0.07	0.04	-1.0	-1.9, -0.15	0.02
ω-6	25.8	-11.8, 63.5	0.18	31.3	2.6, 60.1	0.03
(ω-6)^2	-0.37	-0.90, 0.15	0.16	-0.45	-0.85, -0.05	0.03

1. PUFAs - polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 18 to 22 carbon atoms in this study.
2. Linear regression was used to examine maternal PUFAs concentrations in relation to child brain volumes. β's represent volumetric differences per 1 %, wt:wt increase in ω-3 or ω-6 PUFAs concentrations. Model 1 was adjusted for child sex and age at neuroimaging. Model 2 was additionally adjusted for maternal age at enrollment, educational level, smoking and alcohol use in pregnancy, diet quality in pregnancy, and family income. Model 3 was additionally adjusted for maternal plasma total fatty acids concentration (mg/L). All models were weighted by inverse probability to count for attrition. N=1397.
3. Using likelihood-ratio test, non-linear model shows better model fit than the linear model.

Table S5. Maternal PUFAs concentrations during pregnancy and child white matter microstructure at age 10 years[1,2]

PUFAs concentrations	Global Fractional Anisotrophy			Global Mean Diffusivity		
	β	95% CI	p-value	β	95% CI	p-value
ω-3						
Model 1	0.06	-0.02, 0.14	0.17	-0.0001	-0.01, 0.01	0.99
Model 2	0.01	-0.07, 0.09	0.80	-0.001	-0.01, 0.01	0.78
Model 3	0.01	-0.06, 0.09	0.77	-0.002	-0.01, 0.01	0.73
ω-6						
Model 1	-0.03	-0.08, 0.02	0.28	-0.003	-0.01, 0.004	0.39
Model 2	-0.01	-0.07, 0.04	0.70	-0.003	-0.01, 0.01	0.43
Model 3	-0.01	-0.07, 0.04	0.72	-0.004	-0.01, 0.01	0.42

1. PUFAs - polyunsaturated fatty acids, referring to polyunsaturated fatty acids with 18 to 22 carbon atoms in this study.
2. Linear regression was used to examine maternal PUFAs concentrations in relation to child white matter microstructure. β's represent differences in the indicators of white matter microstructure per 1 %, wt:wt increase in ω-3 or ω-6 PUFAs concentrations. Model 1 was adjusted for child sex and age at neuroimaging assessment. Model 2 was additionally adjusted for maternal age at enrollment, educational level, smoking and alcohol use during pregnancy, diet quality, and family income. Model 3 was additionally adjusted for maternal plasma total fatty acids concentration (mg/L). All models were weighted by inverse probability to count for attrition. N=1348.

3.4

Prenatal exposure to trans fatty acids in relation to brain development in fetal life and childhood: calendar time as an instrumental variable

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ABSTRACT

Background: Dietary trans fatty acids (TFAs) are primarily industrially produced and have been associated with poor cardio-metabolic health. Little is known about intrauterine exposure to TFAs in relation to brain development. We aimed to investigate the effect of maternal TFAs concentration during pregnancy on offspring brain development in fetal life and childhood.

Methods: In total 3295 mother-child dyads from a prospective population-based study in Rotterdam, the Netherlands were included. Maternal plasma TFAs concentration was assessed using gas chromatography in mid-gestation. Offspring head circumference (HC) in fetal life was repeatedly measured using ultrasonography; brain morphology in childhood was assessed using magnetic resonance imaging. We performed regression and instrumental variable (IV) analyses to examine the association between maternal TFAs concentration and offspring brain development. Our proposed IV leveraged a local policy change at recruitment since 2002-2003.

Findings: After adjusting for covariates, maternal TFAs concentration was inversely related to fetal HC in the third trimester (B=-0.37, 95% CI -0.62, -0.11, cm) and to fetal HC growth from the second to the third trimester (B=-0.04, 95% CI -0.07, -0.02, cm/week). Consistent results were obtained with the IV analyses. Maternal TFAs concentration was not predictive of offspring HC in the second trimester, nor global brain volume at age 10 years.

Interpretation: Higher maternal TFAs concentration during pregnancy was associated with suboptimal offspring brain development such as lower HC and slower HC growth in fetal life. These findings were consistent across regression and IV analyses, strengthening a causal interpretation. Our findings are of important public health relevance as TFAs levels in food remain high in many countries.

INTRODUCTION

Trans fatty acids (TFAs) are unsaturated fatty acids with at least one carbon-carbon double bond in the *trans* configuration (i.e., with hydrogens on opposing sides). The major dietary TFAs are industrially-produced with the goal to bring plasticity as well as emulsion stability to shortenings and enhance the palatability of baked goods and sweets.[1] Consumption of TFAs can be harmful for human's health. The positive associations between TFAs intake and adverse cardiometabolic outcomes such as coronary heart disease and diabetes are well-documented.[2,3] Further evidence has linked TFAs to neuropsychiatric and neurodegenerative outcomes. A Japanese prospective study showed that a higher serum level of elaidic acid (t18:1), a major TFA, was associated with an elevated risk of developing all-cause dementia and Alzheimer disease in community residents aged 60 and older.[4] In a cross-sectional study in South Korea, researchers found that female adolescents with a higher TFAs intake had more attention-deficit/hyperactivity disorder (ADHD) symptoms.[5]

The fetus experiences rapid growth critical for the structure and functioning of many organs and is considered particularly vulnerable to environmental adversities.[6] As potential nutritional adversities, TFAs accumulate in the human placenta during pregnancy, and are transported to the fetus in amounts that depend on maternal dietary intake.[7] In a previous study in the present sample, we showed that a higher maternal plasma t18:1 concentration in mid-gestation was related to maternal pregnancy complications, such as preeclampsia, and to an elevated risk of lower birth weight.[8] In another cohort, higher umbilical TFAs levels were associated with a less optimal neurologic status as assessed by a neurologic examination in children aged 18 months,[9] suggesting that prenatal exposure to TFAs may impact neurodevelopment. Head circumference (HC) can serve as a proxy for fetal brain volume, as brain growth in early life corresponds to the cranial capacity that is largely determined by skull development.[10,11] In the Maastricht Essential Fatty Acid Birth (MEFAB) cohort that included women in 2008-2009, the researchers found no association between maternal or umbilical plasma t18:1 concentration and HC an birth.[12,13] To the best of our knowledge, the association between prenatal TFAs exposure and brain growth has not been examined using repeated measures.

Most countries rely on food producers to voluntarily reduce the content of industrial TFAs in food, and only a few European Union (EU) countries such as Denmark and Iceland have introduced legislative limits.[14] In the Netherlands, large food producers decided to eliminate TFAs from retail products since early 1990s, and by 1996 most retail margarines contained only trace amounts of TFAs. However, fast foods and baked goods remained as the two major sources of TFAs.[15] Starting in 2003, the Product Board for Margarine, Fats and Oils (Dutch abbreviation: MVO) initiated the Respon-

sible Fatty Acid Composition Taskforce to further reduce TFA content in Dutch food, contributing to declines of TFAs concentrations in the population.[16]

In this population-based study, we aimed to examine the effect of prenatal exposure to TFAs on brain growth in fetal life and childhood. We hypothesized that exposure to higher TFAs levels was associated with suboptimal brain development characterized by a smaller HC and lower HC growth rate in fetal life. As a clear decline in maternal gestational plasma TFAs concentrations over the period of recruitment was evident in the current cohort,[8] we introduced the calendar time of maternal TFAs assessment as a potential instrument variable (IV) for the relation between maternal TFA status during pregnancy and child brain development.

METHODS

Setting and participants

This study was embedded in the prospective population-based Generation R cohort. Pregnant women with an expected delivery date between April 2002 and January 2006 living in Rotterdam were eligible for enrollment.[17] The Generation R Study was approved by the Medical Ethics Committee of the Erasmus Medical Center, and written informed consent was obtained from all participants.

A total of 8633 live singletons were born to women recruited in pregnancy. To rule out potential interactions between diet and ethnic variation in anthropometric traits,[18] we excluded 4637 children with mothers of a non-Dutch national origin. Of the remaining 3996 children, 692 were excluded due to missing data on maternal plasma TFAs concentration during pregnancy. After further exclusion of those without ultrasound data on HC in the second or third trimesters, 3295 children constituted the study population. About half of these children (1666) also underwent a brain magnetic resonance imaging (MRI) session in a follow-up at age 9-11 years. Of these, 1374 had usable structural brain morphological data after quality control (see Figure S1 in the supplement for the flow-chart).

Maternal TFA during pregnancy

As previously described,[19] maternal plasma fatty acids concentrations were assessed in mid-gestation (mean 20.6 weeks, SD=1.1) using gas chromatography. The concentrations of individual fatty acids are expressed as weight percentage (%, wt:wt) of all glycerophospholipid fatty acids detected with a chain length between 14 and 22 carbon atoms. For the current study, total TFAs concentration was calculated by summing the concentrations of t16:1, t18:1, and tt18:2 isomers. In our sample, the interquartile

range (IQR) of total TFAs concentration was 0.29%~0.43%, and the t18:1 isomer (IQR 0.16%~0.29%) accounted for the majority (on average 63%) of total TFAs.

Brain development in fetal life and childhood

Fetal HC was measured with ultrasound during each trimester of pregnancy by technicians at the Generation R research center.[17] First trimester ultrasounds were used for pregnancy dating.[20] In our study population, fetal HC data were available in 3211 children in the second trimester (mean=20.6 weeks, SD=1.1), and 3254 children in the third trimester (mean=30.4 weeks, SD=1.0). The intra- and inter-observer correlation coefficients for fetal HC were 0.995 and 0.988, suggesting good reliability.[21] In addition, we calculated fetal HC growth rate (cm/week) from the second trimester to third trimester as the difference between HC divided by the difference in gestational age in 3170 children with HC data at both assessments.

Brain morphology in childhood was assessed using MRI at age 9-11 years. All images were acquired on a 3-Tesla GE Discovery MR750w MRI System (General Electric, Milwaukee, WI, USA) scanner using an 8-channel head coil. High-resolution T_1-weighted sequences were obtained using a 3D coronal inversion recovery fast spoiled gradient recalled (IR-FSPGR, BRAVO) sequence, and images were rated for quality control both during and after the MRI acquisition.[22] Volumetric segmentation and cortical reconstruction were performed with FreeSurfer v.6.0.0 (http://surfer.nmr.mgh.harvard.edu/), and the standard reconstruction stream was applied. The quality of FreeSurfer output was visually inspected, and data with insufficient quality were eliminated. For the current study only intracranial volume and total brain volume were used to quantify global brain size.

Covariates

We included the following maternal and child characteristics as covariates based on prior literature:[8,23] child sex and age at brain assessment (i.e., ultrasound or MRI), maternal age at enrollment, educational level (categorized into 'primary or below', 'secondary', and 'higher'), diet quality, smoking ('never smoked', 'smoked until pregnancy was known', or 'continued smoking throughout pregnancy') and alcohol use ('never drank', 'drank until pregnancy was known', 'continued drinking occasionally', or 'continued drinking frequently' defined as '1 or more glasses of alcohol per week in at least two trimesters'), and family income ('<1200€', '1200-2000€', or '>2000€' per month). Maternal diet quality was assessed in early pregnancy using a semi-quantitative 293-item food frequency questionnaire and quantified by an overall score ranging from 0 to 15, reflecting adherence to Dutch dietary guidelines.[24]

Additional variables

Information on calendar time of maternal TFA assessment during pregnancy was available in 3168 (96.1%) of the children in our sample for the IV analysis. Child nonverbal IQ assessed at age 5-7 years was used in a supplementary analysis to examine the relation of HC in utero with cognitive abilities in childhood.[25]

Statistical analysis

For descriptive purposes, continuous variables were presented as mean (SD) and categorical variables were presented as number (%). In a non-response analysis, we compared the characteristics between children with and without MRI data at age 9-11 years, for which analysis of variance (ANOVA) or the Kruskal-Wallis test was used for continuous variables, and the chi-square test was used for categorical variables.

First, we used multiple linear regression to investigate the association of maternal TFAs concentration with child brain measures at each assessment, including HC in the second and third trimesters assessed with ultrasound, and intracranial volume and total brain volume at age 9-11 years assessed with MRI. We also related maternal TFAs concentration to fetal HC growth rate from the second to the third trimester. These regression analyses were performed using multi-stage models with different levels of confounder adjustment. In the initial model, only child sex and (gestational) age at brain assessment were adjusted for. Maternal age at enrollment, educational level, smoking and alcohol use, diet quality, and family income were additionally adjusted for in a second model. In a third model, we additionally corrected for the concentrations of maternal essential fatty acids (EFAs, i.e., α-linoleic acid and linoleic acid) and long-chain polyunsaturated fatty acids (LC-PUFAs), to explore whether reduced EFAs or LC-PUFAs levels (as a potential consequence of high TFAs intake) explained any associations.[26] In addition, each model with MRI measures as the outcome was weighted by inverse probability weights to account for attrition.[27] In a sensitivity analysis, we excluded 244 children born to mothers with gestational complications (including preeclampsia, diabetes, and/or pregnancy-induced hypertension) to examine whether any brain differences were independent of maternal complications. In supplementary analyses we investigated HC and HC growth rate in utero in relation to nonverbal IQ in childhood.

Next, for brain outcomes that were predicted by maternal TFAs concentration in the above analyses, we further performed an IV analysis using two-stage least squares (TSLS) estimation.[28] Calendar time of maternal TFA assessment was proposed as the IV. We first assessed the basic assumptions of IV analysis (see Supplemental Methods for details). For the 'relevance' assumption, we examined the association between calendar time of maternal TFA assessment and maternal TFAs concentration, both with and without adjusting for covariates (i.e., endogenous variables, including child sex,

maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality, and family income). In addition, we performed an F-test on the instrument in the first stage of the TSLS regression. Since the 'exclusion restriction' and 'exchangeability' assumptions cannot be verified, we followed an empirical approach to explore covariates that might falsify these assumptions. First, we regressed all covariates on child brain measures in one multivariate linear model, and retained those that were at least marginally associated with child brain measures (i.e., $p<0.10$). Second, we performed ANOVA or chi-square tests and excluded covariates that did not vary by calendar time of maternal TFA assessment.[29] Finally, the mean ± se of each remaining covariate over calendar time of maternal TFA assessment was plotted to inspect time trends. These analyses were performed to minimize the risk that a covariate with a similar time trend as maternal TFAs concentration underlies the relation between calendar time of maternal TFA assessment and child brain measures. In the primary analysis, we ran the IV analysis without including any covariates. The sandwich estimator was chosen because it is robust to heteroscedastic errors.[30] Covariates with a potential monotonic time trend were addressed in a sensitivity analysis. Since maternal TFAs concentration was the dependent variable in the first-stage model of the IV analysis, we log-transformed the raw values to obtain a normal distribution.

Missing data on covariates were accounted for by multiple imputation. We generated 10 imputed datasets with 10 iterations, and only report pooled results. Statistical significance was set as $\alpha<0.05$ (two-sided), and a false discovery rate (FDR) correction was performed in primary analyses.[31] All statistical analyses were performed using R version 3.6.2 (R Foundation for Statistical Computing, Vienna, Austria), including the 'ivpack' package for IV analysis.

RESULTS

Descriptive information

Table 1 shows the characteristics of the participating mother-child dyads. Mothers were enrolled at an average age of 31 years, and the majority had completed higher education. The non-response analysis showed differences in maternal demographic characteristics between children with and without MRI data at age 10 years. Compared to children with MRI data, those without MRI data were exposed to higher maternal TFAs concentration during pregnancy, and had larger HC in the second trimester but smaller HC in the third trimester (see Table S1).

Table 1. Descriptive information

	Statistics
Maternal characteristics	
Age at enrollment, mean (SD), years	31.4 (4.4)
Educational level, n (%)	
Primary or below	123 (3.7)
Secondary	1259 (38.2)
Higher	1913 (58.1)
Smoking during pregnancy, n (%)	
Never smoked	2408 (73.1)
Smoked until pregnancy was known	310 (9.4)
Continued smoking	577 (17.5)
Alcohol use during pregnancy, n (%)	
Never drank	1061 (32.2)
Drank until pregnancy was known	531 (16.1)
Continued drinking occasionally	1331 (40.4)
Continued drinking frequently	372 (11.3)
Diet quality in early pregnancy, mean (SD), point	7.9 (1.6)
Family income, n (%), €/month	
<1200	254 (7.7)
1200-2000	451 (13.7)
> 2000	2590 (78.6)
Child characteristics	
Sex (male), n (%)	1665 (50.5)
Age at neuroimaging, mean (SD), years	10.1 (0.6)

Data from the first imputed dataset are reported.

Prenatal exposure to TFA and HC in fetal life

As shown in Table 2, maternal TFAs concentration was not related to fetal HC in the second trimester (B=0.08, 95% CI -0.10, 0.25, cm). However, higher maternal TFAs concentration predicted smaller fetal HC in the third trimester (B=-0.37, 95% CI -0.62, -0.11, cm) even after adjusting for all sociodemographic covariates. Also, higher maternal TFAs levels during pregnancy were related to lower HC growth rate (B=-0.04, 95% CI -0.07, -0.02, cm/week). Both associations remained after additional correction for concentrations of maternal EFAs and LC-PUFAs, and survived the FDR correction. Consistent findings were obtained when maternal TFAs concentration was normalized using logarithm transformation (see Table S2).

In 3051 children without exposure to maternal complications, higher maternal TFAs concentration still predicted smaller HC in the third trimester (B=-0.37, 95% CI -0.63, -0.10, cm) and lower HC growth rate (B=-0.05, 95% CI -0.08, -0.02, cm/week) after adjustment for all covariates.

Table 2. Maternal trans fatty acids concentration during pregnancy in relation to fetal head circumference and head circumference growth

Maternal TFAs concentration	fetal HC at single assessments[1]						fetal HC growth rate across assessments[2] (n=3170)		
	second trimester (n=3254)			third trimester (n=3211)					
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1	0.05	-0.12, 0.22	0.56	-0.53	-0.78, -0.28	<0.001	-0.06	-0.08, -0.03	<0.001
Model 2	0.11	-0.06, 0.29	0.20	-0.37	-0.63, -0.12	0.004	-0.05	-0.08, -0.02	<0.001
Model 3	0.08	-0.10, 0.25	0.40	-0.37	-0.62, -0.11	0.005[3]	-0.04	-0.07, -0.02	0.001[3]

1. B's represent difference in fetal HC (cm) per 1%, wt:wt increase in maternal TFAs concentration. Model 1 was adjusted for child sex and gestational age at HC assessment; Model 2 was additionally adjusted for maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality in early pregnancy, and family income; Model 3 was additionally adjusted for concentrations of essential fatty acids and long-chain polyunsaturated fatty acids.
2. B's represent difference in fetal HC growth rate (cm/week) per 1%, wt:wt increase in maternal TFAs concentration. Model 1 was adjusted for child sex; Model 2 was additionally adjusted for maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality in early pregnancy, and family income; Model 3 was additionally adjusted for concentrations of essential fatty acids and long-chain polyunsaturated fatty acids.
3. These p-values survived a false discovery rate (FDR) correction for multiple comparison.
TFA - trans fatty acid; HC - head circumference.

Prenatal exposure to TFA and MRI measures at age 10 years

There was no evidence for associations of maternal TFAs concentration during pregnancy with intracranial volume or total brain volume in the subgroup of children with MRI assessments at age 10 years (Table 3).

Table 3. Maternal trans fatty acids concentration during pregnancy in relation to child brain volumes at age 10 years

Maternal TFAs concentration[1]	intracranial volume (n=1374)			total brain volume (n=1374)		
	B	95% CI	p-value	B	95% CI	p-value
Model 1	-25.9	-81.6, 29.8	0.36	-30.3	-73.1, 12.6	0.17
Model 2	-3.5	-58.8, 51.9	0.90	-16.4	-59.8, 27.0	0.46
Model 3	1.1	-55.0, 57.2	0.97	-13.1	-57.1, 31.0	0.56

1. B's present volumetric difference (cm^3) per 1%, wt:wt increase in maternal TFAs concentration. Model 1 was adjusted for child sex and age at neuroimaging; Model 2 was additionally adjusted for maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality in early pregnancy, and family income. Model 3 was additionally adjusted for concentrations of essential fatty acids and long-chain polyunsaturated fatty acids. All models were weighted by inverse probability to count for attrition.
TFA - trans fatty acid.

IV analysis

The calendar time of maternal TFA assessment ranged from January 2002 to September 2005. Figure 1 visualizes the change of maternal TFAs concentration (mean ± se) over time. It can be seen that there was an overall monotonically inverse relation between calendar time of TFA assessment and TFAs concentration of the mother, except in the first few months. Figure S2 depicts the logarithm transformed values.

We found that calendar time of maternal TFA assessment was inversely associated with maternal TFAs concentration (B=-0.012, 95% CI -0.012, -0.011 without adjustment for covariates; B=-0.011, 95% CI -0.012, -0.010 after adjustment for covariates), and the heteroskedasticity-robust F-statistic for calendar time of maternal TFA assessment in the covariates-adjusted model was 680.7 ($p<0.001$). These results suggest that

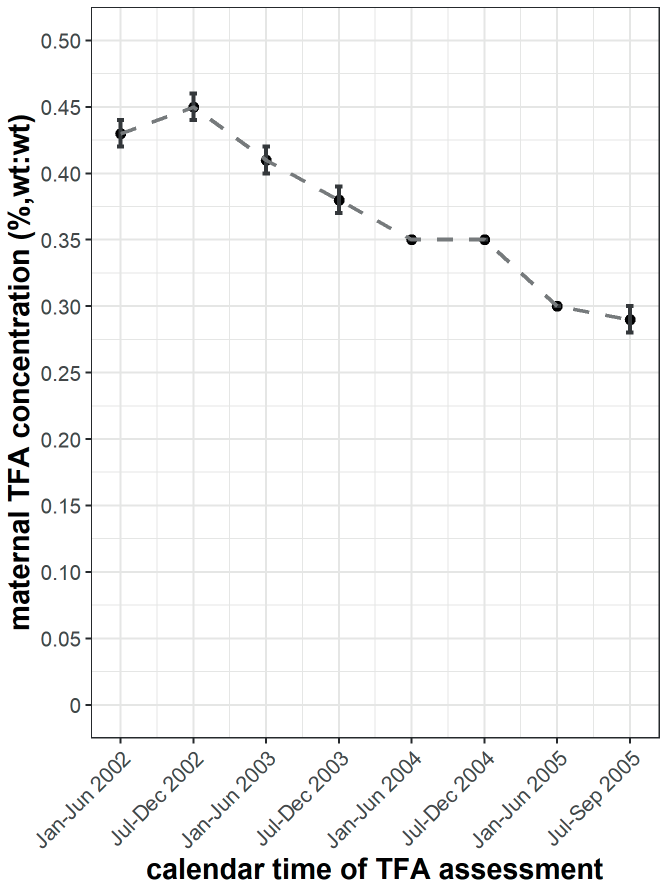

Figure 1. Maternal trans fatty acid concentration (mean ± se) during pregnancy per calendar time of assessment. TFA - trans fatty acid.

calendar time of maternal TFA assessment was not a weak instrument and that the 'relevance' assumption held. Next, of the covariates that might falsify the 'exclusion restriction' and 'exchangeability' assumptions, maternal age at enrollment, education, and alcohol use during pregnancy were dropped because they were not independently related to fetal HC in the third trimester or HC growth rate in the adjusted analysis; child sex was further excluded because it did not differ across calendar time of maternal TFA assessment. Of the remaining covariates, only maternal diet quality and family income showed a trend association with calendar time of maternal TFA assessment (see Figure S3).

Table 4 shows the results of IV analysis. When no covariates were adjusted for, higher maternal TFAs concentration during pregnancy predicted smaller fetal HC in the third trimester and lower HC growth rate from the second to the third trimester. In the sensitivity analysis, when possible confounders (i.e., maternal diet quality and family income) were separately adjusted for in the association, maternal TFAs concentration remained inversely related to fetal HC in the third trimester and HC growth rate; when maternal diet quality and family income were simultaneously adjusted for, higher maternal TFAs concentration still predicted lower HC growth rate, but between-subject differences in HC in the third trimester were slightly attenuated.

Table 4. Instrumental variable analysis

Main analysis						
Covariates included	**fetal HC in the third trimester**			**fetal HC growth rate across assessments**		
	B	95% CI	p-value	B	95% CI	p-value
none	-0.40	-0.74, -0.06	0.02	-0.10	-0.12, -0.07	<0.001
Sensitivity analysis						
Covariates included	**fetal HC in the third trimester**			**fetal HC growth rate across assessments**		
	B	95% CI	p-value	B	95% CI	p-value
family income	-0.35	-0.68, -0.02	0.04	-0.09	-0.12, -0.07	<0.001
maternal diet quality	-0.35	-0.68, -0.02	0.04	-0.10	-0.12, -0.07	<0.001
family income + maternal diet quality	-0.32	-0.65, 0.02	0.06	-0.09	-0.12, -0.07	<0.001

Instrumental variable analysis on maternal trans fatty acids concentration during pregnancy in relation to fetal HC in the third trimester (n=3088) and HC growth across assessments in the second and third trimesters (n=3048) was performed using two-stage least squares estimation. Calendar time of maternal trans fatty acids assessment was used as the instrumental variable. The raw values of maternal trans fatty acids concentration were log-transformed to obtain a normal distribution.
HC - head circumference.

HC in fetal life and non-verbal IQ in childhood

In models fully adjusted for covariates, HC in the third trimester was positively associated with nonverbal IQ at age 6 years (B=1.1, 95% CI 0.61, 1.6, n=2311), while HC growth rate between the second and the third trimesters was not predictive of nonverbal IQ (B=5.1, 95% CI -1.0, 11.2, n=2282).

DISCUSSION

In the current study, maternal TFAs level during pregnancy was inversely associated with fetal HC in the third trimester and with fetal HC growth rate from the second to the third trimester. Analyses utilizing the fact that average TFAs concentration in maternal plasma decreased over the years of enrollment showed consistent results. Prenatal exposure to TFAs was not related to fetal HC in the second trimester or global brain volume of the child at age 10 years.

A few studies related maternal TFAs during pregnancy to birth outcomes such as birth length, birth weight or birth-weight-for-gestational-age,[8,32,33] but the association between exposure to TFAs during pregnancy and brain development has rarely been examined. Dirix et al.[12,13] found neither maternal plasma t18:1 content during pregnancy or at delivery, nor umbilical cord plasma t18:1 concentration was related to neonatal HC. However, these findings cannot easily be compared with those of the present study. In contrast to fetal HC measured by ultrasound that reflects the skull size, postnatal HC measurements also include skin and subcutaneous tissue. Further, the accuracy of postnatal HC is influenced by the measuring tape.[34] To the best of our knowledge, there have been no studies associating prenatal exposure to TFAs with sonographic HC in utero or brain morphology assessed by MRI in childhood.

Our study suggests that the smaller HC in the third trimester of the fetus exposed to higher maternal TFAs levels during pregnancy could be attributed to the slower HC growth from the second to the third trimester. Interestingly, these relations were not accounted for by maternal EFAs and LC-PUFAs concentrations, suggesting that TFAs may influence neurodevelopment independently of the biosynthesis of LC-PUFAs. In addition, the observed associations hardly changed when children born to women with gestational complications were excluded. This suggests that birth outcomes did not underlie the differences in fetal brain development. Several other explanations are possible. First, both randomized clinical trials (RCTs) and observational studies have indicated that TFAs are associated with systematic inflammation, characterized by elevated C-reactive protein (CRP) and pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin 6 (IL-6).[35] Smaller brains in mice offspring exposed to maternal immune activation (as a consequence of inflammation) during

pregnancy have been reported.[36] Second, considerable epigenomic changes take place in the human brain during fetal brain development, and DNA methylation plays a crucial role in epigenetic modification.[37] Robinson et al.[38] found maternal TFAs at preconception and in pregnancy were related to hypomethylation of about 50% of CpG sites in the newborn. Similarly, high TFA doses were related to global hypomethylation in a rodent model.[39] Third, human HC experiences rapid growth from mid-gestation until 35 weeks of gestation, when the growth velocity starts to decrease.[11,40] Although the effect of TFAs on human bone health remains elusive due to a lack of research, decreased bone volume fraction and bone mineral density were observed in rats fed with diet rich in TFAs.[41] Therefore, it is possible that TFAs also affect the generation and metabolism of bone cells, which in turn disturbs normal skull formation and development of the fetus, and leads to a decreased HC growth rate.

Despite the HC differences in the third trimester, we observed no association between exposure to TFAs levels during pregnancy and global brain size in children at age 10 years. Possibly, this can be explained by the catch-up growth of HC after birth, which has been commonly reported in preterm birth and/or small-for-gestational-age term infants fed with breast milk and nutrient-enriched formula.[42,43] Given the inherent brain plasticity, favorable environmental inducers such as breastfeeding and healthy diet in childhood may gradually revert the differences in brain size in the third trimester due to TFAs exposure.[44] In addition, as shown in the non-response analysis, children who were not included in the analysis of MRI measures were exposed to higher maternal TFAs levels and had lower HC and slower HC growth in utero than those included. Moreover, the sample size for the MRI analyses was less than half of that for the HC analyses, limiting the ability to detect subtle differences.

Causal inference is challenging in most nutritional epidemiological studies using an observational design because of unmeasured or residual confounding often related to other lifestyle choices and socioeconomic factors.[45] RCTs could provide rigorous evidence for causality but are frequently not feasible or ethical to test the negative impact of TFAs. In observation studies, IV analysis can help elucidate the effects of diet or nutrients on health.[46] In the current research context, IV analysis allowed for estimating the effect of maternal TFAs on child brain development with a different set of assumptions than the regression estimates. In this study, the 'relevance' assumption of IV analysis was validated, and the other two were explored to the extent possible with no meaningful evidence for their violation. The agreement between the results from regression and IV analyses can strengthen our confidence in the negative effects of exposure to TFAs on brain development of the fetus.[47] Our work serves as a good example of triangulation in epidemiological research, namely, the practice of integrating results from different approaches, where each approach has different sources of bias that are unrelated to each other.[48] Also, it is important to note the discrepancies in the

effect estimates from the two analyses despite the same effect direction. Most likely, this can be explained by the fact that regression and IV analyses may estimate effects in different populations. Specifically, IV analysis requires a fourth assumption termed homogeneity to obtain a point estimate of the average treatment effect. However, this homogeneity assumption remains difficult to assess and is subject to unmeasured confounder of the exposure-outcome relationship that also severs as an effect modifier.[49,50]

Findings from this study are of important public health implications, because industrial TFA content in food remains noticeable, in particular, in South Asia (e.g., India), and Eastern and South-eastern European countries.[51-54] In addition, previous studies have associated lower HC in utero with suboptimal neurodevelopment such as more autistic traits and sleep problems in childhood.[55,56] The current analyses show that children with a smaller HC during gestation also had worse cognitive abilities at age 6 years.

This study has several limitations. First, we did not assess maternal TFA status in early pregnancy when the basis of embryonal neurodevelopment is substantially formed. However, since the diet patterns of women is reasonably stable across pregnancy,[57] maternal TFAs levels in mid-gestation were likely indicative of those in the earlier stages. Second, we summed the concentrations of maternal plasma TFAs, therefore individual TFAs with different carbon atoms and/or of various isomers were not investigated. However, there is a lack of specificity for sources of TFAs with 16 and 18 carbon atoms,[58] and there is no evidence for specific mechanisms of action of the different TFA isomers.[59] Third, only Dutch children were investigated in the current work, thus our findings may have limited generalizability.

To conclude, exposure to higher TFAs levels during pregnancy predicts lower HC in the third trimester and slower HC growth from the second to the third trimester. These findings justify advocating further TFAs reduction across the globe in the pursuit of optimal child development.

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SUPPLEMENTAL METHODS

Three key assumptions for instrumental variable (IV) analysis:
1) the instrument Z is associated with the exposure X ('relevance')
2) Z affects the outcome Y only through X ('exclusion restriction')
3) Z does not share common causes with Y ('exchangeability').

A valid IV analysis can provide estimate free of bias due to unmeasured confounding factors U (see below the illustrative causal diagram).

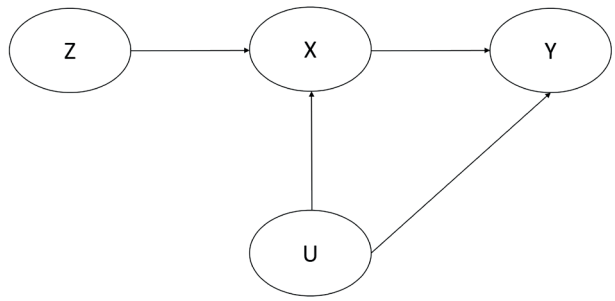

Figure legend:
X-exposure variable (i.e., maternal trans fatty acid concentration in this study);
Y-outcome variable (i.e., fetal head circumference);
Z-instrumental variable (i.e., calendar time of maternal TFA assessment);
U-measured or unmeasured confounding variables.

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SUPPLEMENTAL MATERIALS

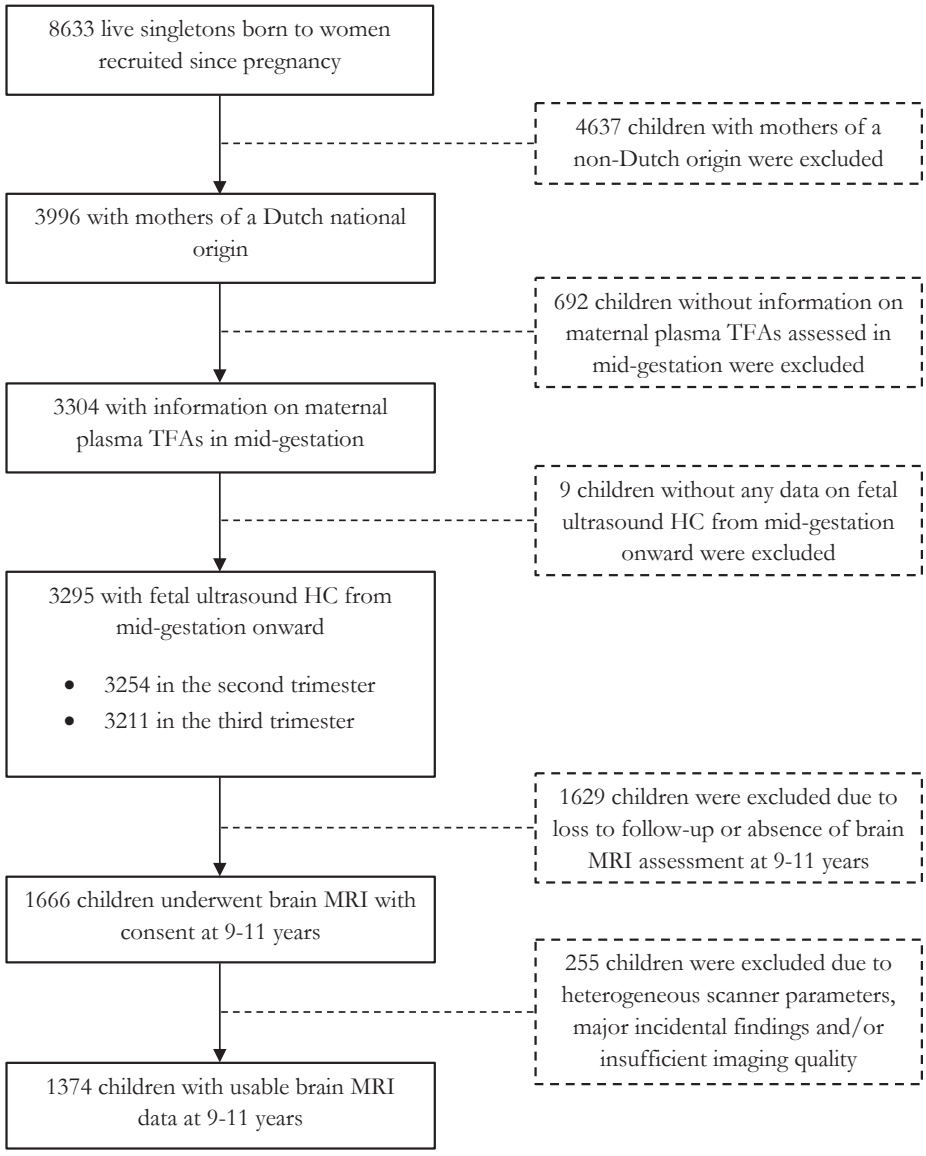

Figure S1. Flow-chart of study population selection

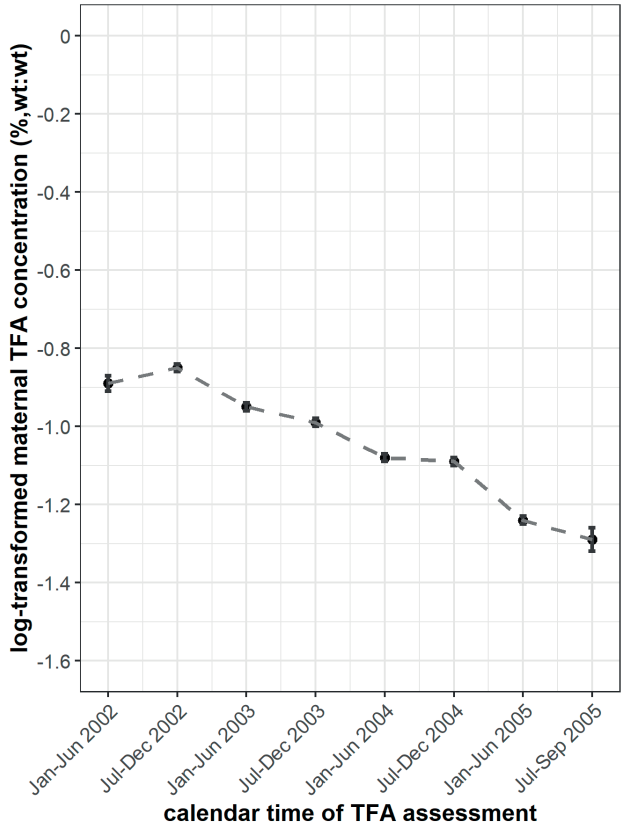

Figure S2. Natural logarithm transformed maternal trans fatty acid concentration(mean ± se) during pregnancy per calendar time of assessment. TFA - trans fatty acid.

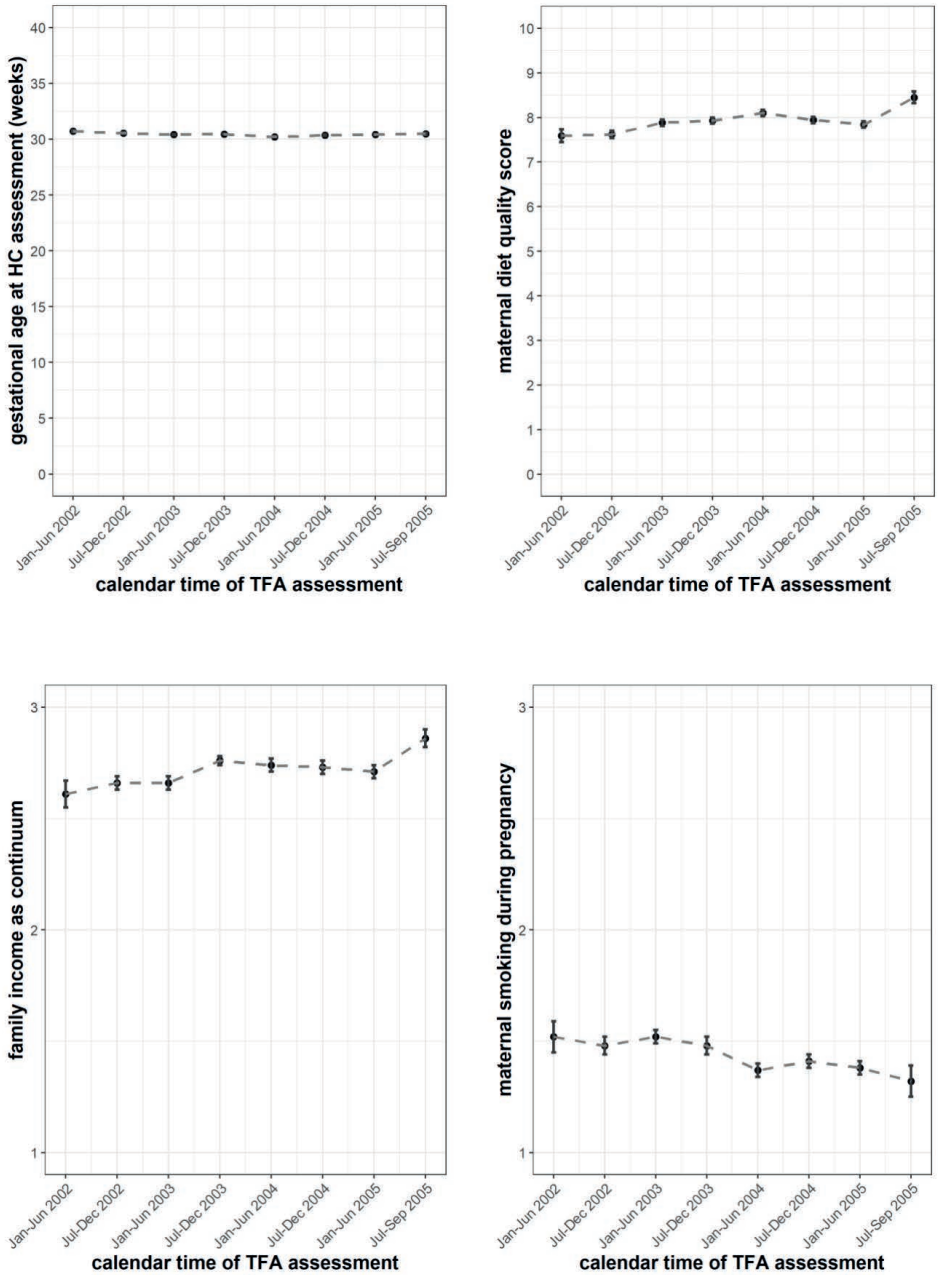

Figure S3. Time trend for covariates (mean ± se). For illustrative purpose, categorical covariates were deemed as ordinary and converted to continuum and depicted as mean ± se. The categories were '1-never smoked', '2-smoked until pregnancy was known', and '3-continued smoking during pregnancy' for maternal smoking; and '1-<1200€', '2-1200-2000€', and '3->2000€' for family income.

Table S1. Non-response analysis comparing children with and without MRI data at age 9-11 years

	Children with MRI (n=1374)	Children without MRI (n=1921)	p-value
Maternal characteristics			
Age at enrollment, mean (SD), years	31.9 (4.0)	31.0 (4.7)	<0.001
Educational level, n (%)			
Primary or below	20 (1.7)	103 (5.4)	
Secondary	460 (33.5)	799 (41.6)	<0.001
Higher	894 (65.1)	1019 (53.0)	
Smoking during pregnancy, n (%)			
Never smoked	1072 (78.0)	1336 (69.5)	
Smoked until pregnancy was known	132 (9.6)	178 (9.3)	<0.001
Continued smoking	170 (12.4)	407 (21.2)	
Alcohol use during pregnancy, n (%)			
Never drank	399 (29.0)	662 (34.5)	
Drank until pregnancy was known	215 (15.6)	316 (16.4)	0.005
Continued drinking occasionally	590 (42.9)	741 (38.6)	
Continued drinking frequently	170 (12.4)	202 (10.5)	
Diet quality in early pregnancy, mean (SD), point	8.1 (1.5)	7.8 (1.6)	<0.001
Family income, n (%), €/month			
<1200	52 (3.8)	202 (10.5)	
1200-2000	165 (12.0)	286 (14.9)	<0.001
> 2000	1157 (84.2)	1433 (74.6)	
Plasma TFA concentrations, mean (SD), %, wt:wt	0.36 (0.12)	0.39 (0.13)	<0.001
Child characteristics			
Sex (male), n (%)	691 (50.3)	974 (50.7)	0.84
HC in the second trimester, mean (SD), cm	17.8 (1.3)	18.0 (1.4)	0.02
HC in the third trimester, mean (SD), cm	28.7 (1.2)	28.5 (1.2)	0.003
HC growth rate, mean (SD), cm/week	1.09 (0.09)	1.08 (0.1)	<0.001

Data from the first imputed dataset are reported. TFA - trans fatty acid; HC - head circumference.
Analysis of variance or the Kruskal-Wallis test was used for comparison of continuous variables; the chi-square test was used for comparison of categorical variables.

Table S2. Log-transformed maternal trans fatty acids concentration during pregnancy in relation to fetal head circumference and head circumference growth

log-transformed maternal TFAs concentration	fetal HC at single assessments[1]						fetal HC growth rate across assessments[2] (n=3170)		
	second trimester (n=3254)			third trimester (n=3211)					
	B	95% CI	p-value	B	95% CI	p-value	B	95% CI	p-value
Model 1	0.01	-0.06, 0.08	0.73	-0.20	-0.30, -0.10	<0.001	-0.02	-0.03, -0.01	<0.001
Model 2	0.04	-0.03, 0.11	0.30	-0.14	-0.24, -0.03	0.01	-0.02	-0.03, -0.01	<0.001
Model 3	0.02	-0.05, 0.09	0.55	-0.13	-0.23, -0.03	0.01	-0.02	-0.03, -0.005	0.005

1. B's represent difference in fetal HC (cm) per one unit increase in natural logarithm transformed maternal TFAs concentration. Model 1 was adjusted for child sex and gestational age at HC assessment; Model 2 was additionally adjusted for maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality in early pregnancy, and family income; Model 3 was additionally adjusted for concentrations of essential fatty acids and long-chain polyunsaturated fatty acids.

2. B's represent difference in fetal HC growth rate (cm/week) per one unit increase in natural logarithm transformed maternal TFAs concentration. Model 1 was adjusted for child sex; Model 2 was additionally adjusted for maternal age at enrollment, education, smoking and alcohol use during pregnancy, diet quality in early pregnancy, and family income; Model 3 was additionally adjusted for concentrations of essential fatty acids and long-chain polyunsaturated fatty acids.

TFA - trans fatty acid; HC - head circumference.

Chapter 4

Gestational Duration and Brain Development in Childhood

4.1

Association of gestational age at birth with brain morphometry

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ABSTRACT

Importance: Preterm and post-term births are associated with adverse neuropsychiatric outcomes. However, it remains unclear whether variation of gestational age within the 37- to 42-week range of term deliveries is associated with neurodevelopment.

Objective: To investigate the association of gestational age at birth (GAB) with structural brain morphometry in children aged 10 years.

Design, setting, and participants: This population-based cohort study included pregnant women living in Rotterdam, the Netherlands, with an expected delivery date between April 1, 2002, and January 31, 2006. The study evaluated 3079 singleton children with GAB ranging from 26.3 to 43.3 weeks and structural neuroimaging at 10 years of age from the Generation R Study, a longitudinal, population-based prospective birth cohort from early pregnancy onward in Rotterdam. Data analysis was performed from March 1, 2019, to February 28, 2020, and at the time of the revision based on reviewer suggestions.

Exposures: The GAB was calculated based on ultrasonographic assessment of crown-rump length (<12 weeks 5 days) or biparietal diameter (≥12 weeks 5 days) in dedicated research centers.

Main outcomes and measures: Brain structure, including global and regional brain volumes and surface-based cortical measures (thickness, surface area, and gyrification), was quantified by magnetic resonance imaging.

Results: In the 3079 children (1546 [50.2%] female) evaluated at 10 years of age, GAB was linearly associated with global and regional brain volumes. Longer gestational duration was associated with larger brain volumes; for example, every 1-week-longer gestational duration corresponded to an additional 4.5 cm^3/wk (95%CI, 2.7-6.3 cm^3/wk) larger total brain volume. These associations persisted when the sample was restricted to children born at term (GAB of 37-42 weeks: 4.8 cm^3/wk; 95%CI, 1.8-7.7 cm^3/wk). No evidence of nonlinear associations between GA and brain morphometry was observed.

Conclusions and relevance: In this cohort study, gestational duration was linearly associated with brain morphometry during childhood, including within the window of term delivery. These findings may have marked clinical importance, particularly given the prevalence of elective cesarean deliveries.

INTRODUCTION

Gestational age at birth (GAB) is an important determinant of child health and development. Worldwide, approximately 13 million newborns are born preterm (GAB<37weeks) annually.[1] Prematurity is associated with morbidity and mortality,[2,3] including neurodevelopmental problems, such as cerebral palsy, intellectual disability, learning disability, and poor motor development.[4-6] Preterm birth is reportedly associated with increased risks of attention-deficit/ hyperactivity disorder (ADHD), autism spectrum disorder,[7-9] and psychiatric disorders in adulthood.[10] In some countries, post-term birth (GAB≥42 weeks) accounts for up to 10% of births[11] and is associated with adverse birth outcomes, increased neonatal mortality, cognitive impairment, and increased risk of ADHD.[12]

Few studies[13,14] have investigated the associations of GAB with brain structures despite the dynamic neurodevelopment that occurs during early life. During the third trimester of gestation, there is a 4-fold increase in brain size accompanied by marked growth of brain surface area, resulting in the emergence of sulci and gyri.[15,16] Thus, birth before the presumed optimal gestational duration (approximately 40 weeks) may be associated with disruption of neurodevelopmental processes in late pregnancy that persist during postnatal life.

Prior studies[17-22] often focused on children born extremely preterm (<28weeks of gestation) or very preterm (<34 weeks of gestation) and found less gray and white matter volumes in premature children and adolescents. These studies[17-22] used categorical indexes for GAB based on somewhat arbitrary cutoffs (e.g., extremely, very, or late preterm) rather than gestational duration as a quantitative trait of fetal maturity. Few studies[23,24] have examined the associations of gestational duration with developmental outcomes in children born at term. Longer gestation in term-born children has been associated with higher scores of cognitive and motor development early in life. In addition, 2 neuroimaging studies that used a group of approximately 100 term-born children (aged 6-10 years) found that longer gestational duration was associated with larger gray matter volumes, in particular temporal and parietal regions,[25] and linear associations of larger GAB with enhanced local and global network efficiency of structural networks.[26]

Given the novelty of investigating brain morphometric outcomes among children born at term, we examined the prospective association of GAB as a quantitative trait with brain morphometry assessed 10 years later using structural neuroimaging in a large population-based cohort. We used an exploratory approach that involved both global and regional metrics of brain development without defining specific regions of interest. We hypothesized that higher GAB would be associated with larger global and regional brain volumes, even within the term range of gestational duration. Mechanistically, we hypothesized that a higher GAB is positively associated with cortical surface

area and gyrification (i.e., cortical folding) because of rapid expansion of the cerebral cortex in the third trimester of pregnancy.

METHODS

Setting and Design

This cohort study was embedded in the Generation R Study, a population-based cohort in Rotterdam, the Netherlands. Pregnant women living in Rotterdam with an expected delivery date between April 1, 2002, and January 31, 2006, were invited to participate.[27] Enrolled children were followed up from fetal life onward. Data analysis was performed from March 1, 2019, to February 28, 2020, and at the time of the revision based on reviewer suggestions. Written informed consent was obtained from all participants, and all data are deidentified. The Generation R Study was approved by the Medical Ethical Committee of the Erasmus Medical Center, Rotterdam. All procedures were conducted in accordance with the World Medical Association Declaration of Helsinki.[28]

Study Population

The study enrolled 9778 mothers who gave birth to 9749 live born children (eFigure 1 in the Supplement). Twin pregnancies were excluded because of the increased risk of prematurity and twin-related complications,[29] resulting in 9418 singletons with information on GAB. Of these, 8270 children were invited to the research center at 10 years of age. All 5669 children visiting the center were invited to undergo brain magnetic resonance imaging (MRI). In total, 3857 children underwent neuroimaging. The final study population comprised 3079 children after excluding 23 children with major incidental findings, 89 with missing T1-weighted MRIs or artifacts because of braces or retainers, 44 with heterogeneous scanning parameters, and 622 with insufficient image quality (based on visual inspection of T1-weighted MRIs and FreeSurfer reconstructions).[30,31]

Gestational Age at Birth

Gestational age was determined by ultrasonography during prenatal visits at the research center. Standard methods of fetal ultrasonographic measurements have been previously described.[32] Inter-observer and intra-observer intra-class correlation coefficients were greater than 0.98.[32] Crown-rump length was used for pregnancy dating until a gestational age of 12 weeks 5 days (<65mm, n = 902). Biparietal diameter was used for pregnancy dating from12 weeks 5 days onward (>23 mm, n = 1790). For the 387 women who did not attend the prenatal visits, we retrieved gestational age from the Netherlands National Obstetric Register. Preterm birth was defined as delivery occur-

ring at less than 37 weeks of gestation (n = 138), term birth as GAB in the window of 37 weeks 0 days through 41 weeks 6 days (n = 2718), and post-term birth as GAB of 42 weeks or greater (n = 223).[33] For illustrative purposes, in eFigure 2 in the Supplement, we subdivided term birth into categories of early, full, and late term.[33]

Image Acquisition and Processing

Brain images were acquired on a 3.0-T MRI scanner (Discovery MR750, GE Healthcare) using an 8-channel head coil. After a localizer, T1-weighted structural images were acquired with an inversion recovery-prepared fast spoiled gradient recalled sequence. Sequence parameters (option BRAVO) were as follows: repetition time, 8.77 milliseconds; echo time, 3.4 milliseconds; inversion time, 600 milliseconds; flip angle, 10°; field of view, 220 × 220 mm; acquisition matrix, 220 × 220; slice thickness, 1mm; number of slices, 230; voxel size, 1 × 1 × 1 mm; and arc acceleration, 2.[34] Images were processed using FreeSurfer, version 6.0, an open source software for analyzing brain images.[35,36] Global and regional volumes, including total brain, cerebral and cerebellar gray, and white matter volumes, as well as subcortical gray matter volumes, including the thalamus, amygdala, hippocampus, putamen, pallidum, caudate, and nucleus accumbens, were measured. A surface-based stream quantified cortical thickness, cortical surface area, and gyrification. FreeSurfer morphometry had good test-retest reliability across scanners and field strengths.[37,38] FreeSurfer reconstructions were visually inspected,[30,31] and images not suitable for analysis were excluded. An automated quality metric[39] found no correlation with GAB ($r = -0.01$, $P = .53$).

Covariates

Potential confounding variables were selected based on the existing literature.[40,41] Self-reported questionnaire data and medical record data measured before child birth provided information on maternal ethnicity,[42] age at intake, marital status, educational level, psychopathologic symptoms,[43,44] smoking and alcohol use during pregnancy, and family income. Medical registries provided information on obstetric variables, including fetal distress, mode of delivery, 5-minute Apgar score, and calendar year of birth. Child sex and age at neuroimaging were also included.

Statistical Analysis

For descriptive purposes, children were categorized as preterm(< 37weeks), term(≥ 37and < 42 weeks), and post-term(≥ 42weeks) delivery. Linear regression was used to investigate the association of the quantitative trait GAB with global and regional brain volumes (eFigure 3 in the Supplement). Subcortical volumes were standardized to enable comparison of the effect estimates of GAB between subcortical structures. Nonlinear associations of GAB with brain morphometry were examined using quadratic

models and models with natural cubic splines with knots at 30, 32, 34, 37, 38, 39, 40, and 42 weeks. We performed additional analyses among children born at term to examine whether associations were also present in this narrow range of gestational duration. To investigate the association between GAB and surface-based cortical measures, we used vertex-wise linear regression models with a custom in-house QDECR package. To compare the results with existing studies[17-22] using clinically defined categories, we also applied linear regression models with the preterm, term, and post-term categories; term-born children served as the reference group.

Model 1 was minimally adjusted for child sex and age at neuroimaging assessment. Model 2 was further adjusted for maternal characteristics, including ethnicity, age at intake, marital status, educational level, psychopathologic symptoms, smoking and alcohol use during pregnancy, and family income. Calendar year of birth was not included as a covariate because it was not associated with GAB or brain characteristics. Models with subcortical outcomes were additionally adjusted for intracranial volume.

In addition, we examined whether the association of GAB with brain morphometry was moderated by sex. To ensure that the associations of GAB with brain volumes were not driven by adverse perinatal events, children exposed to perinatal complications, including maternal preeclampsia, diabetes, pregnancy-induced hypertension, urgent cesarean delivery, intrauterine growth restriction, low birth weight (<2500 g), suspected fetal distress, a 5-minute Apgar score below 7, and premature or post-term birth, were excluded in sensitivity analyses.

Missing values of the covariates were imputed using multivariate imputation by chained equations with 10 imputations.[45] We report pooled results. Statistical significance was defined as $\alpha < .05$ (2-sided). In the primary analyses, a false discovery rate correction was applied to minimize false-positive findings attributable to multiple comparisons.[46] Surface-based analyses were corrected for multiple testing using built-in Gaussian Monte Carlo simulations.[47] The cluster-forming threshold was set to $P = .001$ because this value corresponds closely to a false-positive rate of 0.05,[48] with further Bonferroni correction for independent analysis of each hemisphere ($P < .025$ cluster-wise). All statistical analyses were performed using R statistical software, version 3.5.1 (R Foundation for Statistical Computing).

For nonresponse analyses, we used analyses of variance or the Kruskal-Wallis test for continuous variables and the $\chi 2$ test for categorical variables to compare maternal and child characteristics between responders and non-responders at follow-up.

RESULTS

Descriptive Statistics

This study evaluated 3079 children (1546 [50.2%] female) at 10 years of age. Table 1 presents information on the study population. Mothers of preterm-born children less frequently had a partner (109 [79.0%] vs 2405 [88.5%], $P = .001$), had a lower educational level (53 [38.4%] vs 1411 [51.9%], $P = .01$), and had a lower family income (<€1200 [US $1550] per month: 33 [23.9%] vs 423 [15.6%], $P < .001$) than did mothers of children born at term. Mothers of children born preterm more often had preeclampsia, diabetes, and pregnancy-related hypertension (22 [15.9%] vs 179 [6.6%], $P < .001$) and more often underwent cesarean delivery (33 [23.9%] vs 306 [11.3%], $P < .001$). Maternal sociodemographic or lifestyle factors did not differ between the post-term and the term-born groups. However, children born post-term more often were delivered via cesarean (43 [19.3%] vs 306 [11.3%], $P < .001$) and more often had signs of fetal distress (27 [12.1%] vs 195 [7.2%], $P = .01$).

Table 1. Demographic characteristics of the study population[a]

	Term birth N=2718	Preterm birth N=138	Post-term birth N=223
Maternal characteristics			
Age at intake, mean (sd), year	31.0 (4.9)	30.7 (5.1)	31.4 (4.5)
Multiparous (%)	11.9	12.3	10.3
Pregnancy complications (%) [b]	6.6	15.9	4.9
Ethnicity (%)			
Dutch	57.7	50.7	59.2
Non-Dutch Western	11.9	11.6	16.1
Non-Dutch Non-Western	30.4	37.7	24.7
Educational level (%)			
Primary or lower	7.2	9.4	5.8
Secondary	40.9	52.2	36.8
Higher	51.9	38.4	57.4
Family income, per month (%)			
Less than €1200	15.6	23.9	15.2
€1200 to €2000	17.0	24.6	10.3
More than €2000	67.4	51.4	74.4
Marital status, married or with partner (%)	88.5	79.0	90.6
Smoking during pregnancy (%)			
Never in pregnancy	77.3	75.4	78.9
Until pregnancy was known	8.6	7.2	10.3
Continued in pregnancy	14.2	17.4	10.8

Table 1. Demographic characteristics of the study population[a] (*continued*)

	Term birth N=2718	Preterm birth N=138	Post-term birth N=223
Alcohol use during pregnancy (%)			
Never in pregnancy	40.2	38.4	39.9
Until pregnancy was known	14.0	15.9	11.2
Continued in pregnancy, occasionally	36.2	35.5	38.1
Continued in pregnancy, frequently [c]	9.6	10.1	10.8
Psychopathology, mean (sd)	0.3 (0.4)	0.4 (0.4)	0.3 (0.4)
Birth and child characteristics			
Cesarean delivery (%)	12.3	23.4	20.2
Suspected fetal distress (%)	7.2	10.9	12.1
Apgar at 5 minutes below 7 (%)	1.0	2.9	0.9
Gestational age at birth, mean (sd), week	40.0 (1.1)	34.6 (2.7)	42.3 (0.3)
Range	37.0 – 41.9	26.3 – 36.9	42.0 – 43.4
Birth weight, mean (sd), gram	3472.6 (485.9)	2345.6 (654.7)	3785.7 (475.6)
Sex, boy (%)	49.3	47.1	57.4
Age at MRI scan, mean (sd), year	10.1 (0.6)	10.2 (0.6)	10.1 (0.6)

Abbreviation: MRI, magnetic resonance imaging.

[a.] No data were missing for these variables because they were imputed using multiple imputation methods. Categorization was based on gestational age at birth: term birth (gestational age of 37-42 weeks), preterm birth (gestational age <37 weeks), and post-term birth (gestational age of ≥ 42 weeks).

[b.] Pregnancy complications included preeclampsia, diabetes, and/or pregnancy induced hypertension.

[c.] Frequent continued alcohol use is defined as one or more glasses of alcohol per week in at least two trimesters.

Global and Regional Brain Volumes

Table 2 and Figure 1 report a positive association of GAB with total brain volume (B = 4.5 cm^3/wk; 95% CI, 2.7-6.3 cm^3/wk). In addition, GAB was positively associated with cerebral gray matter (2.8 cm^3/wk; 95% CI, 1.8-3.7 cm^3/wk), cerebral white matter (1.2 cm^3/wk; 95% CI, 0.3-2.0 cm^3/wk), cerebellar gray matter (0.4 cm^3/wk; 95% CI, 0.2-0.5 cm^3/wk), cerebellar white matter (0.1 cm^3/wk; 95% CI, 0.1-0.2 cm^3/wk), and subcortical gray matter (0.2 cm^3/wk; 95% CI, 0.1-0.2 cm^3/wk) volume. These associations remained after correction for multiple comparisons. Of importance, the associations and effect estimates of GAB with global and regional brain volumes remained intact when restricting the sample to children born at term with the exception of cerebellar gray matter (Table 2 and Figure 1). Furthermore, we did not observe any interaction of sex in the association of GAB with global brain volumes (eTable 1 in the Supplement).

Post hoc analyses found that longer gestational duration was associated with larger volumes of the thalamus, caudate, putamen, and pallidum (eFigure 4 in the Supplement). No associations were observed between GAB and amygdala, hippocampus, or nucleus accumbens volume.

Table 2. Association of gestational age at birth with global brain volumes[a]

	Model	Total brain volume		Cerebral gray matter volume		Cerebral white matter volume	
		Difference (95% CI)	P-value	Difference (95% CI)	P-value	Difference (95% CI)	P-value
GAB, week	**All children (n=3079)**						
	Model 1	6.1 (4.2 to 8.0)	<.001	3.7 (2.7 to 4.7)	<.001	1.7 (0.9 to 2.6)	<.001
	Model 2	4.5 (2.7 to 6.3)	<.001[c]	2.8 (1.8 to 3.7)	<.001[c]	1.2 (0.3 to 2.0)	.006[c]
	Term children (n=2718)						
	Model 1	6.7 (3.6 to 9.7)	<.001	3.8 (2.2 to 5.4)	<.001	2.3 (0.9 to 3.7)	.002
	Model 2	4.8 (1.8 to 7.7)	.002[c]	2.8 (1.2 to 4.3)	<.001[c]	1.6 (0.3 to 3.0)	.02[c]

	Model	Cerebellar gray matter volume		Cerebella white matter volume		Subcortical gray matter volume[b]	
		Difference (95% CI)	P-value	Difference (95% CI)	P-value	Difference (95% CI)	P-value
GAB, week	**All children (n=3079)**						
	Model 1	0.5 (0.3 to 0.7)	<.001	0.2 (0.1 to 0.2)	<.001	0.2 (0.1 to 0.2)	<.001
	Model 2	0.4 (0.2 to 0.5)	<.001[c]	0.1 (0.1 to 0.2)	<.001[c]	0.2 (0.1 to 0.2)	<.001[c]
	Term children (n=2718)						
	Model 1	0.4 (0.1 to 0.8)	.01	0.1 (0.05 to 0.2)	.003	0.1 (0.01 to 0.2)	.03
	Model 2	0.2 (-0.1 to 0.6)	.13	0.1 (0.03 to 0.2)	.01[c]	0.1 (0.01 to 0.2)	.03[c]

Abbreviations: GAB, gestational age at birth.

[a] In the results of these regression models, the effect estimates represent the difference in cubic centimeters for brain volumes per 1-week-longer gestational duration. Model 1 is a minimally adjusted model corrected for child sex and age at the neuroimaging assessment. Model 2 is a fully adjusted model, corrected for child sex and age at the neuroimaging assessment, maternal ethnicity, age at intake, marital status, educational level, psychopathologic condition, smoking and alcohol use during pregnancy, and family income.

[b] Intracranial volume was additionally adjusted for in both models.

[c] The associations survived a false discovery rate correction for multiple testing (applied model 2 only).

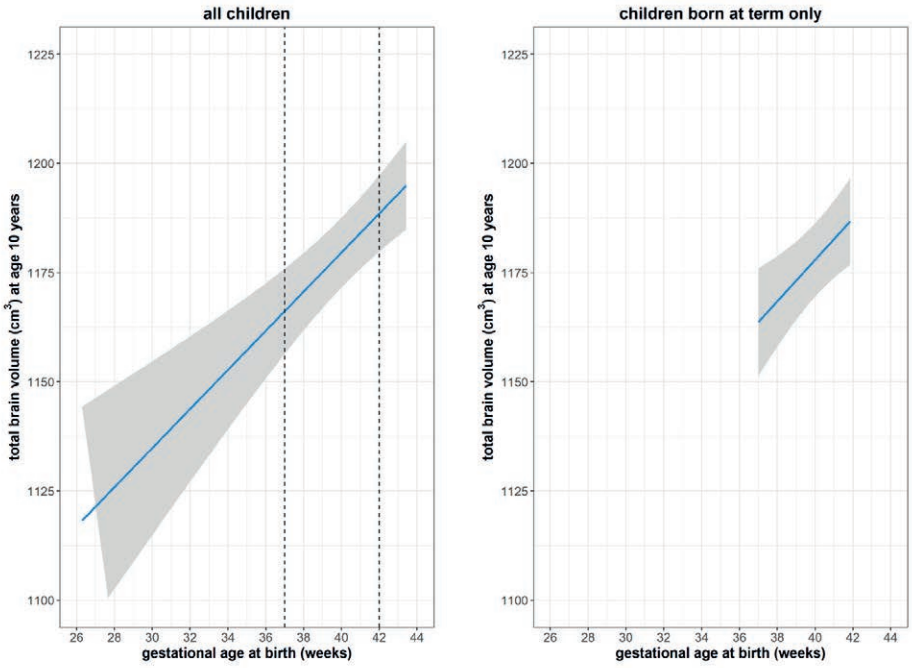

Figure 1. Linear relationship between gestational age at birth and total brain volume in all children (left, n=3079) and only the term birth children (right, n=2718) at age 10 years. Models were fully adjusted and corrected for child sex, age at the neuroimaging assessment, and maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income. Shaded areas indicate the 95%CIs of the predicted values.

Cerebral Cortex Morphometry

Cortical thickness and GAB were associated in a few small regions of the superior temporal lobe, cuneus, and inferior parietal lobe, exhibiting local reduction of cortical thickness (Figure 2A). In contrast, the association of GAB with cortical surface area was widespread across the neocortex (Figure 2B). Longer gestational duration was associated with larger surface area in the inferior and superior parietal regions, inferior and middle temporal regions, middle frontal and orbitofrontal regions, rostral anterior cingulate, and fusiform gyrus. Likewise, widespread positive associations of GAB and neocortical gyrification, including superior parietal lobe postcentral region, fusiform gyrus, insular cortex, and anterior cingulate cortex, were observed (Figure 2C). Results were similar in children born at term (Figure 3). Gestational age at birth was associated with a thinner cortex in the superior temporal region (Figure 3A), with larger surface area and gyrification in the frontal, parietal, and temporal regions (Figure 3B and C). Specific information on the associated brain regions (size, Talairach coordinates, and *P* values) are presented in eTables 2 and 3 in the Supplement.

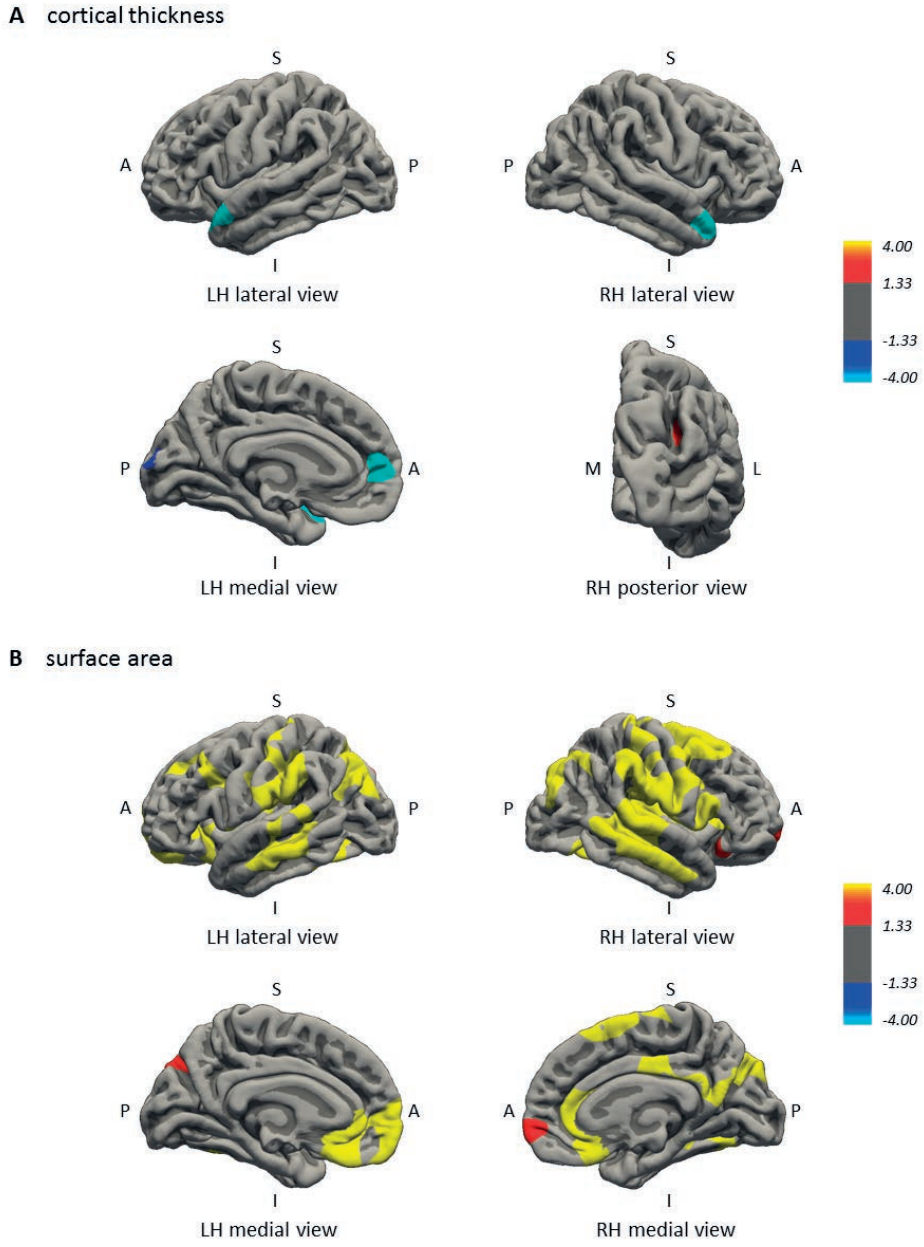

Figure 2. Gestational age at birth and cortical thickness, surface area, and gyrification in all children.

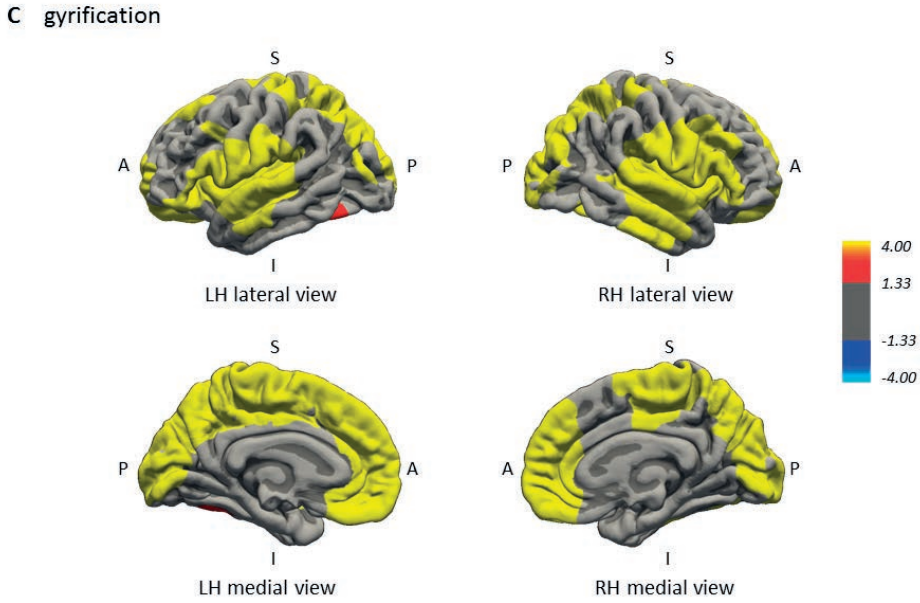

Figure 2. Gestational age at birth and cortical thickness, surface area, and gyrification in all children (continued). Surface-based analysis was performed for 3065 children with a gestational age at birth ranging from 26.3 to 43.4 weeks. The model was adjusted for child sex, age at neuroimaging, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income. The color bar shows the -log p-value of the cluster. Colored clusters represent regions of the brain that were positively (red to yellow) and negatively (dark to light blue) associated with gestational age at birth that survived the cluster-wise (Monte Carlo simulation with 5,000 iterations) correction for multiple comparisons ($P < .001$) (eTable 2 in the Supplement). A indicates anterior; I, inferior; L, lateral; LH, left hemisphere; M, medial; P, posterior; RH, right hemisphere; S, superior.

Nonlinear Associations

We found no evidence of nonlinear associations of GAB with brain volume and morphometry. Models with quadratic or natural cubic splines did not improve model fit compared with linear models (eTable 4 in the Supplement).

Categorical Analyses

With the use of a categorical approach, children born preterm had a smaller total brain volume, cerebral gray matter volume, subcortical gray matter volume, cerebellar gray matter volume, and cerebellar white matter volume. Total brain volume of children born preterm had an adjusted difference of 26.5 cm^3 compared with children born at term (B = −26.5; 95% CI, −42.1 to −11.0). Children born post-term had a larger cerebral gray matter volume and subcortical gray matter volume (eTable 5 in the Supplement).

Sensitivity Analyses

Sensitivity analyses of children born at term without perinatal complications (n = 2264) showed similar associations between GAB and total brain matter (5.5 cm^3/wk; 95% CI, 2.2-8.7 cm^3/wk), cerebral gray matter (3.1 cm^3/wk; 95% CI, 1.4-4.8 cm^3/wk), cerebral white matter (2.1 cm^3/wk; 95% CI, 0.5-3.6 cm^3/wk), cerebellar white matter (0.1 cm^3/wk; 95% CI, 0.02-0.2 cm^3/wk), and subcortical gray matter (0.1 cm^3/wk; 95% CI, 0.01-0.2 cm^3/wk) volumes. However, in this subgroup, GAB was not associated with cerebellar gray matter volume (0.1 cm^3/wk; 95% CI, −0.2 to 0.5 cm^3/wk).

Nonresponse Analyses

Respondents differed from nonrespondents (eTable 6 in the Supplement). Specifically, respondents were older, were more likely of Dutch origin, had a higher educational level, and were less likely to smoke during pregnancy.

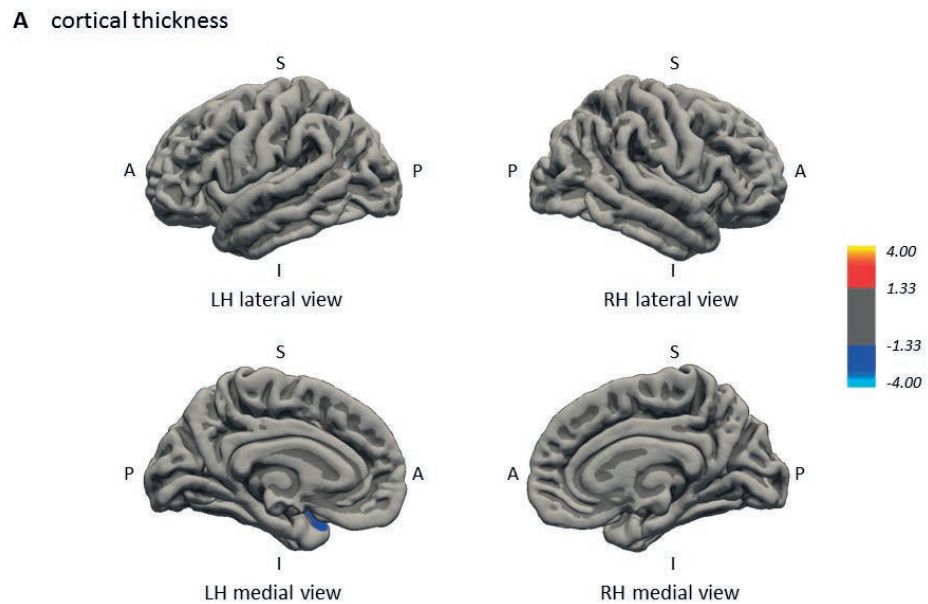

Figure 3. Gestational age at birth and cortical thickness, surface area, and gyrification in children born at term.

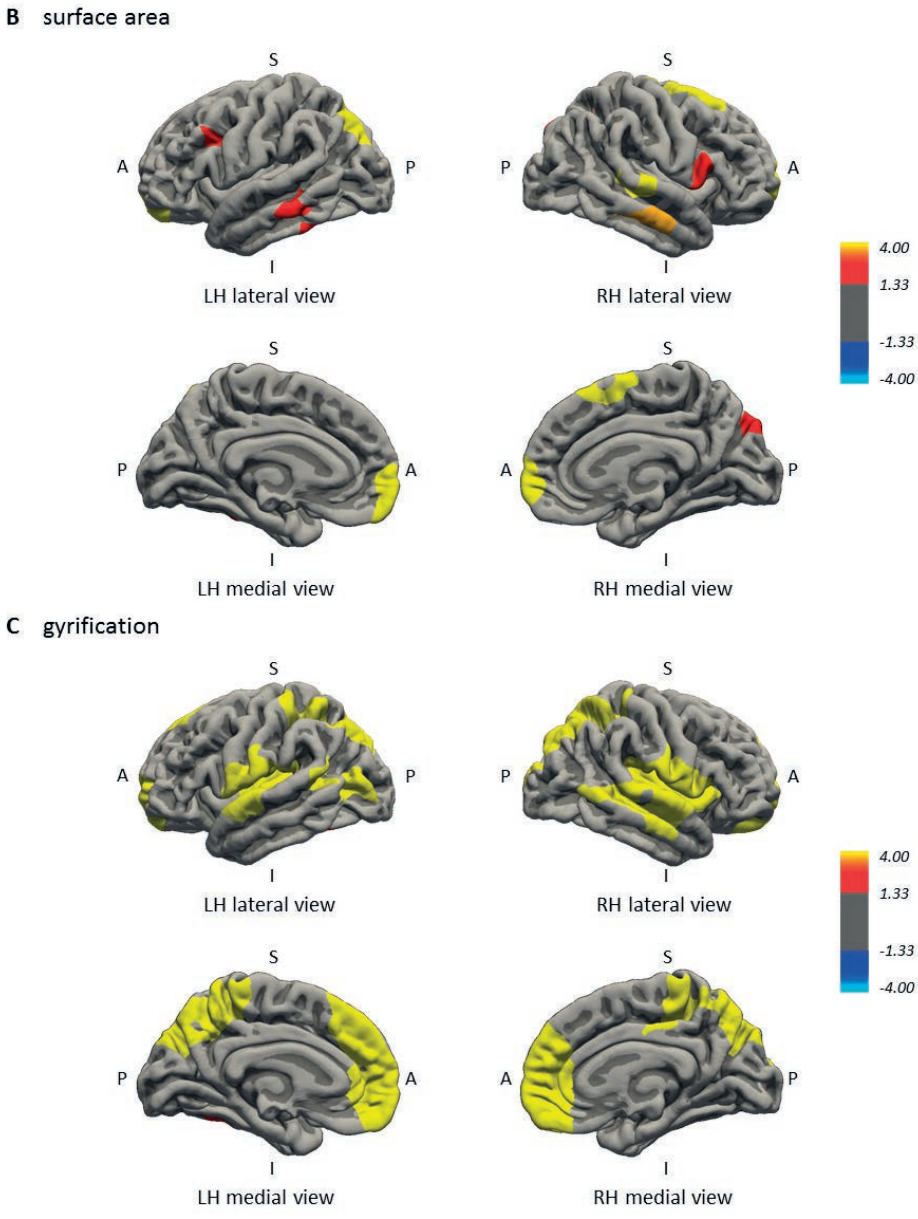

Figure 3. Gestational age at birth and cortical thickness, surface area, and gyrification in children born at term *(continued).* Surface-based analysis was performed for 2706 children with a gestational age at birth ranging from 37.0 weeks to 41.9 weeks. The model was adjusted for child sex, age at neuroimaging, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income. The color bar shows the -log p-value of the cluster. Colored clusters represent regions of the brain that were positively (red to yellow) and negatively (dark to light blue) associated with gestational age at birth that survived the cluster-wise (Monte Carlo simulation with 5,000 iterations) correction for multiple comparisons (P < .001) (eTable 3 in the Supplement). A indicates anterior; I, inferior; L, lateral; LH, left hemisphere; M, medial; P, posterior; RH, right hemisphere; S, superior.

DISCUSSION

In this longitudinal, population-based cohort study, we found that GAB was positively associated with global and regional brain volumes in children at 10 years of age. Our findings suggest that the volumetric association with GAB is a consequence of larger cortical surface area and gyrification in the absence of widespread differences in cortical thickness. Our results were robust to confounding by several sociodemographic and lifestyle characteristics. Of importance, we found that these associations remained present when restricting the analyses exclusively to children born at term, which supports a model in which gestational duration should be viewed as a continuum of development throughout pregnancy. Moreover, we observed no evidence of nonlinear associations using spline regression analyses, a flexible and sensitive method for assessing nonlinearity. In addition, child sex did not moderate the association of GAB with brain morphometry. Overall, these findings suggest that a cutoff for the designation of preterm birth as less than 37 weeks of gestation may not be consistent with brain development.

By reporting associations of GAB with global and regional brain volumes, our findings complement previous neuroimaging studies demonstrating that longer gestational duration was associated with larger temporal and parietal gray matter volumes[25] and reporting linear associations of higher GA with enhanced local and global network efficiency.[26] These results are further supported by our findings that GAB was associated with brain regions that are functionally and structurally integrated (e.g., the cerebellum and thalamus) (eFigure 4 in the Supplement). In addition, temporal and parietal regions (Figure 2 and Figure 3) are involved in higher-order cognitive processes, including auditory perception and processing, language production and perception, and declarative memory. Even though larger brain size typically has been associated with enhanced cognitive functioning (including general cognitive ability, working memory, reading, vocabulary, and set-shifting tasks) in children and adolescents,[49,50] a previous longitudinal study[51] found that neuroanatomical correlates of intelligence, for example, are dynamic and change throughout life. The association between psychiatric disorders and brain size is less evident, but clinical studies[52-54] in children with ADHD found smaller volumes in frontal, parietal, and cerebellar structures. Furthermore, individual variations in brain size and shape occur.[55] Caution is warranted regarding potential functional consequences of the observed association between GAB and brain morphometry.

Several mechanisms are possible. First, the largest absolute volumetric increase in brain volume during fetal development occurs during the third trimester, in particular, the expansion of cortical gray matter.[13,56-58] The growth rate of the cerebellum also peaks during the third trimester.[59] Therefore, on the basis of the observed linear association

with global brain volume across the full range of GAB, a parsimonious explanation could be that birth is necessary and sufficient to attenuate the rapid expansion of the neocortex in the late third trimester. In contrast, we did not observe extensive differences by GAB in cortical thickness. Cortical neurogenesis and proliferation are largely complete by the middle of gestation.[13,14] In addition, cortical lamination (i.e., inside-out layering of the cortex) is already well established by the eighth month of pregnancy, although timing appears to differ slightly among brain regions.[14]

Second, GAB is often associated with multiple factors,[60] including psychological distress, maternal age, substance use, poor nutrition, or fetal growth restriction. Such stress-associated factors may affect the timing of birth and fetal maturation. In line with the developmental origins of health and disease framework, the current study suggests that even small variations in GAB may be associated with fetal programming differences in neurodevelopment.[61] Our results suggest that among healthy term-born children, GAB may be linearly and positively associated with brain volume, surface area, and gyrification when assessed in childhood at 10 years of age. Although we considered a variety of confounding factors, we cannot exclude the possibility of residual confounding.

Third, our results could potentially be explained by genetic predisposition. Previous studies[30,62-65] reported genetic variations that are associated with gestational duration. For instance, Adkins et al[62] found polymorphisms of insulin and insulin-like growth factor 2 associated with an increased risk of being small for gestational age. Moreover, several maternal and fetal genes have been identified through recent genome-wide association studies[63,64] of gestational duration and preterm birth. Genetic factors associated with global and specific brain volumes have also been reported.[30,65] Thus, our findings potentially can be explained by a shared genetic susceptibility that is associated with gestational duration and brain morphometry. However, it is likely that both genetic and environmental factors explain the observed associations.

Further research is needed to investigate underlying mechanisms and causal pathways of the association between GAB and childhood brain structure and function. The results would be particularly important for obstetricians, neonatologists, and pediatricians. The findings of the present study have substantial potential clinical importance, considering the discussions regarding expectant management vs labor induction[66] and the increasing prevalence of elective cesarean deliveries worldwide, which are typically planned 1 or 2 weeks before the estimated full-term date.[67] In line with recommendations of the World Health Organization, our results cautiously support a reduction of elective cesarean deliveries.

Strengths and Limitations

Study strengths were the prospective design, which enabled a temporal association between GA and brain morphometry; measurement of GAB by ultrasonography; adjustment for multiple confounders; and large-scale pediatric neuroimaging (>3000 children), allowing for detection of small effect sizes.

Several limitations should also be mentioned. First, brain morphometry was measured only once, at 10 years of age; thus, whether the observed differences were transient or persistent is unknown. Second, although childhood brain structure has been associated with a diversity of cognitive, emotional, and sensorimotor functions in the general population,[68] we cannot yet address the functional implications of the observed morphologic differences. Although the current study was specifically focused on examining brain morphometry, future studies should investigate the associations of GAB with repeated assessments of brain morphometry and multimodal imaging in combination with behavioral and cognitive outcomes in large population-based cohorts. Third, the nonresponse analyses suggested a possible selection bias. Fourth, because this was an observational study, unmeasured residual (genetic and environmental) confounding limited the ability to establish causal inferences.

CONCLUSIONS

The findings of this study suggest that GAB is associated with widespread differences in childhood brain morphometry in children born at term. Longer gestational duration was associated with larger brain volume, cortical surface area, and cortical gyrification. Thus, physiologic processes during in utero development may have an enduring influence across the life span. Our findings highlight the importance of the last few weeks of pregnancy in association with neurodevelopment for which additional studies are warranted to evaluate the potential effect on international clinical guidelines for elective cesarean delivery.

Authors Contributions: Dr. El Marroun and Mr. Zou contributed equally to this work. Dr. El Marroun and Mr. Zou had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.
Concept and design: El Marroun, Steegers, Kushner, Tiemeier.
Acquisition, analysis, or interpretation of data: All authors.
Drafting of the manuscript: El Marroun, Zou, Leeuwenburg, Reiss, Muetzel.
Critical revision of the manuscript for important intellectual content: El Marroun, Zou, Steegers, Reiss, Muetzel, Kushner, Tiemeier.
Statistical analysis: El Marroun, Zou.
Obtained funding: El Marroun, Zou, Muetzel, Tiemeier.
Administrative, technical, or material support: Reiss, Muetzel.

Supervision: El Marroun, Steegers, Kushner, Tiemeier.

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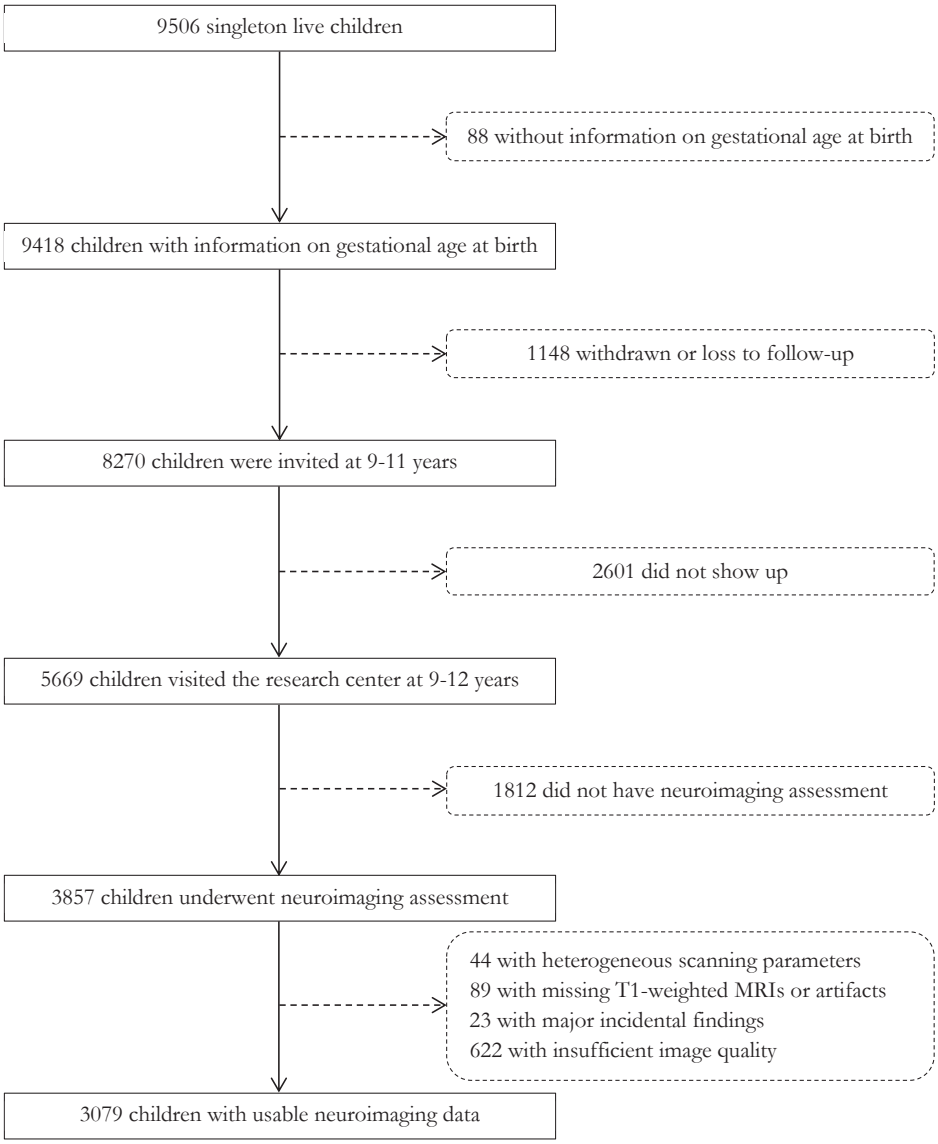
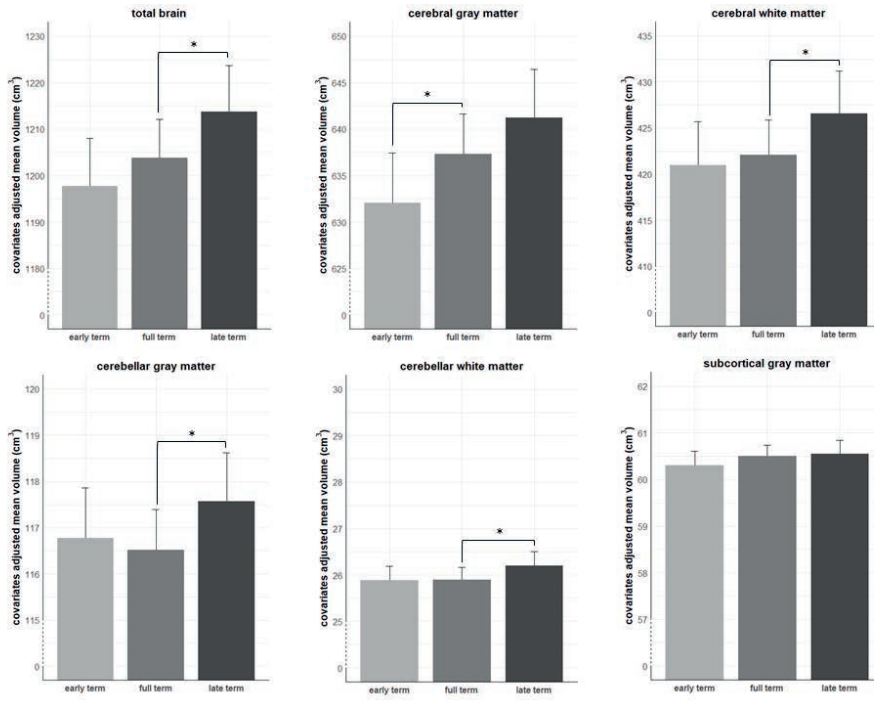
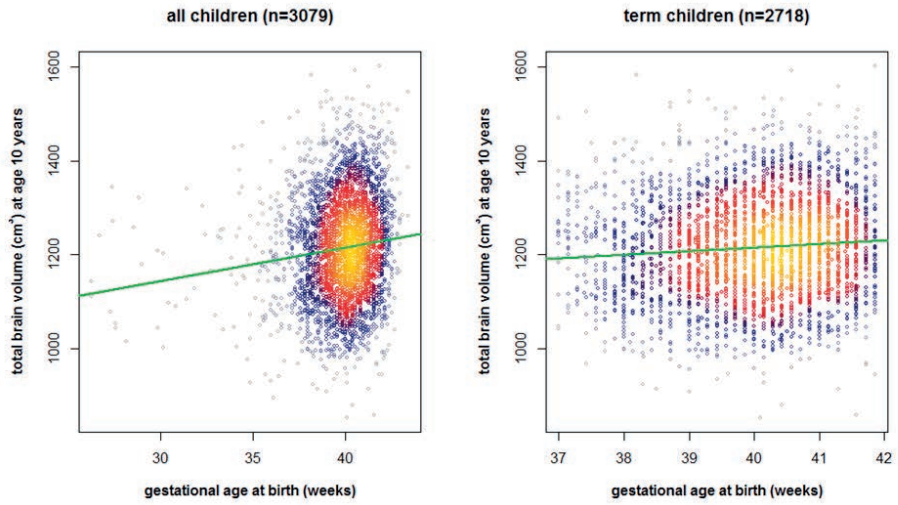
SUPPLEMENTAL MATERIALS



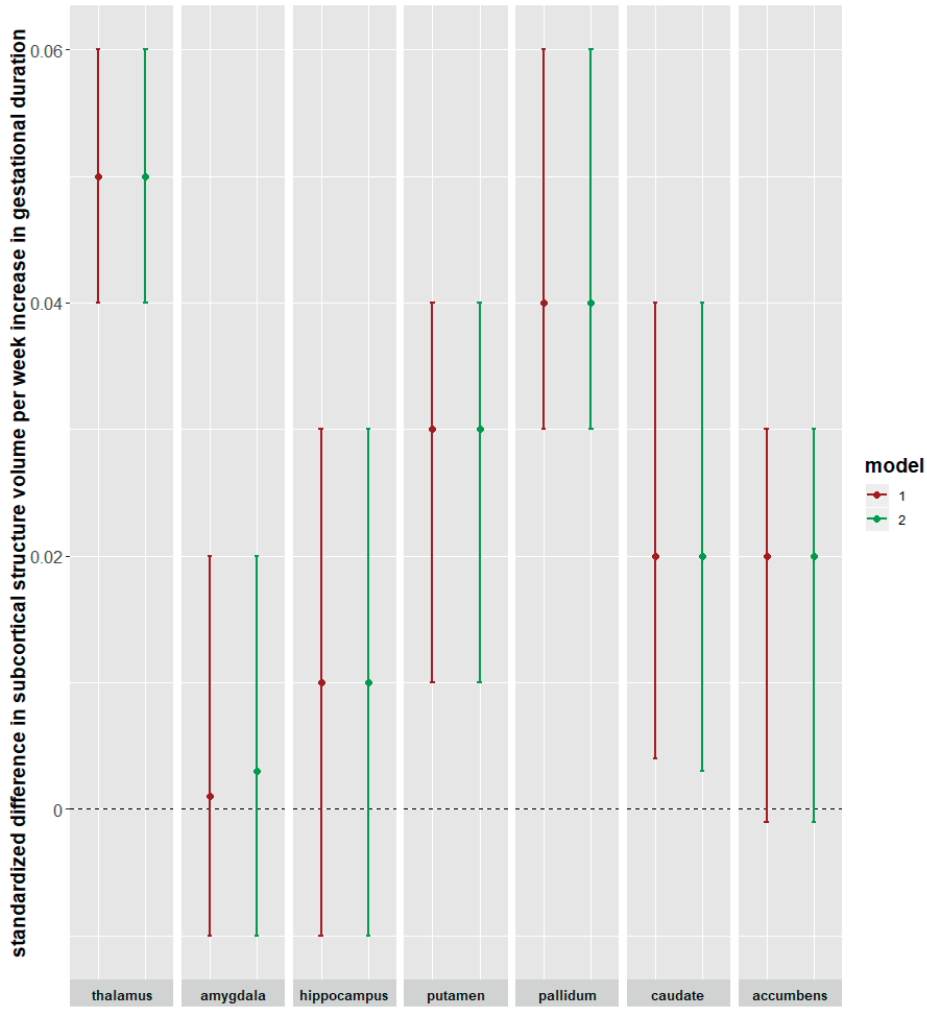
eFigure 1. Flow diagram



eFigure 2. Global brain volumes in children born early term, full term and late term. Brain volumes of children born at term (n=2718) are represented in three subgroups (early term, n=506; full term, n=1599; and late term, n=613). Early term are births occurring between 37 weeks 0 days and 38 weeks 6 days; full term children are those at 39 weeks 0 day through 40 weeks 6 days; late term refers to those at 41 weeks 0 day through 41 weeks 6 days. Covariates including child sex, age at MRI scan, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income were adjusted. The model with subcortical gray matter as the outcome was additionally adjusted for intracranial volume. Statistical differences as indicated by the * p<.01 or p<.05 are comparisons of the three groups with children born full term as the reference.



eFigure 3. Linear relationship between gestational age at birth and total brain volume in all children (left, n=3079) and only the term birth children (right, n=2718) at age 10 years visualized with an additional heatmap. Models were fully adjusted, corrected for child sex, age at the neuroimaging assessment, and maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income.



eFigure 4. The association of gestational age at birth with subcortical gray matter volumes in children. Estimated differences (with 95% confidence intervals) in subcortical gray matter structures (in standardized z-scores) per week increase in gestational age at birth were computed using regression analyses in n=3079 children. Model 1 was a minimally adjusted model, corrected for child sex, age at the neuroimaging assessment, and intracranial volume. Model 2 was a fully adjusted model, corrected for child sex, age at the neuroimaging assessment, and intracranial volume, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income.

eTable 1. Gestational age at birth and brain volumes at age 10 years: interaction of GA with sex

Global brain volumes	Statistics of GAB × sex (interaction term)		
	Difference	95% CI	P-value
Total brain	0.3	-3.3 to 3.9	0.88
Cerebral gray matter	0.3	-1.6 to 2.2	0.74
Cerebral white matter	0.1	-1.5 to 1.8	0.89
Cerebellar gray matter	-0.2	-0.6 to 0.2	0.37
Cerebellar white matter	0.003	-0.1 to 0.1	0.96
Subcortical gray matter *	0.01	-0.1 to 0.1	0.85

The linear regression model examined the association of gestational age at birth (GAB) with global brain volumes in n=3079, in combination with an interaction term of GAB and sex. The model was adjusted for child sex and age at MRI scan, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income.

* Intracranial volume was additionally adjusted for.

eTable 2. Gestational age at birth and cortical morphology at age 10 years: surface-based clusters in all children

Cortical surface metric	Brain region #	Size brain region (mm^2)	Talairach coordinates			Cluster-wise P-value
			X	Y	Z	
Thickness	Left superior temporal	446.2	-45.7	6.2	-22.3	.0001
	Left superior frontal	404.0	-12.5	53.9	4.2	.0001
	Left cuneus	230.0	-5.8	-96.7	11.5	.003
	Right superior temporal	371.7	46.6	12.1	-27.1	.0001
	Right inferior parietal	171.0	33.2	-68.8	26.6	.02
Surface area	Left post central	5831.4	-58.1	-17.4	19.3	.0001
	Left inferior parietal	3132.5	-32.9	-76.5	21.4	.0001
	Left rostral anterior cingulate	2442.3	-5.3	16.9	-6.8	.0001
	Left middle temporal	2220.1	-60.8	-17.8	-16.3	.0001
	Left caudal middle frontal	1347.2	-33.3	11.2	27.2	.0001
	Left inferior temporal	937.1	-46.0	-50.6	-12.4	.0001
	Left superior parietal	403.0	-16.0	-86.0	36.5	.007
	Right middle temporal	16130.7	57.8	-16.1	-17.7	.0001
	Right inferior parietal	5130.1	34.3	-71.5	23.9	.0001
	Right fusiform	1176.6	39.5	-65.3	-15.6	.0001
	Right rostral anterior cingulate	866.8	5.4	19.6	-7.2	.0001
	Right lateral orbitofrontal	457.5	41.1	26.3	-14.6	.004
	Right medial orbitofrontal	320.0	8.7	61.5	-4.6	.02
Gyrification	Left superior parietal	27509.9	-14.6	-86.2	36.0	.0001
	Left post central	11987.8	-46.7	-11.8	17.6	.0001
	Left fusiform	642.5	-40.6	-55.4	-14.2	.002
	Right insula	35998.0	37.9	-13.7	21.5	.0001
	Right rostral anterior cingulate	4780.7	10.2	40.2	4.8	.0001

Brain regions represent colored areas in Figure 2A, 2B and 2C.

A surface-based analysis was performed in n=3065 children. The model was adjusted for child sex, age at MRI scan, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income. The list of clusters represent brain regions of surface-based metrics that were associated with gestational age at birth, which survived the cluster-wise correction for multiple comparisons.

eTable 3. Gestational age at birth and cortical morphology at age 10 years: surface-based clusters in children born at term

Cortical surface metric	Brain region #	Size brain region (mm^2)	Talairach coordinates			Cluster-wise P-value
			X	Y	Z	
Thickness	Left superior temporal	242.3	-41.4	8.4	-27.5	.003
Surface area	Left inferior parietal	1995.7	-30.8	-70.7	29.2	.0001
	Left lateral orbitofrontal	865.1	-11.5	58.3	-17.6	.0001
	Left middle temporal	419.4	-61.9	-35.9	-14.1	.006
	Left caudal middle frontal	345.6	-35.2	14.2	30.2	.01
	Left inferior temporal	342.8	-51.4	-42.9	-24.0	.02
	Right superior frontal	2270.4	19.4	16.8	58.2	.0001
	Right superior temporal	820.6	52.6	-26.7	-0.7	.0001
	Right frontal pole	781.9	7.8	63.7	-5.9	.0001
	Right middle temporal	672.1	63.0	-12.4	-18.5	.0002
	Right pars opercularis	535.9	50.4	8.7	1.5	.001
	Right superior parietal	458.4	10.4	-84.2	37.0	.004
	Right superior parietal	308.1	30.8	-59.0	45.2	.02
Gyrification	Left precuneus	8230.8	-7.7	-51.2	52.5	.0001
	Left post central	4942.1	-40.8	-15.9	20.7	.0001
	Left superior frontal	4024.7	-14.3	46.5	2.0	.0001
	Left inferior parietal	1138.2	-45.3	-75.9	12.5	.0001
	Left supra marginal	913.1	-56.3	-52.9	18.0	.0001
	Left fusiform	501.0	-39.4	-45.1	-19.0	.005
	Right superior parietal	9412.3	24.3	-74.1	39.9	.0001
	Right insula	8625.0	36.7	-27.0	6.2	.0001
	Right medial orbitofrontal	3335.2	9.3	56.5	-3.3	.0001

Brain regions represent colored areas in Figure 3A, 3B and 3C.

A surface-based analysis was performed in 2706 children born at term. The model was adjusted for child sex, age at MRI scan, maternal ethnicity, age at intake, marital status, educational level, psychopathology during pregnancy, smoking and alcohol use during pregnancy, and family income. The list of clusters represent brain regions of surface-based metrics that were associated with gestational age at birth, which survived the cluster-wise correction for multiple comparisons.

eTable 4. Comparison of linear and nonlinear (quadratic and natural cubic spline) models using the likelihood ratio test

Global brain volumes	All children (N=3079)			Children born at term (N=2718)		
	Linear	Quadratic	Natural cubic splines	Linear	Quadratic	Natural cubic splines
	Reference	P-value	P-value	Reference	P-value	P-value
Total brain	-	.33	.31	-	.25	.31
Cerebral gray matter	-	.17	.16	-	.50	.63
Cerebral white matter	-	.52	.50	-	.23	.26
Cerebellar gray matter	-	.90	.86	-	.06	.06
Cerebellar white matter	-	.26	.27	-	.26	.23
Subcortical gray matter*	-	.84	.75	-	.90	.86

In all children (n=3079) and children born at term (n=2718), nonlinear models with a quadratic term or natural cubic splines were compared to linear models using likelihood ratio test. P-value <.05 would have suggested improved model fit of the non-linear models. For the natural cubic splines model the knot was set at 37 weeks (for all children) or 40 weeks (for children born at term). All models were corrected for child sex and age at the neuroimaging assessment, maternal ethnicity, age at intake, marital status, educational level, psychopathology, smoking and alcohol use during pregnancy, and family income.

* Intracranial volume was additionally adjusted for.

eTable 5. The association of gestational age at birth in categories with global brain volumes

Group	Model	N	Total brain volume		Cerebral gray matter volume		Cerebral white matter volume	
			Difference (95% CI)	P-value	Difference (95% CI)	P-value	Difference (95% CI)	P-value
Term	Model 1	2718	Reference	-	Reference	-	Reference	-
Preterm		138	-35.4 (-51.6 to -19.1)	<.001	-22.1 (-30.7 to -13.5)	<.001	-8.8 (-16.1 to -1.4)	.02
Post-term		223	15.0 (2.0 to 28.0)	.02	10.2 (3.3 to 17.0)	.004	3.3 (-2.6 to 9.1)	.28
Term	Model 2	2718	Reference	-	Reference	-	Reference	-
Preterm		138	-26.5 (-42.1 to -11.0)	<.001[#]	-17.3 (-25.5 to -9.1)	<.001[#]	-5.8 (-12.9 to 1.4)	.12
Post-term		223	10.5 (-1.9 to 22.8)	.10	7.7 (1.2 to 14.2)	.02[#]	1.6 (-4.1 to 7.3)	.58

Group	Model	N	Cerebellar gray matter volume		Cerebellar white matter volume		Subcortical gray matter volume	
			Difference (95% CI)	P-value	Difference (95% CI)	P-value	Difference (95% CI)	P-value
Term	Model 1	2718	Reference	-	Reference	-	Reference	-
Preterm		138	-3.5 (-5.3 to -1.8)	<.001	-0.9 (-1.3 to -0.4)	<.001	-0.8 (-1.3 to -0.4)	<.001
Post-term		223	1.1 (-0.3 to 2.5)	.12	0.4 (0.03 to 0.8)	.03	0.7 (0.3 to 1.0)	<.001
Term	Model 2	2718	Reference	-	Reference	-	Reference	-
Preterm		138	-2.6 (-4.3 to -1.0)	.002[#]	-0.8 (-1.2 to -0.3)	.002[#]	-0.8 (-1.3 to -0.4)	<.001[#]
Post-term		223	0.7 (-0.6 to 2.0)	.31	0.4 (-0.01 to 0.7)	.06	0.7 (0.3 to 1.0)	<.001[#]

In the results of these regression models, the effect estimates represent the difference in cm^3 for brain volumes in preterm or post-term born children compared to term born children. Model 1 is a minimally adjusted model corrected for child sex and age at the neuroimaging assessment. Model 2 is a fully adjusted mode, corrected for child sex and age at the neuroimaging assessment, maternal ethnicity, age at intake, marital status, educational level, psychopathology, smoking and alcohol use during pregnancy, and family income.

* Intracranial volume was additionally adjusted for in both models.

[#] The associations survived a false discovery rate correction for multiple testing (applied model 2 only).

eTable 6. Non-response analysis

	Respondents (n=3079) [a]	**Non-respondents** (n=6339)	P-value
Maternal characteristics			
Age at intake, mean (sd), year	31.1 (4.9)	29.3 (5.5)	<0.001
Multiparous (%)	11.8	16.3	<0.001
Pregnancy complications (%) [b]	6.4	7.3	0.14
Ethnicity (%)			
Dutch	57.5	46.0	<0.001
Non-Dutch Western	12.2	11.1	
Non-Dutch Non-Western	30.3	43.0	
Educational level (%)			
Primary or lower	7.2	13.8	<0.001
Secondary	41.1	48.8	
Higher	51.7	37.4	
Family income, per month (%)			
Less than €1200	15.9	24.8	<0.001
€1200 to €2000	16.9	20.2	
More than €2000	67.2	55.1	
Marital status, married or with partner (%)	88.2	83.9	<0.001
Smoking during pregnancy (%)			
Never in pregnancy	77.3	71.3	<0.001
Until pregnancy was known	8.6	8.3	
Continued in pregnancy	14.1	20.4	
Alcohol use during pregnancy (%)			
Never in pregnancy	40.1	52.0	<0.001
Until pregnancy was known	13.9	12.6	
Continued in pregnancy, occasionally	36.3	29.3	
Continued in pregnancy, frequently [c]	9.7	6.1	
Psychopathology, mean (sd)	0.3 (0.4)	0.3 (0.4)	<0.001
Birth and child characteristics			
Cesarean delivery (%)	12.4	12.6	0.96
Suspected fetal distress (%)	7.7	7.8	0.88
Apgar at 5 minutes below 7 (%)	1.1	1.4	0.31
Gestational age at birth, mean (sd), week	39.9 (1.8)	39.7 (2.0)	<0.001
Range	3444.8 (554.1)	3391.4 (566.9)	<0.001
Birth weight, mean (sd), gram	49.8	51.1	0.25
Sex, boy (%)	10.1 (0.6)	10.2 (0.8)	0.68
Age at MRI scan, mean (sd), year	31.1 (4.9)	29.3 (5.5)	<0.001

Abbreviation: MRI, magnetic resonance imaging

Non-respondents are participants with data on gestational age at baseline, but no neuroimaging data at follow-up. P-values were derived from t-tests for or Wilcoxon tests for continuous variables and chi-square tests for categorical variables.

a. Imputed data were reported.

b. Included occurrence of preeclampsia, diabetes, and/or pregnancy induced hypertension.

c. Frequent continued alcohol use is defined as 1 or more glasses of alcohol per week in at least two trimesters.

Chapter 5

General Discussion

DISCUSSION

Investigations of child brain development that could underlie the associations of maternal adversities during the perinatal period with child cognitive and neuropsychiatric outcomes remain scarce. This thesis examined several perinatal determinants including maternal depressive symptoms, nutrient status, and gestational duration in relation to child brain morphology and white matter microstructure in a population-based neuroimaging study. In this chapter, the main findings are summarized and elucidated, methodological considerations for relevant studies are suggested, and clinical implications and future scientific directions are provided.

Main findings and interpretation

Perinatal determinants of child brain morphology

In chapter 2, we first investigated prenatal and postnatal maternal depressive symptoms in relation to offspring brain morphology, including white matter microstructure, in childhood and preadolescence. We showed that exposure to prenatal maternal depressive symptoms was associated with lower fractional anisotropy (FA) and higher mean diffusivity (MD) in specific tracts of the limbic system in children aged 6-9 years, although the lower FA in the cingulum could potentially be explained by genetic predisposition and shared environmental family factors. In the second study, we presented single-time-point analysis and trajectory analysis of maternal depressive symptoms, showing that maternal depressive symptoms during the perinatal period predicted lower total gray matter and total white matter volumes as well as lower FA in children at age 9-11 years. Maternal depressive symptoms at child age of 2 months likely play a more prominent role for offspring brain development than symptoms during pregnancy. Further, no evidence for associations of maternal depressive symptoms in childhood (i.e., at 3 years and 10 years) with offspring brain development at age 6-11 years was observed.

In chapter 3, multiple nutrients of the mother during pregnancy in relation to child brain development were examined. In chapter 3.1, no evident associations between maternal gestational vitamin D status at single assessments (i.e., in mid-gestation and upon delivery) and child brain morphology were found. However, compared to children with persistently sufficient vitamin D levels [i.e., 25(OH)D ≥ 50 nmol/L] from mid-gestation to birth, those persistently exposed to deficient vitamin D status [i.e., 25(OH)D < 25 nmol/L] displayed lower brain volumes and cortical differences, e.g., less surface area in the frontal lobe in the right hemisphere and less gyrification in the temporal lobe in the left hemisphere. In chapter 3.2, we reported that children exposed to maternal folate deficiency (i.e., <7 nmol/L) during pregnancy had lower total brain volume and white

matter volume. We also performed an analysis on repeated brain measures, showing that the global volumetric differences originated from the third trimester and persisted across childhood. In chapter 3.3 and 3.4, we examined maternal fatty acid concentrations during pregnancy and child brain development. The results suggested an inverted U-shaped association between maternal ω-3 polyunsaturated fatty acids (PUFAs) levels and child total gray and total white matter volumes. Further, we showed that higher maternal trans fatty acids (TFAs) concentrations during pregnancy predicted smaller fetal head circumference (HC) in the third trimester as well as lower fetal head growth rate across the second and third trimesters, and these findings were supported by an instrumental variable (IV) analysis using calendar time as the IV (i.e., TFAs concentration decreased with time due to the TFAs reduction initiatives in the Netherlands). We found no evidence for associations between maternal TFAs concentration during pregnancy and child global brain volumes at age 9-11 years.

Finally, we showed a linear association between gestational duration and child brain morphology in chapter 4.1, whereas most previous studies had focused on preterm children only. Specifically, children with a longer gestational duration had larger global and regional brain volumes, as well as larger cortical surface area and more gyrification at age 9-11 years. Consistent results were obtained when children born preterm and/or with maternal or obstetric complications were excluded from the analysis, suggesting that every week of gestational duration counts for brain development on the long term. These findings are in line with the WHO's recommendation on reducing unnecessary cesarean sections.

Table 5.1 presents an overview of the main findings of this thesis.

Some key messages conveyed from these findings deserve interpretation beyond the specific study context. First, in epidemiological studies, commonly reported statistics such as p-value suggest the presence of an effect rather than its magnitude, while effect size describes the strength of an effect that is independent of sample size and thus provides more insight into the clinical importance of the finding.[1] Depending on the independent variable of interest (i.e., continuous vs. categorical), Cohen's f^2 or Cohen's d (Hedges' g when the sample size is below 20) can be used to measure effect size in a regression model, with Cohen's $f^2 < 0.02$ or Cohen's $d < 0.2$ representing small effect sizes.[2,3] In chapter 2.2, we reported the Cohen's f^2 measures for the association of maternal depressive symptoms at postnatal 2 months with offspring total gray matter volume and white matter integrity. Both estimates suggested small effect sizes (Cohen's f^2=0.004 and 0.003, respectively). When focusing on child total brain volume (TBV) as an outcome of interest in the other studies, exposure to persistent 25(OH)D deficiency from mid-gestation to delivery (chapter 3.1, Cohen's d=0.35) suggested a small to medium effect, while gestational folate deficiency (chapter 3.2, Cohen's d=0.015) and gestational duration (chapter 4.1, Cohen's f^2=0.008) both suggested small effects.

Table 5.1. Overview of main findings

Determinant	Measurement	Time of assessment	Brain volumes	Cortical metrics	White matter microstructure	Cognitive and Neuro-psychiatric outcomes
maternal depressive symptoms	BSI	mid-gestation	TGM (-), TWM (-)	NA	individual tract FA↓, MD↑	NA
		postnatal 2 months	TGM↓, TWM (-)	NA	global FA↓, global MD (-)	attention problems at 10 years↑ (mediated by TGM volume)
		(perinatal high)	TGM↓, TWM↓	NA	global FA↓, global MD (-)	NA
		childhood (3 years and 10 years)	TGM (-), TWM (-)	NA	-	NA
vitamin D	25(OH)D, in nmol/L	mid-gestation (maternal plasma)	TB (-), CGM (-), CWM (-), CB↑	CT (-), SA (-), GR (-)	NA	NA
		at delivery (cord blood)	TB (-), CGM (-), CWM (-), CB (-)	CT (-), SA (-), GR (-)	NA	NA
		(persistently low)	TB↓, CGM↓, CWM↓	CT (-), SA↓, GR↓	NA	NA
folate	folate, in nmol/L	early gestation (maternal plasma)	TB↑, CGM(-), CWM↑, SGM (-)	CT (-), SA (-), GR (-)	NA	internalizing score (-), externalizing score (-), at 10 years
PUFAs	ω-3 PUFAs, in wt:wt %	mid-gestation (maternal plasma)	TGM↑, TWM↑ (U-Shaped relation)	NA	global FA (-), global MD (-)	academic performance at 12 years↑
	ω-6 PUFAs, in wt:wt %	mid-gestation (maternal plasma)	TGM (-), TWM (-)	NA	global FA (-), global MD (-)	NA
TFAs	wt:wt %	mid-gestation (maternal plasma)	3rd trimester HC↓, IC (-), TB (-)	NA	NA	NA
GAB	week	conception to birth	TB↑, CGM↑, CWM↑, CBGM↑, CBWM↑, SGM↑	CT (-), SA↑, GR↑	NA	NA

BSI, Brief Symptom Inventory; PUFAs, polyunsaturated fatty acids; TFAs, trans fatty acids; GAB, gestation age at birth; IC, intracranial; TB, total brain; TGM, total gray matter; TWM, total white matter; CGM, cerebral gray matter; CWM, cerebral white matter; CB, cerebellum; SGM, subcortical gray matter; CBGM, cerebellar gray matter; CBWM, cerebellar white matter; HC, head circumference; CT, cortical thickness; SA, surface area; GR, gyrification; FA, fractional anisotropy; MD, mean diffusivity; NA, not applicable (not investigated). ↑ indicates positive association; ↓ indicates inverse association; (-) indicates null association.
Brain measures were collected in children aged 9-12 years unless otherwise specified. Only primary findings are shown.

Adjusting for covariates in observational studies contributes to better control of confounding bias with the price of reduced effect estimates. This is particularly relevant in the current work, where we adjusted for multiple factors on socioeconomic status (SES, e.g., maternal education and family income) and lifestyle (e.g., maternal diet, smoking, and alcohol use), which are commonly correlated with each other. In addition, the small effect sizes of maternal depressive symptoms and gestational duration are not surprising, because we analyzed all determinants along a continuum and that has the advantage of enabling researchers to detect subtle effects.[4] It is also important to note that birth weight can be a potential intermediate in the relation between maternal depressive symptoms and child brain development, thus adjusting for birth weight in the regression (chapter 2.1 and 2.2) takes away the indirect effect from the total effect.[5] The population-based nature of the Generation R Study may also explain the small effect sizes we reported, because the effects of exposure to adversities in pregnancy may be underestimated in a relatively healthy population with few subjects being exposed. However, importantly, small effect sizes cannot be interpreted as being ignorable.[6] This can be particularly true for the health of a population level. In addition, it is likely that exposure to multiple adversities during the perinatal period exerts cumulative effects on child brain development, and the cumulative effects can be of particular clinical relevance for low- and middle-income countries where undernutrition is still common among pregnant women.[7,8] Furthermore, characterizing effect sizes as small, medium, or large using the global conventions has been questioned, especially when differences between disciplines and between study designs are dramatic.[9] Instead, it is recommended to relate findings to comparable studies with similar characteristics.[10] This can be implemented in the future with the advance of large-scaled neuroimaging studies.

Second, due to limited prior knowledge, we used an exploratory and hierarchical approach that started with examining global brain morphological and white matter microstructure measures followed by investigating specific regional structures. Overall, our studies showed that the investigated perinatal determinants were related to global brain measures including TBV, total gray and white matter volumes, and global FA rather than regional measures such as the volumes of the amygdala and hippocampus. Most likely, the global effects can be explained by the rapid and tremendous brain growth in fetal life that is not specific to certain structures. In addition, because nutrients such as vitamin D and folate are involved in multiple physiological functions, the impact of exposure to insufficient levels during fetal life on brain development can be systematic, i.e., not limited to the generation and growth of specific brain cells. Moreover, methods used to investigate effects on regional brain structures independent of global effects deserve discussion. Three methods are commonly used to adjust for head size (e.g., intracranial volume or TBV) to remove global effects: 1) the proportion approach, namely dividing the region of interest (ROI) volumes by the measure of

head size; 2) the analysis of covariance (ANCOVA) approach, namely adjusting for the head size as a covariate in the regression model; and 3) the residual approach, namely regressing the ROI volume on the head size and extracting the individual residuals as the new dependent variable in the regressions of primary interest. Although aiming at the same purpose, these methods are not necessarily interchangeable in all settings. For example, the ANCOVA approach is superior to the proportion approach when the ROI volumes grow at a proportionally slower rate than the global brain volume.[11] Therefore, different adjustment approaches may yield heterogeneous results, although we only used the ANCOVA approach without comparing results using all strategies in the current work. Also, notably, by removing global effects, the research question changes from 'whether there is an absolute volumetric difference of the ROI' to 'whether there is a relative volumetric difference of the ROI (i.e., the volumetric difference of the ROI is not proportional to that of global brain volume)', and their interpretations are also different. Here I use subcortical gray matter volume as an example. In chapter 3.2, we could conclude that prenatal exposure to folate deficiency was related to an absolute lower subcortical gray matter volume that could be completely explained by the lower global brain volume, thus intrauterine low folate levels likely exert a global effect on long-term brain development. In contrast, gestational duration was positively related to subcortical gray matter volume regardless of adjustment for intracranial volume, suggesting that the absolute larger subcortical gray matter also counts for a larger proportion of the whole brain. Absolute subcortical volumes are often collected when establishing the volume trajectory across the lifespan,[12] while relative subcortical volumes are mostly used in clinical research to investigate the morphological basis for neuropsychiatric outcomes.[13-15] In this thesis, we chose not to adjust for intracranial volume in analyses of global measures such as TBV and total gray and white matter volumes given their high correlations (i.e., around or above 0.9) that are commonly seen in pediatric populations. We decided not to adjust for intracranial volume when examining less global measures such as cerebellar volume and frontal lobar volume either, because they are moderately-to-highly correlated with intracranial volume (i.e., 0.6 to 0.8). However, there could be some space for augment and debate concerning these structures.

Child brain morphology and neurocognitive outcomes

One central goal of neuroimaging studies is to translate findings to clinical outcomes.[1] While our primary goal was to identify perinatal determinants of child brain morphometry, we also examined if the observed brain differences were associated with cognitive and neuropsychiatric outcomes using mediation analysis.[16,17] In chapter 2.2, we reported that total gray matter volume mediated the relation between maternal depressive symptoms at postnatal 2 months and child attention problems at age 10

years. However, in the other studies of this thesis there was no evidence for mediation by brain morphology in the associations of the various determinants with cognitive and behavioral outcomes after adjustment for covariates.

Several explanations for the few associations with neuropsychiatric outcomes mediated by brain morphological differences are possible. First, null findings of the fully corrected mediation analyses may be attributed to insufficient statistical power. Although we had large sample sizes for neuroimaging analyses, not all children had neuropsychiatric data that could be used for mediation analyses. Except for chapter 2.2 where the Brief Problem Monitor (BPM) data were available in 1760 children, we commonly ended up with much smaller sample sizes for mediation analyses. For example, we showed in chapter 3.3 that an indirect effect was only found in the unadjusted model with a sample size of 484. Covariates might explain the heterogeneous findings, but the possibility that the mediation analysis became underpowered due to the inclusion of model parameters (i.e., covariates) could not be ruled out.[18] Second, we used child-report BPM in chapter 2.2 and maternal-report Child Behavior Checklist (CBCL) in chapter 3.2 to assess child emotional and behavioral problems, and the CITO (Centraal Instituut voor Test Ontwikkeling, English: Central Institute for Test Development) test (chapter 3.3) to indicate child cognitive performance. These specific measurements obtained from a certain informant were selected in accordance with the study design to reduce bias due to single informant (i.e., systematically biased responses), because other informants had reported on the determinant.[19] However, it is important to note that significant within and between-subject variation of behavior and cognition may be observed in the specific contexts.[20] Indeed, previous work in our research group has shown divergent scores when using child emotional and behavioral data reported by different informants.[21] Therefore, a null finding from the mediation analysis can be attributed to child neurocognitive measures of inadequate validity due to not combining multi-informant data.[22] Third, since most of the neuropsychiatric outcomes used in the current thesis were collected at the same age as the neuroimaging assessment (i.e., 9-11 years), it is possible that offspring mental disorders with elevated incidence in adolescence and early adulthood, such as depression and schizophrenia, can only be predicted by brain morphological differences occurring earlier in life, thus with a latency period.[23,24] Importantly, the ongoing data collection of the Generation R Study using a clinical psychiatric interview between the age of 17-19 years will enable such follow-up investigations.

Methodological considerations

Assessment of nutrients

Nutritional data in most large epidemiological studies in humans are based on questionnaire-based food intake measurements, which are inherently challenged by information bias.[25] In chapter 3.2, we observed a positive relation between maternal plasma folate concentration and child total brain volume, whereas no association was found when examining maternal folic acid supplementation use after adjusting for all covariates. Despite the self-report nature that affects reliability, folic acid supplementation use is correlated with SES indicators including education and employment status (a proxy of income).[26] Consequently, adjusting for SES indicators in the regression model likely also takes away the effects of folic acid supplementation. In addition, absorption function of the gastrointestinal tract and genetic variance play vital roles in the metabolism of nutrients such as folate and fatty acids, leading to a low correlation between dietary questionnaire assessments and circulating biomarkers.[27-29] Therefore, biomarkers of food intake should be preferred when the goal is to validly assess the bio-availability of nutrients with minimal misclassification.[30]

Moreover, it is common that various biomarkers can be used to measure one nutrient, and biomarkers can be categorized into short-term (reflecting intake over past hours/days), medium-term (reflecting intake over weeks/months) and long-term markers (reflecting intake over months/years) depending on the type of sample used.[31] For example, maternal folate was assessed from venous blood and expressed as plasma concentration in our study (chapter 3.2), but folate availability can also be quantified using red blood cell (RBC, or erythrocyte) folate assay. Compared to plasma folate, RBC folate is less correlated with homocysteine and may give a better assessment of folate status in special cases such as after hemodialysis and between pregnancies, but its assay has higher costs.[32] There is evidence showing that RBC folate measurements and plasma folate measurements provide equivalent information on identifying folate deficiency,[33] although the relation between RBC and plasma folate concentrations can be modified by factors such as BMI and genotype.[34] In addition, plasma folate concentrations are more sensitive to folic acid supplementation than RBC folate concentrations.[35] Given a lack of indications, we see no superiority of using one measure over the other in our study context. Similarly, both plasma fatty acids and RBC fatty acids can be assessed to quantify fatty acid availability, and they are moderately to strongly correlated with fatty acid intake assessed by food frequency questionnaire (FFQ). However, RBC fatty acids are superior to plasma fatty acids as biomarkers reflecting long-term fatty acids intake given the much longer half-life of RBC than that of plasma lipoproteins.[36] Since fatty acids were only assessed once during pregnancy in our studies (chapter 3.3 and 3.4),

maternal RBC fatty acids might be a better measure than plasma fatty acids to reflect the overall fatty acids levels throughout gestation.

Longitudinal data modeling

Longitudinal data with repeated measurements over time are common especially in psychological and clinical research. Analysis associating determinants assessed at each time point with the outcome separately (or vice versa) can be easily performed to examine cross-sectional associations. However, modeling repeatedly measured determinants or outcomes simultaneously provides valuable insight on potential interaction of the determinant with time and/or cumulative effects. Due to the correlation between repeatedly measured data, conventional regressions that are subject to the 'independence' assumption cannot be used for obtaining valid results. In this section, three techniques used for modeling repeated measurements in this thesis are elaborated, namely growth mixture model (GMM)/latent class growth analysis (LCGA, chapter 2.2), linear mixed model (LMM, chapter 3.2), and two-stage mixed effect model (chapter 3.4).[37] Other methods such as generalized additive mixed model (GAMM) and clustering are also available to handle repeatedly measured data,[38] but they are not discussed here in detail.

For illustrative purpose I start with methods based on linear regression. LMM contains both fixed effect (i.e., predictions on population level) and random effects (i.e., predictions accounting for individual variance) which allow for modeling dependent data, and an interaction term between determinant and time variable can be included to explore whether the effect of the determinant at baseline on the outcome of interest changes over time along the follow-up. In chapter 3.2, we applied LMM to investigate maternal folate status during pregnancy in relation to offspring brain developmental trajectories across fetal life and childhood, and found no interaction between maternal gestational folate status and child age, suggesting that the differences in global brain volume we observed in late childhood originated from the third trimester and persisted throughout childhood, thus there was no evidence for accelerated brain growth of children exposed to deficient folate status in gestation at this stage. In addition, LMM can be extended to generalized linear mixed model (GLMM) to cope with outcome data from non-normal distributions (e.g., binomial or Poisson).

However, one caveat of LMM is that it cannot be used when the determinants, instead of the outcomes, are repeatedly measured. In such case, two-stage mixed effects model relating individual patterns of determinant to outcome is a viable approach that can be easily implemented. The main idea of this method is to model the repeatedly measured determinant X_{ik} using a random intercept and random slope: $X_{ik} = \theta_{0i} + \theta_{1i} \times t_{ik} + \varepsilon_{ik}$, where k represents the measurement occasion. The person-specific random intercepts θ_{0i} and slopes θ_{1i} jointly describe the pattern of exposure for individual i, which can be then associated with the outcome.[37] Although this approach is mostly developed for

repeatedly measured determinants, it can be used for repeatedly measured outcomes as well. For example, we applied this method in chapter 3.4 to only examine the individual slopes of fetal HC repeatedly measured in the second and third trimesters (i.e., fetal HC growth rate between the two assessments) because we found no differences in the intercepts (i.e., fetal HC in the second trimester), and we found children exposed to higher maternal TFAs concentrations during pregnancy had slower HC growth. Using LMM yielded consistent results in our study, but whether results from two-stage mixed effects model and LMM are also comparable when modeling outcome data of three or more time points warrants further investigations. It is also important to note that, we used these approaches in the above studies because brain growth across mid-gestation and childhood is approximately linear.[39-41] When non-linear relations are suspected, GAMM and/or addition of polynomial terms (or splines) should be considered, with the price of complicating the interpretation.

GMM and LCGA are latent variable mixture modeling (LVMM) used for longitudinal data (latent profile analysis or latent class analysis is commonly used for cross-sectional data).[42,43] Conventional regression belongs to variable-centered approaches with the assumption that all individuals come from a single population with common parameters. In contrast, LVMM is a person-centered approach focusing on similarities and differences among people. For example, although allowing for random effects, LMM assumes that the growth trajectory of all individuals can be well described using a single estimate. However, GMM/LCGA assumes heterogeneity of growth trajectories within the whole population.[44] In addition, GMM/LCGA applies to both determinant and outcome data with repeated measurements and is very feasible to capture non-linear relations by adding polynomial terms based on model fit comparison. In chapter 2.2, given the complexity of the course of depression and previous evidence on heterogeneity of maternal depressive symptoms during the perinatal period, we applied LCGA to model maternal depressive symptoms trajectories from gestation to childhood. Interestingly, we identified a group of women with persistently clinically relevant depressive symptoms across all four assessments, which was related to smaller brain and altered white microstructure of the offspring. This finding complements the single-time-point analyses, suggesting the potential cumulative effect of exposure to maternal depression during the perinatal period on brain development in childhood. LCGA is a special case of GMM whereby within-class variance is set to zero, assuming homogeneous individual growth trajectories within classes. We used LCGA in this study because it contributes to clearer identification of classes, which is in line with our focus on between-group difference. In addition, LCGA has substantially less computational burden than GMM, especially in large samples with numerous model parameters where GMM can easily fail due to statistical problems.[44] Further, LVMM determines the most likely membership based on posterior probability, thus uncertainty in grouping raises a

Table 5.2. Comparison of the three methods for modeling repeatedly measured data

Methods	Determinant	Outcome	Linear assumption
LMM	no	yes	yes*
two-stage mixed effects model	yes	yes	yes*
GMM/LCGA	yes	yes	no

* Polynomial terms or splines can be used for non-linear relations.

threat of misclassification of individuals and biased estimates of the relations between the latent classes and the auxiliary variables.[45] Several approaches are proposed to address this issue, but there is a lack of universal solutions.[46]

The basic features of the three methods discussed in this section are summarized in Table 5.2.

Positivity violation in epidemiological studies

In epidemiological studies using observational data, the importance of adequate control for confounding variables for causal inference is well-known. Moreover, although perhaps less-recognized, sufficient variability in treatment (or exposure) assignment within strata of confounders, termed as the positivity assumption, is also required to obtain valid effect estimates. Violations and near violations of the positivity assumption can increase both the variance and bias of causal effect estimates.[48] Positivity violations in observational data may be deterministic or random. Deterministic violation occurs when participants at 1 or more levels of the confounders cannot receive at least 1 level of the exposure due to study design or theoretical impossibility, while random violation takes place by chance and is commonly seen when the sample size of specific treatment groups is small given a relatively large number of confounders.[49] Chapter 3.2 gives a typical example of random positivity violation: when maternal plasma folate levels were dichotomized using 7 nmol/L, only 103 children were exposed to folate deficiency during pregnancy. The estimated propensity score (i.e., the probability of being exposed to folate deficiency) given all covariates was very small, indicating near violation of positivity.

Considering the trade-off between bias due to positivity violations and bias due to confounding is recommended.[50] Practical approaches for this aim include restricting the adjustment set (i.e., excluding covariates), restricting the sample (i.e., discarding classes of subjects), changing the intervention of interest, and utilizing causal effect estimators that are robust to positivity violations.[48] Compared to naïve regression, G-computation and inverse probability of treatment weighting (IPTW), target maximum likelihood estimation (TMLE) is a semiparametric method estimating double-robust estimators that are less subject to bias due to positivity violation as well as model misclassification.[51] Therefore, in the sensitivity analysis of chapter 3.2, we applied TMLE

with the *SuperLearner* package that improves the performance of TMLE by adaptively combining different machine-learning algorithms, and obtained consistent results with the primary analysis, suggesting the validity of our findings. However, the slightly different effect estimates (and confidence intervals) obtained from the naïve regression and TMLE may be worth noting, especially when the precision of effect estimation is crucial. Moreover, since TMLE is an emerging causal inference technique that has not been extensively used in epidemiological studies, further empirical investigations are needed to evaluate its performance in addressing positivity violation in comparison with other approaches.

Clinical implications

Although it is often challenging to utilize group-level differences identified in neuroimaging studies to aid decision making for an individual in clinical practice,[52] several clinical and public health implications can be delivered from the findings of this thesis.

Needs for perinatal maternal depression screening and intervention

Our studies show that maternal depressive symptoms during the entire perinatal period may contribute to suboptimal brain development of the offspring, and that the substantial brain morphological differences may underlie neuropsychiatric outcomes in childhood. These findings highlight the importance of screening and intervening maternal depression throughout the perinatal period to prevent abnormal brain development of the offspring, which can hardly be performed with current medical technologies. A systematic review shows that the prevalence of maternal depression is significantly higher in low- and middle-income countries compared to high income countries in both the prenatal and postnatal periods.[53] However, most of the screening and interventions programs targeting perinatal maternal depression are conducted in high-income countries. Furthermore, even in these high-income countries, the uptake rates of referrals to on-site assessment or treatment after screening with positive results are less than 50%.[54] Therefore, policy makers and public health practitioners should increase investments on facilitating cost-effective maternal depression screening and interventions in low and middle income countries, and it is critical for physicians and mental health professionals to promote engagement in perinatal depression interventions after screening among women.

Hints on nutritional recommendations

Chapter 3 of this thesis investigated several nutrients of the mother during pregnancy in relation to child brain development, shedding lights on making proper recommendations on nutrients for clinicians and nutritionists. First, we showed that although maternal vitamin D levels at single assessments during pregnancy were barely related

to child brain development at 10 years, brain morphological differences such as lower volume and less gyrification were observed in children exposed to persistently low (i.e., insufficient or deficient) vitamin D levels from mid-gestation to birth, and these children counted for 57.8% of those with data at both assessments. These findings suggest cumulative effects of exposure to inadequate nutrients in utero, advocating maintaining sufficient levels of essential nutrients, including but not limited to vitamin D, throughout the entire pregnancy for offspring brain development. Second, in addition to using dichotomized folate status using the cut-off value established based on hematologic outcomes or risk of neural tube defects (NTDs), we found a positive linear association between maternal plasma folate concentrations along a continuum and child total brain volume at age 10 years. Despite the exploratory nature of our study, and the fact that amending nutritional recommendations can be a sophisticated process involving enormous work such as randomized controlled trials (RCTs) and economic assessments,[55,56] it may be worth thinking about developing cut-off values of nutritional biomarkers that benefit the overall health in both short and long terms. Third, industrial trans fatty acids content in food is a global public health concern because it is well-known to increase the risk of adverse cardiovascular events. Our study showed that higher trans fatty acids levels of the mother during pregnancy also predicted smaller HC and slower HC growth of the fetus, justifying the importance of reducing trans fatty acids for an optimal neurodevelopment of the offspring. This message is particularly relevant in countries and regions where industrial trans fatty acids are still widely used.

Reduce cesarean sections for a natural gestational duration

Cesarean sections have become increasingly common worldwide since 1985, when the ideal rate for cesarean sections was considered to be between 10% and 15%.[57] Latin America and the Caribbean region had the highest rates of cesarean section (i.e., more than 40%) in 2015, while the eastern Europe and central and south Asia regions experienced the most rapid increase between 2000 and 2015.[58] In the most updated recommendation, the WHO concludes that cesarean sections are only required for medical indicated reasons.[57] Despite the previous findings on the long-term outcomes such as increased risk of asthma and obesity in children with a cesarean delivery,[59] in chapter 4.1 of this thesis we showed that shortened gestational duration as a byproduct of cesarean sections was associated with a smaller brain in children, even among those born after 37 weeks of gestation. In line with the current WHO recommendations on reducing unnecessary cesarean sections, these findings provide evidence for long-term impact of shortened gestational duration on child brain development. However, with the current evidence it remains controversial to advocate late intervention on delivery, because post-term birth has been related to negative cognitive outcomes and more

emotional and behavioral problems in childhood and adolescence.[60,61] In addition, a recent meta-analysis of RCTs suggests better perinatal outcomes such as lower mortality and fewer neonatal macrosomia in nulliparous women undergoing induction of labor at 41 weeks than those receiving expectant management until 42 weeks.[62] Further investigations, preferably RCTs, may contribute to determining a potential gestational duration that optimizes neurodevelopment in childhood and beyond while taking into account other health aspects.

Future scientific directions

Based on the Developmental Origins of Health and Disease (DOHaD) theoretical frame, this thesis focused on disentangling the research gap: little is known about the perinatal determinants of child brain development. Further studies are warranted to address caveats of the current studies and examine follow-up questions in this scientific direction. First, the studies in this thesis primarily focused on brain morphology at age 9-11 years. The growth of the human brain continues across childhood and early adulthood with a non-linear developmental pattern as a result of cortical thinning that typically accelerates in adolescence.[63] To track the subsequent brain development over a life course, studies examining neuroimaging outcomes at later ages are needed. Also, diffusion tensor imaging (DTI) measures of individual tracts deserve more research because they may provide more information on specific brain functions and conditions than the global measures. Second, we could not conclude an inverse relation between prenatal exposure to high ω-3 PUFAs levels and brain volume in childhood because of limited subjects being exposed that resulted in wide confidence intervals of the estimates. Follow-up studies should recruit sufficient pregnant women with high ω-3 PUFAs concentrations to justify whether the inverse relation exists, or there are simply ceiling effects. In addition, previous studies on ω-3 PUFAs mostly focused on a few ω-3 PUFAs such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), but our findings show that neither EPA nor DHA counts for the relation between prenatal ω-3 PUFAs exposure and total gray matter volume in childhood. Therefore, the role of other individual ω-3 PUFAs in brain development awaits further investigations. Third, the observational nature impeded us from inferring causal relations. Future studies using an RCT design (if feasible) or implementing methods such as IV analysis and target trial emulation in observational studies can help justify causality, based on which policies, guidelines and recommendations can be developed.[64] Last but not the least, in addition to environmental determinants, genetic and epigenetic mechanisms cannot be ignored in brain development and functioning.[65,66] Genetic variants can also be used in Mendelian randomization for causal inference. Our attempts to incorporate genetic and epigenetic variables into the present work were not successful because of

insufficient power due to limited subjects with complete information. Future studies including adequate genetic and/or epigenetic data are needed.

Conclusion

The human brain is such a complex organ of our body that I, perhaps also many others, constantly wonder whether it is a paradox that the brain is studied by the human whose mind is produced by the brain itself. Our exploration of the brain may never end, and I would be pleased if this work contributes to a small step forward in this brain research marathon. Regardless of all caveats, and despite the fact that some questions remain not fully answered and that new knowledge makes new questions arise, our work highlights the importance of maintaining a good mental and nutritional status during the perinatal period not only for the health of the mother, but also for optimal brain development of the next generation.

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Chapter 6

Summary / Samenvatting

SUMMARY

Based on previous evidence suggesting associations of exposure to adversities during the perinatal period with cognitive and neuropsychiatric outcomes, this thesis investigated perinatal determinants of brain development in a prospective population-based cohort of children and their parents.

Following a brief overview of the human brain structure and developmental process, chapter 1 elaborates the 'Developmental Origins of Health and Disease' (DOHaD) hypothesis that serves as the theoretical framework for the current efforts, and illustrates prior knowledge on the topics of interest. The general and specific aims and setting of this thesis are described at the end of this chapter.

In chapter 2, we investigated maternal depressive symptoms in relation to child brain development. Chapter 2.1 shows that maternal depressive symptoms during pregnancy were related to differences in white matter microstructure in the uncinate fasciculus and the cingulum bundle in children aged 6-9 years, but some of these associations could be confounded by genetic predisposition or shared family factors (as paternal exposure to depressive symptoms also showed association, albeit with smaller effect estimates). In chapter 2.2, we used data on maternal depressive symptoms assessed repeatedly from pregnancy to late childhood and child neuroimaging data at age 9-11 years. The single-time-point analysis showed that maternal depressive symptoms at 2 months postnatally predicted lower total gray matter volume in children aged 9-11 years, which also mediated the relation between postnatal maternal depressive symptoms (2 months) and child attention problems assessed at the 9-11 years visit. The latent growth class analyses further suggested that children born to mothers with persistent elevated depressive symptoms across the perinatal period had lower total gray and white matter volumes as well as reduced global fractional anisotropy. Maternal depressive symptoms assessed at 3 years or 10 years were not related to child brain development.

In chapter 3, we examined the associations between intrauterine exposure to several nutrients and brain development in childhood. In chapter 3.1, although we found little evidence for associations between maternal vitamin D [quantified by 25(OH)D] concentrations at single assessments and child brain morphology, children exposed to persistently deficient vitamin D levels [25(OH)D < 25 nmol/L] showed less total gray and total white matter volumes, as well as regional cortical differences such as smaller surface area and less gyrification. These findings suggest the importance of maintaining adequate vitamin D levels throughout the second half of gestation on favorable brain development in childhood. As a follow-up of the previous work of our research group, chapter 3.2 investigated maternal folate levels during pregnancy and child brain morphology assessed at 9-11 years, showing that children exposed to folate deficiency (i.e., maternal plasma folate concentration < 7 nmol/L) in utero had lower total brain

volume and smaller cerebral white matter compared to the control group. Moreover, the analysis of repeated measurements showed that the reduced global brain volume originated from the third trimester and persisted until late childhood. In chapter 3.3 and 3.4, we explored maternal fatty acids status in relation to child brain development. First, we found an inverted U-shape relation between maternal plasma ω-3 polyunsaturated fatty acids (PUFAs) concentrations and child total gray and white matter volumes at age 9-11 years, albeit the inverse association at the high-end was not clear given the broad confidence intervals. A similar relation was found between maternal docosahexaenoic acid (DHA) concentrations and child total white matter volume. No associations were observed between maternal PUFAs levels during pregnancy and child white matter microstructure. Next, we focused on trans fatty acids (TFAs), a group of fatty acids that are mainly artificial and mostly well-known for their adverse effects on cardiovascular and metabolic health. Using conventional regression, we found that higher maternal TFAs concentrations predicted smaller fetal head circumference in the third trimester and slower head growth from the second to the third trimester, although no differences in global brain volumes of the child at age 9-11 years were observed. These findings were further supported by an instrumental variable analysis using the calendar time of maternal TFAs assessment as the instrumental variable.

In chapter 4, we investigated the association of gestational age at birth along a continuum with child brain morphometry at age 9-11 years. The results showed that gestational age at birth was positively and linearly associated with global and regional brain volumes, as well as widespread surface area and gyrification of the child. These associations remained consistent, even in the absence of preterm birth or complications. This study suggests that every gestational week counts for brain development and thus unnecessary cesarean sections should be reduced, which is line with the WHO recommendation.

Finally, chapter 5 summarizes and interprets the main findings from the above studies, elucidates methodological considerations, highlights clinical implications, and gives future scientific directions.

SAMENVATTING

Op basis van eerder bewijs dat blootstelling aan moeilijkheden tijdens de perinatale periode en cognitieve en neuropsychiatrische uitkomsten geassocieerd zijn, onderzocht dit proefschrift perinatale determinanten van hersenontwikkeling in een prospectief populatiecohort van kinderen en hun ouders.

Na een kort overzicht van de structuur en het ontwikkelingsproces van de menselijke hersenen, wordt in hoofdstuk 1 de 'Developmental Origins of Health and Disease' (DOHaD) -hypothese uitgewerkt die dient als het theoretisch raamwerk voor de huidige studies en beschrijf ik bestaande kennis over de besproken onderwerpen. De algemene en specifieke doelen en setting van dit proefschrift worden aan het einde van dit hoofdstuk beschreven.

In hoofdstuk 2 onderzochten we depressieve klachten van de moeder in relatie tot de hersenenontwikkeling van kinderen. Hoofdstuk 2.1 laat zien dat depressieve klachten van de moeder tijdens de zwangerschap gerelateerd waren aan verschillen in de microstructuur van witte stof in de uncinate fasciculus en de cingulum-bundel in kinderen van 6-9 jaar. Sommige van deze associaties kunnen vertroebeld worden door genetische aanleg of gedeelde familiefactoren (blootstelling van vaders aan depressieve klachten vertoonde bijvoorbeeld ook associaties, hoewel de grootte van het effect kleiner was). In hoofdstuk 2.2 hebben we gegevens over depressieve klachten van de moeder, die herhaaldelijk zijn gemeten vanaf de zwangerschap tot de late kinderjaren, en neuroimaging-gegevens van kinderen op de leeftijd van 9-11 jaar gebruikt. De analyse waarin één tijdspunt meegenomen is, toonde aan dat maternale depressieve klachten op 2 maanden postnataal een lager totaal grijze stof volume voorspelden in kinderen van 9-11 jaar. Dit lagere grijze stof volume medieerde ook de relatie tussen postnatale maternale depressieve klachten (2 maanden) en aandachtsproblemen in kinderen op 9-11 jaar. De latent class growth analyses suggereerden verder dat kinderen van moeders met aanhoudend verhoogde depressieve klachten gedurende de perinatale periode lagere totale grijze en witte stof volumes, en ook verminderde globale fractionele anisotropie hadden. Maternale depressieve klachten, beoordeeld op 3 jaar of 10 jaar, waren niet gerelateerd aan de ontwikkeling van de hersenen van kinderen.

In hoofdstuk 3 onderzochten we de associaties tussen intra-uteriene blootstelling aan verschillende voedingsstoffen en de ontwikkeling van de hersenen tijdens de kindertijd. In hoofdstuk 3.1 vonden we weinig bewijs voor associaties tussen maternale vitamine D [gekwantificeerd door 25(OH)D] concentraties (gemeten op één moment) en de hersenmorfologie van kinderen. Kinderen die werden blootgesteld aan aanhoudende vitamine D deficiëntie [25(OH)D <25 nmol/L] vertoonden lagere totale grijze en totale witte stof volumes, maar ook regionale corticale verschillen zoals een kleiner oppervlak en minder gyrificatie. Deze bevindingen suggereren dat het belang van is gezonde

vitamine D spiegels te behouden gedurende de tweede helft van de zwangerschap voor een gunstige hersenontwikkeling tijdens de kindertijd. Als vervolg op eerder werk van onze onderzoeksgroep, onderzocht hoofdstuk 3.2 de foliumzuur spiegels van de moeder tijden de zwangerschap en de hersenmorfologie van het kind beoordeeld op 9-11 jaar. Hierin werd aangetoond dat kinderen die werden blootgesteld aan een foliumzuurdeficiëntie (d.w.z., foliumzuur concentratie van de moeder <7 nmol/L) in utero, een lager totaal hersenvolume en lager cerebraal witte stof volume hadden, vergeleken met de controlegroep. Bovendien toonde de analyse met herhaalde metingen aan dat het verminderde globale hersenvolume ontstond in het derde trimester en bleef tot in de late kinderjaren bestaan. In hoofdstuk 3.3 en 3.4 hebben we de maternale vetzuurstatus onderzocht in relatie tot de hersenenontwikkeling van kinderen. Ten eerste vonden we een omgekeerd U-vormige relatie tussen maternale plasmaconcentraties van ω-3 meervoudig onverzadigde vetzuren (Engelse afkorting: PUFAs) en de totale grijze en witte stof volumes van kinderen op de leeftijd van 9-11 jaar, hoewel de omgekeerde associatie aan de bovenkant van het spectrum niet duidelijk was door de brede betrouwbaarheidsintervallen. Een vergelijkbare relatie werd gevonden tussen de docosahexaeenzuur concentraties van de moeder en het totale witte stof volume van het kind. Er werden geen associaties gevonden tussen maternale PUFA spiegels tijdens de zwangerschap en de microstructuur van witte stof in kinderen. Vervolgens hebben we ons gericht op transvetzuren (Engelse afkorting: TFAs), een groep vetzuren die voornamelijk kunstmatig is en vooral bekend staat om nadelige effecten op de cardiovasculaire en metabolische gezondheid. Door conventionele regressie te gebruiken, ontdekten we dat hogere TFA concentraties van de moeder een kleinere hoofdomtrek van de foetus in het derde trimester en een langzamere groei van het hoofd tussen het tweede en het derde trimester voorspelden, hoewel er geen verschillen werden waargenomen in het globale hersenvolume van het kind op de leeftijd van 9-11 jaar. Deze bevindingen werden verder ondersteund door een analyse van instrumentele variabelen waarbij de kalendertijd van de meting van TFAs bij de moeder als instrumentele variabele werd gebruikt.

In hoofdstuk 4 onderzochten we de associatie van zwangerschapsduur bij de geboorte als een continuüm met de morfometrie van de hersenen van kinderen op de leeftijd van 9-11 jaar. De resultaten toonden aan dat de zwangerschapsduur bij de geboorte positief en lineair geassocieerd was met globale en regionale hersenvolumes, maar ook met oppervlakte en gyrificatie in een groot deel van de hersenen van het kind. Deze associaties bleven consistent, zelfs zonder vroeggeboorte of complicaties. Deze studie suggereert dat elke zwangerschapsweek aan de ontwikkeling van de hersenen bijdraagt en dat dus onnodige keizersneden verminderd moeten worden, wat in overeenstemming is met de WHO-aanbeveling.

Hoofdstuk 5 vat de belangrijkste bevindingen van de bovenstaande studies samen, interpreteert de methodologische overwegingen, belicht de klinische implicaties en geeft aanwijzingen voor toekomstig wetenschappelijk onderzoek.

Chapter 7

Appendices

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Cortes Hidalgo AP, Delaney S, Kourtalidi SA, Neumann A, **Zou R**, Muetzel RL, Bakermans-Kranenburg MJ, van IJzendoorn MH, Tiemeier H, White T. Prenatal and Childhood Adverse Life Events and Child Brain Morphology: A Population-Based Study. Submitted.

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The 66th Annual Meeting of American Academy of Child & Adolescent Psychiatry, Chicago, USA (poster presentation)	2019	0.5
Generation R Research Meetings, Erasmus MC (oral presentation)	2018	0.5
Wetenschapscafé KJPP, Erasmus MC (oral presentation)	2019	0.5
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1 ECTS (European Credit Transfer System) is equal to a workload of 28 hours

WORDS OF GRATITUDE

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Runyu Zou was born on 1st July, 1989 in Shenyang, Liaoning, P. R. China. He has developed interest in medical sciences since childhood. During his senior high school education in Liaoning Province Shiyan High School, he won a second-class prize in China High School Biology Olympiad in 2017. From 2008 to 2013, he followed the specialization Preventive Medicine in Dalian Medical University, where he received his bachelor of medicine degree. In September 2013, he started a master program at the Department of Preventive Medicine, School of Public Health, Fudan University. Under the supervision of Prof. dr. Hua Fu, his research spectrum covered occupational and environmental health, chronic disease control and management, and health promotion and health communication. After obtaining his Master of Public Health degree in June 2016, he was granted a Erasmus University Rotterdam-China Scholarship Council collaborative fellowship (No. 201606100056) to support his PhD program supervised by Prof. dr. Henning Tiemeier, Prof. dr. Hanan El Marroun, and Dr. Tonya White, at the Department of Child and Adolescent Psychiatry, Erasmus MC, Rotterdam, the Netherlands. Embedded in the Generation R Study, his PhD project focused on perinatal determinants of child brain development using modern epidemiological methodologies, longitudinal data modellings, and population-based neuroimaging techniques, and the study results are presented in this thesis. During his PhD, he received the Frank Verhulst Award in 2020 for his outstanding academic achievement in child and adolescent psychiatry research. As a part of his PhD training, he also followed a master program at the Netherlands Institute for Health Sciences (NIHES), and obtained a Master of Science degree in Epidemiology in 2018. He plans to advance his scientific research career as a postdoc after this PhD, and his ultimate ambition is to become a bridge promoting academic communication and collaboration between China and abroad.