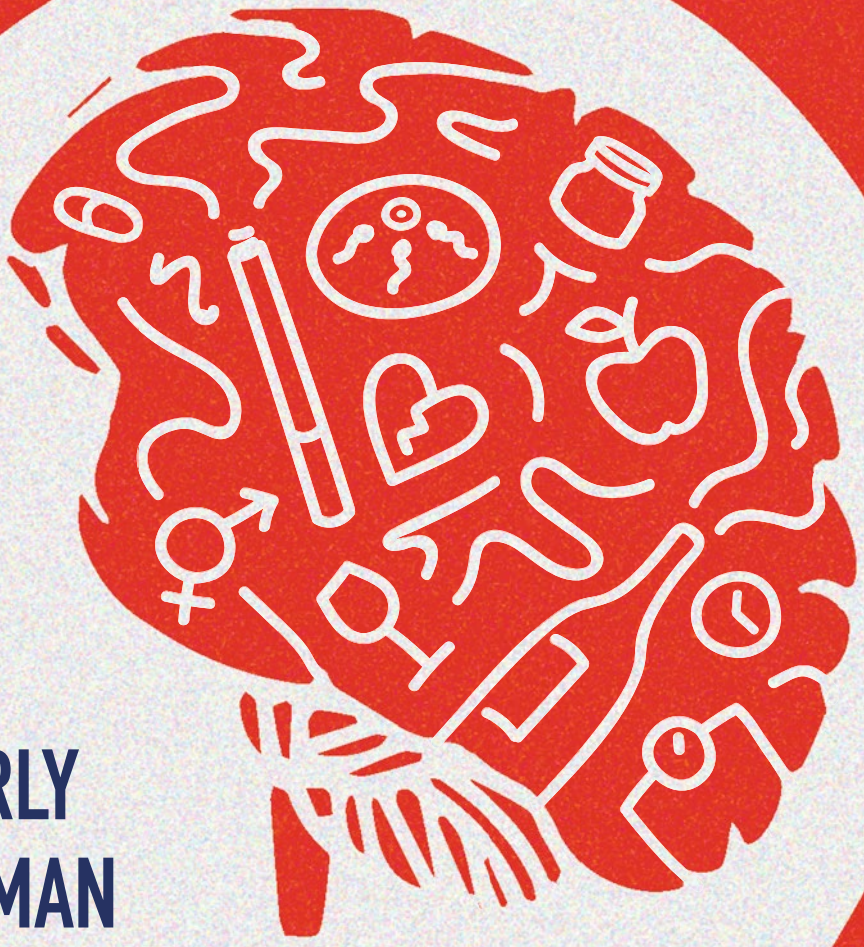

EARLY HUMAN BRAIN DEVELOPMENT

The Impact of Periconceptional Maternal and Fetal factors

Irene V. Koning

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Early Human Brain Development

The impact of periconceptional maternal and fetal factors

VROEGE ONTWIKKELING VAN HET MENSELIJK BREIN

De invloed van periconceptionele maternale en foetale factoren

Proefschrift

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ADDENDUM

1

Introduction

During the first 9 months of our life more significant changes happen to man than in the 70 years that follow – Samuel Taylor Coleridge

RATIONALE

The complex architecture of our brain has baffled scientists for centuries. Development of the human brain is an extremely complex process which begins with the differentiation of neural progenitor cells resulting in the formation of a simple tube. The neural tube differentiates further into an organ that distinguishes man from all the other species on our planet (Figure 1). Neurodevelopmental processes follow a strictly orchestrated sequence driven by an complex interplay between genetic and environmental input [1]. Individual brain regions develop asynchronously. Therefore, disruption of early brain development caused by harmful environmental exposures and altered gene expression results in disorders specific for the time window during which they occur [2]. In addition, the type and severity of the harmful exposure also contributes to specific alterations and the range of subsequent neurodevelopmental consequences [4,5].

Neurodevelopmental disorders carry a heavy economic and emotional burden for societies. One in six children in the industrialized world is affected and prevalence rates are rising [6-8]. Subtle deviations in size of the brain and individual brain structures (cerebellum, cerebral ventricular system, white matter) serve as markers of brain development and predispose to neurodevelopmental impairment and psychiatric disorders [9-11]. Therefore, there is a need to increase knowledge on disruptive conditions and to unravel underlying mechanisms causing alterations in early brain development in order to early diagnose and predict neurodevelopmental disorders. Ultimately, this allows the development of neuroprotective strategies and interventions to improve neurodevelopmental outcome in fetuses at risk for abnormal brain development.

The paradigm of the developmental origins of health and disease (DOHaD) postulates that an adverse intra-uterine environment leads to developmental adaptations that permanently program the structure, physiology and metabolism of fetal organs [14-16]. Mal programming can result in fetal growth deviations and increased susceptibility for adult diseases. The Dutch Famine birth cohort study has substantiated this hypothesis demonstrating the impact of famine during pregnancy on the occurrence of diseases in adult life [18]. Other large cohort studies (ALSPAC, the Barwon infant study, Generation R, Norwegian mother and Child study) have been conducted worldwide and investigated the impact of maternal environmental exposures on outcomes in offspring from the second trimester of pregnancy until early adulthood [19-22]. Evidence is rising however that in particular the periconceptional period (14 weeks before until 10 weeks after conception) is essential for programming, growth and development of the embryo as well as fetal and postnatal health [23]. Therefore, most developmental

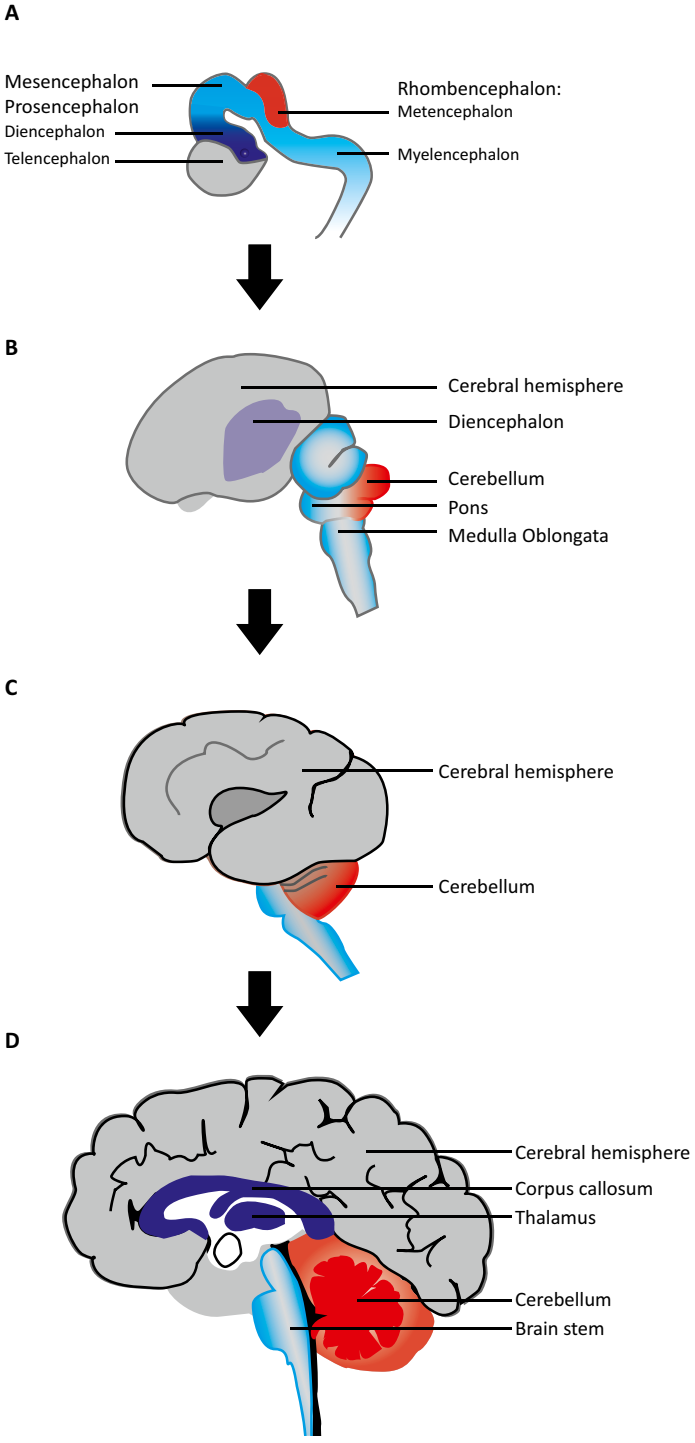


Figure 1 — Early human brain development

From an ectodermal neural plate the neural tube forms and closes in the third week after conception [1]. During the following week this tube is organized n three major subdivisions; ***prosencephalon****,* ***mesencephalon****, rhombencephalon (***A****: embryonic brain at 7 weeks GA) [1]. After 5 weeks, division of the rhombencephalon into the* ***metencephalon*** *and myelencephalon becomes evident when the* ***cerebellum*** *and the pons develop [2]. The* ***cerebellum*** *precedes most rapidly developing brain regions and can already be visualized by ultrasound from 7 weeks gestational age (GA) [3]. After gestational week 12, most fundamental structures of the brain are already formed (****B****: early fetal brain at 12 weeks GA). From this point on the amount of brain tissue is expanding rapidly. Outgrowth of the* ***cerebral cortex*** *starts in caudal direction bending ventrally and then rostrally giving shape to the temporal lobe and thereby the* ***insula*** *and* ***Sylvian fissure*** *(****C****: fetal brain at 26 weeks GA) [2]. From mid-gestation onwards neurogenesis peaks and new neuronal cells migrate to their target destination where they acquire characteristics specific to that area of the brain. Connectivity between the two cerebral hemispheres is assured when the* ***corpus callosum*** *has been formed around 24 weeks of gestation. The perinatal period, from 24 weeks until delivery, is characterized by neuronal maturation, synaptogenesis, cortical folding and volume expansion of the cerebrum [12,13]. At the end of gestation the 300-400 grams weighing newborn brain has been formed (****D****: neonatal brain at term age) [17].*

disorders and deviations in growth occurring later in pregnancy are likely to originate in this period [14-16]. Although the DOHaD paradigm includes all windows of sensitivity to environmental stressors across the life span, only few cohort studies have included the periconceptional window [24,25].

The DOHaD paradigm is also most relevant to early human brain development and its consequences for neurodevelopmental functioning in later life. As the brain starts developing rapidly during very early gestation, its complex architecture is particularly susceptible to the intra-uterine environment. Environmental factors can influence early brain development directly and indirectly by impacting other organ systems or fetal growth as a whole. In this regard, particularly fetuses with a congenital heart defect (CHD), fetal growth restriction (FGR) and preterm born infants are at risk for abnormal brain development and postnatal neurodevelopmental impairment during later life [26-28]. So far, only a few studies have investigated the impact of prenatal exposures on early prenatal brain development. Ekblad et al. has reviewed the literature on the impact of maternal smoking during pregnancy and reported reduced brain growth and regional brain volumes as well as altered microstructure in offspring with consequences for functional outcome in the child [29]. Moreover, maternal intoxications such as the use of cocaine during pregnancy are associated with decreased grey matter volume in the offspring which was most profound in the prefrontal and frontal regions [30]. Social alcohol consumption is widely tolerated in women of reproductive age. This is particularly alarming because prenatal alcohol exposure has been associated with decreased total brain volumes and grey and white matter volumes in children [31,32]. In contrast to these harmful impacts, are the reported positive associations between maternal folate levels and fetal head size and growth [33].

NEUROSONOGRAPHY

Although most of our knowledge of prenatal brain development is still based on post-mortem studies, improvements in sophisticated imaging techniques have changed the scope to *in vivo* evaluation of human brain development. Ultrasound is an unique imaging technique as it is non-invasive, easily accessible and safe for the small embryo [34]. Therefore, it is very suitable for longitudinal use and follow-up. The greatest challenge of ultrasound is that the image quality is mostly influenced by the sonographers' skills and the quality of the equipment [34]. Nevertheless ultrasound has been the first choice diagnostic during pregnancy ever since it made its debut in obstetrics in 1958 [34]. The widespread use of two-dimensional ultrasound (2D-US) for prenatal diagnosis came with the invention of real time scanning during the early 1970s. In the Netherlands however, structural 2D-US evaluation of fetal anatomy as standard second trimester obstetrical care was not implemented until 2006. Three-dimensional ultrasound (3D-US) has been introduced during the 1980s. This technique largely has two modules: the multiplanar mode and volume rendering which enables the reconstruction of precise orthogonal planes and a rendered surface image. Both modules have shown to be practical tools for prenatal neuro-sonography; a detailed ultrasound evaluation of the prenatal central nervous system.

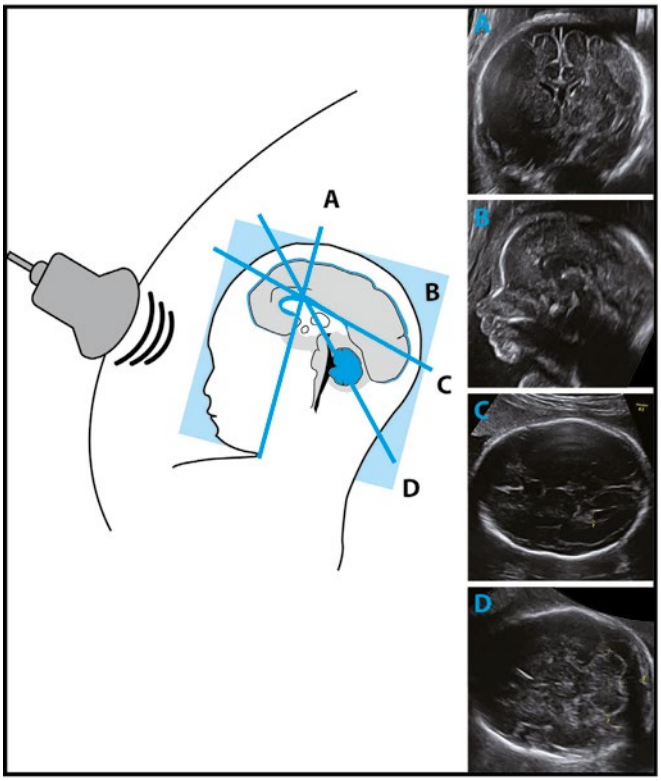


Figure 2

Sectional planes with images of the fetal brain extracted from a 3D-US volume; A: coronal, B: mid-sagittal, C: transventricular and D: transcerebellar planes. Images with permission.

3D-US is useful in addition to conventional 2D-US as it ensures precise measurements and can improve diagnostic accuracy for a number of structural malformations. The use of high-resolution transvaginal transducers allows detailed imaging during the early first trimester of pregnancy [35]. As a result reference charts of several embryonic brain structures have been created to study normal and abnormal brain development [36,37]. Moreover, display of 3D-US images requires desktop applications which offer interactive visualization tools (Figure 2). In addition, virtual reality technologies such as the Barco I-Space (Barco N.V., Kortrijk, Belgium) offer depth perception with a volume-rendered 3D dataset. It enables visualization and quantification of morphology as well as biometric and volumetric measurements of an embryo *in vivo* [38,39]. However, a virtual reality system is less suitable for analysis of 3D-US volumes of the fetal brain later in pregnancy. These commonly used desktop applications can be used more easily to analyse these volumes.

HYPOTHESIS

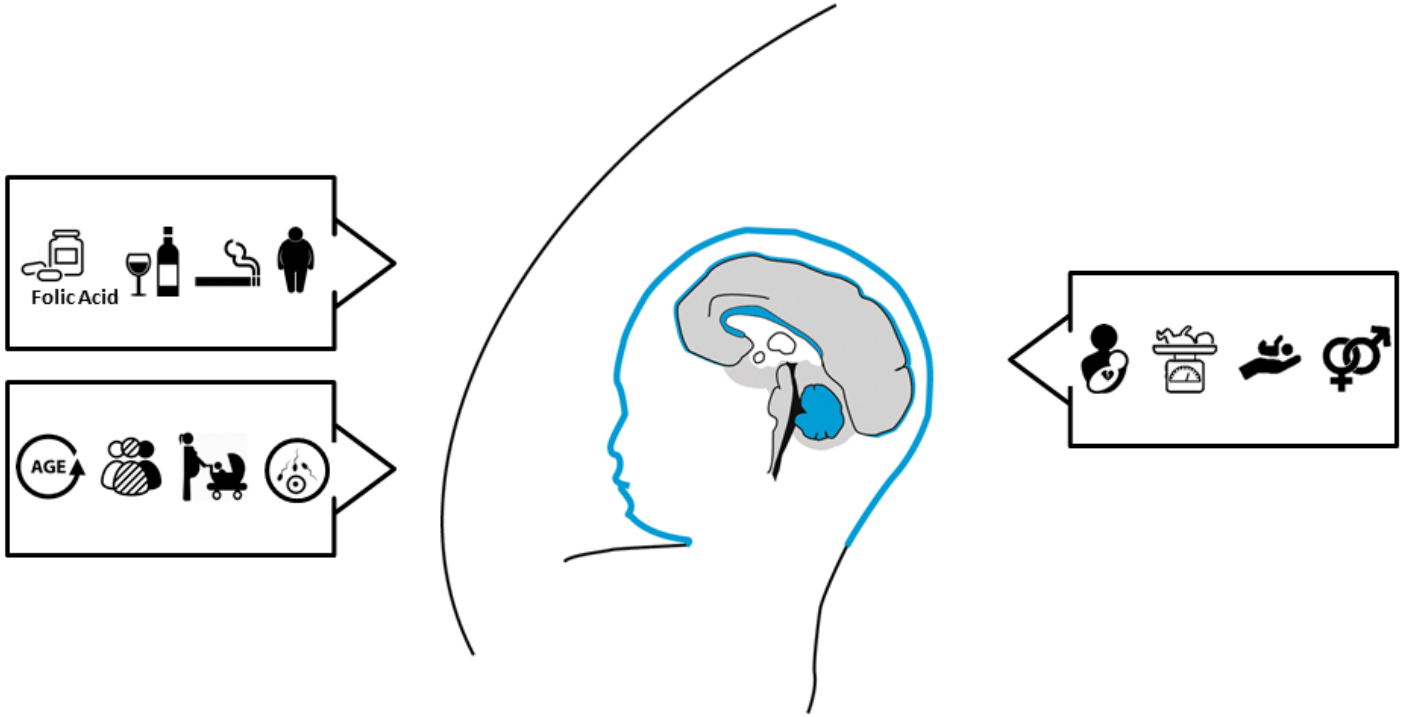
The development of reliable markers of early brain development is essential to detect early deviations in human brain development. Such measures also provide the opportunity to study associations between periconceptional maternal and fetal factors and the sizes and growth patterns of several brain structures. Identifying potential disruptors and protective agents of brain development will give rise to improvement of preconception counselling and antenatal strategies to prevent neurodevelopmental impairments in early and later life.

AIMS OF THE THESIS

In this thesis we aim to investigate the impact of periconceptional maternal and fetal factors on early human brain development (Figure 3). The main objectives of this thesis are:

1. To develop protocols and establish reliability of embryonic, fetal and neonatal brain measurements using ultrasound techniques.
2. To investigate the impact of periconceptional maternal factors on early development of the human brain
3. To study the association between early human brain development and fetal characteristics such as congenital heart defects, fetal growth restriction and gender.

Figure 3 — Aims of the thesis



Are periconceptional maternal behaviors (folic acid supplement use, alcohol consumption, smoking, obesity) and characteristics (age, ethnicity, parity and mode of conception) and fetal factors (congenital heart defect, fetal growth restriction and gender) associated with brain development? Brain structures of interest are depicted in blue: head volume, cerebellum, corpus callosum and cortical folding.

SETTING

The studies described in this thesis were performed in the Dream study and the Submarine study.

The Dream study is a longitudinal ultrasound cohort embedded in the ongoing Rotterdam Periconception Cohort (Predict study >2009), a hospital-based cohort from the preconceptional period with follow up after birth, conducted at the Erasmus MC University Medical Center in Rotterdam [24]. Pregnant women are enrolled during the first trimester and received 3D-US examinations at 7, 9, 11, 22, 26 and 32 weeks GA. Ultrasound scans were performed using a 1-7 MHz transabdominal transducer and a 6-12 MHz transvaginal transducer of the Voluson E8 system (General Electrics Medical Systems, Zipf, Australia). Standard biometric measurements were performed online and measurements of various brain structures were performed offline on 3D-US volumes using 4D View software (General Electrics Medical Systems, Zipf, Australia) and the Barco I-Space virtual reality system. After delivery, at approximately 42 weeks post-menstrual age all newborns were invited for one last visit to collect anthropometric data and perform a cranial ultrasound scan (CUS) using a MyLab 70 scanner (Esaote, Genoa, Italy), with a convex neonatal probe (7.5 MHz). Repeated measurements of embryonic, fetal and neonatal brain structures were combined to create growth trajectories. At study entry data on maternal characteristics, medical obstetrical history and lifestyle were obtained from self-reported questionnaires and verified by a researcher. Data on pregnancy course and neonatal outcomes were obtained from self-reported questionnaires and validated by ultrasound reports and medical records.
The Submarine study is a prospective observational cohort conducted at the Neonatal Intensive Care Unit of the Sophia Children's Hospital, Erasmus MC in Rotterdam. Preterm born infants were included between 2010 and 2012 and CUS were performed according to the standard local protocol using a MyLab 70 scanner (Esaote, Genoa, Italy), with a convex neonatal probe (7.5 MHz). Scans were performed on the day of birth; on days 1, 2, 3, and 7 of life; and then weekly until discharge. Measurements were performed off-line by using the Mylab software (Esaote).

OUTLINE OF THE THESIS

In **PART I** we study the early development of the human cerebellum. In **Chapter 2** we present the results from a systematic review of literature on the impact of parental environmental exposures and intrinsic factors on prenatal growth and development of the human cerebellum. The associations between periconceptional maternal folate status and cerebellar growth in the first trimester of pregnancy are described in **Chapter 3**. In **Chapter 4** cerebellar growth trajectories from 9 weeks to 32 weeks GA were created for the investigation of associations with periconceptional maternal and fetal factors.

In **PART II** we investigate the feasibility of other markers of early human head and brain development. In **Chapter 5**, we introduce embryonic head volume as a new marker for head size during the first

trimester and demonstrate associations with periconceptional maternal and fetal factors. In **Chapter 6,** the impact of fetal congenital heart defects (CHD) on brain fissure depth measurements is addressed. We present a new ultrasound marker for monitoring of preterm brain growth; corpus callosum – fastigium length in **Chapter 7**. The use of this marker for the evaluation of fetal brain growth of fetuses with CHD, fetal growth restriction and controls is investigated in **Chapter 8**.

PART III includes the general discussion and summary.

PART I

EARLY DEVELOPMENT OF THE CEREBELLUM

2

Impacts on prenatal development of the human cerebellum: A systematic review

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Ginette M. Ecury – Goossen, Irwin K.M. Reiss,
Régine P.M. Steegers-Theunissen, Jeroen Dudink

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ABSTRACT

Background — The cerebellum is essential for normal neurodevelopment and is particularly susceptible for intra-uterine disruptions. Although some causal prenatal exposures have been identified, the origin of neurodevelopmental disorders remains mostly unclear. Therefore, a systematic literature search was conducted to provide an overview of parental environmental exposures and intrinsic factors influencing prenatal cerebellar growth and development in humans.

Methods — The literature search was limited to human studies in the English language and was conducted in Embase, Medline, Cochrane, Web of Science, Pubmed and GoogleScholar. Eligible studies were selected by three independent reviewers and study quality was scored by two independent reviewers.

Results — The search yielded 3872 articles. We found 15 eligible studies reporting associations between cerebellar development and maternal smoking (4), use of alcohol (3), *in vitro* fertilization mediums (1), mercury (1), mifepristone (2), aminopropionitriles (1), ethnicity (2) and cortisol levels (1). No studies reported on paternal factors.

Conclusions — Current literature on associations between parental environmental exposures, intrinsic factors and human cerebellar development is scarce. Yet, this systematic review provided an essential overview of human studies demonstrating the vulnerability of the cerebellum to the intra-uterine environment.

INTRODUCTION

One in six children in industrialized countries are affected by neurodevelopmental problems, including cognitive defects, motor disabilities and psychiatric diseases [6]. Most of these aberrations cause lifelong problems with severe societal and economic impact [40]. Harmful prenatal environmental exposures can alter epigenetic programming and structural development, potentially inducing neurodevelopmental short-comings [23,41,42]. The exact contribution of environmental exposures to neurodevelopmental problems is poorly understood. Since individual brain regions develop asynchronously, the exact alterations are not only determined by the type or severity of harmful exposure but also the critical time windows [4,5].

Of the rapidly developing brain regions, the cerebellum precedes most brain structures and shows very early connectivity. It also shows a steep growth curve during fetal life, increasing up to 500% in cerebellar volume between 24-40 weeks of gestation [43-45]. Harmful environmental exposures during pregnancy may therefore pose a risk to disrupt prenatal cerebellar development [46]. Postnatally, the cerebellum is involved in a wide variety of sensorimotor tasks as well as cognitive, emotional and language behaviour [47-49]. This may explain why an altered prenatal cerebellar development or injury may be associated with an increased risk for neurodevelopmental impairment and mental health issues [46,47,50-52].

Overwhelming evidence from animal studies have established the impact of an adverse intra-uterine environment compromised by toxic agents, environmental exposures, infection, inflammation, hypoxia, imbalances in vitamin or hormonal status on cerebellar development [5,46,53]. Human studies on this issue are scarce, predominantly focusing on postnatal cerebellar functions disregarding prenatal deviances in growth and development.

We believe that studying the impact of the intra-uterine environment on human prenatal cerebellar growth and development may provide more insight for better understanding consequences for neurodevelopmental functioning. Therefore, we provide an overview of the literature on parental environmental exposures and intrinsic factors influencing prenatal growth and development of the human cerebellum. The ultimate aim is to identify risk factors for impaired prenatal cerebellar growth and development with potential consequences for neurodevelopmental outcome.

MATERIALS AND METHODS

Search Strategy and Selection Criteria

A literature search according to a predefined protocol was generated by an experienced medical information specialist from the Medical Library of the Erasmus MC University Medical Center. All

relevant literature up to September 4th 2015 was searched in Embase, Medline, Cochrane central, WebofScience, Pubmed and GoogleScholar. The complete search strategy (Appendix A) combined controlled vocabulary terms (in Medline and Embase) and free text words in title and/or abstract related to the exposure (e.g. alcohol, nutrition, ethnicity), the outcome (prenatal cerebellum in context of growth, development and abnormalities), and the studied population (e.g. pregnancy, pregnant women, maternal, paternal). The search was limited to human studies in the English language.

All randomized controlled trials, intervention studies, cohort studies, case-control studies and case reports were eligible for inclusion when they reported the human cerebellum as prenatal outcome measure, in terms of size, histology, morphology or any other measurement of the cerebellum during pregnancy. Eligible studies should report on any environmental or intrinsic factor as exposure, including maternal conditions or characteristics, environmental toxicities, occupational and lifestyle exposures. Exclusion criteria were (1) no or irrelevant exposure defined as an exposure that is not environmental or intrinsic; (2) postnatal cerebellar outcome only; (3) results obtained from animal or *in vitro* studies; (4) articles with no full text available; (5) book chapters and reviews lacking unique data analysis.

Working in pairs, three independent reviewers (IVK, MJT, GMEG) screened the titles and abstracts on the selection criteria. All differences in the study selection were resolved through discussion with the first reviewer (IVK) who read all abstracts. For all selected articles, full text was retrieved and evaluated to decide whether the study met the inclusion criteria. Reference lists of all included studies were checked for potential eligible articles.

Quality assessment

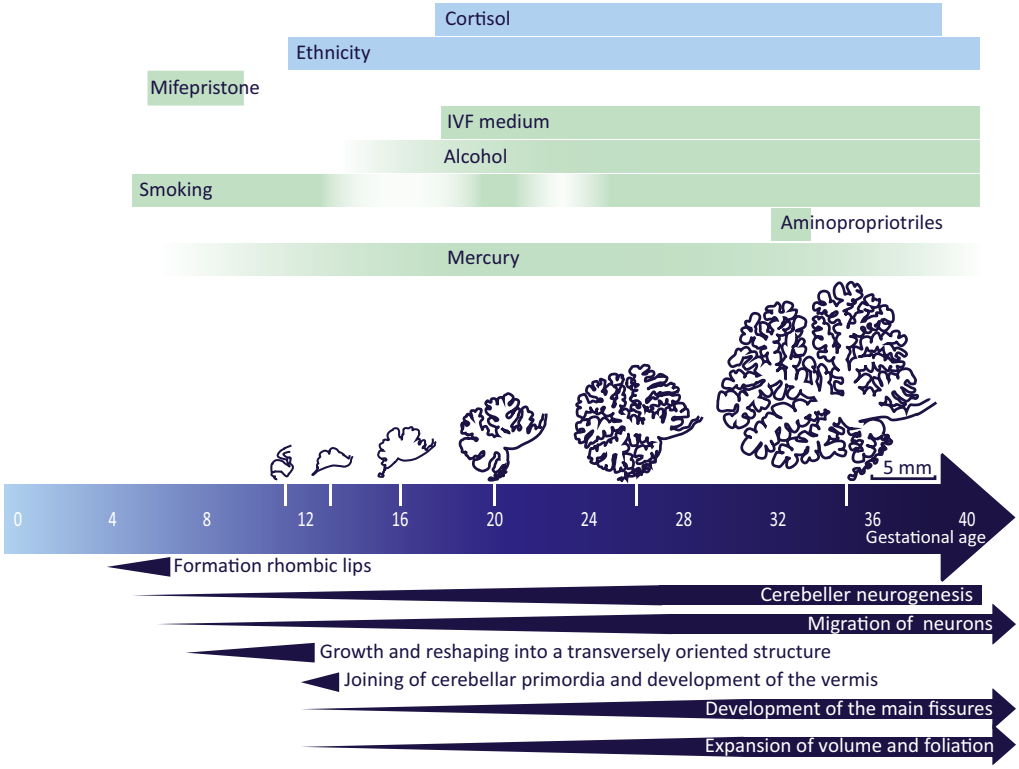
Two reviewers (IVK, MJT) independently scored the quality of the included studies according to a predefined quality score (Appendix B) based on five items namely the study design, population size, exposure and outcome measurements and confounder handling. Each item could score zero, one or two points, resulting in a maximum of ten points representing the highest quality. Case reports were not assessed on quality. Discrepancies in assigned quality scores were discussed until the reviewers agreed. As the quality score was not used as an exclusion criterion we did not define a cut-off score.

RESULTS

The literature search resulted in 3872 unique articles. During title and abstract screening we excluded 3817 articles. Full texts of 55 studies were reviewed for relevance. We excluded studies because of irrelevant outcome (7), irrelevant study population (10), no or irrelevant exposure (9), irrelevant study type or no available full text (7), animal or *in vitro* models (6), duplicate data (1) or the manuscript was not written in the English language (1). Additionally, we included one study through hand search of reference lists of the included studies. In total, 15 studies were included in this systematic review.

Table I, presents details of the included articles and quality scores. In the selected literature we found eligible case reports, cross-sectional, intervention and cohort studies on maternal smoking (4), maternal use of alcohol (3), endemic mercury exposure (1), the influence of *in vitro* fertilization (IVF) mediums (1), mifepristone (1) ethnicity (2) and maternal cortisol levels (1). Two case reports described fetuses with cerebellar anomalies after exposure to mifepristone and aminopropionitriles. No studies reported on paternal exposures or factors influencing cerebellar development. We identified environmental exposures defined as environmental toxicities, occupational and lifestyle exposures and intrinsic factors defined as factors that characterize the origin of a person including ethnicity and stress levels (Figure 1). Findings are presented per documented exposure.

Figure 1 — Impact of maternal environmental exposures and intrinsic factors on prenatal development of the human cerebellum



A schematic display of the evidence found in this systematic review of the impact of maternal factors, divided in environmental exposures (green) and intrinsic factors (blue) on prenatal development of the human cerebellum with respect to gestational age and the most essential developmental stages of the cerebellum. Adjusted from Rakic et al. 1970 [54].

Table 1 — Study selection, characteristics, quality score and main effects

First Author	Year	Study design	Quality score	n	Method	GA at outcome assessment	Exposure window	Exposure	Main results
Roza	2007	pC	8	7042	US	25-40	Preconceptional & prenatal	Smoking:	TCD: Smoking < Non-smoking
Lavezzi	2013	CS	5	30	H	25-40	Preconceptional & prenatal	Smoking	Abnormal number and morphology of Purkinje cells in SIUDS, mostly smoking mothers
Lavezzi	2007	CS	4	21	H	20	Preconceptional & prenatal	Smoking	Smoking vs. non-smoking: Abnormal cytology and increased cell death
Falk	2005	CS	3	12	H	5-12	Prenatal	Smoking	Smoking vs. non-smoking: Increased expression of nicotinic and muscarinic receptors
Wass	2001	pC	6	167	US	18-42	Prenatal	Alcohol	TCD: Alcohol = no alcohol
Handmaker	2006	pC	5	255	US	17-40	Preconceptional & prenatal	Alcohol	TCD: Heavy < no alcohol & early abstainers
Kfir	2009	pC	4	166	US	13-38	Prenatal	Alcohol	TCD: Alcohol = no alcohol
Nelissen	2013	I	7	294	US	17-40	Preconceptional	IVF medium	TCD: Vitrolife > Cook medium
Lapham	1995	CS	7	40	H	NR	Preconceptional & prenatal	Mercury	Endemic vs non-endemic mercury exposure: Elevated levels in cerebellar white matter and cortex, no pathologic abnormalities
Sitruk-Ware	1998	rC	3	71	M	6-9	1st trimester	Mifepristone & Prostaglandin	One case of cerebellar atrophy
Afadapa	2006	CR	NA	1	M	17	1st trimester	Mifepristone & Prostaglandin	Right sided cerebellar agenesis
Dembinski	1997	CR	NA	1	M	32	Preconceptional & prenatal	Aminopionitriles	Cerebellar hypoplasia
Li	2012	pC	7	432	US	17-39	3rd trimester	Cortisol	TCD: Inverse relation with cortisol levels
Jacqemyn	2000	CS	6	549	US	20-32	NA	Ethnicity	TCD: Moroccan > Turkish & Belgian
Araujo Junior	2007	pC	5	52	US	12-42	NA	Ethnicity	Cerebellar volume: Brazilian > Taiwanese

The study selection and study characteristics per included study identified by name of the first author and year of publication. Ref= reference number. Study type categorized as; CR= Case report, CC= Case control study, C= cohort study, p= prospective, r= retrospective. Methods: H= Histology, US= Ultrasound. n= number of participants. GA at assessment= Gestational age in weeks. NR= Not reported. NA= Not applicable. TCD= transcerebellar diameter. SIUDS= Sudden intra-uterine death syndrome.

Environmental exposures and intrinsic factors

Maternal smoking

Four studies evaluated the association between maternal smoking and fetal cerebellar development [55-58]. A prospective cohort described a significantly smaller fetal transcerebellar diameter (TCD) in addition to smaller head parameters in fetuses of mothers who continued smoking throughout pregnancy compared to non-smoking mothers (-0.08mm, 95%CI= -0.15;-0.00) when adjusting for known confounders [58]. Cerebellar growth did not differ significantly [58]. In addition, morphological associations between tobacco and cerebellar development have been studied by Lavezzi et al., suggesting defective maturation and migration of Purkinje cells in histology specimen of fetuses with sudden intra-uterine death [56]. In 91% of these cases at least one developmental alteration was shown, suggesting a strong correlation between tobacco exposure and prenatal cerebellar insults. Moreover, in a similar study, 92% of fetuses had bio-pathological alterations in the cerebellar cortex ($p<0.05$) [57]. A fourth study showed that maternal smoking during pregnancy, compared to non-smoking, significantly increased the expression of nicotinic and muscarinic acetylcholine receptors in human cerebellum, pons and medulla oblongata tissue of fetuses between 5 and 12 weeks gestation obtained after routine abortion procedures [55].

Maternal use of alcohol

Three studies evaluating the association between maternal alcohol consumption and fetal cerebellar growth measured by TCD, showed inconsistent results [59-61]. The study with the highest quality investigated the influence of maternal alcohol consumption measured in average ounces of absolute alcohol consumption per day and fetal brain measurements in 167 women. Maternal alcohol consumption was reported to be associated with a reduction of the frontal cortex and not to cerebellar size (Pearson= -0.040, $p\geq0.10$) [61]. This finding is supported by a prospective cohort which compared mothers using alcohol with mother not using alcohol in the second (mean difference TCD= 0.7mm, $p>0.05$) and third trimester (mean difference TCD= 0.2mm, $p>0.05$) [60]. In this study alcohol consumption was recorded by validated self-administered questionnaires [62,63]. In contrast, using the same questionnaires another study reported smaller cerebellar size ($p= 0.087$) and significantly decreased cerebellar growth ($p= 0.008$) in heavy drinking women who continued drinking compared to early abstainers and non-drinkers [59].

Environmental toxicities

Five studies reported on the impact of maternal exposure to environmental toxicities on prenatal cerebellar development [64-68]. An intervention study reported differences in fetal TCD (mean difference= 0.4mm, $p= 0.008$) in fetuses exposed as an embryo to different commercially available culture media (Vitrolife G1.3, Göteborg, Sweden vs. Cook K-SICM, Brisbane, Australia), establishing the culture medium as a direct environment for the growing embryo outside of the uterus [67]. In fetal brain tissue from the Seychelles endemically exposed to mercury demonstrated a correlation of dietary mercury exposure and levels in the brain. These levels were highest in posterior fossa structures and

subcortical nuclei [66]. Although this study identified no structural pathological alterations, histological alterations were found in cerebellar white matter of which clinical significance remains unclear [66]. Two cases of cerebellar anomalies after failed induction of abortion with mifepristone and prostaglandins have been reported, including cerebellar atrophy and half-sided cerebellar agenesis [64,68]. Teratogenic effects of aminopropionitriles have been suggested in a case report of an exposed fetus with multiple congenital anomalies including cerebellar malformations [65].

Intrinsic maternal factors

Li et al. conducted a cross-sectional study in relatively low risk singleton pregnancies to evaluate associations of maternal stress levels measured by serum cortisol before delivery and fetal growth measures. A negative correlation between maternal serum cortisol and mid-gestation TCD (R^2= 0.76, p= 0.014) not late TCD was demonstrated, adjusting for maternal body mass index, age and body weight at delivery, moment of the ultrasound, infant gender, moment of cortisol measurement and maternal uterine contraction states [69]. Two studies reported ethnicity in relation to cerebellar size [70,71]. TCD measurements were dependent of ethnicity when comparing measurements from Moroccan, Turkish and Belgian fetuses [71]. Additionally, cerebellar volume of Taiwanese fetuses compared Brazilian fetuses showed a significantly increased cerebellar growth curve (mean difference= 0.366mm, p<0.001) [70].

DISCUSSION

This review provides a complete overview of the current published literature of the effects of parental environmental exposures and intrinsic factors on prenatal human cerebellar growth and development. Notable, we did not find any evidence of paternal factors affecting cerebellar development. Although the available data were collected from heterogenic study populations with various outcomes, they provide ample evidence to support the particular susceptibility of the human cerebellum to maternal environmental exposures and intrinsic factors [47]. We found associations between maternal smoking, use of alcohol, type of IVF culture medium, mercury, mifepristone, aminopropionitriles, ethnicity, maternal cortisol levels and cerebellar growth or development. We discuss these findings in detail and aim to guide future studies investigating impacts on prenatal development on the human cerebellum.

Main findings

Maternal smoking

We found evidence in histological and ultrasound studies that maternal smoking has a harmful effect on prenatal cerebellar development, complementing previously documented reduced cerebellar volumes in prematurely born infants of smoking mothers [72]. So far, direct influences of maternal smoking have only been demonstrated in animal studies reporting alterations in various receptors and cellular processes [73,74]. Human studies have only reported indirect evidence for harmful

influences of nicotine on behavioural and cognitive outcome in the offspring [75]. Previous imaging studies also showed that maternal smoking was associated with reduced head growth and total brain volume measured in the second half of pregnancy [29,58,76]. However, whether the relationship between maternal smoking and neurodevelopmental outcome is mediated by derangements in brain development needs to be elucidated. However as the brain overall is affected, one could reason that the cerebellum as a separate entity is not spared. In addition, reduced cerebellar growth might be a secondary network injury effect due to lack of innervation. Toxicity of tobacco may alter epigenetic mechanisms and vascular processes which in turn may directly and/or indirectly influence cerebellar growth and maturation [29].

Maternal use of alcohol

Although previous studies reported that cerebellar neurons are particularly susceptible to alcohol-induced developmental disruptions [77-79], ultrasound studies did not demonstrate consistent associations between alcohol exposure and cerebellar size. One study provided evidence for reduced prenatal cerebellar growth in relation to heavy alcohol exposure compared to non-alcohol exposure or early-abstainers [59]. These findings may support the possible benefit of early abstinence for fetal growth measures including cerebellar growth. Although there is no comparable animal data available, decreased cerebellar weight and cerebellar neuronal loss was recorded due to ethanol induced neurotoxicity [80]. Alcohol-induced cerebellar alterations may result from underlying mechanisms, such as excitotoxicity, nutritional deficiencies, growth factor alterations, glial abnormalities, apoptosis, oxidative stress and compromised energy production [81]. However, adverse effects of maternal alcohol exposure on cerebellar growth were not detected in the other studies [60,61]. This may be due to the relatively small effect sizes and considerable variability in study populations, quality and methodology. In addition, reliance of self-reported alcohol consumption is limited. We speculate that the impact of maternal alcohol intake on the developing cerebellum may be revealed in larger ultrasound cohorts using more precise measures for alcohol consumption in contrast to the relatively crude measures for maternal alcohol intake at conception used previously [61]. However, to date prenatal ultrasound evaluation of fetal brain development in women using alcohol during pregnancy receives little attention.

Environmental toxicities

Environmental toxicities including IVF-mediums, mercury, mifepristone and aminopropionitriles were associated with a variety of cerebellar anomalies and altered development [64-68]. However, quality of the evidence was relatively low, based on two case-reports and studies with quality scores between 3 and 7. One study reported the interesting finding that IVF culture mediums could influence cerebellar growth during the second trimester of pregnancy [67]. This suggests that already during *in vitro* stages, embryonic growth can be influenced, which also affects the cerebellum and connecting brain structures. This may indicate that the embryo's own epigenetic programming and early neurogenesis by modulation of neurotrophic factors can be directly influenced by its environment, being a culture medium or the womb [23,41].

Only one study reported on the neurotoxic effects of mercury *in utero* on the human cerebellum showing elevated levels of mercury in the brains of endemically exposed fetuses, in the absence of clear pathologic developmental alterations [66]. Although the evidence from this small sample is rather scarce, animal models provide clear evidence for neurotoxic effects of mercury including dose-related cerebellar damage, anomalous cerebellar development and altered Purkinje cell migration [82-86]. In addition, mercury levels in the cerebellum among other posterior fossa structures were reported higher than in the cerebral cortex. This finding is consistent with a rat model showing the highest mercury concentrations in the cerebellum and hippocampus after *in utero* exposure [84]. Potentially this indicates greater susceptibility of these structures to destructive neurotoxic effects of mercury [66]. Although the results of this prenatal study remained inconclusive, previously significantly reduced neonatal cerebellar measurements were reported after antenatal mercury exposure [87].

Considering that only two studies reflected on the potential teratogenic effects of mifepristone, this finding seemed of little significance as failed termination of pregnancy was reported in fewer than 0.02% of cases [64]. In addition, causal relation between occupational aminopropionitriles exposure and cerebellar development cannot be concluded from one fetus diagnosed with Cantrell-sequence. Although the evidence of a neurotoxic impact of aminopropionitriles on cerebellar morphology seems limited, effects on a finer scale or on cerebellar connectivity cannot be excluded.

Intrinsic maternal factors

Two studies demonstrated that cerebellar size measured with TCD and cerebellar volume varied among different ethnicities. Ethnical differences in transcerebellar size are highly interesting, as this may impact obstetrical care and management, seeing that the TCD measurement is part of routine ultrasound examinations and used for second trimester estimation of gestational age [88]. Therefore, universal reference curves disregarding ethnical differences of cerebellar size may not be accurate for pregnancy dating in later pregnancy. However, to investigate a spectrum of ethnical backgrounds in association with fetal cerebellar growth warrant larger longitudinal cohorts.

Although large-scale studies have provided evidence on the impact of maternal stress on prenatal brain development and neurodevelopmental outcomes [89-92], the evidence for a detrimental impact on cerebellar development was limited to one study. Although Li et al. demonstrated a negative association between pre-labour cortisol levels and TCD [69], we note that the cortisol level was only determined once in this cross-sectional study. Previously this was demonstrated to be an unreliable recording of maternal stress because of its daily and pregnancy-related fluctuations [93-95]. Therefore, adjustment for these fluctuations in the statistical analyses, which was performed in this study, seemed insufficient. Animal studies provided more evidence for the neurotoxic effects of prenatal stress on Purkinje cell morphology but show no effects on cerebellar growth measurements [96,97]. To crystallize this issue in humans, prospective studies with repeatedly measured stress hormone levels could provide more robust conclusions.

Based on our review we conclude that cerebellar growth and development showed signs of susceptibility to maternal environmental exposures and intrinsic factors. For each factor that influenced the developing cerebellum, the level of impact may vary with the timing, severity and type of insult [47]. Potentially, effects may also differ among specific cerebellar regions [4,5]. Subsequent derangements in cerebellar development potentially affect downstream connectivity and development of other brain structures.

Despite our extensive literature search, we stress that the amount of evidence and its quality was relatively low. From the current literature no conclusions on causal relations can be drawn. Our search yielded the whole spectrum of developmental deformations and abnormal growth of the cerebellum. However we did not specifically include Arnold Chiari malformations often co-occurring with myelomeningocele. Although these malformations are often considered as cerebellar anomalies, they typically result from mechanical forces, without which the cerebellum otherwise would have developed normally. Furthermore, only a minority of the studies adjusted for confounding factors. Therefore, residual confounding may have influenced the reported associations. Because of the numerous potential confounders in human research, findings from animal studies may contribute to a great extent to the understanding of environmental effects on cerebellar development [46]. Nevertheless, human research is indispensable as the human cerebellum is significantly different from animals, engaging in a more extensive and complex set of tasks and functions and neuro-embryology follows different timelines. Caution is needed when translating animal experimental results to the human setting. Therefore, we did not incorporate animal data in this systematic literature search.

Currently, most human evidence concerns cerebellar development during the second half of pregnancy. However, new evidence indicates that embryonic growth can predict fetal growth with consequences for health and disease in later life [24,98]. Therefore, research needs to incorporate these earlier developmental stages to study causes of derangements in cerebellar development which may originate early in the embryonic period. More precise and reliable measures of cerebellar growth, as well as maternal environmental exposures and intrinsic factors are warranted to investigate the small cerebellar effects of these impacts. This kind of epidemiological evidence may be provided by large prospective cohort studies using three-dimensional ultrasound, biomarkers and epigenetic patterns [20,24].

3

Periconception maternal folate status and human embryonic cerebellum growth trajectories: The Rotterdam Predict study

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ABSTRACT

Background — We aimed to investigate whether periconceptional maternal folate status affects human embryonic cerebellar size and growth trajectories.

Methods — In a prospective periconceptional cohort participants filled out questionnaires and received weekly transvaginal 3D-ultrasounds between 7+0 and 12+6 weeks gestational age (GA). Viable non-malformed singleton pregnancies were selected for cerebellar measurements; transcerebellar diameter, (TCD), left and right cerebellar diameters (LCD, RCD). Linear mixed models were performed to estimate associations between questionnaire data on the timing of maternal folic acid supplement initiation and longitudinal cerebellar measurements as a function of crown-rump length (CRL) and GA. Maternal red blood cell folate concentrations were analysed before 8 weeks GA to validate the associations.

Results — A total of 263 serial high quality three-dimensional ultrasound scans of 135 pregnancies were studied. Preconceptional compared to postconceptional initiation of folic acid use was associated with slightly larger cerebellar diameters per millimetre increase of CRL (TCD: β=0.260mm, 95%CI=0.023-0.491, $p<0.05$; LCD: β= 0.171mm, 95%CI= 0.038-0.305, $p<0.05$; RCD: β= 0.156mm, 95%CI= 0.032-0.280, $p<0.05$) and with proportional cerebellar growth (TCD/CRL:β= 0.015mm/mm, 95%CI=0.005-0.024, $p<0.01$; LCD/CRL:β= 0.012mm/mm, 95%CI= 0.005-0.018, $p<0.01$; RCD/CRL:β= 0.011mm/mm, 95%CI= 0.005-0.017, $p<0.01$). Cerebellar growth was significantly highest in the third quartile of maternal red blood cell folate levels (1538-1813nmol/L).

Conclusions — These first findings show that periconceptional maternal folate status is associated with human embryonic cerebellar development. Implications of these small but significant variations for fetal cerebellar growth trajectories and the child's neurodevelopmental outcome are yet unknown and warrant further investigation.

INTRODUCTION

Last decades our understanding of the complex functions of the human cerebellum has expanded immensely. Previous literature demonstrated cerebellar involvement in motor and non-motor functions including perception, cognition and emotion [48,49]. Yet, prenatal studies of the cerebellum have mainly been focussing on abnormal cerebellar development and morphology in the second half of pregnancy when ultrasonography landmarks of development can be visualized [99-101]. However, the first trimester of pregnancy is known to be essential for organogenesis and deviations in growth and development are likely to originate during this particular period. Nowadays, ultrasound measurements of embryonic structures are more easily accessible through the improvements of ultrasound techniques. Growth charts of embryonic brain structures including the cerebellum were created with measurements performed between 7^{+0} and 12^{+6} weeks of gestation using three-dimensional ultrasound and virtual reality ultrasound visualization [37]. Three-dimensional techniques provide very precise information on variations in first trimester cerebellar growth trajectories.

Development of the cerebellum starts from the 6^{th} week of gestation and extends to the first years of life [102]. Due to this protracted process, the cerebellum is extremely vulnerable to disturbances in its development caused by a complex interplay of genetic and environmental factors [45,103]. Perinatal environmental and genetic risk factors act at particular time windows when cerebellar development is most vulnerable to derangements [50]. Maternal exposures and conditions during the periconceptional period are known to influence embryonic growth [104-106]. These factors may also affect embryonic cerebellar growth with potential implications for fetal cerebellar growth and neurodevelopmental functions in later life [107,108].

Periconceptional maternal folate status plays a vital role in biological processes involved in cellular growth and differentiation [109]. Maternal folate deficiency is associated with a broad spectrum of reproductive failures [23]. In addition, maternal periconceptional folic acid supplement use is known to reduce the risk of congenital anomalies such as neural tube defects and probably also congenital heart disease and oral facial clefts and offspring born small for gestational age (SGA) [110-112]. Furthermore adequate maternal periconceptional folic acid use seems beneficial to early and late human neurodevelopmental outcome and may reduce the risk of neurodevelopmental disorders including autism spectrum disorder in which the cerebellum seems to play a key role [113-118]. Animal models show dramatic alterations in both prenatal and postnatal cerebellar growth and development when derangements of the folate pathway and changes in folate status occurred [119,120]. So far no human studies reported on consequences of maternal folic acid supplement use on the growth and development of the human cerebellum. From this perspective we aim to investigate whether the timing of maternal folic acid supplement initiation during the periconceptional period as measure of periconceptional maternal folate status affects human embryonic cerebellar size and growth trajectories in pregnancies ending in live births without congenital malformations.

MATERIALS AND METHODS

Study Population

This study is embedded in the Rotterdam Predict study, an ongoing prospective periconceptional cohort investigating the influence of gene-environment interactions and underlying mechanisms on reproductive, (extra-) embryonic and pregnancy outcome at the Erasmus MC University Medical Centre, Rotterdam, the Netherlands [24,106]. Pregnant women of at least 18 years of age with a gestational age (GA) of less than 8 weeks were eligible for participation and were followed until 1 year after delivery. All pregnant women and their partners gave written informed consent before participation. The Central Committee of Human Research in The Hague and the regional Medical Ethical and Institutional Review Board of the Erasmus MC University Medical Centre approved the study (MEC 2004-227, 15 October 2004).

We selected singleton pregnancies conceived spontaneously or through assisted reproductive techniques using biological oocytes from the participating mother with reliable GA only. GA was defined as reliable when; (1) GA was calculated using the first day of the last menstrual period (LMP) confirmed by first trimester ultrasound evaluation for spontaneously conceived pregnancies with regular menstrual cycles of approximately 28 days and was adjusted when the cycle was prolonged (>31 days) or shortened (<25 days). (2) In pregnancies assisted with *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI) and intra-uterine insemination, GA was calculated from the date of oocyte retrieval plus 14 days, from the day of embryo transfer plus 17 or 18 days cryopreserved embryo transfers, or from insemination date plus 14 days respectively.

In our statistical analyses, we used Crown-rump Length (CRL) as outcome variable. Therefore when there was no reliable recall of the (LMP) or CRL measurements differed more than 7 days from the expected GA, pregnancies were dated on CRL and consequently excluded. Because this study aimed to investigate the initiation of periconceptional maternal folic acid supplement intake in pregnancies ending in live births without congenital malformations, we also excluded pregnancies ending in miscarriages, ectopic pregnancy and termination of pregnancy or pregnancies with adverse foetal outcome including minor and major congenital malformations, foetal or neonatal death. When data from questionnaires or maternal folic acid status was missing, pregnancies were also excluded from further analysis.

Measurements

The study visit before 8 weeks GA was used to verify self-administered questionnaires to obtain information on the current pregnancy with regard to maternal age, preconceptional weight and height, ethnicity, educational level, medical history, gynaecological and obstetric history, familial hereditary or congenital disorders and diseases, diet, lifestyle and the use of folic acid and/or other (multi)vitamin supplements. The accuracy of the self-reported questionnaires was enhanced by an

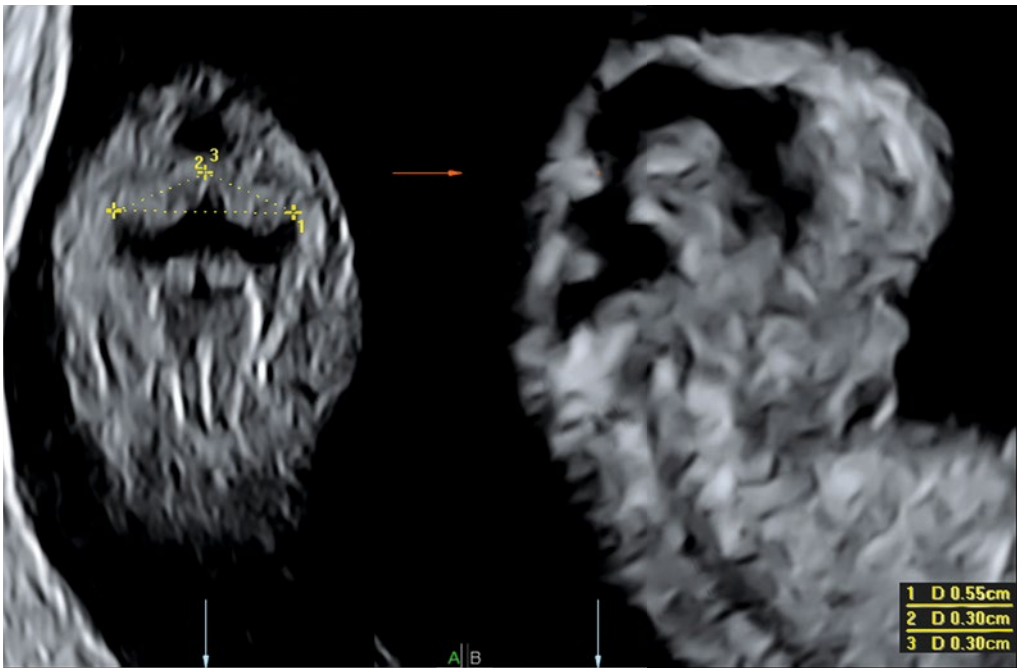
intake consultation at enrolment discussing unclear topics and questions. Follow-up on pregnancy, fetal and infant outcomes comprised of questionnaires validated by the structural ultrasound scan at 20 weeks GA and delivery reports. Periconceptional was defined as the period of 4 weeks before until 8 weeks after conception. Timing of initiation of folic acid use was defined as the initiation at any time before (pre) or after (post) conception of (multi)vitamin supplements containing folic acid either the standard low dosage of 0.4-0.5mg/day or high dosage of 5mg/day. Timing of folic acid initiation served as measure for duration of folic acid supplement use and implicitly the folate status during the first trimester of pregnancy. In a small subgroup (n= 57) a venous blood sample was collected at enrolment for determination of red blood cell folate according to the study protocol representing maternal folate status of 2-4 months previously [121]. Thereby red blood cell folate reflects the folate status of the periconceptional period and specifically the preconceptional period. As red blood cell folate is significantly higher in preconceptional folic acid users ($p<0.01$), we demonstrated that red blood cell folate concentration is an accurate indicator of the timing of initiation and duration of folic acid supplement use in our study population.

High resolution transvaginal three-dimensional ultrasounds were performed weekly between 6^{+0} weeks and 12^{+6} weeks of gestation using a 4.5-11.9 MHz transvaginal probe of a Voluson E8 system (GE Medical Systems, Zipf, Austria). Series of three-dimensional ultrasound sweeps were obtained for biometry measurements focusing on the whole embryo or the head separately. Scans were evaluated off-line using specialised 3D-software (4D View, version 7.0, GE Medical Systems). Only high quality images of the fossa posterior in which the cerebellum could be clearly demarcated were accepted for further analysis. We selected only three-dimensional ultrasound scans between 8^{+0} weeks and 12^{+6} weeks gestation, as success rates of cerebellar measurements before 8 weeks GA were very low [37]. Three-dimensional ultrasound scans were displayed in the orthogonal multiplanar mode in a standardized format ensuring that the left and right side corresponded to the viewer's perspective (Figure 1). Cerebellar measurements were performed in a coronal section of the head at the level of the rhombencephalon enabling the visualisation of cerebellum, fourth ventricle and choroid plexus of the fourth ventricle as previously described [37]. In order to obtain a clearly defined image of the cerebellum at 12 weeks GA, the coronal plane was rotated slightly over the x-axis. The following cerebellar measurements were performed: Transcerebellar diameter (TCD) and left and right cerebellar diameters (LCD, RCD). The 'distance two points' function was used to measure the greatest diameter. All measurements were repeated three times in all eligible 3D sweeps, the mean of these measurements was used for analyses. The proportional cerebellar growth was calculated for all cerebellar parameters by dividing the parameter by CRL.

Two observers (IG and AG) measured the cerebellar parameters in the dataset conducted between 2009 and 2010. Inter-observer reliability and agreement were calculated for 35 cerebellar measurements on randomly selected 3D volumes independently repeated by both measurers. Intra-observer reliability and agreement was evaluated by repeating measurements in 30 randomly selected volumes.

Reliability calculations included measurements from all gestational ages, with a minimum of 5 scans per gestational week. The observers were blinded to each other's and previous results.

Figure 1 — Protocolled display of the three-dimensional ultrasound scan.



Three-dimensional ultrasound image of an embryo at 9+2 weeks GA with the standardized cerebellum measurements TCD (1), LCD (2) and RCD (3) in 4D view.

Statistical analysis

Statistical analysis was performed using SPSS software version 21.0 (SPSS for Windows, SPSS Inc., Chicago, Illinois, USA) and SAS software version 9.3 (SAS Institute Inc., Cary, NC, USA). Results with p-values of 0.05 or lower were considered statistically significant. General characteristics were calculated for all pregnancies and compared between preconceptional and postconceptional folic acid supplement users. Continuous data of maternal age, BMI, birth weight and GA at birth were compared using the Mann Whitney U-test. Categorical data of ethnicity, education, primigravida, nulliparous, mode of conception, periconceptional use of tobacco and alcohol and infant gender were compared using the Pearson Chi-square test.

To assess the association between timing of initiation of folic acid supplement use during the periconceptional period and first trimester cerebellar growth trajectories we performed linear

mixed model analyses. The linear mixed model takes into account the existing correlation between the measurements within one pregnancy. Interaction terms of the timing of initiation of folic acid supplement use with potential confounders were studied, but not included in the model as they yielded no significant contribution. A random intercept only was used to model the within subject correlation, a random slope did not improve the model fit. An unadjusted mixed model was estimated using TCD, LCD and RCD measurements as response variables and CRL and GA as predictors. Timing of initiation of folic acid supplement use was used as independent predictor for cerebellar growth. For the adjusted model we used maternal age, mode of conception, parity, preconceptional smoking, periconceptional alcohol use, BMI, ethnicity, and the measurer as potential confounders, based on literature and significant differences between the preconceptional and postconceptional user groups [106,122]. Stepwise backwards elimination of variables with $p>0.20$ was conducted to determine the final model. Additionally, quartiles of red blood cell folate were calculated and used to perform linear mixed models investigating the association with cerebellar growth trajectories. Including the interaction term between red blood cell folate and GA we were able to study the slope between the four quartiles using the third quartile (Q3) as reference [105].

RESULTS

In 2009 and 2010, 259 viable singleton pregnancies were eligible for this study. We excluded 2 pregnancies conceived from oocyte donations, 43 miscarriages, 1 ectopic pregnancy, 12 pregnancies dated by CRL, 8 pregnancies with abnormal foetal outcome and 7 pregnancies with missing data. This resulted in a final evaluation of 186 pregnancies of 186 women. In 135 (73%) of all pregnancies scans were eligible for cerebellar measurements. All 135 women started folic acid supplements before or after conception. Only 7 women were administered folic acid in a high dosage of 5mg/day, 4 in the preconceptional users and 3 in the post conception users.

In Table 1 the baseline characteristics of the total group of pregnancies, pregnancies with measurements, and the groups of preconceptional and postconceptional initiation of folic acid supplement use are depicted. No significant differences were found between pregnancies with and without cerebellar measurements, except for BMI. Comparing preconceptional and postconceptional folic acid users, general characteristics showed significant differences in ethnicity, primigravida, nulliparous, periconceptional alcohol use, periconceptional smoking and mode of conception via IVF/ISCI procedures. Red blood cell folate is significantly higher in the preconceptional folic acid user group ($p<0.01$). Education level, BMI, neonatal birth weight, GA at birth and gender were comparable between both groups.

A total number of 880 three-dimensional ultrasounds between 8^{+0} weeks and 12^{+6} weeks gestation were available for detailed cerebellar measurements. The number of measurements per pregnancy

is shown in Table 2 Cerebellar measurements could be performed in 263 volumes, demonstrating an overall success rate of 29.9%, with the highest success rate of 41.0% in gestational week 8. Table 3 shows the means of all measurements per gestational week with the corresponding SD values, and the success rate per gestational week.

Table 1 — General Characteristics of the study groups

Characteristics	All pregnancies (n=186)	Pregnancies with measurements (n=135)	Preconceptional Folic acid users (n=108)	Postconceptional Folic acid users (n=27)
Maternal				
Age, years	32.1 (18.9-42.7)	32.1 (18.9-42.3)[1]	32.1 (20.3-42.1)	32.7 (18.9-42.3)
Ethnicity				
Dutch	144 (77.8)	99 (73.3)[2]	84 (77.8)	15 (55.5)*
Western-other	16 (8.6)	14 (10.4)	11 (10.2)	3 (11.1)
Non-western	25 (13.5)	20 (14.8)	11 (10.2)	9 (33.3)
Education				
Low	15 (8.5)	12 (8.9)[3]	10 (9.3)	2 (7.4)
Intermediate	54 (30.5)	40 (29.6)	31 (28.7)	9 (33.3)
High	108 (61.0)	77 (57.0)	63 (58.3)	14 (51.9)
BMI, kg/m^2	23.8 (18.6-38.3)	23.5 (18.6-34.9)**	23.2 (18.6-34.9)	24.1 (19.1-28.7)
Primigravida	69 (37.1)	48 (35.6)	45 (41.7)	3 (11.1)**
Nulliparous	119 (64.0)	83 (61.5)	73 (67.6)	10 (37.0)**
Periconceptional use of alcohol	85 (45.7)	67 (49.6)	49 (45.4)	18 (66.6)*
Periconceptional smoking	31 (16.7)	24 (17.8)	14 (12.9)	10 (37.0)**
Red blood cell folate, nmol/L	1500 (814-2936)	1537 (814-2936)	1626 (844-2936)	1063 (814-1815) **
Mode of Conception IVF/ICSI	57 (30.6)	40 (29.6)	40 (37.0)	0 (0)**
New-born outcome				
Birth weight, grams	3378 (450-4700)	3430 (1715-4700)	3410 (1715-4700)	3440 (2150-4110)
Gestational age at birth, days	276 (187-294)	276 (228-294)	277 (228-294)	276 (258-291)
Gender, male	88 (47.3)	62 (45.9)	50 (46.3)	12 (44.4)

*Continuous data is presented as median, range, categorical data as n (%). BMI, body mass index; IVF/ICSI, in vitro fertilization with or without intra-cytoplasmic sperm injection; Missing data was due to incomplete questionnaires. Missing: [1] n= 3, [2] n= 2, [3] n= 6, * p<0.05, ** p<0.01*

Table 2 — 3D-Ultrasound data and the success rate of measurements

	TCD	RCD	LCD
Images	880	880	880
Measurements	263 (29.9)	259 (29.4)	259 (29.4)
Pregnancies with measurements	135	135	135
≥ 2 measurements	77 (57.1)	75 (55.6)	75 (55.6)
1 measurement	58 (42.9)	60 (44.4)	60 (44.4)
2 measurements	37 (27.4)	36 (26.7)	36 (26.7)
3 measurements	31 (22.9)	31 (22.9)	31 (22.9)
4 measurements	7 (5.1)	6 (4.4)	6 (4.4)
5 measurements	2 (1.5)	2 (1.5)	2 (1.5)

Presented is the number of images (%) eligible for measurement per pregnancy. TCD, transcerebellar diameter; LCD, left cerebellar diameter; RCD, right cerebellar diameter.

Intra-observer reliability analysis showed no significant differences in the mean cerebellar parameters (TCD= 0.020mm, 95%CI= -0.054 to 0.094, LCD= 0.007mm, 95%CI= -0.043 to 0.057, RCD = 0.030mm, 95%CI= -0.027 to 0.087). ICC values for all parameters were above 0.99, representing an excellent reliability between measurements. Analysis of the inter-observer agreement showed a significant mean difference between the two measurers (TCD= 0.501mm, 95%CI= 0.260 to 0.742, LCD= 0.310mm 95%CI= 0.134 to 0.485, RCD= 0.200mm 95%CI= 0.063 to 0.337). A good reliability was established with ICC for TCD, LCD and RCD of respectively 0.92, 0.84, 0.89.

Table 3 — Measurements per gestational week, with corresponding mean and SD values

Ultrasound characteristics	Number of measurements	CRL, mm	TCD, mm	RCD, mm	LCD, mm
GA 8 weeks	73/178 (41%)	18.65 (3.44)	4.38 (0.74)	2.58 (0.35)	2.58 (0.35)
GA 9 weeks	70/177 (40%)	26.15 (4.50)	5.56 (0.97)	3.06 (0.43)	3.07 (0.47)
GA 10 weeks	44/178 (25%)	36.45 (5.82)	7.18 (1.17)	3.78 (0.56)	3.79 (0.54)
GA 11 weeks	39/181 (22%)	48.43 (6.70)	8.97 (1.03)	4.62 (0.48)	4.61 (0.57)
GA 12 weeks	37/166 (22%)	61.46 (6.95)	10.34 (1.16)	5.20 (0.57)	5.21 (0.58)

Presented are the number of images eligible for analysis relative to the total number and the cerebellar parameters per GA. CRL, crown-rump length; TCD, transcerebellar diameter; LCD, left cerebellar diameter; RCD, right cerebellar diameter; GA, gestational age.

Table 4 depicts the results of both crude and adjusted linear mixed model analyses for all 3 cerebellar diameters and the proportional cerebellar growth trajectories by the timing of initiation of folic acid supplement use. Maternal age, mode of conception, periconceptional smoking, periconceptional alcohol use, ethnicity and the measurer were designated confounders in the final model. Subsequently, models for TCD, LCD, RCD and proportional growth (cerebellar diameters divided by CRL) were all

adjusted for these final confounders. Both crude and adjusted linear mixed models showed small but significant effects on cerebellar size and proportional cerebellar growth by the timing of initiation of folic acid supplement use as function of CRL Repeating the same analysis for all cerebellar diameters and the timing of folic acid supplement use initiation as a function of GA, no significant differences were observed in either crude or adjusted models. Both crude and adjusted models showed significantly increased proportional cerebellar growth for all three cerebellar diameters when folic acid supplement use was initiated preconceptionally.

Table 4 — Preconceptional compared to postconceptional initiation of folic acid supplement use and embryonic cerebellar size and growth trajectories as function of gestational age and crown-rump length

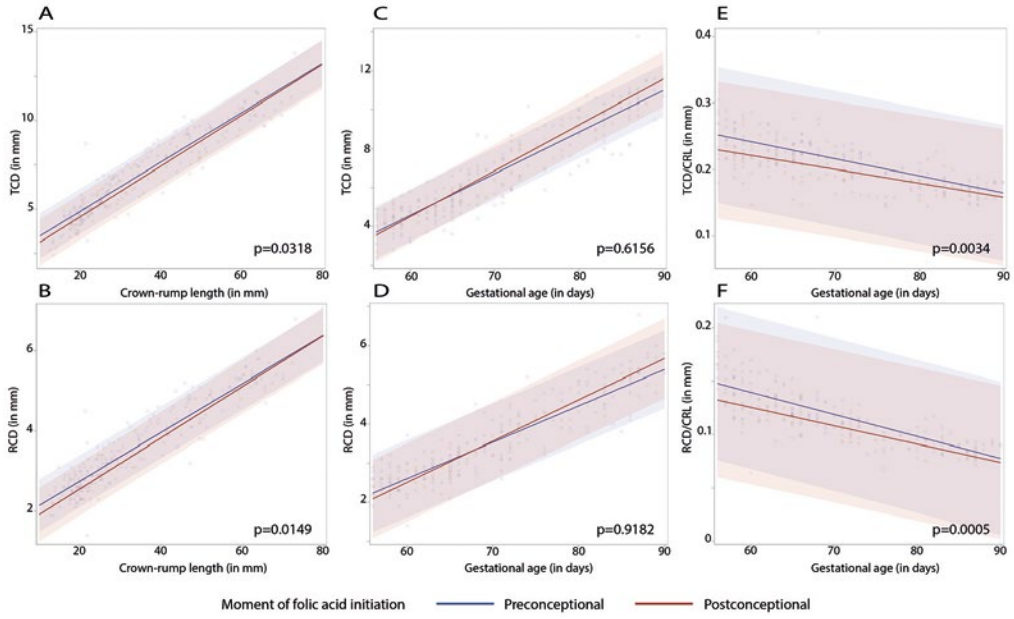
Model		β (se)	95%CI	p-value
TCD vs. CRL	Crude	0.286 (0.098)	0.090; 0.482	0.005
	Adjusted	0.257 (0.117)	0.023; 0.491	0.032
LCD vs. CRL	Crude	0.153 (0.057)	0.038; 0.268	0.010
	Adjusted	0.171 (0.067)	0.038; 0.305	0.013
RCD vs. CRL	Crude	0.132 (0.054)	0.024; 0.240	0.018
	Adjusted	0.156 (0.062)	0.032; 0.280	0.015
TCD vs. GA	Crude	0.112 (0.142)	-0.174; 0.398	0.436
	Adjusted	-0.078(0.154)	-0.388; 0.232	0.616
LCD vs. GA	Crude	0.093 (0.069)	-0.045; 0.232	0.183
	Adjusted	0.041 (0.078)	-0.115; 0.197	0.601
RCD vs. GA	Crude	0.048 (0.068)	-0.090; 0.185	0.490
	Adjusted	0.008 (0.076)	-0.144; 0.159	0.918
TCD/CRL ratio vs. GA	Crude	0.015 (0.004)	0,007; 0,024	0.001
	Adjusted	0.015 (0.005)	0.005; 0.024	0.003
LCD/CRL ratio vs. GA	Crude	0.010 (0.003)	0.004; 0.016	0.002
	Adjusted	0.012 (0.003)	0.005; 0.018	0.001
RCD/CRL ratio vs. GA	Crude	0.010 (0.003)	0.004; 0.015	0.001
	Adjusted	0.011 (0.003)	0.005; 0.017	0.001

Crude and adjusted linear mixed models comparing effect estimates of preconceptional with postconceptional folic acid use of cerebellar growth trajectories. Estimates are depicted as embryonic cerebellar measurement (TCD, LCD and RCD) in mm per mm increase in CRL or days increase in GA. Crude model: Unadjusted model. Adjusted model, adjusted for designated confounders after stepwise backward elimination. TCD, transcerebellar diameter; LCD, left cerebellar diameter; RCD, right cerebellar diameter; CRL, crown-rump length; GA, gestational age; CI, confidence interval.

In Figure 2 we graphically display the results of the adjusted linear mixed models as regression lines. The coloured area represents the ninety-five percent prediction interval for both lines. Comparable results for the right and left cerebellar hemisphere were depicted; therefore we only display the TCD and RCD graphs. There is a clear linear display of data points showing slightly increased cerebellar

diameters as a function of CRL for TCD (A) and RCD (B). The linear functions of preconceptional and postconceptional initiation of folic acid supplement use as function of GA cross for both the total (C) and unilateral (D) cerebellar diameters. Figures 2E and 2F show the proportional growth trajectories as function of GA, with the associated prediction interval, which decreases with advancing gestation.

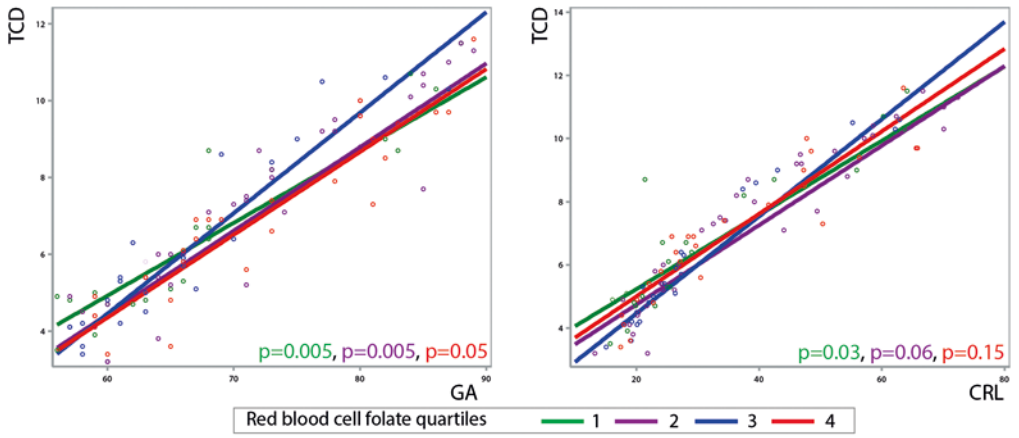
Figure 2 — Longitudinal cerebellar measurements and proportional growth by folic acid initiation



Adjusted linear mixed models represent the effects of preconceptional compared to postconceptional initiation of folic acid supplement use on cerebellar size and growth trajectories as function of CRL in millimetres (A and B) and GA in days (C and D) and proportional cerebellar growth trajectories (E and F).

In our subgroup of 57 patients quartiles for red blood cell folate were calculated (Q1: 814-1242nmol/L, Q2: 1243-1537nmol/L, Q3: 1538-1813nmol/L and Q4: 1814-2936nmol/L). Linear mixed models showed the highest cerebellar growth throughout all models of total, left and right cerebellar diameter in the third quartile of red blood cell folate levels compared to lowest first and second and highest fourth quartiles as a function of GA (Q1= -0.0721mm/day, 95%CI= -0.119 to -0.0250, p<0.01; Q2= -0.0438mm/day, 95%CI= -0.0865 to -0.0011, p<0.01; Q4= -0.0459mm/day, 95%CI= -0.0927 to 0.0010, p= 0.05) and CRL (Q1= -0.0364mm/mm, 95%CI= -0681 to -0.0047, p<0.05; Q2= -0.0280mm/mm, 95%CI= -0.0568 to 0.0008, p>0.05; Q4= -0.0232mm/mm, 95%CI= -0.0554 to 0.0089, p>0.05). The same accounts for proportional cerebellar growth which was the highest in Q3 (Q1= -0.0017mm/mm/day, 95%CI= -0.0039 to 0.0001, p>0.05; Q2= -0.0010mm/mm/day, 95%CI= -0.0026 to 0.0006, p>0.05; Q4= -0.0459mm/day, 95%CI= -0.0018 to -0.0001, p<0.05). Figure 3 depicts cerebellar growth trajectories associated with the four quartiles of maternal red blood cell folate levels.

Figure 3 — Associations between periconceptional red blood cell folate quartiles and cerebellar growth trajectories



Linear mixed models represent the effects of red blood cell folate quartiles on cerebellar growth trajectories as a function of CRL in millimetres and GA in days. The blue line represents the third quartile used as reference.

DISCUSSION

This study shows increased, albeit slightly, embryonic cerebellar diameters and proportional cerebellar growth trajectories in ongoing non-malformed pregnancies in a tertiary hospital setting when maternal folic acid supplement use was initiated before conception compared to post conception. Hereby, we discriminate between overall embryonic growth and embryonic cerebellar growth. This data are validated in a subgroup by maternal red blood cell folate levels before 8 weeks GA which is a more reliable marker for periconceptional folate status. It is intriguing that the third quartile of red blood cell folate levels (1538-1813nmol/L) seems to be the optimum for embryonic cerebellar growth of which the implications for fetal cerebellar growth and future neurodevelopmental outcome needs further investigation. This first study investigating associations between periconceptional folate status and human embryonic cerebellar size and growth trajectories may substantiate to previous animal studies showing alterations in cerebellar growth and development in a folic acid deficient environment [119,123,124]. Besides, our results are in agreement with previous human studies demonstrating significant positive effects of periconceptional maternal folic acid supplement use and folate status on embryonic and fetal size [105,112,122] and DNA methylation of the growth gene IGF2 DMR [16,125].

By establishing a prospective dataset of serial first trimester three-dimensional ultrasound images together with periconceptional exposure data and follow-up information, we were able to study associations between periconceptional folic acid supplement use and embryonic cerebellar growth. We substantiate the validity of our questionnaire data by verification at the intake visit and

measurements of red blood cell folate concentrations in a subgroup. Although data on the precise day of folic acid initiation was not available, red blood cell folate levels were significantly higher when the timing of initiation of folic acid supplement use was defined as preconceptional. This agrees with the assumption that the timing of folic acid use initiation serves as measure for periconceptional folate status which may involve the embryonic environment as well. First trimester red blood cell folate levels follow an optimum curve in which the third quartile is associated with the highest cerebellar growth rates compared to the first two and upper quartile which corresponds to the available evidence of its effects on overall embryonic growth [105]. Because of the prospective and observational character of our study we were able to study associations, causality however could not be shown, since this warrants a randomised controlled trial.

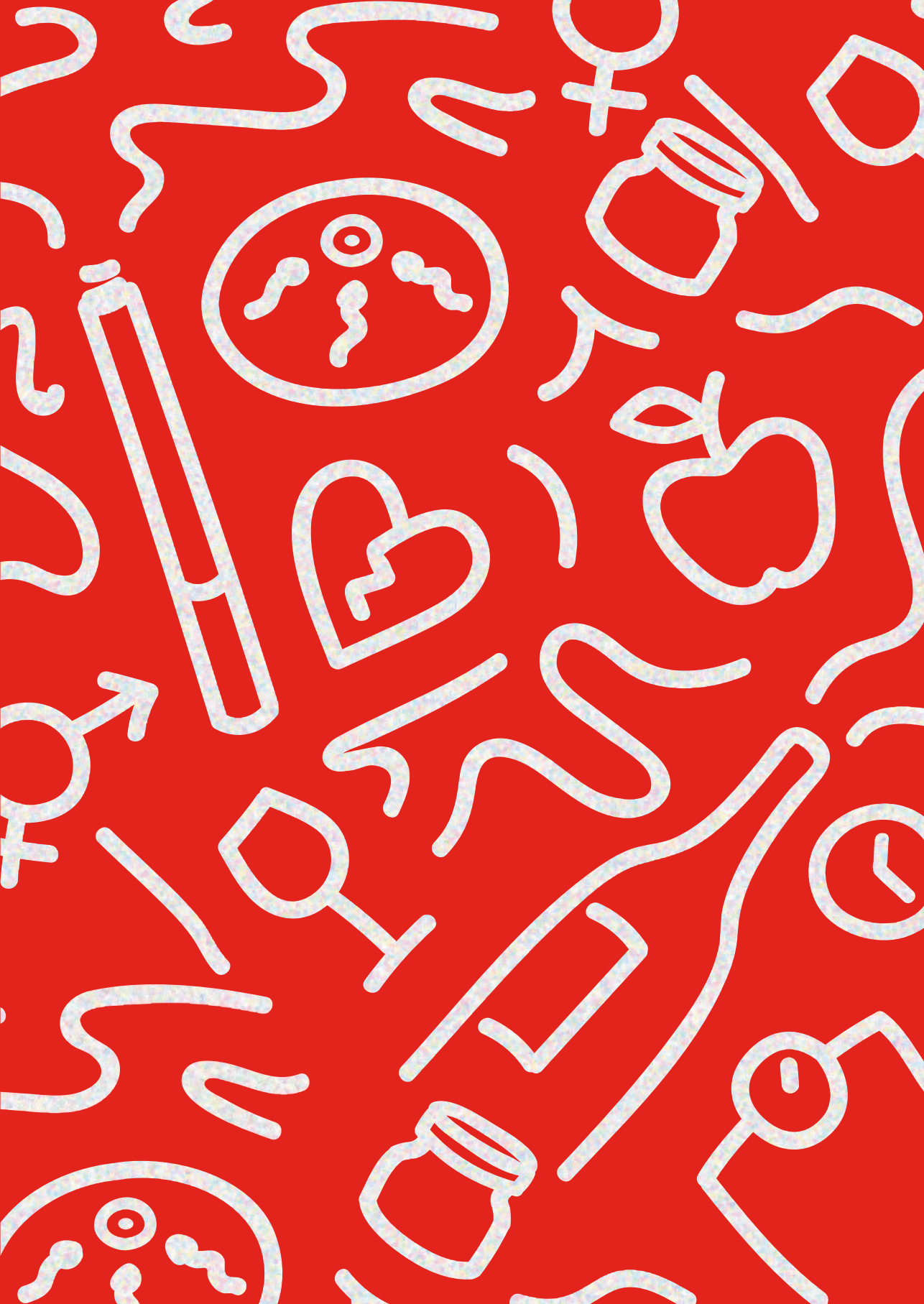
The low success rates of the cerebellar measurements are comparable to previous publications and are due to non-targeted scanning for the purpose of embryonic biometry and volumetric measurements, motion artefacts, acoustic shadowing and maternal factors, such as BMI and an unfavourable position of the uterus [37]. Although differential confounding may be an issue, it seems unlikely as these factors are unrelated to the selected population, the timing of folic acid initiation, red blood cell folate levels and cerebellar outcome. Low success rates could compromise the longitudinal setting of the study. Yet, 57% of the patients had 2 or more measurements. Since measurements within subjects are strongly correlated, linear mixed models are the most appropriate method for the statistical analyses of our data. Preconceptional and postconceptional folic acid users are in many ways not totally comparable. Hence, for statistical analyses we adjusted for known confounders of embryonic growth and maternal characteristics to minimize confounding. Demonstrating comparable effects in both crude and adjusted models for all cerebellar diameters strengthened the model fit.

Differences in cerebellar size and proportional growth trajectories may be explained biologically by folates' vital role in synthesis of DNA, RNA and proteins for cellular growth and differentiation processes. It could be hypothesized that in early embryonic development epigenetic modification of genes including those regulating the development of the cerebellum as part of the central nervous system is altered by the use of folic acid amongst other factors [16,126]. Also, the proportion of cerebellar size in relation to CRL increased when folic acid use was initiated before conception. This is either the result of a larger cerebellum, a smaller CRL, or a combination of the two. Since previous literature demonstrated larger embryonic and fetal biometric measurements in association with optimal folate status, proportional cerebellar growth seems to be increased due to a relatively larger cerebellum in particular [105,122]. However we did not find significant differences in cerebellar measurements between preconceptional and postconceptional folic acid users using GA as predictor. We assume that this is the result of inaccurate pregnancy dating, despite using strict criteria based on reliable LMP only. Inaccurate determination of GA is a recurrent issue in embryonic and foetal growth studies [122]. In this context, it is important to consider that our methodology for pregnancy dating is

stricter than methods used routinely for clinical practice. Hence, our method enables designation of very early growth deviations corrected by pregnancy dating using CRL.

Prenatal modification of cerebellar growth potentially has consequences for neurodevelopmental outcome [107,108]. One could speculate that early changes in embryonic size affect neurodevelopmental functions in early and later life. However, exploring the association between the small differences of embryonic cerebellar growth and its possible clinical implications is beyond the scope of this study. This warrants larger periconceptional cohorts including biomarkers and pre- and postnatal neuroimaging combined with long term standardized neurodevelopmental follow-up. Also, considering the tertiary hospital setting, external validity of our results should be studied in a general population cohort.

In conclusion, our findings show small albeit significant associations between preconceptional initiation compared to postconceptional initiation of folic acid supplement use, used as measure of folate status in the embryonic environment, and increased size and growth trajectories of the embryonic cerebellum. Although the effects of these small differences for prenatal and postnatal neurodevelopment are unknown, this study further supports the importance of periconceptional maternal folic acid supplement use.



4

Prenatal cerebellar growth trajectories and the impact of periconceptional maternal and fetal factors

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ABSTRACT

Background — The cerebellum is essential for normal neurodevelopment and has been associated with neurodevelopmental impairments and psychiatric diseases. Cerebellar development is particularly susceptible to harmful exposures during the prenatal period, including maternal folate status, smoking and alcohol consumption. Can we assess human prenatal cerebellar growth from the first until the third trimester of pregnancy and create growth trajectories to investigate associations with periconceptional maternal and fetal characteristics?

Methods — From 2013 until 2015, we included 182 singleton pregnancies during the first trimester as a subgroup in a prospective periconception cohort with follow-up until birth. For the statistical analyses, we selected 166 pregnancies ending in live born infants without congenital malformations. We measured transcerebellar diameter (TCD) at 9, 11, 22, 26 and 32 weeks gestational age (GA) on ultrasound scans. Growth rates were calculated and growth trajectories of the cerebellum were created. Linear mixed models were used to estimate associations between cerebellar growth and maternal age, parity, mode of conception, geographic origin, pre-pregnancy and first trimester body mass index (BMI), periconceptional smoking, alcohol consumption, timing of folic acid supplement initiation and fetal gender.

Results — 166 pregnancies provided 652 (87%) ultrasound images eligible for TCD measurements. Cerebellar growth rates increased with advancing GA by 0.1691mm/day in the first trimester, 0.2336mm/day in the second trimester and 0.2702mm/day in the third trimester. Pre-pregnancy BMI, calculated from self-reported body weight and height, was significantly associated with decreased cerebellar growth trajectories (β= -0.0331mm, 95%CI= -0.0638; -0.0024, p= 0.035). A similar association was found between cerebellar growth trajectories and first trimester BMI, calculated from standardized measurements of body weight and height (β= -0.0325, 95%CI= -0.0642; -0.0008, p= 0.045 respectively).

Conclusions — Prenatal growth trajectories of the human cerebellum between 9 and 32 weeks gestational age (GA) were created using three-dimensional ultrasound (3D US) and show negative associations with pre-pregnancy and early first trimester Body Mass Index (BMI) calculated from self-reported and standardized measured weight and height, respectively. The cerebellum may act as a mediator in the association between obesity and impaired neurodevelopmental outcome. Our findings further substantiate previous evidence for the detrimental impact of increasing maternal BMI on neurodevelopmental health of offspring in later life. As the study population largely consisted of tertiary hospital patients, external validity should be studied in the general population. Whether small differences in prenatal cerebellar growth due to increasing pre-pregnancy and first trimester BMI have consequences for neurodevelopmental outcome needs further investigation.

INTRODUCTION

The cerebellum originates from 6 weeks gestational age (GA) onwards, preceding most human brain structures and expanding rapidly during the second half of pregnancy [43-45]. It is not surprising that this very early, fast and protracted course of development is particular susceptibility to prenatal disturbances caused by complex gene-environment interactions [45,103]. Impaired cerebellar development has been associated with several periconceptional and prenatal exposures including maternal folate status, smoking habit, alcohol consumption and environmental toxins [58,59,87,127,128].

Prenatal cerebellar alterations and injury are associated with an increased risk for neurodevelopmental impairment [46,51,52]. Recently, MRI studies demonstrated the relation between a reduced cerebellar diameter and adverse neurodevelopmental outcomes, including general motor function, mental development and major neurologic disability, in the young child [28,129].

In the clinical setting, prenatal cerebellar growth is usually evaluated during a routine or extended mid-trimester ultrasound scan by measuring the transcerebellar diameter (TCD) [130,131]. Nowadays, TCD can even be measured reliably from the early first trimester onwards using three-dimensional ultrasound (3D-US). This facilitates longitudinal assessment of cerebellar size providing precise information on variations in prenatal growth trajectories from early gestation onwards [37,127]. To provide new insights in the dynamic process of (ab) normal cerebellar growth, we conducted a prospective longitudinal 3D-US study including the first, second and third trimester with follow-up until birth.

The primary aim of the study was to create a reference curve and growth trajectories of TCD from the first trimester up to the third trimester of pregnancy. Second, we aimed to confirm the hypothesis that derangements in prenatal cerebellar growth may be associated with one or more periconceptional, maternal and fetal characteristics such as age, parity, *in vitro* fertilization / intra cytoplasmic sperm injection (IVF/ICSI), geographic origin, folate status, alcohol consumption, smoking habit and fetal gender.

MATERIALS AND METHODS

Study design and ethical approval

This study was conducted in the setting of the Rotterdam Periconceptional Cohort (Predict study); an ongoing prospective cohort study at the Department of Obstetrics and Gynaecology of the Erasmus MC, University Medical Center, Rotterdam, the Netherlands [24]. The study protocol was approved by the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam, the Netherlands (MEC 2004-227). All participants and partners signed written informed consent at enrolment, also on behalf of their unborn child.

Study population

From November 2013 until March 2015, pregnant women who volunteered for the Predict study during the first trimester with ongoing singleton pregnancies were invited to undergo another set of 3D-US examinations. For the current study, we selected pregnancies without congenital malformations, conceived either spontaneously or through assisted reproductive techniques using oocytes from the participating mother only. Exclusion criteria were oocyte donation and all minor and major fetal congenital malformations. For this study we excluded pregnancies without fetal 3D-US or questionnaire data due to withdrawal, termination of pregnancy or intra-uterine fetal death.

Study parameters and endpoints

Data on maternal characteristics, medical and obstetrical history, BMI and lifestyle behaviours were obtained through self-reported questionnaires upon enrolment before 12 weeks GA. A researcher verified the questionnaires and also standardized measured height and weight to calculate BMI at the study entry visit before 12 weeks GA. Educational level was categorized as low, middle or high and geographic origin was categorized as Dutch, Western and Non-western according to Statistics Netherlands [132]. Questionnaires filled out in the second and third trimesters provided follow-up information on pregnancy course and neonatal outcomes. This data was validated by obstetric medical records and by ultrasound reports of the routine anomaly scan performed between 18 and 22 weeks GA.

Estimation of GA was based on protocolled clinical methods using first trimester crown-rump length (CRL) measurements before 13 weeks GA [133]. In pregnancies conceived through IVF/ICSI procedures, the GA was calculated from the date of oocyte retrieval plus 14 days. In pregnancies conceived through intrauterine insemination, the GA was calculated using the insemination date. The GA in pregnancies conceived after the transfer of cryopreserved embryos was calculated from the day of embryo transfer plus 17 or 18 days, depending on the number of days between oocyte retrieval and cryopreservation.

Ultrasound

All participants received serial 3D-ultrasound examinations at 9, 11, 22, 26 and 32 weeks GA and were followed until delivery. All ultrasound examinations were performed by one certified sonographer (IVK). All ultrasound scans were performed with the Voluson E8 system (GE Medical Systems, Zipf, Australia). During first trimester 3D-US scans a 6-12 MHz transvaginal transducer was used, during fetal ultrasounds we used a 1-7 MHz transabdominal transducer or a 6-12 MHz transvaginal transducer. All 3D volumes were stored digitally as Cartesian and 4DView volumes. First trimester measurements of the transcerebellar diameter (TCD) were performed offline using 4D View Version 5.0 (GE Medical Systems) according to the protocol which was previously described [37,127]. Only 3D-US volumes at 9 and 11 weeks GA of good quality without motion artefacts were used. Standardized biometric measurements of the TCD were obtained online during fetal ultrasound examinations. All first, second and third trimester measurements were repeated three times and the mean values of the three measurements were used for statistical analyses.

Statistical analysis

For data analyses we used SPSS (SPSS release 21 for Windows, IBM, USA). All results with p-values <0.05 were considered statistically significant. Mean values and success rates of the TCD measurements were calculated per GA in weeks. To differentiate between first, second and third trimester cerebellar growth rates, we calculated the differences of two transcerebellar diameters and divided these by the difference in GA in days between the ultrasound examinations following the equations below:

$$\text{First trimester cerebellar growth} = \frac{TCD11 - TCD9}{GA11 - GA9}$$

$$\text{Second trimester cerebellar growth} = \frac{TCD26 - TCD22}{GA26 - GA22}$$

$$\text{Third trimester cerebellar growth} = \frac{TCD32 - TCD26}{GA32 - GA26}$$

To assess the associations between the periconceptional maternal and fetal characteristics and cerebellar growth we performed linear mixed model analyses, taking into account the existing correlation for repeated measurements within one pregnancy. A random intercept only was used to model the within subject correlation. Linear mixed models were estimated using the longitudinal cerebellar measurements as response and GA as predictor. Likelihood ratio tests were used to test which polynomials of GA best described the trajectory of the repeated cerebellar measurements. The quadratic polynomial model performed best; therefore both GA and GA squared were included in the final model. After designating the best model fit, an equation was calculated representing the relationship between the repeated measurements of TCD and GA. Linear mixed models were estimated to investigate the associations between TCD, GA and the individual maternal conditions (Model 1). As covariates we considered documented maternal conditions from our previous studies and literature including maternal age, BMI, geographic origin, moment of periconceptional initiation of folic acid supplement use, periconceptional smoking, periconceptional use of alcohol, parity, and IVF/ICSI as mode of conception and fetal gender. All covariates were entered simultaneously in the fully adjusted model (Model 2).

RESULTS

Study population

Between November 2013 and March 2015, 182 pregnancies were included in the first trimester of pregnancy. We excluded pregnancies resulting from oocyte donation (3); pregnancies complicated with fetal congenital malformations (5); pregnancies without fetal ultrasound examinations due to withdrawal (4) intra-uterine fetal death (1) or termination of pregnancy (1) and when questionnaire data were missing (2). Table 1 lists the general characteristics of the included study population of 166 pregnancies. In total 652 of 762 (86%) of ultrasound scans was eligible for TCD measurements. Mean values and success rates of TCD measurements per GA are shown in Table 2.

Table 1 — General Characteristics

Characteristics	All (n= 182)	Selected (n= 166)	Missing
Maternal			
Age, year	32 (21-48)	32 (21-45)	3
Nulliparous	76 (43)	70 (42)	0
Mode of Conception IVF/ICSI	53 (30)	49 (30)	0
Geographic origin			1
Dutch	133 (75)	124 (75)	
Western other	12 (7)	10 (6)	
Non-western	33 (19)	31 (19)	
Educational level			1
Low	21 (12)	20 (12)	
Intermediate	65 (37)	64 (39)	
High	91 (51)	81 (49)	
BMI, pre-pregnancy, self-reported, kg/m^2	23 (15-40)	23 (15-40)	10
BMI, first trimester, measured, kg/m^2	24 (16-43)	24 (16-43)	36
Preconception folic acid initiation	124 (72)	114 (71)	6
Periconception alcohol consumption	51 (29)	45 (28)	3
Periconcepion smoking	29 (16)	28 (17)	2
New born			
Gestational age at birth, days	273 (182-292)	273 (182-292)	2
Birth weight, grams	3293 (665-4380)	3285 (665-4380)	2
Gender, male	90 (50)	83 (50)	0

Data are presented as median and range or number (n) and percentage (%). BMI, body mass index; IVF/ICSI, in vitro fertilization with or without intra-cytoplasmic sperm injection; Missing data was due to incomplete questionnaires.

Table 2 — Ultrasound examinations and transcerebellar diameter per gestational age

	Number US scans	Number of measurements	Success rate, %	TCD (±SD) mm
GA 9^{+0} to 9^{+6}, weeks	114	78	68	6.41 (±0.64)
GA 11^{+0} to 11^{+6}, weeks	162	99	61	8.60 (±0.67)
GA 21^{+3} to 23^{+3}, weeks	166	166	100	24.08 (±0.94)
GA 25^{+0} to 27^{+6}, weeks	165	163	99	30.67 (±1.34)
GA 31^{+4} to 34^{+0}, weeks	155	146	94	41.89 (±1.56)

Presented are the number of images eligible for TCD measurements from the total available 3D-US scans per GA. Measurements per gestational week, with corresponding mean and SD values. TCD, transcerebellar diameter in millimetres; GA, gestational age in weeks.

Figure 1A presents a spaghetti plot in which every line corresponds to the TCD measurements of each pregnancy. The mean growth rate of TCD increased during gestation with the highest growth rate during the third trimester (1st trimester= 0.1691mm/day, 95%CI= 0.0727 to 0.2655; 2nd trimester= 0.2336 mm/day, 95%CI= 0.1731 to 0.2941; 3rd trimester= 0.2702 mm/day, 95%CI= 0.2216 to 0.3175).

The relation between repeated TCD measurements and GA was estimated as the following equation: $TCD = -2.8248 + 0.1124 * GA + 0.0004 * GA^2$. This resulted in an mean TCD of 3.58mm at 49 days GA, 5.74mm at 56 GA and 8.04mm at 77 days GA. The reference curve for the cerebellum between 9 and 32 weeks GA is presented in Figure 1B.

The results of the linear mixed models estimating the associations between the repeated cerebellar measurements and periconceptional maternal characteristics are shown in Table 3. A significant negative association between pre-pregnancy BMI and cerebellar growth was found in the crude model (Model 1). The associations sustained in the fully adjusted model (Model 2) in which the cerebellar growth trajectories are 0.033mm lower for every point increase in BMI. To validate the self-reported data of pre-pregnancy BMI, we repeated the analysis with standardized first trimester maternal BMI measurements. These results were comparable both in the crude (β= -0.0278, 95%CI= -0.0549 ; -0.0008, p= 0.044) and fully adjusted model (β= -0.0325, 95%CI= -0.0642 ; -0.0008, p= 0.045).

The analyses showed a trend towards a positive association between male gender and cerebellar growth. No significant associations were demonstrated between growth trajectories of the cerebellum and maternal age, parity, mode of conception, geographic origin, moment of folic acid supplement initiation, periconceptional alcohol consumption and smoking.

To illustrate our findings we created a categorical variable of pre-pregnancy BMI according to the WHO classification: Underweight: <18.5kg/m^2; normal: 18.5-24.99kg/m^2; overweight: 25-29.99kg/m^2; severe obesity: >30 kg/m^2 [134]. Figure 1C shows the associations between cerebellar growth trajectories and these four categories (Blue= underweight, green= normal, yellow= overweight, red= severe obesity). Cerebellar growth trajectories are lowest in the overweight group (β= -0.298, 95%CI= -0.597 ; 0.002, p= 0.05) and the obese group (β= -0.097, 95%CI= -0.526 ; 0.332, p= 0.66) and highest in the underweight group (β= 0.258, 95%CI= -0.220 ; 0.736, p= 0.29) all compared to the group with a normal BMI.

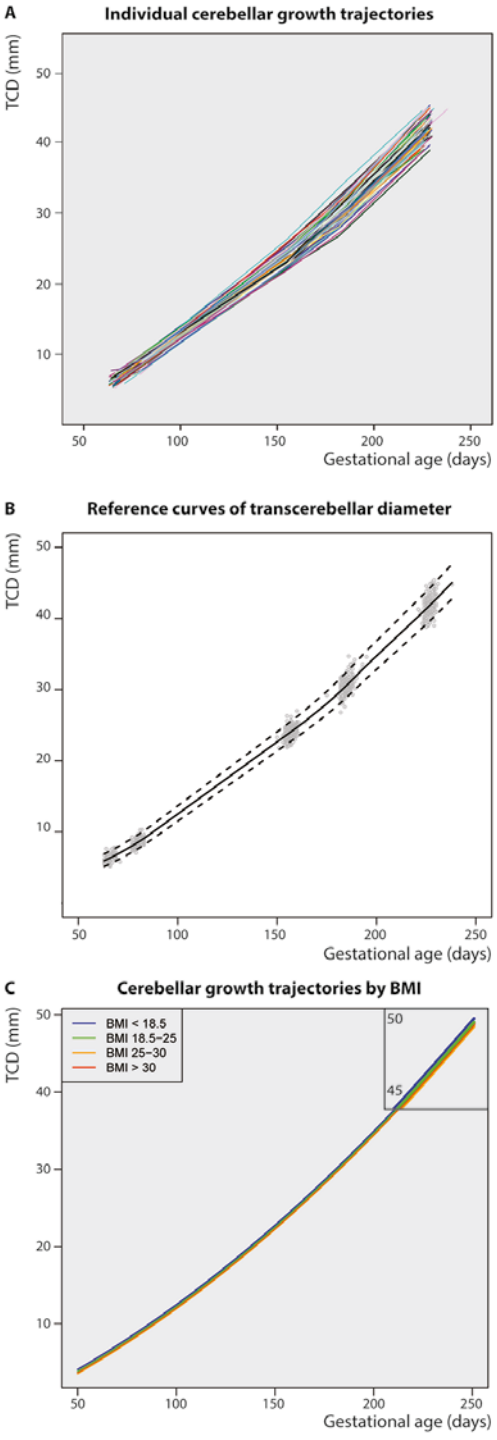

Figure 1 — A reference curve and prenatal growth trajectories of transcerebellar diameter

(A) Growth trajectories of TCD measurements in millimetres are presented as a function of gestational age per individual. (B) The reference curve of TCD is with the 5th (dashed line), 50th (solid line) 95th (dashed line) percentile. (C) Mean growth trajectories per category of BMI. TCD, transcerebellar diameter; BMI, body mass index in kg/m^2.

Table 3 — Linear mixed models: Associations between periconceptional maternal and fetal factors and cerebellar growth trajectories

	Model	β	95%CI	p
Maternal age	1	-0.0039	-0.0296 ; 0.0219	0.768
	2	-0.0013	-0.0319 ; 0.0293	0.932
Nulliparous	1	0.0280	-0.2162 ; 0.2724	0.821
	2	-0.0558	-0.3458 ; 0.2342	0.704
Mode of conception (IVF/ICSI)	1	0.0259	-0.2347 ; 0.2865	0.845
	2	0.0309	-0.2890 ; 0.3508	0.849
Geographic background (Dutch)	1	0.0020	-0.3138 ; 0.3178	0.990
	2	-0.0761	-0.4403 ; 0.2881	0.680
Geographic background (Western other)	1	-0.1104	-0.6873 ; 0.4666	0.706
	2	-0.5315	-1.1811 ; 0.1181	0.108
Pre-pregnancy BMI	1	-0.0323	-0.0596 ; -0.0050	0.021
	2	-0.0331	-0.0638 ; -0.0024	0.035
Folic acid (preconceptional)	1	0.2115	-0.0655 ; 0.4884	0.134
	2	0.2277	-0.1141 ; 0.5695	0.190
Periconception alcohol (Yes)	1	-0.0144	-0.2882 ; 0.2594	0.917
	2	-0.0494	-0.3806 ; 0.2819	0.769
Periconception smoking (Yes)	1	0.0593	-0.2665 ; 0.3851	0.720
	2	0.1210	-0.2693 ; 0.5113	0.541
Gender (male)	1	0.1720	-0.0697 ; 0.4137	0.162
	2	0.2349	-0.0338 ; 0.5036	0.086

Data is presented in ß values with corresponding 95%CI and p-values. Significant results are in bold. Model 1 represents the crude models with only gestational age and the covariate of interest as predictor. Model 2 represents the fully adjusted models with all covariates entered simultaneously. ß, beta value; 95%CI, ninety-five percent confidence interval; BMI, body mass index in kg/m²; IVF/ICSI, in vitro fertilization/intra-cytoplasmic sperm injection.

DISCUSSION

We successfully created cerebellar growth trajectories using repeated TCD measurements from the first, second and third trimester in a tertiary hospital population. Data analysis revealed that maternal BMI is negatively associated with cerebellar growth between 9 and 32 weeks GA. Interestingly, no significant associations were found between cerebellar growth and maternal age, parity, IVF/ICSI treatment, timing of initiation of folic acid supplements, periconceptional alcohol consumption and smoking. Male gender showed a trend towards a positive association with cerebellar growth. In agreement with previous literature, we demonstrated a nonlinear growth pattern (Figure 1A) with cerebellar growth rates increasing with advancing GA, thus being highest in the third trimester [135,136].

Strengths and weaknesses

Our longitudinal study design enabled us to study cerebellar growth as a dynamic process during pregnancy. In contrast to a cross-sectional approach we studied actual growth patterns as opposed to size at one time point. To draw more robust conclusions on the impact of maternal BMI we analysed associations between cerebellar growth and self-reported BMI as well as measured BMI. Epidemiological studies consider self-reported pre-pregnancy BMI an adequate measure; however standardized BMI measurements are more precise and not influenced by recall bias. Our periconceptional cohort study is conducted in a tertiary hospital setting; therefore the results may not be valid for the general population. Moreover, residual confounding inherent to the observational design cannot be excluded. As TCD is strongly correlated to GA, small errors in pregnancy dating may be a confounder of cerebellar growth. Although a standardized clinical method was used to calculate GA, estimation of GA remains a challenge in prenatal growth studies as well as clinical practice [24].

Main findings

Our results add to the growing recognition that maternal obesity has an impact on neurodevelopmental health in offspring. We studied cerebellar growth in a tertiary hospital population without congenital malformations including abnormalities or hypoplasia of the cerebellum, and showed a negative association with periconceptional maternal BMI. This suggests that a higher BMI is associated with a slight decrease in cerebellar growth within the normal ranges of which the consequences for neurodevelopment in postnatal life need further investigation. Our findings are in agreement with the reported altered in white matter development in children of obese mothers [137]. Another study reported that every increase of 1 unit pre-pregnancy BMI was associated with a significant reduction of the intelligence coefficient in the child [138]. Several studies demonstrate that children born to obese mothers are at higher risk for intellectual disability or cognitive deficit [139-141] as well as impaired neurodevelopmental outcomes including attention-deficit hyperactivity disorder, autism spectrum disorders and other behavioural problems [142-145]. Interestingly, these particular impairments and mental health issues have also been associated with abnormal development of the cerebellum [51,52,146]. The cerebellum has been recognized as a key player in a spectrum of neurodevelopmental complications as it is involved in a variety of sensorimotor tasks and cognitive, emotional and language behaviour [47-50]. Therefore, we hypothesize that the cerebellum acts as an intermediate in the association between maternal BMI and neurodevelopmental outcome. A harmful intra-uterine environment resulting from an increasing BMI would then cause deviations in cerebellar growth which subsequently causes an increased risk for impaired neurodevelopment. Such a hypothesis is supported by accumulating evidence linking epigenetic modifications to early brain development and neurodevelopmental functioning [147,148]. More clues on such a relationship can be found in human and mouse models of autism disorders demonstrating similar patterns of cerebellar DNA damage and methylation [149].

The association between maternal BMI and decreased cerebellar growth trajectories may be attributable to BMI as a composite determinant of several mechanisms. Potential mechanisms include dietary intake, nutritional status, chronic inflammation and excessive oxidative stress [150-155]. These mechanisms may act on the level of epigenetics causing derangements in DNA methylation in genes which play a crucial role in fetal, neonatal and adult health [156,157]. BMI can be a strong determinant of such epigenetic derangements [158]. In addition, neurodevelopment could also be influenced by downstream effects of gestational weight gain, fetal growth alterations and the increased risk of pregnancy complications [139]. Whether these mechanisms act alone or as a component of a multifactorial process influencing prenatal neurodevelopment needs to be elucidated. Nevertheless, a higher maternal BMI offers an altered genetic, hormonal and biochemical environment for the fetus and its developing brain.

No other significant associations between cerebellar growth and periconceptional maternal and fetal factors were demonstrated. The lack of an association with folate status in particular is in contrast with our previous results [127]. Possibly, the impact of the moment of periconceptional folic acid initiation on the growing cerebellum attenuates over this larger growth trajectory. Fetal head growth was previously reported to be negatively associated with maternal smoking [58,159]. We speculate that the cerebellum in comparison to overall brain growth might be relatively protected when harmful exposures pose a risk for causing alterations. Since the cerebellum is also mostly unaffected in growth restricted fetuses, [160] normal cerebellar growth seems preferential to head growth. Hence, finding a relation between BMI and cerebellum growth strengthens our case of an actual effect.

Future implications

As the proportion of obese women of reproductive-age worldwide has increased dramatically, the potential adverse effects on prenatal brain development deserve more attention. In particular, it would be interesting to know whether minor deviations in prenatal cerebellar growth affect long-term neurodevelopmental outcome. Serial measurements of the cerebellum will be of additional value for studying the protracted process of cerebellar development. Future prospective birth cohorts with a focus on serial cerebellar growth measures and specific functional cerebellar outcome data are warranted. Preconception counselling and lifestyle interventions may encourage future mothers to attain a healthy weight prior to conceiving and thereby potentially decreasing the risk for impaired neurodevelopmental outcome in the new generation.

PART II

EARLY HEAD AND BRAIN DEVELOPMENT

5

Growth trajectories of the human embryonic head and periconceptional maternal conditions

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ABSTRACT

Background — Fetal growth is influenced by periconceptional maternal conditions. We aim to investigate whether growth trajectories of the human embryonic head can be created using three-dimensional ultrasound (3D-US) and virtual reality (VR) technology, and are associated with second trimester fetal head size and periconceptional maternal conditions?

Methods — Bi-parietal diameter (BPD) and occipital frontal diameter (OFD) to calculate head circumference (HC), head volume (HV) and crown-rump length (CRL) were measured weekly between 9^{+0} and 12^{+6} weeks gestational age (GA) using 3D-US and VR. Fetal HC was obtained from second trimester structural anomaly scans. Growth trajectories of the embryonic head were created with general additive models and linear mixed models were used to estimate associations with maternal periconceptional conditions as a function of GA and CRL, respectively.

Results — We selected 149 singleton pregnancies with a live born non-malformed fetus from the Rotterdam periconception cohort. 303 3D-US images of 149 pregnancies were eligible for embryonic head measurements (intra-class correlation coefficients (ICC) >0.99). Associations were found between embryonic HC and fetal HC (ρ= 0.617, $p<0.001$) and between embryonic HV and fetal HC (ρ= 0.660, $p<0.001$) in Z-scores. Maternal periconceptional smoking was associated with decreased, and maternal age and IVF/ICSI treatment with increased growth trajectories of the embryonic head measured by HC and HV (All $p<0.05$).

Conclusions — Serial first trimester head circumference (HC) and head volume (HV) measurements were used to create reliable growth trajectories of the embryonic head, which were significantly associated with fetal head size and periconceptional maternal smoking, age and *in vitro* fertilization (IVF)/intra-cytoplasmic sperm injection (ICSI) treatment. The consequences of the small effect sizes for neurodevelopmental outcome need further investigation.

INTRODUCTION

Fetal health determined by growth and development has consequences for health and disease in later life [15,16,41,161]. New evidence reveals that embryonic growth and development can be an early predictor of fetal health [15,24,98]. Neurodevelopmental health in particular may already originate during neurogenesis in the first weeks of gestation [162]. Therefore it is essential to establish measures for the earliest possible prenatal detection of neurodevelopmental disorders.

So far, measurements of fetal head circumference (HC) are performed to monitor growth and development of the fetal head and brain with suggested implications for neurodevelopmental outcome [9,163]. In contrast to actual brain volume measurements, which are notoriously difficult to assess *in utero*, HC is a feasible measure used from the first trimester until the neonatal period. However, HC measurements can be imprecise and associations with neurodevelopmental outcome remain subject to discussion [164,165]. During the last decade, three-dimensional (3D) ultrasound (US) techniques provide accurate and precise markers for actual growth, most accurately defined as an increase in volume, in addition to 2D-US crown-rump length (CRL) measurements [166-168]. Furthermore, virtual reality (VR) systems visualizing 3D volumes offer depth perception and interaction with projected images enabling precise semi-automated volume measurements [168]. In this context, to evaluate (ab) normal head growth using VR, head volume (HV) compared to conventional HC measurements may serve as a more accurate measure for prenatal neurodevelopment. Previous studies already demonstrated that 3D-US embryonic volume measurements provide more information and enable a more sensitive detection of altered or impaired growth including fetal growth restriction and chromosomally abnormal fetuses in the first trimester of pregnancy [169-172].

Prenatal head and brain development can be disrupted during pregnancy. As the mother serves as a direct environment for her growing fetus, maternal conditions, such as obesity, smoking and use of alcohol are associated with deviations in growth of the head and brain with consequences for neurodevelopment [29,58,152,173,174].

Monitoring small deviations in growth of the embryonic head by ultrasonography is still challenging. Therefore, we hypothesize that assessment of growth trajectories of embryonic HV using new sophisticated 3D-US and VR techniques may serve as a more accurate marker for embryonic head growth compared to the conventional HC measurements. Moreover, these precise embryonic head growth trajectories may enable us to study small deviations in head growth and associations with periconceptional maternal conditions and exposures. Therefore, we aim to create growth trajectories and reference curves of the embryonic head and investigate associations with fetal head size and periconceptional maternal conditions.

MATERIALS AND METHODS

This study is embedded in the Rotterdam Predict Study a prospective periconception cohort investigating the influence of gene-environment interactions and underlying epigenetic mechanisms on embryonic parameters and pregnancy outcome at the outpatient clinic of the Department of Obstetrics and Gynaecology, Erasmus MC University Medical Centre in Rotterdam, the Netherlands [24]. All pregnant women and their partners enrolled in 2009 and 2010 and gave written informed consent before participation.

Study population

Enrolment of pregnant women was aimed before the 6th week of gestation. We selected viable singleton pregnancies conceived spontaneously or conceived through assisted reproductive techniques using biological oocytes from the participating mother. Exclusion criteria were oocyte donation, miscarriages, ectopic pregnancy, intra-uterine fetal death, neonatal death, minor and major congenital anomalies and termination of pregnancies. We also excluded pregnancies with unreliable gestational age (GA). GA was defined as unreliable, when pregnancy dating relied on CRL measurements only, in case of; (1) Unknown or unreliable recall of last menstrual period (LMP), (2) irregular menstrual cycle (cycle <25 or >31 days), or (3) discrepancy of more than 6 days between expected GA based on LMP and first trimester CRL measurement according to the Robinson curve [133]. Pregnancies with missing questionnaires, unknown maternal folic acid status and ultrasound scans with low quality were also excluded.

Maternal and pregnancy characteristics

Gestational age was calculated strictly according to the first day of the LMP in spontaneously conceived pregnancies with regular menstrual cycles. In case of *in vitro* fertilization (IVF), intra-cytoplasmic sperm injection (ICSI) or intrauterine insemination, GA was calculated from the conception date plus 14 days, plus 18 days in cryopreserved embryos or from the insemination date plus 14 days respectively. Information on maternal characteristics, dietary and lifestyle behaviours and medical and obstetrical history was obtained through a self-reported questionnaire upon enrolment before 8 weeks of gestation. From reports of the routine second trimester structural anomaly scans, which are carried out between 18 and 22 weeks GA as standard obstetrical care, we obtained fetal biometric measurements including HC. Data on pregnancy complications, congenital malformations and neonatal outcome were obtained from medical records of the hospitals registries.

Ultrasound data

3D-Ultrasound examinations were performed using a 4.5-11.9 MHz transvaginal probe of the Voluson E8 system (GE Medical Systems, Zipf, Austria). Weekly transvaginal 3D-ultrasound examinations were performed between 6^{+0} and 12^{+6} weeks gestational age, obtaining 3D volumes encompassing the whole embryo. All 3D-US volumes were stored as Cartesian volumes and analysed in the BARCO I-Space a

four walled CAVE$_{TM}$-like VR system in which investigators explore 3D images with depth perception [175]. Only high quality images, eligible for embryonic volume measurements were used for the measurements of the head. Images without clear reference points or without a clear delineation of the borders of the head were excluded for further analysis. We only performed measurements on scans between 9+0 and 12+6 weeks GA because the selected reference points to define the cutting plane needed for HV measurements only become visible at the end of gestational week 8.

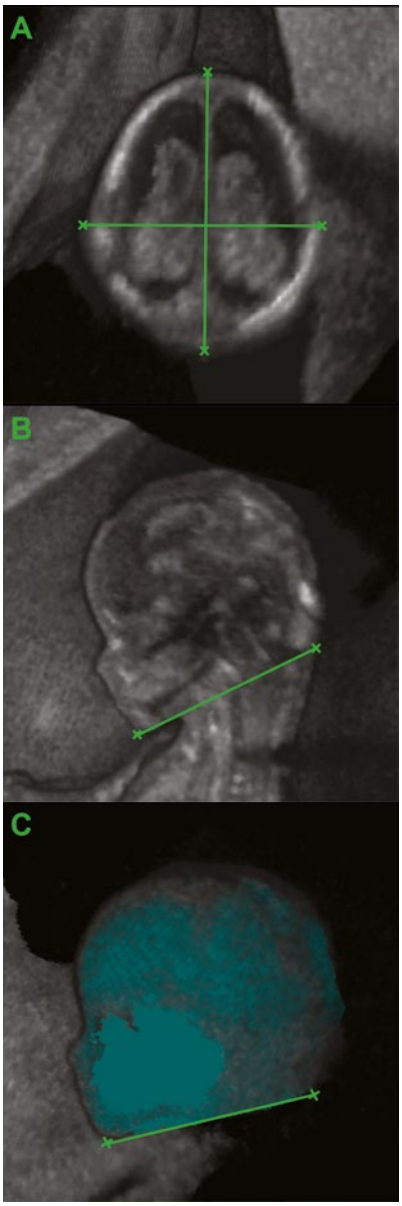


Figure 1 — Three dimensional ultrasound images for the measurements of human embryonic head parameters using the VR technique of the BARCO I-Space

Image of OFD and BPD measurement of an embryo at 12+4 gestational age (A). Reference points for embryonic HV measurement (B). Embryonic HV after segmentation of the head (C). OFD, occipital frontal diameter; BPD, bi-parietal diameter; HV, head volume; VR, virtual reality. Images with permission.

The following parameters were measured: CRL (mm), bi-parietal diameter (BPD) (mm), occipital frontal diameter (OFD) (mm) and HV (cm^3). The BPD and OFD measurements were performed in an axial plane of the embryonic head with visualization of both lateral ventricles and a vertical midline (Figure 1A). In scans of gestational week 9 and 10, this plane also includes visualization of diencephalon and mesencephalon. Both diameters were measured by placing callipers on the outer borders of the skull; BPD perpendicular to the midline and OFD along the midline [35]. To verify correct placement of the callipers, the embryo was turned in a coronal plane for BPD measurements and to a mid-sagittal plan for OFD measurements. The HC was calculated using the formula from GE medical systems:

$$HC = \pi\left[0.75(BPD+OFD) - \sqrt{\frac{BPD+OFD}{4}}\right]$$

HV measurements were performed using the V-scope application in a standardized manner as described in detail before [171,176]. Prior to measuring HV, the cutting plane was defined. The lowest point of the chin and the lowest point of the fourth ventricle in the mid-sagittal plane were selected as reference points. Next, a line was drawn between these two points to indicate the cutting level below which the embryonic body could be 'erased' (Figure 1B). Firstly, in order to measure HV, hyperechoic structures were segmented using a region growing approach, selecting an upper (255) and lower (60) grey level threshold and an upper (80-90) threshold for the standard deviation, before placing the seed point. Secondly, hypo-echoic regions including the brain ventricles were segmented by directly marking the voxels using a virtual 'brush'. Volume measurements were performed by two independent examiners (S.C.H and L.B.). Inter- and intra-observer reliability and agreement were calculated for a subset of 34 randomly selected images of 34 pregnancies including all GA from 9 to 12 weeks. For analyses measurements were repeated by two examiners who were blinded to previous and each other's results.

Statistical analyses

For data analyses we used SPSS (SPSS release 21 for Windows, IBM, United States of America) and R (R: A language and Environment for Statistical Computing, version 3.1.3 2015 for Windows, R Core Team, Vienna, Austria). All results with p-values below 0.05 were considered statistically significant. General characteristics were calculated for all pregnancies and pregnancies with and without volume measurements. A sensitivity analysis was performed to compare pregnancies with and without measurements using Mann Whitney U-test for continuous data and Chi-square test for categorical data.

To assess intra- and inter-observer reproducibility of HV measurements mean difference and mean percentage difference with corresponding 95% limits of agreement (mean (percentage) difference ± 1.96SD) are calculated. To assess agreement between and within the two examiners Bland-Altman plots were created. Intra-class correlation coefficients (ICCs) were calculated to quantify inter- and intra-observer reliability.

Embryonic head growth trajectories and regression equations were calculated and visualized for HV as a function of GA and CRL. Fourth root transformations of HC and HV were calculated to approach linearity for the regression lines in relationship to GA. A root transformation for HV was used for the regression line in relationship to CRL. Square of HC was used for the regression line with CRL. Reference curves for HV were created based on the Generalized Additive Model for Location, Scale and Shape [177]. Based on Akaike Information Criterion (AIC) and Worm plots we observed that the use of a normal model after Box-Cox transformation was the optimal model [178]. With these reference curves we calculated the average first trimester Z-score for HC and HV and second trimester HC. For each pregnancy, the mean of the longitudinal embryonic Z-scores of HC and HV was plotted against the second trimester Z-scores of the HC-measurements.

Linear mixed models were estimated using PROC MIXED. To study associations with growth of the embryonic head and maternal conditions we considered maternal age, moment of periconceptional initiation of folic acid supplement use, periconceptional smoking, periconceptional use of alcohol, parity, and IVF/ICSI as mode of conception [106]. Linear mixed models were estimated using the longitudinal embryonic head measurements as response and GA or CRL and maternal conditions as covariates. The same transformations for HV and HC were used to approach linearity. In the model we used random intercept and random coefficients of GA and CRL as well as a power variance function to fit the covariance structure [179]. Firstly we performed univariate analyses for both GA and CRL and all individual maternal conditions. Secondly, in the multivariable model we entered all maternal conditions simultaneously in the fully adjusted model.

Ethical Approval

The Central Committee on Research Involving Human Subjects in The Hague and the regional Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Centre in Rotterdam approved the study (MEC 2004-227).

RESULTS

In total, 259 pregnant women underwent longitudinal 3D-US scans. We excluded pregnancies according to the exclusion criteria because of oocyte donation (n= 2), miscarriage (n= 43), ectopic pregnancy (n= 1), unreliable gestational age (n= 12), fetal or neonatal death and termination of pregnancy (n= 5), congenital anomaly (n= 3), missing data (n= 7). Of the 186 remaining pregnancies, 37 more pregnancies were excluded because the quality of the 3D-US did not allow volume measurements. General characteristics of the total study population, our selected study group with embryonic head measurements and the excluded pregnancies without measurements are shown in Table 1. No significant differences were found except for a higher maternal age and BMI in the excluded group.

Table 1 — General Characteristics

Characteristics	Pregnancies		
	All (n = 186)	**With measurements (n = 149)**	**Without measurements (n=37)**
Maternal			
Age, years[1]	32.0 (4.8)	31.6 (4.8)	33.7 (4.6)*
Ethnicity[2]			
Dutch	144 (77.4)	116 (78.4)	28 (77.8)
Western-other	16 (8.6)	12 (8.1)	3 (8.3)
Non-western	25 (13.4)	20 (13.4)	5 (13.9)
Education[3]			
Low	15 (8.1)	9 (6.0)	6 (17.6)
Intermediate	54 (29.0)	43 (28.9)	11 (32.4)
High	108 (58.1)	91 (61.1)	17 (50.0)
BMI, median (range), kg/m^2	23.8.(18.6-38.3)	23.5 (19.1-35.0)	25.5 (18.6-38.3)*
Primigravida	69 (37.1)	57 (38.3)	12 (32.4)
Nulliparous	119 (64.0)	96 (64.4)	23 (62.2)
Mode of conception IVF/ICSI	57 (30.6)	45 (30.2)	12 (32.4)
Periconceptional use of alcohol	85 (45.7)	72 (48.3)	13 (35.1)
Periconceptional smoking	31 (16.7)	27 (18.1)	4 (10.8)
Preconceptional initiation of folic acid	150 (80.6)	122 (81.9)	28 (75.7)
Neonatal outcome			
Birth weight, median (range), grams	3378 (450-4700)	3390 (450-4700)	3235 (1540-4045)
GA at birth (range), days	276 (187-294)	276 (187-294)	275 (220-290)
Infant gender, male	88 (47.3)	68 (45.6)	20 (54.1)

*Data is presented as mean and standard deviations (SD) or number (%) unless otherwise specified. Missing data was due to incomplete questionnaires. [1] missing n= 3; [2] missing n= 1; [3] missing n= 9; *p<0.05. BMI; Body Mass Index; IVF/ICSI, in vitro fertilization/intra-cytoplasmic sperm injection; GA, gestational age; CRL, Crown-rump length.*

Table 2 depicts the success rates and means of HV and HC measurements per gestational week with the corresponding SD values. A total of 415 ultrasound scans between gestational ages 9^{+0} to 12^{+6} weeks from 149 pregnancies were available for volume measurements. We were able to perform 303 (73%) HV measurements and 305 (74%) HC measurements, achieving the highest success rates (77%) in gestational week 10. Two or more measurements were available in 100 (67%) pregnancies.

Intra-observer and inter-observer reliability analyses show no significant differences for the mean HV (intra: -0.013 cm^3, 95%CI= -0.039; 0.014, p= 0.334; inter: 0.012 cm^3, 95%CI= -0.029; 0.053, p= 0.544). The mean percentage differences for the intra-observer and inter-observer are respectively 0.455% (95% limits of agreement -4.940 ; 5.850) and -0.411% (95% limits of agreement -7.564 ; 6.742). Both

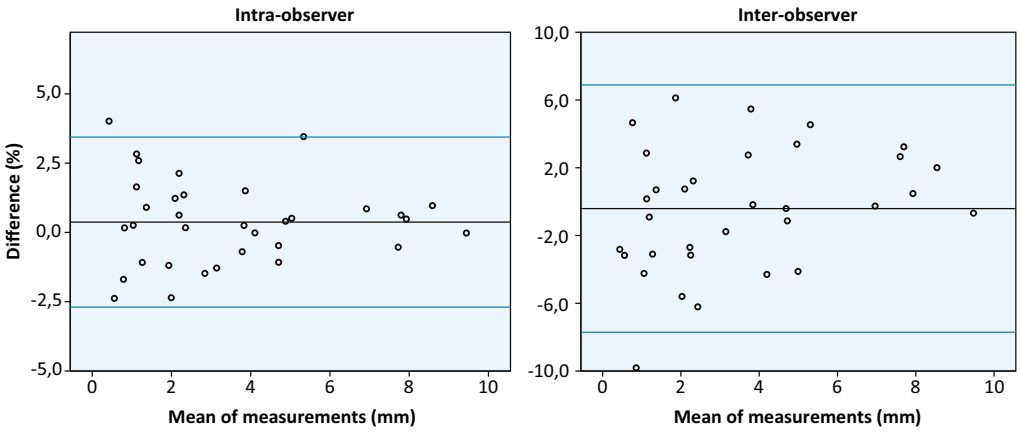
ICC values were >0.99, representing excellent reliability. The Bland-Altman plots for both intra-observer and inter-observer reliability (Figure 2) show excellent agreement.

Table 2 — Embryonic head measurements and success rates per gestational age

	Embryonic Head Volume			Embryonic Head Circumference		
GA	**N (%)**	**Mean (cm³)**	**SD**	**N (%)**	**Mean (mm)**	**SD**
Total	303/415 (73)			305/415 (74)		
9	97/135 (72)	0.954	0.398	98/135 (73)	37.135	5.828
10	90/117 (77)	2.049	0.766	90/117 (77)	48.676	5.694
11	76/100 (76)	4.010	1.360	76/100 (76)	59.506	6.217
12	40/63 (64)	6.750	1.721	41/63 (65)	70.395	7.261

Number of measurements (N), success rates (%), means and standard deviations (SD) of embryonic head volume and head circumference. GA, gestational age.

Figure 2 — Bland Altman plots



Intra-observer and inter-observer agreement in percentages with corresponding 95% limits of agreement for embryonic head volume measurements

Reference curves and regression lines

Figure 3 displays the individual growth trajectories, reference curves and regression lines of HV as a function of GA and CRL. Growth patterns of HV are non-linear and variance seems to increase with advancing gestational age in all models. In order to approach linearity to model regression lines for HV and HC we performed transformations as below, resulting in the following equations:

$HV^{1/4}$ = -1.0947 + 0.0315 x GA (days)

$HV^{1/2}$ = -0.27713 + 0.04761 x CRL (mm)

$HC^{1/4}$ = 1.0738 + 0.0214 x GA (days)

HC^{2} = -1320.03 + 105.01 x CRL (mm)

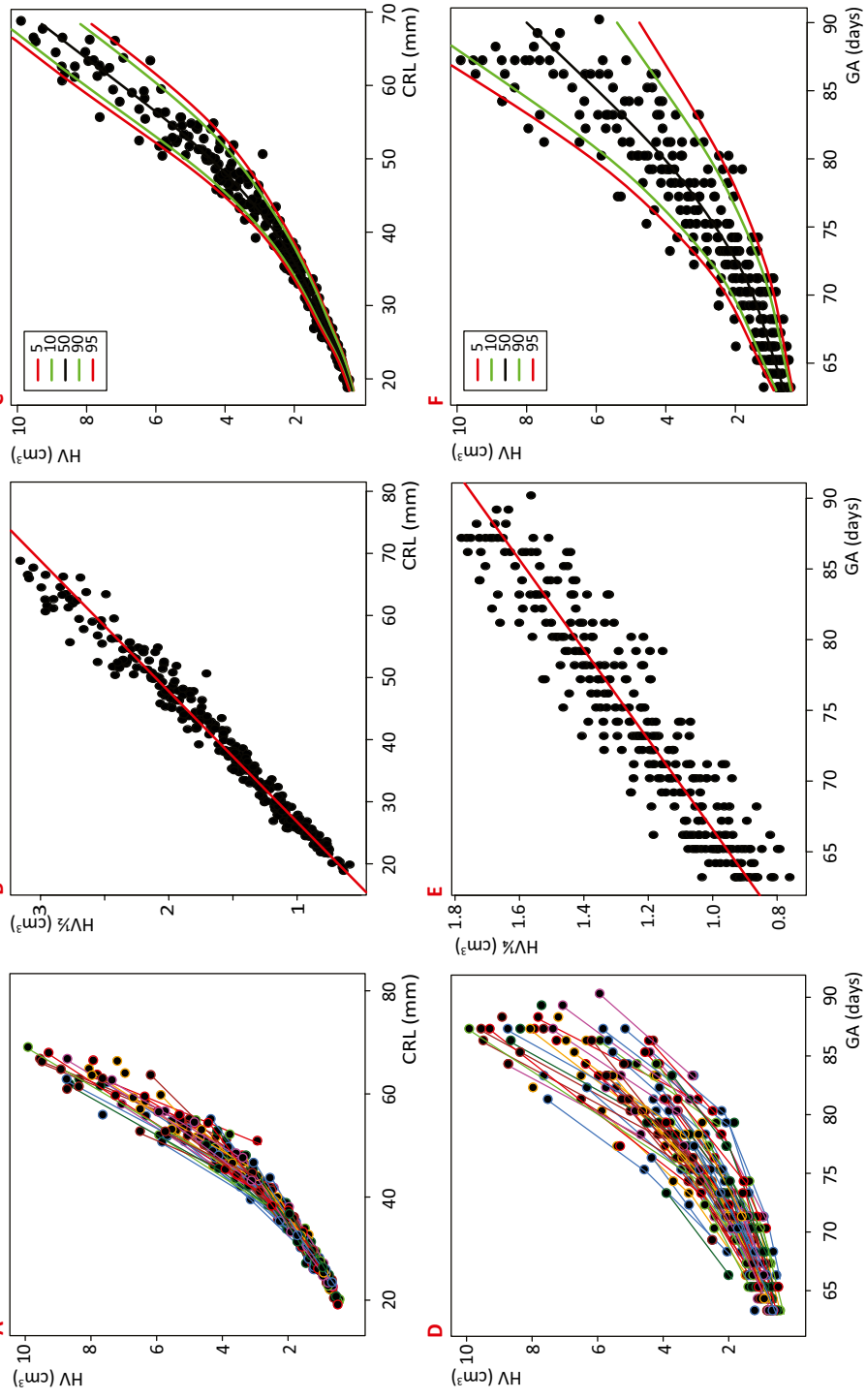


Figure 3 — Growth trajectories of embryonic head volume as a function of gestational age and crown-rump length

Individual growth trajectories as a function of CRL (A). Regression line accompanying regression equation: $HV^{1/2} = -0.27713 + 0.04761 \times CRL$ (mm) (B). Reference curves with 5, 10 and 50 percentiles lines as a function of CRL (C). Individual growth trajectories as a function of GA (D). Regression line accompanying regression equation: $HV^{1/4} = -1.0947 + 0.0315 \times GA$ (days) (E). Reference curves with 5, 10 and 50percentiles lines as a function of GA (F). CRL, Crown-rump length in millimetres; GA, Gestational age in days; HV, head volume.

The correlation coefficients between embryonic and fetal head measurements in Z-scores are depicted in Figure 4 (HC vs fetal HC: ρ= 0.617, p<0.001; HV vs fetal HC: ρ= 0.660, p<0.001).

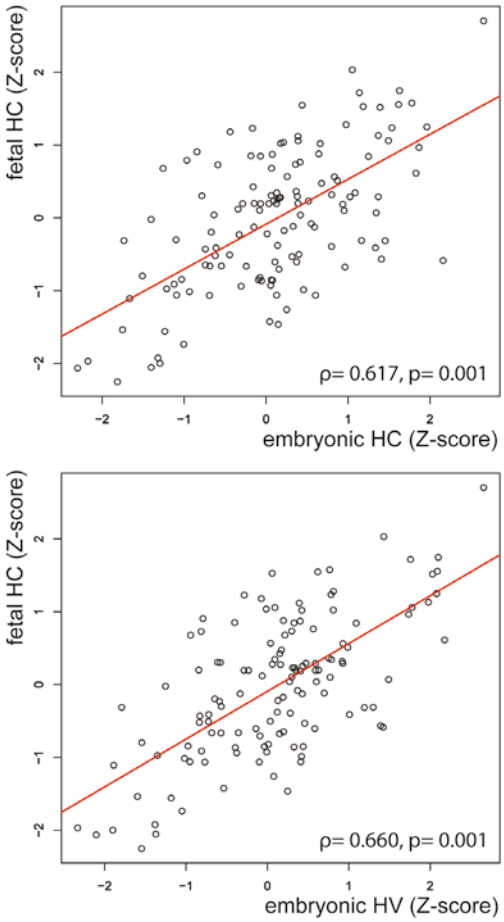


Figure 4 — Associations between embryonic and fetal head parameters in Z-scores

Associations between first trimester embryonic head circumference and head volume, and second trimester fetal head circumference in Z-scores. HV, head volume; HC, head circumference.

Linear mixed models

The effect estimates, standard errors and corresponding p-values of the linear mixed models of HV and HC as a function of GA and CRL and the association with maternal conditions are depicted in Table 3. Maternal age is associated with significantly increased growth trajectories of HV and HC as a function of GA. This effect is attenuated to non-significant, modelling HV as a function of CRL. Maternal periconceptional smoking is associated with decreased head growth trajectories of both HV and HC in all models. We show significantly decreased HV in univariate and multivariable models as a function of CRL and in the multivariate model for HC as a function of CRL. Significantly increased embryonic head growth trajectories were assessed in IVF/ICSI pregnancies as a function of GA, although these associations were attenuated to non-significant associations in the models as a function of CRL.

Table 3 — Linear mixed models: Associations between maternal conditions and growth trajectories of embryonic head volume and head circumference as a function of gestational age and crown-rump length

Model		$HV^{1/4}$ vs GA			$HV^{1/2}$ vs CRL			$HC^{1/4}$ vs GA			HC^{2} vs CRL		
		β	SE	P	β	SE	P	β	SE	P	β	SE	P
Maternal age	Univariate	0.0043	0.0014	**0.003**	-0.0003	0.0009	0.765	0.0027	0.0011	**0.013**	1.0766	2.0459	0.600
	Multivariate	0.0037	0.0015	**0.017**	-0.0009	0.0010	0.371	0.0023	0.0011	**0.041**	0.3801	2.2170	0.864
Preconceptional initiation of folic acid	Univariate	0.0155	0.0179	0.387	-0.0125	0.0105	0.235	0.0215	0.0134	0.111	32.7300	24.1997	0.178
	Multivariate	0.0366	0.0189	0.055	-0.0111	0.0115	0.335	0.0428	0.0143	**0.003**	46.4534	26.1357	0.077
Periconceptional smoking	Univariate	-0.0280	0.0179	0.121	-0.0251	0.0096	**0.010**	-0.0180	0.0135	0.183	-28.2974	21.2214	0.185
	Multivariate	-0.0178	0.0183	0.334	-0.0276	0.0107	**0.011**	-0.0153	0.0137	0.265	-51.7020	23.8256	**0.032**
Periconceptional use of alcohol	Univariate	-0.0152	0.0138	0.273	0.0091	0.0082	0.268	-0.0125	0.0103	0.228	29.8525	18.0489	0.100
	Multivariate	-0.0126	0.0142	0.378	0.0118	0.0086	0.173	-0.0086	0.0106	0.421	30.8990	19.0732	0.108
Nulliparous	Univariate	0.0091	0.0145	0.531	0.0031	0.0086	0.717	0.0000	0.0109	0.999	-10.7078	19.1669	0.577
	Multivariate	0.0028	0.0154	0.858	0.0006	0.0094	0.950	-0.0066	0.0115	0.568	-33.1358	20.8924	0.115
Conception IVF/ICSI	Univariate	0.0342	0.0148	**0.022**	-0.0080	0.0094	0.400	0.0291	0.0110	**0.008**	-9.8440	22.3081	0.660
	Multivariate	0.0301	0.0169	0.078	-0.0089	0.0107	0.406	0.0295	0.0125	**0.020**	-4.2604	24.7550	0.864

Here we show the effect estimates of maternal conditions and HV and HC in both univariate and multivariate linear mixed models. The effect estimates (β), standard errors (SE) and p-values are depicted for 4 separate models. Significant findings are bolt. GA, gestational age; CRL, Crown-rump length; HV, head volume; HC, head circumference; IVF/ICSI, in vitro fertilization / intra-cytoplasmic sperm injection.

Preconceptional folic acid use was associated with significantly increased growth trajectories of HC in the multivariable model as a function of GA only. There were no significant associations between periconceptional use of alcohol, parity and embryonic head growth trajectories.

DISCUSSION

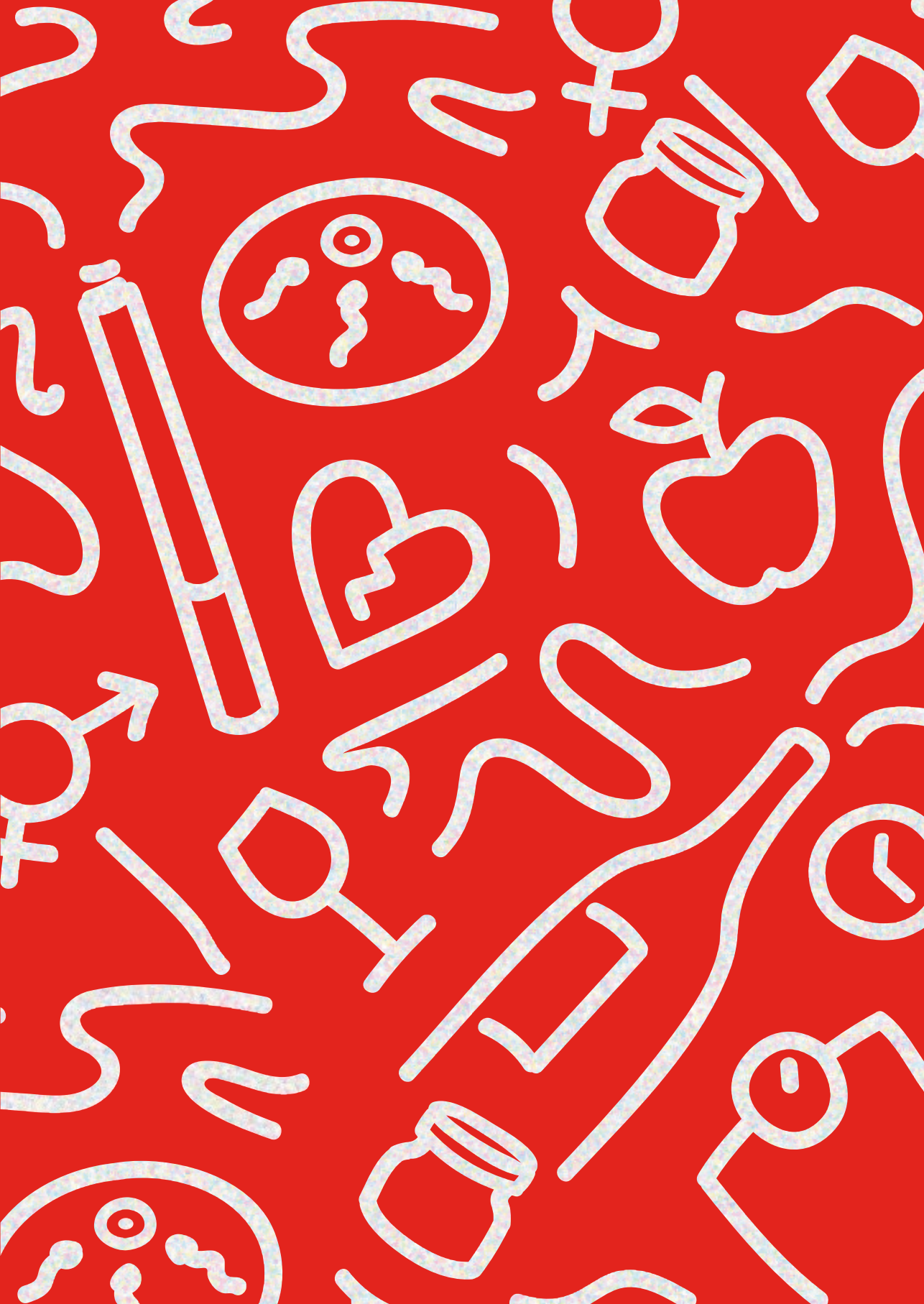
In this periconceptional cohort we show that growth trajectories of HV and HC can serve as possible and reproducible marker for detecting small deviations in human embryonic head growth in the first trimester of pregnancy. Interesting findings are the establishment of associations between maternal periconceptional smoking, maternal age and IVF/ICSI treatment and growth trajectories of the embryonic head. Furthermore, embryonic head measurements are associated with fetal head size, thereby supporting our hypothesis that embryonic growth trajectories may serve as early marker of fetal head growth.

Since the study population of our periconceptional cohort is largely derived from a tertiary hospital setting, external validity is a limitation to consider. Moreover, inherent to the observational cohort design residual confounding cannot be excluded completely. Duration of pregnancy is a strong confounder in studies on embryonic growth; therefore we used a very precise method for pregnancy dating. Nevertheless, we observe a larger variance of growth trajectories as a function of GA compared to CRL. Hence, it cannot be excluded that GA is still a confounder in the studied associations between embryonic head growth and maternal conditions. As growth of the embryo depends on physiological processes involved in implantation and programming, it is challenging to develop an optimal measure to estimate GA for research and clinical practice. Also, we are aware that the results of significant associations with small effect estimates which were not always consistent in the GA and CRL models should be assessed with care. The study was challenged by moderate success rates of embryonic head measurements, partially due to the use of 3D-US sweeps comprising the entire embryo, as no specific sweeps of the embryonic head were available. Moreover, high Body Mass Index (BMI), unfavourable position of the uterus, acoustic shadowing and motion artefacts affect ultrasound image quality and consequently affected these moderate success rates. In the majority of pregnancies however, we were able to measure embryonic HV and HC repeatedly (≥2), therefore linear mixed models could be applied for longitudinal data analysis. We are aware that the interpretation of the effect estimates may be complicated by the use of different transformations in the various models and that clinical interpretation requires a larger sample size and further follow up studies. Our study was not aimed to investigate associations between embryonic and neonatal head measurements and therefore we cannot draw conclusions on associations between maternal conditions and neonatal head size, although it is an interesting issue for future research.

Considering these limitations, this study is the first to provide reliable growth trajectories of embryonic head volume obtained from longitudinal high resolution transvaginal 3D-ultrasound data. We observe a more than six fold increase of the trajectory of HV and doubling of the HC between 9 and 12 weeks GA, which is supported by embryonic volume measurements in chromosomally normal and abnormal fetuses [172,180,181]. We found a non-linear growth trajectory of the HV, which is consistent with previous growth trajectories of embryonic volume [169,182]. Linear growth of embryonic volume measured between 11 and 13 weeks of gestation demonstrated by Falcon et al. could be due to the short span of 3 weeks [181].

Our finding of the association between maternal periconceptional smoking and decreased embryonic head growth is supported by other studies investigating head growth from the first trimester onwards [29,58]. In addition, a Magnetic Resonance Imaging study also showed that maternal smoking was associated with reduced brain volume in the second half of pregnancy [76]. Toxicity of tobacco influences epigenetic mechanisms and vascular processes [29,183] which may directly influence growth trajectories of the embryonic head. In addition smoking is often accompanied by other poorer lifestyles as well, which may enhance the detrimental effects. Although we observed positive association between maternal age and IVF/ICSI pregnancies on both embryonic head parameters, these findings were not consistent in all models. Nevertheless, maternal age has previously been reported in association with an increased embryonic growth [15,106,184]. We hypothesize that this finding may be explained by epigenetic changes due to aging including global DNA hypomethylation or hypermethylation of specific genes essential for embryonic growth [16]. The significantly increased embryonic head growth trajectories in IVF/ICSI pregnancies are probably due to an overall larger embryo. This is in line with Eindhoven et al. showing positive associations, albeit not significant, between IVF/ICSI treatment and CRL or embryonic volume as a function of GA [182]. The hypothesis of an overall larger embryo is further supported by the attenuation of the β's in the CRL models. Although the differences are very small, we consider IVF/ICSI treatment to be another environmental exposure that interferes in epigenetic programming of embryonic growth genes, such as insulin growth factor 2 (IGF2) differentially methylated region (DMR) and other biological mechanisms as a result of ovarian stimulating hormones, use of progesterone and other maternal conditions and exposures [16,23]. The association between preconceptional folic acid supplement use and growth trajectories of HC is in line with previous embryonic studies [105,106]. Correlation between first and second trimester head measurements is in correspondence with previous results revealing associations between embryonic growth and estimated fetal weight and birth weight [98]. This may establish embryonic HV as a separate entity in search for prenatal measures for (ab) normal head and brain development. As such, HV may add new insight in our understanding of prenatal neurodevelopment.

In conclusion, growth trajectories of the embryonic head can be precisely assessed as early as the first trimester. Future research is recommended to establish whether first trimester head parameters can contribute to the early detection of deviations in head growth with consequences for neurodevelopmental outcome.



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Congenital heart defects and trajectories of cortical folding of the human fetal brain by three-dimensional ultrasound

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ABSTRACT

Background — Brain abnormalities in newborns with congenital heart defects (CHD) may well originate in the prenatal period. We hypothesized that CHD are associated with prenatal derangements in cortical folding. In this study we used a three-dimensional ultrasound (3D-US) method for measuring fetal brain fissure depths to investigate growth trajectories of cortical folding in fetuses with CHD and controls.

Methods — In a subgroup of 227 women recruited from a prospective cohort, we performed longitudinal 3D-US examinations of the fetal brain at 22, 26 and 32 weeks gestational age (GA). The Sylvian, insula and parieto-occipital fissure (POF) depths were measured. Intra- and inter-observer reliability and agreement were evaluated with intra-class correlation coefficients (ICC's) and the Bland-Altman method. Doppler pulsatility indices of the umbilical artery and middle cerebral artery were measured to calculate the cerebro-placental ratio (CPR). The association between CHD and cortical folding was estimated using linear mixed models with adjustment for potential confounders.

Results — We included 213 pregnancies (20 CHD fetuses and 193 controls) providing 44 and 532 3D-US scans, respectively. Brain fissure measurements were successful in over 80% of 3D-US scans, except for the POF at 32 weeks GA (65%). All measurements showed a good reliability (ICCs>0.84). After adjustment for maternal alcohol consumption, fetal head circumference (HC) and gender, growth trajectories of the left insula depth (β= -2.753, 95%CI= -5.375 ; -0.130, p= 0.040) and right POF (β= -3.762, 95%CI= -7.178 ; -0.346, p= 0 .031) were significantly decreased in CHD compared to controls, whereas their growth rates were slightly increased (β= 0.014, 95%CI= 0.001 ; 0.027, p= 0.036 and β= 0.024, 95%CI= 0.007 ; 0.041, p= 0.006). In contrast to controls, we found no associations between CPR and cortical folding in CHD.

Conclusions — Cortical folding can be evaluated reliably by measuring brain fissure depths. We showed that regional trajectories of cortical folding were delayed in CHD compared to controls but showed accelerated growth rates. In CHD, no associations were found between cerebro-placental redistribution and cortical folding, which suggests that besides hemodynamic mechanisms other mechanisms play a role in the prenatal origin of CHD-related neurodevelopmental disorders.

INTRODUCTION

Brain abnormalities occur in up to 49% of new borns with congenital heart defects (CHD) [26]. Accumulating evidence support a prenatal origin for these brain abnormalities [185]. As the majority of these infants today reach adulthood, efforts are directed to decrease morbidity and improve long-term neurodevelopmental outcome [186,187]. In order to contribute to these objectives research should focus on unravelling the underlying pathophysiologic mechanisms contributing to alterations in brain development in CHD fetuses.

In this context, the effects of deranged intra-uterine hemodynamic conditions on the developing brain in CHD fetuses have received much attention. In severe CHD, specifically left side obstructive lesions fetal hemodynamics, can be drastically changed, with as a consequence cerebro-placental redistribution to maintain cerebral perfusion [188-190]. However, this fetal autoregulation response preserving cerebral blood flow may not be sufficient to spare to brain from adapting.

Prenatal CHD-related brain abnormalities include alterations in white matter microstructure and volume, reduced (sub) cortical grey matter volume amongst others. These alterations are spread over time and are preceded by derangements in cortical folding [191-194]. Cortical folding is a complex process strongly correlated to gestational age (GA) [195-197]. Gender differences and left-right asymmetry in cortical folding have been documented and are considered physiologic phenomena [198-200]. However, derangements in both global and regional cortical folding can also be a sign of pathological processes observed in neuropsychiatric disorders [201,202]. Therefore, it was suggested that prenatal cortical folding parameters are associated with neurodevelopmental outcome.

Previous ultrasound studies used a scoring system based on the degree of tortuosity to evaluate the development of various brain fissures, which appeared to be feasible and reliable [203,204]. Recently however, simple depth measurements of brain fissures including the Sylvian, parieto-occipital (POF), cingulate and calcarine fissures have been demonstrated to be applicable with two-dimensional and three-dimensional (3D) ultrasound (US) [200,205-207]. In contrast to MRI, ultrasound is readily available, cheap and allows serial examinations of cortical folding [208].

From this background we aimed to study (1) the reliability of measuring fetal brain fissure depths for the non-invasive evaluation of fetal cortical folding using 3D-US and (2) the influence of CHD on trajectories of prenatal cortical folding.

MATERIALS AND METHODS

Study design and population

From the Rotterdam periconception cohort (Predict study), a prospective observational study conducted at the Erasmus MC University Medical Center, a subgroup of women with a singleton pregnancy was recruited for longitudinal 3D-US examinations of the fetal brain at set moments [24]. All participants signed written informed consent at enrolment and also on behalf of their unborn child. The Central Committee of Human Research in The Hague and the Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam approved the study (MEC 2004-227, date of approval 25-01-2013).

Pregnant women were enrolled before 12 weeks of GA, from November 2013 until March 2015. Additionally, women with a pregnancy complicated by a case of isolated fetal CHD confirmed before 32 weeks GA by an extended structural US examination were recruited from the outpatient clinic at the Erasmus MC University Medical Center, Rotterdam, the Netherlands. For this study exclusion criteria were withdrawal before 3D-US examinations, intra-uterine fetal death, termination of pregnancy, multiple congenital anomalies or malformations other than CHD.

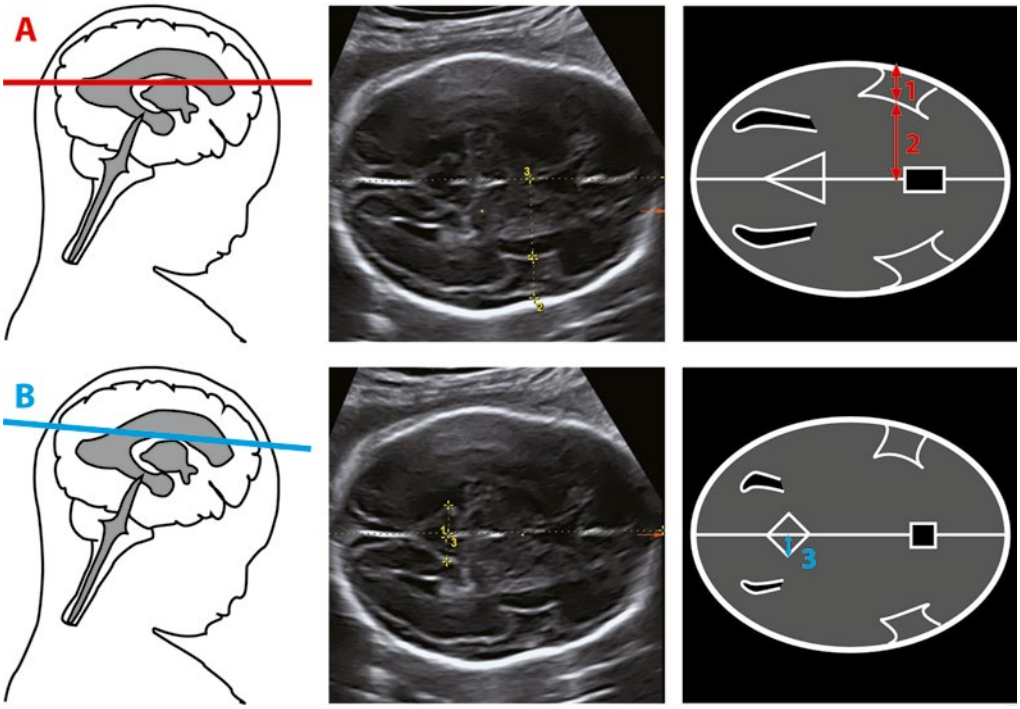
Study parameters and endpoints

Maternal characteristics and data on medical and obstetrical history were obtained through self-reported questionnaires upon enrolment and verified at the study entry visit. Data on pregnancy course and outcomes were obtained from questionnaires filled out in the second trimester and around delivery. The reports of the second trimester structural US examination and the delivery were used to validate the questionnaire data. Pregnancies were dated at the routine clinical intake visit using crown-rump length (CRL) measurements before 13 weeks GA [133]. Pregnancies resulting from artificial reproductive techniques were dated using the oocyte retrieval date plus 14 days or date of the embryo transfer plus 17-18 days in cryopreserved transfers.

Ultrasound and measurements

All participants were scheduled to undergo serial 3D-US examinations at 22, 26 and 32 weeks GA. Ultrasound scans were performed with the use of a 1-7 MHz transabdominal transducer and a 6-12 MHz transvaginal transducer of the Voluson E8 system (GE Medical Systems, Zipf, Australia). Detailed neurosonography was performed by one certified sonographer (IVK) using a transabdominal approach primarily; a transvaginal approach was considered when the fetus was in vertex presentation. Standard 3D-US volumes according to the ISUOG guidelines were acquired of the transventricular plane and a plane directly above the transventricular plane [130,209]. In addition, we performed biometric measurements of the bi-parietal diameter (BPD) and head circumference (HC). Doppler measurements of the umbilical artery (UA) and middle cerebral artery (MCA) were obtained and cerebro-placental ratio (CPR) was calculated as a measure of brain sparing [210,211].

Figure 1 — Brain fissure measurements using 3D-Ultrasound



Brain fissure measurements and corresponding ISUOG standard planes. The insula and Sylvian fissure depths were measured in the transventricular plane. The Sylvian fissure (1A) was measured from the midpoint of the internal face of the fissure to the inner table of the parietal bone. The insula depth (2) was measured from the interhemispheric fissure to the midpoint of the external face of the fissure roof. The POF was measured in the plane directly above the transventricular plane slightly changing the angle and measured from the interhemispheric fissure to the outer border of the fissure's apex. Adapted from ISUOG guidelines [130]. ISUOG, International Society of Ultrasound in Obstetrics and Gynaecology; CHD, congenital heart defect; POF, Parieto-occipital fissure.Images with permission.

Brain fissure depth measurements were performed offline using 4D View Version 5.0 (GE Medical Systems). All measurements were performed by one observer (AWG) to exclude inter-observer variability and enhance precision. Measurements were performed perpendicular to the midline fissure. Perpendicularity was ensured by drawing a guiding line over the midline fissure. The insula and Sylvian fissure depths were measured in the transventricular plane and included the following reference points; cavum septum pellucidum, posterior horns of the lateral ventricles, the choroid plexus and the atrium [130]. The insula depth (Figure 1A) was measured from the interhemispheric fissure to the midpoint of the external face of the fissure roof [205,206]. The Sylvian fissure (Figure 1A) was measured from the midpoint of the internal face of the fissure to the inner table of the parietal bone [200,205,206]. After positioning the reference dot in the cavum septum pellucidum, the head was rotated along the z-axis until the maximal depth of the POF was visualized. The POF was measured in the plane directly above the transventricular plane slightly changing the angle (Figure 1B), using the cavum septum pellucidum

as reference point [130,212]. The distance was measured from the interhemispheric fissure to the outer border of the fissure's apex [200,206,207]. Differentiation between the left and right side was ensured by reorientation of the 3D-US image according to a standard approach [209].

Statistical analysis

For data analyses we used SPSS (SPSS release 21 for Windows, IBM, USA). Differences in general characteristics between the case and control groups were assessed using Mann Whitney U-tests for continuous data, and Chi-square tests for categorical data. Reliability analyses were performed on 30 randomly selected ultrasound examinations of 30 different fetuses at all three time points. Two observers, blinded to the outcomes, independently performed the measurements in threefold. Both intra-observer and inter-observer reliability was analysed with intraclass correlation coefficients (ICCs). The extent of agreement was examined according to Bland Altman. All results with p-values <0.05 were considered statistically significant.

In all 3D-US scans, measurements of the Sylvian fissure, insula and POF were repeated three times per time point. We calculated the means of the three measurements to use in the statistical analyses. We calculated success rates, medians and ranges for all brain fissure depth measurements per time point (Supplemental data – Table A). Trajectories of brain fissure measurements per individual comprised of the repeated measurements at 22, 26 and 32 weeks of gestation. Linear mixed models were estimated to investigate the association between CHD and trajectories of cortical folding, taking into account the subject correlation for repeated measurements. In the model we used a random intercept only. A maximum likelihood approach was applied to assess whether polynomials contributed to the best model fit. The linear mixed models were estimated using GA and GA squared as predictors and the longitudinal brain fissure depths as response. In the final models, the relationship between CHD and cortical folding were investigated by analysing the associations with (1) the height of the trajectory and (2) the growth rate (slope) of the trajectory represented by the interaction term (CHD*GA). Potential confounders of cortical folding were entered simultaneously as covariates and their independent associations with trajectories of cortical folding were investigated in addition. Potential confounders were derived from literature and were selected when there were significant differences in general characteristics between CHD cases and controls [189,200]. In addition, associations were investigated between Doppler pulsatility indices and trajectories of brain fissures in CHD and controls.

RESULTS

Fourteen of the 227 pregnant women were excluded from the analysis because of withdrawal (n= 6), intra-uterine fetal death (n= 1), termination of pregnancy (n= 1), multiple congenital anomalies (n= 1) or anomalies other than CHD (n= 3) and trisomy 21 (n= 2). The study population of 213 pregnancies comprised of 20 cases and 193 controls. CHD cases included Tetralogy of Fallot (n= 6), transposition of

the great arteries (n= 6), hypoplastic left heart syndrome (n= 2), perimembranous ventricular septal defect (n= 3), atrio-ventricular septal defect (n= 1), truncus arteriosus (n= 1) and aortic coarctation with tricuspid valve insufficiency (n= 1). Table I lists the general characteristics of the study group stratified for CHD and controls. The only significant difference between the groups concerned periconceptional alcohol consumption, which was higher in the CHD group. CPR values were lower than 1.0 in only 3 fetuses at 32 weeks GA, which were all controls.

Table 1 — General characteristics

Population	Total (n= 213)	CHD (n= 20)	Control (n= 193)	p-value	Missing
Maternal characteristics					
Maternal age, years	31.9 (21-48)	33.0 (22-48)	31.8 (21-45)	0.81	7
Nulliparous	96 (46)	11 (58)	85 (45)	0.29	6
Geographic background					6
Western	169 (82)	18 (90)	151 (81)	0.46	6
Non-western	38 (18)	2 (10)	36 (19)		
Pre-pregnancy BMI, kg/m^2	23.1 (15.2-43.4)	23.4 (18.0-35.8)	23.0 (15.2-43.4)	0.87	19
Educational level					8
low	25 (12)	0 (0)	25 (13)	0.20	
middle	83 (40)	7 (39)	76 (40)		
high	97 (47)	11 (61)	86 (46)		
Mode of conception (IVF/ICSI)	56 (27)	2 (11)	54 (28)	0.10	3
Periconception folic acid use	195 (95)	18 (95)	177 (95)	0.99	7
Periconception smoking	34 (17)	3 (16)	31 (17)	0.92	8
Periconception alcohol use	60 (29)	10 (53)	50 (27)	**0.02**	9
Neonatal characteristics					
Birth weight, grams	3200 (400-4380)	3420 (1650-4140)	3190 (400-4380)	0.47	2
Gestational age at birth, days	272 (182-292)	274 (200-292)	271 (182-292)	0.12	2
Gender (male)	111 (52)	13 (65)	98 (51)	0.44	0

General characteristics of the total study population and the two subpopulations, CHD fetuses and controls. Data is presented as median and range or number (n) and percentage (%). Significant differences are in bold font. BMI, body mass index in kilograms/square meter; IVF/ICSI, in vitro fertilization/intra-cytoplasmic sperm injection.

Reliability analysis

The mean number of 3D-US scans in the case group was 2.2 and 2.8 in the controls. Overall success rates of the insula and Sylvian fissure depth measurements were above 82%. Success rates of the POF decreased from 96% at 22 weeks to 65% at 32 weeks gestation. The intra- and inter-observer reliability and agreement statistics are shown in Table 2. The means differences of the Sylvian fissure are significantly different when repeated by one observer and between the two observers, although

the mean percentage differences are 2.8% and 5.0%, respectively. The intra-observer analysis does not show significant differences between the measurements of the same observer. All ICC values were above 0.84, representing good reliability. Intra- and inter-observer agreement of the Sylvian fissure and insula were good, the agreement of the POF was moderate.

Table 2 — Intra- and inter-observer reproducibility for measurements of the Sylvian fissure, insula and POF

		Mean difference (mm)	95%CI mean difference (mm)	95% limits of agreement (mm)	Mean difference (%)	95% limits of agreement (%)	ICC
Intra-observer	Sylvian	-0.282	-0.563 ; -0.002	-2.298 ; 1.733	-2.8	-22.3 ; 16.8	0.902
	Insula	0.229	-0.082 ; 0.540	-2.007 ; 2.465	1.2	-10.9 ; 13.4	0.944
	POF	0.070	-0.237 ; 0.377	-2.091 ; 2.232	2.1	-25.2 ; 29.5	0.955
Inter-observer	Sylvian	0.508	0.127 ; 0.890	-2.176 ; 3.193	5.0	-19.7 ; 29.7	0.846
	Insula	0.308	-0.124 ; 0.739	-2.734 ; 3.349	1.9	-14.4 ; 18.2	0.879
	POF	-0.480	-1.048 ; 0.089	-4.441 ; 3.913	-5.0	-60.0 ; 50.4	0.841

Intra- and inter-observer reliability analyses for all brain fissure depth measurements in a random selection of 30 3D-US. POF, parieto-occiptial fissure; ICC, intraclass correlation coefficient; %, percentage.

Longitudinal analyses

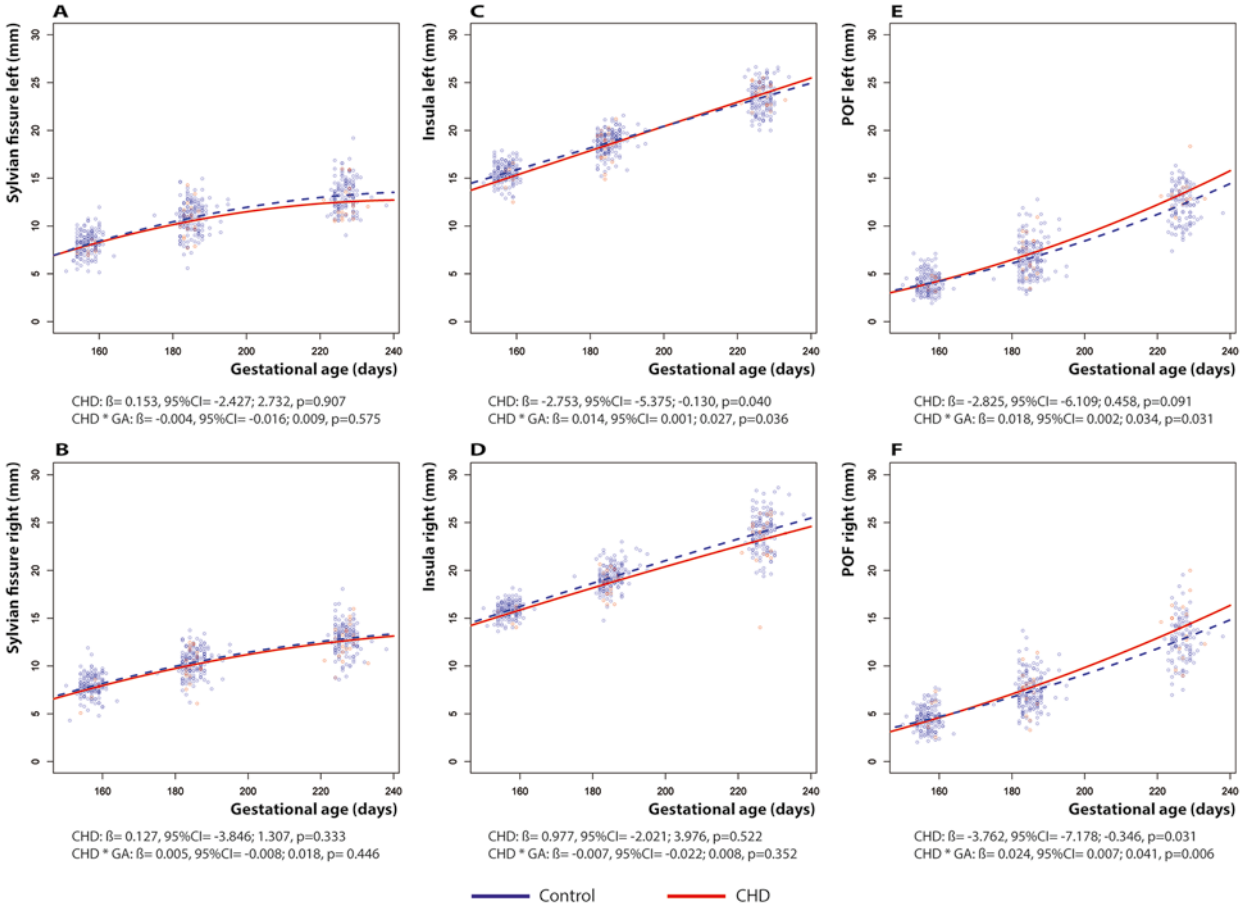
Table 3 shows the results of the crude and multivariate linear mixed models. Trajectories of the left insula and right POF were significantly decreased between 22 and 32 weeks GA whereas the growth rates (slope) in millimetres per day (CHD*GA) were slightly increased in CHD compared to controls (Model 1). Adjustment for periconceptional alcohol consumption, fetal HC and gender did not change the estimates considerably (Model 2). In addition, CHD was significantly associated with an increased growth rate of the left POF. No significant associations were demonstrated between CHD and the trajectories of the left and right Sylvian fissures and the right insula. Figure 2 graphically displays the results of the multivariate linear mixed models (Table 3. Model 2). The mean trajectories of the left and right POF start smaller in CHD fetuses but due to the significantly increased growth rates cross the trajectories of the controls around 24 weeks GA. The same is shown for the left insula around 27 weeks GA. Moreover, significantly positive associations were revealed between HC and all brain fissures. Male gender was significantly positively associated with the Sylvian fissure and insula depth; although in the multivariate model this association only remained significant in trajectories of the left insula.

Table 3 — Linear mixed models: Associations of CHD in the fetus and longitudinal brain fissure measurements

		Model 1			Model 2		
Fissure		**β**	**95%CI**	**P**	**β**	**95%CI**	**P**
Sylvian L	CHD	1.040	-1.575 ; 3.6456	0.435	0.153	-2.427 ; 2.732	0.907
	CHD * GA	-0.008	-0.021 ; 0.004	0.198	-0.004	-0.016 ; 0.009	0.575
	Gender male	0.420	0.075 ; 0.765	**0.017**	0.164	-0.127 ; 0.455	0.267
	Alcohol	0.136	-0.254 ; 0.525	0.493	0.028	-0.293 ; 0.348	0.865
	HC	0.078	0.065 ; 0.090	**<0.001**	0.076	0.062 ; 0.090	**<0.001**
Sylvian R	CHD	-0.422	-3.003 ; 2.158	0.748	-1.270	-3.846 ; 1.307	0.333
	CHD * GA	0.001	-0.012 ; 0.013	0.929	0.005	-0.008 ; 0.018	0.446
	Gender male	0.393	0.072 ; 0.714	**0.017**	0.171	-0.105 ; 0.446	0.224
	Alcohol	0.099	-0.256 ; 0.454	0.583	0.007	-0.293 ; 0.307	0.964
	HC	0.067	0.054 ; 0.079	**<0.001**	0.063	0.050 ; 0.077	**<0.001**
Insula L	CHD	-2.959	-5.601 ; -0.316	**0.028**	-2.753	-5.375 ; -0.130	**0.040**
	CHD * GA	0.014	0.001 ; 0.028	**0.031**	0.014	0.001 ; 0.027	**0.036**
	Gender male	0.475	0.236 ; 0.715	**<0.001**	0.385	0.154 ; 0.616	0.001
	Alcohol	-0.092	-0.367 ; 0.183	0.509	-0.194	-0.448 ; 0.059	0.132
	HC	0.041	0.030 ; 0.053	**<0.001**	0.036	0.024 ; 0.048	**<0.001**
Insula R	CHD	0.770	-2.217 ; 3.756	0.613	0.977	-2.021 ; 3.976	0.522
	CHD * GA	-0.006	-0.021 ; 0.008	0.391	-0.007	-0.022 ; 0.008	0.352
	Gender male	0.297	0.006 ; 0.588	**0.046**	0.234	-0.040 ; 0.507	0.094
	Alcohol	0.038	-0.281 ; 0.357	0.813	-0.033	-0.330 ; 0.264	0.827
	HC	0.052	0.039 ; 0.065	**<0.001**	0.048	0.034 ; 0.063	**<0.001**
POF L	CHD	-2.520	-5.823 ; 0.783	0.134	-2.825	-6.109 ; 0.458	0.091
	CHD * GA	0.016	-0.000 ; 0.033	0.051	0.018	0.002 ; 0.034	**0.031**
	Gender male	0.206	-0.173 ; 0.585	0.284	-0.070	-0.439 ; 0.298	0.707
	Alcohol	0.181	-0.241 ; 0.603	0.399	0.050	-0.351 ; 0.452	0.805
	HC	0.059	0.042 ; 0.076	**<0.001**	0.057	0.039 ; 0.075	**<0.001**
POF R	CHD	-3.529	-6.941 ; -0.117	**0.043**	-3.762	-7.178 ; -0.346	**0.031**
	CHD * GA	0.022	0.005 ; 0.039	**0.010**	0.024	0.007 ; 0.041	**0.006**
	Gender male	0.290	-0.133 ; 0.713	0.178	-0.010	-0.414 ; 0.395	0.963
	Alcohol	0.095	-0.373 ; 0.562	0.690	0.091	-0.531 ; 0.349	0.683
	HC	0.062	0.044 ; 0.081	**<0.001**	0.058	0.038 ; 0.078	**<0.001**

Data of the linear mixed models is presented in β values with corresponding 95%CI and p-values. Significant results are in bold. Model 1 represents the univariate models investigating all covariates separately and model 2 is the multivariate model adjusted for periconception alcohol consumption, fetal gender and HC. β, beta value; 95%CI, ninety-five percent confidence interval; P, p-value; L, left; R, right; CHD, congenital heart defect; GA, gestational age; HC, head circumference.

Figure 2 — Trajectories of fetal brain fissure depths of CHD and controls



*Data points and regression lines are depicted for the mean trajectories of the three brain fissures of CHD fetuses (red) and controls (blue) as a function of gestational age in days corresponding to the multivariate linear mixed models (model 2). Beta values CHD (growth) correspond to the mean difference between the CHD trajectory compared to controls. Beta values of CHD*GA (growth rate) correspond to the difference in mean slope per day gestational age of the CHD trajectory compared to controls. POF, Parieto-occipital fissure; CHD, congenital heart defect; GA, gestational age; ß, beta value, 95%CI, ninety-five percent confidence interval; p, p-value.*

No significant associations between CHD and pulsatility indices of UA, MCA and CPR were found (UA: β= -0.026, 95%CI= -0.105 to 0.053, p= 0.519, MCA: β= 0.006, 95%CI= -0.074 to 0.086, p= 0.876; CPR: β= 0.001, 95%CI= -0.-0.126 to 0.128, p= 0.984). Associations between the CPR and trajectories of brain fissures were analysed in the same manner (Table 4). These analyses showed significant associations between the CPR and the trajectories of the right Sylvian, left insula, right and left POF in controls. No significant associations between the CPR and brain fissures were found in CHD cases.

Table 4 — Associations between the cerebro-placental ratio (CPR) as measure of 'brain sparing' and longitudinal brain fissure measurements

	Controls			CHD		
Fissures	**β**	**95%CI**	**P**	**β**	**95%CI**	**P**
Sylvian L	0.3260	-0.0548 ; 0.7067	0.093	-0.4136	-1.869 ; 1.0418	0.569
Sylv R	0.5937	0.2444 ; 0.9431	**0.001**	-0.0375	-1.5338 ; 1.4588	0.960
Insula L	0.4293	0.0943 ; 0.7643	**0.012**	-0.3682	-1.8151 ; 1.0787	0.610
Insula R	0.3577	-0.0031 ; 0.7186	0.052	-0.9370	-3.1205 ; 1.2465	0.391
POF L	0.7265	0.2584 ; 1.1945	**0.002**	0.2037	-1.7125 ; 2.1199	0.830
POF R	0.8290	0.3426 ; 1.3154	**0.001**	1.0570	-1.0850 ; 3.1990	0.324

Data is presented in β values with corresponding 95%CI and p-values. Significant results are in bold. β, beta value; 95%CI, ninety-five percent confidence interval; P, p-value; L, left; R, right; CHD, congenital heart defect.

DISCUSSION

From this study we conclude that measuring brain fissure depths by 3D-US is reliable for evaluating cortical folding. Trajectories of the left insula and right POF in CHD were significantly decreased compared to controls whereas their growth rates were slightly increased in both crude and fully adjusted models. The delay in cortical folding is in agreement with recent imaging studies [189,191-194,213-215]. Yet, we are the first to describe the accelerated growth rates of brain fissures in the second half of pregnancy. No associations were demonstrated between CHD and the left and right Sylvian fissures and the right insula. Most interestingly, the CPR was significantly associated with trajectories of cortical folding in controls, but not in CHD. Moreover, HC and male gender were positively associated with cortical folding.

Strengths and limitations

Measuring three brain fissures allows conclusions on regional patterns of cortical folding only. The reliability of our measurements was good but not excellent and intra- and inter-observer agreement was largely comparable with previous studies [200,204,207]. The prevalence of CHD is low and therefore we were only able to include a small number of cases. This number was too small to study trajectories of

cortical folding in the separate phenotypes. Moreover, residual confounding due to the observational study design cannot be excluded. External validity is limited because the study was conducted in a tertiary hospital setting. Strengths of our study are the prospective and longitudinal design, providing insight in cortical folding as a dynamic process as opposed to a cross-sectional approach [203,216]. Using multivariate linear mixed model analyses enabled drawing conclusions without confounding of HC, alcohol consumption and gender. In particular, by adjusting for HC we were able to discriminate between smaller brain fissures due to the CHD itself and smaller brain fissures due to delayed head growth [189,215]. The 3D-US method using standard axial ultrasound planes facilitated simple but precise measurements by the reconstruction of orthogonal planes for longitudinal evaluation of brain fissures [216-218].

Main findings

This study showed that increased growth rates of brain fissures can co-occur with a delayed trajectory [189,191-194,213-215]. This implies a delay in cortical folding, which paradoxically may even result in increased fissure depths at the end of pregnancy in CHD (Figure 2). These findings contradict most literature except a study by Masoller et al. which showed an increased left insula depth between 36 and 38 weeks GA in CHD [191,215,219]. Since the precise mechanisms driving this strictly organized process of gyrification remain largely unknown, we can only speculate about the origin of these findings. Considerable changes in the normal process of cortical folding occur between 25 and 30 weeks GA, with the steepest growth around 30 weeks GA, coinciding with white matter development [13]. This suggests that the most distinct changes in cortical folding occur during our study period. As decreased cortical thickness is inversely related to cortical folding one might argue that cortical thinning may lead to deeper brain fissures. Consequently, deeper brain fissures in CHD fetuses do not necessarily imply that the cortex is more or better developed. In addition, atypical cortical development has been described in infants with Tetralogy of Fallot, with deeper, broader and more simplistic sulcal patterns [194]. Nevertheless, the increased growth rates in addition to the delayed regional cortical folding emphasize that longitudinal evaluation of cortical folding is recommended.

The associations between CHD and brain fissures depths appear to be regional and asymmetrical which is in line with studies investigating the impact of SGA on cortical folding [206,220]. Our results in CHD are substantiated by a MRI study showing significantly decreased depths of the POF and left insula among other fissures and another study showing profound morphological differences in the POF and Sylvian fissures and other regions [189,191]. The wide variety of measures and methods evaluating cortical folding may explain the observed discrepancies in the reported findings [221]. We suggest that the susceptibility to adverse prenatal conditions, such as hemodynamic variations, varies among different brain regions, since every region of the cortex follows a different developmental timeline [196,197,222].

Of interest is the finding that trajectories of cortical folding were significantly associated with CPR in controls, but not in CHD. We speculate that besides adaptive hemodynamic mechanisms other mechanisms may play a role in the origin of abnormal brain development in CHD [215,223]. An explanation may be sought in the simultaneous development of the fetal heart and brain which share morphogenetic programs. Moreover, the small derangements in cortical folding without finding associations between hemodynamic features of cerebro-placental redistribution in CHD suggest that derangements in brain development, can arise as a consequence of subtle undetected prenatal hemodynamic derangements [223]. This supports the assumption that normal Doppler indices do not guarantee proper cerebral perfusion, metabolism, oxygen delivery and uptake in the brains of CHD fetuses [189,215,224].

The associations of delayed cortical folding and CHD were independent of head growth in CHD. However, the significant associations between cortical folding and head circumference confirm that brain fissure measurements are dependent on head size. Therefore, adjustment for fetal head size in future research is recommended. Furthermore, trajectories of cortical folding were also positively associated with male gender, which is in agreement with physiology and previous literature [198,200,221]. Still, the contribution of gender seems rather small as these associations attenuate to non-significant after full adjustment.

Future implications

Our results suggest that fetal CHD impact prenatal brain development. However, whether the small regional derangements in trajectories of cortical folding have implications for neurodevelopmental outcome needs to be studied further in larger prenatal cohorts with postnatal neurodevelopmental follow-up. Such studies may elucidate whether the clinical type of CHD is an independent predictor of the level of impact on cortical folding [215,225]. Therefore, future prenatal care should focus on the assessment of the dynamic process of prenatal neurodevelopment in CHD as pre-operative neurologic conditions are an important determinant of neurodevelopmental outcome [226].

7

New ultrasound marker for bedside monitoring of preterm brain growth

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ABSTRACT

Background — Preterm infants are at risk for neurodevelopmental impairment, but reliable, bedside-available markers to monitor preterm brain growth during hospital stay are still lacking. The aim of this study was to assess the feasibility of corpus callosum fastigium (CCF) length as a new cranial ultrasound (CUS) marker for monitoring of preterm brain growth.

Methods — In this longitudinal prospective cohort study, CUS was planned on day of birth, day 1, 2, 3 and 7 of life, and then weekly until discharge in preterm infants born before 29 weeks of gestation. Reproducibility and associations between clinical variables and CCF growth trajectories were studied.

Results — One to eight CUS were performed in 140 infants (median gestational age at birth 27^{+2} weeks (interquartile range 26^{+1}-28^{+1}), 57.9% male infants). CCF measurements showed good to excellent agreement for inter- and intra-observer reproducibility (intraclass correlation coefficients>0.89). Growth charts for preterm infants between 24-32 weeks of gestation were composed. Male gender and birth weight SD score were positively associated with CCF growth rate.

Conclusions — CCF length measurement is a new reproducible marker that is applicable for bedside monitoring of preterm brain growth during neonatal intensive care stay.

INTRODUCTION

Brain growth is an important predictor of neurodevelopmental outcome in preterm infants [28,227-229]. In neonatal intensive care units (NICU) brain growth is usually monitored by manual measurement of the head circumference (HC). However, HC measurement has a low interrater agreement and does not correspond well with actual brain development [230,231]. Therefore, there is a need for a new reliable bedside marker for monitoring preterm brain growth in clinical practice.

Brain structures measured by cranial ultrasonography (CUS) could provide clinically applicable markers for brain growth. A few ultrasound markers of brain growth have been used in the past, mainly measuring the corpus callosum (CC) or cerebellum, thereby reflecting growth of a small part of the brain only [163,232-235]. In addition to currently available markers of preterm brain development, we propose that the length between genu of the CC and the fastigium (roof of the fourth ventricle) could serve as a new marker for brain growth.

The aim of this study was to evaluate the usefulness of corpus callosum – fastigium (CCF) length, and that of CC length, an existing marker, as markers for monitoring of brain growth in preterm infants during NICU stay. We assessed the reproducibility of CC and CCF length measurements, developed growth charts for preterm infants between 24-32 weeks of gestation, and evaluated prenatal and postnatal characteristics possibly associated with CC and CCF growth trajectories. We hypothesize that both measurements are highly reproducible. Furthermore, we hypothesize that CCF and CC growth trajectories are associated with prenatal and postnatal determinants of neurodevelopmental outcome in preterm infants.

MATERIALS AND METHODS

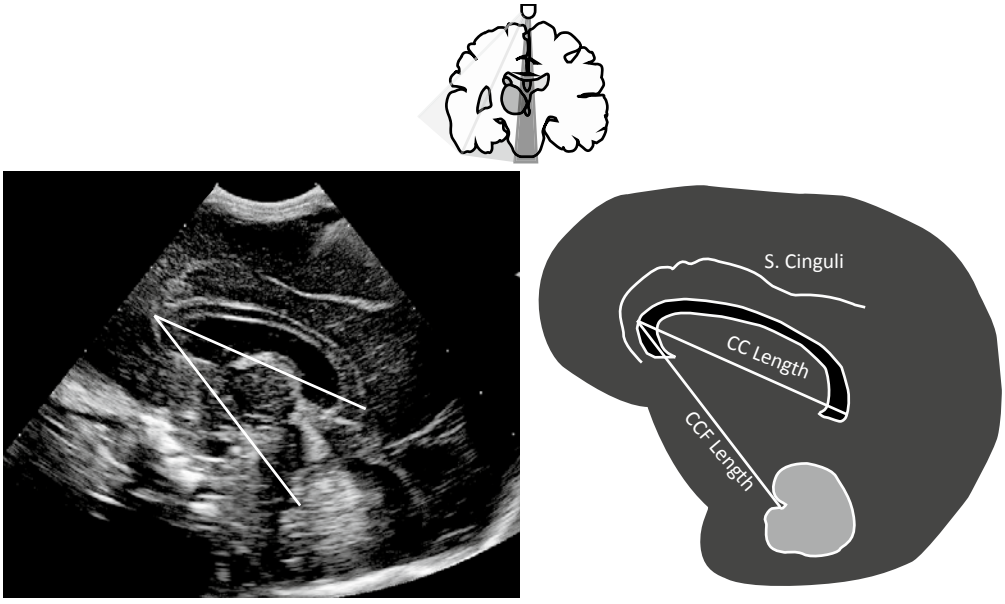
This prospective observational cohort study was performed at the level III NICU of the Sophia Children's Hospital, Erasmus MC, Rotterdam, the Netherlands. The local medical ethics review board approved this study. Written parental consent was obtained before participation. Between 2010 and 2012, all newly admitted singleton, preterm infants born before 29 weeks of gestational age (GA) were eligible for enrolment. The following exclusion criteria were applied; (1) unknown GA at birth; (2) major congenital abnormalities and (3) extensive brain injury (including IVH grade III, post haemorrhagic ventricular dilatation and venous infarction). The latter complications are expected to influence the validity of the measurements due to possible midline shift and expected altered brain growth. GA at birth was dated using the first day of last menstrual period and was confirmed by first trimester crown rump length measurement on ultrasound. Postnatal age was expressed by post menstrual age, calculated as GA at birth + weeks and days of postnatal age. Pregnancy and neonatal characteristics were collected prospectively. Maternal characteristics were collected retrospectively from medical

records. Pregnancy complications, including intra-uterine growth retardation, pre-eclampsia and HELLP syndrome, are obtained from obstetrical records and are defined based on clinical definitions according to national guidelines [236].

Cranial ultrasound and measurements

CUS was performed according to the standard local protocol on the day of birth, on day 1, 2, 3 and 7 of life and then weekly until discharge. The protocol was only violated on clinical grounds (e.g. hemodynamic instability). One researcher (MR) performed all CUS using an Esaote MyLab 70 (Genoa, Italy), with a convex neonatal probe (7,5 MHz). Measurements were performed off line using Mylab software of Esaote. Measurements of CC and CCF length were performed on a standard sagittal plane. In this plane a complete corpus callosum (genu to splenium) and distinct vermis of the cerebellum, including the fastigium, had to be visualized. CCF length was measured from the genu of the corpus callosum (outer border) to the fastigium. CC length was measured from outer to outer border (genu to splenium, Figure 1). All measurements were performed by one investigator (MR). To establish the reliability, 30 randomly selected scans of varying quality and of infants with different GA were measured by a second investigator (JR) who was blinded for previous results.

Figure 1 — An ultrasound and schematic image of the corpus callosum and corpus callosum-fastigium length measurements



In the upper part, we show the coronal view of the brain and the position of the sonography probe for assessment of the corresponding correct sagittal plane below. Measurements of the corpus callosum-fastigium and corpus callosum length are displayed in the sagittal sonography view (left) and schematically (right). S. Cinguli indicates sulcus cinguli.

Statistical methods

Data were analysed using SPSS (SPSS release 21 for Windows, IBM, USA) and R (R: A language and Environment for Statistical Computing, version 3.1.3 2015 for Windows, R Core Team, Vienna, Austria). P-values below 0.05 were considered statistically significant. Median value and (inter quartile) range ((IQ)R) and mean and standard deviations (SD) were used as appropriate.

Intra-observer and inter-observer agreements for CC and CCF lengths were evaluated using intraclass correlation coefficient (ICC) and Bland-Altman plots [237]. ICC was analysed using a two-way mixed model. Cut-off values were in accordance to Landis et al. [238]. Growth charts were developed for CCF and CC growth as a function of post menstrual age (weeks) and weight (gram). To model the relation between the measured CCF and CC lengths and a predefined list of covariates linear mixed models were estimated using lme (in the R nlme package) [239]. To account for the within-subject correlation a random intercept and random coefficient of GA as well as a power variance function to model the residual covariance have been used. The predefined covariates were GA at birth, birth weight (BW) standard deviation score (SDS), gender, intra-uterine growth retardation (defined as expected fetal weight <p10), pre-eclampsia/HELLP, chorioamnionitis, death, sepsis and days on mechanical ventilation. In all models both GA and GA^2 (square of GA) were used as covariates. To this basic model the additional predictors were added separately (termed univariable models below) and also all at once (the multivariable model).

Figure 2 — Reproducibility analyses

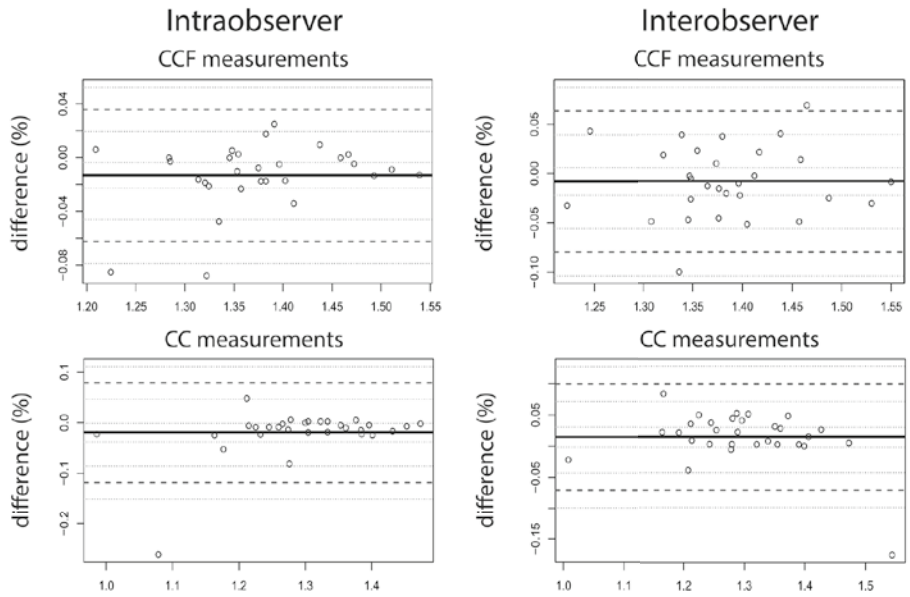


Figure 2 shows the Bland Altman plots of the intra- and inter-observer reliability.

RESULTS

Of 336 infants admitted to our NICU during the study period, 152 were eligible for inclusion. Twelve infants were excluded as they met the exclusion criterion of extensive brain injury, resulting in a sample size of 140 infants. Baseline maternal and neonatal characteristics are listed in Table I. The median gestational age at birth was 27^{+2} weeks (IQR 26^{+1}-28^{+1}), the median birth weight was 955 (IQR 780-1125) gram. The number of ultrasound scans per infants ranged from one to eight.

Table 1 — Baseline characteristics

	n= 140	Missing[b]
Maternal characteristics		
Maternal age, years (mean) (SD)	30 (5.6)	0
Ethnicity		0
Dutch	74 (52.9%)	
Other Western	9 (6.4%)	
Non-western	57 (40.7%)	
Maternal smoking during pregnancy	26 (18.6%)	17
IVF/ICSI	9 (6.4%)	0
IUGR	42 (30%)	4
PE/ HELLP syndrome	37 (26.4%)	0
Chorioamnionitis	37 (26.4%)	0
PPROM	32 (22.9%)	0
Neonatal characteristics		
GA at birth, weeks + days	27^{+2} (26^{+1} -28^{+1})	0
Male gender	81 (57.9%)	0
Birth weight, gram	955 (780-1125)	0
Use of antenatal steroids	127 (90.7%)	2
Apgar score at 5' minutes	8 (7-9)	0
CRIB score	3 (1-6)	1
Death	17 (12.1%)	0
Days on mechanical ventilation	5 (1-14)	3
Days to regain birth weight	9 (7-12)	14
Sepsis	67 (47.9%)	0
IVH grade I or II	32 (22.9%)	0
Severe BPD	15 10.7%)	33

Baseline maternal and neonatal characteristics are presented as median (interquartile range) or n (%) unless otherwise specified. IVF/ICSI, in vitro fertilization with or without intra-cytoplasmic sperm injection; IUGR, intra-uterine growth retardation; PE, pre-eclampsia; PPROM, prolonged premature rupture of membranes; CRIB, clinical risk index for babies; IVH, intra-ventricular haemorrhage; BPD, bronchopulmonary disease. [b] Missing data was mainly due to early transfer to a secondary hospital.

The mean inter-observer difference was -0.3207 (SD 1.4527) mm for CCF (p= 0.244) and 0.4600 (SD 1.8463) mm for CC length (p= 0.183). The ICCs for inter-observer and intra-observer analysis showed excellent agreement for both CCF and CC length (respectively intra: 0.958 (95%CI 0.912-0.980), inter: 0.885 (95%CI 0.770-0.944) and intra: 0.922 (95%CI 0.844-0.962) and inter: 0.893 (95%CI 0.783-0.948)). Figure 2 shows Bland-Altman plots of inter-observer and intra-observer agreements for both measurements.

The CC and CCF measurements

The mean CCF length was 40.9 (SD 2.97) mm, with a range from 34.0 to 54.3 mm. The mean CC length was 36.3 (SD 3.33) mm, with a range from 26.6 to 48.8 mm. Growth charts of CCF and CC lengths by post menstrual age and by weight are shown in Figure 3. Results of univariable analyses are shown in Table II for CC and CCF growth. The multivariable analysis confirmed a positive association between BW SDS and CCF growth rate, and a negative association between female gender and CCF growth rate. For CC growth rate, a positive association was found with BW SDS using multivariable analysis.

Figure 3 — Reference curves of CC and CCF length as a function of post menstrual age and weight

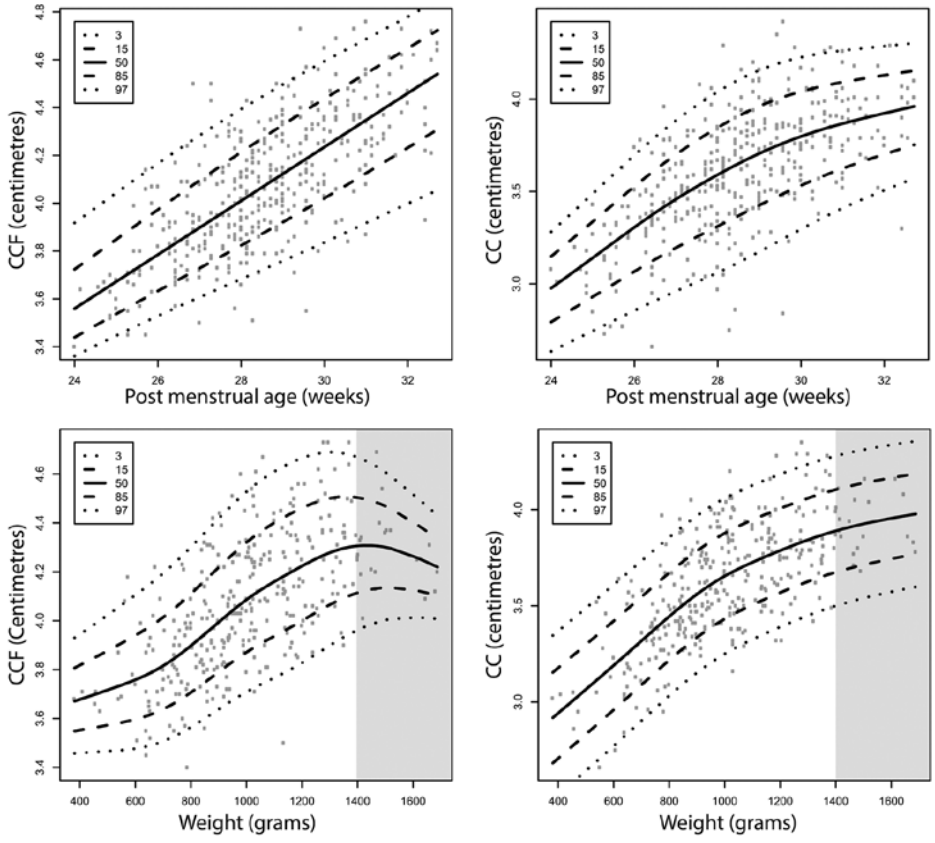


Table 2 — Linear mixed models

	Corpus callosum – fastigium growth						Corpus callosum growth					
	Univariable			Multivariable			Univariable			Multivariable		
	β	(SE)	P	β	(SE)	P	β	(SE)	P	β	(SE)	P
Gestational age at birth	0.029	(0.012)	**0.022**	0.011	(0.017)	0.518	0.024	(0.017)	0.146	0.004	(0.021)	0.857
Birth weight, SDS	0.053	(0.009)	**<0.001**	0.050	(0.014)	**<0.001**	0.094	(0.011)	**<0.001**	0.075	(0.017)	**<0.001**
Gender (female)	-0.109	(0.030)	**<0.001**	-0.070	(0.029)	**0.018**	-0.066	(0.043)	0.124	-0.003	(0.035)	0.938
IUGR (no)	0.094	(0.033)	**0.005**	-0.034	(0.045)	0.451	0.267	(0.041)	**<0.001**	0.046	(0.054)	0.390
PE/HELLP (yes)	-0.064	(0.035)	0.068	0.000	(0.038)	0.992	-0.200	(0.045)	**<0.001**	-0.052	(0.046)	0.260
Chorioamnionitis (yes)	0.030	(0.035)	0.397	0.031	(0.035)	0.370	0.136	(0.047)	**0.004**	0.069	(0.042)	0.106
Death (yes)	-0.103	(0.048)	**0.033**	-0.061	(0.046)	0.186	-0.200	(0.064)	**0.002**	-0.105	(0.054)	0.057
Sepsis (yes)	-0.034	(0.031)	0.272	-0.021	(0.029)	0.477	-0.050	(0.042)	0.239	-0.043	(0.035)	0.218
Mechanical ventilation, days	-0.001	(0.002)	0.432	0.002	(0.002)	0.340	-0.003	(0.002)	0.160	0.002	(0.002)	0.397

Presented are the effect estimates of maternal and neonatal characteristics on CF and CC growth in both univariable and multivariable linear mixed models. The effect estimates (β), standard errors (SE) and p-values are given. Significant findings are in bold font. GA indicates gestational age; BW, birth weight; SDS, standard deviation score; IUGR, intra-uterine growth retardation; PE, pre- eclampsia.

DISCUSSION

In this report we demonstrated that CCF length, measured using CUS, is a reproducible and feasible marker that could serve as a new bedside tool to monitor preterm infants' brain growth during NICU stay. We provided growth charts of CCF and CC length for preterm infants, from 24 to 32 weeks post menstrual age. We identified that a higher BW SDS results in increased CCF and CC growth rate during hospital stay, while female infants have a slower CCF growth compared to male infants.

Previous ultrasound studies have evaluated only a limited number of brain structures as potential markers for brain growth or predictors for neurodevelopmental outcome in preterm infants [163,232-234]. One explanation for this is the fact that the brain has few easily recognizable and consistent landmarks for reliable measurements on CUS. The CC, a flat bundle of white matter, that connects the left and right hemispheres, is one of the brain structures that are easily visualized and recognizable on CUS [240]. Prematurity is known to affect CC development, by the early transition from intra-uterine to extra-uterine life and by postnatal stress and injury [241], leading to both structural and functional impairment [242,243]. Associations have been found between length and thickness of the CC and brain volumes and neurodevelopmental outcome [235,244,245]. Further studies should elucidate if CC length can be considered a proxy of telencephalon development, creating an impression of white matter development and brain maturation. The advantages of using CCF length in the monitoring of brain growth rely on anatomic and practical issues. CCF length may be considered a marker of diencephalon and mesencephalon development and vermis growth. The diencephalon includes the thalamus, a neural relay centre crucial for adequate cognitive function [246]. Altered development of the thalamus, and thus of the diencephalon, may lead to adverse neurodevelopmental outcome. Several studies showed impaired thalamus volume and extreme vulnerability of the thalamus to neonatal risk factors after preterm birth [247,248]. Whether thalamic injury or growth impairment directly influences CCF length needs to be further studied.

One of the other advantages of CCF length measurement is the use of CUS instead of MRI or HC measurement. In Table 3 the pros and contras of every method are depicted. Although volumetric MRI is increasingly used for growth assessment of the preterm brain, its use for serial assessment is still very limited [228]. HC measurement has a low interrater agreement, limited association with long-term outcome and it does not measure actual brain growth, but also growth of the skull and of the subarachnoid spaces, which are frequently enlarged in preterm infants [230,231,249]. Measurement of CCF length is not considered a burden compared to HC measurement as it can be performed on routine CUS, which are often recommended to perform weekly in preterm infants [250]. Both CCF length and CC length can already be measured prenatally, as the CC and the fastigium are already visible on ultrasound around 18 weeks of gestation, allowing to use the same marker prenatally and postnatally for monitoring of brain growth [251].

Table 3 — Pros and contras of different methods for assessment of brain growth

	HC	CUS	MRI
Patient friendly	++	++	-
Bedside available	++	++	-
Serial measurement possible	++	++	-
Fast measurement	++	+	-
Reproducible	+-	+	++
Reflecting actual brain growth	-	+	++
Low costs	++	+	-
Dimension	1D	2D	3D

++= very good; += acceptable; -= bad agreement with the corresponding item. HC, head circumference; CUS, cranial ultrasound.

In accordance with previous studies, we showed satisfactory reproducibility for CC length [232]. CCF reproducibility was excellent too, suggesting that both measurements are feasible for longitudinal evaluation of brain growth. Increasing lengths with increasing ages and weights, as shown in the growth charts, support the use of these markers in clinical practice.

We observed a non-linear growth pattern for CC and CCF length. Previous studies found an intra-uterine constant growth rate of 0.20-0.22mm/day of the CC [252,253]. Also in preterm infants a constant, though slower, growth rate was observed [232]. In contrast to previous studies, we performed longitudinal measurements (1-8 scans per infant), allowing for a more reliable estimation of CC growth. Other brain structures, such as the vermis of the cerebellum, show a non-linear growth pattern as well [233]. As we are the first to evaluate the use of CCF length, no literature is available for comparison. We did expect a non-linear growth pattern, based on current literature.

In Figure 3 parts of the weight charts are coloured grey, as we would advise not to use these parts of the curves as a reference curve. We chose to analyse and present the complete original data of infants with a post menstrual age between 24-32 weeks and not to select ideal reference cases. The drawback is seen in in the upper part of the weight charts; the curves appear to go down above 1400 gram and, despite the very small numbers, the confidence interval narrows. This, of course, does not reflect an incline of brain size, but rather selection and censoring. These data are not “first measurements” (reflecting intrauterine accomplished growth), but are follow-up data of patients with prolonged NICU admission, representing the most complex cases, e.g. with severe chronic lung disease, that were not stable enough to be discharged early. In conclusion, we state that the last part of this curve depicts valid data you would expect in a NICU population but we consider these not representative for normal growth in preterm infants.

The decreased growth rate of CCF length in female infants is in accordance with previous studies, which identified gender differences in brain structures and neurodevelopmental outcome [254,255]. The positive association between BW SDS and CCF and CC growth rate is also in accordance with current literature [256].

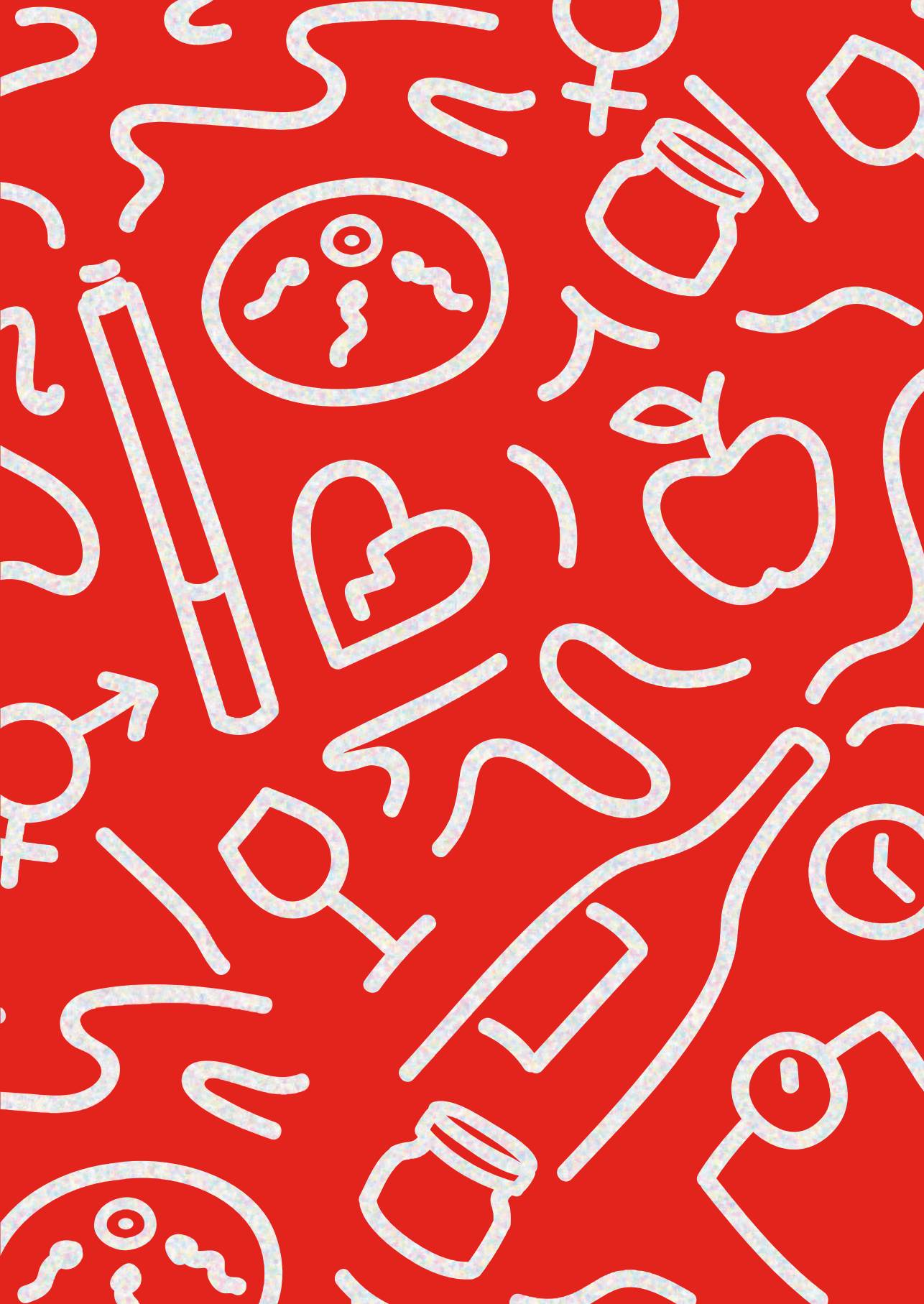
One investigator who was trained in visualizing a standard sagittal plane performed all scans. This likely improved the quality of the scans and may have enhanced the reproducibility. We realize therefore that the clinical applicability is probably overestimated in our cohort. Reliable measurements and a correct sagittal plane using CUS depend on the experience of the observer but are easy to learn. Recently developed software to identify the sagittal plane automatically may further increase the reproducibility and clinical applicability [257].

This study has some limitations. First of all, in the Netherlands preterm infants are transferred to a secondary hospital relatively early, accounting for very few data in our cohort of infants born at 29 weeks of gestation and limited data of infants after 30 weeks of gestation. Although white matter injury is already visible on scans after a few days, brain atrophy is often only noticeable after weeks to months [258]. Our short follow up time could explain why we did not find an association between expected clinical variables, such as sepsis and days on mechanical ventilation, and CCF or CC growth rate. Second, including all scans between 24-32 weeks PMA may have influenced the reliability of the growth charts; it is a common finding that preterm infants lose weight after birth and start to grow days later. Brain growth may be limited before regain of birth weight (usually after 10 days). This may have increased variation in CC and CCF lengths. Extremely preterm and clinically unstable infants have a longer NICU stay and are likely to receive more CUS. This might have biased our growth charts. On the other hand, our data reflect clinical practice in a neonatal intensive care setting.

In future studies it would be interesting to compare fetal and preterm CCF growth. Currently, we are scanning fetuses in the second and third trimester of pregnancy to develop reference curves for fetal brain growth, which could also serve as an ideal growth curve for preterm infants. We were not able yet to assess the association between feeding regimens and growth during NICU stay and CCF growth trajectories. This is of interest as it may have clinical implications for nutritional practices. Moreover, CCF length can possibly be used as an outcome measure in nutritional, and other, intervention studies. It would be of main interest to assess whether CCF length, possibly combined with other available markers of brain growth such as CC length, could serve as predictor for neurodevelopmental outcome. The clinical applicability may extend beyond the NICU stay into the outpatient follow-up period as the anterior fontanelle can be used as an acoustic window until approximately 6 months in most infants.

CONCLUSION

There is a lack of bedside markers for brain growth in preterm infants during NICU stay. We propose a feasible, new ultrasound measurement entitled 'corpus callosum – fastigium length' with high reproducibility for monitoring of brain growth in preterm infants during hospital stay. This marker may help clinicians to determine if preterm infants show adequate postnatal brain growth and may eventually be used as outcome measure in nutritional and other intervention studies. Further research is warranted to assess whether this marker could also serve as an early predictor for short-term and long-term neurodevelopmental outcome.



8

New ultrasound measurements to bridge the gap between prenatal and neonatal brain growth

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S.P. Willemsen, I.K.M. Reiss, P. Govaert,
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Submitted for publication

ABSTRACT

Background — Most ultrasound markers for monitoring brain growth can only be used in either the prenatal or the postnatal period. We investigated whether corpus callosum (CC) length and corpus callosum-fastigium (CCF) length could be used as markers for both prenatal and postnatal brain growth.

Methods — In the setting of a prospective cohort, we performed a three-dimensional ultrasound study in fetuses with fetal growth retardation (FGR), fetuses with congenital heart defects (CHD) and controls at 22, 26 and 32 weeks gestational age (GA). Postnatally, cranial ultrasound was performed at 42 weeks GA. CC and CCF measurements were performed offline and reliability was evaluated. Associations between prenatal and postnatal CC and CCF length were investigated. We created reference curves and compared growth trajectories of CC and CCF length of controls with those of fetuses with FGR and CHD.

Results — We included 199 fetuses; 22 FGR fetuses, 20 CHD fetuses and 157 controls. Reliability of both measurements was excellent (ICC≥0.97). CC growth trajectories were significantly decreased in FGR and CHD (β= -2.295, 95%CI= -3.320 ; -1.270, p<0.01; β= -1.267, 95%CI= -0.972 ; -0.562, p<0.01 respectively). CCF growth was decreased in FGR fetuses (β= -1.295, 95%CI= -2.595 ; 0.003, p= 0.05).

Conclusions — CC and CCF length may serve as reliable markers for monitoring brain growth from the prenatal to the postnatal period. Clinical applicability of these markers for monitoring brain growth was established by the significantly different CC and CCF growth trajectories in fetuses at risk for abnormal brain growth compared to controls.

INTRODUCTION

In preterm and small-for-gestational age (SGA) infants, brain growth is an important predictor for long-term neurodevelopmental outcome [28,227-229]. Although prenatal growth often predicts postnatal growth [98], there is a traditional division between fetal and neonatal growth charts. This is probably due to the lack of consistent measures of brain growth which can be used in both the prenatal and postnatal period.

Markers of brain growth that can theoretically be used in both the prenatal and postnatal period include head circumference (HC) and some ultrasound (US) or MRI measures. HC measured postnatally however, lacks precision and does not correspond well with neurodevelopmental outcome [230,231]. Prenatal and postnatal US markers are largely based on individual brain structures, only reflecting on growth of a specific part of the brain [163,232-235]. Moreover, these brain structures are not measured consistently during the prenatal and postnatal period due to the discrepancy between standard US planes. Although MRI provides more precise measures of brain growth, volume and development, this technique is expensive and therefore not suitable for serial measurements.

Recently, we demonstrated that corpus callosum-fastigium length (CCF) is a reliable bedside-available US marker that can be used to monitor brain growth during NICU stay in preterm infants [259]. CCF length is considered a composite marker of both diencephalon and mesencephalon size and adds information to the more widely used corpus callosum (CC) length [259]. We hypothesized that these two postnatal cranial ultrasound (CUS) markers are feasible to use during second and third trimester US examinations. Thereby, these markers would provide a continuum for monitoring brain growth bridging the period before and after birth.

Our main aim is to investigate whether CC and CCF length can be used as reliable US markers for monitoring both fetal and neonatal brain growth. First, we assessed the reliability of the measurements. Second, we created reference curves from 22 weeks to 42 weeks gestational age (GA) by combining fetal and neonatal measurements. Finally, as a first step to establish the clinical applicability of these US markers, we investigated CC and CCF growth trajectories in fetuses at risk for abnormal brain growth compared to controls.

METHODS

Study design and populations

This three-dimensional (3D) US study was conducted in the setting of the Rotterdam Periconceptional Cohort (Predict study); an ongoing prospective cohort study at the Department of Obstetrics and Gynaecology, Erasmus MC University Medical Center, Rotterdam, the Netherlands [24]. At enrolment,

all participating women and their partners gave written informed consent on behalf of themselves and their unborn child. This study was approved by the regional Medical Ethical and Institutional Review Board of the Erasmus MC, University Medical Center in Rotterdam approved the study (MEC 2004-227, date of approval 25-01-2013).

Pregnant women enrolled between November 2013 and July 2015. They were either enrolled before 12 weeks GA or between 22 and 32 weeks GA. Controls enrolled before 12 weeks GA and were defined as fetuses without FGR before 32 weeks GA, who were born after 37 weeks GA and without congenital malformations. The cases included those pregnancies referred to our outpatient clinic with fetal growth restriction (FGR) or isolated fetal congenital heart defect (CHD). Diagnosis was confirmed by an extended structural US examination at our hospital. FGR was defined as abdominal circumference (AC) or estimated fetal weight (EFW) percentile of less than 5 according to Hadlock [260]. For this analysis we excluded pregnancies ending in intra-uterine death, termination of pregnancy or preterm birth. We also excluded fetuses with congenital anomalies other than CHD, trisomy 21 and without US images.

Study parameters

According to Dutch clinical practice, GA in spontaneously conceived pregnancies was calculated based on first trimester crown-rump length (CRL) measurements before 13 weeks GA [133]. In pregnancies conceived through *in vitro* fertilization (IVF), with or without intra-cytoplasmic sperm injection (ICSI) procedures, GA was calculated from the date of oocyte retrieval plus 14 days or from the day of embryo transfer plus 17 or 18 days after cryopreserved embryo transfer, depending on the number of days between oocyte retrieval and cryopreservation.

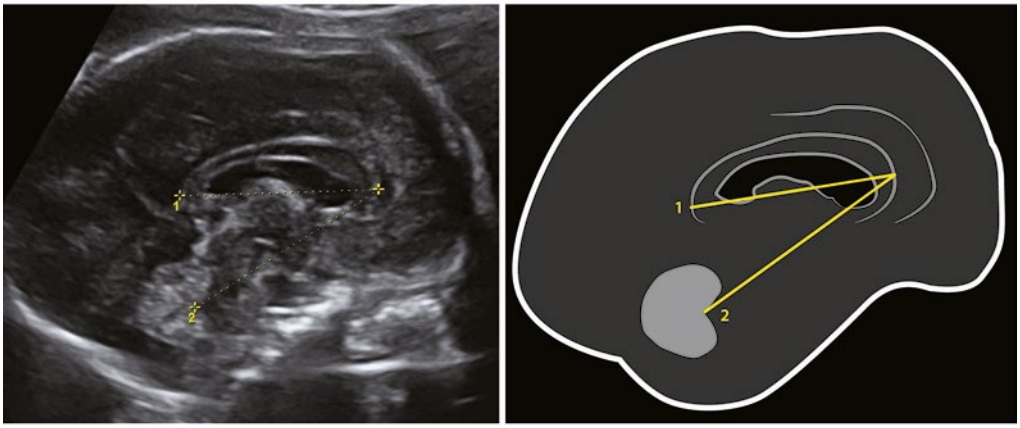
Data were collected on maternal characteristics, medical and obstetrical history, pregnancy course and neonatal outcome from self-administered questionnaires in the first trimester, second trimester and around delivery. Follow-up data on pregnancy outcomes were validated based on US report of the routine second trimester anomaly scan and on the obstetric medical records.

Prenatal ultrasound

Prenatal 3D US examinations were performed on the Voluson E8 system (GE Medical Systems, Zipf, Australia) using a 1-7 MHz transabdominal transducer or a 6-12 MHz or MHz transvaginal transducer. Primarily, we used an abdominal approach, but a transvaginal approach was considered when the fetus was in head-down presentation. Serial prenatal 3D-US examinations and measurements were performed at 22, 26 and 32 weeks of gestation by one certified sonographer (IVK). Standard biometry was measured including bi-parietal diameter (BPD), HC, AC and femur length. An estimation of fetal weight was calculated using the Hadlock equation [260]. Biometry was followed by detailed 3D-US neuro-sonography. The mid-sagittal plane and two bilateral parasagittal oblique planes were obtained according to ISUOG guidelines [209]. CC and CCF length measurements were performed offline in an exact mid-sagittal plane (Figure 1). CC length is measured from genu to splenium, outer-outer border

and CCF length represents the length between the genu of the CC and the fastigium (roof of the fourth ventricle) [259]. CCF length was only measured in images in which the CC measurement was successful. Manipulation of the 3D-US volume to ensure an exact mid-sagittal plane for the measurements was performed in 4D View Version 5.0 (GE Medical Systems).

Figure 1 – CC and CCF length measured on three-dimensional ultrasound



Prenatal measurements of Corpus callosum length (1) and Corpus callosum – Fastigium length (2). Image with permission.

Postnatal assessments

After birth, CUS was planned between 42^{+0} to 42^{+6} weeks postmenstrual age, independent of GA at birth. CUS were performed by an experienced team of researchers using an Esaote MyLab 70 (Genoa, Italy) with a convex neonatal probe (7.5 MHz). CC and CCF length were performed offline by one researcher (JR) according to the method described above, using MyLab software of Esaote.

To enhance precision, all prenatal and postnatal measurements were repeated three times. The mean values were used in the statistical analyses.

Statistical analysis

For data analyses we used SPSS (SPSS release 21 for Windows, IBM, United States of America) and R (R: A language and Environment for Statistical Computing, version 3.1.3, 2015 for Windows, R Core Team, Vienna, Austria). Results with p-values ≤0.05 were considered statistically significant.

Previously, we demonstrated that postnatal measurements of CC and CCF length had good intra- and inter-observer agreement [259]. To evaluate reliability and reproducibility of prenatal measurements, we randomly selected 30 US examinations of 30 different fetuses from the whole study population divided over the three prenatal time points. CC and CCF measurements were then performed in

threefold by two independent observers (1) IVK and 2) JAR). Reliability analyses for intra- and inter-observer reliability were performed, calculating the mean differences with 95%CIs and intraclass correlation coefficients (ICC). Moreover, the extent of agreement was examined with the Bland-Altman method.

Generalized Additive Models for Location and Scale were used to create reference ranges of CC and CCF measurements between 22 and 42 weeks GA in controls [177]. To investigate whether cases are associated with deviations in CC and CCF growth, we created growth trajectories of the serial measurements of CC and CCF length between 22 and 42 weeks GA. A maximum likelihood approach was used to test whether polynomials of GA contributed to the best model fit. In the same manner, we tested the contribution of random and fixed effects of the intercept and slopes for all included polynomials. A quadratic model of GA with random intercept and slopes was designated as the best model. We placed the origin of the GA scale at 140 days GA. In this model was used the variable indicating whether a fetus was FGR, CHD or control as the covariate of interest (Model 1). Finally, the final model (Model 2) was adjusted for serial measurements of fetal weight and gender as potential confounders.

RESULTS

In total 227 pregnant women were enrolled prenatally. After excluding pregnancies ending in intra-uterine fetal death (1), termination of pregnancy (1), preterm birth (14), congenital anomalies other than CHD (4), trisomy 21 (2) and withdrawals (6), the study population consisted of 199 pregnancies. Of those 199 fetuses, 22 fetuses were FGR, 20 had CHD and 157 were controls. The general characteristics of the study populations including 157 controls, 22 FGR and 20 CHD infants are listed in Table 1.

Success rates and reliability analyses

Of 542 prenatal 3D US scans 377 contained a high quality mid-sagittal plane eligible for CC and CCF measurements. Means and success rates of CC and CCF measurements per gestational age are listed in Table 2. Success rates of prenatal CC and CCF measurements ranged between 61 and 75% and between 59 to 72%, respectively. Postnatally, CC and CCF measurements were successful in 97%. In 83% of the subjects CC length was measured at least at two time points during the whole study period and CCF length in 65%. The intra- and inter-observer reliability and agreement are shown in Table 3. CC lengths measured by observer 1 were slightly smaller (mean difference= -1.109mm, mean percentage difference= -3.4%) than those measured by observer 2. Ninety-five percent limits of agreement for all measurements represent excellent agreement when the CC and CCF measurements were repeated by the same observer and good agreement when repeated by a second observer. ICC values of both intra- and inter-observer were ≥0.97, which represents excellent reliability.

Table 1 — General characteristics

Characteristics	Controls (n= 157)	FGR (n= 22)	CHD (n= 20)	Missing
Maternal				
Age at enrolment, years	32.3 (21-44)	29.7 (21-41)	33.0 (22-48)	7
Nulliparous	69 (44)	13 (68)	11 (58)	6
Mode of Conception (IVF/ICSI)	48 (31)	2 (10)	2 (11)	3
Geographical background				6
Western other	126 (81)	15 (79)	18 (90)	
Non-western	29 (19)	4 (21)	2 (10)	
Educational level				8
Low	20 (13)	4 (20)	0	
Intermediate	56 (36)	12 (60)	7 (39)	
High	79 (51)	4 (20)	11 (61)	
Pre-pregnancy BMI, kg/m^2	22.9 (15.2-39.7)	22.9 (17.6-43.4)	23.4 (18.0-35.8)	19
Periconception folic acid initiation (yes)	149 (96)	15 (79)	18 (95)	6
Periconcepion smoking (yes)	25 (16)	3 (16)	3 (16)	8
Periconception alcohol consumption (yes)	44 (29)	4 (21)	10 (53)	9
Neonatal				
Birth weight, grams	3345 (2035-4380)	1400 (400-2900)	3420 (1650-4140)	2
Gestational age at birth, days	274 (259-292)	240 (185-276)	274 (200-292)	2
Gender, male	82 (52)	11 (50)	13 (65)	0

Data are presented as median and range or number (n) and percentage (%). BMI, body mass index; IVF/ICSI, in vitro fertilization with or without intra-cytoplasmic sperm injection; Missing data was due to incomplete questionnaires.

Table 2 — Intra-observer and inter-observer reproducibility for prenatal measurements of the corpus callosum and corpus callosum fastigium length

		Mean difference (mm)	95%CI mean difference (mm)	95% limits of agreement (mm)	p-value	Mean difference (%)	95% limits of agreement (%)	ICC
Intra observer	CC	0.011	-0.228 ; 0.250	-1.373 ; 1.396	0.923	0.1	-4.1 ; 4.3	>0.99
	CCF	0.180	-0.157 ; 0.517	-1.711 ; 2.071	0.284	0.4	-4.7 ; 5.4	>0.99
Inter observer	CC	-1.109	-1.702 ; -0.515	-4.546 ; 2.329	0.001	-3.4	-14.9 ; 8.1	0.97
	CCF	-0.125	-0.741 ; 0.492	-3.589 ; 3.340	0.684	-0.4	-9.5 ; 8.6	0.97

Intra- and inter-observer reliability analyses for prenatal CC and CCF measurements in a random selection of thirty 3D-US. CC, Corpus Callosum length; CCF, Corpus Callosum – Fastigium length; mm, millimetres; 95%CI, ninety-five percent confidence interval; %, percentage; ICC, intraclass correlation coefficient.

Table 3 — Linear mixed models: Growth trajectories of CC and CCF influenced by fetal growth restriction and congenital heart defects compared to controls

		Model 1			Model 2		
		β	**95%CI**	**p-value**	**β**	**95%CI**	**p-value**
CC	FGR	-2.384	-3.262 ; -1.505	<0.01	-2.295	-3.320 ; -1.270	<0.01
	CHD	-1.252	-1.954 ; -0.549	<0.01	-1.267	-1.972 ; -0.562	<0.01
CCF	FGR	-1.413	-2.500 ; -0.326	0.01	-1.295	-2.595 ; 0.003	0.05
	CHD	0.012	-0.829 ; 0.963	0.98	0.000	-0.835 ; 0.835	0.99

Data is presented in β values with corresponding 95%CI and p-values. Significant results are in bold. Model 1 represents the crude model using GA and its polynomials as predictor and type of case as covariate of interest. Model 2 is the fully adjusted model adjusted for serial measurements of fetal weight and gender. β, beta value; 95%CI, ninety-five percent confidence interval.

Linear mixed model analyses

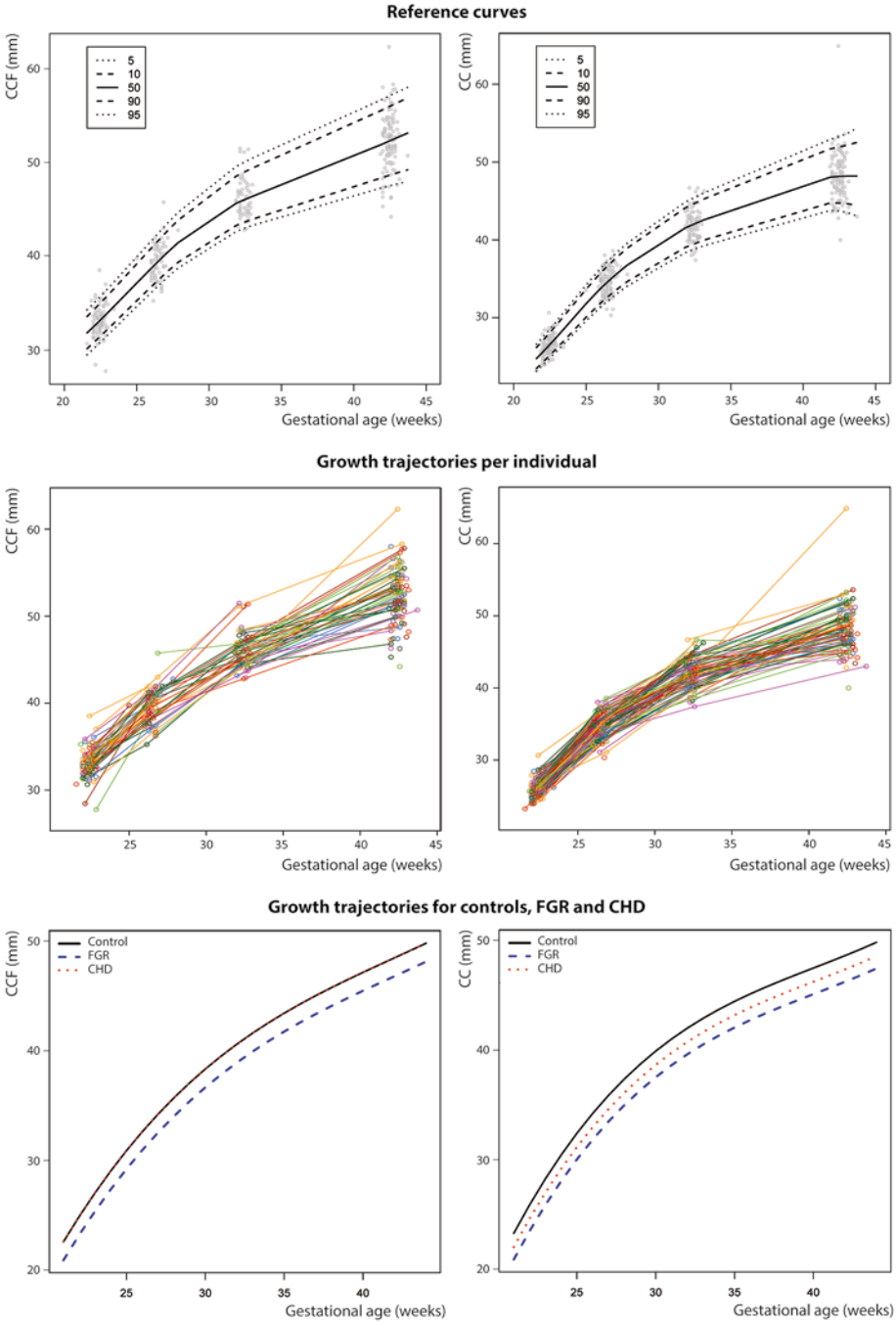
In Figure 2A the reference curves and individual growth trajectories of the CC and CCF measurements are shown. The results of the linear mixed models estimating differences in the mean growth trajectories of CC and CCF length between controls, fetuses with FGR and CHD are shown in Table 4. Growth trajectories of CC length are significantly decreased in FGR and CHD fetuses compared to those of controls. CCF growth trajectories were only significantly decreased in FGR fetuses compared to those of controls. In Figure 2C this is graphically displayed.

DISCUSSION

Here, we demonstrate that CCF and CC length are reliable markers for monitoring pre- and postnatal brain growth. By combining fetal and neonatal measurements in one reference chart we created a practical US tool to bridge the gap between the fetal and neonatal period when monitoring brain growth. FGR fetuses showed decreased growth of both CC and CCF while CHD showed only decreased CC growth between 22 and 42 weeks GA.

Our results show that we are able to bridge the traditional division between fetal and neonatal US growth charts. To date, studies that combine fetal and neonatal US markers of brain growth in a single cohort are scarce. One explanation for this can be that standard prenatal US planes containing easily recognizable landmarks of the brain do not correspond well to the standardized planes accessible by CUS. This results in differences in prenatal and postnatal measures and measuring methods. For example, HC assessed prenatally, calculated from the bi-parietal diameter and occipital frontal diameter, correlates poorly to direct postnatal measurement using tape [231,261]. This difference is further amplified by changes in head shape, e.g. due to skull moulding, oedematous swelling and hematoma's.

Figure 2 — Reference curves and growth trajectories of transcerebellar diameter from 9 until 32 weeks gestational age



In contrast to other markers, we showed excellent reliability for prenatal measurements which is comparable to the postnatal findings [259]. Based our data we suggest that CCF length is the most reliable and relevant measure for monitoring brain growth. Compared to CC length, CCF length is assumed to be a composite marker of brain structures with different embryological origins including the diencephalon and mesencephalon. Therefore CCF length may be a better representative of global brain growth than previous sonographic markers based on individual brain structures [163,232-235].

Growth trajectories of CC and CCF were decreased in fetuses at risk for abnormal brain growth and long-term neurodevelopment. While CCF length has not been published in literature before, the CC findings are in line with previous literature. The decreased CC growth trajectories in FGR are in accordance with findings of a recent MRI study which showed significantly reduced CC length in FGR fetuses compared to appropriate-for-gestational-age controls [262]. Results from our previous study in preterm infants demonstrated a similar association between CCF length and birth weight SD-score [259]. There are, to the best of our knowledge, no publications on CC length in CHD fetuses to compare our results with, although previous studies did report anomalies of the CC and reduction of CC volume in CHD children [263,264]. The decreased growth trajectory of CC length in CHD fetuses is, however, supported by accumulating evidence reporting that CHD fetuses are at risk for abnormal brain growth and development [26,189,265,266]. However to our knowledge there are no publications on CC length in CHD fetuses to compare our results with.

Brain growth is an important predictor for long-term neurodevelopment [9,227,229]. Therefore, we hypothesize that decreased CC and CCF growth in cases may have consequences for neurodevelopmental outcome. In preterm infants, a shorter CC length is related to a higher risk for adverse neurodevelopmental outcome at 2 years corrected age [28]. Moreover, a significantly smaller corpus callosum was found in individuals with schizophrenia and autism [267,268]. CCF length represents diencephalon and includes thalamus development which is crucial for normal cognitive functioning. Derangements in thalamus development are associated with adverse neurodevelopmental outcome [246]. Yet, the clinical relevance of the differences in CC and CCF growth trajectories for neurodevelopmental outcome needs further investigation.

Clinical applicability

Landmarks for CC and CCF measurements are relatively easy to distinguish on US images. Prenatally, the main challenge is obtaining an exact mid-sagittal US plane. Predominantly, prenatal success rates are influenced by both acoustic shadowing and fetal position. 3D US can enhance precision by manipulating volumes to reconstruct the exact mid-sagittal plane [216-218]. When a mid-sagittal plane is obtained, both measurements take less than 1 minute in experienced hands. Postnatally, a standard mid-sagittal plane is easy to obtain, and the offline measurement of both CC and CCF take less than 1 minute. Newly developed software which enables the identification of the midline automatically could still improve the measurements for clinical practice [269].

Strengths and limitations

Some considerations should be taken into account. First, our study was conducted in a tertiary hospital setting, with a relatively high maternal age, women mainly of western origin and a high educational level. Therefore, replication of the data is warranted to validate our findings for the general populations. Second, the small number of cases limits the conclusions of our study. Therefore, we cannot exclude that absence of statistical significant findings may be due to a lack of power. Third, growth charts are based on measurements at 4 time points and may improve by validation in a cohort that included intermediate time points to further smooth the curves. Finally, the US scans and measurements were performed by experienced observers which potentially enhanced quality of the mid-sagittal images and thereby success rates and reliability. Clinical applicability may be overestimated as a consequence. Success rates of the measurements was mostly influenced by fetal position, therefore selection bias due to the success rates seems less likely. We consider the prospective and longitudinal study design as strength of our study. Combining prenatal and postnatal measurements in one reference curve is an innovative method to facilitate monitoring brain growth in fetuses at risk for impaired brain growth.

Future implications for clinical care and research

Tight collaborations between obstetrical and neonatal researchers and caregivers are needed for bridging the gap when monitoring fetal and neonatal brain growth. This is of great importance for optimizing neurodevelopmental care in fetuses and infants at risk for abnormal brain growth and neurodevelopmental impairment. Easily applicable US tools that can be used without constraints of a prenatal or postnatal environment will have clinical implications. We consider our reference curves useful for age-equivalent preterm infants as they are largely comparable to the postnatal reference curves between 24 and 32 weeks in preterm infants from Roelants et al. [259]. In addition, CC and CCF measurements may be applicable from mid-gestation onwards and may theoretically be prolonged until closure of the anterior fontanelle in the first year of life. Future research should explore the link between the growth measures and functional neurodevelopmental outcome.

CONCLUSIONS

In this prospective cohort we demonstrated that CC and CCF measurements are reliable markers for brain growth from the fetal until the early neonatal period. By combining prenatal and postnatal CC and CCF measurements in one reference curve a continuum for monitoring brain growth was created irrespective of an intra- or extra-uterine environment. We demonstrated that fetuses at risk for abnormal brain growth, i.e. CHD and FGR, showed significantly decreased CC and CCF growth between 22 and 42 weeks GA. Whether these markers could serve as an early predictor for neurodevelopmental outcome in later life warrants further research.

PART III

9

General Discussion

This thesis provides evidence for the feasibility and validity of the assessment of early growth and development of a spectrum of human brain structures by three-dimensional ultrasound (3D-US) techniques. Growth trajectories of the cerebellum, head volume (HV), brain fissures, corpus callosum (CC) and corpus callosum – fastigium (CCF) have been associated with several periconceptional maternal and fetal characteristics (Figure 1). In this chapter, I will discuss the main findings and their clinical implications, some methodological considerations and future perspectives.

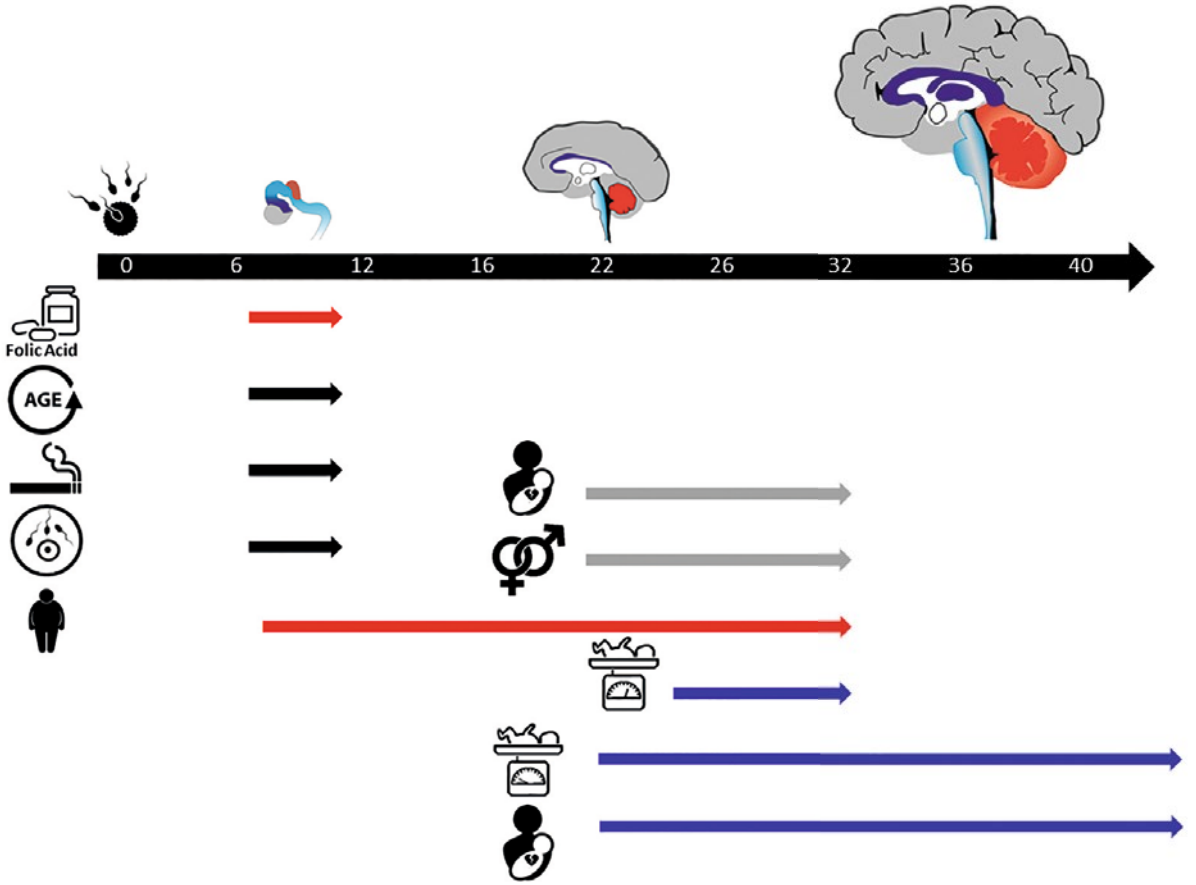
MAIN FINDINGS

The vulnerable human brain

The complexity of the structural development of the human brain implicates a high susceptibility for developing disorders. The developing brain is subject to adverse environmental influences, affecting early growth and developmental programming as a blue print for a lifelong susceptibility to neurodevelopmental disorders and psychiatric disease [270]. During pregnancy an adequate intake of nutrition, avoidance of harmful maternal behaviours and adequate oxygenation are essential for optimal growth and development of the rapidly developing human brain [270]. In this thesis we show that periconceptional maternal conditions that influence overall embryonic and fetal growth, are also associated with growth of the human cerebellum and head (**Chapter 3**, **4** and **5**) [105,106].

The evolutionary expansion of cerebellar size in humans is thought to be fundamental to the human's unique cognitive abilities [271]. The human cerebellum is involved in motor and non-motor functions essential for normal neurodevelopment [48,49]. Although overwhelming evidence from animal studies establishes the particular susceptibility of the cerebellum to adverse intra-uterine environmental conditions [5,46,53], human evidence for such impacts are scarce (**Chapter 2**). In addition to the evidence from the reviewed literature, we demonstrated that first trimester cerebellar growth trajectories are influenced by the periconceptional folate status of the mother (**Chapter 3**). Similar evidence was demonstrated in animal models showing that the cerebellum is susceptible to a folate rich or deficient environment [119,123,124]. Underlying mechanisms of the impact of maternal folate status may lie in epigenetic processes relating to the one-carbon pathway including DNA methylation of embryonic growth genes [16,125]. Our findings add to the widely known positive effects of periconceptional maternal folic acid supplement use, reducing the occurrence of neural tube defects and other congenital malformations [110-112]. However, our data show that mothers-to be can achieve a folate status optimal for cerebellar growth. In addition to similar findings for embryonic size, the highest levels of folate may not relate to the most optimal cerebellar growth [105]. This implies that we need to be cautious with high dosages of synthetic folic acid intake in the absence of clinical need. However, **Chapter 4** shows that the impact of folate attenuates as cerebellar growth progresses into the fetal period. This supports that folate is particularly important during very early gestation [23]. Furthermore, we demonstrated a negative association between maternal body mass

Figure 1 — A schematic presentation of the main results from this thesis



Significant associations between the periconceptional maternal characteristics and fetal factors and the individual brain structures are presented on the timeline of gestation. Cerebellum (red), Cortex (grey), Corpus callosum (Dark blue) and head volume (Black). Icons from top to bottom and left to right represent; folic acid supplement initiation, mode of conception, maternal age, smoking, body mass index, fetal congenital heart defect, gender, fetal growth restriction.

(BMI) and cerebellar growth between 9 and 32 weeks gestational age (GA). An increasing maternal BMI can reflect derangements in a spectrum of biological pathways including inflammation, oxidative stress, metabolism and vascularization which continuously expose the developing brain [150-154,272]. Specifically, the increased inflammatory state and circulating cytokines play a key role in fetal brain inflammation inducing neuronal damage and mal programming of neonatal brain genes and subsequent abnormal neurodevelopment in the offspring [150,152]. To intervene in these pathways and improve neurodevelopmental health in offspring of obese mothers, the effect of metformin treatment, nutrient supplementation, lifestyle and dietary changes were explored [273-277]. However to date, evidence is insufficient to recommend any of these exploratory therapies.

This thesis also reveals that embryonic HV is positively associated with maternal age and IVF/ICSI treatment and negatively associated with periconceptional smoking (**Chapter 5**). Although interpretation of these results is complicated by the use of transformations in the models, there are several explanations. Decreased head size due to maternal smoking could be explained by direct toxic effects on embryonic tissues [29]. Also, epigenetic alterations in DNA methylation and down regulation of genes that are important for normal brain development could play a role [278,279]. Moreover, maternal aging is related to global hypomethylation potentially leading to decreased DNA methylation in the embryo. When this involves growth genes, it may also explain why in general embryonic growth is increased in aging mothers [106]. Furthermore, IVF/ICSI treatment may be considered as an environmental factor that influences programming and gene expression patterns in the embryo as well [182,280].

Our data is important as a large proportion of women of reproductive-age struggle daily with one or more of these potentially harmful periconceptional behaviours [148,281,282]. Since the demonstrated associations involve periconceptional maternal factors that have been related to epigenetic pathways, our findings may even have transgenerational effects. Therefore, we speculate that preconceptional counselling and public health policies targeting these modifiable factors, can contribute to the improvement of neurodevelopmental health in the next generations. However, as time passes potential new threats including limited physical exercise, recreational drug use, phthalates and the use of mobile phones invade our daily lives and warrant our attention.

The early brain adapts

The critical window of exposures is not restricted to the periconceptional and embryonic period. The fetal and neonatal brain adapts to its prenatal and postnatal environment. In addition, an adverse environment may contribute to developmental disorders, growth restriction or preterm birth and indirectly affect brain development. Literature indicates that fetuses with congenital heart defects (CHD) growth-restricted fetuses and preterm born infants are at risk for abnormal brain development and consequently impaired neurodevelopment [26,283,284]. Cerebro-placental blood flow can be redistributed in these cases to preserve an adequate cerebral oxygenation as an auto-

regulative response [188,190,285]. Such an adaptive process favours short-term survival but can be disadvantageous for long-term functional outcome [270,285]. Therefore, it seems obvious to monitor early brain development already during pregnancy in these infants at risk. However, reliable markers to monitor brain growth during prenatal and neonatal life are lacking.

From this perspective, we investigated new early markers of head and brain development including cortical folding, CC length and CCF length in **Chapter 6**, **7** and **8** in addition to the previously discussed HV (**Chapter 5**). HV was measured using the Barco I-Space virtual reality system. We proposed HV as a new measure for first trimester head size and possible early marker of fetal head size. Since the CAVE-like virtual reality system is bound to a spacious room and measurements warrant extensive training, clinical implementation of this technique is limited. A desktop version of the same virtual reality software, which can be used next to the ultrasound equipment, may facilitate this in the future.

Fetal cortical development can be evaluated with a wide variety of measures and methods from both ultrasound and magnetic resonance imaging studies [200,203,205,207]. In **Chapter 6** we showed the feasibility of fetal brain fissure depth measurements using 3D-US. Our findings of altered cortical folding in CHD fetuses are supported by accumulating evidence that brain abnormalities co-occurring with CHD have a prenatal origin [26,185]. However, our conclusions may be limited because it is not clear whether the three brain fissure depths represent the complex process of global cortical folding. We demonstrated that cortical folding can be altered without signs of hemodynamic adaptation. Therefore, other mechanisms seem to play a role in the prenatal origin of CHD-related brain abnormalities. This also supports why growth restricted fetuses show a different patterns of cortical folding alterations [206]. The simultaneous development and shared morphogenetic programs of the heart and brain may bring a new perspective placing more emphasis on early gestation [286]. This creates a window of opportunity for prenatal diagnostic screening. Furthermore, we need to consider that the brains of these fetuses are challenged to overcome a series of events including hemodynamic changes, delivery, closure of the ductus arteriosus and cardiac surgery that all contribute to derangements in brain development. Therefore, it is recommended to monitor brain development, not only cerebro-placental blood flow indices, and screen for adverse functional outcome [187].

Monitoring brain growth in the neonatal period is mostly limited to imprecise HC measurements. CCF length, a new cranial ultrasound (CUS) marker that we proposed in **Chapter 7** was demonstrated to be a reliable tool for monitoring brain growth in preterm infants. The marker was relatively stable as no associations were found with gestational or neonatal complications. However, the short follow-up period and little data after 30 weeks GA should be considered when interpreting these non-significant findings. In **Chapter 8** we confirmed that the CCF length could also be measured reliably using prenatal 3D-US. In addition prenatal and neonatal CC and CCF measurements were combined in one reference chart. The reference charts from **Chapter 7** and **8** were largely comparable. Therefore, we suggest that these new curves can be useful for both fetuses and age-equivalent preterm infants. Clinical value

of these markers is established by the decreased CC and CCF growth trajectories in fetuses at risk for abnormal brain growth and adverse neurodevelopmental outcome. In theory, applicability of CC and CCF measurements can be prolonged from mid-gestation until closure of the anterior fontanelle. However, before implementing these practical, relatively cheap and reliable markers in clinical practice, we need to explore its predictive value and relationship with neurodevelopmental outcome.

METHODOLOGICAL CONSIDERATIONS

We conducted a longitudinal 3D-US study (Dream study) in the setting of the Rotterdam periconception cohort (Predict Study) and a longitudinal ultrasound study in preterm infants during hospital stay (Submarine study). In these studies we combined first, second, third trimester and postnatal brain measurements. The submarine study collected serial postnatal brain measurements in preterm infants. Previously, ultrasound studies predominantly studied growth and developmental disorders with a cross-sectional approach, which limited conclusions on actual growth of brain structures. In addition, embedding the dream study in the Predict study had the advantage that data was obtained from the periconceptional period until the early neonatal period. This is in contrast to most studies limiting data collection to the third trimester of pregnancy and delivery [24]. However, interpretation of our findings should be done with caution as the observational study design limits the ability to demonstrate causation. Additionally, the study was carried out in a tertiary hospital setting which contributed to our population consisting of high risk pregnancies and a large proportion of pregnancies resulting from IVF/ICSI treatment. Therefore, external validity of the findings needs to be established. During the study we were often challenged by moderate or even low success rates of 3D-US measurements. This could have compromised the longitudinal setting of the study. However, in all analyses more than half of the subjects did have repeated measurements, which supports that the linear mixed models which we have estimated are the most suitable statistical method. How the small deviations in the growth of the investigated brain structures are related to the child's functional outcome needs to be assessed in follow up studies. Small derangements in early brain development potentially lead to larger functional effects in later life. However, conclusions on neurodevelopmental consequences are beyond the scope of this thesis. Replication of our study is warranted to draw more robust conclusions on the observed associations.

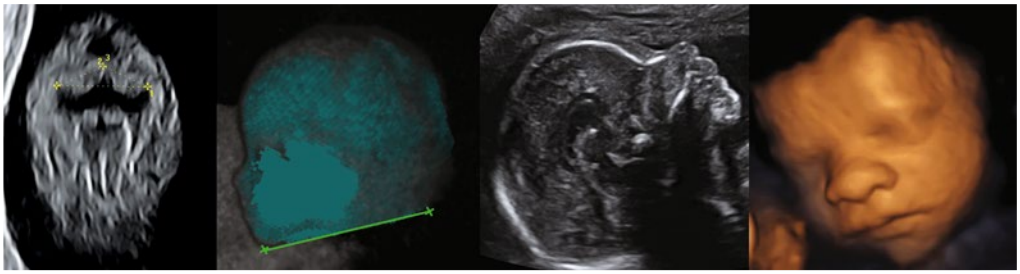
In studies with embryonic and fetal growth outcomes, GA remains an important confounder. Although all statistical analyses were adjusted for GA, inaccuracies in pregnancy dating may still have influenced the observed associations despite the strict method of pregnancy dating in the Predict study. However, in the Netherlands reference charts of CRL measurements from Robinson et al. 1975 are still used in daily clinical practice [133]. One may argue that these charts are outdated and lack the image quality which can be ensured by current ultrasound equipment. Innovative methods for pregnancy dating by Carnegie stages may enhance precision of designating the true developmental age of the embryo

[287]. However, this is a time consuming method and currently not ready for clinical implementation. Until we have found a more optimal method, we need to take into account inaccuracies of pregnancy dating (both in clinical practice and for research purposes), as studying trajectories of growth warrant a time scale.

FUTURE PERSPECTIVES

The DOHAD paradigm is a hot topic in research, demonstrating accumulating new evidence for the prenatal origins of non-communicable diseases in later life. However, the contribution of adverse conditions during pregnancy on the developmental programming of neurodevelopmental and psychiatric disorders has comparatively been ignored. Nevertheless, it is an important issue as one in six children in the industrialized world are at risk for neurodevelopmental disorders and the prevalence of ADHD and ASD is rising [6-8].

Figure 2 — Examples of the use of three-dimensional ultrasound



(A) measurements of the embryonic cerebellum, (B) segmentation of the embryonic head for head volume measurements in the Barco I-Space, (C) Manipulating 3D-US volumes for a precise mid-sagittal plane for corpus callosum measurements and (D) Render mode of 3D image of a fetus of 32 weeks gestational age. Images with permission.

Evaluating early human brain development may contribute to a better understanding of developmental phases in which the brain is most vulnerable to specific environmental insults. Redirecting our focus to even earlier prenatal measures of brain growth is of great importance and creates new windows of opportunity for prenatal diagnostics. Although we demonstrated that 3D-US measures of a spectrum of brain structures were feasible and reliable, measuring small brain structures remains a challenge and warrant a trained neurosonographer. Therefore, clinical implementation of such complex measurements at this moment is rather limited. One may argue that MRI provides more detailed and sophisticated measures to evaluate brain development. Yet, in a society in which we constantly deliberate on health care costs and the burden for the patient, ultrasound will remain the first choice for imaging, especially during pregnancy. The multiplanar mode of 3D-US shows the three orthogonal planes enabling a precise reconstruction of the plane needed for measurements with

minimal deviation [216-218,288]. Improvements in 3D-US applications will enhance image quality and usability and thereby clinical implementation of early human brain measurements. Then, monitoring brain development from the first trimester onwards may become within reach.
\It is crucial for clinical practice that we use precise and reliable measures of early human brain development. The question is however, what is precise and reproducible? What is an acceptable level of measurement error? In literature we find a wide variety of statistical analyses to calculate reliability and to classify the 'extend of agreement'. Throughout this thesis we used the same methods for the reliability analysis including intraclass correlations and the Bland Altman approach. Although clinical applicability of measurements depends on the level of agreement, literature provides no consensus on the level of agreement required for specific measurements.

Another important issue is the traditional division between fetal and neonatal reference charts for monitoring weight and head circumference for example. This leaves a gap when monitoring brain growth in fetuses and infants at risk for abnormal neurodevelopment. To provide sustainable neurodevelopmental care for these high risk patients, it is essential that we find continuous measures for brain growth that can be used irrespective of an intra or extra uterine situation. Extending our focus to a more protracted perspective on neurodevelopment of the embryo, fetus and neonate will open up a whole new field of research and clinical care. Such a perspective however requires a strong collaboration between obstetrical and neonatal health care professionals and researchers. Combining knowledge of these fields will enable monitoring the unborn child from conception until the first weeks of life irrespective of an intra or extra-uterine environment.

Large follow-up studies need to elucidate whether the deviations in size and growth of the studied brain structures have consequences for neurodevelopmental functioning. Functional outcome tests that specifically target the exact set of functions of the individual brain structure are essential. In this context, human research is mandatory as the human brain is significantly different from animals, following different embryologic timelines and engaging in a more extensive and complex set of functions. However, to provide evidence for causal relations between adverse intra-uterine conditions for prenatal brain development, animal studies are indispensable. Human trials in this context are neither ethical nor feasible. Combining evidence from both animal and human studies may lead to an increasing understanding of the underlying mechanisms driving derangements in neurodevelopment due to periconceptional behaviours, maternal characteristics and fetal factors subsequently influencing postnatal neuro-psychological functioning.

This thesis provides evidence for the impact of periconceptional maternal and fetal factors on early human brain development. Hopefully this knowledge will contribute to more awareness of the periconceptional and prenatal period as window of opportunity for the modification of preventable risk factors that affect early neurodevelopment. Ultimately, future research will contribute to efficacious

periconceptional counselling supporting a healthier lifestyle for parents-to-be which will contribute to an optimal environment for the developing fetus and its brain.

10

Summary / Samenvatting

SUMMARY

Early human brain development is an extremely complex process which is highly susceptible to genetic and environmental conditions. These factors may cause subtle changes in early brain development and subsequent neurodevelopmental impairment. The main objective of this thesis is to provide more insight in early human brain development during the embryonic, fetal and early neonatal period and factors that disrupt this process.

In **Chapter 1** the background this thesis is described. We also introduce the three-dimensional ultrasound (3D-US) technique which facilitates neuro-sonography; the evaluation of embryonic, fetal and neonatal brain development. Standardized 3D-US measures of various brain structures were developed to create reference curves. Moreover, we investigate the impact of periconceptional, maternal and fetal characteristics on early human brain development. In this thesis we describe the results from three studies; the Rotterdam periconception cohort, the DREAM study and the Submarine study.

In **PART I** we focus on early human development of the cerebellum. A systematic literature search is conducted to provide an overview of parental environmental exposures and intrinsic factors influencing prenatal cerebellar growth and development in humans (**Chapter 2**). We have found 15 eligible studies reporting associations between maternal smoking, use of alcohol, *in vitro* fertilization mediums, mercury, mifepristone, aminopropionitriles, ethnicity, cortisol levels and cerebellar development. We conclude that current literature on factors impacting the human cerebellar development is scarce and not always consistent.

Therefore, a 3D-US study is conducted to study the development of the cerebellum during the first, second and third trimester of pregnancy. In **Chapter 3** we show that preconceptional maternal folic acid supplement initiation compared to postconceptional initiation is associated with increased embryonic cerebellar size and growth trajectories. Although the variations in cerebellar growth are small, we validated our findings by establishing an optimal level of red blood cell folate. Growth trajectories of the cerebellum between 9 and 32 weeks of gestation were created in **Chapter 4**. By creating these growth trajectories we are able to study associations with a spectrum of periconceptional behaviours, maternal characteristics and fetal gender. We demonstrate that maternal pre-pregnancy body mass index is negatively associated to cerebellar growth from the first until the third trimester. As maternal obesity has previously been associated to increased risks for neurodevelopmental impairment, we hypothesized that prenatal cerebellar growth acts as a mediator in this association.

To evaluate early brain development, new and reliable early imaging markers are warranted. In **PART II** several serial measurements of embryonic, fetal and neonatal brain structures are studied. In **Chapter 5**

we demonstrate that first trimester head volume measured with a virtual reality technique (Barco I-Space) are positively associated with fetal head size. This supports the hypothesis that embryonic head growth may serve as early marker of fetal head size. Furthermore we demonstrate that head growth is negatively associated with periconceptional maternal smoking and positively associated with maternal age and *in vitro* fertilization (IVF)/intra-cytoplasmic sperm injection (ICSI) treatment. Implications of these small but significant variations in growth of the embryonic head for the child's neurodevelopmental outcome are yet unknown and warrant further investigation.

Chapter 6 describes the 3D-US method to evaluate brain fissure depths as a measure for prenatal cortical folding. Congenital heart defects show delayed growth trajectories of the left insula and right parieto-occipital fissure while their growth rates were slightly increased compared to controls. These findings cannot be explained by hemodynamic adaptation as the cerebro-placental ratio is not significantly associated to cortical folding in CHD, which is in contrast to controls. In addition we show a positive association between male gender and growth trajectories of the Sylvian fissure and the insula depths.

In the Submarine study, we study the feasibility of a new bedside available ultrasound marker for preterm brain growth; corpus callosum – fastigium (CCF) length. Reference curves of this new marker are presented in **Chapter 7**. In addition we show that CCF length was positively associated to birth weight and male gender. No other associations with gestational complications including pre-eclampsia, chorioamnionitis, ventilation days, perinatal death or sepsis are demonstrated. Thereafter, we investigate whether the same marker is feasible for evaluating fetal brain growth (**Chapter 8**). Furthermore we provide reference curves of CC and CCF length between 22 weeks until 42 weeks gestational age to monitor fetal and neonatal brain growth as continuum. Reference curves of CC and CCF in fetuses (**Chapter 8**) are largely comparable to those in preterm infants (**Chapter 7**). Furthermore, CC and CCF growth trajectories of CHD and FGR fetuses are decreased compared to those of controls.

In the general discussion we address the main findings and their clinical implications, some methodological considerations and future perspectives. In addition, recommendations for future neurodevelopmental research in the field of the DOHaD paradigm are discussed.

SAMENVATTING

Vroege ontwikkeling van het menselijk brein is een uiterst complex proces waarbij zowel genetische factoren als omgevingsfactoren een belangrijke rol spelen. Deze factoren kunnen subtiele veranderingen in vroege breinontwikkeling tot gevolg hebben. Dit zou kunnen leiden tot neurologische ontwikkelingsstoornissen. Het doel van dit proefschrift is meer inzicht te krijgen in de vroege ontwikkeling van het brein gedurende de embryonale, foetale en vroege neonatale periode en factoren die dit proces kunnen verstoren.

In **Hoofdstuk 1** wordt de achtergrond van dit proefschrift beschreven en de driedimensionale echografietechniek (3D-US) geïntroduceerd die gebruikt wordt voor neurosonografie, de evaluatie van embryonale, foetale en neonatale breinontwikkeling. Gestandaardiseerde 3D-US metingen van verschillende breinstructuren zijn ontwikkeld en referentiecurves zijn gemaakt. Bovendien wordt de invloed bestudeerd van periconceptionele maternale en foetale factoren op de vroege breinontwikkeling. In dit proefschrift worden de resultaten beschreven van de studies die zijn uitgevoerd in het Rotterdam periconceptie cohort, de DREAM studie en de Submarine studie.

In **DEEL I** ligt de focus op de vroege ontwikkeling van het cerebellum bij de mens. Een systematische literatuurstudie is uitgevoerd waarin een overzicht wordt gegeven van ouderlijke omgevingsinvloeden en intrinsieke factoren die de prenatale groei en ontwikkeling van het cerebellum beïnvloeden (**Hoofdstuk 2**). Er worden 15 geschikte studies gevonden die associaties rapporteerden tussen roken, alcoholconsumptie, media die gebruikt worden voor *in vitro* fertilisatie (IVF), kwik, mifepristone, amino-propaannitrilen, etniciteit en cortisolspiegels en cerebellaire ontwikkeling. De conclusie is dat de huidige literatuur over factoren die de cerebellaire ontwikkeling bij de mens beïnvloeden schaars en bovendien niet altijd consistent is.

Dit is aanleiding geweest voor het opzetten van een 3D-US studie waarin de ontwikkeling van het cerebellum wordt bestudeerd gedurende het eerste, tweede en derde trimester van de zwangerschap. In **Hoofdstuk 3** wordt preconceptioneel starten met foliumzuursupplementen door de aanstaande moeder geassocieerd met een groter cerebellum en toegenomen cerebellaire groeitrajecten in het eerste trimester vergeleken met postconceptioneel starten. Hoewel de variaties in de groei van het cerebellum klein zijn, werden de bevindingen gevalideerd met het vaststellen van een optimum concentratie van maternaal foliumzuur in erytrocyten. In **Hoofdstuk 4** zijn de groeitrajecten van het cerebellum tussen 9 en 32 weken zwangerschap gecreëerd. Hierdoor werd het mogelijk om associaties met verschillende periconceptionele gewoonten, maternale kenmerken en het foetale geslacht te bestuderen. In dit hoofdstuk laten wij zien dat de body mass index van de moeder vóór de zwangerschap en gedurende de vroege zwangerschap negatief geassocieerd is met de groei van het cerebellum

van het eerste tot en met het derde trimester. Aangezien maternale obesitas eerder geassocieerd werd met een verhoogd risico op neurologische ontwikkelingsstoornissen, veronderstellen wij dat de prenatale groei van het cerebellum optreedt als een intermediair in deze associatie.
Om de vroege breinontwikkeling te beoordelen zijn nieuwe betrouwbare vroege markers nodig. In **DEEL II** bestuderen we verschillende seriële metingen van embryonale, foetale en neonatale breinstructuren. In **Hoofdstuk 5** tonen wij aan dat het hoofdvolume in het eerste trimester, gemeten met de Barco I-Space virtual reality-techniek, positief geassocieerd is met de foetale hoofdomtrek. Dit ondersteunt de hypothese dat de embryonale groei van het hoofd een vroege marker kan zijn voor de foetale hoofdgroei. Bovendien wordt de groei van het hoofd negatief geassocieerd met periconceptioneel roken en positief geassocieerd met de leeftijd van de moeder en het ondergaan van een IVF/intracytoplasmatische sperma-injectie behandeling. De consequenties van deze kleine, doch significante variaties in de groei van het embryonale hoofd voor de neuropsychologische ontwikkeling van het kind zijn nog onbekend en moeten verder onderzocht worden.

Hoofdstuk 6 beschrijft de 3D-US methode opgesteld voor het evalueren van foetale breinfissuren als een maat voor de prenatale corticale ontwikkeling. Foetussen met een congenitale hartafwijking (CHA) tonen vertraagde groeitrajecten van de linker insula en rechter parieto-occipitale fissuur met een verhoogde groeisnelheid vergeleken met een controlegroep. In tegenstelling tot de controles is de cerebro-placentaire ratio niet significant geassocieerd is met de corticale ontwikkeling van CHA-foetussen. Dit suggereert dat hemodynamische mechanismen hier niet verantwoordelijk voor lijken te zijn. Daarnaast vinden wij een significant positieve associatie tussen het mannelijke geslacht en groeitrajecten van de insula en fissuur van Sylvius.

In de Submarine studie, wordt de toepasbaarheid onderzocht van een nieuwe aan-het-bed-beschikbare echomarker voor premature breingroei; corpus callosum – fastigium (CCF) lengte. Referentiecurves van deze nieuwe meting worden gepresenteerd in **Hoofdstuk 7**. CCF lengte is positief geassocieerd met het geboortegewicht en het mannelijke geslacht. Er worden geen andere associaties aangetoond met zwangerschapscomplicaties zoals pre-eclampsie, chorio-amnionitis, aantal dagen mechanische ventilatie, perinatale sterfte of sepsis. Vervolgens wordt onderzocht of dezelfde marker bruikbaar is voor het evalueren van de groei van het foetale brein (**Hoofdstuk 8**). Referentiecurves tussen de 22 en de 42 weken zwangerschapsduur worden gemaakt zodat breingroei als continuüm kan worden gemonitord in de foetale en neonatale periode. Deze foetale referentiecurves komen grotendeels overeen met die van de prematuren beschreven in **Hoofdstuk 7.** Bovendien blijkt dat CC and CCF groeitrajecten van foetussen met groeirestrictie en CHA verlaagd waren ten opzichte van de controlegroep.

In de algemene discussie gaan wij in op de belangrijkste bevindingen van dit proefschrift. We bespreken de klinische implicaties, methodologische aspecten en het toekomst perspectief. Daarnaast doen we aanbevelingen voor toekomstig onderzoek naar de vroege ontwikkeling van het menselijk brein in het kader van het DOHaD paradigma.

ADDENDUM

Supplementary materials

References

Abbreviations

Authors & affiliations

Bibliography

PhD portofolio

About the author

Acknowledgements

SUPPLEMENTARY MATERIALS

CHAPTER 3 – APPENDIX A

Literature search 4 September 2015

Embase	2226
MedLine OvidSP	279
Web-of-science	1091
PubMed publisher	95
Cochrane	0
Google Scholar	181
Total	**3872**

Embase

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MedLine OvidSP

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cochrane

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Web-of-science

TS=(((((cerebell* NEAR/3 (agenes* OR atroph* OR hypoplas*)))) OR (((cerebell* OR Purkinje OR transcerebel*)) AND ((growth* OR develop* OR matur* OR malform* OR defect* OR deficit* OR anomal* OR damag* OR formation* OR size OR dysmorphogen* OR dysgenes* OR abnormal* OR measure* OR ((cerebel* OR transcerebel*) NEAR/3 (weight OR volume* OR diameter* OR small* OR large*)) OR hypoplas* OR hypogen*)))) AND ((((risk* OR nutrition* OR malnutrit* OR malnourish* OR underfe* OR undernourish* OR undernutrit* OR macronutrient* OR micronutrient* OR nutrient* OR supplement* OR additive* OR deficien* OR behav* OR obes* OR overweight* OR adipos* OR (weight NOT "birth weight") OR cigarette* OR smok* OR nicotin* OR lifestyle OR "life style" OR "folic acid" OR folate OR alcohol* OR ethanol OR addict* OR abuse OR substance* OR drug* OR cannabis OR cocaine OR opiate* OR opioid* OR methadone* OR intoxicat* OR abus* OR exposure OR ethnic* OR race OR racial OR parity OR multipar* OR primipar* OR nullipar* OR ((social OR socio OR socioeconomic) NEAR/3 (status* OR rank OR class* OR background*)) OR poverty OR income OR ((parent* OR matern* OR patern* OR mother* OR father*) NEAR/3 (age OR old* OR young* OR "birth weight" OR birthweight OR SGA OR small OR LBW OR VLBW OR ELBW)))) AND ((pregnan* OR "child bearing" OR gravid* OR primigravid* OR fetus* OR foetus* OR fetal* OR foetal* OR embryo* OR gestation* OR prenatal OR perinatal OR offspring* OR newborn* OR mother* OR intrauter* OR trimester))) OR ((maternal* OR paternal*))) NOT ((animal* OR rat OR rats OR sheep OR mouse OR mice OR ewe OR cattle OR pig OR swine OR rodent* OR rabbit*) NOT (human* OR wom?n OR child* OR newborn*)))

PubMed publisher

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Google Scholar

intitle: Cerebellum|Cerebellar (growth|development|malformation|defect|anomaly) (risk|obesity|nutrition| malnutrition|deficiency|macronutrient|smoking|lifestyle|alcohol|drugs|ethnicity|"social status"|"parental age") pregnancy|pregnant human|humans

CHAPTER 3 – APPENDIX B

Quality score Systematic review on cerebellar growth and environmental factors
This quality score can be used to assess the quality of studies included in systematic reviews and meta-analyses and is applicable to both interventional and observational studies, excluding case reports. The score was designed based on previously published scoring systems (Carter et al, 2010 and the Quality Assessment Tool for Quantitative Studies*)*. The quality score is composed of 5 items, and each item is allocated 0, 1 or 2 points. This allows a total score between 0 and 10 points, 10 representing the highest quality.

1. **Study design**
   - **0** for studies with cross-sectional data collection.
   - **1** for studies with longitudinal data collection (both retrospective and prospective).
   - **2** for intervention studies.

2. **Study size**

   Imaging studies
   - **0** small population for analysis (n<200)
   - **1** intermediate population for analysis (n= 200 to 1000).
   - **2** large population for analysis (n >1000).

   Histological studies
   - **0** small population for analysis (n<20).
   - **1** intermediate population for analysis (n= 20 to 100).
   - **2** large population for analysis (n >100).

3. **Exposure**

   Observational studies
   - **0** if the study reported no or unclear exposure in terms of quality or quantity.
   - **1** if the study used categorical measurements of the exposures.
   - **2** if the study used quantitative measurements for the exposures.

4. **Outcome**

   Imaging studies
   - **0** when using no, unclear or no appropriate method for measuring or describing the cerebellum. Or when description is without a clear clinical definition.
   - **1** when using a method for describing or measuring the cerebellum without information on validation, reliability or reproducibility.
   - **2** when using a standardized and validated cerebellar measurement (volume, diameter, etc.) or protocolled description of ultrasound anomaly.

Histological

0 if the study used no appropriate, unclear outcome measurement or if not reported.

1 if the study used descriptive observations.

2 if the study used quantitative observations/measurements of histological findings.

5. Adjustments

0 if findings are not adjusted.

1 if findings are controlled for:

- Gestational age
- Occurrence of congenital malformations

2 if an intervention study is adequately randomized **or** if findings are additionally controlled for GA, and **at least two** of the following covariates†:

- Maternal body characteristics (age, BMI, ethnicity, socio-economic status)
- Obstetrical characteristics (Gravidity, parity, PE/HELLP, IUGR, stage of delivery)
- Medical characteristics (Medication, disease, subfertility)
- Fetal characteristics (Fetal growth, EFW, gender)
- Other behavioral factors (Drugs, smoking, alcohol, exercise, etc)

† Either adjusted for in the statistical analyses; stratified for in the analyses; or not applicable (e.g. a study in nulliparous women only does not require controlling for parity)

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ABBREVIATIONS

2D-US	Two-dimensional ultrasound
3D-US	Three-dimensional ultrasound
AC	Abdominal circumference
BMI	Body mass index
BPD	Biparietal diameter
BW	Birth weight
CC	Corpus callosum
CCF	Corpus callosum – fastigium
CHD	Congenital heart defect
CI	Confidence interval
CPR	Cerebro-placental ratio
CRL	Crown-rump length
CUS	Cranial ultrasound
EFW	Estimated fetal weight
FA	Folic acid
FGR	Fetal growth restriction
FL	Femur length
GA	Gestational age
HC	Head circumference
HV	Head volume
ICSI	Intracytoplasmic sperm injection
ICC	Intraclass correlation coefficient
IQR	Interquartile range
IVF	*In vitro* fertilisation
LCD	Left cerebellar diameter
LMP	Last menstrual period
MCA	Middle cerebral artery
MRI	Magnetic resonance imaging
NICU	Neonatal intensive care unit
OFD	Occipital frontal diameter
PMA	Post menstrual age
POF	Parieto-occipital fissure
SD	Standard deviation
SD(S)	Standard deviation (score)
SGA	Small for gestational age
RCD	Right cerebellar diameter
UA	Umbilical artery
TCD	Transcerebellar diameter

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This thesis

Periconception maternal folate status and human embryonic cerebellum growth trajectories: The Rotterdam Predict study.
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A new ultrasound marker for bedside monitoring of preterm brain growth.
J.A. Roelants, **I.V. Koning,** M. Raets, S.P. Willemsen, M.H. Lequin, R.P.M. Steegers-Theunissen, I.K.M. Reiss, M.J. Vermeulen, P.P. Govaert, J. Dudink.
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Congenital heart defects and trajectories of cortical folding of the human fetal brain by three-dimensional ultrasound.
I.V. Koning, A.W. van Graafeiland, I.A.L. Groenenberg, S.C. Husen, A.T.J.I. Go, J. Dudink, S.P. Willemsen, J. Cornette, R.P.M. Steegers-Theunissen.
Submitted for publication

Prenatal cerebellar growth trajectories and the impact of periconceptional maternal and fetal factors.
I.V. Koning, I.A.L. Groenenberg, J. Dudink, I.K.M. Reiss, R.P.M. Steegers-Theunissen.
Submitted for publication

New ultrasound measurements to bridge the gap between prenatal and neonatal brain growth.
I.V. Koning, J.A. Roelants, M.J. Vermeulen, I.A.L. Groenenberg, I.K.M. Reiss, P.P. Govaert, R.P.M. Steegers-Theunissen, J. Dudink.
Submitted for publication

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Effect of preterm birth on echogenicity in basal ganglia.
Robbin de Goederen, Marlou M.A. Raets, R.J.C. de Jonge, L.A. Ramenghi, F.E. Hoebeek, **I.V. Koning**, P. Govaert, J. Dudink.
Submitted for publication

Mid-gestational fetal fractional thigh volume: an early ultrasound marker of neonatal fat mass.
J.A. Roelants, M. Vermeulen, **I.V. Koning,** I.A.L. Groenenberg, S.P. Willemsen, A.C. Hokken-Koelega, K.F.M. Joosten, I.K.M. Reiss, R.P.M. Steegers-Theunissen.
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S*ubmitted for publication*

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	Prof.dr. I.K.M. Reiss
Co-promotoren:	Dr. J. Dudink
	Dr. I.A.L. Groenenberg

PHD TRAINING	**YEAR**	**ECTS**
General courses		
e-BROK course (NFU BROK Academy)	2016	1.0
Integrity in Science (Erasmus MC)	2014	0.3
Repeated Measurements in Clinical Studies (NIHES)	2014	1.4
Biostatistical Methods II: Classical Regression Models (NIHES)	2013	4.3
Biostatistical Methods I: Basic principles (NIHES)	2013	5.7
Systematic literature search in Pubmed (Erasmus MC)	2013	0.3
Attended, seminars, conferences and workshops		
Hospital management, Academie voor Medisch Specialisten	2016	0.6
8th DOHaD Congres. Singapore, Singapore	2013	1.0
Masterclass Fetal Anomaly Ultrasound Scan, Erasmus MC	2013	2.0
Annual Sophia Research Day	2013-2016	0.5
Erasmus MC PhD day	2013-2016	0.5
Annual Wladimiroff award Meeting, department of obstetrics and gynaecology	2013-2016	0.5
Three-monthly research meetings Rotterdamse Gynaecologen Opleidings Cluster (RCOG)	2013-2016	0.5
Weekly research meeting of the department of obstetrics and gynaecology	2013-2016	0.5

	YEAR	ECTS
Presentations at (inter)national conferences		
ISUOG 26th World Congress, Rome, Italy. Oral	2016	1.0
Sophia Research Day, Rotterdam, the Netherlands. Oral	2016	1.0
7th Dutch Neonatal Fellow Meeting, Leiden, the Netherlands. Oral	2016	1.0
3rd European Congress on Periconception Health and Care, Uppsala, Sweden. Poster	2016	1.0
9th DOHaD Congress, Cape Town, South Africa. Two orals	2015	2.0
ISUOG 25th World Congress, Montreal, Canada. Oral	2015	1.0
ISUOG 24th World Congress, Barcelona, Spain. Oral Nomination Young investigator Award	2014	1.0
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ABOUT THE AUTHOR

At 01:15 on April the 3rd 1986, Irene Victoria Koning was born in Amstelveen the Netherlands. She weighed 3875 grams. The youngest daughter of Gerrit Jan Koning and Francisca Pauline van den Berg van Saparoea completed the family of five.

During primary and secondary school her family moved from Amstelveen to Rotterdam to Eindhoven. She was a creative child who enjoyed dancing and singing. From the age of three until twenty she danced semi-professionally with the Royal Academy of Dance training courses. She obtained her VWO diploma in 2003 at the Lorentz Casimir Lyceum in Eindhoven. Thereafter, she studied 'Dance Studies' at Roehampton University in Surrey, London, United Kingdom for a year.

Although she did not see herself as a future medical doctor, she started studying medicine at the Erasmus University Rotterdam, the Netherlands. During her studies she looked beyond the borders of our national health care system, which took her on a trip to Zimbabwe where she studied prevention of mother to child transmission of the HIV virus. She started working at the department of radiology where her love for imaging techniques became evident. After an active student life and obtaining experience in several committees, she started her internships. This took her abroad once more for an internship at the Academic Hospital Paramaribo, Suriname. Although she did not start of her studies to become a medical doctor, she graduated as one on the 13th April 2012.

Over the years her enthusiasm for obstetrics and gynaecology expanded. For a year she worked as a resident (ANIOS) at the gynaecology department of the Albert Schweitzer Hospital in Dordrecht. Exactly after one year, Irene was appointed as PhD student on the DREAM Study. She conducted the study under the guidance of Prof.dr. R.P.M. Steegers-Theunissen, Prof.dr. I.K.M. Reiss, Dr. J. Dudink and Dr. I.A.L. Groenenberg. She obtained her Standard Ultrasound Examination (SEO) certificate and independently performed over 600 three-dimensional ultrasounds for her research project.

This work resulted in her PhD thesis: '*Early human brain development: The impact of periconceptional maternal and fetal factors*'.

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A DREAM DOES NOT BECOME REALITY THROUGH MAGIC;
IT TAKES SWEAT, DETERMINATION AND HARD WORK – COLIN POWELL

236 droomkindjes
608 uren in de echokamer
21283 echobeelden
74 besprekingen
7 manuscripten
1 proefschrift.

Soms was het een pittige wetenschappelijke discussie, een slechte grap, een woord van vertrouwen of gewoon een kop koffie die er voor gezorgd heeft dat mijn proefschrift nu uiteindelijk 'omkaft' hier voor me ligt. Grote dank gaat uit naar alle lieve moeders en vaders uit de DREAM studie; zonder hen was er niemand om te echoën en zou mijn proefschrift geen bestaansrecht kennen. Natuurlijk zijn er een aantal mensen die ik in het bijzonder wil bedanken.

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