THE NEONATAL BRAIN

early connectome development and childhood cognition

Kristin Keunen

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HET NEONATALE BREIN
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(met een samenvatting in het Nederlands)

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To my dad, who managed to make me feel
his love every day

even though he never got the chance
to know me

Never give in.
Never give in.
Never, never, never,
never - in nothing,
great or small, large or
petty. Never give in,
except to convictions of
honour and good
sense. Never yield to
force. Never yield
to the apparently
overwhelming might
of the enemy.

Winston S. Churchill

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CHAPTER 1

General introduction

BACKGROUND

The human brain is a puzzling system that has fascinated neuroscientists and neurologists since time immemorial. This intricate organ of neurons interconnected by dendrites and axons that synapse onto each other and are supported by glia cells emerges during pregnancy. By the time of normal birth, the brain's layout verges on the adult human brain. All major structures have come into place, including white matter pathways, and the cortical mantle has started to exhibit secondary and early tertiary folds. The rapid and complex nature of the neurobiological processes underlying brain development renders them at particular risk of developmental adversity. One such risk is extremely preterm birth, defined as gestational age <28 weeks.

Advances in neuroimaging techniques have enabled researchers to study early brain development exhaustively and have fuelled the field of connectomics. Connectomics refers to research effort into depicting and studying the elements (e.g., neurons, Brodmann areas) and connections (e.g., axons and dendrites, major white matter fiber bundles) of a neural system, the connectome. The human connectome is most accessible at the macroscale level. Hence, when applied to the human brain, the connectome typically encompasses the large-scale wiring diagram of brain regions and their interconnections (e.g., white matter pathways or correlations of functional time-series). Human connectomics may also comprehend micro- and mesoscale neural circuits, yet these investigations are beyond the scope of the present work.

Recent years have witnessed a rapid surge of interest in connectomics to study early brain development. In this thesis, we contribute to this literature, advancing our understanding of early macroscale brain network development and how this process relates to brain function. The aim of this thesis is binary. First, we aim to unravel early developmental processes of brain network formation. Secondly, we aim to investigate brain-behavior relationships following preterm birth. To this end, we relate metrics of brain network organization in the neonatal brain to childhood cognitive performance in preterm born children. Next, we widen the scope of neuroimaging markers to brain volumes and cortical parameters and relate these parameters to neurodevelopmental outcome in late infancy and early childhood.

In what follows, we will provide a structured summary of embryonic and fetal brain development in order to contextualize the studies presented in this thesis. Subsequently, we will preface connectomics to study early brain development, followed by a generic background on developmental implications of preterm birth. Finally, the outline of this thesis is presented, introducing its chapters.

We should be taught not to wait for inspiration to start a thing.
Action always generates inspiration. Inspiration seldom generates action.
- Frank Tibolt -

Embryonic through neonatal brain development

The human brain is a vastly complex system that enables us to walk and talk, perceive the world surrounding us, empathize and interact, be creative, think, and play. As such, our brain defines who we are. This elaborate structure is largely formed and shaped during human gestation, which typically spans only 280 days. During this timeframe, the vast majority of the brain's estimated 86 billion neurons as well as their ~10^{15} synaptic connections are formed and the brain's overall layout transforms from a seemingly simple cylindrical structure into the biological masterpiece that has intrigued scientists for hundreds of years [1-3].

Embryonic brain development and corticogenesis

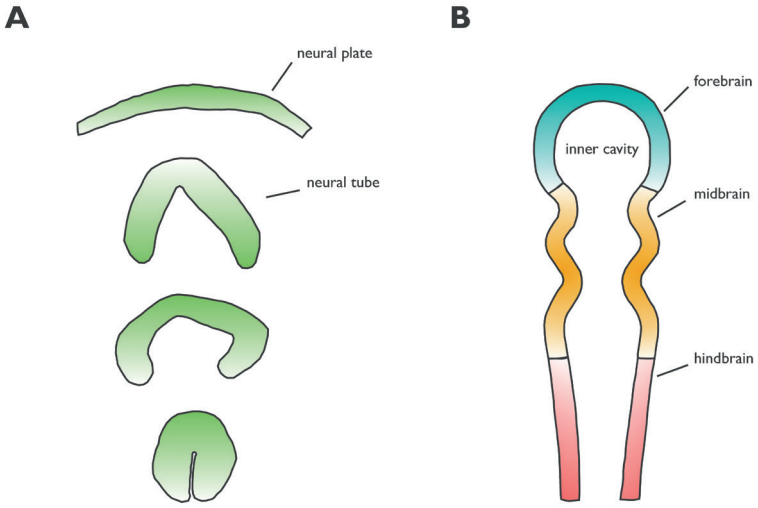
The brain's emergence originates in the embryonic process of *gastrulation*, reflecting the movement of undifferentiated embryonic cells to specific positions and which results in remodeling of the embryo into a three-layered structure. The anterior part of this structure progresses into the *endoderm*, the middle portion continues into the *mesoderm*, and the posterior layer becomes the *ectoderm*. It is the latter layer that will eventually generate the central nervous system, including the brain [2].

At around postconceptional day 21-27 the ectoderm moves inward, forming the neural tube [2-4] (Figure 1a). Closure of the neural tube occurs in an anterior to posterior fashion, which is beautifully illustrated in the review by Darnell & Gilbert [2]. Before neural tube formation is completed, the anterior portion undergoes exuberant changes and balloons into three key vesicles: 1. the forebrain that propagates the cerebral hemispheres, thalamus, hypothalamus and retina; 2. the midbrain which differentiates into the brainstem including the tectum and the motor pathways of the

basal ganglia and 3. the hindbrain which will bring about the cerebellum, pons and medulla oblongata (Figure 1b). Once the neural tube has emerged, neural stem cells start to proliferate and differentiate, deriving neurons and supporting glia cells [2,3,5]. Neural stem cells are located at the inner surface of the neural tube, lining its inner cavity, which will later constitute the cerebral ventricles. This layer is referred to as the ventricular zone [2,3].

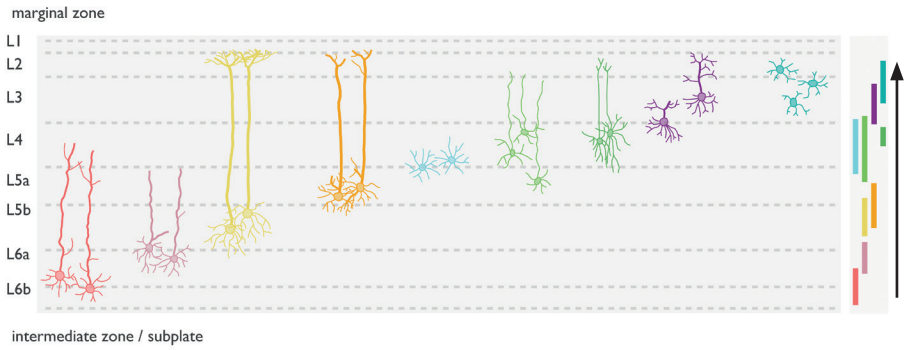
Once neurons are formed, they migrate to their designated location following an inside-out sequence: earliest-born neurons become cortical layer six adjacent to what is destined to become the cerebral white matter, while neurons originating last migrate to the outer border of the cortical plate and form layer two, passing their older peers in the developing cortex. Accordingly, the six layers of the human cerebral cortex come into existence (Figure 2) [3,6].

Figure 1 Neurulation



Panel A schematically captures the process of neural tube formation between days 20-28 of embryonic development. Choreographed by a complex interplay of signaling factors, the neural plate folds inward, transforming itself into the neural tube. Panel B illustrates the ontogeny of cerebral vesicles, which derive from the anterior portion of the neural tube at around embryonic days 22-23 [2]. Hence, primary vesicles emerge before closure of the neural tube is completed.

Figure 2 Neuronal layers of the developing cortex



Graphical depiction of the six major cortical layers and their neurons. When neurons have originated from neural stem cells, they migrate through the lower layers; adjacent to the cerebral structure bound to become white matter (intermediate zone), to their age-appropriate peers to form a new layer approximating the marginal zone. Figure is based on [2,94].

Fetal brain development

As soon as neurons have reached their destination in the cortical plate, they start projecting axons and apical dendrites, which sets the stage for neural circuit formation [3,6]. In **chapter two** of this thesis we will provide a generic overview of embryonic and fetal brain development in light of functional brain network formation. Axons have been identified from 8-9 gestational weeks [3,7]. Early human development has now entered the fetal stage and the brain's precursor further proliferates to encompass six distinct zones, including the ventricular zone, subventricular zone, intermediate zone, subplate, cortical plate and marginal zone in an inside-out manner [3]. While the cortical plate ultimately matures into the cerebral cortex, the intermediate zone is progressively invaded by developing axons and will eventually transform into the cerebral white matter [3].

Post mortem studies have detected axonal fiber bundles from as early as 13 postconceptional weeks and described the sequence through which their pathways proceed [8-10]. Limbic fibers have been noted to emerge first, followed by thalamocortical projection fibers and commissural fibers interconnecting both cerebral hemispheres. Intra-cortical association fibers derive last and continue to be formed in the first postnatal weeks [6,11,12]. A graphical depiction of developmental trajectories of major white matter pathways is provided in **chapter two**.

Cortical development

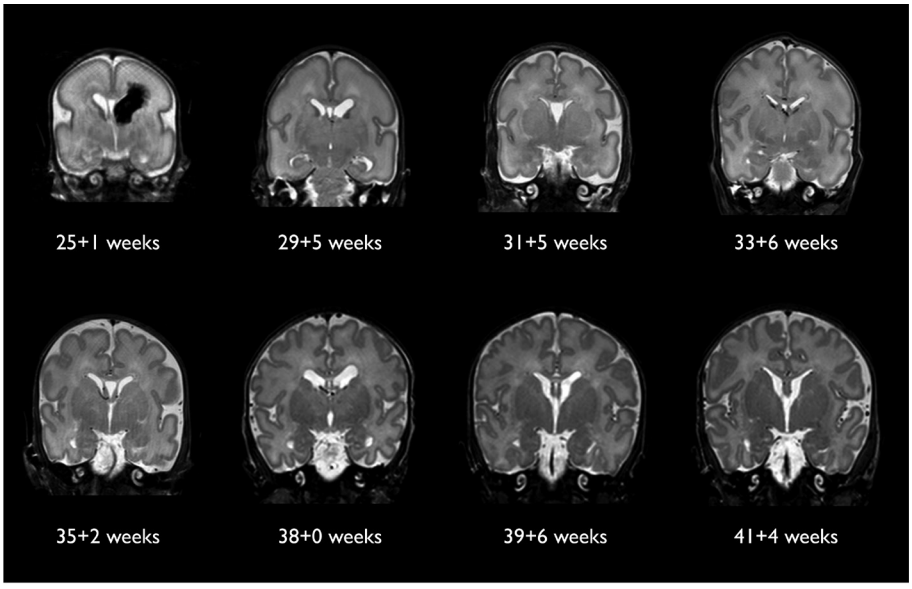
Development of the fetal cortex *in vivo* has been investigated from as early as 20 weeks of gestation and has been noted to follow a quadratic expansion curve with the peak growth rate at around 30 weeks of gestation [13-15]. Gyrification arises in the second trimester of pregnancy, transforming the brain's smooth surface into a complex and highly convoluted structure that approximates the morphology of the adult human brain by the time of normal birth (Figure 3) [16,17].

Many premises of cortical folding have been proposed and although the exact mechanisms underlying this complex phenomenon remain largely elusive, the potential paradigm has recently been captured in an umbrella framework of radial intercalation of neurons and subsequent tangential expansion of the cortical plate [18]. In this theorem, radial migration of young neurons into the cell-dense outer layer of the cortical plate is postulated to lead to its tangential expansion and because the expansion of the cortical mantle - which is intricately attached to its underlying structures - outpaces the extension of these deeper layers, the cortex folds [18,19]. As such, cortical surface area and related gyrification increase vigorously, while cortical thickness remains relatively constant. Several factors are thought to influence the process of cortical folding, including differences in neural proliferation rate, migration distance and axonal connectivity [18].

Cerebellar development

While the cerebral wall is developing rapidly during human gestation, the cerebellum undergoes even more dramatic development that outpaces all other developmental processes in the prenatal brain [20]. Cerebellar development is mounted by the formation of the primordia and pontine structures from the rostral hindbrain in the first month of pregnancy. Next, crucial events include the formation of two proliferative zones, the dorsomedial ventricular zone, which gives rise to interneurons of the deep cerebellar nuclei and to Purkinje cells, and the dorsolateral ventricular zone, which will propagate granular cells and projection neurons of the deep nuclei. The prior neurons constitute the most prominent neuronal cell population in the human brain: granular cells account for 95% of neurons in the cerebellum and exceed four times the number of neurons in the cerebral cortex [20]. The rapid increase in granular cell count causes the cerebellar surface to expand tangentially and foliate. Between 24 and 40 weeks postconceptional age, the cerebellum increases five-fold in volume and shows an astonishing 30-fold expansion in surface area.

Figure 3 Brain growth and cortical development between 25-41 PCW



Coronal T2-weighted images portray the dramatic expansion of cortical surface area between 25 postconceptional weeks and term age, resulting in the emergence of primary and subsequent secondary and tertiary complex folds. The image in the left upper panel shows a periventricular hemorrhagic infarction on the left. Between 29-41 weeks postconceptional age the brain growths substantially, causing it to increase its size 2.9 times (based on data from **chapter three**).

Given the velocity and complexity of cerebellar development during the prenatal period, this structure is particularly vulnerable to early developmental insults. Such insults may have a protracted impact on subsequent neurodevelopment [21-23]. Consequently, when investigating associations between brain metrics and neurodevelopmental outcome - as will be performed in part two of this thesis - it is important to take putative effects of cerebellar injury into account.

Connectomics to study brain development

In the footsteps of the genome, the term 'connectome' was introduced in 2005, describing a comprehensive map of the elements and connections of a neural system [24,25]. Foreshadowing major advances in cognitive and computational neuroscience to

unravel the mysteries of brain structure and function, several approaches to assemble the human connectome were put forward [24]. Such approaches include multiple levels of detail and organization (micro-, meso-, and macroscale), of which the macroscopic level is most intuitive in the human brain because it is most accessible. Conversely, mapping all estimated 86 billion neurons and their approximated 10^{15} connections at the microscale is not only computationally strenuous, it may also be redundant. Reasons for the latter include the following. Firstly, the human connectome is a scale-free network. Neurons operate in orchestras and therefore it is unlikely that single-cell or single-synapse alterations would exert macroscopic effects [24,26,27]. Secondly, neuronal connections are highly plastic and evermore modified through alterations in synaptic weights, dendritic spines and synaptic boutons [24,28]. As such, the study of mesoscale connectome wiring is slightly more innate. Neurons aggregate forming subpopulations of 80-100 neurons that span all cortical layers and are connected to each other through axonal projections [24,26]. At present, ongoing research efforts are compiling the mesoscale mouse connectome, Drosophila nervous system and zebrafish connectome [27,29-31]. The human brain network is most accessible at the macroscopic level and the years that followed the proposal of connectomics to study brain structure and function have witnessed an exponentially expanding literature of human connectome studies. Such studies have revealed that integrated information flow across the brain network is fundamental for healthy brain function and that connectome organization is related to intelligence, creativity, working memory, personality traits, and executive functions [32-38].

Several techniques can be used to study the macroscale human connectome and the most commonly employed modalities include diffusion weighted imaging (DWI) and resting-state functional MRI (rs-fMRI). Diffusion weighted imaging makes use of the principle of Brownian motion of water as proposed in one of the seminal papers by Albert Einstein published in 1905 [39]. Brownian motion describes that particles - and water itself - move randomly when suspended in water because of the kinetic energy of water molecules. When applied to DWI in the human brain, water will move freely in cerebrospinal fluid, yet will be mitigated in highly organized structures such as the white matter. In white matter fiber bundles, water can diffuse relatively easily in the direction parallel to the fibers, while diffusion perpendicular to the fibers is hindered [40,41]. Extending these measurements to all voxels in the brain, one is able to obtain *in vivo* estimates of macroscopic wiring of the brain's white matter [24]. In turn, rs-fMRI derives from spontaneous fluctuations in blood oxygen level dependent (BOLD) contrast over time [42]. When brain regions are active, their regional blood flow increases, which leads to a local increase in oxygenated hemoglobin levels. Consequently, the

ratio between oxygenated and deoxygenated hemoglobin changes. Since the latter two components both have different magnetic susceptibility, BOLD contrast changes. As such, BOLD is an indirect measure of local (voxel-wise) brain activity. When measured across the entire brain over time, brain regions are considered functionally connected when their fluctuations in BOLD signal are temporally correlated above a certain threshold [42,43].

In order to reconstruct a brain network, one needs estimates of the macroscopic connections within the brain - as described in the previous section - as well as a definition of the brain's elements (i.e., brain regions). To that end, several parcellation schemes have been adopted including atlases specifically designed for the neonatal brain [44,45]. In **chapter two**, we will provide an overview of different parcellation templates used in neonatal connectome studies and touch on challenges and difficulties of neonatal neuroimaging data acquisition to study connectomics. These topics will be further elaborated on in **chapter seven**.

After acquisition of neuroimaging data to obtain estimates of macroscale brain network wiring, data are processed and elements (i.e., brain regions) and their connections (i.e., white matter pathways or temporal correlations) are defined. Next, this complex collection of data can be decomposed into a 'connectivity matrix', derived from a graph that mathematically describes brain regions as nodes and their structural or functional connections as edges. The topology of this brain network can be investigated by means of *graph theory*. The adoption of graph theory is not unique to connectome studies and is widely applied across other complex systems, including online social networks (e.g., Facebook and Twitter), aviation and genomics [46-49]. Research efforts in the field of connectomics have revealed a set of topological attributes in the human brain that are universal across species [29-50]. These features can essentially be condensed into a single paramount principle of optimal tradeoff between network cost and adaptive capacity [51]. The human brain - as well as other complex systems - features high *global efficiency*, facilitating fast information transfer across spatially distributed brain regions in combination with high levels of local organization marked by *clustering* and *modularity* [52]. Clustering measures whether the neighbors of a node to which that node is connected are also connected to each other. Hence, it measures the tendency of nodes to form triangles or clusters [50]. Modularity describes to what degree the network is divided into subnetworks of nodes that are strongly connected to each other but have limited connections to nodes outside their subnetwork [50,52]. As such, clustering and modularity provide information about network segregation.

An illustrated **glossary** of network metrics that are commonly employed across this thesis is provided at the end of this chapter. The distinct combination of high levels of integration, facilitating global network communication and segregation, favoring minimal wiring cost and localized information processing, constitute a so-called *small world* topology. In parallel with other biological systems, the human brain is a small world network [53]. Furthermore, the human connectome has a heavy-tailed degree distribution, in which a small number of brain regions exhibit a disproportionately large number of connections. These brain regions are referred to as *hubs* [51,52,54]. Being connected to many other brain regions, hubs play a key role in the network's communication capacity. One can compare brain hubs to major transport gateways including London Heathrow Airport, Paris Gare du Nord, and Port Rotterdam. Such comparisons easily draw attention to the Achilles heel of brain network organization: hubs have high metabolic demands [51,55,56] and akin the profoundly disruptive effects of signal failure at Utrecht Central Station to the Dutch railway network, brain disease or developmental disorders affecting hub regions may substantially deteriorate global brain functioning [57-61]. Indeed, brain disorders, including schizophrenia and Alzheimer's disease have been noted to primarily target hub regions and degrade the integrative capacity of the brain network [57]. Finally, brain hubs feature another pivotal characteristic. Next to having large numbers of connections, neural hubs are considered strongly connected to each other. Hub regions display a larger number of interconnections than one would expect based on their degree alone. This set of hub regions exhibiting higher than chance-level interconnectedness is referred to as the *rich club* [54,62].

In summary, the human brain - like other biological systems - negotiates between wiring cost and efficiency, resulting in small world topology, which balances out efficient long-range communication and high levels of local organization. Other key features of such an optimal trade-off include a heavy-tailed degree distribution with hub regions that are more strongly connected to each other than one would expect from their number of connections and therefore display a rich club organization. While these key features had been identified and confirmed across species, one of the outstanding questions that remained was *when* these attributes emerge during brain development. Answers were provided by neonatal neuroimaging studies published in the first five years of this decade [63-69]. These reports demonstrated that the neonatal brain network exhibits substantial overlap with adult connectome organization and that hallmark features including a rich club and small world topology can be detected as early as 30 weeks postconceptional age, soon after connectome genesis and in the earliest phases that allow *in vivo* neuroimaging [63,64,69]. In **chapter two** we provide

a comprehensive overview of both structural and functional network development in the fetal and neonatal brain, including a collation of graph theoretical analyses in the context of early brain network formation. In **chapter three** we zoom in on early macroscopic connectome development in the neonatal brain between 29-45 weeks postconceptional age. Employing DWI and graph theory, we map trajectories of early postnatal brain network development in 44 typically developing preterm and full-term born infants.

The notion that healthy brain function is founded on efficacious brain wiring alleges that charting early connectome development is of cardinal importance for our understanding of healthy brain development. Furthermore, this concept puts the research question forward how early connectome organization relates to brain function including intelligence, memory and social skills. Contributing to a dawning knowledge about brain-behavior correlates of early connectome wiring, we relate structural brain network organization in the neonatal brain to cognitive functioning measured at early school age in a cohort of 30 preterm born children in **chapter four**.

Ultimately, gaining insight into early trajectories of connectome development is crucial to pinpoint deviances thereof. Prenatal brain development lays the foundation for brain structure and function throughout the lifespan and if (epi-)genetic changes and/or environmental influences lead to disruptions in the brain's blueprint, their effects are potentially protracted [70-73]. A number of putatively perturbing factors that are under active study include maternal schizophrenia, intrauterine substance exposure, prenatal malnutrition, congenital heart disease and preterm birth [72-79]. The latter factor is comprehensively studied in this thesis and although the other factors are as relevant in terms of developmental risk, we will only elaborate on preterm birth in the remainder of the introduction.

Preterm birth

Preterm birth, defined as a gestational age <37 weeks occurs in approximately 8% of births in the Netherlands [80]. The total costs of prematurity include 154 million euro, accounting for 0.2% of the national healthcare spending [81]. The majority of this expenditure is on very preterm birth (gestational age <32 weeks and/or birth weight <1500 grams), which is associated with significant morbidity. The developing brain, however, is at most substantial risk if the infant is born between 24-28 weeks gestational age, at the verge of the third trimester of pregnancy. Extremely preterm birth is a major risk factor for neurodevelopmental deficits that persist throughout the lifespan.

At present, these impairments predominantly constitute attention and socialization deficits, behavioral problems, working memory impairments and impaired cognitive functioning [82]. An accumulating literature has demonstrated that preterm born children and adolescents are at increased risk to go on and develop psychiatric disorders, including depression, attention deficit hyperactivity disorder (ADHD), autism spectrum disorder, and psychosis [83-85]. Collectively, developmental adversity following preterm birth places a major burden on society and healthcare spending, as special needs- and remedial education, rehabilitation therapy and consumption of healthcare are frequently required.

The major contributors to developmental sequelae of prematurity constitute brain injury, including white matter injury, intraventricular hemorrhage and cerebellar injury. A detailed description of these pathologies falls beyond the scope of this introduction and we refer the reader to the seminal paper by Joseph Volpe for an in-depth discussion on preterm white matter disease [82]. Here, intraventricular hemorrhage is also touched on, while cerebellar injury is elaborated on in Volpe's 2009 paper on cerebellar development [20]. Whereas preterm brain injury accounts for a substantial proportion of lifetime developmental deficits, an ever-growing number of preterm born children exhibits developmental impairments without macroscopic brain pathology [82].

Neurodevelopmental outcome prognostication following preterm birth has been one of the major challenges in neonatology for the past 15 years. Enhanced image resolution and the widespread application of MRI in the preterm population have enabled researchers to identify imaging markers for neurodevelopmental deficits, predominantly on cognitive and motor domains. Such markers include brain volumes, measures of cortical maturation, brain injury scores and diffusion metrics of white matter maturation, including fractional anisotropy [86-91]. In this thesis, we add to this literature by demonstrating a relationship between volumes of the ventricles, cortical gray matter, white matter and cerebellum in the preterm brain at term-equivalent age and neurodevelopmental outcome in late infancy and early childhood (**chapter five**). Additionally, we focus on the association between white matter fractional anisotropy and related network metrics and cognitive functioning at age 5.5 years in **chapter four**.

The significant problems we have cannot be solved at the same level of thinking with which we created them.
- Albert Einstein -

Outline of the thesis

The main objective of this thesis is twofold. Firstly, we aim to unravel early developmental processes of connectome wiring. In part I, we present findings of a narrative review collating results from structural and functional neuroimaging studies as well as electroencephalography reports on brain network formation in the fetal and early postnatal brain (**chapter two**). Here, we set out to investigate the trajectories of healthy functional brain network development and how these courses are affected in high risk or disease states. Focusing on the emergence of functional brain networks in **chapter two**, we shift gears in **chapter three**, where we concentrate on structural connectome development between 29-45 postconceptional weeks. Following the 2014 paper on the neonatal connectome during preterm brain development [63], we consolidate the developmental period that corresponds with the third trimester of normal pregnancy and neonatal period in typically developing infants, further elaborating on developmental trajectories of key network attributes and specific white matter pathways. We explore the hypotheses that primary and higher-order connections follow divergent maturational pathways, that diffusion properties delineate distinct features of neonatal white matter development and that early white matter maturation results in alterations in brain network topology that will facilitate integration and reduce segregation.

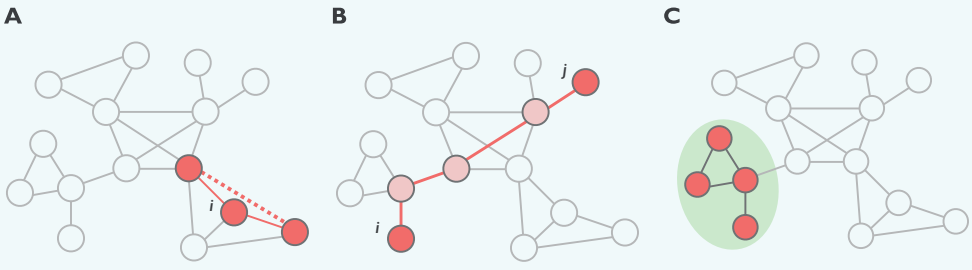
Part two revolves around the brain-behavior interplay in preterm infants. Here, we investigate the second objective of this thesis, which is to identify neuroimaging markers for neurodevelopmental outcome in preterm infants, with a particular focus on cognitive functioning since this domain has been notoriously difficult to predict [92,93]. In **chapter four** we study the relationship between neonatal structural white matter organization and cognitive functions at early school age in preterm born children. Similarly, we investigate the association between brain volumes measured at term-equivalent age and neurodevelopmental outcome at age two, 3.5 and 5.5 years in **chapter five**. In **chapter six** we further contemplate the preterm population, targeting differences and similarities in brain volumes and cortical maturation between preterm twins and singletons in an attempt to pinpoint the influence of the intrauterine environment on early brain development in preterm infants. In line with the brain-behavior interplay studied in this part of the thesis, cortical parameters and brain volumes are related to cognitive and motor performance in late infancy. Finally, **chapter seven** covers a general discussion of the chapters presented in this thesis and **chapter eight** amounts to a summary in Dutch.

GLOSSARY

The *clustering coefficient* is a metric of network segregation. It measures whether the neighbors of a given node *i* are connected to each other, which would result in triangles of nodes (panel A). If the clustering coefficient is measured in weighted networks (e.g., fractional anisotropy weighted networks), the *clustering coefficient* is defined as the average intensity over all possible triangles that node *i* is involved in. The intensity of a single triangle is taken as the geometric mean of the weights of its edges, with higher weights reflecting 'stronger' connections. If connections are absent, i.e. in case of incomplete triangles, their weight is considered to be zero. The *clustering coefficient* is typically normalized before analyses are performed. In this thesis, the normalized clustering coefficient is calculated as the ratio of the *clustering coefficient* of each subject to the average *clustering coefficient* of 1000 random networks per subject with the same number of connections and a similar degree distribution.

Global efficiency provides an estimate of the network's integrative capacity and is closely related to the average shortest *path length*. Shortest *path length* measures the number of connections that must be traversed when traveling from node *i* to node *j* (panel B). In weighted networks, nodes with higher weights reflect 'faster' connections. Hence, average shortest *path length* of weighted networks is defined as the sum of weights that need to be passed to travel from node *i* to node *j*, calculated for all pairs of nodes within the network. Similarly, *global efficiency* is computed as the harmonic mean of the inverse average shortest *path length* between all pairs of nodes. Normalized global efficiency is computed as the ratio of global efficiency to the average global efficiency of 1000 random networks while keeping the number of connections and degree distribution intact.

Modularity is another metric of network segregation. It measures to what degree the network is divided into subnetworks of nodes that are strongly connected to each other but have limited connections to nodes outside their subnetwork (panel C). In this thesis, modularity is computed using the Newman modularity score.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PART 1

Early connectome development

The greatest challenge to any thinker is stating the problem in a way that will allow a solution

Bertrand W. Russell

CHAPTER 2

The emergence of functional architecture during early brain development

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HIGHLIGHTS

- interhemispheric functional coupling has been noted in the fetal brain from 24 PCW
- the overall framework of mature brain wiring is established by the time of birth
- development of functional architecture follows a primary-to-higher order sequence
- prematurity disrupts long-range connectivity of primarily thalamocortical pathways
- prenatal substance exposure affects receptor regions and amygdala-frontal circuits

ABSTRACT

Early human brain development constitutes a sequence of intricate processes resulting in the ontogeny of functionally operative neural circuits. Developmental trajectories of early brain network formation are genetically programmed and can be modified by epigenetic and environmental influences. Such alterations may exert profound effects on neurodevelopment, potentially persisting throughout the lifespan. This review focuses on the critical period of fetal and early postnatal brain development. Here we collate findings from neuroimaging studies, with a particular focus on functional MRI research that interrogated early brain network development in both health and high-risk or disease states. First, we will provide an overview of the developmental processes that take place from the embryonic period through early infancy in order to contextualize brain network formation. Second, functional brain network development in the typically developing brain will be discussed. Third, we will touch on prenatal and perinatal risk factors that may interfere with the trajectories of functional brain wiring, including prenatal substance exposure, maternal mental illness and preterm birth. Collectively, studies have revealed the blueprint of adult human brain organization to be present in the neonatal brain. Distinct attributes of human brain architecture have even been detected in the developing fetal brain from as early as 24 postconceptional weeks. During postnatal brain development, the brain's wiring pattern is further sculpted and modulated to become the full facsimile of the adult human brain, with functional brain network refinement being more rigorous than structural brain network maturation. Advances in neuroimaging techniques have paved the way towards a comprehensive understanding of the maturational pathways of brain network development and of *how* early developmental adversity may affect these trajectories. Such insights are fundamental for our understanding of human brain functioning, for early identification of infants at risk, as well as for future neuroprotective strategies.

Keywords
functional MRI, brain networks, neonatal, fetal, connectivity

INTRODUCTION

The human brain is arguably the most complex system in biology and yet its macroscopic layout is nearly complete by the time of term birth. The neonatal cerebral cortex displays a complex, adult-like gyrification pattern and in the underlying white matter all large-scale connections are already in place [1-6]. Historically, much of what we know about the intricate processes of early brain development came from post mortem studies in human fetuses, neonates, and non-human primates [7-11]. With the increasing availability of high-quality neuroimaging techniques, including anatomical sequences customized to the developing neonatal and fetal brain, diffusion weighted imaging (DWI) and functional MRI, as well as electrophysiology recordings including electroencephalography (EEG), it has now become feasible to study early human brain development in unprecedented detail *in vivo* [4,5,12-19]. These advances have led to exciting new insights into both healthy and atypical macroscale brain network development and have paved the way to bridge the gap between the brain's neurobiological architecture and its behavioral repertoire. Such cross-correlation studies are invaluable for improving our understanding of *how* and *when* neural circuit establishment supports cognitive function and increasingly complex behavior.

Given that many psychiatric and neurological disorders may have a neurodevelopmental origin, mapping the brain's anatomical and functional trajectories is crucial for early identification of altered development [20]. These disturbances in brain development may be genetically programmed, epigenetically mediated or environmentally influenced and early detection may provide a window of opportunity for preventive strategies.

The present review aims to consolidate recent findings from prenatal and neonatal brain network studies that have helped move the field forward, with a focus on the ontogeny of functional brain network architecture. Many techniques are currently available to study functional brain connectivity, including EEG, magnetoencephalography (MEG), functional near-infrared spectroscopy (NIRS), and task-related functional MRI [16,21,22] (see for review of the most commonly employed techniques [23]); however this review will primarily address resting state functional connectivity studies. Resting state functional MRI (rs-fMRI) employs spontaneous fluctuations in blood oxygen level dependent (BOLD) signal at low frequencies (0.01-0.1 Hz) across the entire brain at a millimeters' resolution. Brain regions are considered functionally connected if they display synchronous activity, i.e. if their temporal fluctuations in BOLD signal are highly correlated [24].

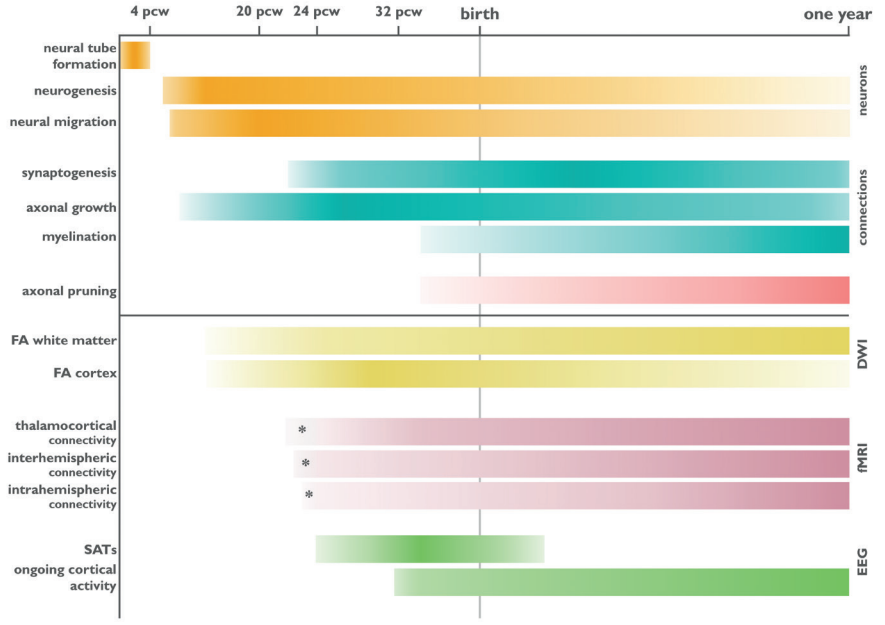
The first section of this review comprises a general overview of the major processes delineating prenatal and early postnatal brain development in order to provide a conceptual framework to build on. Developmental processes are compared against MRI and EEG correlates as illustrated in Figure 1. The second section represents a summary of functional connectivity studies examining healthy early development of functional brain network architecture. In the third section, we will discuss neuroimaging findings of deviating brain network development that occurs as a result of identifiable risk, including prenatal exposure to substances, maternal mental illness and preterm birth. Fourth, we will touch on important methodological considerations that specifically apply to imaging fetal and infant populations. Finally, we will present our conclusions based on the collated findings and discuss the remaining gaps that deserve attention for future research.

Developmental processes underlying early brain network formation

Embryonic and early fetal period

The human brain comprises 86 billion neurons and an even more staggering number of synapses, dendrites, axons and glia cells connecting and supporting them [25]. The vast majority of these cells are formed during prenatal development [26]. The brain originates from the anterior portion of the neural tube [27,28]. Following neurulation (i.e. closure of the neural tube), which starts at embryonic day 21 and is finished by day 27 [2,28], the precursor of the human brain rapidly expands and undergoes a series of processes including proliferation, neurogenesis and differentiation, orchestrated by an intricate genetic program [2,28,29]. By mid-gestation neurogenesis is largely complete [2] and during this phase, i.e. between the third and fifth month of gestation, neuronal migration peaks with the first neurons appearing in the cortical plate by 15 postconceptional weeks (PCW) [28,29] (Figure 1). Once neurons have reached their destination in the cortical plate, they start extending axons and dendrites [29]. Early afferent fibers appear from as early as 8-10 PCW forming the cerebral stalk at the level of the thalamus [30]. High field DWI studies have demonstrated the emergence of projection- and commissural fibers, including the internal capsule and corpus callosum from as early as 13 and 15 PCW respectively [3,31] (Figure 2).

Figure 1 Gantt chart of developmental processes and corresponding neuroimaging and electroencephalography findings from the embryonic phase until age one year



Schematic overview based on findings by [2,8,14,28,30,35,71,89,103,147-150]. Upper panel indicates developmental processes and lower panel portrays corresponding neuroimaging and electroencephalography findings. Intensity of color bars illustrates the course of developmental trajectories. Asterisks indicate that earlier prenatal imaging data are not available. Therefore, exact timing of emergence is as yet unclear. Axonal elimination (pinkish-red bar) is a predominantly postnatal process, yet axons in the subplate begin to diminish from 32-34 pcw. DWI = diffusion weighted imaging, EEG = electroencephalography, FA = fractional anisotropy, fMRI = resting state functional MRI, pcw = postconceptional weeks, SATs = spontaneous activity transients.

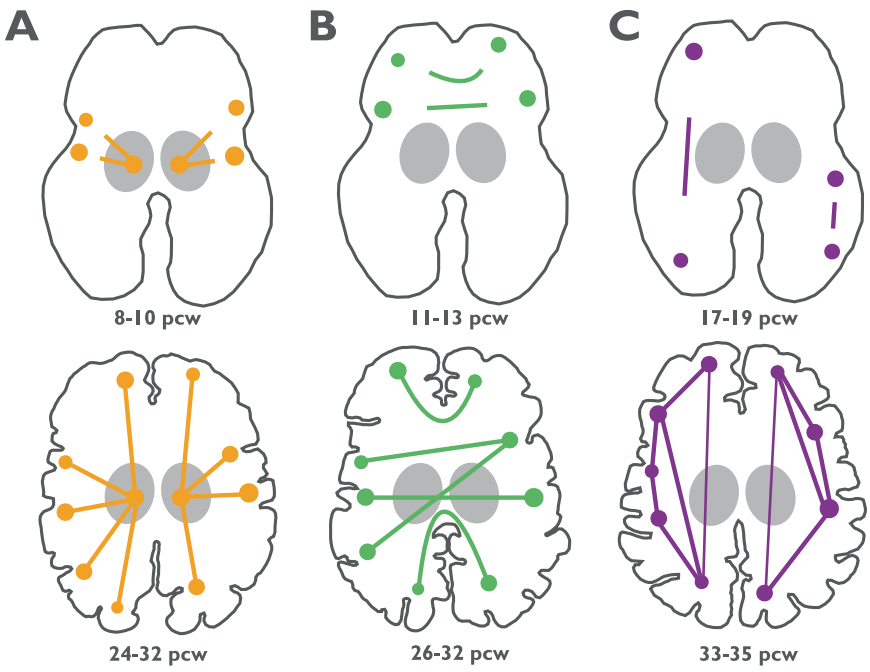
Midfetal period

The second trimester of pregnancy is characterized by exuberant synaptogenesis, dendritic sprouting and axonal path finding and thus by neural circuit formation, thereby setting the stage for functional communication [29]. Thalamocortical and cortico-thalamic fibers form transient circuits with subplate neurons before growing into the developing cortex and thalamus respectively [32,33]. The subplate is located below the cortical plate and serves as a 'waiting' compartment for afferent fibers. During the waiting period, which lasts approximately four weeks, afferents receive instructive input and start projecting onto their cortical targets. Concurrently, the corpus callosum extends in an anterior to posterior fashion [31,34], first synapsing on neurons in the transient subplate zone before establishing its definitive connections in the cortical plate [32,33]. Once thalamocortical connections are established, which has been noted to occur between 24-32 PCW (Figure 2), sensory stimuli including visual and auditory input can reach the developing cortex [32]. The formation of transient thalamocortical-subplate circuits has been linked to the emergence of *spontaneous activity transients* (SATs) on electroencephalography recordings in extremely preterm infants [32,35]. SATs are endogenous bursts of neuronal activity, which are either autonomously generated in the cortex, or in response to input from the subplate and are thought to drive neural circuit formation before sensory stimuli come online [35,36]. Conversely, sensory driven activity is thought to be reflected in *ongoing cortical activity*, which gradually increases with advancing postconceptional age and reaches fully continuous levels by 45-50 PCW [35,36] (Figure 1).

Late fetal period

In the third trimester thalamocortical connections are consolidated, while commissural and long-range association fibers including the corpus callosum, fronto-occipital fasciculus and inferior longitudinal fasciculus leave the subplate to extend into the cortical plate [32]. The notion that thalamocortical connectivity is established by the time of birth was recently illustrated in a rs-fMRI study, showing a high level of overlap between the distribution of thalamocortical projections in the thalamus in the neonatal brain and their topographical organization in the adult human brain [13]. While axons continue to grow, facilitating major connections in the white matter to find their cortical targets, the overlying cortex transforms its relatively smooth surface into a highly convoluted mantle with secondary and tertiary sulci that resembles the adult human brain [2,37]. MRI studies in preterm infants have reported a five-fold increase in cortical surface area and doubling of cortical curvature measures in the timeframe that coincides with the third trimester of pregnancy [38-41]. Concomitantly, a number of

Figure 2 Graphic representation of major white matter pathway development



Panel A. Projection fibers have been traced from 8-10 postconceptional weeks (PCW) and include the corticospinal tract that expands from the internal capsule. Thalamocortical projection fibers reach their destination in the developing cortex between 24-32 PCW. Panel B. Commissural fibers of the corpus callosum are detectable from 11-13 PCW and reach the cortex in the late second to early third trimester (26-32 PCW), closely following the developmental trajectory of thalamocortical projection fibers. Panel C. Association fibers appear at different stages. The initial formation of the inferior fronto-occipital peduncle and inferior longitudinal fasciculus occurs at around 17-19 PCW. Long-range association fibers do not undergo significant development until the third trimester of pregnancy (33-35 PCW), although the superior longitudinal fasciculus is not yet prominent at birth. Schematic overview based on findings by [3,30-32].

processes take place that extend well into the postnatal period, including myelination, synaptogenesis and the formation of dendrites and associated dendritic spines. Myelination emerges as a result of maturation of oligodendrocytes in the white matter and progresses in a caudal to rostral, central to peripheral, and posterior to anterior fashion with brain areas involved in primary functions myelinating before association areas [26,42-44]. Mature oligodendrocytes produce myelin, forming fatty sheaths around developing axons, thereby vigorously enhancing conduction speed. White matter maturation, including axonal growth, increasing axonal coherence and myelination can be clearly delineated using DWI [6,17,19,45-47].

Postnatal brain development

With all major white matter tracts being in place by the end of normal gestation, it is not surprising that structural connectome studies - examining whole brain connectivity on a macroscopic level (Box 1) - have revealed structural network organization in the neonatal brain to show great similarity to the adult human brain [4,5,48-52].

Key features of adult connectome architecture have been demonstrated in the neonatal brain, including a *small world* organization - combining local specialization with long-range efficiency -, a *modular* topology - meaning that the network constitutes smaller subnetworks for specialized information processing -, and a heavy tailed *degree* distribution with brain regions which show the highest number of connections forming a central core or *'rich club'* (Box 1). A number of these studies included preterm subjects and preterm birth is known to impact on brain development, with potentially detrimental effects on brain network architecture, which will be discussed in the third section of this review. However, alterations in the global framework of brain wiring have not been observed and are unlikely in the absence of major brain lesions [53]. The adverse effects of preterm birth have predominantly been noted to affect the quality of connections rather than their overall layout [4,13,54-58].

Whilst macroscale white matter connections have been established and long-range inter- and intrahemispheric projections are no longer formed in the early postnatal brain, short-range cortico-cortical connections continue to develop and have been traced through the fourth month after birth [59]. The postnatal period is further marked by refinement of existing intracortical connections, ongoing dendriting arborization and an explosive increase in synaptogenesis, eventually resulting in an abundance of connections [2,8,32,60,61]. Overproduction of synapses and associated dendrites and axons is observed across all mammalian species and is followed by pruning (i.e. selective

elimination of connections) [8,62]. The latter process spans childhood and adolescence and occurs as a result of competition for neurotrophic factors including brain-derived neurotropic factor (BDNF) and the need for afferent input to stabilize immature, labile connections [2,8,26,63].

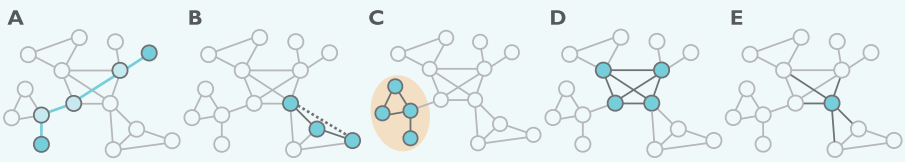
A number of DWI studies have evaluated postnatal structural brain network development [6,48,64,65] and demonstrated increasing integration and decreasing segregation (or *clustering*, Box 1) as hallmark features of childhood connectome maturation. Similarly, modules become increasingly interconnected and are further shaped, especially in the earliest postnatal years [6,64]. Yet, major reorganization of their configuration has not been observed [6]. Consistent with the persistence of the modular configuration in the developing postnatal brain, the localization of *hub nodes* (i.e. brain regions that take up a central position in the network) has been noted to remain largely stable [64,66].

Although present-day findings of neuroimaging *in vivo* cannot be directly translated to microscopic neural circuit development, not least because of the substantial gap in spatial scale on which macro- and microscale brain network development take place, there are considerable similarities in the biological principles that both systems seem to adhere to [5,67]. Myelination and increases in axonal diameter of white matter connections promote efficiency of axonal information transfer and may therefore be reflected in increasing integration capacity of the macroscale connectome. Decreasing clustering may be the macroscopic representation of pruning, leading to modulation of the network from a relatively 'random' topology to a specialized organization, maintaining and stabilizing meaningful connections.

Box 1

Neural network formation in the developing human brain can be studied at different levels. On the microscale, pioneering work has been done to delineate neural circuit establishment in the cortex, subplate, thalamus and cerebellum [8,147,151]. On a macroscopic level, an increasing literature converges upon the principles of whole-brain network development: the connectome. Structural brain wiring can be investigated using diffusion weighted imaging techniques. Connections reflect white matter pathways consisting of axonal fiber bundles and supporting glia cells (astrocytes, oligodendrocytes, microglia). Similarly, brain regions are considered functionally connected when their activation signals (e.g. blood oxygen-level dependent 'BOLD' signal or electroencephalography trace) follow a similar pattern over time and are temporally correlated. When these structural and functional connections are charted for all brain regions, such reconstructions result in a connectome map. Next, the topological features of the brain's connectivity matrix (connectome map) can be examined (see Rubinov & Sporns [152] for a comprehensive review of network measures). Here we will discuss a number of key attributes of the human connectome that have been studied in the early developing brain. Complementary illustrations are provided in the toy network below.

Similar to the adult connectome, the neonatal brain has repeatedly been found to display short characteristic path length, which is computed as the average number of edges that need to be traversed to travel between nodes of the network (panel A). Concomitantly, newborn infants exhibit a high level of clustering. Clustering reflects the tendency of the neighbors of a node to form connections, resulting in triangles of interconnected nodes (panel B). Together, short characteristic path length and high clustering compose 'small worldness', a hallmark of human brain organization that is already present during the earliest phases of brain development, as soon as paramount connections have come into place. Modularity (panel C) measures the number of subnetworks ('modules') that the connectome encompasses. Functional modules identified during early development are spatially proximate, involving primary order brain regions. In the neonatal brain, clustering and modularity as metrics of segregation prevail, while levels of integration are relatively limited. Another important feature of the infant brain network is a rich club organization (panel D), meaning that its highest degree nodes (i.e. hub nodes (panel E)) are more strongly connected to each other than one would expect based on chance alone, in accordance with adult brain



organization. The rich club coefficient is computed based on comparison against the organization of random networks. The overall spatial layout of structural hub nodes in the neonatal connectome shows substantial overlap with the adult human brain, while the configuration of functional hubs is still immature. Functional brain hubs are essentially confined to primary brain regions (e.g. sensorimotor and primary visual cortex). Hence, postnatal trajectories of functional brain wiring are more profound than maturational courses of structural brain network development. Structural refinements revolve around improvement of communication efficiency and connection strength, resulting in shorter characteristic path length, decreasing radial and axial diffusivity and increasing fractional anisotropy. During postnatal development, functional brain architecture transforms its spatial arrangement with brain hubs shifting from predominantly primary order brain regions to higher-order association areas.

Functional network development

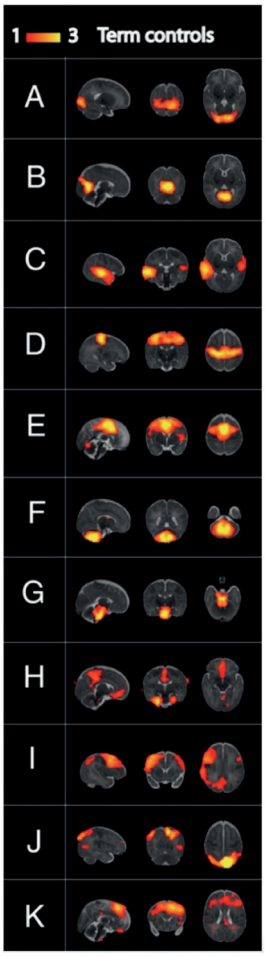
The past decade has witnessed a rapid surge of interest in rs-fMRI as an imaging technique to investigate functional connectivity in the earliest stages of human development. The first paper on the presence of functional brain networks in the neonatal brain was published in 2007 [12] and a number of research groups have now committed to the challenging task of functional imaging *in utero* [14,68-70]. Collectively, these efforts have led to exciting insights into functional brain network architecture in the earliest phases following its emergence. As such, these findings have furthered our understanding of functional network development in the typically developing brain and may prove invaluable for early identification of infants at risk of neurodevelopmental disorders. In what follows, we will collate findings from functional connectivity studies during healthy fetal and neonatal brain development.

Interhemispheric connectivity has been investigated in the fetal brain from as early as 24 PCW and has been noted to increase with advancing gestational age, following a medial to lateral trajectory [14]. Similarly, long-range thalamocortical and intrahemispheric connectivity are also strengthened with increasing fetal age (24-39 PCW) [71] (Figure 1). Fetal imaging is challenging due to motion of the fetus and surrounding tissues because of maternal respiration, requiring specific acquisition and analysis frameworks for this population [69,70]. Furthermore, image resolution and signal-to-noise ratio are frequently reduced compared to that acquired in postnatal fMRI. Collectively, these differences impact the comparability of fetal and infant studies and are therefore relevant for their interpretation, which will be further elaborated in the final section of this review.

A number of studies have focused on the emergence of resting-state networks in fetuses and newborn infants [12,56,68,71-78]. In healthy adults, a distinct set of resting-state networks has been described encompassing brain regions involved in primary functions (i.e. sensorimotor, auditory and visual processing network), and higher-order functions including self-awareness, memory, attention and executive functioning [79,80]. Networks engaged in complex cognitive functioning are spatially distributed across the cortex, typically spanning multiple brain regions. In contrast, resting state networks governing primary functions tend to be more localized with their functional connections mainly limited to homologous counterparts [79-82].

Comparable functional network architecture has been revealed in the neonatal brain, albeit in an immature state [56,58,72,75-78,83] (Figure 3). Primary networks can be clearly

Figure 3 Resting state networks in the neonatal brain



Resting state networks as detected in the neonatal brain employing probabilistic independent component analysis on resting-state functional MRI data. Color bar indicates Z-statistic of functional connectivity strength. Adapted from Doria *et al.* PNAS 2010 [58], with permission.

depicted in newborn infants and display a mature configuration whilst higher-order networks are largely fragmented [56,58,72,75,76]. These higher order networks are merely restricted to their core regions in the newborn brain, exhibiting relatively limited functional connections with spatially distant homologues. The default mode network for instance - that has been associated with self-awareness, future planning, mind

wandering and conceiving the perspectives of others ('theory of mind') in healthy adult brain functioning -, extends the posterior cingulate cortex, ventral and dorsal medial prefrontal cortex, inferior parietal lobule, lateral temporal cortex and the hippocampus regions in the adult human brain [81,84,85]. In neonates, the default mode network is immature and incomplete, encompassing frontal and association cortices including the medial prefrontal cortex and posterior cingulate cortex [56,58,76,78] (Figure 4). A fragmented precursor of the default mode network, comprising the medial prefrontal cortex and posterior cingulate cortex, was recently also identified in the developing fetal brain from 35 PCW [71].

In line with the primary-to-higher-order maturational sequence of functional networks, immature forms of the sensorimotor, visual and auditory network have been detected from an earlier gestational age, i.e. approximately 30 PCW in healthy fetuses (n=32) [71]. A recent study elegantly mapped the maturational trajectories of resting state networks in the first postnatal year at three-month intervals in 65 typically developing infants [76]. The sensorimotor network, visual processing network and auditory/language network demonstrated adult-like topology at birth and showed minimal topological changes during the first postnatal year. The dorsal attention network and default mode network followed the development of primary networks and became increasingly synchronized with spatially remote within-network brain regions, exhibiting mature topology at one year of age. Higher order cognitive networks, including the salience network and bilateral frontoparietal networks - involved in executive control, decision-making and working memory - were noted to mature latest and still displayed an incomplete configuration at the end of the first postnatal year [75,76].

Interestingly, not only does the maturational sequence of functional resting state networks mimic the developmental pattern of myelination and synaptogenesis, it also parallels the order in which behavioral functions are achieved. Developmental milestones in visual and sensorimotor function are most prominent in the first postnatal year, when their corresponding functional brain networks are complete [86,87]. Conversely, higher order cognitive functions such as executive control and social cognition develop well into adolescence and early adulthood [88]. Their associated brain networks become operational last.

Functional connectivity and behavioral counterparts

Although the developmental trajectories of resting state networks and neurodevelopmental milestones display remarkable similarities, cross-correlation

studies linking findings on functional networks to behavioral measures during early development are sparse [74,89]. A longitudinal study assessing functional brain network development and their cognitive correlates in 74 infants found functional connectivity between the thalamus and the immature salience network at age one year to be associated with working memory performance at age two [89].

Much of our present-day knowledge about brain-behavior relationships shortly after birth comes from task-based fMRI studies [21,73,90-100]. These studies have provided important background on the brain's responses to sensory input during the earliest phases of development of brain-behavior interactions. Adult-like activation patterns were observed in response to a variety of sensory stimuli, including tactile and proprioceptive stimulation (passive hand movement) [73,90,100], auditory [93], olfactory (the odor of infant formula) [21] and visual input. fMRI studies in two- to three-month-old infants demonstrated left-lateralized activation of perisylvian regions including the superior temporal gyrus, angular gyrus and Broca's area in response to native-language speech [91,99]. The response followed a hierarchical pattern, with auditory regions being activated first, followed by superior temporal regions and the temporal poles and Broca's area in the inferior frontal cortex; a pattern that is highly consistent with language organization in the mature brain. Excitingly, the infant brain seemed capable of distinguishing speech from music and exhibited signs of early learning. Music induced activation of bilateral auditory areas (posterior temporal regions), while speech showed a left-hemispheric preference. Furthermore, listening to the mother's voice resulted in differently modulated responses in both language and emotion areas than when infants were exposed to a stranger's voice [92]. Together, these findings point towards the early existence of functionally operable brain networks that are genetically dictated and sculpted by environmental input and learning. The observation of brain activity in language areas including Broca's area well before the onset of produced speech, further underscores the complex interplay between brain and behavior during development, which is continuously modified by environmental and (epi)genetic influences. Further studies are required to improve our understanding of these brain-behavior relationships.

Functional connectome

A number of reports described functional network architecture in neonates on a whole-brain level [101-103]. In line with network attributes of the neonatal structural connectome and adult human brain organization, functional brain networks in newborn infants exhibit *small-world* topology, a *modular* organization and a heavy-tailed *degree*

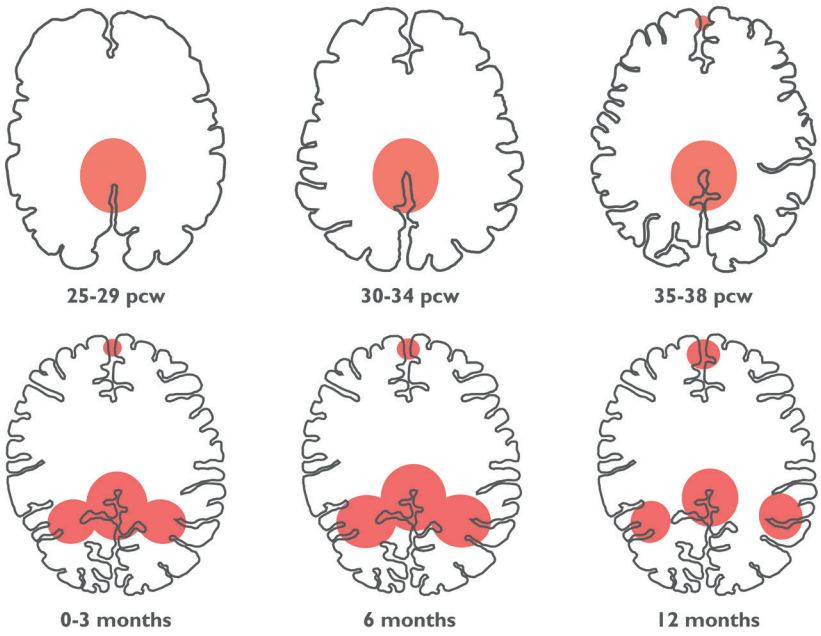
distribution with functional *hubs* (Box 1). A recent MRI study in the developing preterm brain observed substantial overlap between structural and functional brain network organization in 17 neonates [5]. Given the small sample size and that preterm infants are at considerable risk of neurodevelopmental deficits, further studies are required that extend these investigations to the healthy term-born infant brain. Differences between neonates and adults have also been noted. Functional hubs were found to be predominantly confined to brain regions supporting primary functions (e.g. sensorimotor and visual cortex) and only a few hubs have been observed in association cortices including the insula and posterior cingulate cortex [101-103]. Notably, the configuration of functional brain hubs as observed in the neonatal brain is highly consistent with the outline of resting state networks during this period.

Whole-brain network topology has also been examined in the prenatal period, with a focus on modularity. In a pioneering study including 33 pregnant women, fetal brain network architecture demonstrated a modular decomposition, which was more pronounced in younger fetuses (24-31 PCW) compared with older fetuses (31-39 PCW). These findings suggest functional brain wiring to become increasingly integrated during prenatal development [104].

Electrophysiological connectivity

Brain activity, which can be measured using EEG is considered crucial for consolidation of immature neural circuits and therefore plays a pivotal role in early brain network formation. A number of studies have explored functional connectivity patterns of early electrical brain activity as extracted from EEG recordings. The neonatal brain displayed a marked bimodal connectivity pattern, characterized by strong functional coupling of spatially remote brain regions during periods of high levels of brain activity (mostly reflecting SATs) and minimal functional synchrony during periods of relative quiescence (low amplitude bands). This bimodality is thought to govern early functional brain wiring and diminishes with advancing postnatal age, when young neurons mature and endogenous brain activity (SATs) is progressively replaced by sensory driven oscillations (*ongoing cortical activity*) [16]. Other prominent features of neonatal brain activity encompass distinct differences between sleep states, well before the onset of mature sleep-wake EEG representations and highly dynamic signature patterns of functional neural synchrony that mature rapidly during the first few postnatal weeks [105]. In summary, the rapid and complex ontogeny of structural brain wiring during the second and third trimester of human gestation facilitates functional neural circuit establishment. The emergence of long-range projection, commissural and association

Figure 4 Schematic illustration of default mode network development



Development of the default mode network in the prenatal period (top row) and the first postnatal year (bottom row) as revealed by resting state fMRI studies. Illustration based on [56,58,71,76]. Please note that preprocessing and region of interest (ROI) selection differed between studies (see section on Methodological considerations). The fetal imaging study by Thomason *et al.* [71] adopted the ROIs provided in Smyser *et al.* [56]. Doria *et al.* [58] also performed manual ROI placement and Gao *et al.* [76] employed adult BrainMap data to define seed-regions [80]. PCW = postconceptional weeks.

fibers initiates the onset of thalamocortical, as well as inter- and intrahemispheric functional connectivity. These early functional connections are consolidated by SATs, which are crucial for survival and maturation of neurons shortly after their genesis. In line with the notion that early electrical brain activity is fundamental for the establishment of operational neural circuits, a recent study in 21 preterm infants revealed early endogenous brain activity as measured during the first three postnatal days to be positively related to brain growth between 30 and 40 postmenstrual weeks. Strongest associations were demonstrated with deep gray matter structures [106].

Present-day neuroimaging studies have detected functional brain networks from as early as 26 PCW [69,71]. These networks follow a primary-to-higher order maturational sequence, consistent with their behavioral correlates. By the time of term birth, primary networks including the systems involved in visual, auditory and sensorimotor functioning are largely complete, while higher order networks display fragmented, immature connectivity patterns. The notion that the blueprint of functional brain network organization is present in the neonatal brain is emulated by whole-brain connectome findings, which have revealed adult-like network attributes including *small-world* organization, *modularity* and a *rich club* of high degree *hub* nodes, although this early connectome architecture is similarly immature. Crucial first steps have been taken toward improving our understanding of the cognitive and behavioral implications of early functional brain wiring. Significant caveats remain that deserve attention from the field. Moreover, longitudinal studies are required to answer important questions regarding developmental trajectories of healthy functional network formation.

Prenatal and perinatal risk factors affecting functional network development

Risk factors for altered functional network development include environmental and epigenetic risk, such as prenatal drug exposure, maternal mental illness and preterm birth. Although these conditions and risk factors are of different origin, their common denominator is that they exert effects in the earliest stages of human brain development, potentially permanently altering the developmental pathways of neural circuit formation. Behavioral sequelae are observed across myriad domains, including academic achievement, executive functioning, attention, conduct and social-emotional skills from childhood throughout the lifespan. Connectivity studies have begun to take on the challenge of mapping developmental trajectories in the earliest phases of human life, when the brain is most plastic and modifiable, and are paving the way towards an understanding of how prenatal and perinatal risk alter these trajectories. In this section, we will provide an overview of the growing literature on the impact of identifiable risk on the formation of functional brain organization and touch on the mechanisms underlying these effects.

Prenatal drug exposure

Prenatal exposure to both licit and illicit substances is a major public health concern. In the US, prevalence estimates of illegal drug use during pregnancy vary between 4.4 - 5.1% [107]. Self-reported legal substance use is even more prevalent, with 16.3% of pregnant women reporting cigarette smoking and 10.8% disclosing alcohol consumption [107]. Prenatal substance exposure may elicit teratogenic effects in the

embryonic stage. During fetal development, substances may affect the developing brain in a number of direct and indirect ways. Psychoactive drugs and nicotine target monoaminergic neurotransmitter systems, including dopamine, epinephrine and serotonin signaling, thereby modifying the intricate orchestration of neural circuit establishment. Other neural processes susceptible to substance exposure include neural proliferation and migration, axonal growth and dendritic branching. Fetal brain development may be indirectly affected by reduced oxygen and nutrient supply as a result of placental and/or umbilical vasoconstriction. Additionally, substance abuse is frequently associated with poor maternal health and nutrition, which may further compromise the developing fetus [108].

Until recently, the impact of maternal substance use on early brain wiring - i.e. before significant environmental influences come into play - remained largely unexplored. Two studies investigated the effects of prenatal exposure to cocaine and marijuana on functional connectivity in the neonatal brain using a well-controlled design [109-111]. The first study evaluated 45 cocaine-exposed neonates with or without in utero exposure to other substances, including marijuana, opioids, alcohol, nicotine and serotonin reuptake inhibitors (SSRIs), 43 newborn infants exposed to the latter substances without cocaine and 64 drug-naive control infants. Cocaine-specific alterations were observed in functional connectivity strength between the amygdala and medial prefrontal cortex and between the thalamus and anterior cortex regions [109,110]. The amygdala, insula and thalamus were preselected as regions of interest, because of their involvement in reward- and control systems, and because of high levels of dopamine expression in the thalamus. The second study was performed in a subset (n=63) of this sample, with a focus on marijuana. Marijuana exposure was associated with disrupted connectivity of the caudate and insula; brain structures that display high expression levels of the type 1 cannabinoid receptor [111]. Furthermore, diffuse patterns of both hyper- and hypoconnectivity were observed between the insula, amygdala and thalamus and widespread cortical areas including medial frontal cortices, sensorimotor regions and the medial visual cortex in neonates who were prenatally exposed to substances 109-11 [109-111].

Maternal mental illness

Maternal mental health disorders may affect the establishment of neural circuits in the developing brain owing to maternal genetic risk factors, alterations in the intrauterine environment (e.g. increased cortisol levels or exposure to disease related medication) and environmental risk including low socio-economic status, poor nutrition and prenatal

substance exposure. Confounding factors pose a particular challenge to disentangling hereditary susceptibility that may be conferred to the developing fetus *in utero* from environmental factors that may be versatile or that conversely may enhance risk.
Maternal depression (and related SSRI use) has been associated with disturbances in brain connectivity of newborn infants across a number of imaging modalities. A small rs-fMRI study in six-month old infants (n=24) reported stronger functional connectivity of the amygdala seed region with distributed brain areas of the limbic system and medial prefrontal cortex when their mothers had reported more severe depressive symptoms during the second trimester of pregnancy. Details on maternal psychoactive drug use that may have influenced the observed effects were not reported [112]. A recent DWI study described widespread reductions in white matter microstructural maturation of predominantly corticofugal and corticothalamic projection fibers in 20 newborn infants of depressed mothers who had used SSRIs during pregnancy compared to healthy matched control infants. These alterations were not observed in neonates born to depressed mothers who had not been exposed to SSRIs [113]. Complementing this literature, a recent study reported both local and global changes in EEG recordings of 22 neonates prenatally exposed to SSRIs because of maternal mood- or anxiety disorder. These alterations persisted beyond the acute withdrawal period and were noted to be independent of the underlying maternal mental condition. Abnormalities included lower interhemispheric synchronicity and shorter interburst intervals (i.e. periods of relative rest in brain activity) during quiet sleep and lower cross-frequency integration during active sleep, the latter indicating reduced coordination of oscillations from spatially linked neural networks (e.g. the subplate and cortex) [114]. Taken together, these findings suggest that SSRI use in pregnant women with mood disorders affects early brain wiring in the developing fetus, although evidence remains scarce and heterogeneous at present. The unique contribution of maternal depression to these perturbations remains poorly understood. Future research of longitudinal design is thus needed to determine whether maternal mood disorders and related drug therapy (SSRIs) have distinct neural substrates and to monitor if the observed deviances are persistent.

To our knowledge only one study has investigated the impact of maternal schizophrenia on neonatal brain network organization. The authors reported distributed changes in structural covariance networks of the cortex - based on synchronous variation in cortical morphology measures as derived from structural MRI - in 26 neonates born to mothers with schizophrenia or schizoaffective disorder, whereas the structural connectome of white matter connections was largely unaffected. Alterations in structural covariance

were related to the brain's overall communication capacity, including reduced global efficiency, longer connection distance and less hub nodes and edges in high-risk infants [50]. To date, the influence of maternal schizophrenia on early-life *functional* connectivity remains to be elucidated.

Preterm birth

Preterm birth occurs at a developmental time when crucial processes of neural circuit formation are taking place, including synaptogenesis, axonal growth and late neuronal migration [33]. Besides the event of preterm birth itself posing substantial risk to the establishment of healthy and fully operational brain network organization, prematurity is frequently accompanied by postnatal illness that may amplify the detrimental effects on early brain wiring. Not surprisingly, preterm birth is associated with impaired brain development reflected in reduced brain volumes, diminished cortical gyrification and delayed maturation of gray and white matter structures [33,115-118]. These deficits are also present in the absence of focal brain injury and have been noted to persist through childhood [119-121]. Behavioral sequelae of these structural disturbances in brain development are observed across a wide spectrum of neurodevelopmental outcomes and include cognitive delay, working memory impairment, learning disabilities, executive functioning deficits, internalizing and externalizing behavioral problems and developmental psychopathology including autism and attention deficit hyperactivity disorder [120-124]

Accumulating literature converges on pervasively disrupted thalamocortical connectivity as a result of preterm birth. Both structural [54,55,125] and functional deficits [13,56,58] have been reported. Disturbances in *structural* connectivity were revealed to be most widespread, involving thalamocortical connections distributed across frontal, temporal, occipital and parietal lobes [54,125]. Complementing these findings, reduced structural connectivity between the thalamus and extensive cortical regions as measured at term equivalent age was found to be related to poorer cognitive performance at age two years in 57 preterm born children [55]. The neurobehavioral link as observed in the latter study, has stressed the profoundly disruptive effects of preterm birth on brain wiring for the first time. Two studies have charted the development of resting state networks in the preterm brain evaluating 74 [58] and 100 datasets respectively [56]. Consistent with findings in typically developing infants, primary functional networks appeared first, followed by precursors of higher order networks. A number of developmental algorithms that seemed to dictate resting state network genesis could be identified. These rules included medial to lateral expansion

of interhemispheric functional connectivity, strengthening of interhemispheric connectivity before anteroposterior intrahemispheric connectivity, increasing within-network coherence and progression from diffuse, yet spatially proximate connectivity patterns to focused local connectivity with emerging connections to spatially distant regions. Facsimiles of these developmental patterns were observed in a recent EEG study. EEG recordings were obtained in 20 preterm infants between 30 and 44 weeks postmenstrual age and revealed strong increases in interhemispheric synchrony and less pronounced increases in intrahemispheric connectivity. In addition, anterior brain regions showed stronger synchrony compared with posterior brain regions and most subjects displayed higher correlations within the left hemisphere than the right, suggesting an anteroposterior maturation gradient and early left lateralization [126].

Differences between preterm infants and healthy full-term neonates were also noted in numerous reports and were predominantly confined to the strength of connections. Preterm infants exhibited reduced interhemispheric connectivity [56,127] and impaired lateralization of language areas [128]. Resting state networks as observed in typically developing neonates were present in preterm infants scanned at term equivalent age, yet displayed reductions in connectivity strength of primarily higher order networks [58,129]. Similarly, on a whole brain level hallmark network attributes including *small world* organization, *modularity* and *rich club* architecture were found to be preserved while differences were revealed in the quality of connections [57]. A recent functional connectome study observed reductions in *clustering* and *rich club* connectivity in 12 preterm infants at term equivalent age compared with 25 full-term controls [57]. Conversely, in a DWI study of structural whole-brain connectivity (46 preterm infants and 17 healthy term controls), prematurity was associated with relatively intact *rich-club* organization but altered cortical-subcortical connectivity and short-distance connections outside the core network [4]. Contrasting findings may be the result of methodological, clinical and technical differences.

Finally, the impact of white matter injury resulting from extensive intraventricular hemorrhage - a typical pattern of preterm brain injury [130] - on functional connectivity was investigated in two reports that employed rs-fMRI and EEG [127,131]. Both inter- and intrahemispheric functional connectivity were diminished. Evidence from the EEG study suggests widespread reductions in functional connectivity in the presence of white matter injury as well as disruptions of the bimodal connectivity pattern. The latter constitutes a distinct feature of healthy early brain development [131].

Collectively, these findings consolidate previous research on the impact of preterm birth on early brain development and add substantially to our understanding of its neural underpinnings. Adverse effects of prematurity have been revealed to predominantly target thalamocortical connectivity, with major implications for long-term cognitive functioning and behavior. Given the developmental processes of thalamocortical axonal pathfinding that peak during the period of (extremely) preterm birth, these widespread disruptions and their ongoing adversity are intuitive [33]. In parallel, reductions in interhemispheric and long-range intrahemispheric connectivity strength have repeatedly been reported, indicating comparable impact on the functionality of commissural and association fibers. On a global level these disturbances seem to affect communication capacity, while the overall layout of resting state networks and functional whole-brain network architecture appears to remain intact.

Methodological considerations

There are a number of technical concerns that need to be taken into consideration when interpreting rs-fMRI and DWI data. Important topics include false positives, false negatives, test-retest reliability and acquisition protocols [132-134]. However, an in-depth discussion of these methodological considerations falls beyond the scope of this review. Here, we will highlight some of the key methodological concerns that specifically apply to fetal and neonatal neuroimaging, particularly focusing on issues regarding rs-fMRI.

Head motion is of particular concern in fetal and neonatal neuroimaging. Most infants are scanned during natural sleep or after administering mild sedation (25-60 mg/kg oral chloral hydrate) [5,13,73] and although appropriate measures are generally taken to reduce motion artifacts including feeding and swaddling (where infants are scanned during natural sleep), wrapping the infant in a vacuum fixation pillow and applying hearing protection devices to reduce exposure to acoustic noise, even subtle head movement remains a significant problem. Furthermore, the use of sedative medication - if administered - is generally confined to infants scanned in a clinical setting and may thus introduce a bias in terms of the effect of motion, as well as differences in physiological parameters and sleep state/level of consciousness between health and disease states. Preventing motion is even more challenging in fetal imaging, where large displacements of the fetal head are not uncommon and maternal respiration and potential movement present additional challenges [69]. Head motion introduces spurious, yet systematic noise into rs-fMRI data, reducing long-range connectivity between distant brain regions (primarily along anterior-posterior and vertical axes)

and increasing the strength of functional correlations between nearby voxels in the brain (predominantly left-right connectivity) [135,136]. Worryingly, these structured motion artifacts coincide with reported developmental effects: young children display a segregated communication framework, with strong connections between approximating brain regions and relatively limited long-range connectivity. Over the course of development, the brain modulates its layout and enhances long-range connections while diminishing local connectivity [137,138]. Hence, efforts to disentangle developmental effects from noise are vitally important in any rs-fMRI preprocessing pipeline. Evaluating motion (e.g. using frame-wise displacement or DVARS (Derivative of Root-mean-square VARiance over voxelS) and incorporating strategies to account for motion artifacts that go beyond rigid body realignment and regression of motion parameters are required [139-141]. Data censoring or scrubbing, i.e. removal of motion-contaminated data, which has been proposed by a number of research groups, may be particularly appealing for fetal and infant imaging and has been applied to most present-day rs-fMRI datasets (i.e. publications from ~ 2013 and beyond). However, concerns have also been raised about temporal concatenation of non-consecutive time-points and how such approaches may affect the data as well as about how excessive data removal may impact correlations between time-series [20,141]. Analysis strategies designed specifically to accommodate fetal movement by addressing motion on a slice-by-slice timescale and correcting for signal variations due to changes in the position of the fetal head with respect to the receiver coil are now available and enable most (potentially all) of the acquired rs-fMRI data to be retained [69].

Another relevant aspect that limits correlations between studies of early development is the use of different atlases for rs-fMRI data registration. Some studies employ adult templates [78,89,103,109,110,142] whereas others adopt group-level clustering to design a population-specific template based on similarity between voxels [14,57,143] or make use of available neonatal atlases (e.g., Oishi *et al.* [144]). Manual region of interest (ROI) placement is also performed in a number of resting-state network studies [56,58,71]. Altogether, optimization and standardization of the preprocessing pipeline and acquisition protocols are essential to allow for reliable comparisons across study populations and enable multi-center studies to be conducted. Such efforts will form the groundwork to further our understanding of early healthy and aberrant functional brain network development.

Future perspectives and conclusion

In recent years, neuroimaging research has shown that the overall framework of functional brain architecture, including functional networks and whole-brain functional connectome organization emerges in synchrony with the ontogeny of structural brain wiring during the mid through late fetal period. In the neonatal period both systems encompass the blueprint of their adult analogues, albeit in an immature state. Postnatal trajectories of functional brain network development appear more comprehensive than maturational courses of structural brain wiring. While structural refinements revolve around improvement of communication efficiency and connection strength, functional brain architecture transforms its spatial arrangement. Functional brain hubs shift from essentially primary order brain regions to higher-order association areas during postnatal development. Accordingly, functional resting state networks mature according to principles of increasing complexity. Networks involved in primary functions are largely complete in the neonatal brain, while higher order networks display a fragmented layout in the early postnatal period and continue to mature during the postnatal phase, with networks involved in the most intricate cognitive processes being the last to mature. A comprehensive understanding of the trajectories of typical early brain wiring is fundamental to the study of disrupted connectivity, and although crucial steps have been taken, further work is required. Additional studies of longitudinal design would be of particular relevance, as well as research linking functional brain network organization to cognitive and behavioral functions that are attained later in development. Such studies may help identify relevant imaging biomarkers.

Functional connectivity studies have begun to elucidate the impact of early developmental adversity. Preterm birth disrupts thalamocortical connectivity. Reductions in inter- and intrahemispheric connectivity have also been revealed. These widespread perturbations impair the brain's overall communication efficiency. Intrauterine exposure to psychoactive drugs seems to exert more targeted attacks, firstly confined to specific receptor-regions and secondly leading to accelerated engagement of the amygdala-prefrontal circuit. Whether maternal psychopathology intrinsically affects early brain wiring remains elusive and warrants further study.

There is an urgent need for studies investigating gene-environment interactions in relation to early brain development in both health and high-risk or disease states. Important first steps have recently been taken, cross-correlating common genetic variation analysis with DWI findings in preterm neonates [145,146]. These studies illustrated

genes mostly involved in lipid pathways to be related to the degree of maturation of distributed white matter tracts at term equivalent age.

Functional connectivity research is on the verge of elucidating developmental trajectories of healthy early human brain organization as well as of portraying departures from these typical trajectories that may pervasively affect brain functioning. Ongoing multidisciplinary and multimodal efforts are needed to unravel the inception of complex brain wiring and to help identify windows of opportunity for future interventions for those who experience serious developmental risk during the earliest phases in life.

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All things are difficult before they are easy

Thomas Fuller

CHAPTER 3

Early human brain development: insights into macroscale connectome wiring

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HIGHLIGHTS

- thalamocortical projection fibers mature at a faster pace than heteromodal association fibers during perinatal brain development
- fractional anisotropy matures before radial diffusivity between 29-45 postconceptional weeks
- radial diffusivity reflects an early marker of subcortical white matter myelination in the neonatal brain
- early developmental trajectories of the white matter may delineate valuable parameters of typical perinatal white matter development and may therefore assist in the designation of deviances thereof

ABSTRACT

Early brain development is closely dictated by distinct neurobiological principles. We investigated structural connectome development between 29-45 postmenstrual weeks in 44 preterm (n=23) and full-term (n=21) newborns scanned soon after birth (postnatal age 16±10 days). Diffusion weighted imaging data were combined with cortical segmentations derived from T2 data to construct neonatal connectome maps. Projection fibers interconnecting primary cortices and deep gray matter structures were noted to mature faster than connections between higher-order association cortices (fractional anisotropy (FA) $F=58.9$, $p<.001$, radial diffusivity (RD) $F=28.8$, $p<.001$). Neonatal FA-values resembled adult FA-values more than RD, while RD approximated the adult brain faster ($F=358.4$, $p<.001$). Maturational trajectories of RD in neonatal white matter pathways revealed substantial overlap with what is known about the sequence of subcortical white matter myelination from histopathological mappings as recorded by early neuroanatomists (mean RD 68 regions $r=0.45$, $p=.008$). Employing postnatal neuroimaging we reveal that early maturational trajectories of white matter pathways display discriminative developmental features of the neonatal brain network. These findings provide valuable insight into the early stages of structural connectome development.

Keywords
diffusion weighted imaging, brain development, connectome, neonatal, preterm

INTRODUCTION

Human gestation marks the onset of comprehensive development of the human brain. Fundamental processes of brain development including neurogenesis, neuronal migration, synaptogenesis and axonal pathfinding take place during the earliest gestational phases, i.e. from the fetal period commencing at eight postmenstrual weeks (PCW) continuing through the early postnatal period [1-4]. By the time of term birth, all major white matter pathways are in place [5,6] and the neonatal cortex approximates the shape of the adult human brain [4,7].

Diffusion weighted imaging (DWI) studies in post mortem fetal brains have identified axonal projections from as early as 13 PCW [5,8]. Major white matter pathways develop in a hierarchical order, predominantly during the second trimester of pregnancy, with limbic tracts and thalamocortical projection fibers emerging first, followed by commissural tracts that interconnect both hemispheres and intrahemispheric association pathways originating last [6,8,9]. Structural connectome studies in preterm infants have revealed an adult-like connectivity framework as early as 27 weeks gestational age [10-13]. By this time, pivotal brain network attributes are already present, including short communication paths favoring efficiency, clustering of brain regions for specialized information processing, and a central core of brain hubs, reflecting brain regions that are connected to a large number of other brain regions and therefore play a crucial role in communication facilitation, referred to as the rich club [10-15].

To date, developmental trajectories of the earliest stages of macroscale brain network development remain largely unexplored. Here, we set out to investigate early structural development of the neonatal brain during the earliest phases that allow postnatal *in vivo* neuroimaging, i.e. during the developmental period that coincides with the last trimester of pregnancy. To this end, we derived structural connectivity matrices from DWI scans and T2-weighted images acquired in 44 preterm and full-term infants scanned shortly after birth between 29 and 45 PCW. We postulated that thalamocortical projection fibers interconnecting deep gray matter structures and primary cortices (i.e., primary sensory and motor areas) would follow different maturational trajectories than association fibers between heteromodal association cortices (i.e., cortical regions governing higher cognitive functions and involved in integration of information from sensory and decision-making brain regions) well before they are myelinated. To test this hypothesis, we mapped their trajectories in the developmental timeframe of our study. In addition, we compared early developmental changes in white matter diffusion

properties against historical mappings of subcortical myelination as performed by pioneering neuroanatomists in the 1900s [16-18]. As a secondary objective, we studied whether brain network attributes crucial for human brain functioning exhibit early developmental changes and explored their association with measures of cognitive functioning in infancy. Third, we aimed to investigate the resemblance of the neonatal brain network to the adult connectome.

METHODS

Study population

In this study, we set out to investigate typical macroscale connectome development in the earliest phases of human development that allow *in vivo* delineation of these processes. Because of ethical restrictions, we were not able to include healthy fetuses or healthy full-term infants. We therefore aimed to approximate typical brain development by selecting infants who underwent neuroimaging for clinical reasons but that did not display overt brain injury. Infants eligible for participation in this study had been admitted to the level III or level II Neonatal Intensive Care Unit of the Wilhelmina Children's Hospital, University Medical Center Utrecht, Netherlands between June 2013 and June 2015. Infants were included in this prospective cross-sectional observational study if the following criteria were met: 1) no focal brain injury 2) no genetic syndrome, congenital infection of the central nervous system, or inborn error of metabolism, 3) good quality MRI data including T2-weighted imaging, DWI and resting state-functional MRI (not reported here) and 4) MRI acquisition as soon as clinically feasible after birth in order to minimize the likelihood of introducing - potentially detrimental - effects of the extra-uterine environment on the developing brain. Based on these criteria, 47 infants were eligible for inclusion in this study. Processing of the MRI data failed in three subjects owing to suboptimal cortical parcellations (described in the section on brain tissue segmentation and connectome reconstruction). The final cohort therefore comprised 44 neonates.

Neurodevelopmental outcome was formally evaluated at age 18 months corrected age using the Griffiths Mental Development Scales and/or at age 24 months corrected age using the Bayley Scales of Infant and Toddler Development, Third Edition (BSITD-III) in 34 (77%) of the infants. The BSITD-III assessment could not be completed in one other child because of hospital-related anxiety. The development quotient at age 12 months was 96 in this child. Neurodevelopmental follow-up was not indicated in the

remaining nine infants at the discretion of the attending physician. The majority of infants (n=27, 79%) had cognitive outcome scores within the normal range, defined as one standard deviation from the normative mean (normative mean BSITD-III 100±15 and Griffiths Mental Development Scales 100±12, mean in this population 103±12). The remaining seven infants had either higher cognitive scores (119, 120, 120 and 129) or lower cognitive composite scores. The three children scoring below the normal range had a cognitive composite score of 82 and motor composite scores of 95, 115 and 118 on the BSITD-III. Cognitive scores on the Griffiths Mental Development Scales were computed by taking the average of the personal-social, hearing and language, and performance subscales.

The Institutional Review Board of the University Medical Center Utrecht, Netherlands (IRB) gave approval for use of the clinically acquired data for scientific examinations as conducted in this study. Since we only made use of clinically obtained data, written informed parental consent for participation in the study was waived by the IRB. Clinical characteristics of the study population are outlined in Table 1.

MRI data acquisition

All scans were acquired on a 3 tesla Philips Achieva Clinical Scanner. Preterm infants were sedated using oral chloralhydrate (n=14) 35-60 mg/kg. Nine preterm infants were scanned during natural sleep and did not receive sedative medication prior to scanning at the discretion of the attending physician. Full-term infants were either sedated using oral chloralhydrate (n=12) 35-60 mg/kg or an intramuscular combination of pethidine 2 mg/kg, chloropromazine 0.5 mg/kg and promethazine 0.5 mg/kg (n=5) or did not receive sedative drugs (n=4) and were scanned during natural sleep. Heart rate, oxygen saturation and respiratory rate were monitored throughout the scanning session in all infants and two pairs of earmuffs were used for hearing protection (Natus Medical Inc. San Carlos, CA, USA; Em's 4 Kids LLC, Culver City, CA, USA). All MRI procedures were supervised by a neonatologist or physician assistant. Preterm infants were scanned in an MR compatible incubator (LMT Medical Systems GmbH, Luebeck, Germany). Full-term infants were scanned in a vacuum fixation pillow (Kohlbrat and Bunz GmbH, Radstadt, Austria). Additional foam padding was applied to reduce the noise of the scanner. The scan protocol included a coronal T2-weighted image: preterm infants 29-35 weeks: TR 10085 ms, TE 120 ms, voxel size 0.64 x 0.53 x 2.0 mm (reconstructed voxel size 0.34 x 0.34 x 2.0 mm); full-term infants 36-45 weeks: TR 4847 ms, TE 150 ms, voxel size 0.89 x 0.78 x 1.2 mm (reconstructed voxel size 0.35 x 0.35 x 1.2 mm);

Table 1 Characteristics of the study population

	Very preterm infants (<32 weeks) n = 17	Late preterm infants (32 - 36 weeks) n = 6	Full-term infants n = 21
Gestational age (weeks)	27.4 (26.1 - 31.9)	33.4 (32.0 - 36.7)	40.0 (37.0 - 41.6)
Birth weight (grams)	1145 (590 - 2050)	1808 (1205 - 4300)	3296 ± 636
Sex (female), no (%)	9 (53)	1 (17)	12 (57)
Postmenstrual age MRI (weeks)	31.0 (29.7 - 34.7)	35.1 (34.1 - 38.4)	41.4 ± 1.8
Postnatal age MRI (days)	23 ± 8	9 (5 - 23)	9 (2 - 31)
Neurodevelopmental outcome[#]	108 (82 - 121)	115 (91 - 129)	101 (82 - 120)
Age at outcome assessment (months)	20.5 (17.4 - 28.9)	24.9 (17.5 - 26)	24.1 (17.6 - 24.8)

Data are depicted in no (%) and mean, standard deviation (±) in case data are normally distributed or median, range otherwise.
[#]Neurodevelopmental outcome was defined as a composite of either the cognitive composite score extracted from the Bayley Scales of Infant and Toddler Development, Third Edition (n=25) performed at age 24 months or a composite of the personal-social, hearing and language and performance subscales on the Griffiths Mental Development Scales at age 18 months (n=9) [58,59]. Follow-up data was not available for 10 infants (see methods section).

and a DWI image: spin-echo EPI sequence obtaining 45-50 near-axial slices, 2.0 mm isotropic voxels, b-value 800 s/mm^2, 45 diffusion-weighted directions and four averaged non-diffusion weighted scans.

Brain tissue segmentation and DWI processing

T2-weighted images were automatically segmented into eight tissue classes, i.e., cortical gray matter, unmyelinated and myelinated white matter, deep nuclear gray matter, ventricles, extracerebral cerebrospinal fluid, cerebellum, and brainstem [19]. The segmentation method is a deep learning algorithm that employs a multi-scale convolutional neural network and was previously demonstrated to provide good accuracy when applied to the neonatal brain (30 weeks gestational age averge Dice coefficient 0.87, 40 weeks gestational age average Dice coefficient 0.82) [19]. A detailed description of the segmentation algorithm is provided by Moeskops *et al*. [19].

Next, the cortical mantle of each individual subject was reconstructed and parcellated into 68 distinct anatomical brain regions (34 per hemisphere) and 14 deep gray matter regions (seven per hemisphere) with the FreeSurfer image analysis suite (V5, http://surfer.nmr.mgh.harvard.edu) [20]. Prior to the automated Freesurfer process, surrogate T1-weighted images were reconstructed from the brain tissue segmentations by assigning tissue intensity values to each tissue class in the neonatal segmentation that correspond with expected adult T1-weighted signal intensity. Hence, brain tissue segmentations now displayed similar tissue contrast as adult T1-weighted images, which is a prerequisite for the analysis with Freesurfer (Figure 1). The first step of the Freesurfer reconstruction pipeline was adapted as such that resampling was performed using a nearest neighbor approach, instead of linear interpolation (default).

Preprocessing of the DWI data included the following steps: first, data quality of each subject's DWI dataset was visually inspected. DWI images were corrected for eddy-current distortions and small head movements [21]. Secondly, they were realigned to the b=0 image and third, a tensor was fitted to the diffusion signal within each voxel using a robust tensor fitting algorithm (RESTORE) [22]. DWI data were registered to the parcellation results using the tkregister tool in FreeSurfer. Registration results were visually inspected and manually optimized.

Structural connectome reconstruction

The complete connectivity wiring pattern of each infant was reconstructed using deterministic streamline tractography (FACT) [23]. To this end, 27 seeds were started

in each white matter voxel and fiber tracking was terminated when the curvature angle exceeded 45 degrees, when fractional anisotropy (FA) was lower than 0.01 or when fibers left the brain mask. A proportional fiber length threshold was employed at 8 mm * 3^√ (intracranial volume) in order to account for the substantial increase in head size during this developmental period. The minimum relevant fiber length was considered to be 8 mm. The cube root of intracranial volume was taken because of the ratio between fiber length and intracranial volume. Given the low FA of the largely unmyelinated and immature neonatal brain with relatively high water content, a minimum FA threshold of 0.01 was chosen [5,12,24-26]. Next, for each individual infant a connectivity matrix was obtained comprising 82 brain regions (nodes) including 68

Figure 1 Overview of the pipeline for neonatal connectome reconstruction

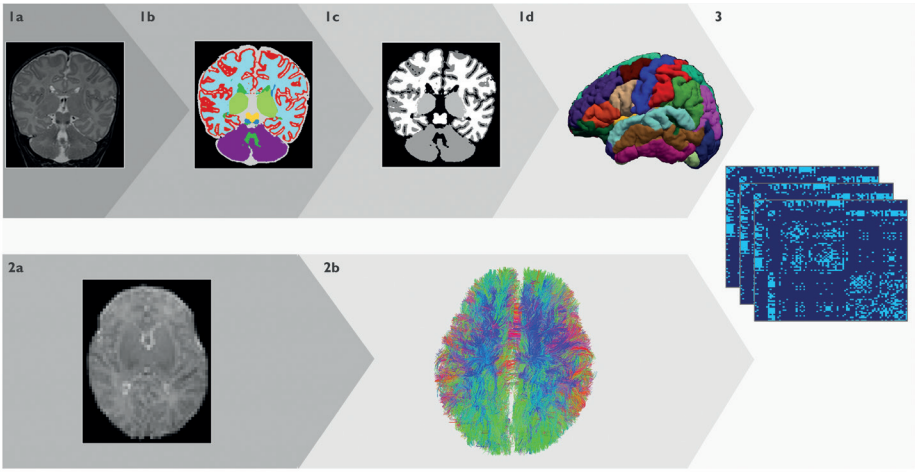


Figure 1 represents an illustration of the processing steps of neonatal connectome reconstruction. 1a. Coronal T2-weighted images are segmented into eight tissue types [19] as illustrated in 1b. Next, surrogate 'T1-weighted' images are created by assigning similar tissue intensity to the brain tissue segmentations as adult T1-weighted images (1c). Parcellation of the cortical mantle is performed using Freesurfer [20] for which these surrogate T1-weighted images serve as input (1d). DWI-images are realigned to the b=0 image (2a), corrected for small head movements and EPI-distortion. Next, DWI-images are automatically registered to the segmentation results from Freesurfer and manually adjusted when appropriate. 2b. Whole-brain streamline tractography is performed with the following settings: FA 0.01, maximum angle 45 degrees, 27 seeds per voxel, minimum fiber length 8 mm * 3^√ (intracranial volume). Streamlines are terminated when they leave the white matter mask. 3. Finally, whole-brain streamline tractography results and the results from the cortical parcellation are collated, resulting in connectivity matrices of 82x82 regions, including 68x68 cortical regions as defined by the Desikan-Kiliany atlas [27].

cortical regions and 14 subcortical regions as provided in the Desikan-Kiliany (DK) atlas [27] and white matter pathways (edges) interconnecting them. The complete processing pipeline of neonatal structural connectome reconstruction is outlined in Figure 1.

Data analyses were performed on binary and weighted connectivity matrices (FA and radial diffusivity [RD]) using two distinct approaches. First, individual binary and weighted matrices were computed employing a number of streamlines (NOS)-threshold ≥ three streamlines. This streamline threshold was set in order to account for false positives [28,29]. Second, subjects were sorted by postmenstrual age at time of scan and subdivided into groups of five individual infants. A sliding window was employed with steps of two subsequent subjects and a subgroup prevalence threshold of 60% [30]; i.e. connections were included when they were present in at least three out of five infants within a specific subgroup.

Connection-wise analyses

We zoomed in on developmental trajectories of specific groups of edges, focusing on primary and heteromodal association fibers. Primary connections were defined as white matter pathways interconnecting the subcortical gray matter and precentral gyrus (i.e. primary motor cortex), postcentral gyrus (i.e. somatosensory cortex) and pericalcarine cortex (i.e. primary visual cortex). Heteromodal association fibers were defined as connections between the frontal cortex including the superior-frontal cortex, rostral-middle frontal cortex, pars opercularis, pars triangularis and pars orbitalis, the superior-parietal cortex, supramarginal gyrus, precuneus, anterior-cingulate cortex and middle temporal gyrus. FA and RD of these connections were calculated on individual connectome maps (employing a NOS-threshold of ≥ three streamlines).

Flechsig atlas

In the early 1900s neuroanatomist Paul Flechsig published his findings on 'medullary substance' expansion in the postnatal subcortical white matter [16,17]. His atlas that divided the cortex into specific areas based on their sequence of subcortical myelination was modified by Von Bonin in the 1950s [18]. Here, we adopted the modified Flechsig and Von Bonin atlas as reported previously [31,32]. Regions were manually mapped to the DK atlas [20,27] employing a winner-takes-it-all approach: the myelination number as provided by Flechsig and Von Bonin that comprised the greatest surface area of the cortical region as defined by the DK atlas was assigned to that specific brain region for each of the 68 cortical areas.

Graph theory

Network metrics were calculated on individual weighted connectome maps (employing a NOS-threshold of ≥ three streamlines) using the Brain Connectivity Toolbox [33]. Connectome maps consisting of 82 brain regions (including 14 subcortical regions) were entered in these analyses.

Network density describes the proportion of connections that are present within the network out of all possible connections. Node *degree* measures the number of connections that each node has.

Global efficiency is used as a measure of network integration and is computed as the harmonic mean of the inverse average shortest path length between all pairs of nodes in the network. Path length measures the sum of weights that need to be traversed to travel from node *i* to node *j*, computed for all nodes in the network, with higher weights reflecting 'faster' connections. Therefore, RD was inverted, log normalized and scaled by the maximum occurring weight before computing global efficiency. No preparative computations were performed on FA before calculating global efficiency. Next, *normalized global efficiency* was computed as the ratio of global efficiency to the average global efficiency of 1000 random networks. To this end, 1000 random weighted networks (FA and RD) were computed while keeping the number of connections and their degree distribution intact. Additionally, global efficiency was also compared against 1000 reference networks with rearranged *weights* while keeping binary network topology intact.

Weighted clustering coefficient is defined as the average intensity over all possible triangles that a specific node is involved in. The intensity of a single triangle is taken as the geometric mean of the weights of its edges. RD was inverted, log normalized and scaled by the maximum occurring weight before computing the clustering coefficient. The normalized clustering coefficient is calculated as the ratio of the clustering coefficient of each subject to the average clustering coefficient of its 1000 random networks (as described in the section on global efficiency).

Modularity (Newman modularity score [34]) measures to what degree the network is divided into subnetworks of nodes with strong connections within their subnetwork and limited connections outside the subnetwork. Modularity was measured in FA- and RD-weighted networks. Modularity and clustering are considered measures of network segregation.

Resemblance to the adult connectome

In order to investigate the overlap between the neonatal brain network and the adult human connectome and validate findings from our previous report on the neonatal connectome in a different sample [12], we compared neonatal networks against connectome maps derived from high-quality DWI data from 487 adult subjects (males and females, aged 22-35 years) as provided by the 500 Subjects Release from the Human Connectome Project [35,36]. DWI data were acquired using generalized *q*-sampling imaging (GQI) that allows reconstruction of multiple fiber directions within one voxel (GQI parameters: 1.25 mm isotropic voxels, TR 5520 ms, TE 89.5 ms, 270 diffusion directions with diffusion weighting 1000, 2000, or 3000 s/mm^2). The white matter connectivity framework was reconstructed using deterministic streamline tractography and combined with cortical parcellations derived from T1-weighted imaging data (0.7 mm isotropic voxels) to obtain connectome maps [37,38]. Similar to the neonatal data, adult T1-weighted images were segmented and parcellated into 82 distinct brain regions (i.e., 34 cortical regions per hemisphere and 14 subcortical regions) provided by the DK atlas [27] using Freesurfer [20]. A group-averaged connectivity matrix was computed based on connections that were present in at least 60% of adult subjects. The average connectivity weight of existing connections was taken as the weighted adult connectome map. Next, the relative difference between mean FA of all reconstructed white matter fibers (global mean FA) was computed as follows:

$$\frac{\textit{(global mean FA neonates - global mean FA adults)}}{\textit{(global mean FA adults)}}$$

These calculations were repeated for RD and axial diffusivity (AD).

A modified Mantel test was used to compute the overlap between binary brain networks of each individual newborn infant and the group-averaged adult human connectome [12]. The distance between each of the cell entries in the neonatal and adult binary brain network was calculated. For comparison, the distance between the cell entries of the adult connectome and 1000 random neonatal brain networks was computed by redistributing the connections of each neonatal brain network while keeping their degree distribution intact. The binary connectivity overlap was expressed as the ratio between the number of overlapping cell entries and the total number of cell entries in the adult connectome, with 1 indicating perfect overlap between the neonatal and adult connectome and 0 reflecting the opposite [12].

Statistical analysis

This study is a prospective observational cohort study. In what follows, we will describe the statistical analyses used in our study. Means and standard deviations were reported for normally distributed data. Data that were not normally distributed were reported as median and their corresponding range. The relationship between postmenstrual age and brain connectivity measures, including white matter diffusion properties (FA and RD) or primary and association fibers, higher-order brain network metrics, and resemblance parameters between the neonatal brain and adult connectome was investigated using Pearson correlations for linear relationships and Spearman rank correlations for non-linear relationships. The significance level (two-sided p-value) was set to an alpha of .05 in all analyses, unless otherwise specified.

Network density increased substantially with age ($r=0.83$, $p<.001$). Therefore, a number of approaches were used to account for its potential effect on higher order network metrics [39] (i.e., normalized global efficiency, modularity and normalized clustering in our study). First, network cost (i.e., density) was added as a covariate to a general linear model investigating the association between postmenstrual age and relevant network metrics. Conversely, density was selected as the variable of interest and postmenstrual age and relevant network metrics were entered as independent variables. Second, a prevalence threshold was employed and set as follows. The prevalence of connections was calculated in the youngest age group. Next, the lowest prevalence threshold was chosen at which density was not significantly correlated with postmenstrual age. The group mask formed based on this threshold was applied to the connectivity matrices of all infants.

Mean FA and RD of primary and higher-order association connections were compared using paired t-tests. The effect of postmenstrual age was analyzed using ANCOVA. Post-hoc paired samples t-tests were performed when the ANCOVA yielded significant results employing a Bonferroni correction for statistical significance. P-values less than .05/2 were thus considered statistically significant.

The relationship between white matter maturation and subcortical myelination order as provided by Flechsig and Von Bonin was investigated as follows. First, node-wise FA and RD were calculated 1) by taking the mean of their non-zeros components and 2) by taking the sum of their components. The latter analysis was included to take differences in node degree into account. These metrics were computed on connectome maps of age-consecutive subgroups. Second, node-wise alterations in FA and RD

were calculated, defined as the difference in node-wise mean FA and RD between the youngest (n=5) and most mature subgroup (n=5). Subgroups were extended to n=10 youngest and most mature infants. Node-wise changes were related to Flechsig mappings using a Spearman rank correlation. Alterations of the relative difference in RD, AD and FA between the neonatal white matter of individual subjects and the adult brain over the 16 weeks time-period were analyzed using ANCOVA entering postmenstrual age as a covariate and employing a post-hoc Bonferroni correction. Additionally, a general linear model was performed for each metric to estimate its slope.

RESULTS

Primary and heteromodal association fibers

First, we investigated whether thalamocortical projection fibers between deep gray matter structures and primary cortices displayed divergent trajectories from association fibers interconnecting higher-order cortices. To this end, we compared FA and RD as measures of white matter microstructural maturation between these fiber categories over the 16-week developmental period. FA of primary connections was significantly higher than FA of heteromodal association fibers ($t(43)=5.9$, $p<.001$, paired t-test) and increased significantly faster with postmenstrual age ($F(1, 84)=58.9$, $p<.001$, ANCOVA). Conversely, RD of primary connections was significantly lower ($t(43)=-9.0$, $p<.001$) and decreased significantly faster than RD of higher-order association fibers $F(1, 84)=28.8$, $p<.001$). Results are outlined in Figure 2. In order to account for the difference in number of connections, with heteromodal association fibers being larger in number than primary connections, analyses were repeated on random selections of an equal number of primary and heteromodal association connections. These analyses yielded similar results (Supplemental Materials).

Flechsig mappings

Next, region-wise alterations in RD and FA were compared against what is known about the sequence of subcortical myelination from post mortem examinations as performed by early neuroanatomists. Differences in RD between the youngest and most mature neonatal subgroup were correlated with Flechsig mappings of subcortical myelination (mean RD Spearman rank $r=0.45$, $p=.008$, sum RD $r=0.50$, $p=.002$). Results of the correlation between mean RD and subcortical myelination as provided by Flechsig and Von Bonin are portrayed in Figure 3. Similar results were found when extending

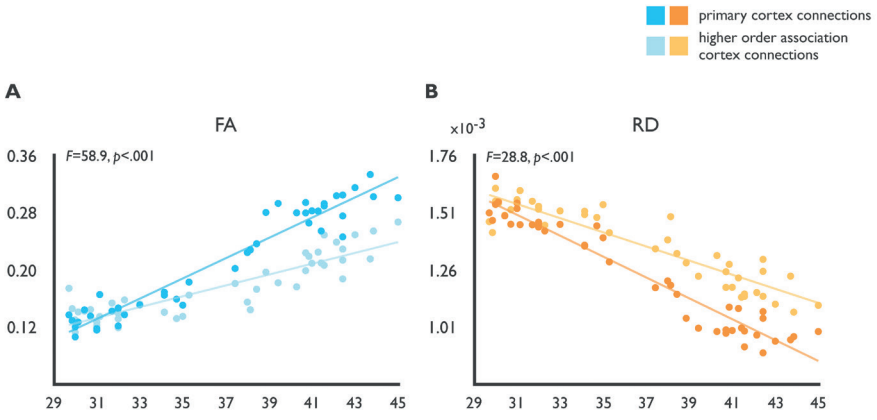
the age groups to n=10 youngest and n=10 most mature infants (mean RD Spearman rank r=0.45, p=.008, Spearman rank sum RD r=0.47, p=.005). Correlations of mean and cumulative node-wise FA were not significantly related to Flechsig mappings.

Network metrics

Third, we investigated the developmental trajectories of the structural connectivity framework in the neonatal brain between 29 and 45 PCW. Normalized global efficiency of FA-weighted brain networks significantly increased with advancing postmenstrual age (Pearson r=0.38, p=.01). Results are displayed in Figure 4. Modularity and normalized clustering did not significantly change over the 16 weeks study period. Furthermore, RD-weighted network metrics did not significantly change with increasing postmenstrual age.

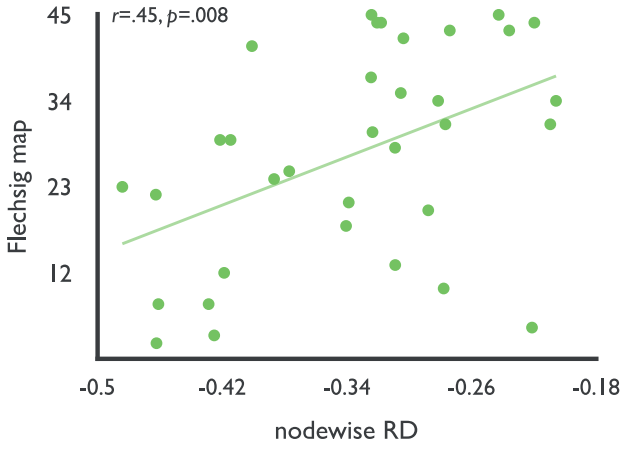
To explore the effect of density on the association between postmenstrual age and normalized global efficiency, density was added as a covariate to a general linear model investigating their relationship. The model was statistically significant (F[1,2]=3.64, p=.04). Next, the association between postmenstrual age and density was examined; entering normalized global efficiency as a covariate in the model. The model was statistically significant (F[1,2]=41.1, p<.0001) and postmenstrual age was the major

Figure 2 Maturational trajectories of primary and heteromodal association fibers



Alterations in mean FA (left panel), and mean RD (right panel) with postmenstrual age are outlined. Primary connections are depicted in bright colors and reveal significantly faster trajectories. Multimodal connections are illustrated in pale colors.

Figure 3 RD changes related to historical Flechsig mappings



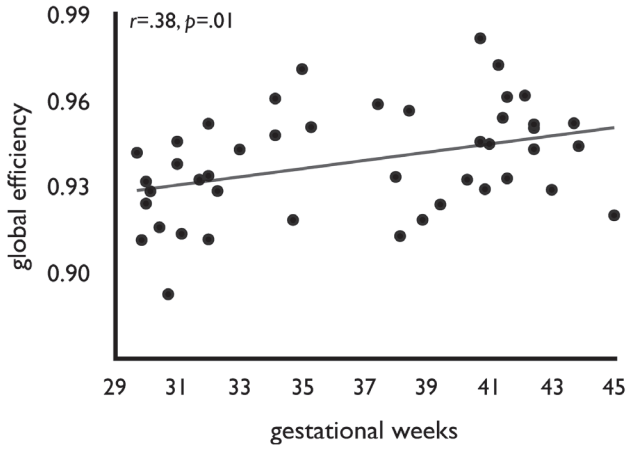
Scatter plot outlines the correlation between changes in RD and histopathological recordings of subcortical myelination as performed by Flechsig in the early 1900s and modified by Von Bonin in the 1950s. Cortical regions showing the greatest early change in RD were significantly correlated (Spearman rank correlation) to the sequence of subcortical myelination as provided in the modified Flechsig atlas. Change in RD was defined as the difference in mean RD between the youngest and most mature subgroup for each region, averaged over the two hemispheres.

contributor to the model (t=8.2, p<.0001), while normalized global efficiency did not add significantly (t=0.6, p=.57). Subsequently, a prevalence threshold was employed based on the presence of connections in 80% of subjects in the youngest age group. Normalized global efficiency was not significantly correlated with postmenstrual age (r=0.29, p=.06) (Supplemental Figure 1). Network metrics were not significantly correlated with early cognitive functioning as measured at age 18-24 months.

Resemblance adult connectome

The relative difference in diffusion properties between neonates and adults was smallest for FA and largest for RD. The relative distance of FA in the neonatal brain to FA in the adult brain varied between -0.73 and -0.68 at the earliest time-points (29-30 PCW) and decreased to -0.52 through -0.44 by 42-45 PCW. RD was 2.11-2.45 times higher in the youngest infants than RD in the adult brain and declined to 1.37-1.67

Figure 4 Developmental changes in global efficiency of the neonatal brain network

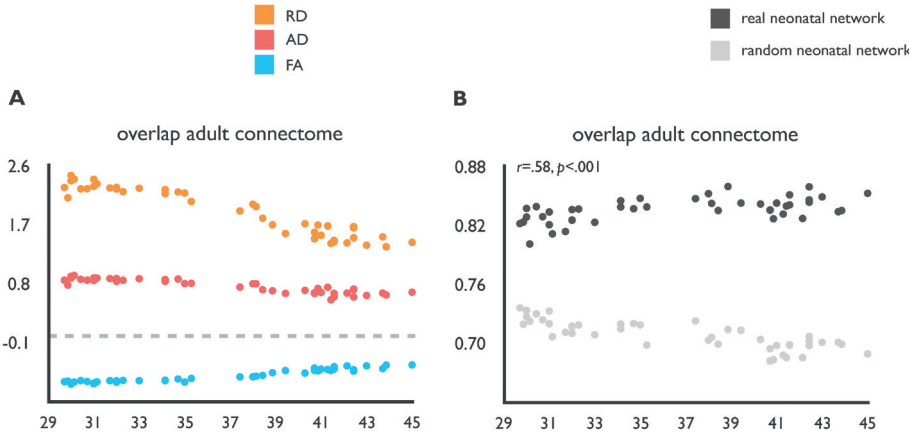


Alterations in global efficiency over 16 developmental weeks that reflect the third trimester of normal pregnancy and first postnatal weeks are illustrated. Normalized global efficiency increased significantly (Pearson correlation) with postmenstrual age as measured on FA-weighted connectome maps employing a NOS-threshold of three streamlines.

times higher values in the most mature group of infants. Distilling the components attributing to FA, the relative difference in AD between the neonatal brain and adult brain decreased least (Figure 5, panel A). The relative difference in RD, AD and FA was significantly different between the groups (ANCOVA $F(1,2)=358.4$, $p<.001$) and the relative difference in RD declined fastest with postmenstrual age (coefficient RD -0.046 versus FA 0.041, AD 0.005).

The modified Mantel test revealed that the binary connectome showed substantial overlap with the adult brain network (>0.80 at all developmental time-points) thereby reconfirming findings from our previous report on the neonatal brain network in a different neonatal sample [12]. Similarities between the neonatal and adult connectome increased moderately with postmenstrual age (Pearson $r=0.58$, $p<.001$) (Figure 5, panel B.

Figure 5 Differences and similarities between neonates and adults



Relative differences in FA (blue), AD (reddish-pink) and RD (orange) are portrayed in panel A. Dotted grey line indicates adult level (i.e., no difference). The relative difference between neonates and adults is smallest in FA, while RD-differences decrease at a faster pace (beta-coefficient RD -0.07 versus FA 0.02). Panel B illustrates the results of the modified Mantel test (see Methods section), which revealed substantial overlap of the neonatal connectome and the adult human brain network. Similarity exceeded 0.80 at all time-points and increased gradually with postmenstrual age.

DISCUSSION

Combining data from DWI and T2-weighted imaging in 44 newborn infants, we provide insight into early white matter development between 29-45 PCW *in vivo*, revealing distinct early developmental features. Development of premyelinated primary projection fibers preceded maturation of heteromodal association fibers. In addition, early white matter changes in RD were significantly correlated with histopathological mappings of subcortical myelination as performed by early neuroanatomists [17,18]. FA did not show such correlations, yet verged on the adult human brain most. Collectively, these findings provide evidence that diffusion properties feature distinct developmental trajectories, with FA approximating the adult brain and RD reflecting an early marker of subcortical white matter myelination. Consequently, these early

developmental trajectories may delineate valuable parameters of typical perinatal white matter development and may therefore assist in the designation of deviances thereof.

Using clinically applicable DWI, we were able to reconfirm the well-documented maturational trajectories of white matter fibers, which have been established through elaborate research efforts on post mortem brain tissue employing both histopathological examinations and high-resolution diffusion imaging techniques [5,6,8,40-43]. Limbic and projection fibers are known to originate first, followed by commissural fibers of the corpus callosum and association fibers deriving last [1,6,8]. Nearly a century ago, pioneering neuroanatomists mapped the sequence of subcortical myelination and outlined their concept of the neurobiological principles that orchestrate this developmental process [16,17,44]. These rules correspond substantially to the order of white matter tract formation, with projection fibers myelinating before projection tracts. Here, we demonstrate primary connections to exhibit a faster maturational course than multimodal association fibers from as early as ~32 weeks PCW onwards. In addition, we reveal that early maturational changes in RD, which are present well before fibers are myelinated correspond to the sequence of myelination as mapped in post mortem histopathological recordings of peri- and postnatal myelination [17,44,45]. Conversely, FA did not show such correlation.

The notion that RD and FA depict distinct maturational features is in agreement with previous DWI studies and post mortem reports [41,46,47]. Increasing FA has been noted to predominantly reflect progressive fiber coherence, decreasing membrane permeability and myelination, while decreasing RD is thought to result from reductions in brain water content, advancing fiber organization, functional maturation of intracellular compartments including the cytoskeleton and microtubules and increases in membrane density owing to proliferation of maturing oligodendrocytes and subsequent myelination of white matter fibers. RD reductions also coincide with current knowledge about the decline in radial glial fibers, which govern neuronal migration during embryonic and fetal brain development and proliferate to become radial glial cells that support axons when their role in neuronal migration is completed [25,41,47,48]. Although our analyses do not allow direct comparisons with their underlying neurobiology, they point toward biologically meaningful divergent trajectories, with RD being an early marker of subsequent myelination and FA - as a reflection of increasing fiber coherence - resembling the adult human brain more. As such, these findings are clinically relevant for the designation of early developmental white matter changes

that may occur as a consequence of perinatal brain injury, preterm birth or congenital heart disease [49-51].

We observed a significant increase in global efficiency of FA-weighted networks with advancing postmenstrual age. The observation of a neonatal brain network that increasingly supports global communication consolidates previous findings on structural connectivity in preterm infants [10-12,43]. However, its interpretation is not straightforward. Network cost (density) was found to similarly increase with postmenstrual age and the latter measure is known to substantially influence network metrics describing the brain's topology, including global efficiency [39]. Here, we aimed to control for density changes using normalization, additionally employing a prevalence threshold and interchanging density and global efficiency as a covariate in the statistical model. These analyses confirmed the hypothesis that residual impact of network cost cannot be precluded after normalization of network metrics, if density differences are present in a study population. The multivariable regression analysis of postmenstrual age and density revealed that normalized global efficiency did not significantly contribute to the model. Furthermore, postmenstrual age was not significantly correlated with normalized global efficiency using a prevalence threshold.

To date, studies investigating early developmental trajectories of structural brain network organization remain scarce, but the few earlier reports that we are aware of consistently demonstrated decreasing path length between ~30 PCW and term age, denoting increasing integration capacity [10-12,43]. Concomitantly, these studies also uncovered density changes with age [10,11,43]. Similar to our study, Ball *et al.* [10] and Brown *et al.* [11] used diffusion tensor imaging and demonstrated increasing density with advancing age during a developmental period that coincides with the timeframe of our study. Notably, Batalle and colleagues [43] reported decreasing density with increasing age employing DWI with multiple shells, which allowed constrained spherical deconvolution tractography. Together, these findings stress the complexity of the concept of density and that inferences about the implied formation of novel fibers cannot be drawn from diffusion tensor imaging *in vivo* alone. Consequently, the clinical DWI protocol that we used in our study is a limitation. The tradeoff between state-of-the-art DWI imaging and clinical feasibility including limited scan time and the infant's comfort, which naturally comes first, resulted in the decision to use this clinical DWI protocol which lasted little over five minutes and was successful in all infants included in the study. However, it inherently imparted limitations on the reconstruction of complex fiber orientations (i.e. crossing fibers in single voxels) and resulted in

neonatal connectivity matrices of different density. Another important consideration is that we were not able to include healthy fetuses or healthy full-term infants owing to ethical objections by the IRB of our institution at the time the study was designed. We thus aimed to approximate typical brain development by selecting infants who did not exhibit brain injury and were scanned as soon as clinically feasible after birth in case of preterm birth. None of the infants included in the follow-up program (n=35, 80%) showed signs of neurodevelopmental delay on formal assessments in infancy. Regardless, we included preterm infants in our study to obtain estimates of third trimester white and gray matter development and prematurity may have impacted their designated trajectories [43,52-54]. Such effects may have resulted in delayed white matter maturation owing to developmental insults including neonatal illness, malnutrition and the administration of neuro-suppressive drugs, but may have also facilitated maturation because of exposure to sensory stimuli [6,55-57]. A recent resting-state fMRI study revealed functional connectivity reductions to be present in the fetal brain even *before* preterm birth in 14 fetuses that were to be born prematurely [53]. Hence, we cannot rule out that prematurity and its consequences may have affected the relationship between postmenstrual age and white matter maturation in our study. It would thus be of particular interest to repeat the present analyses in healthy fetuses and neonates, granted that methodological hurdles innate in fetal neuroimaging are overcome.

CONCLUSION

In conclusion, we reveal that premyelinated white matter fibers in the neonatal brain have specific developmental trajectories; with primary connections being strengthened before heteromodal association fibers and FA verging on the adult human brain prior to RD, while RD advanced at a faster pace. Our findings corroborate earlier notions of pioneering neuroanatomists who revealed that connections playing a primary role in brain functioning are favored to mature during early brain development and add that these distinct developmental trajectories can be identified *in vivo* well before myelination becomes apparent. Collectively, these findings provide valuable insight into developmental trajectories of the human brain network shortly after its genesis and are coherent with the neurobiological principles they adhere to. Future research should focus on functional correlates of these structural neurodevelopmental trajectories and is urged to include healthy fetuses to obtain unobscured estimates of typical brain development.

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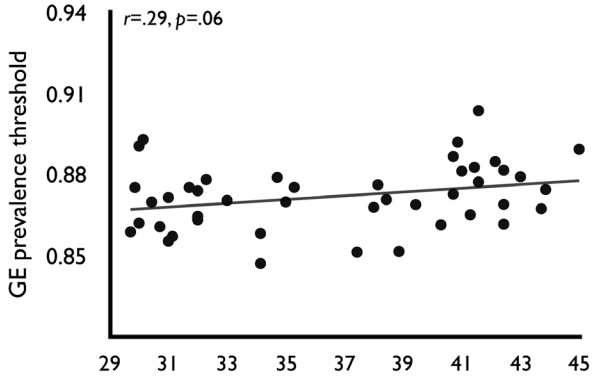
SUPPLEMENTAL MATERIALS

Connection wise analyses of primary and higher-order fibers

In the analyses of white matter maturation of primary and heteromodal association connections, the number of connections differed between the two edge categories, with primary connections comprising 86 potential entries and association connections consisting of 190 potential elements. Therefore, we randomly selected n=50 (primary and association fibers) and n=75 connections. These analyses yielded similar results, as described below.

FA of primary connections was significantly higher than FA of heteromodal association fibers (n=50 and n=75 randomly selected connections t=5.9, p<.001, paired t-test) and increased significantly faster with postmenstrual age (n=50 randomly selected connections F (1, 87)=60.1, p<.001, ANCOVA, n=75 F (1, 87)=59.1, p<.001, ANCOVA). Reciprocally, RD of primary connections was significantly lower (n=50 and n=75 randomly selected connections t=-9.0, p<.001, paired t-test) and decreased significantly faster than RD of multimodal association fibers (n=50 randomly selected connections F (1, 87)=28.9, p<.001, ANCOVA, n=75 F (1, 87)=29.0, p<.001, ANCOVA).

Supplemental Figure 1 Exploration of density effect on neonatal global efficiency



Network density was found to increase substantially with postmenstrual age (r=.83, p<.001, Pearson correlation). Therefore, analysis of the association between postmenstrual age and global efficiency was repeated employing a prevalence threshold. The prevalence threshold was set to the lowest prevalence in the youngest age group at which density was not significantly correlated with postmenstrual age (here 0.8). The scatter plot outlines the results of this analysis, revealing no significant correlation between postmenstrual age and global efficiency.

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3

PART 2

Neonatal neuroimaging markers of childhood cognitive functioning

To avoid situations in which you might make mistakes may be the biggest mistake of all

Peter A. McWilliams

CHAPTER 4

White matter maturation in the neonatal brain is predictive of school age cognitive capacities in children born very preterm

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HIGHLIGHTS

- neonatal white matter maturation is related to performance IQ at school age
- neonatal brain connectivity represents a valuable predictor for long-term cognitive outcome

ABSTRACT

The organization of human brain wiring is related to intellectual performance, with high performing individuals exhibiting more efficient connectivity patterns. How early connectome formation is related to intellectual achievement remains unclear. We examined the putative link between neonatal connectome organization and cognitive capacities at early school age in a cohort of 30 preterm born children (mean gestational age 27.5±1.2 weeks, 18 (60%) boys). For each infant, a connectome map was derived from diffusion weighted imaging acquired on a 3T system in the neonatal period (41.3±0.8 weeks) and combined with data from formal cognitive testing performed using the Wechsler Preschool and Primary Scale of Intelligence at age 5.5 years (mean 5.7±0.2 years). Structural connectivity maps were reconstructed using deterministic streamline tractography. Network metrics of global and local efficiency and mean fractional anisotropy of white matter pathways were related to intelligence quotient (IQ) and processing speed at age 5.5 years using linear regression analyses. Mean fractional anisotropy was significantly related to performance IQ at age 5.5 years ($F=8.48$, $p=.007$). These results persisted after adjustment for maternal education level. Our findings provide evidence that the blueprint of later cognitive achievement is already present at term equivalent age, and add to our understanding of the complex interplay between early brain development and cognitive functioning later in life.

Keywords
diffusion weighted imaging, preterm, connectome, neonatal brain, cognition

INTRODUCTION

Recent years have witnessed the widespread application of MRI in the neonatal brain following preterm birth to assess brain injury, evaluate brain development, and make predictions about neurodevelopmental outcome. So far it has remained difficult to accurately predict the full spectrum of neurodevelopmental deficits following preterm birth. Several imaging markers of cognitive and motor impairment have been proposed, including brain volumes and maturation of large white matter structures such as the corpus callosum and corticospinal tracts, in addition to white and gray matter injury scores [1-4]. Although these metrics have been demonstrated to be reliable for prediction of moderate and severe impairments, they tend to lack accuracy in predicting mild cognitive delays and behavioral problems. Today, mild developmental deficits are as clinically relevant as more profound delays, because they constitute the majority of adverse neurodevelopmental sequelae in modern neonatal medicine and have myriad functional implications such as educational underachievement, attention deficits, impaired executive functioning and socialization problems [5].

The limited predictability of mild cognitive impairments using imaging parameters proposed so far may be due to the integrative character of cognitive functioning. Cognition has been hypothesized to be the result of orchestrated information flow between neurons across white matter pathways, rather than of processes restricted to certain regions of the brain. This postulate has been corroborated by studies in adults that reported on the relationship between global brain network organization and intellectual performance [6,7]. Employing network science, a direct relationship was demonstrated to exist between characteristic path length as a measure of brain network efficiency and intelligence in both structural and functional brain network analyses, indicating that higher efficiency of global information transfer corresponds with higher intelligence [6-8]. In preterm infants, white matter connectivity between the thalamus and entire cortical mantle as measured at term-equivalent age (TEA) has been associated with cognitive outcome scores at age two years [9]. Investigating structural connectivity in the preterm brain in relation to childhood cognitive performance may help identify valuable imaging markers for long-term cognitive outcome prognostication.

In the present study we investigated the putative link between structural organization of the white matter at term equivalent age and cognitive functioning at early school age (5.5 years) in a cohort of very preterm infants. We hypothesized that white matter connectivity strength, reflected in global mean fractional anisotropy (FA) would be

associated with cognitive achievement 5.5 years later. In addition, we postulated that associated global communication efficiency of the neonatal brain network would be related to measures of intelligence at age 5.5 years.

METHODS

Study population

30 very preterm infants (18 [60%] males) born at <31 weeks of gestation at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Netherlands between January 2007 and March 2009 were included in this study. In total, 48 infants were eligible for inclusion. In these infants, diffusion weighted MR images had been acquired at TEA and evaluation of cognitive functioning had been performed at age 5.5 years. DWI data and/or atlas registration was of insufficient quality in 18 infants (see section on Image processing), resulting in a final sample of n=30 infants. Preterm infants selected for this study were part of either one of two prospective cohort studies conducted in the NICU during that period [4,10]. Exclusion criteria for participation in these studies were the presence of congenital anomalies, inborn errors of metabolism, and/or congenital infections of the central nervous system. The Institutional Review Board of the University Medical Center Utrecht granted ethical permission for both study protocols. Written informed consent was obtained from the parents. Clinical characteristics of the study population are outlined in Table 1.

MRI data acquisition

MR investigations were performed on a 3 tesla MR system (Philips Healthcare, Best, Netherlands) using an 8-channel sense head coil. Infants were sedated with 50-60 mg/kg oral chloralhydrate prior to scanning. Infants were placed in a vacuum fixation pillow and earmuffs (Natus Medical Inc. San Carlos, CA, USA) were applied for hearing protection. Heart rate, transcutaneous oxygen saturation and respiration rate were continuously monitored and a neonatologist was present throughout the examination. During the study period, the following T1- and T2-weighted imaging sequences were acquired: axial 3D T1-weighted image (TR=9.4 ms; TE=4.6 ms; voxel size 0.94x0.94x2.0 mm; no gap), coronal 3D T1-weighted image (TR 9.5=ms; TE=4.6 ms, voxel size 0.78x0.91x1.2 mm; no gap), axial T2-weighted image (TR=6293 ms; TE=120 ms; voxel size 0.54x0.61x2.0 mm; no gap), and coronal T2-weighted image (TR=4847 ms; TE=150 ms; voxel size 0.78x0.89x1.2 mm; no gap). T2 and T1-weighted images were used to evaluate focal brain injury.

Table 1 Clinical characteristics of the study population

	N = 30
Gestational age at birth (weeks)	27.5 (25.5 - 29.5)
Birth weight (grams)	923 (593 - 253)
Sex (male), no	18
Singleton, no	22
Full course of antenatal corticosteroids, no*	24
Apgar score at 5 min	9 (8 - 10)
Days of mechanical ventilation	5.5 (0 - 15.5)
Blood culture proven sepsis, no†	15
Patent ductus arteriosus requiring intervention, no	11
Bronchopulmonary dysplasia, no‡	7
Necrotizing enterocolitis (≥ Bell's stage II), no	1
Periventricular hemorrhagic infarction, no	2
Large unilateral/bilateral cerebellar lesions, no	1
Mild white matter injury, no§	20
Moderate white matter injury, no§	4
Moderate white matter injury, no§	0
Postmenstrual age at time of scan	41.3 (40.0 - 42.6)

Abbreviations: no = number. Numbers are reported as median, interquartile range unless indicated otherwise. *Full course of antenatal corticosteroids was defined as administration of betamethasone >48 hours prior to delivery. †13 children were diagnosed with a coagulase-negative Staphylococci sepsis during the NICU course, the other two pathogens were: Bacillus cereus and Candida Albicans. ‡Bronchopulmonary dysplasia was defined as requirement for supplemental oxygen at 36 weeks postmenstrual age and data was unavailable for seven infants. §White matter injury was assessed according to Woodward *et al*. [34].

DWI included 32 weighted diffusion scans (b=800 s/mm^2) and a non-weighted scan (b=0 s/mm^2); single shot echo planar imaging with 50 slices covering the entire brain (TR=5736 ms; TE=70 ms; field of view 180 x 146 mm; acquisition matrix 128 x 102 mm; voxel size 1.4x1.4x2.0 mm; no gap; total scan duration 4:33 min).

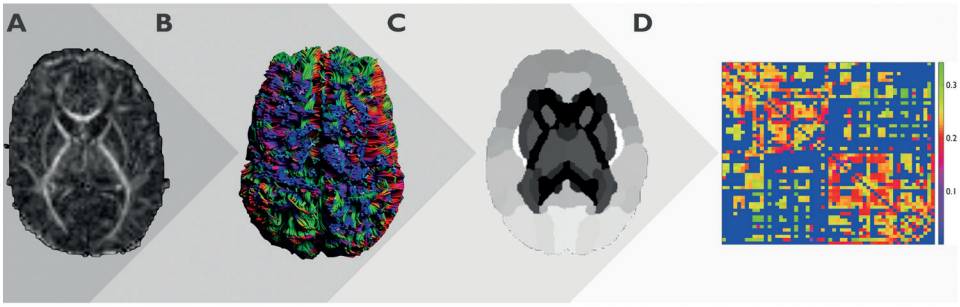
Image processing

Processing of the DWI data was performed using ExploreDTI [11] as described in Kersbergen *et al.* [10] and included the following steps: DWI images were corrected for eddy-current distortions and small head movements. For each voxel the diffusion tensor was estimated using the reweighted linear least squares method after excluding outliers with a robust fitting approach (REKINDLE) [12]. Data quality was visually inspected and datasets were excluded in case five or more volumes (i.e. diffusion directions) had motion artifacts. An automated neonatal atlas [13] was registered to the FA-maps of the DWI data using affine and elastic registration (Elastix [14]). This neonatal atlas was previously applied to the developing preterm brain to study white matter maturation [10] and the architecture of the neonatal brain network [15]. A detailed description of the anatomical neonatal template including illustrations of the regions and an open access copy of the template is presented in the paper by Oishi and colleagues [13]. Adequate registration of the neonatal template to the DWI image was visually verified and resulted in a final sample of n=30 good quality datasets.

Structural brain network reconstruction

Whole brain tractography was performed using deterministic streamline tractography with the following settings: FA threshold = 0.08, angle threshold = 45°, fiber length 20-200 mm, and 2.0 mm uniform seed point resolution throughout the brain. Next, the total set of reconstructed fiber streamlines was combined with the FA co-registered neonatal atlas in order to mathematically describe the infant's brain network as a graph, consisting of a set of nodes (i.e. brain regions of interest) connected by edges (i.e., fiber streamlines). For each infant, 56 regions of interest were defined (25 cortical regions per hemisphere, together with bilateral amygdala, hippocampus, and cerebellum) as predefined by the neonatal template [13]. Regions of interest were taken as nodes of the reconstructed neonatal network. Structural connectivity was assessed for each pair of brain regions (nodes) by determining whether streamlines (edges) were present interconnecting them. Connections were included when at least three streamlines were present between their nodes and connections comprising <3 streamlines were omitted in order to reduce the likelihood of false positives [7,8]. The processing pipeline for neonatal connectome reconstruction is illustrated in Figure 1.

Figure 1 Processing steps for structural network reconstruction of the neonatal brain



Panel A. Representative example of an FA map extracted from the DWI data obtained at term equivalent age. Panel B. Tractography in an example neonate. Deterministic streamline tractography was performed to reconstruct the complete connectivity wiring of the neonatal brain. Panel C. Neonatal template as reported by Oishi and colleagues [13]. The neonatal template was registered to the FA-map of the DWI data. Next, for each infant a connectivity matrix was formed by selecting 56 cortical regions of interest [13] in each hemisphere and by measuring the connections between these brain regions using each infant's whole brain tractography. Panel D shows a representative example of a reconstructed structural connectivity matrix of the connections between the 56 x 56 cortical regions of the neonatal atlas. The color bar represents connectivity strength (FA).

Graph theory

After having reconstructed the neonatal connectome of each individual subject, we investigated its topological organization by calculating a number of graph metrics [16]. Graph metrics were computed based on FA-weighted networks and included white matter connectivity strength, clustering coefficient, and global efficiency. These metrics were selected because they were previously related to cognitive functioning [6-8] in adults and pre-adolescent children and because they reflect both the integrative capacity of the human brain network (i.e. connectivity strength and global efficiency) and local efficiency (clustering coefficient). For a detailed description of the graph metrics included in our study we refer the reader to the paper by Rubinov & Sporns [16].

White matter connectivity strength provides information about the global level of connectivity of the network and was calculated as the average FA-value of all connections.

Global efficiency is computed as the harmonic mean of the inverse average shortest path length between all pairs of nodes in the network, with a weighted shortest path between two nodes computed as the minimization of the sum of weights (here taken as 1/FA) of edges that had to be passed to travel from one node within the network to another. Normalized global efficiency was computed as the ratio of each subject's global efficiency to the mean global efficiency of 1000 random networks. These random networks were obtained by randomizing the connections while keeping the number of connections and degree distribution intact.

Clustering coefficient is a metric of local connectedness and measures whether the neighbors of a given node are connected to each other, which would result in triangles of interconnected nodes. The clustering coefficient measures the ratio of the number of triangles a given node is involved in to the possible number of triangles. Here, we used the weighted clustering coefficient, defined as the average intensity of these triangles. Normalized clustering coefficient is computed as the ratio of clustering coefficient and the average clustering coefficient of a set of 1000 random networks with identical density and degree distribution.

Neurodevelopmental outcome assessment

Preterm born children were included in the standard of care neurodevelopmental follow-up program at the Wilhelmina Children's Hospital spanning from infancy through early school age. Cognitive functioning was assessed at age 5.5 years using a validated intelligence test: the Wechsler Preschool and Primary Scale of Intelligence, Third Edition (WPPSI-III) assessment [17]. Children are referred to the Department of Clinical Psychology for an intelligence test (WPPSI-III) in case they are born at less than 28 gestational weeks (n=21) and/or if clinically indicated at the discretion of the attending neonatologist at the outpatient clinic (n=9). The WPPSI-III includes the following scores: verbal intelligence quotient (IQ), performance IQ, full-scale IQ, and processing speed. The mean and standard deviation (SD) of each composite score is 100 SD 15. If WPPSI-III assessment was undertaken, performance IQ and verbal IQ could be calculated for every child. Full-scale IQ could not always be calculated owing to a discrepancy between the components (performance IQ and verbal IQ) (n=2). The Child Behavior Checklist and Teacher Report Form were administered at age 5.5 years to evaluate behavioral and emotional problems [18]. Behavioral and/or emotional problems were defined as suspected internalizing or externalizing behavioral problems on either or both questionnaires (score p>97) and/or a psychiatric diagnosis of a behavioral disorder. Finally, post discharge history of neurological disease, the use

of central nervous system medication and psychological stress defined as parental death or divorce, domestic violence or abuse and re-hospitalization >one day was collected based on retrospective chart review if recorded. The latter information was not systematically evaluated. Post discharge history, and details about cognitive performance and behavior at age 5.5 years are presented in Table 2.

Statistical analysis

Statistical analyses were undertaken in Matlab (MathWorks, Natick, MA, United States) and SPSS Statistics (IBM Corporation, Armonk, NY, United States). The relationship between graph metrics and intellectual functioning was assessed using linear regression analyses. First, a univariate regression analysis was computed for each metric. Second, multivariate linear regression analysis was performed entering the level of maternal education as a covariate. This factor was classified as low, middle or high, according to the Dutch Central Bureau for Statistics classification [9] and is known to have a significant impact on cognitive achievement in infancy and early childhood. One could however argue that maternal education level may be considered a surrogate measure of genetic make-up, so that adjustment for its effect would result in overcorrection, masking a true effect of white matter organization on intelligence. We thus carried out the analyses both with and without maternal education level included in the model.

In order to control for the potential impact of brain injury, focal brain injury was assessed on the basis of T1 and T2 images, binarized and added to the linear regression model as a covariate. Three of the 30 infants showed focal brain lesions including periventricular hemorrhagic infarction (n=2) and unilateral cerebellar hemorrhages (n=1). Gestational age at birth has been related to white matter maturation and cognitive development, with lower gestational age being associated with reduced FA at TEA and lower cognitive scores [9,19]. The impact of gestational age at birth on the postulated brain-behavior relationship was therefore investigated by adding this factor to the linear regression model. In an additional, exploratory analysis network metrics were correlated with behavioral problems using logistic regression analysis. The strength of the association between connectivity strength and cognitive outcome parameters was reported in terms of F-statistics with corresponding p-values. For the multivariable general linear models, t-statistics and corresponding p-values of graph metrics were reported. Two-tailed p-values ≤ 0.05 were considered statistically significant.

RESULTS

Median gestational age of the study population was 27.5 (interquartile range (IQR) 25.5-29.5 weeks) and 18 infants (60%) were boys (Table 1). Median full-scale IQ was 99 (IQR 78-120). 13 children (43%) exhibited behavioral problems defined as a psychiatric diagnosis of a behavioral disorder and/or suspected internalizing or externalizing behavioral problems based on the Child Behavior Checklist and Teacher Report Form (p>97). Symptoms of internalizing behavior include withdrawal, anxiety, somatic complaints and/or emotional reactivity, whereas externalizing symptoms comprise aggressive behavior and attention problems. One child was diagnosed with attention-deficit hyperactivity disorder and another child was referred to a child psychiatrist because of a suspected pervasive developmental disorder. None of the children developed cerebral palsy (Table 2).

White matter connectivity strength (global mean FA) of the neonatal brain was significantly related to performance IQ (F=8.48, degrees of freedom (dF) 28, p=.007, Figure 2). Entering gestational age as a covariate in the general linear model did not influence the relationship between connectivity strength and performance IQ at age 5.5 years (connectivity strength t(dF 27)=2.83, p=.009). Adjusting for focal brain injury (n=3) yielded similar results in terms of white matter connectivity strength in relation to performance IQ (t(dF 27)=2.91, p=.007). The model including maternal education level explained 23% of the variance in performance IQ at age 5.5 years (F(dF 25)=3.16, p=.03, adjusted r-squared 0.23), whereas global mean FA alone explained 21% of variance in performance IQ (adjusted r-squared 0.21).

Global efficiency was significantly associated with performance IQ (F=10.4, p=.003). In contrast, normalized global efficiency showed no significant association with performance IQ (F=0.55, p=.47), suggesting that effects were predominantly related to overall connectivity strength (i.e., mean FA) of the brain network. Similar findings were noted for network clustering. The clustering coefficient was significantly associated with performance IQ (F (dF 28)=8.48, p=.007), while the normalized clustering coefficient was not significantly related to performance IQ (F=0.81, p=.38).

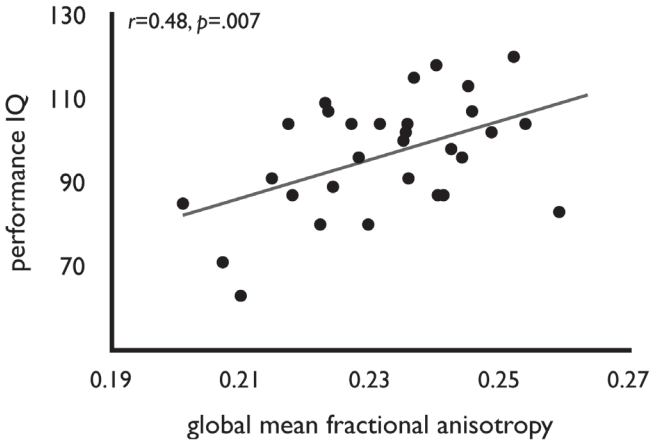
Network metrics were not significantly associated with verbal IQ or processing speed. Exploratory analysis of brain network metrics in relation to behavioral problems, comprising internalizing and externalizing symptoms revealed no significant associations.

Table 2 Neurodevelopmental outcome

	Outcome assessment	Score below - 1 SD
WPPSI, age (years)	5.7 (5.4 - 5.9)	n/a
Full-scale IQ*	99 (78 - 120)	6
Verbal IQ	100 (77 - 123)	5
Performance IQ	99 (82 - 116)	4
Processing speed*	91 (75 - 107)	7
Behavioral problems†, no	13	n/a
Repeated a grade at school‡, no	8	n/a
Special educational needs‡, no	1	n/a
Receives educational assistance‡, no	5	n/a
Cerebral palsy, no	0	n/a
Hearing impairment requiring aids‡, no	1	n/a
IQ <70§, no	1	n/a
Post discharge central nervous system medication#	1	n/a
Post discharge hospitalization#	1	n/a
Psychological stress#&	2	n/a

Abbreviations: n/a = not applicable, no = number. Numbers are reported as median, interquartile range unless indicated otherwise. *Data available for 28 children. †Defined as suspected internalizing or externalizing behavioral problems based on the Child Behavior Checklist and Teacher Report Form (p>97) and/or psychiatric diagnosis of a behavioral disorder [18]. §Full-scale IQ, verbal IQ and/or performance IQ <70 [17]. #Information was extracted from the medical records if available, yet not systematically evaluated. &Psychological stress was defined as early life trauma, including parental divorce, domestic violence, abuse and/or parental death and recorded if this information was available.

Figure 2 Neonatal white matter connectivity strength and performance IQ at age five



Scatter plot shows the observed relationship (F-statistic of linear regression model) between global mean FA as a reflection of white matter connectivity strength in the neonatal brain and performance IQ at age 5.5 years.

DISCUSSION

This study provides evidence that overall white matter connectivity strength in the neonatal brain is related to childhood cognitive functioning following very preterm birth. Measuring global mean FA in all major white matter pathways in very 30 preterm infants as assessed by means of 3T diffusion weighted imaging at TEA and combining these measurements with information about cognitive functioning at early school age revealed neonatal white matter connectivity strength to be significantly associated with performance IQ at age 5.5 years. Graph metrics measuring communication capacity (i.e., global efficiency) and segregation of the brain network (i.e., clustering) were also significantly related to performance IQ, while their normalized counterparts did not show a significant association with measures of intelligence at school age. The latter finding points toward overall white matter integrity - here measured as FA - being the major contributor to the observed relationship. Together, these results indicate that global maturation of the white matter may play an integral role in childhood cognitive functioning.

Our observations are in line with a growing literature of DWI studies in preterm infants focusing on the relationship between white matter microstructure in the neonatal period and neurodevelopmental outcome [2,4,9]. Numerous studies performed tract based spatial statistics, manual region-of-interest placement in specific large white matter structures including the corpus callosum and corticospinal tracts or tractography thereof and related their measurements to cognitive and motor functioning in infancy and childhood [2,4,9]. Employing these measurements, FA in the corpus callosum and corticospinal tracts have been consistently related to cognitive and motor performance in infancy and early childhood (i.e., 18-24 months corrected age) [2,4]. In addition, a recent study demonstrated structural thalamocortical connectivity across the entire brain to be related to cognitive outcome at age two years in preterm born children. A model including gestational age at birth, socio-economic status and thalamocortical connectivity at TEA explained nearly 40% of the variance in cognitive scores [9]. In our study, maternal education as a surrogate for socio-economic status added 2% to the variance in performance IQ explained by white matter connectivity strength of cortico-cortical connections. Gestational age was not related to mean FA or cognitive functioning at school age.

Recent reports in school-age children revealed FA in the corpus callosum and FA-weighted global efficiency of the entire brain network to be related to measures of intelligence and mathematical skills in both healthy full-term and preterm born children [8,20,21]. The latter connectivity measure reflects how well the white matter framework is able to support global information transfer across different sites of the brain. These studies had a cross-sectional design, measuring both DWI and cognitive measures in childhood. Our study now adds to the discussion about how white matter maturation in the neonatal brain may help predict cognitive functioning later in life by showing an association between global white matter connectivity strength at TEA and cognitive performance at school age, a relationship that thus appears to be sustainable. The notion that white matter connectivity was related to performance IQ, while such association was not observed with verbal IQ or behavioral problems may be attributed to the small sample size. Alternatively, these differences may reflect biologically meaningful dimorphism. Performance IQ has been associated with efficiently orchestrated information flow across the brain while verbal functioning may be restricted to specific brain regions [22]. In support of this postulate, white matter disorders in both children and adults have been related to diminished cognitive performance and executive functioning deficits, while language remains relatively intact [23,24]. Similarly, behavioral correlates may originate in specific structures as well [25].

Clinical implications of the present findings are twofold. Firstly, neonatal white matter connectivity strength may be of interest for cognitive outcome prognostication following preterm birth, which may be particularly relevant for the milder spectrum of impairments, including low average IQ to borderline intellectual functioning, and educational difficulties. Mild cognitive delays are notoriously difficult to predict. Here we provide evidence that mean FA, reflecting whole-brain white matter connectivity strength is related to cognitive achievement at school age. Accordingly, this key feature may serve as a valuable marker to identify preterm infants at risk of mild cognitive deficits and educational underachievement. Secondly, our findings emphasize the importance of early intervention strategies aimed at protecting the developing preterm brain from detrimental effects on neurodevelopment as a consequence of being born prematurely. Stem cell treatment, erythropoietin, magnesium sulfate, and nutritional interventions, such as probiotics and lactoferrin consistute promising candidates [26,27]. Given the notion that crucial processes of brain network formation take place before 32 weeks postmenstrual age [5,28] and that global white matter maturation by TEA is related to cognitive performance, it is likely that a critical window of opportunity for neuroprotective intervention exists even earlier [29]. Elaborating on this postulate, it would be of particular interest to replicate the present findings in the neonatal brain network at an even earlier stage of brain development. Meanwhile, white matter connectivity analysis of the preterm brain around TEA may serve as a valuable surrogate endpoint for evaluation of neuroprotective therapies. Furthermore, longitudinal studies evaluating the relationship between the brain's structural white matter organization and cognitive functioning and how this relation is modulated by epigenetic and environmental influences through the course of development would also be particularly relevant to expand our understanding of how the brain's microstructural organization supports function. Finally, we believe that the putative impact of white matter injury on the brain-behavior relationship as observed in our study deserves further attention. White matter injury was limited in the present cohort (Table 1), yet is well-known to exert detrimental effects on white matter maturation and subsequent neurodevelopment [5]. Perinatal conditions, including inflammation, malnutrition and respiratory disease may have also impacted on white matter development in the absence of macroscopic white matter lesions [5,26,29].

Several limitations need to be taken into account when interpreting the results of the present study. First, the sample size was small because of the limited availability of good quality DWI data at TEA and the design of the standard-of-care follow-up program in which intellectual performance was evaluated at age 5.5 years only

if preterm children were born at less than 28 weeks gestational age or if clinically indicated. Therefore, we focused on a small number of network metrics measured in FA-weighted connectome maps including global mean FA as a representation of white matter microstructural maturation and measures of global and local communication capacity. These measures were selected because they were previously related to cognition in late infancy, childhood and adulthood [4,6-8]. Our study was underpowered to detect the full impact of potential confounders and mediators, including brain injury, sex, gestational age, maternal education level, and post discharge injurious events. Extending the present analyses to a larger sample would thus be relevant for future studies. In order to obtain representative estimates (at a power of 0.8) of the relationship between neonatal brain network organization and childhood cognitive functioning in very preterm children, while accounting for relevant variables one would require a sample size of approximately 150 children (based on power calculation using G*Power). In addition, corroborating our findings in a sample of healthy term-born infants would be valuable to elaborate our understanding of this brain-behavior relationship in the typically developing brain. Second, the use of various atlases across different studies may limit the interpretation of findings. Here, we employed the neonatal template provided by Oishi and colleagues [13]. We refer the reader to the review by Keunen *et al*. [28] for a discussion of this issue. A third limitation is that scan time of the neonatal MRI procedure was limited. A DWI protocol was used that included 32 non-collinear directions of diffusion-weighted images and a single b-value of 800 s^2/mm, which imparted limitations on the streamline tractography and did not allow accurate reconstruction of complex fiber orientations [30]. Nevertheless, neonatal connectome organization derived from DWI data obtained using the same DWI protocol was found to exhibit large overlap with the adult structural connectome in an earlier study [15]. Furthermore, studies making use of a variety of imaging modalities and diffusion models have demonstrated attributes of brain network organization with a high degree of consistency over cohorts and species [31,32]. Future research should consider employing DWI protocols with multiple shells including high b-values in order to account for complex fiber orientations, given that available timeslots and the infant's comfort during the MRI session allow such protocols [33].

CONCLUSION

In conclusion, our study shows that connectivity strength of the neonatal structural brain network is associated with cognitive capacities at early school age in children born very preterm. This result provides evidence for a fundamental relationship between white matter organization and long-term cognitive achievement and strengthens the view that the level of white matter maturation at term age may serve as a valuable predictor for long-term cognitive outcome.

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4

If everything seems under control, you're not going fast enough

Mario G. Andretti

CHAPTER 5

Brain volumes at term-equivalent age in preterm infants: imaging biomarkers for neurodevelopmental outcome through early school age

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HIGHLIGHTS

- larger volumes of the ventricles are consistently associated with poorer neurodevelopmental outcome in very preterm infants
- unmyelinated white matter volumes are positively related to motor performance at age two years and processing speed at age five
- brain injury significantly impacts on the relationship between cerebellar volume and cortical gray matter volume and neurodevelopment following preterm birth
- a contemporary population of very preterm infants has relatively favorable outcome without severe mental disabilities

ABSTRACT

Brain volumes measured in the neonatal period have been associated with neurodevelopmental outcome in preterm infants across various domains. In this prospective observational cohort study we set out to investigate the relationship between brain volumes measured at term in a contemporary cohort of preterm infants and neurodevelopment until early school age assessed at different time points. To this end, we included 112 infants (mean GA 28.6±1.7 weeks) studied with MR-imaging at mean 41.6±1.0 weeks. T2- and T1-weighted images were automatically segmented and volumes of six tissue types were related to neurodevelopmental outcome assessed using the Bayley Scales of Infant and Toddler Development (cognitive, fine and gross motor scores) at 24 months corrected age (n=112), Griffiths Mental Development Scales (developmental quotient) at age 3.5 (n=98), Movement Assessment Battery for Children (n=85), and Wechsler Primary and Preschool Scale of Intelligence at age 5.5 (n=44). In order to control for well-known confounders, corrections were made for intracranial volume, maternal education, and severe brain lesions. Ventricular volumes were negatively related to neurodevelopmental outcome at age 24 months, 3.5 years, and processing speed at age 5.5 years. Unmyelinated white matter (UWM) volume was positively associated with motor outcome at 24 months and with processing speed at age 5.5. Cortical gray matter (CGM) volume demonstrated a negative association with motor performance and cognition at 24 months and with DQ at age 3.5. Cerebellar volume was positively related to cognition at these time-points. Adjustment for brain lesions attenuated the relations between cerebellar and CGM volumes and cognition. Our findings suggest that brain volumes of ventricles, UWM, CGM, and cerebellum may serve as biomarkers for neurodevelopmental outcome in preterm infants. The relationship between larger CGM volumes and adverse neurodevelopment may reflect disturbances in neuronal and/or axonal migration at the UWM-CGM boundary and warrants further investigation.

Key words
preterm infants, MRI, segmentation, brain volumes, cognition, neurodevelopment

INTRODUCTION

Very preterm infants exhibit impaired brain growth and delayed maturation compared with full-term infants, which is likely the result of a combination of primary injury and secondary maturational disturbances [1]. Both white and gray matter structures are affected, and at term-equivalent age (TEA) preterm infants have been reported to display volume changes in cortical gray matter (CGM), basal ganglia and thalami (BGT), cerebellum, cerebral white matter, and in particular in corpus callosum size, compared with term born controls [2-4]. In preterm born children, the association between smaller brain volumes and cognitive impairments, such as lower overall intelligence, memory deficits, and impairments in executive functioning has been well established [5]. A recent study in extremely preterm born (<28 weeks) adolescents also showed an association between brain tissue reductions and deficits in basic educational skills such as word reading, math computation, and spelling at 18 years of age. Total brain tissue volume accounted for 20-40% of the variance in cognitive and educational performance [6]. Although research investigating brain volumes measured in childhood and adolescence in relation to cognition and neuromotor performance is abundant [5,6], the relationship between brain tissue volumes of preterm infants measured at TEA and neurodevelopmental outcome has been studied less extensively. Both global and regional brain tissue reductions have been described. The cerebellum, CGM, hippocampus, white matter, BGT, ventricles, and cerebrospinal fluid volumes have all been related to poorer cognitive and motor outcome in infancy and early childhood, up to 30 months corrected age (CA) [3,7-10]. Bora and colleagues recently reported that reductions in total brain tissue volume and increases in cerebrospinal fluid volume at TEA were related to persistent inattention/hyperactivity problems during childhood. Specific reductions were found in the dorsal prefrontal region in very preterm infants with problems compared with their preterm peers without problems (7% cerebral tissue volume reduction) or term born controls (16% reduction) [11]. Hence, brain volume alterations in the neonatal period seem to be associated with neurodevelopment across a wide range of functional outcomes (cognition, behavior, as well as neuromotor performance).

The aim of this study was to expand our understanding of the relationship between early brain growth and long-term neurodevelopmental outcome following very preterm birth. Here we report findings on the association between brain tissue volumes measured at term in a contemporary cohort of preterm infants and neurodevelopment until early school age assessed at different time points.

METHODS

Study population

Preterm infants born <31 weeks of gestation and admitted to the Neonatal Intensive Care Unit (NICU) at the Wilhelmina Children's Hospital, University Medical Center Utrecht, Netherlands were recruited for this prospective cohort study between October 2006 and March 2008. Infants with congenital anomalies, genetic disorders, inborn errors of metabolism, or congenital infections of the central nervous system were excluded. The study was approved by the local Institutional Review Board. Written informed parental consent was obtained for all infants. Other findings of this cohort were previously published [9,12-14].

MRI acquisition and image analysis

All MR investigations were performed on a 3.0 Tesla MR system (Philips Healthcare, Best, Netherlands) using an 8-channel sense head coil. Infants were sedated with 50-60 mg/kg oral chloralhydrate. Next, infants were placed in a vacuum fixation pillow (Kohlbrat & Bunz GmbH, Radstadt, Austria) and earmuffs (Natus Medical Inc. San Carlos, CA, USA) were applied for hearing protection. Heart rate, transcutaneous oxygen saturation and respiration rate were continuously monitored and a neonatologist was present throughout the entire examination. The scan protocol included sagittal T1-weighted images (repetition time (TR)=886 ms; echo time (TE)=15 ms; slice thickness, 3.0 mm), axial 3D T1-weighted images (TR=9.4 ms; TE=4.6 ms; slice thickness, 2.0 mm, no gap) and axial T2-weighted images (TR=6293 ms; TE=120 ms; slice thickness, 2.0 mm, no gap). Axial T1- and T2-weighted images were used for brain tissue segmentation which was subsequently performed using an automatic algorithm, described by Anbeek . [15]. The method segments the neonatal brain into eight different structures: CGM, BGT, ventricles, cerebrospinal fluid in the extracerebral space (CSF), myelinated white matter (MWM), unmyelinated white matter (UWM), brainstem and cerebellum. The method was able to segment all brain structures accurately (achieved Dice coefficients 0.75-0.92), except for MWM (Dice Similarity Index 0.47) [15]. Therefore, MWM volume was excluded from further analyses. In addition, brainstem volumes were omitted from the analyses with neurodevelopmental outcome because the brainstem was not considered relevant for this specific evaluation, resulting in six different cerebral tissue types that were entered in the final analyses. The reader is referred tothe report by Anbeek . [15] for a detailed description of the segmentation process. Four expert observers visually inspected all automatic segmentations and confirmed the accuracy thereof. However, in two datasets automatically obtained results had to be manually corrected. The MRI

of one infant displayed misclassification of voxels in the region of the occipital lobe because of a large bilateral cerebellar hemorrhage and another infant presented with the same problem in the temporal lobe caused by a temporal hemorrhage. White matter injury was assessed according to Woodward . [16] using axial T2-weighted images and axial and sagittal T1-weighted images. Cerebellar hemorrhages were classified as punctate unilateral, punctate bilateral, extensive unilateral, extensive bilateral, and vermis involvement, according to the cerebellar injury score reported by Kidokoro . [17]. Brain injury was evaluated by two neonatologists with extensive experience in the field of neonatal neuro-imaging (LdV and MB) and a pediatric radiologist (RN). In case of disagreement, a fourth reader (FG) was consulted and consensus was achieved by discussion.

Neurodevelopmental outcome assessment

Neurodevelopmental outcome was assessed at the outpatient clinic at three different time points; two years CA using the Bayley Scales of Infant and Toddler Development, Third Edition (BSITD-III) [18]; 3.5 years chronological age using the Griffiths Mental Development Scales (GMDS) [19]; and age 5.5 years using the Movement Assessment Battery for Children, second edition (MABC-2) [20] and the Wechsler Preschool and Primary Scale of Intelligence, Third Edition (WPPSI-III) [21]. Normative means for each outcome score were 100, standard deviation (±) 15 unless stated otherwise. Developmental specialists (child physiotherapist, child behavior specialist and/or neonatologist) assessed children at at age two, 3.5 and 5.5 years. Child psychologists administered the WPPSI-III assessment. All preterm infants were included in the long-term follow-up program until age 5.5 years. Only infants born at <28 gestational weeks or infants with an indication for psychological assessment were referred to the department of psychology for an intelligence test at the discretion of the attending phyisician. Because of the limited attention span of two-year-old children, only the cognitive and fine and gross motor subtests of the BSITD-III were administered. Composite scores were corrected for prematurity. The GMDS consists of six different subscales (locomotor, personal-social, language, eye and hand coordination, performance, and practical reasoning) and yields a developmental quotient (DQ) (100±12). The MABC-2 is a standardized test to identify impairments in motor performance and yields scores on three different subscales (manual dexterity, aiming and catching, and balance) (10±3). The WPPSI-III includes the following scores: verbal IQ (VIQ), performance IQ (PIQ), full-scale IQ (FSIQ), and processing speed. If WPPSI-III assessment was undertaken, PIQ and VIQ could be calculated for every child. FSIQ could not always be calculated due to a discrepancy between the components (PIQ and VIQ).

Statistical analysis

SPSS software package version 20.0 (SPSS INC, Chicago, Illinois, USA) and R version 2.15.3 (www.r-project.org) were used for data analyses. Volumes of the different brain structures were related to neurodevelopmental outcome scores at two years CA, age 3.5, and 5.5 years. First, exploratory analyses were performed using univariable linear regression between absolute brain volumes and neurodevelopmental outcome scores (Model a). Next, brain volumes were adjusted for intracranial volume (ICV) in order to account for potential effects of head size, as a consequence of later PMA at scan and/or greater body weight. As maternal education level is known to influence neurodevelopmental outcome, this was considered a covariate in multivariable analysis (Model b). Maternal education was classified as low, middle, or high according to the CBS classification (Statistics Netherlands, The Hague, Netherlands; http://www.cbs.nl/en-GB/menu/home/default.htm). In order to investigate whether associations between brain volumes and neurodevelopmental outcome were mediated by severe brain injury, additional adjustments were made for severe brain lesions (Model c), divided into a) severe supratentorial lesions, defined as cystic periventricular leukomalacia, intraventricular hemorrhage (IVH) with post-hemorrhagic ventricular dilatation (PHVD) requiring neurosurgical intervention, and periventricular hemorrhagic infarction (PVHI), and b) severe cerebellar lesions, defined as large unilateral or bilateral cerebellar hemorrhages. CGM volume was significantly associated with PMA at scan, even after adjustment for ICV. Therefore, relative CGM volume was additionally adjusted for PMA, by calculating the CGM-ICV-PMA ratio. Regression analyses were carried out in R and interactions between brain volumes and severe brain lesions were investigated. Subjects with missing outcome data were omitted from the analyses. Coefficients with corresponding 95%-confidence intervals (95%-CI) were reported for the effect size of absolute brain tissue volume (Model a) or relative brain volumes (i.e., percentage of ICV) (Model b and Model c) or – applicable to CGM volume only – relative brain volume-PMA ratio (Model b† and Model c†). Because the design of this study was exploratory in nature - i.e., we aimed to identify brain structures related to neurodevelopmental outcome that would be relevant for future studies - a two-sided p-value of $<.05$ was considered statistically significant.

Table 1 Clinical characteristics

	N = 112 infants
GA (weeks)	28.4 ± 1.7
Birth weight (grams)	1128 ± 324
Birth weight z-score	0.23 ± 0.89
Sex (male), no (%)	61 (55)
Singleton, no (%)	82 (73)
Full course of antenatal corticosteroids[*], no (%)	80 (71)
Apgar score at 5 min	8 ± 1.5
Days of mechanical ventilation, median (range)	5 (0 – 42)[†]
Administration of postnatal hydrocortisone, no (%)	16 (14)[‡]
Bronchopulmonary dysplasia, no (%)	19 (17)[§]
Blood culture proven sepsis, no (%)	53 (47)
Necrotizing enterocolitis (all stages), no (%)	3 (3)
Cystic periventricular leukomalacia, no (%)	1 (1)
IVH grade I, no (%)	8 (7)
IVH grade II, no (%)	15 (13)
IVH grade III, no (%)	8 (7)
PVHI[±,#], no (%)	3 (3)
PHVD requiring neurosurgical intervention[#], no (%)	2 (2)
Punctate cerebellar lesions, no (%)	12 (11)
Large unilateral/bilateral cerebellar lesions[±], no (%)	0/2 (2)
No white matter injury[$], no (%)	11 (10)
Mild white matter injury[$], no (%)	87 (78)
Moderate white matter injury[$], no (%)	14 (13)

Abbreviations: GA = gestational age; IVH = intraventricular hemorrhage; no = number; PVHI = periventricular hemorrhagic infarction; PHVD = post-hemorrhagic ventricular dilatation. Data are displayed as mean, standard deviation (±) unless indicated otherwise. [*]Full course of antenatal corticosteroids was defined as administration of betamethasone >48 hours prior to delivery; [$]white matter injury was assessed according to Woodward *et al.* [16]. [±]One infant showed a combination of large cerebellar hemorrhages and a small PVHI and was therefore counted twice. [#]Another infant displayed PVHI with subsequent porencephaly and PHVD requiring a ventriculoperitoneal drain. Data unavailable for: [‡]=1, [†]=2, [§]=19.

RESULTS

124 preterm infants were eligible for inclusion in the present study. The final sample consisted of 112 preterm infants born <31 weeks GA; reasons for exclusion included motion artifacts (n=3), acquisition of MR images in the coronal plane (n=6), MR imaging beyond 44 weeks PMA (n=2), and unsuccessful segmentation because the brain was not completely included in the field of view (n=1). There were no significant differences in GA, BW, gender, or illness severity (including days of mechanical ventilation, patent ductus arteriosus requiring treatment, or blood culture proven sepsis) between infants included in this study and those excluded. Clinical characteristics of the study population are presented in Table 1. Seven infants (6%) developed severe brain injury during their NICU course, including PVHI (n=3); with subsequent porencephaly in two, periventricular leukomalacia with focal WM cysts (n=1), PHVD requiring neurosurgical intervention (n=2), and large cerebellar hemorrhages (n=2). Two infants showed a combination of lesions; PVHI and severe cerebellar injury in one and PVHI with subsequent porencephaly and PHVD requiring a ventriculoperitoneal drain in the other. None of the infants displayed severe white matter abnormalities according to Woodward [16] on MRI at TEA.

Neurodevelopmental outcome

Table 2 shows the results of neurodevelopmental outcome using four age-specific assessment tools at three different time points. Outcome data were available for all children at two years CA (n=112) and 98 children (88%) at age 3.5 years. At age 5.5, MABC-2 scores were obtained in 85 children (76%) and WPPSI-III scores in 44 children (39%). There were no significant differences in clinical characteristics including sex, GA, BW and illness severity between children that were followed through age 5.5 years (n=85) and children that were lost-to-follow-up between age two and 5.5 years. However, preterm born children that underwent the WPPSI-III assessment were younger (GA 27.4±1.4 weeks versus 29.1±1.5 weeks, p<.001), smaller (BW 931±189 grams versus 1255±330 grams, p<.001), and more severely ill (days of mechanical ventilation 9.9±6.8 days versus 4.6±7.3 days, p<.001; blood culture proven sepsis 28 (64%) versus 25 (37%), p=.007; and patent ductus arteriosus requiring treatment 21 (48%) versus 17 (25%), p=.02) compared with children that were not evaluated by a child psychologist. One child developed mild bilateral dyskinetic cerebral palsy (GMFCS level 1) during follow-up and one child was diagnosed with attention deficit hyperactivity disorder by a child psychiatrist. Another three children were being evaluated because of suspected attention deficits and/or pervasive developmental disorder.

Table 2 Neurodevelopmental outcome

	Outcome assessment	Score below -1 SD (%)
BSITD-III, age at time of assessment (months CA) (n = 112)	24.2 ± 0.6	n/a
Cognitive composite score	102 ± 12	4 (4)
Fine motor scaled score	13 ± 3	n/a
Gross motor scaled score	10 ± 2	n/a
Total motor composite	107 ± 12	2 (2)
GMDS, age at time of assessment (years) (n = 98)	3.6 ± 0.2	n/a
DQ	98 ± 7	9 (9)
WPPSI-III, age at time of assessment (years) (n = 44)	5.6 ± 0.3	n/a
Full-scale IQ	95 ± 16	10 (23)
Verbal IQ	100 ± 17	9 (19)
Performance IQ	95 ± 15	8 (17)
Processing speed	88 ± 12	14 (34)
MABC-2, age at time of assessment (years) (n = 85)	5.5 ± 0.3	n/a
Standard score	7 ± 3	34 (41)
IQ <70[†]	2 (5)	n/a
Repeated a grade at school[*‡], no (%)	15 (17)	n/a
Receives special educational needs[*‡], no (%)	10 (11)	n/a
Receives educational assistance[*‡], no (%)	8 (9)	n/a
Cerebral palsy	1 (1)	n/a
Cortical visual impairment[*‡], no (%)	1 (1)	n/a
Hearing impairment requiring aids[*‡], no (%)	3 (3)	n/a

Abbreviations: CA = corrected age; n/a = not applicable. Data are displayed as mean, standard deviation (±) unless indicated otherwise. [*]assessed at age 5.5 years. [†]Full-scale IQ, verbal IQ and/or performance IQ <70. [‡]Data available for 90 children.

Brain tissue volumes and neurodevelopmental outcome

In what follows, we will describe the findings on the relationship between brain volumes at term and neurodevelopmental outcome. Results are outlined in Table 3. Ventricular volumes were negatively associated with all included subscales of the BSITD-III at 24 months CA (absolute volumes: cognitive composite score p=.01, fine motor scaled score p=.01, gross motor score p=.01; adjusted for ICV and maternal education level: cognitive composite score p=.006, fine motor scaled score: p=.007, gross motor scaled score: p=.01), DQ at age 3.5 years (p=.01) (absolute volumes p=.01; adjusted for ICV and maternal education level p=0.01), and with processing speed at age 5.5 years (p=.02; p=.004 respectively). No significant relationship was found between ventricular volumes and IQ-scores or MABC-2 scores at age 5.5 years. After additional adjustment for severe brain lesions, the association with cognitive composite score, fine motor scaled score (p=.04) at two years CA (p=.02) and with processing speed at age 5.5 years persisted (p=.01). Associations with gross motor scaled score at 24 months CA and DQ at 3.5 years were attenuated and no longer statistically significant. UWM volume was related to fine and gross motor performance at 24 months CA (absolute UWM volume: fine motor scaled score p=.002, gross motor scaled score p=.009) and to processing speed at age 5.5 (p=.009). These findings persisted after controlling for ICV, maternal education level and severe brain lesions, except for the association with fine motor outcome. CGM volume demonstrated an unexpected inverse relationship with all included subscales of the BSITD-III at two years CA in multivariable analysis (cognitive composite score p=.009, fine motor scaled score p=.002, gross motor score p<.001) and with DQ at age 3.5 years (p=.03), although effect sizes were small. The inverse association with cognition at both time-points was mediated by severe brain lesions (Table 3). Cerebellar volume showed a positive relation with cognition at 24 months CA and at age 3.5. The effect appeared to be partially mediated by severe injury, as adjustment for severe brain lesions attenuated these findings (cognitive composite score p=.06, DQ p=.03). No significant interaction was observed between brain lesions and brain volumes. Absolute and relative volumes of BGT and cerebrospinal fluid (CSF) were not related to neurodevelopmental outcome. Additional adjustments were therefore not performed. No correlations were found between brain volumes at TEA and intellectual performance or MABC-2 scores at age 5.5.

Table 3 Relationship between brain tissue volumes and neurodevelopmental outcome

Coefficient		Cognitive composite score	Fine motor scaled score	Gross motor scaled score	Developmental Quotient	Processing speed	Verbal IQ	Performance IQ	Movement ABC
	24 months CA			Age 3.5 years	Age 5.5 years				
CGM	Model[a]	-0.03 (-0.14-0.07)	-0.007 (-0.03-0.02)	-0.02* (-0.03- -0.0002)	-0.02 (-0.08-0.05)	0.04 (-0.12-0.20)	0.05 (-0.16-0.26)	0 (-0.18-0.18)	0.01 (-0.02-0.04)
	Model[b†]	-1.50* (-2.62- -0.40)	-0.40** (-0.65- -0.15)	-0.33** (-0.49- -0.16)	-0.84* (-1.57- -0.10)	-0.65 (-2.75-1.45)	1.22 (-1.55-3.98)	-1.26 (-3.66-1.14)	-0.31 (-0.64-0.01)
	Model[c†]	-1.25 (-2.46- -0.04)*	-0.32* (-0.59- -0.05)	-0.27** (-0.45- -0.10)	-0.53 (-1.34-0.28)	0.58 (-1.74-2.89)	0.47 (-2.65-3.59)	-0.20 (-2.86-2.45)	-0.16 (-0.51-0.18)
Ventricles	Model[a]	-0.59* (-1.04- -0.14)	-0.13* (-0.23- -0.03)	-0.09* (-0.16- -0.02)	-0.33* (-0.63- -0.04)	-0.78* (-1.39- -0.16)	0.19 (-0.61-1.0)	-0.61 (-1.29-0.07)	-0.03 (-0.16-0.1)
	Model[b]	-3.64** (-6.2- -1.08)	-0.81** (-1.40- -0.23)	-0.50* (-0.89- -0.11)	-2.22* (-3.90- -0.53)	-6.41** (-10.5- -2.32)	0.15 (-5.14-5.45)	-3.49 (-7.98-0.99)	-0.59 (-1.37-0.18)
	Model[c]	-3.13* (-5.83- -0.44)	-0.64* (-1.25- -0.03)	-0.36 (-0.77-0.04)	-1.65 (-3.44-0.14)	-5.64* (-9.73- -1.55)	-1.33 (-6.98-4.31)	-2.10 (-6.86-2.66)	-0.41 (-1.16-0.35)
Cerebellum	Model[a]	0.48 (-0.02-0.10)	0.10 (-0.006-0.21)	0.03 (-0.05-0.10)	0.34* (0.04-0.64)	0.22 (-0.63-1.07)	0.13 (-1.01-1.28)	0.54 (-0.45-1.54)	0.13 (-0.008-0.28)
	Model[b]	5.54* (1.32-9.75)	0.82 (-0.15-1.80)	0.30 (-0.36-0.96)	4.04** (1.58-6.51)	1.73 (-10.98-14.4)	-0.36 (-16.4-15.7)	7.62 (-6.19-21.4)	1.03 (-0.94-2.99)
	Model[c]	4.53 (-0.22-9.23)	0.33 (-0.76-1.41)	-0.15 (-0.86-0.57)	3.24* (0.46-6.02)	-6.99 (-21.0-7.04)	4.23 (-13.0-21.4)	2.85 (-11.8-17.5)	-0.16 (-2.24-1.92)
UWM	Model[a]	0.10 (-0.01-0.21)	0.04 (0.02-0.06)**	0.02** (0.006-0.04)	0.02 (-0.05-0.09)	0.27** (0.08-0.46)	-0.009 (-0.28-0.27)	0.06 (-0.18-0.30)	0.02 (-0.01-0.05)
	Model[b]	0.26 (-0.41-0.94)	0.15 (-0.004-0.30)	0.13* (0.03-0.22)	0.03 (-0.42-0.47)	1.5* (0.30-2.66)	0.02 (-1.52-1.56)	-0.28 (-1.62-1.06)	-0.05 (-0.25-0.15)
	Model[c]	0.20 (-0.47-0.87)	0.13 (-0.02-0.28)	0.11* (0.02-0.21)	-0.03 (-0.47-0.40)	1.2* (0.05-2.4)	0.17 (-1.37-1.71)	-0.48 (-1.78-0.83)	-0.11 (-0.31-0.09)
BGT	Model[a]	0.88 (-0.13-1.89)	0.13 (-0.09-0.35)	0.06 (-0.9-0.21)	0.30 (-0.32-0.93)	0.74 (-0.89-2.37)	-0.67 (-2.83-1.48)	0.78 (-1.10-2.66)	0.32 (0.06-0.59)
	Model[b]	3.59 (-4.44-11.61)	-0.40 (-2.23-1.43)	0.82 (-0.39-2.03)	1.62 (-3.33-6.58)	-4.57 (-20.5-11.3)	-13.5 (-33.7-6.7)	2.87 (-15.1-20.8)	0.60 (-1.66-2.86)
CSF	Model[a]	0.01 (-0.12-0.14)	0 (-0.03-0.03)	-0.01 (-0.03-0.006)	0.004 (-0.08-0.08)	-0.004 (-0.20-0.19)	-0.01 (-0.27-0.23)	0.10 (-0.11-0.32)	0.03 (-0.003-0.06)
	Model[b]	0.15 (-0.88-1.17)	-0.06 (-0.29-0.17)	-0.08 (-0.23-0.08)	0.10 (-0.52-0.72)	-1.62 (-3.58-0.34)	-0.05 (-2.26-2.16)	1.42 (-0.46-3.29)	0.16 (-0.10-0.42)

Model[a] (univariable analyses): absolute brain volume; Model[b] (multivariable analyses): brain volume adjusted for ICV and maternal education level; Model[c] (multivariate analyses): brain volume adjusted for ICV, maternal education level, and severe brain lesions. Coefficients and corresponding 95%-CIs are displayed for absolute brain volume (Model[a]) and relative brain volume (i.e., percentage of ICV) (Model[b] and Model[c]); †CGM-volume was additionally adjusted for PMA at scan in multivariable analyses, using the CGM-volume-ICV-PMA ratio because PMA at scan was significantly related to CGM-volume even after adjustment for ICV; *p<.05, **p<.01, ***p<.001.

DISCUSSION

In the present study, the relationship between brain tissue volumes at TEA and long-term neurodevelopmental outcome in preterm born children was evaluated. Larger ventricular volumes appeared to have the most significant and consistent adverse effect, because ventricular volumes were negatively related to all aspects of neurodevelopment and associations persisted through early school-age. UWM volume showed a positive association with motor performance at 24 months CA and with processing speed at age 5.5 years. Larger cerebellar volume was related to better cognitive performance in early childhood, but not at age 5.5 years and the relationship seemed to be partially mediated by brain injury. Interestingly, we found an inverse relationship between CGM volume and neurodevelopment at age two and 3.5 years. The association with cognition could be attributed to brain injury. The observation of larger CGM volume at TEA to be related to poorer neurodevelopmental outcome in infancy and early childhood is in contrast with earlier reports on brain volume alterations in preterm infants, although regional increases in CGM volume were previously reported [22-24]. Nosarti and colleagues found larger CGM volume in the temporal lobe in cognitively impaired preterm adolescents. Subgroup analysis revealed largest alterations in both cortical and subcortical volumes in preterm individuals with severe WM injury, defined as periventricular hemorrhagic infarction or ventricular dilatation on cranial ultrasound. The authors reasoned that severe insults to the developing preterm brain would lead to extensive plastic processes in order to compensate for the effects of injury-related cell loss. These newly generated neurons and synapses may fail to connect and function optimally because of alterations in synaptic pruning [24]. Studies assessing brain volumes at TEA in relation to neurodevelopmental outcome in preterm infants have largely confirmed perinatal findings of reduced CGM compared with term-born infants [8,25,26]. Inder demonstrated smaller CGM volumes in preterm infants with moderate to severe disabilities at 12 months CA [25]. However, the segmentation method used was not optimized to separate cerebellar volume from CGM volume in the cerebellar region, which may have led to non-optimal classification of CGM volume [27]. Regardless, Skiöld recently confirmed a positive association between CGM volume and both language and motor composite scores at 30 months CA, using an automatic segmentation method that differentiated between CGM and cerebellar volumes [8].

Visual assessment of the border between CGM and UWM of infants with larger CGM volumes showed a gradual transition of the signal intensity of CGM to UWM on both T2 and T1-weighted images instead of a clear border. This so-called 'blurry cortex'

phenomenon may reflect migration disturbances of axons emerging from the subplate zone and of late-migrating neurons that enter the cortex between 24-32 weeks of gestation and travel to its upper layers. The latter constitute 20-30% of all cortical neurons and play a key role in coordination and integration of cortical functions [1]. Consequently, disruption of their migration trajectory may exert profound effects on cognitive functioning. The subplate is a transient zone of neurons, axons, and glial cells located between the cortical plate and intermediate zone during fetal brain development. It serves as a reservoir for 'waiting' cortical afferents that form temporary synaptic circuits, before growing into the cortical plate where they reach their final target [2,28-30]. Injury to the subplate may disrupt thalamocortical and corticocortical pathfinding, thereby resulting in incomplete migration of axons into the cortex. Differences in cell patterning are likely to influence MRI signal intensities in the gray and WM border and the subsequent, algorithm-dependent classification of voxels in this area. Alternatively, the phenomenon of poorer outcome with increasing CGM volume as observed in our study may be due to cortical overgrowth, although TEA might be somewhat early to observe such an effect. Increases in CGM volume have been described in fetuses and neonates with isolated ventriculomegaly, in children with neurofibromatosis type 1, and in children with autism [31-35]. It has been hypothesized that cortical overgrowth may be due to lack of apoptosis in the cortical layers. Apoptosis is an integral part of normal cortical development that occurs during late gestation and in the postnatal period; a disruption thereof may have long-lasting consequences for cortical development.

Van Kooij *et al.* previously reported on the association between cerebellar volume, metabolism, and cognitive outcome at two years CA in this cohort of preterm infants [9].Here we show that these findings persist over time and are associated with severe cerebellar injury. The impact of cerebellar injury on cognitive development in preterm infants is increasingly being recognized [7,36,37]. Several studies have demonstrated associations between cerebellar lesions and neurodevelopmental deficits, such as cognitive, behavioral and language delays, and neurologic abnormalities [36-38]. These sequelae seem to be limited to large cerebellar lesions with detrimental effects on cerebellar growth and development that are readily visible on cranial ultrasound; the consequences of small cerebellar lesions have been demonstrated to be milder [36-39]. Our data support these findings. The exact mechanisms of how cerebellar injury and growth failure may affect cognition have yet to be elucidated. Disruption of the interplay between cerebellar and cerebral connections has been postulated to be of key relevance [2].

Our longitudinal follow-up data delineate the concept of 'growing into deficits', that is well known in the preterm population [40]. The number of infants with neurodevelopmental impairments (defined as outcome scores more than one SD below the population mean) increased as a function of age. Mild to moderate impairments become more apparent when functional demands increase, which specifically holds true for cognitive skills. Subtle impairments affecting higher cognitive functioning may not yet be manifest at two years but may lead to educational difficulties at school age. BSITD-III scores have been reported to correlate only modestly with measures of neurodevelopment at later time-points, including IQ, emphasizing the importance of long-term neurodevelopmental follow-up beyond early childhood [41,42].

This study has several limitations that need to be addressed. First, we were unable to include healthy term controls for comparison owing to ethical constraints. Second, our automatic segmentation method was unable to accurately segment MWM volume and this structure was therefore excluded from the analysis. Furthermore, the type of (MRI) measurements and level of detail thereof did not allow evaluation of the underlying mechanism of the observed association between larger CGM volume and poorer neurodevelopmental outcome in infancy and early childhood. These findings could reflect a true biological phenomenon of disrupted migration at the CGM-UWM boundaries [2,28-30], cortical overgrowth [31-35] or they could be the result of difficulties segmenting blurry cortical boundaries. Finally, the cohort studied here represents a relatively healthy sample of the preterm population; there were no infants with a GA <25 weeks and only 6% exhibited severe brain injury. At two years CA, only four children scored outside the normal range for cognitive performance (<85) and an even smaller number of children displayed a motor composite score <85 (73 and 82 respectively in two children). Dutch normative values for the BSITD-III have only recently been validated and outcome scores were consequently calculated based on American reference standards. Studies performed in Australia and the United Kingdom have reported an overestimation of neurodevelopmental performance when using the BSITD-III [43]. A similar observation was made in this study. Finally, WPPSI-III scores were only available for a small and selective proportion of preterm children due to the design of our follow-up program; all preterm born children are followed-up until age 5.5 as part of routine clinical care. Yet, psychological assessment is only performed in infants born <28 weeks and/or if clinically indicated. Additional long-term follow-up studies should therefore be undertaken to corroborate our findings. A comprehensive follow-up study at age 9-10 years has been scheduled for this cohort.

CONCLUSION

In conclusion, brain volumes of the ventricles and UWM are related to neurodevelopment through early school age, with larger UWM volumes and smaller ventricular volumes being related to better motor performance, cognition and improved processing speed. CGM volume demonstrated a negative association with motor performance and cognition at 24 months and DQ at age 3.5 years, while cerebellar volume was positively related to cognition at age two and 3.5 years. The latter associations with cognition were partially mediated by brain injury. The notion of larger CGM volumes to be related to poorer neurodevelopmental outcome scores may reflect migration disturbances in CGM-UWM boundaries and warrants further investigation. Our findings suggest that brain volumes may serve as early imaging markers for neurodevelopmental outcome in preterm infants and brain tissue segmentation may therefore offer a valuable tool for evaluation of future neuroprotective strategies in the preterm population.

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Once you make a decision, the universe conspires to make it happen

Ralph W. Emerson

CHAPTER 6

Brain volumes, cortical maturation and early neurodevelopment are comparable in extremely preterm twins and singletons

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HIGHLIGHTS

- extremely preterm multiple birth is not associated with impaired morphological brain development in the neonatal period
- preterm twins in our study did not display poorer cognitive functioning and motor performance in late infancy than preterm singletons
- preterm infants manifest significantly smaller white matter volumes, larger cerebrospinal fluid volumes and a reduced gyrification index compared with full-term neonates
- consistent with chapter five, our contemporary population of extremely preterm infants has relatively favorable outcome at age two years without severe mental disabilities

ABSTRACT

Preterm twins are considered at risk of aberrant brain development. Yet, it remains unclear whether developmental adversity is intrinsically related to twin pregnancy or mainly accounted for by lower gestational age (GA) and birth weight (BW), which are associated with an increased risk of neonatal morbidity. Here, we tested the hypothesis that extremely preterm twins exhibit delayed brain development and poorer neurodevelopmental outcome at age two years compared with preterm singletons. T2-weighted images of 240 extremely preterm infants (mean GA 26.4 SD 1.0 weeks, BW 879 SD 182 grams; 31 individuals from monochorionic (MC) twin pairs, 51 infants from dichorionic (DC) twins, 158 singletons) were segmented using an automatic brain tissue segmentation method. Next, brain volumes and parameters of cortical morphology were computed, including cortical gray matter volume, unmyelinated white matter volume (UWM), cerebellar volume, ventricles and cerebrospinal fluid volume (CSF), gyrification index, mean curvature and surface of the neonatal cortex. Cognitive and motor functioning were formally assessed using the Bayley Scales of Infant and Toddler Development, Third Edition at two years corrected age. Preterm twins were matched with preterm singletons by gender, GA and BW. A reference group of typically developing full-term infants was added (n=15). Comparisons of brain metrics and neurodevelopmental outcome measures were made between preterm twins and singletons using paired t-tests. Brain metrics and neurodevelopment of preterm twins (MC and DC), singletons and full-term infants were compared using ANOVA. There were no significant differences in brain volumes, cortical morphology, cognitive functioning and motor performance between surviving preterm twins and singletons. Preterm infants displayed significantly increased CSF volume ($p<.001$), decreased relative UWM volume ($p<.001$) and a reduced gyrification index ($p=.005$) compared with full-term neonates. In conclusion, morphological brain development at term-equivalent age and early neurodevelopmental outcome do not differ between preterm twins and singletons with similar GA and BW. Our findings suggest that preterm multiple birth is not fundamentally associated with impaired brain development.

Key words
preterm, twins, cortical morphology, brain volumes, neurodevelopment

INTRODUCTION

Preterm twins are postulated to be at risk of developmental deficits as a result of increased neonatal morbidity [1-3]. It remains unclear whether developmental adversity is primarily related to multiple birth in preterm infants or whether increased risk is mediated by lower gestational age (GA), lower birth weight (BW) and an increased risk of pre- and perinatal complications, which are frequently observed in extremely preterm twins [1,4,5]. It would thus be of interest to compare early trajectories of brain development in preterm twins and singletons, taking these factors into account.

To date, little is known about putative differential brain development in preterm twins. Scientific evidence on neurodevelopmental outcome is somewhat inconsistent, with some studies demonstrating increased risk of impairment in infancy and early school age years [1,2] while others reported no significant differences between preterm twins and singletons [3]. Comparing early brain development between preterm twins and singletons is highly relevant to neonatologists, pediatric neurologists and other health care providers involved in the care for these vulnerable infants. Early identification of infants at risk is pivotal for prognostication and to target therapies that may be rehabilitative or neuroprotective. Such comparisons would also be valuable to researchers interested in the heritability of neurodevelopmental processes. Twin studies have long been performed to determine heritability of traits. However, concerns have been raised about the generalizability of twin data to a population level because of fundamental differences in the intrauterine and family environment between twins and singletons [6-8]. These concerns may specifically hold true for *preterm* twins whose pregnancies are typically influenced by prenatal factors more common in or even unique to twin pregnancies. Such factors and complications include fetal growth restriction, medically assisted reproduction and twin-twin transfusion syndrome.

Here, we investigated brain morphology in terms of brain volumes and measures of cortical morphology at term-equivalent age (TEA) in preterm twins that were matched to singletons by GA, BW and sex. A reference group of full-term infants was added for comparison. As a secondary objective, we evaluated neurodevelopmental outcome at two years corrected age. We hypothesized that preterm twins would display smaller brain volumes, more cerebrospinal fluid (CSF) and delayed cortical maturation at TEA compared with preterm singletons. Secondly, we postulated cognitive and motor functioning at two years corrected age to be poorer in preterm twins than in singletons.

METHODS

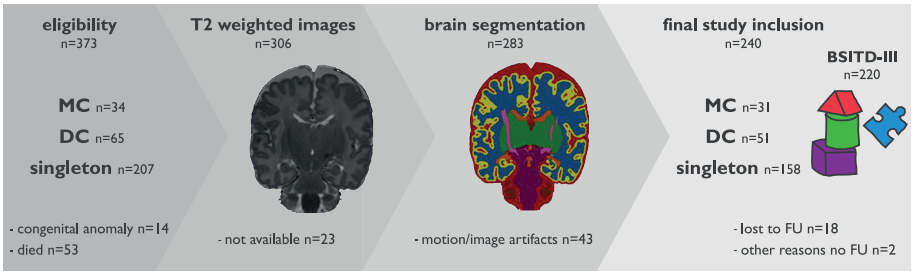
Study population

306 very preterm infants born between May 2008 and February 2015 were eligible for inclusion (n=34 individuals from monochorionic (MC) twin pairs, n=65 infants from dichorionic (DC) twins and n=207 singletons). During the study period a total number of 373 preterm infants were admitted to the Neonatal Intensive Care Unit (NICU) of the Wilhelmina Children's Hospital, University Medical Center Utrecht, The Netherlands. Infants that died during the neonatal period, infants with congenital anomalies, inborn errors of metabolism, congenital infections of the central nervous system and/or genetic disorders and infants for whom a standard-of-care MRI at TEA was not available were excluded (Figure 1). Infants were also excluded if the T2-weighted image and/or the related segmentation dataset (see section below) contained severe motion or image artifacts (n=43) or if the T2-data could not be segmented (e.g., owing to angulation, data conversion errors or because the field of view did not completely cover the neonatal brain) (n=23), which resulted in a final sample of 240 preterm infants (31 individuals from MC twin pairs, 51 infants from DC twins and 158 singletons). Twins were matched to eligible preterm singletons that met the same inclusion criteria by sex, GA and BW. Finally, a reference group of full-term infants (n=15) without focal brain injury was added. Full-term infants were scanned for clinical reasons; scan indications are outlined in Supplemental Table 1. The Institutional Review Board (IRB) of the University Medical Center Utrecht, The Netherlands gave approval for use of the clinically acquired data for study purposes. Since clinically obtained data were employed, written informed parental consent for participation in the study was waived by the local IRB.

MRI acquisition and image analysis

Imaging data were obtained using an eight-channel head coil on a Philips 3 tesla MR system (Philips Healthcare, Best, Netherlands). Preterm infants were sedated using oral chloralhydrate (50-60 mg/kg) 15 minutes prior to scanning. Full-term infants were sedated with 50-60 mg/kg oral chloralhydrate (n=8) or a combination of 2 mg/kg pethidine, 0.5 mg/kg chlorpromazine, and 0.5 mg/kg promethazine per intramuscular injection five minutes before the scanning session (n=5). Sedation was administered at the discretion of the attending physician. Two full-term infants did not receive sedative medication and were scanned during natural sleep. Infants were placed in a vacuum fixation pillow (Kohlbrat an Bunz GmbH, Radstadt, Austria) and two pairs of earmuffs were applied for hearing protection (Natus Medical Inc. San Carlos, CA, USA;

Figure 1 Flowchart



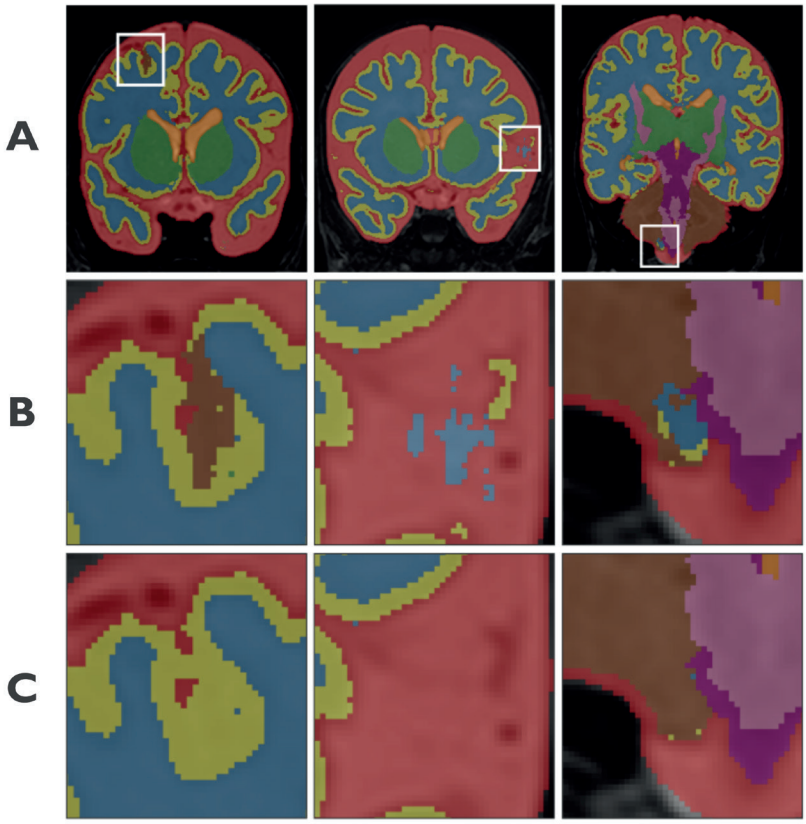
Flowchart of study inclusion. MC = monochorionic, DC = dichorionic. Left panel shows all eligible infants and reasons for exclusion. Middle left panel shows a representative example of a T2-weighted image as acquired on a 3T system at term-equivalent age and the number of infants for whom T2-weighted imaging was available. Images were automatically segmented into eight tissue classes as described in the Methods sections (middle right panel). Segmentation results were visually inspected to assure data quality. Datasets with severe motion artifacts or image artifacts (~ >10% voxel misclassification) were excluded. Remaining segmentation results were manually edited in case of minor voxel misclassification. Right panel shows the final study sample. Neurodevelopmental outcome at two years corrected age assessed using the Bayley Scales of Infant and Toddler Development, Third Edition was available for 220 infants.

Em's 4 Kids LLC, Culver City, CA, USA). A neonatologist or an experienced physician assistant was present throughout the examination and heart rate, transcutaneous oxygen saturation and respiratory rate were continuously monitored while the infant was breathing spontaneously.

During each scanning session, three-dimensional T2-weighted images were acquired in the coronal plane. Details of the scanning protocol are listed in the report by Išgum *et al*. [9]. Coronal T2 weighted images were automatically segmented into eight tissue classes (Figure 2) - i.e. cortical gray matter (CGM), basal ganglia and thalami (BGT), cerebellum, white matter that displayed low signal intensity on T2-weighted images and was therefore considered (largely) unmyelinated (UWM), myelinated white matter, (i.e., high signal white matter), brainstem, ventricles and CSF in the extracerebral space - using a deep learning method as described in detail in the report by Moeskops *et al*. [10]. This method accurately segments all brain structures (Dice coefficients - reflecting the overlap with manual reference segmentations - 0.78-0.93), except for myelinated

white matter (Dice coefficient 0.56). Myelinated white matter was therefore excluded from the analyses. Automatic segmentation results were visually inspected and manually edited in case of minor voxel misclassification (<10% of the dataset based on visual assessment). A representative example of automatic segmentation results and manual edits are provided in Figure 2.

Figure 2 Segmentation results



Coronal T2 weighted images were segmented into cortical gray matter (yellow), basal ganglia and thalami (green), cerebellum (brown), unmyelinated white matter (blue), myelinated white matter (light purple), ventricles (orange), brainstem (dark purple) and cerebrospinal fluid surrounding the brain (red) [10]. Panel A shows a representative example of automatic segmentation results. Panel B illustrates an example of voxel misclassification. Panel C delineates the final result of a manually edited segmentation.

Extracerebral CSF volume and ventricle volumes were combined in the analyses (from here on referred to as CSF). Cerebellar volume, total intracranial volume, CGM, UWM, BGT and CSF volume were considered relevant for analyses with respect to the hypotheses. Relative brain volumes were computed as the percentage of intracranial volume for each brain structure. Measures of cortical morphology included the gyrification index, cortical thickness and inner surface area. Gyrification index describes the level of folding of the area covering the border between the CGM and UWM segmentation. Computational methods are described by Moeskops *et al*. [11].

Neurodevelopmental outcome assessment

All preterm infants were included in a standard-of-care neurodevelopmental follow-up program and cognitive and motor functioning were formally assessed at two years corrected age using the Bayley Scales of Infant and Toddler Development, Third edition (BSITD-III) according to Dutch normative scores (cognitive composite score: mean=100, SD 15; fine and gross motor scaled score: mean, 10 SD 3) [12]. Follow-up at two years corrected age was available for n=75/82 twins (27 MC and 48 DC) and n=145/158 singletons (Figure 1). Only nine full-term infants were enrolled in a long-term follow-up program as clinically indicated at the discretion of the attending physician and eight infants had outcome scores within the normal range. One child was not cooperative enough for the BSITD-III assessment to be completed because of hospital-related anxiety. This child had scores within the normal range at age one year. In the remaining six full-term infants, neurodevelopmental follow-up was not indicated because they were considered typically developing infants.

Statistical analysis

Data analyses were performed using SPSS (version 22.0, IBM Corp, Armonk, NY). Preterm twins were automatically matched to eligible singletons using the following matching criteria: sex, BW (±10%) and GA (±7 days). Preterm twin A of twin pair one was thus matched to a preterm singleton of the same sex, similar BW and GA. Preterm twin B of twin pair one was matched to another preterm singleton according to the same criteria. Automatic matching was performed using SPSS and successfully performed in n=80 infants. Remaining infants (n=2) were manually matched.

Exploratory analyses were performed to test the distribution of continuous variables. Data are reported as mean and standard deviation (±) for normally distributed variables and median and range for non-normally distributed data. A chi square test was used for nominal and ordinal variables. Subsequent analyses were performed using paired

t-tests and ANOVA if data were normally distributed. When the assumption of normality was violated, related non-parametric tests were performed (i.e., Wilcoxon signed rank test and Kruskal Wallis test). A paired t-test was performed to evaluate differences in brain volumes and measures of cortical morphology between preterm twins and matched singletons. Next, an ANOVA was performed to evaluate differences in brain metrics between MC twins, DC twins, singletons and full-term infants. Bonferroni post hoc analysis was performed to identify differences between individual groups.

Neurodevelopmental outcome at two years corrected age was compared between the groups using a paired t-test for the matched samples and an ANOVA for all four groups. Outcome measures included the cognitive composite score, fine and gross motor scaled score of the BSITD-III. Neurodevelopmental outcome data of all eligible preterm infants, i.e., preterm twins and singletons with and without available segmentation data, were evaluated in an additional analysis employing an ANOVA (from here on referred to as 'all infants'). Missing data were omitted from the analyses and p-values were considered statistically significant at an alpha level <.05 (two-tailed tests).

RESULTS

First, prenatal characteristics and measures of postnatal morbidity were compared between preterm twins (n=31 MC, n=51 DC) and singletons n=158). The results are displayed in Table 1. BW was significantly different between the groups ($F(2,237)=3.5$, $p=.03$). Post hoc analysis revealed that singletons had significantly lower BW than DC twins ($p=.03$). Pre-eclampsia (PE) was significantly more frequently the reason for preterm birth in singletons than in twins ($X^2(2)=16.0$, $p<.001$). PE was strongly associated with BW (beta -165.1 confidence interval (CI) -226.6 - -103.6, $p<.001$) and may thus have accounted for lower BW in preterm singletons. Fetal growth restriction defined as being small for gestational age (BW $<10^{th}$ percentile) was not significantly different between the groups. Severe IVH was significantly more prevalent in MC twins ($X^2(2)=10.1$, $p=.006$). There were no significant differences in other pre- and postnatal characteristics between preterm twins and singletons (Table 1). Baseline comparisons of the matched pairs revealed no significant differences in GA, BW, sex, PE and severe IVH. Results are outlined in Supplemental Table 2.

Table 1 Clinical characteristics

	MC twins (n = 31)	DC twins (n = 51)	Singletons (n = 158)	Full-term infants (n = 15)
GA (weeks)	26.3 ± 1.0	26.4 ± 1.0	26.5 ± 1.1	40.0 ± 1.1***
BW (grams)	891 ± 187	935 ± 181	859 ± 179*	3509 ± 568***
SGA*, no (%)	2 (4)	2 (7)	18 (11)	1 (7)
Sex (female), no (%)	12 (39)	30 (59)	78 (49)	8 (53)
Full course of antenatal CCS, no (%)	18 (58)	33 (65)	93 (60)	-
Pre-eclampsia, no (%)	2 (7)	-	34 (22)***	-
Chorioamnionitis†, no (%)	9 (43)	24 (60)	67 (48)	-
Apgar score at 5 min	8 (4 - 10)	7 (3 - 10)	8 (2 - 10)	10 (8 - 10)***
Blood culture proven sepsis‡, no (%)	17 (55)	17 (33)	60 (38)	1 (7)*
Days of mechanical ventilation	11 (0 - 51)	10 (0 - 41)	6 (0 - 47)	1 (0 - 13)***
NEC Bell's stage > II, no (%)	1 (3)	6 (12)	12 (8)	1 (7)
PDA requiring treatment, no (%)	19 (61)	34 (67)	80 (51)	-
Administration of morphine, no (%)	20 (65)	34 (67)	90 (57)	7 (47)
Days of parenteral nutrition	15 (7 - 68)	14 (7 - 28)	13 (7 - 185)	-
BPD§, no (%)	11 (37)	13 (29)	44 (31)	-
ROP ≥ stage 2 with plus disease, no (%)	4 (13)	4 (8)	7 (5)	-
Duration NICU stay (days)	49 ± 16	41 ± 20	42 ± 18	5 (0 - 23)***
IVH grade I-II, no (%)	8 (26)	14 (28)	45 (29)	-
IVH grade III requiring treatment and grade IV, no (%)	4 (13)**	1 (2)	3 (2)	-
Moderate-severe WMI±, no (%)	4 (14)	9 (18)	17 (11)	-
Cerebellar hemorrhage#, no (%)	1 (3)	2 (3)	3 (2)	-
Postmenstrual age at time of MRI (weeks)	41.1 (40.4 - 43.3)	41.1 (40 - 43.3)	41.1 (38.9 - 45.9)	41.1 ± 1.1
Weight at time of MRI (grams)	3345 (2045 - 4040)	3466 ± 410	3373 ± 437	3591 ± 602

Abbreviations: BPD = bronchopulmonary dysplasia; IVH = intraventricular hemorrhage; CCS = corticosteroids; NEC = necrotizing enterocolitis; PDA = patent ductus arteriosus; ROP = retinopathy of prematurity. SGA = small for gestational age. Data are depicted in no (%) and mean, standard deviation (±) in case data are normally distributed or median, range otherwise. Significant difference at an alpha level of * p<.05, ** p<.01, *** p<.001. *SGA was defined as BW <10th percentile. †Diagnosis of chorioamnionitis was based on histopathological examination of the placenta if such investigation was available (n=200; 21 MC infants, 40 DC infants and 139 singletons). ‡Blood culture proven sepsis including coagulase negative staphylococcus sepsis. §BPD was defined as need for supplemental oxygen at 36 weeks postmenstrual age. ±White matter injury was assessed according to Woodward *et al.* [40]. #Cerebellar hemorrhage was defined as a large unilateral or bilateral hemorrhage (>4 mm).

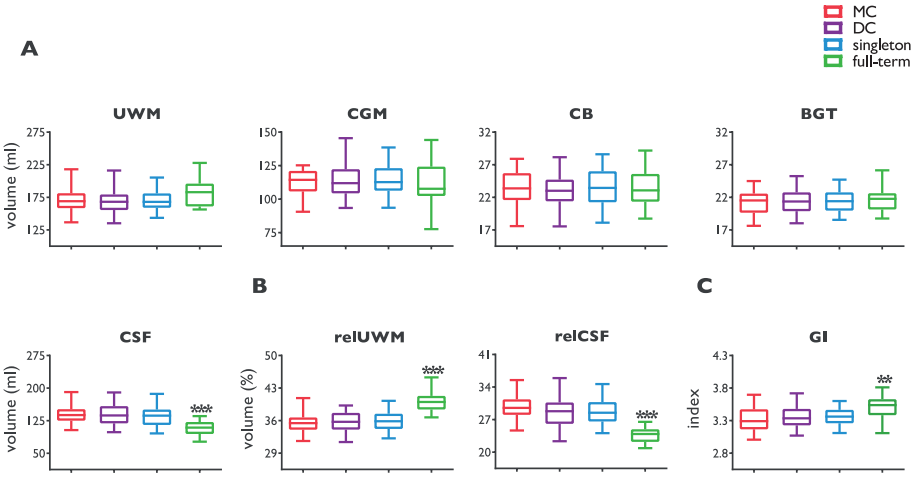
Next, we explored differences in postnatal mortality rates, incidence of congenital anomalies, BW, GA and sex between preterm twins and singletons in all preterm infants that were admitted to the NICU during the 7-year study period (n=373 infants). There were no significant differences in mortality rates or the occurrence of congenital anomalies between preterm twins and singletons. BW was significantly different between the preterm groups ($F(2,371)=3.9$, $p=.02$). Similar to the eligible cohort, post hoc analysis revealed that preterm singletons had significantly lower BW than DC twins. There were no significant differences in sex and GA.

Brain volume and cortical descriptors

Paired comparisons between preterm twins (n=82) and their singleton matches (n=82) revealed no significant differences in terms of brain volumes or measures of cortical morphology. Analyzing MC twins, DC twins, singletons and full-term infants by employing an ANOVA showed significant differences in both absolute and relative CSF volume (absolute $F=6.8$, $p<.001$; relative $F=13.3$, $p<.001$). Post hoc analyses revealed that full-term infants exhibited significantly smaller CSF volumes than all preterm groups but that preterm MC twins, DC twins and singletons did not display significant differences in CSF volumes. Significant differences were also noted for relative UWM volume ($F=14.9$, $p<.001$) and gyrification index ($F=4.5$, $p=.005$). Similarly, post hoc analyses revealed significantly larger relative UWM volumes and a greater gyrification index in full-term infants than in preterm infants and no significant differences in brain volumes or measures of cortical morphology between the preterm groups. There were no significant differences in cerebellum volumes, CGM volumes and BGT volumes between the groups. Results are outlined in Figure 3.

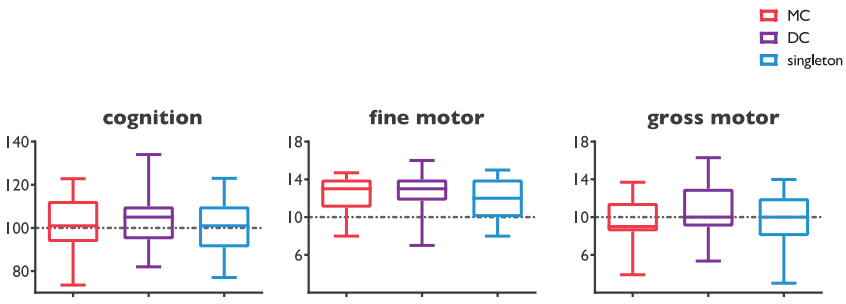
Because of the significant differences in PE between preterm twins and singletons, we investigated the relationship between PE and brain metrics in a univariable regression analysis. PE was significantly associated with smaller UWM volumes (beta -8.9 CI -15.8 - -2.0, $p=.01$) and larger relative cerebellum volumes (beta 0.3 CI 0.1-0.5, $p=.009$). PE was not associated with absolute cerebellum volume. Additionally, we analyzed the data excluding infants with PE (n=51 DC twins, n=29 MC twins and n=124 singletons). The latter subgroup analysis revealed that relative CGM volume was significantly different between preterm twins and singletons ($F=3.1$, $p=.047$). Post hoc analysis showed that MC twins had significantly smaller relative CGM volumes than singletons. No significant differences were observed for absolute brain volumes or cortical parameters.

Figure 3 Brain volumes and measures of cortical morphology



Boxplots indicate median and 95% confidence intervals of preterm MC twins, DC twins, preterm singletons and full-term infants. Panel A reveals absolute brain volumes of unmyelinated white matter (UWM), cortical gray matter volume (CGM), cerebellum (CB), basal ganglia and thalami (BGT) and cerebrospinal fluid in the ventricles and around the brain (CSF). Absolute CSF volumes were significantly different between the groups: preterm MC twins 141 SD 22 ml, DC twins 139 SD 27 ml, singletons infants 137 SD 26 ml versus full-term infants 108 SD17 ml. Panel B shows significant differences in relative brain volumes of UWM (relUWM) and CSF (relCSF) between preterm infants and full-term infants. relUWM: preterm MC twins 36% SD 2.4, DC twins 36% SD 2.5, singletons 36% SD 2.3 versus full-term infants 40% SD 2.3; relCSF: preterm MC twins 29% SD 3.0, DC twins 29% SD 3.3, singletons 29% SD 3.2 versus full-term infants 24% SD 1.7. Panel C portrays significant differences in the gyrification index (GI) between the study groups (MC twins 3.32 SD 0.19, DC twins 3.36 SD 0.17, singletons 3.36 SD 0.15 versus full-term infants 3.50 SD 0.19. Asterisks indicate significant differences at an alpha level of ** $p<.01$, *** $p<.001$.

Figure 4 Neurodevelopmental outcome



Cognitive composite score (left panel) and fine and gross motor scaled scores (middle and right panel) (median and 95% confidence interval) at two years corrected age. Dotted lines indicate normative mean (100 for cognitive composite score and 10 for fine and gross motor scaled score). There were no significant differences between preterm twins and preterm singletons.

Neurodevelopmental outcome

Preterm twins did not differ from preterm singletons with respect to cognitive functioning and fine and gross motor performance at two years corrected age. No significant differences in level of maternal education were observed between preterm twins and singletons, indicating that findings were not accounted for by maternal education level. Furthermore, cognitive and motor scores were not significantly different between preterm infants and full-term infants that were included in the neurodevelopmental follow-up program (Figure 4). Because neurodevelopmental outcome was only assessed if clinically indicated in full-term infants (n=8, 53%), these results may have been biased.

Additional analyses (ANOVA) of data from all eligible preterm infants for whom neurodevelopmental outcome data was available (n=275) - including infants without (good quality) segmentation data - revealed similar results. Gross motor outcome showed a trend-level significant difference: F=2.82, p=.06. Post-hoc analyses demonstrated that MC twins had lower scores (mean 9 SD 3, singletons mean 11 SD 3, DC twins mean 10 SD 3); these results were not significantly different either (p=.08). Finally, PE was not significantly associated with any of the neurodevelopmental outcome measures. Subgroup analysis excluding infants with PE yielded similar results.

DISCUSSION

In the present study, we observed no significant differences in brain volumes and measures of cortical morphology between preterm twins and singletons at TEA. In line with findings of morphological brain development, cognitive functioning and motor performance at two years corrected age were neither significantly different. Similar results were obtained when all preterm infants were evaluated who survived through age two years. PE was more prevalent in singleton pregnancies and was associated with lower BW, smaller UWM volumes and smaller relative cerebellum volumes, but not with neurodevelopmental outcome in late infancy. Results of our study thus suggest similar trajectories of morphological brain growth in the neonatal period and early neurodevelopment in preterm twins and singletons when GA and BW are comparable. Furthermore, they draw attention to the potential impact of the intrauterine environment on brain development. The latter is likely particularly substantive if it prompts growth restriction [13,14]. Since PE did not affect cognitive and motor functioning in late infancy in our study, its putative long-term effect on neurodevelopmental abilities remains to be awaited.

Previous large population-based cohort studies reported both increased risk of neurodevelopmental impairment in preterm twins and comparable neurodevelopment during early childhood in preterm twins and singletons [1-3]. Different from our study, these population-wide cohort studies were conducted 10-20 years ago. Poorer neurodevelopmental outcome in preterm twins as reported in a number of these studies [1,2] may thus have resulted from poorer perinatal care in the late 1990s and early 2000s as compared to present-day NICU standards. In the National Institute of Child Health and Human Development network study conducted in the United States between 1997 and 2005, over 50% of twin survivors were diagnosed with bronchopulmonary dysplasia - a severe pulmonary complication of prematurity and an overall estimate of clinical disease severity - and nearly 25% of preterm twins displayed signs of severe brain injury (IVH grade III-IV or periventricular leukomalacia) [1]. Conversely, in our study approximately one in three preterm twins developed bronchopulmonary dysplasia and <15% showed severe IVH. Our results may thus more closely represent neurodevelopmental trajectories in preterm twins born in contemporary NICU settings. Additionally, advances in antenatal care including intrauterine therapy in case of twin-twin transfusion syndrome have contributed substantially to improved outcomes in MC twins [15].

Comparisons of brain morphology between preterm twins and singletons are scarce. In a small MRI study including 12 preterm twins, measures of cortical morphology including cortical surface area, mean curvature and cortical volume were noted to be reduced shortly after birth and around TEA compared with preterm singletons [16]. We recently demonstrated multiple birth to be modestly related to smaller surface area of the insula and superior temporal sulcus in a cohort of preterm infants that showed substantial overlap with infants included in the present cohort [17]. The latter study focused on development of specific sulci. We now extend these measurements to a whole-brain level, demonstrating no significant differences in volumes of global brain structures and overall cortical morphology.

Notably, in our study absolute volumes of major brain structures did not differ between preterm infants scanned at TEA and full-term infants scanned shortly after birth. Yet, the composition of the neonatal brain was significantly different, with preterm infants showing more CSF and proportionately less UWM compared with their full-term peers. These findings correspond with a number of reports in literature that also observed discrete differences in CSF volumes at TEA without changes in volumes of cerebral structures between preterm infants and full-term newborns [18,19]. However, most studies have noted reduced brain volumes of major cerebral structures including UWM, cortical and deep gray matter [20-25]. These studies all included preterm infants that were born approximately 15 years ago and may thus not be a fair representation of present-day NICU graduates. Furthermore, weight at scan was significantly lower in preterm infants in a number of these reports [21,23,24], which may have accounted for some of the observed differences between preterm infants and full-term controls. In contrast, neonates included in our study displayed similar weight and age at scan and reflected a contemporary NICU population. Improved nutritional care as well as reduced illness severity may have contributed to greater weight gain and consequent head growth in our preterm infants [26-28], yet further research is needed to decipher which factors played a pivotal role in body growth. Finally, full-term infants included in our study were scanned because of suspected or increased risk of brain injury and although all infants at risk were typically developing in late infancy, one cannot rule out that some of the full-term infants displayed impaired brain morphology compared to healthy full-term neonates. Although brain volumes did not display significant differences between preterm and full-term infants in our study, the preterm cortex was significantly more immature as reflected in a reduced gyrification index. These findings confirm what has been observed in previous studies comparing cortical morphology of preterm infants and full-term controls. Cortical maturation has consistently been demonstrated to be

delayed in preterm infants at TEA [29-31], which suggests a specific vulnerability of cortical development to prematurity. The long-term impact of these deficits awaits further investigation and is likely to affect higher cognitive functions [32-35].

There are methodological limitations to be considered in our study. First, neurodevelopmental follow-up data was limited to the first two years after birth. Preterm born children have been noted to grow into their deficits with advancing age [36-38]. Long-term follow-up into childhood and adolescence is thus key to evaluate the full spectrum of potential neurodevelopmental impairments in these children as well as to provide definite answers on whether or not extremely preterm twins and singletons show differential trajectories throughout the developmental course of the lifespan. Second, we were not informed about intrauterine fetal demise in our population specifically. Nationwide numbers show that stillbirth is more prevalent in multiple pregnancy than in singleton pregnancy [39]. Our results are thus only applicable to surviving, liveborn extremely preterm infants. Finally, genetic data were not available in this sample. Identical twins could therefore not be distinguished from fraternal twins, which precluded quantification of heritability of brain morphology.

CONCLUSION

In conclusion, our findings suggest that preterm multiple birth is not associated with altered brain morphology at TEA in terms of brain volumes and cortical maturation in a contemporary setting. Consistent with the observation of comparable brain morphology at TEA, preterm twins and singletons displayed similar cognitive and motor functioning at two years corrected age. PE was significantly more prevalent in singleton pregnancies and associated with lower BW and smaller (relative) brain volumes. PE was not related to neurodevelopmental outcome at two years corrected age. Our findings support the notion that preterm twins and singletons exhibit similar trajectories of early morphological brain development and related neurodevelopment in late infancy when GA and BW are comparable. Furthermore, they underscore the putative impact of the intrauterine environment on brain development with its long-term effects remaining to be awaited.

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SUPPLEMENTAL MATERIALS

Supplemental Table 1

Reason for MRI	No of full-term infants
Neonatal surgery	9
Clinically suspected neonatal seizures without electrophysiological evidence of seizure on 48-hour electroencephalography recordings	2
Hyperbilirubinemia above exchange transfusion levels without clinical signs of kernicterus	1
Apneas of unknown origin	1
Inspiratory stridor	1
Extracranial hemangioma	1

Supplemental Table 2

	Matched pair 1 (n = 82)	Matched pair 2 (n = 82)
GA (weeks)	26.4 ± 1.0	26.4 ± 1.0
BW (grams)	911 ± 174	918 ± 184
Sex (female), no (%)	42 (51)	42 (51)
Pre-eclampsia, no (%)	2 (2)	8 (10)
IVH grade III requiring treatment and grade IV, no (%)	1 (1)	5 (6)

Abbreviations: IVH = intraventricular hemorrhage. Numbers are depicted in no (%) and mean, standard deviation (±). Significant difference at an alpha level of * p<.05, ** p<.01, *** p<.001.

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7

CHAPTER 7

Summarizing discussion and future directions

BACKGROUND

In this final chapter we will summarize the findings of the studies presented in this thesis and compare them against the background of relevant recent literature. In addition, we will shed light on methodological considerations of the employed imaging techniques in the context of the neonatal population. Finally, we will touch on future directions for connectome research in the developing brain during the earliest life stages and for the role of neonatal neuroimaging in neurodevelopmental outcome prognostication following preterm birth. In this final section, we will transcend the field of neonatal neuroimaging and propose some cross-modality investigations that we believe are particularly promising in this context.

Aim of this thesis

Neonatal MRI has been widely adopted to study early brain development, evaluate brain injury and identify early imaging markers for cognitive, motor, and behavioral outcomes later in life. The objective of this thesis was twofold. Firstly, we aimed to delineate early developmental processes of brain network formation. Secondly, we set out to investigate the brain-behavior interaction in preterm infants in order to identify neuroimaging markers for neurodevelopmental outcome in this population of vulnerable individuals who are at particular risk of developmental deficits across myriad domains. This array includes cognition, working memory, social skills, executive functioning, and attention that may each individually and collectively have protracted impact on the futures of these infants and those of their families. This final chapter is structured according to the two parts of this thesis. In the first part, we will discuss collated literature findings on early connectome development in light of functional brain circuit formation. Next, we will provide a comprehensive discussion of our prospective diffusion-weighted imaging (DWI) study into structural connectome development between 29-45 weeks postconceptional weeks, which spans the earliest phases following macroscale connectome genesis. Part two builds on these neonatal connectome explorations, linking its postulated relevant metrics to intelligence at school age in an attempt to address the research question that neonatal connectome organization is related to childhood cognitive functioning. Further exploring potentially predictive neonatal neuroimaging parameters, we extend the scope of our brain metrics to brain volumes and measures of cortical morphology, considering their association with neurodevelopmental outcome in (specific populations of) preterm infants.

Doesn't it really astonish you that you are this fantastically complex thing?
- Alan Watts -

Connectome development

In 2007, Fransson and colleagues published their paper on resting-state functional networks in the neonatal brain. Reporting on resting-state functional MRI (rs-fMRI) data in 12 preterm born infants scanned at term-equivalent age, they provided evidence for the early existence of resting state networks as reported in adults, including sensory and motor networks and a fragmented precursor of the default mode network [1]. The default mode network has received considerable attention from the neuroscience and neuroimaging community and is involved in internal modes of cognitive functioning (e.g., future planning, reminiscing, mind wandering and theory of mind), when the brain is disengaged from externally focused tasks [2]. The compelling notion that this brain network imperative for higher cognitive functions is already established in the early developing brain, has lent to alternative interpretations of how functional brain networks translate into function. Given that immature derivatives of the default mode network have been observed well before its supported functions become operational, has propagated the postulate that functional networks derive from intricate (epi-) genetic programming and will ultimately facilitate function, rather than that these networks are a reflection of brain function itself.

The years that followed have witnessed a rapid surge of interest in large-scale brain network studies to investigate macroscale processes of early brain development [3-12]. Numerous reports confirmed the early presence of resting state networks in the neonatal brain and extended these observations to well before term birth [4,9,11,12]. Primary networks, including auditory, visual, somatosensory and motor networks could already be identified in preterm infants at 29-32 weeks postconceptional age and a herald of the default mode network was detected at this early age as well, albeit in an immature and incomplete state [12]. Recently, researchers have taken up the challenging task of functional neuroimaging *in utero*, revealing similar findings in the developing fetal brain at around 30-35 postconceptional weeks [11].

In **chapter two**, we scrutinized literature findings that have been published since the original paper by Fransson and coworkers [1], extracting a number of key biological rules that seemingly dictate brain network formation across scales (i.e., micro-meso-, and macro). First, brain network development proceeds in a bottom-up,

central-to-peripheral, and primary-to-complex sequence. Limbic fibers adjoining the central gray matter are established first [13-15]. Notably, the limbic system is one of the phylogenetically oldest parts of the brain and of key relevance for emotional behavior including fear, anger, appetite, and sexual behavior [16]. These emotions are all primary in nature and essential for survival of the species. Subsequently, projection fibers are formed, connecting the thalamus to the cerebral cortex and vice versa. Next, commissural fibers of the corpus callosum become apparent and finally, association fibers arrive [13,15,17-19]. The latter tracts (e.g., superior longitudinal fasciculus) are involved in integration of information from sensory, motor, and decision-making brain regions; functions considered to be higher-order and thus complex [16]. When axons have emanated, they are enveloped by developing pre-oligodendrocytes [18,20]. These early derivatives of glia cells mature into oligodendrocytes between 32-36 postconceptional weeks and subsequently start producing myelin, thereby vigorously enhancing axonal conduction speed [18,20]. In accordance with axonal pathway formation, myelination advances in a caudal-to-cranial, central-to-peripheral, and primary-to-complex fashion: primary sensory areas are myelinated before heteromodal association regions and the posterior limb of the internal capsule, which is located centrally in the brain is myelinated prior to the centrum semiovale, situated adjacent to the overlying cortical mantle [21-23]. Similarly, large-scale functional brain networks have been noted to adhere to the same orderly arrangement [5,24]. The formation of microscale neural circuits at around midgestation allows macroscale functional networks to be established. Functional brain networks have been studied in the developing brain from as early as 25-29 postconceptional weeks [11]. Collectively, these studies have confirmed the primary-to-higher-order maturational sequence universal in developmental biology [7,11,12,25,26]. Gao and colleagues carefully charted resting-state network development in the first postnatal year at three-monthly intervals, showing that primary sensory and motor networks are largely completed in the neonatal brain. Their developmental trajectories were followed by the dorsal attention network and default mode network (DMN) that became increasingly synchronized with spatially distant, topologically integrated brain regions. Higher order cognitive networks including the bilateral frontoparietal networks were noted to mature last and still displayed an immature configuration by the end of the first postnatal year [5].

Another key attribute of early brain network development is that brain structure matures before functional circuits and that structural networks are less modifiable. While functional networks are subjected to substantial remodeling during the first postnatal years and beyond, enabling them to support increasingly complex functions, their

structural counterparts undergo only modest refinement, which pertains to enhancing connectivity strength and axonal pruning. These adjustments result from myelination, increasing fiber coherence, and the removal of redundant connections and cater to improved integrative capacity of the brain network as a whole [21,27-30]. Macroscale connectome studies have revealed that structural refinements are not random and serve global communication capacity through enhanced network efficiency and reduced segregation, reflected by increasing global efficiency and decreasing modularity and clustering in infancy and childhood (Figure 1). Meanwhile, the overall layout of structural brain hubs remains unchanged [30,31]. Conversely, adaptations to the functional connectome are more profound, altering its configuration and shifting hub regions from primary brain regions to higher-order association cortices [26,32-35]. Despite this developmental remodeling, signature features of brain network organization are already present in the neonatal brain and remain unaltered during the postnatal processes of developmental sculpting. These attributes comprise the blueprint of brain network organization and include its small-world, scale-free and rich club architecture [26,32.33,36-39].

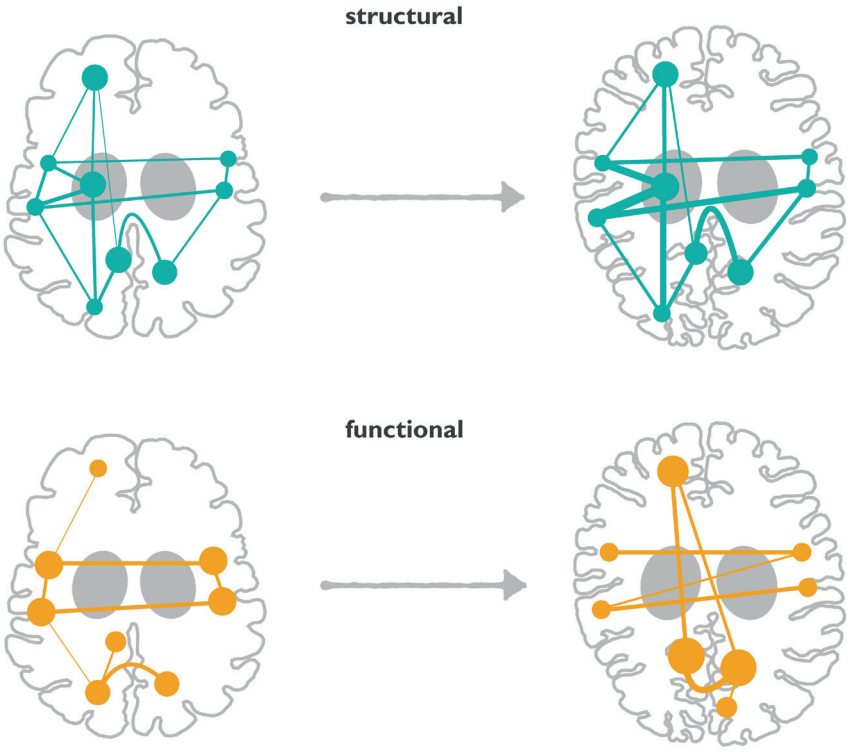
After having charted typical brain network formation during early development, **chapter two** aimed to consolidate findings from studies investigating the effects of developmental risk. Studied conditions and risk factors included maternal mental illness, maternal substance abuse and preterm birth. Although these developmental adversities have distinct origins, their common denominator is that they potentially interfere with early brain network formation, which may have protracted effects on lifelong brain functioning. A recent functional MRI study showed that altered functional connectivity could already be detected in fetuses bound to be born preterm [40]. This observation further illustrates the putatively disruptive impact of developmental risk factors that may go beyond risk factors itself and may have significant (epi-)genetic implications. The developmental neuroscience field is on the verge of incorporating imaging modalities and other data resources including genetic information, which will set the stage for unraveling brain-behavior correlates and developmental mechanisms in unprecedented detail [41,42].

In the review presented in **chapter two** we reported on converging evidence that prenatal substance exposure predominantly exerts targeted effects on receptor regions and leads to accelerated engagement of the macroscale amygdala-prefrontal circuit [43-45]. Conversely, preterm birth has been noted to diminish overall connectivity strength of particularly thalamocortical connections, while the topological layout of the

brain network is preserved given that destructive brain injury including periventricular hemorrhagic infarction and cystic periventricular leukomalacia are absent. The impact of maternal mental illness is less categorical and less systematically investigated [46-50]. Our Medline search yielded a limited number of reports and we concluded that confounding factors pose a considerable challenge to disentangling developmental risk intrinsically related to maternal mental illness from related developmental adversity that is conjointly conveyed to the fetus. Such confounding factors include disease-related medication (e.g., selective serotonin-reuptake inhibitors), poor nutritional status, prenatal substance exposure and low socioeconomic status [46,47]. As such, the potentially disruptive effects of maternal mental illness including affective disorders and schizophrenia have not yet been fully elucidated and warrant further study. Another significant developmental risk factor that has received increasing attention is critical congenital heart disease. Diminished cerebral blood flow resulting from altered cardiac structure and related physiology is considered eminent in the pathophysiology of disrupted brain development in congenital heart disease patients. First reports on structural connectivity in congenital heart disease demonstrated reductions in white matter fractional anisotropy (FA) and alterations in network topology, including increased modularity and small-worldness, that were related to cognitive abilities, inattentiveness and hyperactivity in adolescents with congenital heart disease [51-53].

Having captured the generic processes of early brain development, structural and functional connectome wiring, and the potentially disruptive effects of identifiable risk on early brain network formation, we have paved the way for a detailed analysis of early connectome development, explorations of brain-behavior correlates and further explorations of the impact of disease states on early brain wiring. In **chapter three** we examined early structural connectome development between 29-45 postconceptional weeks in 44 typically developing infants. The developmental timeframe of this study was empirically chosen, marking the earliest stages in which neonatal neuroimaging is clinically feasible, extending through the most mature age at which neonatal imaging is typically performed at our hospital. Because of ethical restrictions that applied to neonatal neuroimaging at time of data collection, typical development was defined as the absence of parenchymal brain lesions including periventricular hemorrhagic infarction, stroke, cystic periventricular leukomalacia and cerebellar hemorrhage. The majority of infants included in our study had cognitive outcome scores within the normal range (n=27), showed accelerated neurodevelopmental trajectories (n=4) or were not included in the neurodevelopmental follow-up program because they were not considered at developmental risk by the responsible physician (n=10).

Figure 1 Connectivity changes during early postnatal brain development



Upper panel depicts developmental changes of postnatal structural connectome maturation.No additional large-scale connections are formed. Yet, existing connections strengthened, thereby facilitating global communication capacity. Conversely, network clustering decreases with development. I.e., fewer triangles of interconnected brain regions can be detected and remaining triangles become less pronounced. The latter *macroscale* finding may result from *microscale* pruning, indicating selective elimination of redundant connections. Lower panel illustrates functional brain network alterations. The functional connectivity framework transforms its layout from a largely segregated network, marked by connections between homotopic counterparts and few long-range connections to an integrated brain network in which distant, yet topologically proximate brain regions are interconnected. Brain hubs modify their spatial localization from primary brain regions to association areas.

The remaining three infants had low average cognitive scores on the Bayley Scales of Infant and Toddler Development (all 82). Reciprocally, their motor scores were well within the normal range or beyond. In this study, we measured a palette of white matter diffusion properties, including FA, radial diffusivity (RD), and their related graph metrics. FA is thought to reflect fiber coherence, membrane permeability and early myelination, while RD measures diffusion perpendicular to the fibers and being predominantly a reflection of myelination, membrane density and maturation of intracellular compartments [54,55]. We uncovered a number of premises of early structural connectome development that are in concert with the primary-to-higher-order developmental sequence that connectome wiring across scales has been consistently noted to adhere to. First, FA and RD of primary connections between deep gray matter structures and primary sensory and motor regions were found to mature before connections between heteromodal association cortices. The latter govern higher cognitive functions and play an important role in integration of information from sensory, motor and decision-making brain regions. Second, we revealed that RD changes in the neonatal brain were significantly correlated with the order of white matter myelination abutting the cortex (from now on referred to as 'subcortical myelination') as depicted by pioneering neuroanatomists. In the early 1900s, Paul Flechsig published his atlas that divided the cortex into distinct regions of subcortical myelination [56,57]. The atlas was based on recordings of postnatal medullary substance expansion in the subcortical white matter and was modified by Von Bonin in the 1950s [58]. Here, we adopted the modified Flechsig-Von Bonin atlas that decomposes the cerebral cortex into 45 unique regions representing myelination order. Integrating modalities (i.e., diffusion weighted imaging and histopathological mappings of postnatal myelination), we attempted to improve our understanding of early white matter development and to gain further insight into the neurobiological underpinnings of early diffusion changes. Interestingly, only RD changes were associated with subcortical white matter myelination, while alterations in FA were not. These results coincide with earlier DWI studies and postmortem data, which denoted that RD and FA delineate distinct features [54,55,59].

Concomitantly, early white matter changes were noted to result in improved FA-weighted global efficiency of the neonatal brain network, whereas measures of network segregation were observed to remain relatively constant. Speculating on the microscale underpinnings of these macroscale findings, our observations are consistent with the postulate that decreasing clustering is dictated by pruning of redundant connections. We did not observe changes in network clustering and the developmental timeframe of our study does not overlap with the stage of axonal

pruning, which typically emanates beyond term-age. Our observation that the overall neonatal brain network becomes increasingly efficient is coherent with earlier reports on neonatal connectome alterations [36,37,60,61]. These studies reported decreasing path length and/or increasing global efficiency in the developing preterm brain between ~30 postconceptional weeks and term age. With path length measuring the number of edges that need to be traversed to travel from one node to another in *binary* networks, or the average weight of those edges in *weighted* networks and with global efficiency being equal to the inverse of average path length, these metrics are strongly interrelated [62,63]. A fourth observation from the study presented in **chapter three** is that the overlap between the neonatal brain network and adult connectome exceeded 80% and increased from the earliest time-points onwards, which confirmed findings from our earlier report [36]. We extended this knowledge with the notion that neonatal white matter FA resembled FA in the adult human brain more, while RD matured at a faster pace.

Collectively, these findings suggest that diffusion properties portray different developmental trajectories. FA-weighted neonatal networks approximated the FA-weighted connectome in the adult human brain most and their related network metrics showed maturational changes resulting in improved communication capacity of the neonatal connectome. Conjointly, changes in RD during the neonatal period were correlated with the sequence of subcortical white matter myelination. We thus provided empirical evidence that RD in the neonatal brain may be a valuable early imaging marker of subsequent white matter myelination, while FA and related global efficiency of the neonatal connectome may inform about brain network maturity. As such, these parameters may represent valuable markers of typical trajectories of white matter development and may help designate deviances thereof. Such deviances include a wide range of developmental disorders and developmental risk, such as preterm birth, critical congenital heart disease, perinatal brain injury, tuberous sclerosis complex, schizophrenia and autism spectrum disorder.

Hence, extending the present mappings to atypically developing infants would be particularly relevant. Furthermore, it would be of interest to corroborate our findings in typically developing fetuses and healthy neonates. Such comparisons require a set of methodological hurdles to be crossed. First, image resolution and subject motion (or rather immobility) need to be comparable. Next, fetal and neonatal templates should be similar, which may be challenging owing to the pronounced morphological changes that characterize cortical development during the third trimester of pregnancy. Third,

increases in head size, which invariably lead to differences in network density, need to be accounted for. The latter is crucial, because density substantially impacts on graph metrics [64]. In **chapter three**, we aimed to take density differences into account by adjusting minimum fiber length of streamline tracings to the factor head size. Graph metrics were normalized by comparing them against 1000 random networks with similar density and degree distribution, although this measure is known to be insufficient to control for density effects [65,66]. Furthermore, we set a proportional density threshold before computing network metrics [62]. These analyses yielded similar results in terms of increases in FA-weighted global efficiency, suggesting that the neonatal connectome is intrinsically improving its integrative capacity.

Brain-behavior correlates

Another relevant implication of the findings presented in **chapter three** would be to relate these developmental changes to behavioral measures (e.g., cognitive functioning, working memory skills, attention, and executive functioning). We made such attempt by correlating network metrics to measures of cognitive functioning in late infancy in the subgroup of 34 (77%) infants that were followed-up through early childhood. Measures of network segregation including clustering and modularity were significantly related to cognitive parameters measured at age 18-24 months. In **chapter four** we expanded these efforts by linking global mean FA - being an estimate of white matter maturation - and related graph metrics in the neonatal brain to cognitive functioning at early school age in a sample of 30 preterm born children. This study showed that global mean FA of the neonatal brain was significantly associated with performance intelligence quotient (IQ) at age 5.7 years. No significant relationship was revealed between related network metrics including normalized clustering and normalized global efficiency and IQ measures. For this study we retrospectively selected very preterm born children (all born <31 weeks gestational age) for whom good quality DWI data were available in the neonatal period and who underwent an intelligence test at the department of Medical Psychology at early school age (5.5-6 years). Following the selection procedure, neonatal connectome maps were derived by combining deterministic whole-brain streamline tractography data with an automatically registered neonatal template [67]. Performance IQ, verbal IQ and processing speed measured using the Wechsler Preschool and Primary Scale of Intelligence were included as primary outcome parameters [68]. Secondary outcome parameters comprised behavioral problems as assessed using a validated behavioral questionnaire for parents and teachers: the Child Behavior Checklist and Teacher Report Form [69]. Linking neonatal white matter estimates to measurements of cognitive

functioning 5.7 years later, global mean FA of macroscale connectome was found to be significantly related to performance IQ, while normalized network metrics were not.

These findings build on an accumulating literature of studies relating brain network attributes to intellectual performance in childhood, adolescence and adulthood [70-74]. Notably, they are in line with numerous reports on white matter measurements in the preterm brain in the neonatal period and cognitive functioning in infancy and early childhood. Such measurements predominantly encompassed FA-estimates, derived from regions of interest in prominent white matter structures such as the corpus callosum and corticospinal tracts, tract-based spatial statistics and tractography results of specific fiber bundles or whole-brain thalamocortical connectivity [75-78]. Together, these studies support the notion that increased neonatal white matter maturation is associated with improved cognitive abilities following preterm birth during infancy and childhood.

Maternal education level and related socio-economic status have frequently been associated with cognitive performance, especially in infancy and early childhood [76,79]. We aimed to explore the effect of maternal education level on the relationship between global mean FA and performance IQ, showing no significantly mediating effect. These findings may suggest that the categorization of maternal education level as used in our study is not distinctive enough, that this parameter is an insufficient marker of socio-economic status (and genetic make-up), that the impact of maternal education is confined to infancy and preschool age or that the sample size should be substantially larger to detect effects of maternal education level. The latter implication is however less likely, because Ball and colleagues demonstrated a significant effect of socio-economic status on cognitive functioning at age two years in a cohort of 57 infants; only nearly twice the sample size of our population [76]. Hence, would a mediating effect of maternal education level on the association between neonatal white matter FA and performance IQ at school age be present, one would expect to note (at least) trend-level evidence for such a relationship. The measurements as performed in **chapter four** should be extended to a larger sample to corroborate this postulate. Although the study was originally designed as a prospective observational cohort study into the association between neonatal brain metrics and neurodevelopmental outcome in late infancy, the complexity of the add-on measurements as well as the extended follow-up that we employed in our study restricted the sample size substantially. Therefore, we advocate repeating the study as presented in **chapter four** using a prospective design. Accordingly, the hypothesis that attributes of neonatal brain network organization are

associated with childhood cognitive functioning, which was not supported in our initial study should be investigated further in a sufficient sample. Furthermore, potentially sexual dimorphism and the impact of post-discharge injurious events should be further explored [80,81]. Finally, given the divergent trajectories of FA and RD - each delineating specific features of early white matter maturation - it would be relevant to add RD and its related graph metrics to the present examinations.

Moving from neonatal connectome measurements to parameters of neonatal brain morphology, we focused on the association between brain volumes of major brain structures and neurodevelopmental outcome in preterm infants in **chapter five**. Automatically obtaining estimates of neonatal brain volumes of the unmyelinated white matter (UWM), cortical gray matter (CGM), cerebellum, ventricles, deep nuclear gray matter, and cerebrospinal fluid surrounding the brain, we investigated their relationship with neurodevelopmental outcome measurements at age two years, 3.5 years and 5.5 years in 112 preterm born children. We found that smaller volumes of the ventricles were related to higher scores on cognitive and motor scales at age two years, higher development quotient at age three years and faster processing speed at age five years. Larger UWM volumes were associated with better gross motor performance at age two years and faster processing speed at early school age. Furthermore, smaller CGM volume and larger cerebellar volume were related to higher outcome scores in late infancy and age three years, although these associations were less robust and partially mediated by neonatal brain injury. Together, these findings provided evidence that volumes of the ventricles and UWM may be added to global mean FA (**chapter four**) as valuable imaging parameters for neurodevelopmental outcome prognostication following preterm birth. These brain metrics each cover specific developmental features, including performance IQ at early school-age (global mean FA), processing speed at early school age (volumes of ventricles and UWM), cognitive and motor performance in late infancy and at age three (ventricles) and gross motor performance (UWM) in late infancy. As such, they may complement each other.

Results of our study support a growing literature that brain volumes in the neonatal period are associated with neurodevelopmental outcome [82-87]. Most reports based their assessments on developmental parameters measured in infancy and early childhood [83,86,87] and only a few studies reported on data at school age [82,85]. Details at school age included overall neurological status and attention- and/or hyperactivity problems. Our study added to this body of work that volumes of the ventricles and UWM may be predictive of processing speed as a measure of cognitive functioning.

Future research is needed to validate the latter premise. Large samples are needed to make intelligible distinctions between typically developing children, those at risk of developmental deficits and those exhibiting profound delays. Prediction of cognitive and behavioral outcome following preterm birth would be most valuable for children at risk of mild-moderate delays and borderline to low average IQ, because these spectra are most difficult to prognosticate. With the availability of structured long-term follow-up, accurate automatic segmentation algorithms [88,89], and ever improving supportive logistics accommodating image processing (including enhancing computational speed), it is now within our scope to perform such invaluable studies.

Except for the sample size precluding a truly prognostic design of the study presented in **chapter five**, there are a number of other relevant considerations. Firstly, at time of recruitment (i.e., 2006-2007) the limit of viability was 25 gestational weeks in the Netherlands. The gestational age (GA) at which active perinatal care is initiated was lowered to 24 weeks in 2010 and is currently under active debate of being further lowered to 23 weeks of gestation. Hence, findings of our study have not been validated in the youngest and most vulnerable infants born at less than 25 gestational weeks. Secondly, assessment of intellectual functioning was only performed if the child was born <28 weeks of gestation or if clinically indicated at the discretion of the attending physician at the outpatient clinic. Consequently, the subgroup of infants for whom IQ-data were available was biased towards the youngest infants (i.e., GA 25-28 weeks) and those at risk of impairments. Provided that financial means for comprehensive follow-up are available, this limitation is to be overcome in future research. Furthermore, extending the volumetric measurements with estimates of cortical maturation would be informative given that cortical gyrification has been consistently related to cognitive abilities in human adults and across species [90,91]. Alterations in cortical thickness have also been observed in preterm born adolescents and have been linked to cognitive functioning [92,93]. However, algorithms to obtain such estimates were not incorporated in the segmentation method used in this study [94]. Elaborating on the observations in **chapter five**, we made use of a recently developed segmentation algorithm that allowed cortical measurements in **chapter six** [89].

Here, we studied a total number of 306 extremely preterm infants (born <28 gestational weeks) and 15 full-term control infants. Addressing the research question that preterm twins would exhibit divergent developmental trajectories from preterm singletons in terms of brain morphology at term age and cognitive and motor functioning at age two years, brain segmentations and subsequent volumes and cortical parameters were

derived for 240 preterm infants. Neurodevelopmental outcome data was available in 275 preterm infants. Preterm infants were divided into dichorionic twins (n=51/n=60), monochorionic twins (n=31/n=30) and singletons (n=158/n=185). We aimed to account for birth weight (BW) and GA, in an attempt to disentangle the prior two from the principal influence of twin pregnancy. To this end, preterm twins were matched to preterm singletons by GA, BW and sex. Finally, a small reference group of 15 full-term control infants was added; cognitive and motor functioning was evaluated in eight of these full-term neonates.

First, clinical characteristics were compared between the preterm groups, revealing that BW was substantially lower and pre-eclampsia (PE) was significantly more prevalent in preterm singletons. PE is a severe pregnancy complication caused by placental and maternal vascular dysfunction, which leads to hypertension and proteinuria in the mother and growth restriction in the fetus [95]. Observed differences in clinical parameters were taken into account in subsequent analyses. Brain metrics and neurodevelopmental outcome parameters were compared between preterm twins and their matched singletons and between the complete preterm groups. Both analyses yielded no significant differences in brain volumes, measures of cortical morphology or neurodevelopmental outcome at age two years. Next, the impact of PE was further explored in a linear regression model including all preterm infants and entering PE as an independent variable in the model. In addition, subgroup analysis was performed excluding infants born to mothers with PE. These analyses revealed PE to be related to smaller brain volumes, while it did not affect cortical maturation or neurodevelopment in late infancy. Finally, morphological brain metrics and early neurodevelopmental outcome parameters were compared between preterm infants and full-term neonates, demonstrating significant differences in cerebrospinal fluid volume, relative UWM volume and gyrification index. Surprisingly, cognitive and motor functions were comparable between the groups.

Together, these findings suggest that preterm twin pregnancy does not impair early morphological brain development and neurodevelopment, if GA and BW are comparable with those of singleton peers. Furthermore, they draw attention to the potentially disruptive impact of a diminished intrauterine environment, although its long-term consequences remain to be awaited. Studies investigating brain morphology and neurodevelopmental outcome in preterm twins and singletons are scarce [96,97]. In an earlier study, multiple pregnancy was noted to be modestly related to smaller cortical surface area of the insula and superior temporal sulcus in a subset (n=71) of

the preterm infants included in the sample studied in **chapter six** [96]. In the present study, we extended measurements of specific sulci to a whole-brain level, revealing no significant differences between preterm twins and singletons. Long-term follow-up of these infants is key, particularly with regards to cognitive and behavioral domains. Should preterm twins display altered trajectories over the course of development, targeting their cerebral underpinnings is highly relevant to further our understanding of the underlying neurobiology and for future prediction. The segmentation algorithm applied in this study provides results that can be automatically parcellated into biologically meaningful cortical regions using the Freesurfer analysis suite [98]. Such analyses were successfully performed in **chapter three**. Consequently, the brain-behavior intersection of putative cognitive deficits and/or behavioral problems that may occur in childhood, when functional demands increase can be pinpointed. Similarly, the brain-behavior relationship as observed in the earlier study can be further investigated omitting manual labor required for those comparisons [96], now enabling the application of similar measurements to large samples.

An important limitation of the study presented in **chapter six** is that genetic data imperative to distinguish identical from fraternal twins was not available. Studies in monozygous (i.e., identical) and dizygous (i.e., fraternal) twins have revealed high heritability of brain volumes - particularly white matter and subcortical structures - and cortical parameters that fluctuate with age [99-102]. Repeating the present study while being informed about zygosity and provided that intrauterine conditions are comparable between monozygous and dizygous twins would allow comparisons of heritability of brain morphology before postnatal influences come into play. Such examination would be relevant to enhance our understanding of the genetic impact on early brain development.

Notably, preterm infants did not display volumetric deficits of the majority of cerebral structures at term age compared to their full term peers and early cognitive and motor functioning was not significantly different either. A number of factors may have contributed to these lacking differences. First, ethical restrictions at time of the study precluded recruitment of healthy full-term newborns. Full-term infants included in this study were therefore typically developing infants without signs of neonatal brain injury who were scanned for various clinical reasons (e.g., neonatal surgery, suspected neonatal seizures without electroencephalographic evidence of seizures and asymptomatic hyperbilirubinemia above exchange transfusion levels). Accordingly, morphological brain development may have been marginally different from healthy

full-term newborns. Ongoing research efforts at our institute aimed at obtaining MRI scans of healthy fetuses and newborn infants will address the latter hypothesis. Second, body growth and related nutrition may have contributed to the absence of significant differences in dry brain volumes. Weight at scan was comparable between preterm infants and full-term neonates. Improved nutritional care as well as reduced neonatal morbidity may have led to enhanced body growth and related brain growth, ultimately resulting in comparable absolute brain volumes at term age [103-105]. Despite that dry brain volumes were not significantly different between preterm and full-term infants, we found evidence that cortical maturation was lagging behind in preterm infants, as their gyrification index was significantly decreased.

Difficulties and challenges of neonatal imaging

There are a number of limitations to neonatal neuroimaging and image processing that we feel deserve consideration. Infant head motion is particularly challenging and should be adequately accounted for as it may profoundly impact data quality and study results. Rs-fMRI studies have demonstrated that motion artifacts induce effects that mimic *inverse* developmental brain network alterations, including decreased connectivity between distant regions and increased short-range connectivity [106-108]. Reciprocally, during development long-range connections are consolidated, thereby enhancing integrative capacity of the brain network and short-range connections are diminished, reducing brain network segregation [29,30,109-111]. Infant movement should be limited by creating a comfortable scanning environment, which involves enough warmth (yet prevents overheating), minimal acoustic noise exposure and a comfortable sleeping position. Measures to meet these criteria include placing the infant in a vacuum fixation pillow, using sound reducing cushions, applying earmuffs, swaddling, soothing, switching off the lights while scanning and making use of an MR incubator in preterm infants. Moderate sedation (oral chloralhydrate) further reduces motion artifacts, yet sedative medication is generally only indicated if the infant is scanned in a clinical setting. In research settings, the MRI procedure is typically undertaken after feeding and swaddling, during quiet sleep. This dichotomy in policies for sedation between healthy newborns and infants at risk may introduce bias. This bias should at least be considered and rather be accounted for by scanning both populations during natural sleep. Another relevant aspect of data acquisition is that voxel size should preferably be isotropic in case data is acquired for three-dimensional measurements, including tensor estimation (DWI), volumetric measurements (T2) and cortical tracings (T2) in order to limit data interpolation.

Finally, we would like to focus attention on some methodological considerations of DWI. Essentially, DWI does *not* measure white matter pathways, yet it relies on water diffusion to obtain indirect estimates of white matter fiber bundles [59,112]. In the studies presented in this thesis we employed diffusion tensor imaging (DTI), which does not allow extraction of information about multiple fiber orientations within one voxel (e.g., kissing or crossing fibers) [112,113]. Instead, a single preferred diffusion direction is extrapolated from the signal within each voxel [112]. In order to estimate the diffusion tensor, the DTI protocol must incorporate at least six different gradients of diffusion directions and in most DTI studies 15 or more directions are used [114]. Here, we used 32 (**chapter four**) and 45 diffusion gradients (**chapter three**). In addition, adopting multiple baseline measurements (i.e., *b*=0 scans) and reversed k-space read-out is often advocated for DWI studies to optimize data quality [114,115]. However, the clinical protocols employed in our studies included one *b*=0 scan (**chapter four**) and an average *b*=0 scan derived from 5 *b*=0 images (**chapter three**). Hence, in future research the prior acquisition strategy should be considered.

After data acquisition, datasets should each be accurately inspected for motion artifacts. DWI and rs-fMRI data are particularly susceptible to their disruptive effects. When processing DWI data, the tensor estimation algorithm should include outlier detection and subsequent exclusion of those outliers [116-118]. Additionally, in our studies in **chapter three** and **chapter four**, datasets were omitted when five or more volumes contained motion artifacts. In rs-fMRI data processing, motion can be evaluated using customized algorithms (e.g., frame-wise displacement or DVARS [Derivative of Root-mean-square VARiance over voxelS]) [119-121] and addressed using data scrubbing (i.e., removal of motion corrupted volumes) or correcting for frame-by-frame signal variations due to changes in head position with respect to the receiver coil [122]. The latter technique has been designed for fetal imaging [122].

Brain volumes can subsequently be derived from T2- and/or T1-weighted images using segmentation algorithms or voxel-based morphometry. In the studies presented in this thesis, we made use of two different automatic segmentation methods. In **chapter five** we adopted the probabilistic segmentation method by Anbeek *et al.* that assigns each of eight manually annotated tissue classes (basal ganglia and thalami, brainstem, cerebellum, cerebrospinal fluid within the ventricles and outside the brain, cortical gray matter, unmyelinated white matter and myelinated white matter) based on spatial features and image intensity of T1- and T2-weighted images [94]. Spatial information was obtained by registering each subject's T2-weighted image to a T2-based average

brain atlas using affine and elastic registration [123]. Next, each voxel within the image was assigned one of the eight predefined tissue classes using a k-nearest neighbour classifier. This method was compared to other segmentation algorithms in the field, revealing robust results that were comparable to the other methods [88]. Most accurate results were obtained for the cerebellum and basal ganglia and thalami, yielding dice coefficients >0.90. The dice coefficient provides information about the similarity of a specific segmentation result to the gold standard, i.e., manual tissue classification and is computed based on true and false positive results and false negative results [94]. In **chapter six** we employed a machine-learning algorithm based on a convolutional neural network. Each voxel of the T2-weighted image was classified into each of the eight manually annotated tissue classes as described above using a convolutional neural network with multiscale information. This information was derived from multiple patch sizes in order to obtain both detailed and global accuracy. Similar to the segmentation method used in **chapter five**, the deep learning algorithm yielded reliable results, with dice coefficients >0.81 for all tissue classes, except for low signal intensity white matter (generally considered myelinated white matter) [89]. The latter tissue type is notoriously difficult to segment, rendering moderate to poor results across segmentation algorithms and therefore frequently excluded from data analyses. Nowadays, neonatal segmentation methods provide accurate results for all other tissue classes with limited variability across algorithms [88]. The segmentation pipeline adopted in **chapter six** was extended with an algorithm to obtain cortical parameters including the gyrification index, cortical thickness, mean curvature and cortical surface area. Comparability of these features across methods is limited because they exhibit substantial variability and should be borne in mind when interpreting the results [124-128]. Results for cortical surface area have been noted to range most, varying from 250 cm^2 to 1200 cm^2 in neonates scanned at term-equivalent age. This wide range is mostly related to the level of detail incorporated in the computations (e.g., because of differences in voxel size and smoothing across algorithms), often referred to as the coastline paradox [126].

In connectome studies, the next step after data processing is image registration. Co-registration of subject data and a certain template that divides the cortex (and subcortical structures) into distinct regions is imperative to derive nodes (i.e., brain regions) of the network. Needless to say, such parcellation schemes can also be employed on segmentation data to obtain local estimates of cortical surface area or thickness. Atlas registration should be carefully evaluated and optimized. Numerous templates have been adopted across neonatal connectome studies, imparting limitations on their comparability [3,6,44,67,129,130]. Data uniformity, both in terms

of acquisition, processing and template registration should be advocated and can be stimulated through data sharing and freely available databases (e.g. WU-Minn Consortium Human Connectome Project) [131-133]. Such initiatives are ample in the adult neuroimaging community and should be extended to infant and neonatal imaging too to make essential advances [134,135].

Beyond the baby brain

We touched on a number of relevant future perspectives in the previous sections of this chapter, including incentive to data sharing and data uniformity, large-scale prognostic studies with structured follow-up to allow prediction of neurodevelopmental outcome and combining data. In this final section we will further elaborate on important outstanding questions and shed light on what we believe will help move the field forward.

In the past 10-15 years, neonatal neuroimaging has largely focused on improving image quality and processing techniques. Now these goals have been widely achieved, attention is shifted to collating data for neurodevelopmental outcome prediction, obtain a better understanding of the underlying neurobiology of macroscale imaging findings and brain-behavior correlates, and to ultimately enable tailored care. In light of the exciting advances that the field is converging on, a number of outlooks are key. These include data sharing as mentioned previously, big data analysis, and cross-modality investigations. In order to ultimately improve neurodevelopmental prospects of newborn infants (and fetuses) at risk, development of neuroprotective and restorative therapies is key.

In the context of this thesis, big data refers to the richness of neuroimaging data, clinical parameters and other relevant metrics (demographics, DNA samples, etc.) that allow researchers to infer neurobiological knowledge or predict outcome by integrating data-driven analysis techniques such as machine learning. Machine learning essentially revolves around distilling knowledge from data. By analogy of how humans learn from experience, the computer musters an algorithm to perform a certain task and improves how it performs that task based on previous runs of the algorithm. Hence, it 'learns' the task from experience. The field of neonatology, especially with respect to preterm birth spawns a scaffold of data from monitoring devices (vital parameters, cerebral oxygenation, electroencephalography recordings, nutrition, etc.), medical records, neuroimaging procedures and neurodevelopmental follow-up. As such, neonatology is particularly well suited to big data approaches. In a recent study we

made a first attempt to combine neuroimaging data and clinical characteristics to predict neurodevelopmental outcome in late infancy employing a supervised machine learning approach [136]. Although the sample size was small (n=173), impinging on the machine learning analysis, this work underscores the present-day feasibility of the exciting paradigm of big data and associated machine learning approaches. Indeed, initiatives are currently underway to set up infrastructure for big data analysis at our department and build on the previous explorations [136].

A next step in the nascent field of big data analysis and machine learning applied to neurodevelopmental outcome prediction in preterm infants would be to considerably increase the *n* and add relevant variables in order to yield biologically informed models for outcome prognostication. Such features are potentially exhaustive given the richness of data resources that one can dispose of (e.g., medical records, neuroimaging procedures, monitoring devices, laboratory measurements, etc.). One interesting, yet largely unexplored avenue in this walk of knowledge from lavish data resources to neurodevelopmental outcome prediction entails genomic profiling. Genomic profiling is increasingly used across fields and first steps have also been taken in neonatology. Two recent studies reported on the association between common genetic variants, derived from genotyping of single nucleotide polymorphisms, and FA across major white matter tracts in preterm infants scanned at term age. Genes involved in lipid pathways were consistently found to be related to white matter FA [41,137]. Notably, Krishnan and colleagues provided inferential evidence for a link between gene expression of identified genes and biological underpinnings of white matter injury [41]. Although circumstantial and preliminary, the latter notion focuses attention on an important future perspective of genetic profiling in neonatal neurology and developmental neuroscience. It provides a novel framework to deduce neurobiological mechanisms underlying neuroimaging findings. As such, neurogenetics may add pivotal pieces to the puzzle reflecting the sheer complexity of early brain development and developmental disease. We will shortly touch on potential implications of neurogenetics to study both typical and aberrant early brain development in the following section of this chapter. Here, we would like to emphasize that establishing links between common genetic variants, gene expression profiles and brain development in preterm infants has strong potential to separating infants at risk from those with prospects of typical development and thus to target neuroprotective and restorative therapies.

Until now, treatment regimens for perinatal brain injury and neuroprotection in preterm infants have been limited. Antenatal magnesium sulfate was implemented in clinical

practice nearly five years ago because of a reported reduction of cerebral palsy when administered to the mother shortly before preterm delivery [138]. However, the efficacy of magnesium sulfate has been under debate ever since its introduction into routine clinical care because of low cerebral palsy rates in present-day NICU graduates [139]. Currently, erythropoietin, melatonin and stem cell treatment are considered promising candidates which are under active investigation in clinical trials and translational studies [140-145].

It follows that a particularly exciting prospect of the incentives of big data analysis and genomic profiling is that they expedite personalized healthcare. Suggested neuroprotective and rehabilitative therapies may be attuned to infants who would benefit most. Patient-centered, tailored care is particularly relevant for preterm infants, who typically spend a prolonged period of time in the neonatal intensive care unit, whose illness and care are markedly stressful and demanding for the infants and their families and whose condition(s) can be profoundly disruptive for their developmental course and functioning throughout the lifespan. Personalized healthcare may facilitate stress-reduction in both infants and parents.

The adverse effects of early-life stress and pain are increasingly recognized. Studies in preterm infants have demonstrated an association between painful procedures, brain development and neurodevelopmental outcome [146-147]. Measures to reduce stress in preterm infants have predominantly encompassed the Newborn Individualized Developmental Care and Assessment Program (NIDCAP) and kangaroo care, widely advocated in the 00s [148]. A meta-analysis of reported randomized controlled trials investigating NIDCAP that included 627 infants did not show significant benefit of NIDCAP on neonatal mortality and disability in late infancy [149]. Perhaps, significant impact of 'soft' measures, aimed at reducing stress and promoting development (i.e., regular skin-to-skin care, lowering ambient light and sound, clustering care, providing aids to stimulate flexion and self-regulation, and involving parents in their child's care) on 'hard' outcomes is less intuitive. These adaptations may not exert effects on death or disability, however they may relieve stress, benefit infant-parent bonding and have potentially protracted yet more subtle impact on development and behavior, which might not become manifest until school age or beyond [150]. At present, a number of other 'soft' measures are under active exploration, including single family rooms, stimulating sound therapy, and promoting infant sleep that may individually and/or cumulatively represent favorable add-on therapies [151,152]. It is likely that the amount of stress as well as the tolerability of stress-relieving measures is dependent on

clinical illness severity of the infant. One advantage of personalized medicine is that tailored treatment plans can be designed and accustomed to the infant's and parent's needs. These personalized care plans may incorporate therapeutic agents such as erythropoietin and stem cell treatment as well as softer add-on measures, including nutritional interventions, kangaroo care, and sleep stimulation.

Implementing big data analysis, genomic profiling and related machine learning brings about practical considerations, including data storage and computational power. Today, investments are urgently needed that will enable us to capitalize on the data trove of tomorrow. Data storage coincides with data security and similar efforts must be made to protect data repositories form unwanted access by unauthorized users. Importantly, this nascent field also paves a novel path in medical-ethics, raising eminent questions with regards to patient privacy, clinical decision-making, and healthcare expenditure. In parallel with the field of neonatology itself: given that what technology allows us to do proceeds apace, we should both carefully and timely attend to what we as clinicians, researchers, and society believe we *should* do.

The third anchor of anticipated advances in the future of developmental neuroscience constitutes cross-modality studies. Multimodal studies, linking neuroimaging data (T2, DWI, rs-fMRI) in the early developing brain to brain function recordings (e.g., electroencephalography, functional near-infrared spectroscopy [NIRS]), cerebral blood flow (e.g., arterial spin labeling and NIRS), brain metabolism (e.g., MR spectroscopy), histopathological mappings, gene expression profiles [42,153] and genomic data [41,137] will advance our knowledge of the brain structure-function interplay, neurobiological underpinnings of early brain wiring and gene-environment interactions in typical early brain development and high-risk or disease states. We briefly alluded to combining genomic research and neuroimaging data on page 194 of this chapter. There are myriad potential corollaries that this emerging field may give way to. In the studies by Krishnan *et al.* and Boardman *et al.* we have seen the application of detecting single nucleotide polymorphisms, pathway analysis and clustering of genes to identify gene-brain interrelations in preterm neonates [41,137,154]. It has been postulated that susceptibility to preterm brain injury is modulated by the cumulative effect of multiple genes in response to environmental influences in the pre- and perinatal period and beyond. Individually, these genes likely exert small effect, whereas their impact is putatively substantial when acting in concert [155,156]. Leaping ahead, polygenic risk scores distilled from relevant candidate genes may potentially relate to preterm brain injury and assist in identifying infants at risk of aberrant brain development.

Particularly exciting in this context as well is the open source availability of the developmental transcriptome of the fetal brain and corresponding neuroimaging data as provided by the Allen Institute for Brain Science and its consortium partners (www.brain-map.org). These data include gene expression maps and 7T *post mortem* MRI data of mid-gestational fetal brains [157]. Capitalizing on this rich data trove, one may distill gene expression profiles of identified risk-genes for preterm brain injury and investigate whether these gene expression patterns correspond with the localization of white matter alterations as reported in earlier the studies that combined neuroimaging with genomic profiling [41,137]. Needless to say, the potential implications of this powerful source of data go far beyond the (epi)-genetic substrates of preterm brain injury. For instance, they allow cluster analysis to pinpoint subsets of co-expressed genes relevant for specific developmental processes, charting transcriptome maps of genes involved in distinct features of brain development (e.g., corticogenesis, myelination, axonal pathfinding etc.) and related neuroimaging findings in both healthy brain development and high risk or disease states, targeting the developmental underpinnings of human-specific brain function and cross-species investigations of evolutionary differences. As such, these data encompass a scaffold of neurobiological knowledge to build on and to bridge important gaps between macroscale neuroimaging findings and their microscale underpinnings.

Shifting gears from large-scale and multimodal research to lower-hanging fruit, we would like to draw attention the elegant study by Gao *et al.* who charted developmental trajectories of resting state networks during the first postnatal year at three-monthly intervals, revealing their maturational order [5]. Charting developmental courses of healthy connectome development at timely intervals (e.g., every 6-9 months) during the first postnatal years as well as during fetal development would be a critical first step forward. These mappings would herald novel insights into healthy brain wiring and should be extended to the diseased or high-risk brain to elucidate how brain network formation is affected in high risk and disease states.

It's better to be a pirate than to join the navy.
- Steve Jobs -

Concluding remarks

In this thesis, we studied brain network development in the earliest stages after connectome genesis, consolidating data from structural and functional brain network studies and building on these earlier findings, exploring unchartered attributes of early structural brain network formation. We scrutinized neuroimaging markers for neurodevelopmental outcome prediction following preterm birth, identifying a set of potentially relevant imaging features for distinct developmental domains. Finally, we revealed brain-behavior correlates in preterm twins and singletons relevant to clinicians and researchers interested in the heritability of traits, and drew attention to the putative impact of the intrauterine environment to early brain development.

Today, we are entering the era of cross-modal research and big data analysis. The increasing availability of open access repositories of neuroimaging data, transcriptome recordings and other pivotal resources pave the way to exciting novel insights and paramount discoveries that will help further our understanding of typical and deviating early brain development and promote the identification of infants at developmental risk. We hope that this thesis helped point toward potentially relevant cross-modal phenomena, imaging markers, and remaining gaps for early developmental neuroimaging research.

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7

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7

CHAPTER 8

Nederlandse samenvatting (Summary in Dutch)

NEDERLANDSE SAMENVATTING
(Summary in Dutch)

Het menselijk brein is een fascinerend complex systeem dat ons in staat stelt te lopen en te praten, de wereld om ons heen waar te nemen, na te denken, creatief te zijn en relaties aan te gaan. Onze hersenen bepalen feitelijk wie we zijn. Deze complexe structuur wordt grotendeels tijdens de zwangerschap gevormd. Een zwangerschap duurt in principe 40 weken (9 maanden) en tijdens deze periode wordt de overgrote meerderheid van de ongeveer 86 miljard zenuwcellen en hun ~10^{15} (biljard) verbindingen gevormd. Dit zijn duizelingwekkende getallen die ons voorstellingsvermogen te boven gaan. Een mooie vergelijking om deze astronomische aantallen inzichtelijker te maken, is die van de zandkorrels en de colaflessen. Als je het aantal zenuwcellen in de hersenen zou uitdrukken in zandkorrels en je zou naar het strand gaan om al die zandkorrels te verzamelen, dan zou je 100.000 literflessen moeten vullen.

Tijdens de zwangerschap transformeren de hersenen van een ogenschijnlijk simpele cilinder tot de ingewikkelde structuur die we allemaal kennen en die uiterlijk weinig verschilt van het volwassen brein. Bij de geboorte zijn alle grote witte stofbanen aangelegd. De witte stof bevat de 'axonale' verbindingen van de hersenen. Zenuwcellen zijn met elkaar verbonden via dendrieten en axonen die op elkaar schakelen via synapsen. Axonen zijn lange zenuwuitlopers en zoals elektriciteitsdraden in hoogspanningskabels zijn gebundeld, zo zijn axonen op hun beurt in bundels georganiseerd: de witte stofbanen. Tegen de tijd dat een kind geboren wordt, vertoont de hersenschors (cortex) reeds secundaire en tertiaire windingen (gyri) en sulci (groeven), eveneens vergelijkbaar met de hersenen van volwassenen. De snelheid en complexiteit van de processen die ten grondslag liggen aan vroege hersenontwikkeling maken hen bijzonder kwetsbaar voor verstorende invloeden. Eén van die verstorende factoren is extreme vroeggeboorte, gedefinieerd als geboorte voor een zwangerschapsduur van 28 weken. In dit proefschrift, hebben we de invloed van extreme vroeggeboorte (prematuriteit) op vroege hersenontwikkeling en de ontwikkeling van cognitieve en motorische vaardigheden onderzocht.

In het KRO-programma 'Anita wordt opgenomen' rekende presentatrice Anita Witzier samen met de programmamakers uit dat een opnamedag op de neonatale intensive care unit (NICU) gemiddeld €2500 kost. In **hoofdstuk zes** hebben we gezien dat een extreem prematuur geboren baby gemiddeld 41-49 dagen op de NICU verblijft. Het zal dan ook niet verbazen dat de totale zorgkosten van prematuriteit €154 miljoen

bedragen en daarmee 0.2% van het totale budget voor de gezondheidszorg in Nederland beslaan. Extreem te vroeg geboren kinderen hebben een groot risico op ontwikkelingsproblemen later in het leven. Deze problemen bevinden zich met name op de gebieden van aandacht en concentratie, sociale vaardigheden, werkgeheugen, executieve functies en gedrag. Steeds meer studies tonen aan dat prematuur geboren kinderen een verhoogd risico hebben op psychiatrische aandoeningen, zoals depressie, autisme en ADHD (aandachtstekort-hyperactiviteitstoornis). De combinatie van langdurige intensieve zorg en chronische zorgbehoefte maken de zorgkosten en maatschappelijke belasting van extreme prematuriteit aanzienlijk. Ex-prematuren hebben vaker dan andere kinderen onderwijsondersteuning nodig, zoals remedial teaching en speciaal onderwijs. Ook maken deze kinderen veelvuldiger gebruik van de gezondheidszorg (denk aan luchtweginfecties, ziekenhuisopnames en revalidatie). De belangrijkste veroorzakers van ontwikkelingsproblemen bij prematuur geboren kinderen zijn hersenbeschadigingen als gevolg van witte stofschade of hersenbloedingen in de grote en kleine hersenen. Het voorspellen van neuropsychologische ontwikkeling na (extreme) vroeggeboorte is de afgelopen 15 jaar één van de grootste uitdagingen binnen het vakgebied van de neonatologie geweest. Met name de verstandelijke ontwikkeling, waaronder we o.a. intelligentie, concentratie, executieve functies en (werk)geheugen rekenen, bleek in een vroeg stadium moeilijk te voorspellen. Dankzij verbeteringen in beeldvormende technieken zoals magnetische resonantie (MRI) zijn wetenschappers erin geslaagd om parameters te vinden die van voorspellende waarde kunnen zijn voor de ontwikkeling later. Voorbeelden van deze hersenmaten zijn hersenvolumes, witte stof rijping en markers van corticale ontwikkeling. In dit proefschrift hebben we een bescheiden bijdrage geleverd aan onze kennis over relevante voorspellers van neuropsychologische ontwikkeling na vroeggeboorte.

Naast het vooruitzicht op nauwkeurige(r) voorspelling van de neuropsychologische ontwikkeling, heeft de vooruitgang in beeldvormende technieken ons in staat gesteld vroege hersenontwikkeling nauwgezet in kaart brengen. Bovendien is deze technologische vooruitgang de belangrijkste voedingsbodem geweest voor 'connectoom'-onderzoek. De term 'connectoom' is geïntroduceerd in navolging van het genoom (de complete vingerafdruk van onze genetische informatie) en beschrijft de wetenschap die de elementen van de hersenen (vb. zenuwcellen, hersenregio's) en de verbindingen daartussen (vb. axonen, witte stofbanen) bestudeert. Complexe hersenfuncties zoals intelligentie, verbeeldingskracht en creativiteit zijn niet terug te voeren op specifieke hersengebieden, maar vloeien voort uit de werking van het hersennetwerk, het connectoom, als geheel. Deze

notie en de mogelijkheid om het hersennetwerk met geavanceerde MRI-technieken af te beelden, hebben connectoom-onderzoek een boost gegeven. Het menselijk brein is het meest toegankelijk op macroscopische schaal, dat wil zeggen als we naar hersengebieden en de witte stofbanen daartussen kijken. Dit staat in contrast met de microscopische schaal, die de individuele zenuwcellen en hun synaptische, dendritische en axonale verbindingen beschrijft. We hebben de duizelingwekkende getallen gezien waar het over gaat als we de menselijke hersenen op microscopische schaal willen onderzoeken. Het zou onvoorstelbaar veel rekenkracht en tijd vergen om het hersennetwerk op microscopische schaal te onderzoeken en daarnaast zijn synaptische verbindingen veranderlijk: ze worden gevormd of versterkt als we nieuwe vaardigheden leren of nieuwe herinneringen opdoen en verdwijnen als we vaardigheden niet gebruiken (het zogenaamde *'use it or lose it'* principe). Bovendien neemt het aantal synapsen in de loop van het leven af: van circa 1 biljard (10^{15}) tot ongeveer 100-500 biljoen bij volwassenen. De vraag is dus of het noodzakelijk en wenselijk is om alle zenuwcellen en hun verbindingen in de menselijke hersenen in kaart te brengen. Als we het over het menselijk connectoom hebben, verwijzen we dan ook meestal naar het bedradingspatroon van het brein op macroscopische schaal. Dit bedradingspatroon kunnen we zowel structureel als functioneel bestuderen. Met het structurele connectoom bedoelen we de witte stofverbindingen tussen verschillende hersengebieden, terwijl we met het functionele connectoom verwijzen naar een benadering van de functionele verbindingen in de hersenen. Het functionele hersennetwerk is dan ook minder intuïtief en daarom lastiger te vertalen. We hebben verschillende technieken tot onze beschikking om het functionele hersennetwerk te bestuderen, zoals elektro-encefalografie (EEG) en nabij infrarood spectroscopie (NIRS). De meest gebruikte techniek is evenwel functionele MRI. Deze techniek maakt gebruikt van het zogenaamde BOLD-signaal (BOLD staat voor blood oxygen level dependent) en de theorie die ten grondslag ligt aan functionele MRI is dat hersengebieden die actief zijn, meer zuurstof verbruiken. Tijdens hersenactiviteit verandert de bloedstroom naar het specifieke hersengebied en daarmee verandert de verhouding tussen zuurstofhoudend en zuurstofarm bloed ter plaatse. Als je over de tijd meet (in de orde van grootte van minuten), kun je fluctuaties in het BOLD-signaal meten. Als de fluctuaties in het BOLD-signaal tussen twee verschillende hersengebieden in een bepaalde - vooraf vastgestelde - mate met elkaar correleren, oftewel met elkaar in verband staan, dan stellen we vast dat deze gebieden functioneel met elkaar verbonden zijn. Om het functionele connectoom te bestuderen, wordt functionele MRI klassiek in rust gemeten, we noemen het dan 'resting-state fMRI'. In dit proefschrift ligt de nadruk op de ontwikkeling van het structurele connectoom.

In **hoofdstuk twee** hebben we evenwel de vroege ontwikkeling van functionele hersennetwerken onderzocht door middel van een literatuuronderzoek.

Dit proefschrift bestaat uit twee gedeelten. In deel één hebben we de vroege ontwikkeling van het hersennetwerk in kaart gebracht, welke is afgebeeld met behulp van geavanceerde MRI-technieken. In deel twee hebben we de relatie tussen hersenstructuur van de neonatale hersenen en neuropsychologische ontwikkeling in de (vroege) kindertijd bestudeerd. In dit gedeelte hebben we verschillende MRI- en beeldverwerkingstechnieken geëxploreerd, waaronder connectoom-onderzoek, om cognitieve en motorische ontwikkeling na vroeggeboorte te voorspellen.

Hoofdstuk twee is gebaseerd op een literatuuronderzoek naar vroege functionele netwerkontwikkeling in het foetale en neonatale brein. We onderzochten hoe het hersennetwerk gevormd wordt en hoe de eigenschappen van het netwerk veranderen in de eerste levensfase. Ook bestudeerden we hoe verstorende invloeden, waaronder blootstelling aan (verboden) middelen zoals cannabis en cocaïne, maternale depressie en prematuriteit de ontwikkeling van het netwerk beïnvloeden. Hoewel deze factoren zeer uiteenlopend van aard zijn, hebben ze ook een belangrijke gemeenschappelijke noemer: ze hebben vroeg in de ontwikkeling een verstorend effect en kunnen daarom het ontwikkelingsplan blijvend beïnvloeden. In deze literatuurstudie hebben we een aantal belangrijke neurobiologische principes geïdentificeerd die ten grondslag lijken te liggen aan hersenontwikkeling op alle verschillende niveaus (micro-, meso- en macroscopisch). De aanleg van het connectoom vindt in een vaste volgorde plaats, van simpel naar complex, van beneden naar boven en van centraal naar perifeer. Limbische verbindingen ontstaan centraal in de hersenen en zijn al zeer vroeg aanwezig. Deze vezels worden vanaf circa 13 weken zwangerschapsduur beschreven. Het limbische systeem is evolutionair één van de oudste hersensystemen en verantwoordelijk voor honger, angst en seksueel gedrag. Het limbische systeem speelt dus een belangrijke rol bij de overleving van de menselijke soort. Vlak nadat de eerste limbische verbindingen verschijnen, ontstaan ook projectievezels tussen de thalamus, een belangrijk schakelstation in de diepe grijze stof, centraal in de hersenen en de cortex. Daarna worden de witte stofbanen van de hersenbalk (corpus callosum) gevormd en ten slotte de intra-hemisferische associatie vezels die betrokken zijn bij de integratie van informatie afkomstig van sensorische, motorische en beslisgebieden in de hersenen. Deze verbindingen zijn dus essentieel voor de totstandkoming van hogere cognitieve functies. Wanneer zenuwcellen zijn gevormd en hun plek van bestemming in de hersenschors, de thalamus of de kleine

hersenen hebben bereikt, brengen ze onderlinge verbindingen tot stand. Dit gebeurt ongeveer vanaf de 20e zwangerschapsweek. Zodra er onderlinge verbindingen zijn gevormd, kunnen functionele netwerken ontstaan. De vroegste metingen van functionele hersennetwerken zijn rond 25-29 weken zwangerschapsduur verricht in gezonde foetussen. Dit onderzoek heeft het bestaan van primaire netwerken, zoals het motorische en visuele netwerk aangetoond en heeft ook een gefragmenteerde voorloper van het 'default mode netwerk' gedetecteerd. Het default mode netwerk is betrokken bij internaliserende cognitieve functies zoals planning, het ophalen van herinneringen en overdenken, wanneer de hersenen gevrijwaard zijn van externe, gerichte taken. Vroege hersennetwerkstudies hebben empirisch bewijs geleverd dat deze functionele netwerken zich ook ontwikkelen volgens de eerder vastgestelde primair-complexe ontwikkelingsvolgorde. Een groep vooraanstaande hersenwetenschappers in de Verenigde Staten heeft bijvoorbeeld aangetoond dat primaire sensorische en motorische netwerken nagenoeg compleet zijn bij de geboorte en slechts weinig postnatale aanpassingen ondergaan, terwijl het default mode netwerk en het dorsale aandachtsnetwerk belangrijke ontwikkelingsgerichte veranderingen doormaken tijdens het eerste levensjaar. De functionele netwerken die in verband worden gebracht met de meest complexe cognitieve functies waren aan het einde van het eerste levensjaar nog onrijp en moesten op dat moment nog belangrijke veranderingen doormaken om op hun volwassen equivalenten te gaan lijken.

Een ander belangrijk kenmerk van vroege netwerkontwikkeling is dat de rijping van structurele netwerken voorafgaat aan die van functionele netwerken en dat functionele netwerken sterkere veranderingen doormaken dan het structurele connectoom. Het belangrijkste verschil tussen structuur en functie is dat de architectuur van structurele netwerken grotendeels onveranderd blijft: de zogenaamde 'hubs', dat zijn hersengebieden die de meest invloedrijke rol in het communicatiesysteem spelen, omdat ze de meeste verbindingen hebben of omdat de meeste transportroutes via deze gebieden gaan, blijven dat gedurende het leven in het structurele hersennetwerk. De precuneus bijvoorbeeld (een gebied centraal, achterin de cortex) is een hub in het neonatale brein en bij volwassenen. De veranderingen die optreden in het structurele netwerk hebben voornamelijk betrekking op het versterken van connecties, het vergroten van de efficiëntie van het netwerk en het verlagen van de segregatie. Deze veranderingen komen tot stand als gevolg van myelinisatie, toegenomen coherentie tussen de witte stofvezels en het selectief verwijderen van overbodige connecties, oftewel 'pruning'. Daartegenover staat dat de aanpassingen aan het functionele

connectoom meer uitgesproken zijn. Hubs in het hersennetwerk veranderen hun lokalisatie van primaire hersengebieden naar associatiegebieden, zoals de precuneus. Ondanks deze onmiskenbare veranderingen, blijft de globale organisatie van het functionele connectoom behouden. De eigenschappen van deze organisatie zijn een small-world en scale-free netwerk met een zogenaamde 'rich club' van centrale gebieden die sterk met elkaar verbonden zijn en daarmee de ruggengraat van het hersennetwerk vormen. Deze kenmerken zijn zowel bij functionele als structurele hersennetwerken aanwezig.

In **hoofdstuk drie** hebben we de vroege ontwikkeling van het structurele hersennetwerk onderzocht met behulp van diffusie tensor imaging (DTI). Deze MRI techniek meet diffusie van water en maakt een inschatting van de oriëntatie van witte stofbanen op basis van de belangrijkste diffusierichting van water. In georganiseerde structuren zoals de witte stof zal water gemakkelijk in de richting van de vezels bewegen (diffunderen), maar veel moeilijker in de richtingen loodrecht op de vezelrichting. Met DTI kunnen we de belangrijkste diffusierichting van water voor alle voxels in de MRI-beelden beoordelen. Op deze manier kan de organisatie van alle macroscopische witte stofbanen dan ook worden afgeleid. We hebben deze metingen bij 44 prematuur en a terme geboren kinderen verricht die tussen 29 en 45 weken werden gescand (40 weken komt overeen met de uitgerekende datum). In deze studie hebben we een aantal kenmerken van vroege structurele connectoomontwikkeling geïdentificeerd die overeenkomen met de eerder beschreven biologische principes van hersenontwikkeling. Verbindingen tussen de diepe grijze stof en primaire cortexgebieden, zoals de primaire motorcortex en de visuele cortex lieten meer rijping zien gedurende de ontwikkelingsperiode van 16 weken dan connecties tussen heteromodale associatiegebieden, zoals de prefrontale cortex, de precuneus en het cingulum. Deze heteromodale associatiegebieden spelen een belangrijke rol bij de integratie van informatie en bij hogere cognitieve functies. We zagen ook dat radiale diffusie (RD), als maat voor myelinisatie, membraandensiteit en de rijping van het intracellulaire compartiment gerelateerd was aan de volgorde van myelinisatie van de subcorticale witte stof zoals deze in het begin van de vorige eeuw is beschreven door Paul Flechsig. Flechsig beschreef een atlas van postnatale myelinisatie, waarbij de nummering en intensiteit van de arcering in zijn atlas de volgorde en de mate van myelinisatie aangaf. Deze atlas was gebaseerd op metingen in de witte stof direct onder de cortex (oftewel subcorticaal) in hersenweefsel van overleden baby's en peuters. In onze studie vonden we een relatie tussen de myelinisatievolgorde zoals beschreven door Flechsig en de veranderingen in RD tussen 29 en 45 weken:

de subcorticale gebieden die de grootste verandering in RD lieten zien, myeliniseren het eerst, terwijl de gebieden met de kleinste veranderingen in RD in de neonatale periode het laatst myeliniseren. Een derde observatie in deze studie was dat veranderingen in fractionele anisotropie (FA), als maat voor membraanpermeabiliteit, coherentie van witte stofvezels en myelinisatie leidden tot een efficiënter hersennetwerk. We zagen geen veranderingen in andere organisatorische eigenschappen van het connectoom, zoals clustering en modulariteit. Laatstgenoemde kenmerken verschaffen informatie over netwerk*segregatie*, terwijl globale efficiëntie informeert over netwerk*integratie*. Ten slotte zagen we dat het neonatale hersennetwerk meer dan 80% overlap vertoonde met de organisatie van het volwassen netwerk en dat de overeenkomsten toenamen met de tijd. FA vertoonde daarbij snellere rijping dan RD.

In deel twee van dit proefschrift hebben we de relatie tussen een drietal kwantitatieve MRI-maten en cognitieve ontwikkeling bij peuters en kleuters onderzocht. Het drieluik van MRI-parameters voor hersenontwikkeling op de neonatale leeftijd omvat FA van de witte stof, hersenvolumes en parameters van corticale rijping. Allereerst beschreven we de associatie tussen FA in het neonatale connectoom (als proxy van witte stofrijping), FA-gerelateerde netwerkmaten (als maat voor netwerkorganisatie) en intelligentie op de vroege schoolleeftijd in 30 prematuur geboren kinderen (zwangerschapsduur <31 weken, van wie de meesten geboren waren <28 weken). We vonden een sterke associatie tussen FA in de witte stof gemeten rond de uitgerekende datum en performaal IQ op de leeftijd van 5 jaar en 7 maanden. Deze resultaten bleven significant na correctie voor neonatale hersenschade, hetgeen bij 3 kinderen in het studiecohort aanwezig was en opleidingsniveau van de moeder. Opleidingsniveau van moeder is een belangrijke mediator van cognitieve en fijne motorische ontwikkeling, met name in de eerste levensjaren en is daarom belangrijk om in ogenschouw te nemen wanneer men de relatie tussen MRI-maten en cognitieve vaardigheden bestudeert. De resultaten van dit onderzoek zijn beschreven in **hoofdstuk vier**.

In **hoofdstuk vijf** onderzochten we de relatie tussen hersenvolumes gemeten rondom de uitgerekende datum en neuropsychologische ontwikkeling bij 2 jaar, 3,5 jaar en 5,5 jaar bij 112 prematuur geboren kinderen (zwangerschapsduur <31 weken). Hier zagen we dat ventrikelvolume een sterke voorspeller was voor ontwikkeling in de kindertijd. We vonden namelijk dat kleinere ventrikels geassocieerd waren met betere cognitieve en motorische vaardigheden bij 2 en 3,5 jaar en met snellere verwerkingssnelheid bij 5,5 jaar. We vonden ook een relatie tussen witte stofvolume en grove motorische ontwikkeling bij 2 jaar en verwerkingssnelheid bij 5,5 jaar. Kinderen die een groter witte

stofvolume hadden rond de uitgerekende datum, hadden hogere scores op de grove motoriekschaal van een gevalideerde ontwikkelingstest voor jonge kinderen (Bayley Scales of Infant and Toddler Development, derde editie) en betere verwerkingssnelheid op de Wechsler Preschool and Primary Scale of Intelligence bij 5,5 jaar. Ten slotte vonden we een relatie tussen corticale grijze stofvolume, cerebellumvolume en neuropsychologische ontwikkeling bij 2 jaar en bij 3,5 jaar. Deze relatie werd echter grotendeels gemedieerd door hersenschade. Het opleidingsniveau van moeder had geen invloed op gevonden relaties. Concluderend kunnen we dus stellen dat ventrikelvolume en witte stofvolume zinvolle hersenmaten lijken te zijn om ontwikkeling van hedendaagse prematuur geboren kinderen in Nederland te voorspellen.

In **hoofdstuk zes** hebben we de morfologische hersenontwikkeling en neuropsychologische ontwikkeling van extreem prematuur geboren tweelingen en eenlingen beschreven. In deze studie brachten we de hersenvolumes en corticale ontwikkeling gemeten rond de uitgerekende datum alsmede cognitieve en motorische vaardigheden op de leeftijd van 2 jaar in kaart in een cohort van 306 extreem prematuur geboren kinderen. Van 240 kinderen was er een goede kwaliteit MRI-scan met bijbehorende segmentatiedata en daaruit volgende hersenvolumes en parameters van corticale ontwikkeling beschikbaar. Neuropsychologische ontwikkelingsdata op de leeftijd van 2 jaar konden bij 275 worden verkregen. Een deel van de kinderen in onze studie had dus alleen een bruikbare MRI scan maar kon niet worden teruggezien (v.b. omdat ouders verhuisd waren of afzagen van follow-up), een ander deel had neuropsychologische ontwikkelingsdata zonder goede MRI data en voor het laatste deel beschikten we over beide data. Ter vergelijking hebben we een groep van 15 a term geboren kinderen toegevoegd die om uiteenlopende redenen een MRI-onderzoek hadden ondergaan maar zich volgens de norm ontwikkelden (in ieder geval tot de leeftijd van 2 jaar) (n=9) of bij wie er geenszins zorgen waren over eventuele ontwikkelingsproblemen (n=6). Een belangrijke vraag die we met onze studie trachtten te beantwoorden, is of premature tweelingen intrinsiek meer risico hebben op afwijkende hersenontwikkeling en ontwikkelingsproblemen, of dat de ontwikkelingsproblemen die vaak in verband worden gebracht met prematuur geboren tweelingen hun origine vinden in een lager geboortegewicht en kortere zwangerschapsduur. Als gevolg van de intra-uteriene omstandigheden, eventuele zwangerschapscomplicaties en/of fertiliteitsbehandelingen is de zwangerschapsduur bij tweelingen namelijk vaak korter en het geboortegewicht lager dan bij eenlingen. Om inzicht te krijgen in deze vraagstelling, werden prematuur geboren tweelingen gematcht met premature eenlingen met een vergelijkbare zwangerschapsduur,

geboortegewicht en een identiek geslacht. Daarnaast onderzochten we de gehele groep, zonder voorafgaande matching en vergeleken we de prematuur geboren kinderen met voldragen kinderen. Hersenvolumes, corticale parameters, cognitieve en motorische ontwikkeling bij 2 jaar waren niet verschillend tussen prematuur geboren tweelingen en eenlingen. Pre-eclampsie, een ernstige zwangerschapscomplicatie die zich kenmerkt door hypertensie en proteïnurie bij de moeder en foetale groeivertraging bij het kind, als gevolg van placentaire en maternale vasculaire dysfunctie, kwam vaker voor bij premature eenlingen. Deze factor was geassocieerd met kleinere witte stofvolumes en wanneer we kinderen van moeders met pre-eclampsie excludeerden, was relatief grijze stofvolume groter bij prematuur geboren tweelingen dan bij eenlingen. Voldragen kinderen hadden complexere gyrering en een andere samenstelling van het brein met relatief meer witte stof en minder liquor dan prematuur geboren kinderen. De cognitieve en motorische ontwikkeling bij 2 jaar waren niet verschillend tussen prematuur en a term geboren kinderen die vervolgd waren. Uit deze studie kunnen we concluderen dat er geen aanwijzingen zijn voor verschillen in afwijkende hersenontwikkeling bij prematuur geboren tweelingen en dat de intra-uteriene omgeving een belangrijke rol lijkt te spelen bij de vroege hersenontwikkeling. De lange termijneffecten van de foetale omstandigheden op de neuropsychologische ontwikkeling van extreme prematuren moeten afgewacht worden.

Tot slot

In dit proefschrift hebben we netwerkontwikkeling in de eerste stadia na de totstandkoming van het hersennetwerk onderzocht, zowel door bestaande literatuurdata te bestuderen als door nieuwe data te verzamelen en analyseren. Op deze manier hebben we een aantal belangrijke facetten van vroege connectoomontwikkeling vastgesteld, waaronder de primair-complexe ontwikkelingsvolgorde. Daarnaast hebben we MRI-parameters voor neuropsychologische ontwikkeling na vroeggeboorte onderzocht en een set markers geïdentificeerd die relevant zouden kunnen zijn als vroege voorspellers voor verschillende ontwikkelingsdomeinen. We hebben de interactie tussen vroege morfologische hersenontwikkeling en hersenfunctie in de vorm van cognitieve en motorische vaardigheden bij prematuur geboren tweelingen en eenlingen onderzocht, hetgeen van belang kan zijn voor clinici betrokken bij de zorg voor extreem te vroeg geboren kinderen en voor (neuro-)wetenschappers met erfelijkheid als aandachtsgebied. In dat onderzoek vroegen we ook aandacht voor de mogelijke (lange termijn) effecten van de intra-uteriene omgeving op de ontwikkeling van het ongeboren kind.

Het tijdperk van *'big data'* breekt aan. Daarbij is er een toenemend aantal mogelijkheden en initiatieven om verschillende onderzoeksmodaliteiten, zoals beeldvormende technieken (vb. om het connectoom in kaart te brengen), histologische data, gegevens over metabolisme, hersenfunctie en genetische data met elkaar te combineren. Deze rijke verzameling aan onderzoeksgegevens stelt ons in staat een nieuwe weg in te slaan om de neurobiologische beginselen die ten grondslag liggen aan gezonde en afwijkende hersenontwikkeling beter te leren begrijpen. Ook biedt het een unieke kans om gegevens te combineren om op deze manier de voorspelling van neuropsychologische ontwikkeling te verbeteren. Genetische profilering en neurogenetica zijn bijzonder interessant in deze context. Dit type onderzoek is relatief nieuw en is tot nu toe slechts sporadisch toegepast in de neonatologie. De samenhang tussen genetische informatie en MRI-maten voor hersenontwikkeling zou weleens een belangrijke ontbrekende schakel kunnen zijn tussen neurobiologische mechanismen enerzijds en het fenotype van hersenontwikkeling (vb. intelligentie, gedrag en taalvaardigheid) anderzijds. Daarbij heeft het de potentie om kinderen die een verhoogd risico hebben op hersenschade en een afwijkende hersenontwikkeling in een vroeg stadium op te sporen. Op deze manier kunnen toekomstige neuroprotectieve en herstellende behandelingen toegespitst worden op zuigelingen en kinderen die daar de meeste baat bij zullen hebben.

De ontzaglijke hoeveelheid data waarover we tegenwoordig beschikken is tegelijkertijd een vloek en een zege. Het roept nieuwe vraagstukken op het gebied van medische ethiek op en vereist adequate dataversleuteling. Samenwerking tussen verschillende partners en disciplines is een sleutelbegrip om de vergaarbak aan gegevens die we *kunnen* verzamelen optimaal te benutten, waarbij ieders kennis en expertise een belangrijke rol speelt. We hopen dat dit proefschrift een bescheiden bijdrage heeft kunnen leveren aan het aanmerken van relevante MRI-markers, multimodale onderzoeksvragen en onontgonnen onderzoeksterrein op het gebied van vroege hersenontwikkeling waar we nog veel kunnen leren en winst kunnen behalen voor onze jongste en meest kwetsbare patiënten.

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LIST OF PUBLICATIONS

Publications included in this thesis are marked by an asterisk (*).

* **Keunen K**, Benders MJ, Leemans A, van Haastert IC, Fieret-Stam PC, Scholtens LH, Viergever MA, Kahn RS, Groenendaal F, de Vries LS, van den Heuvel MP. Predicting school age cognitive capacities from the neonatal connectome in preterm born children. Dev Med Child Neurol 2017; 59(9): 939-946.

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Forthcoming

Keunen K, Benders MJ, Moeskops P, de Reus MA, van der Burgh HK, de Lange SC, Schmidt R, Išgum I, Groenendaal F, de Vries LS, van den Heuvel MP. White and gray matter deficits in the neonatal brain in Down syndrome. Manuscript in preparation.

* **Keunen K**, van der Burgh HK, de Reus MA, Moeskops P, Schmidt S, Stolwijk LJ, de Lange SC, Išgum I, de Vries LS, Benders LS, van den Heuvel MP. Early human brain development: insights into macroscale connectome wiring. Submitted.

* **Keunen K**[**], van Kalken F[**], Moeskops P, van Haastert IC, de Vries LS, Viergever MA, Groenendaal F, Išgum I, Benders MJ. Brain volumes, cortical maturation and early neurodevelopment are comparable in extremely preterm twins and singletons. Manuscript in preparation.

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Senden RE, **Keunen K**, van der Aa NE, Leemans A, Isgum I, Viergever MA, Dudink J, de Vries LS, Groenendaal F, Benders MJ. Cerebellar injury does not affect supratentorial white matter microstructural organization and neurodevelopmental outcome in preterm infants. Submitted.

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ABOUT THE AUTHOR

Kristin was born on April 5 1987 in Varsseveld. After graduating high school *cum laude* at the Isala College in Silvolde in 2005 she started medical school at the University Utrecht. During her medical training she did a research internship at the Brigham and Women's Hospital, Harvard Medical School in Boston. After finishing medical school in 2011, Kristin worked as a resident (ANIOS) at the department of pediatrics at the Amphia Hospital in Breda and the Wilhelmina Children's Hospital, University Medical Center (UMC) in Utrecht. Chasing her ambition to become a scientist, she started a PhD at the department of Neonatology and Brain Center Rudolf Magnus, UMC Utrecht in 2013 supervised by Prof. dr. Manon Benders and Prof. dr. Linda de Vries. She started collaborating with Dr. Martijn van den Heuvel (Dutch Connectome Lab) in 2014, studying neonatal connectome development and its link to cognitive functioning, which resulted in this thesis. Desiring to widen her scope in terms of clinical experience, Kristin is currently working as a resident (ANIOS) in the intensive care unit at the Amphia Hospital in Breda. Besides, volunteering has played an important role in her professional career. During her PhD, she was involved in Stichting Heppie and Stichting Handje Helpen, supporting children with different needs. After completing the PhD program she spent six weeks at Esperanza Verde, a wildlife rescue center in the Peruvian Amazon. Here, she discovered her interest in wildlife preservation and rainforest conservation and decided to continue to be involved in the foundation. Kristin aims to become a pediatrician and combine clinical work with a scientific career.