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Atypical fetal brain development in fetuses with non-syndromic isolated musculoskeletal birth defects (niMSBDs)

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Non-syndromic, isolated musculoskeletal birth defects (niMSBDs) are among the leading causes of pediatric hospitalization. However, little is known about brain development in niMSBDs. Our study aimed to characterize prenatal brain development in fetuses with niMSBDs and identify altered brain regions compared to controls. We retrospectively analyzed in vivo structural T2-weighted MRIs of 99 fetuses (48 controls and 51 niMSBDs cases). For each group (19–31 and >31 gestational weeks (GW)), we conducted repeated-measures regression analysis with relative regional volume (% brain hemisphere) as a dependent variable (adjusted for age, side, and interactions). Between 19 and 31GW, fetuses with niMSBDs had a significantly ($P < 0.001$) smaller relative volume of the intermediate zone ($-22.9 \pm 3.2\%$) and cerebellum ($-16.1 \pm 3.5\%$,) and a larger relative volume of proliferative zones ($38.3 \pm 7.2\%$), the ganglionic eminence ($34.8 \pm 7.3\%$), and the ventricles ($35.8 \pm 8.0\%$). Between 32 and 37 GW, compared to the controls, niMSBDs showed significantly smaller volumes of central regions ($-9.1 \pm 2.1\%$) and larger volumes of the cortical plate. Our results suggest there is altered brain development in fetuses with niMSBDs compared to controls ($13.1 \pm 4.2\%$). Further basic and translational neuroscience research is needed to better visualize these differences and to characterize the altered development in fetuses with specific niMSBDs.

Key words: fetus; fetal brain; prenatal brain development; birth defects.

Introduction

According to the Centers for Disease Control and Prevention, each year in the United States, at least 1 in 1943 infants is born with isolated limb defects ("Leading Causes of Death and YPLL 2001-2020" n.d.). Among those, specific muscle/bone defects, such as clubfoot, reach an incidence as high as 1 in 593 births (Mai et al. 2019). Besides, between 2001 and 2020 in the United States, congenital anomalies, including inborn limb defects, were the 10th leading cause of potential years lost. Congenital anomalies remain one of the leading causes of pediatric hospitalization and medical expenditures (Centers for Disease Control and Prevention (CDC) 1995; Yoon et al. 1997), placing a significant burden on society and individual families. However, despite the relatively high incidence, a national registry for musculoskeletal birth defects does not exist, and the causes remain unknown. As a result, children with isolated congenital limb defects and their families face frequent challenges navigating healthcare systems and finding support groups.

The lack of knowledge about the etiology of limb defects contributes to our inefficacy in preventing and treating them. While some studies suggest that gene abnormalities are linked to musculoskeletal defects in certain syndromes (Mensah et al. 2023), the presence of gene abnormalities in isolated conditions like

clubfoot is found in less than 25% of cases (Huang et al. 2023). As a result, preventative strategies still need to be updated, with a primary emphasis on the administration of prenatal vitamins. On top of that, for many inborn limb defects, treatment strategies primarily focus on early surgery, rehabilitation, and personalized prosthetics/orthotics. With the opening of new venues, the potential for growing organs from organoids is expanding. At the same time, the increase in computing power and the rise of highly sophisticated neuroimaging machines and tools (such as Magnetoencephalography) provide means to develop highly efficient brain-computer interfaces capable of using brain signals to control external devices (Vidal et al. 2016). However, the first step toward utilizing new technology for disease modeling and therapeutic purposes is a better understanding of peripheral and central nervous system reorganization in congenital skeletal abnormalities, which in humans remains largely unexplored.

The adult human body parts are represented topographically in the primary somatosensory cortex, referred to as S1. Different cortical regions participate in the sensation (physical or mental response) of mechanical and proprioceptive stimuli arising from various body parts. Some aspects of how these inputs combine to create such a robust sense at each spinal cord level and, subsequently, in the cortex still need to be better understood. However,

increasing evidence suggests that cortical regions involved in processing these inputs overlap and create a widespread distribution of information content more similar to their motor counterpart, the primary motor cortex, M1 (Schieber 2001; Muret et al. 2022). Recently, M1 was found to have inter-effector regions that connect with a number of other areas of the brain critical in higher-level functioning and coordination, including S1. It was suggested that the homunculus is more complex than we previously thought and contains areas in between the well-known topographical "hand, foot, and mouth" regions. These inter-effector regions are not yet present in newborns but detectable in an 11-month-old infant and integrate body control with higher-level action planning, perhaps identifying a stage of development and interaction between M1, S1, and other brain regions previously unexplored (Gordon et al. 2023).

As the fetal telencephalon evolves during human fetal brain development, the representation of the fetal body is yet to be established. The following fetal compartments constitute the immature fetal brain: proliferative compartments (the ventricular zone and subventricular zone), the intermediate zone (fetal white matter; iz), subplate zone (sp), cortical plate (future cortex; cp), and marginal zone. Within these compartments, characterized by their transient nature, the major neurogenic events (including but not limited to cell proliferation and migration, growth of axon pathways and synaptogenesis, pruning of axons, developmental cell death, and myelination) occur with varying spatiotemporal intensity (for reviews see Bystron et al. 2008; Silbereis et al. 2016). Although elements of corticogenesis take place in each of these compartments, empirical evidence suggests that some compartments have a predominant role as sites for particular neurogenetic events. Specifically, the subplate and the marginal zones are critical sites for early synaptic interaction (Ayoub and Kostovic 2009). Furthermore, because of its rich extracellular matrix, the sp remains pivotal for guiding growing axons and establishing early circuitry (Molnár et al. 2003; Kostović and Jovanov-Milosević 2006). Once the circuits and connections of the telencephalon are pruned, the axons waiting within the sp move to the cp, the primary site of post-migratory cortical neuron differentiation.

The development of the endmost tracts that provide input to cortical S1 occurs in the early stages of fetal growth, beginning at roughly 24 gestational weeks (GW) when thalamocortical axons penetrate the cerebral cortex (Kostović and Judas 2010). In contrast, terminals of the tracts that carry outputs from M1 to the spine finish their anatomic development and reach the lumbar spine around the 8th month of pregnancy (for review, see ten Donkelaar et al. 2004). Therefore, preceding neurogenic events (such as the growth of peripheral nerves and the arrival of spinothalamic tracts) that occur before the establishment of permanent cortical circuitry must be considered when characterizing fetal brain development.

In contrast to a normal fetus, a fetus with congenital musculoskeletal abnormalities may have limited or altered input from the periphery spurring changes in brain developmental trajectories, particularly the somatosensory cortex. Liao et al. (2016) demonstrated the reorganization of afferent tracts along the spinal cord and cerebrum at every hierarchical level in a monkey with a congenitally deformed foot. When tracing the representation of the plantar pads of the foot in the spinal cord and the gracile nucleus on each side of the body, they found that the terminations were spread out along the length of the spinal cord in a continuous manner on both affected and unaffected sides. These results were in contradistinction with segregated areas found in the spinal cord of the normal monkey. The transient compartments of the fetal brain may reveal additional changes in early development above the level of the spinal cord.

The growth trajectories of transient fetal compartments have been characterized in-vitro using magnetic resonance imaging (MRI) methods (Vasung et al. 2016; Pogledic et al. 2021). Recently, these approaches were employed in vivo to characterize growth trajectories of transient fetal compartments in typically developing fetuses (Vasung et al. 2019b, 2020; Stuempflen et al. 2023) and fetuses with congenital heart disease (Feldmann et al. 2020; Rollins et al. 2021) and brain abnormalities (Schwartz et al. 2021). Here we further explored the brain development of fetuses with non-syndromic isolated musculoskeletal birth defects (niMSBDs) and characterized the differences in fetal brain structure compared to controls.

Materials and methods
Materials

For this observational study, we retrospectively screened all pregnant women with fetal MRIs obtained between July 2013 and June 2022 in our institution. From 2623 fetal MRIs, we identified 114 fetuses with non-syndromic isolated musculoskeletal structural birth defects of the body [Congenital malformations of limbs including arthrogryposis, unspecified congenital malformation of limb(s), club foot, polydactyly, syndactyly, reduction defects of upper, lower, or unspecified limbs]. Inclusion criteria were: maternal age between 18 and 45 years, dysmorphic features of the musculoskeletal system of the body on fetal MRI (ICD-10-CM codes Q74.3, Q68.8, Q74.9, Q66.8, Q69, Q70, Q79.9, Q71, Q72, Q73), no serious maternal medical conditions during pregnancy, between 15 and 32 GW of pregnancy, pregnant women that were recruited prospectively as controls for fetal MRI in other research studies but had an incidental fetal niMSBDs findings, and pregnant women referred to fetal MRI after diagnosing fetal niMSBDs on the routine ultrasound (US) in the absence of other abnormal findings. Exclusion criteria were the following: multiple gestation pregnancies, brain malformations or lesions identified on the US or MRI, other identified organ anomalies on the US or MRI, known chromosomal abnormalities, and known congenital infections.

We pooled the control subjects from ongoing or current prospective fetal MRI studies (Gholipour et al. 2017; Vasung et al. 2019b, 2020; Rollins et al. 2021). Eligibility inclusion criteria for controls included maternal age between 18 and 45 years, no record of serious maternal medical conditions during pregnancy, between 15 and 32 GW of pregnancy, pregnant women that were recruited prospectively as controls for fetal MRI in other research studies whose fetal MRI was read as normal by radiologists and pediatric neuroradiologists, and fetuses with MRI acquired for reasons other than suspected malformation or injury of the central nervous system that was read by as normal by radiologists and pediatric neuroradiologists. Exclusion criteria for controls included multiple gestation pregnancies, dysmorphic features on US examination, brain malformations, or brain lesions identified on MRI or US, other identified organ anomalies, known chromosomal abnormalities, known congenital infections, and any central nervous system abnormality on the fetal MRI.

Methods
Magnetic Resonance Imaging

All fetal MRIs were acquired on 3 T Siemens Skyra or Siemens Magnetom Vida MRI Scanners at Boston Children's Hospital.

We included only T2 weighted Half-Fourier Acquisition Single-shot Turbo spin Echo imaging (HASTE) of the fetal head for the analyses. Per clinical MRI protocol, we used the following image acquisition parameters: time to repeat $=\sim 1400$ ms, time to echo $=\sim 93$–103 ms, in-plane resolution $=\sim 1$–1.2 mm, slice thickness $=\sim 2$–4 mm, acquisition matrix $\sim 256 \times 256$, four-slice interleaved acquisition. The total scan time for each subject (including other sequences and the time for adjustments between sequences) did not exceed 60 min.

All HASTE acquisitions with satisfactory quality were preprocessed with a previously validated, in-house processing pipeline (for details, see Gholipour et al. 2017). The pipeline is composed of the following steps: (i) correction of motion with superresolution 3D slice-to-volume reconstruction, (ii) masking of the brain, (iii) correction of bias field using N4 and intensity normalization, and (iv) registration to a spatiotemporal fetal brain MRI atlas (Gholipour et al. 2017). We visually inspected the reconstructed 3D volumes and automatically segmented them into tissue classes. The automatic segmentations were based on the spatiotemporal atlas using probabilistic simultaneous truth and performance level estimation (PSTAPLE) (Akhondi and Warfield 2013; Gholipour et al. 2017). In younger fetuses (19–31 GW), we segmented the following tissue classes (Fig. S1) for each hemisphere: cp, sp, intermediate zone, central regions (including the caudate, lentiform nucleus, thalamus, subthalamic nucleus, and central white matter structures that include internal capsules, fornix, and hippocampal commissure), proliferative zones (ventricular and subventricular zones), ganglionic eminence, lateral ventricles, limbic regions (hippocampus, amygdala), and cerebellum. Because of the lack of a clear border between the intermediate zone and sp after 31 GW, in the older group of fetuses (>31 GW), we grouped these two tissue classes into one tissue class called "fetal white matter" (Fig. S2). See our previous publications for anatomical details and borders to segment the tissue classes mentioned above (Vasung et al. 2019b, 2020, 2022). We included only fetuses with excellent quality automatic segmentation in further analyses (no need for manual correction).

Statistical analyses

Relative volumes (% of the hemisphere, i.e. the ratio between the region's volume and the corresponding hemispheric volume) showed a skewed distribution and were log-transformed for analysis. Younger fetuses (19–31 GW) and older fetuses (32–37 GW) were analyzed separately. We assembled the regional relative volumes within each measured hemisphere into a vector for use as the dependent variable in repeated-measures regression analysis, thus accounting for correlation among regions. The regression model was stratified by hemisphere and adjusted for gestational age (continuous within the age group). We included interaction terms to allow differential effects for all combinations of hemisphere, region, and group (niMSBDs vs. control), then discarded non-significant interactions from the final fitted model. We employed iterative reweighting to identify outliers and reduce the influence of extreme values (Rousseeuw and Leroy 1987).

For reporting, we converted contrasts on the log scale ($x \pm SE$) into percentage differences $[100\% \times (10\times - 1) \pm 100\% \times 10\times(10^{SE}-1)]$. To compensate for multiple comparisons, we applied the Holm step-down procedure to each set of region-specific findings, enforcing a 5% familywise maximum Type I error rate (Holm 1979). For the subset of findings that emerged as statistically significant, we calculated the false discovery rate (Benjamini and Hochberg 1995). We used SAS software (version 9.4, Cary, NC) for all statistical computations.

Table 1. Breakdown of limbs affected by niMSBDs included in the study. Note: Some of the fetuses had conditions that affected multiple joints.

Condition/Extremity	Upper Extremity		Lower Extremity	
Side	Left (N)	Right (N)	Left (N)	Right (N)
Clubfoot			9	11
Arthrogryposis	5	4	3	3
Skeletal Dysplasia	3	2	2	2
Short long bones	9	9	9	9
Unspecified Deformities	5	3	5	7
Absence	2	2		
Rocker Bottom			1	1
Claw Hand	1			
Dactylys (abnormalities of fingers)	4	3	1	
Osteogenesis Imperfecta	1	1	1	1

niMSBDs, non-syndromic isolated musculoskeletal birth defects.
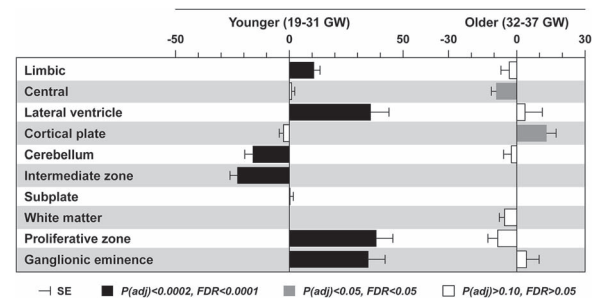

Fig. 1. Summary of results showing % difference in the relative volume of structures between niMSBDs and controls between 19-31GW (left) and 32-37GW (right). Abbreviations: central (central regions including the subcortical gray matter (caudate, lentiform nucleus, thalamus, subthalamic nucleus) and white matter (internal capsule, fornix, and hippocampal commissure).

We conducted post-hoc power calculations for the non-significant results based on the magnitude of their standard errors. In the younger group, we had 80% power to detect a niMSBDs–control difference as small as 5.4% in the cp, 4.2% in the subplate, and 4.1% in the central region. In the older group, among the six regions showing non-significant differences, the detectable effects were smallest for white matter (6.8%) and cerebellum (9.9%) and ranged up to 21.9% for lateral ventricles.

Results

From 2623 fetal MRIs obtained between 2013 and 2022 at our institution, we identified 114 cases with prenatal niMSBDs. All cases were processed using an *in-house* built pipeline developed for an automatic 3D MRI reconstruction and segmentation of fetal brains (see Methods). From 114 cases, 31 fetal MRIs failed to be processed ($\sim$27%). The other 83 cases that were processed were visually inspected by an expert in fetal anatomy and stratified based on the quality of segmentations into bad ($n = 3$), moderate ($n = 15$), good ($n = 14$), and excellent ($n = 51$). Only subjects with excellent anatomical brain segmentations (no need for manual edits) were kept for further statistical analyses (45 younger subjects 19–31 GW and six older subjects > 31 GW).

Clinical characteristics

Fetuses in our cohort had a broad range of conditions (Table 1). The short long bones and clubfoot were the most frequent descriptions of the deformity.

Table 2. The difference in relative volumes (% hemisphere) of brain regions between controls and niMSBDs in younger fetuses (19–31GW).

Region	niMSBDs	Controls	niMSBDs versus Controls	t	P-value	Adjusted P-value	False Discovery rate
Intermediate zone	16.9 ± 0.4	22.0 ± 0.7	−22.9 ± 3.2	6.420	<0.0001	<0.0001	<0.0001
Proliferative Zone	9.4 ± 0.3	6.8 ± 0.3	38.3 ± 7.2	−6.36	<0.0001	<0.0001	<0.0001
Ganglionic Eminence	0.8 ± 0.0	0.6 ± 0.0	34.8 ± 7.3	−5.63	<0.0001	<0.0001	<0.0001
Lateral Ventricles	6.0 ± 0.2	4.4 ± 0.2	35.8 ± 8.0	−5.34	<0.0001	<0.0001	<0.0001
Limbic regions	1.6 ± 0.0	1.5 ± 0.0	10.9 ± 2.6	−4.42	<0.0001	<0.0001	0.000
Cerebellum	3.1 ± 0.1	3.6 ± 0.1	−16.1 ± 3.5	4.240	<0.0001	0.000	0.000
cp	29.8 ± 0.4	30.6 ± 0.4	−2.6 ± 1.8	1.380	0.169	0.506	0.217
Central Regions	7.0 ± 0.1	6.9 ± 0.1	1.0 ± 1.4	−0.73	0.469	0.938	0.528
Subplate	31.8 ± 0.3	31.7 ± 0.4	0.3 ± 1.5	−0.22	0.825	0.825	0.825

niMSBDs, non-syndromic isolated musculoskeletal birth defects.

Between 19 and 31GW fetuses with niMSBDs have significantly smaller volumes of the cerebellum and intermediate zone and greater volumes of proliferative compartments and limbic regions.

We found significant differences in relative regional brain volumes between niMSBDs and controls between 19 and 31GW (Fig. 1, Table 2). Specifically, niMSBDs, on average, showed 22.9% smaller relative volumes of the intermediate zone and 16.1% smaller relative volumes of the cerebellum. In contrast, proliferative zones were, on average, 38.3% larger in niMSBDs as well as the ganglionic eminence (34.8 ± 7.3%) and ventricles (35.8 ± 8.0%). Finally, limbic regions were also found to be significantly larger in niMSBDs, although these differences were relatively small (10.9 ± 2.6%).

Between 32 and 37GW fetuses with niMSBDs have significantly smaller volumes of central regions and greater volumes of cp.

In the older group of fetuses, 32–37GW old, we also found significant differences in relative volumes of brain regions (Fig. 1). However, these differences were less prominent (Fig. 1, Table 3). Specifically, fetuses with niMSBDs showed significantly smaller volumes of central regions (−9.1 ± 2.1%) and larger volumes of the cp (13.1 ± 4.2%).

Discussion

We explored structural differences between fetuses with niMSBDs and healthy controls in a single institution. Even though we included a broad spectrum of conditions, they all share a common anatomic abnormality: an abnormal development of a part or the entire musculoskeletal system. Our results indicate regional differences in fetal brain structure between niMSBDs and controls. Specifically, between 19 and 31GW, fetuses with niMSBDs showed significantly larger proliferative zones, ganglionic eminence, ventricles, limbic regions, and smaller volumes of the cerebellum and intermediate zone (i.e. fetal white matter). Older fetuses (>31 GW) showed significant but small differences in relative volumes of central regions and the cp. We cannot explain the pathophysiology leading to these differences. However, based on the knowledge of developmental anatomy, we suggest that altered input from/to the periphery might lead to altered regional brain maturation.

The development of the peripheral nervous system is tightly coupled with the development of the musculoskeletal system. In certain niMSBDs conditions, abnormal peripheral nervous system development parallels the abnormal development of the musculoskeletal system (e.g. polydactyly, limb reduction). However, this link is less clear in other niMSBDs conditions (such as skeletal dysplasia). New studies suggest that even normal peripheral nervous system development, including peripheral growth and branching of fibers, might be stochastic and not exclusively influenced by specific local guidance cues during different stages of development (Hassan and Hiesinger 2015). To this end, even if peripheral nerves are present in certain niMSBDs like arthrogryposis or polydactyly, the functionality of these connections and synchronized transfer of signals from/to the periphery remain unknown. Furthermore, in monkeys, experimental studies provide clear evidence that niMSBDs congenital foot deformation leads to structural reorganization of the nervous system at every hierarchical level (Liao et al. 2016). Given that we found significant differences between niMSBDs and controls, our results go hand in hand with these findings.

Specifically, between 19 and 31GW, fetuses with niMSBDs showed significantly smaller relative volumes of the cerebellum and intermediate zone, a difference that we did not detect after >31GW. Potential explanations for these structural differences between niMSBDs and controls are innumerable. However, based on the evidence from animal studies (Liao et al. 2016), it is likely that the changes we observe in the volume of the cerebellum and intermediate zone are the result of a spread out and anatomical change of inputs from/to the spine and periphery.

There are three types of ascending sensory pathways: (i) for tactical information, vibration, and position sense, (ii) for pain and temperature, and (iii) for somatosensory information to the cerebellum. The pathways carrying tactile/vibration/position sense and somatosensory information to the cerebellum are particularly interesting for our study.

The first-order primary afferent fibers collecting tactile information from the periphery converge into the dorsal columns of the spinal cord. From there, they project to the second-order neurons located in the dorsal nuclei of the spine. These second-order neurons cross the midline (internal arcuate fibers) and send their afferent axons to the ventrobasal complex of the thalamus via the medial lemniscus. Finally, the third-order afferents from the thalamus project to the postcentral gyrus. The dorsal funiculus is already present in human embryos and developed ~5GW (Müller and O'Rahilly 1989). The medial lemniscus, carrying information from the nucleus gracilis and cuneatus of the spine to the thalamus, is present ~9GW (Müller and O'Rahilly 1990). However, anatomical studies indicate that despite its presence, the medial lemniscus shows protracted maturation and myelinates throughout the third trimester (Bodhireddy et al. 1994). Finally, fibers connecting the thalamus to the somatosensory cortex also show protracted maturation. They show an outgrowth ~9.5GW of age and form massive bundles that pass the diencephalic-telencephalic boundary ~11.5GW (Vasung et al. 2010). Between 14 and 16GW, thalamocortical bundles can be traced from the

Table 3. The difference in relative volumes (% hemisphere) of brain regions between controls and niMSBDs in older fetuses (>31GW).

Region	niMSBDs	Controls	niMSBDs versus Controls	t	P-value	Adjusted P-value	False Discovery Rate
Central Regions	6.1 ± 0.1	6.7 ± 0.1	−9.1 ± 2.1	4.230	0.000	0.001	0.001
cp	37.7 ± 1.2	33.3 ± 0.6	13.1 ± 4.2	−3.42	0.001	0.010	0.005
White Matter	51.7 ± 1.1	54.6 ± 0.7	−5.4 ± 2.3	2.340	0.024	0.142	0.063
Proliferative Zones	3.1 ± 0.1	3.4 ± 0.1	−8.4 ± 4.3	1.900	0.064	0.320	0.128
Limbic	1.2 ± 0.0	1.2 ± 0.0	−3.3 ± 3.7	0.890	0.379	1.000	0.606
Ganglionic Eminence	0.5 ± 0.0	0.4 ± 0.0	4.2 ± 5.6	−0.78	0.437	1.000	0.583
Cerebellum	5.2 ± 0.2	5.4 ± 0.1	−2.4 ± 3.4	0.720	0.475	0.950	0.543
Lateral Ventricles	2.5 ± 0.2	2.4 ± 0.1	3.6 ± 7.6	−0.49	0.623	0.623	0.623

niMSBDs, non-syndromic isolated musculoskeletal birth defects.

thalamus to the intermediate zone, reaching and waiting within the sp between 21 and 24GW (Krsnik et al. 2017). The period between 19 and 22 GW has also been reported as a period when one can identify using MRI both radial and tangential neuronal migration pathways (Kolasinski et al. 2013). Finally, thalamocortical fibers penetrate the somatosensory cortex between 25 and 26GW (Vasung et al. 2010, 2017; Krsnik et al. 2017), when the cerebral cortex starts to show regional patterning and initial lamination (Kostović et al. 2008; Krsnik et al. 2017). From 26GW onwards, the terminals of thalamocortical fibers continue to mature within the cp (Krsnik et al. 2017). Although, at this point, we can only speculate about the link between niMSBDs and brain maturation, our results shed light on this relationship, indicating that between 19 and 31GW, certain afferent pathways bringing input from the periphery might be altered and/or missing and might contribute to smaller volumes of the intermediate zone.

In contrast to dorsal funiculi, the development of pathways carrying somatosensory information to the cerebellum shows a relatively late appearance. Anatomical studies indicate that the spinocerebellar tract appears during the beginning of the second trimester (~14 GW) and is likely absent before (Bayer and Altman 2002). Spinocerebellum, to be distinguished from the spinocerebellar tract, is part of the cerebellum comprised of the vermis and intermediate parts of the cerebellar hemispheres. Spinocerebellum receives spinocerebellar sensory information from the periphery as well as information about the activity of spinal neurons. The most known and studied role of spinocerebellum is in the surveillance and adjustment of movement and proprioception (Bosco and Poppele 2001). We found significantly smaller volumes of the cerebellum between 19 and 31GW in niMSBDs compared to controls. As with the intermediate zone, one should be cautious in interpreting the link between limb malformation and cerebellar volumes at this stage of development. However, the magnitude of our findings and the need for extensive physical therapy that children with niMSBDs show during infancy and childhood indicate that the relationship between the three should be studied more in detail (volume of the cerebellum, motor behavior, and congenital malformations of the limbs).

Interestingly, the only differences we found between niMSBDs and controls after 31 GW are smaller central regions and larger volumes of the cp. It is well-known that myelination of the fetal brain later in gestation occurs from the caudal to rostral sides, and in the telencephalon, it progresses outward from the central sulcus to the poles, with posterior sites preceding anterior frontotemporal sites (for excellent review, see book Inder et al. 2017). While myelination could be in part a driver of our results, it is also highly likely that differences in conditions that we included

in younger versus older groups contributed to discrepancies in our results. Studies in monkeys with congenital foot deformations clearly show changes in the topographic organization of the thalamus and somatosensory cortices, suggesting the strong ability of both the thalamus and somatosensory cortex to reorganize (Florence et al. 1996; Liao et al. 2016). Furthermore, such studies even suggest the reorganization of the brain structures of the hemisphere connected to the non-affected side of the body (Florence et al. 1996; Liao et al. 2016), indicating the additive effect of reorganization on other brain structures. Suppose we were to interpolate some of the findings that were reported in the animal studies. In that case, an exponential growth of such brain reorganization, spanning over different hierarchical levels, could easily lead to changes in regional brain growth, contributing to volume differences between niMSBDs and controls. Yet as the differences between relative volumes in our study are evidently small, we can only speculate about their neuroanatomical substrate at this stage. Finally, our study cannot discuss the potential relevance of these volume differences between niMSBDs and controls. As such, we conclude that certain brain structures in niMSBDs might be different compared to controls, at least regarding their size.

Furthermore, we cannot exclude the possibility of an unidentified genetic abnormality in niMSBDs, as seen in some of the congenital limb malformations (Mensah et al. 2023). However, whether the genetic abnormality of limbs might be tied to the genetic abnormality of certain neuronal/glial brain structures, i.e. before the input from the periphery reaches central structures, is beyond the scope of this article and remains to be determined by experimental studies within the field of basic neuroscience.

Finally, we also found significantly larger ventricles as well as relative volumes of proliferative zones and ganglionic eminence in niMSBDs compared to the controls before 31 GW. In the setting of an increased volume of the ventricles, a parallel increase in the volume of the proliferative zones lining them is expected (i.e. an increase in the diameter of a cavity will lead to an increase in the total surface area lining it). Aside from the mathematical explanation linking the enlargement of lateral ventricles and proliferative zones, recent discoveries from basic neuroscience and neurobiology provide evidence that a substantial prenatal increase in the volume of the lateral ventricles is associated with the dysregulated fate of neuronal stem cells (Duy et al. 2022a, 2022b). Evidence from our group (Vasung et al. 2022) and others (Gilmore et al. 2008; Kyriakopoulou et al. 2014) also indicate altered growth and/or maturation of cerebral cortex in fetuses with isolated ventriculomegaly. However, whether enlarged volumes of lateral ventricles, proliferative zones, and ganglionic eminence represent the remnants of altered early brain

development remains to be determined by future well-designed prospective studies. Moreover, such studies are warranted in our quest to identify early neuroimaging biomarkers of adverse postnatal neurodevelopmental outcomes (Kyriakopoulou et al. 2023).

Although the findings of this study are not definitive, they do provide clear insights into the impact of intrauterine peripheral inputs on the development of the central nervous system. This concept further reinforces the necessity of perinatal interventions to address fetal anomalies. One of the best examples is myelomeningocele (MMC), in which a randomized trial showed that prenatal repair reduced the need for shunting and improved motor outcomes compared to standard postnatal repair (Adzick et al. 2011). It is known that most of the fetuses with MMC have a certain degree of motor dysfunction, and the prenatal repair could ideally prevent further damage (Houtrow et al. 2021). Accordingly, the preservation of fetal limb movement could affect the fetal brain development as well. Another example is the amnioinfusion for patients with oligohydramnios. Because fetal movement requires the free space that amniotic fluid provides, oligohydramnios results in joint contracture regardless of the primary cause(Christianson et al. 1999). Accordingly, amnioinfusion restores the ability of the fetus to move, which is one of the major sensory inputs to the fetal brain. In patients with lower urinary tract obstruction, placing the vesico-amniotic shunt significantly improves the oligohydramnios along with the fetal renal function (Haeri 2015). The third example could be the fetoscopic ablation of amniotic bands, which could be a limb-threatening condition. An amniotic band is an abnormal extension of the membranes that could tighten the fetal limb, prevent its development, with the potential risk of amputation. Prenatal release of the amniotic band salvages the limb, restoring both function and anatomic integrity (Javadian et al. 2013). Lastly, there are fetal interventions, in which the visceral organs are subject to intervention. This includes the fetoscopic endotracheal balloon occlusion for congenital diaphragmatic hernia, in which the anatomy of the abdomen and thorax is changed by moving the contents of the abdomen into the thoracic cavity (Style et al. 2019). The effect of this intervention on the development of visceral proprioception is completely unknown.

It is imperative that our findings are interpreted with caution. The anatomical knowledge that is the basis of our interpretations is limited by the quality of the fetal tissue used for scientific publications, outdated methods, and limited ability to replicate the results [lack of accessible embryonic and fetal banks (Hrabač et al. 2018)]. Furthermore, while certain studies exploring the functionality of brain–body connections exist in preterm infants, they are known numerous technical limitations. These limitations prevent us from translating these biological and physiological concepts from postnatal development to in-utero development (for review, see Vasung et al. 2019a). Finally, little is known about the functionality of connections that carry input from/to the periphery in human embryos and fetuses. Therefore, we are open to additional interpretation of our results and broader discussion within the neuroscientific community.

Limitations

Our study has numerous limitations. As such, the results should not be generalized. First, it is limited by its small sample size and retrospective nature. Because of the lack of additional clinical information, including postnatal MRI, we cannot assess the effects of potential confounders (e.g. maternal conditions during pregnancy, socioeconomic status, genetic abnormalities, etc.), or effect modifiers. Additionally, the absence of postnatal MRI follow-up for all subjects prevents us from ruling out the potential for biased or incomplete results. Besides, we grouped numerous conditions whose etiology and onset differ and/or remain unknown. Therefore, "isolated non-syndromic" in our manuscript refers to the limited information these patients had during diagnostic workups. Moreover, because of our grouping, small volumetric differences of certain brain regions that might be specific to subtypes of niMSBDs were most likely lost. Finally, we conducted two separate analyses (younger and older group), which included different sets of conditions. As such, we cannot compare differences between niMSBDs and controls between younger and older groups.

Furthermore, during fetal development, various bilateral (e.g. archispinothalamic and paleospinothalamic), contralateral, and ipsilateral pathways (e.g. ipsilateral corticospinal tract) between the brain and body emerge. However, the laterality of these pathways during prenatal development and their impact on the maturation of higher subcortical and cortical brain centers is unknown. Furthermore, studies in monkeys (Liao et al. 2016) and rsMRI in human newborns (Gordon et al. 2023) show a significant level of plasticity after congenital injury and even during normal motor development, suggesting a similar somatotopic effect on the structural organization of the involved centers in the ipsilateral and contralateral hemispheres. Again, however, similar to the emergence of laterality of pathways, the neurogenic events that drive this plasticity are yet to be identified.

Therefore, given the multifaceted and heterogeneous conditions included in our study and the assumption that, like in animal models, niMSBDs will affect both hemispheres, we chose not to perform separate hemispheric analyses. Further research is needed to elucidate the effects of altered body input on fetal brain development using homogenous, well-defined prenatal niMSBDs conditions with known timing of the injury, etiology, and genetic background.

Lastly, our study's limitations encompass the quality of clinical fetal MRI, as well as loss of follow-up—specifically, the inability to assess postnatal MRI, potentially introducing selection bias. Moreover, the niMSBDs might be associated with specific patterns of fetal motion resulting in group-specific MRI segmentation errors. Prospective studies with well-defined types of injury, precise detection of the timing of the injury, stricter inclusion, and exclusion criteria, and fetal movement patterns are warranted to detect brain changes relevant to family counseling and clinical decision-making. Finally, the difference in volumes of particular brain structures should also be interpreted with caution, given that we lack an understanding of their underlying biology.

Conclusion

In conclusion, our findings show brain structure differences in niMSBD-affected fetuses compared to controls. In the 19-31GW group of fetuses, niMSBD fetuses had larger proliferative zones, ganglionic eminence, ventricles, and limbic regions, but smaller cerebellum and fetal white matter. In the older than 31GW group, minor volume differences in central regions and the cp were seen. The exact cause underlying these differences remains unknown.

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Supplementary material

Supplementary material is available at *Cerebral Cortex* online.

Author contributions

Esha Ahmad (Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing—original draft, Writing—review & editing), Olivia Brumfield (Conceptualization, Data curation, Formal analysis, Investigation, Writing—original draft, Writing—review & editing), Olivia Masse (Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing—original draft, Writing—review & editing), Clemente Velasco-Annis (Data curation, Formal analysis, Investigation, Methodology, Software, Visualization), Jennings Zhang (Data curation, Formal analysis, Investigation, Methodology), Caitlin Rollins (Data curation, Formal analysis, Investigation, Validation), Susan Connolly (Data curation, Formal analysis, Investigation, Methodology), Carol Barnewolt (Data curation, Formal analysis, Investigation, Methodology), Alireza A Shamshirsaz (Conceptualization, Data curation, Investigation, Writing—original draft, Writing—review & editing), Shohra Qaderi (Conceptualization, Writing—original draft, Writing—review & editing), Ali Javinani (Conceptualization, Investigation, Writing—original draft, Writing—review & editing), Simon. K. Warfield (Formal analysis, Investigation, Methodology, Software, Validation, Writing—review & editing), Edward Yang (Data curation, Formal analysis, Investigation, Methodology), Ali Gholipour (Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Software, Supervision, Validation, Visualization, Writing—review & editing), Henry Feldman (Formal analysis, Methodology, Software, Supervision, Validation, Writing—original draft, Writing—review & editing), Judy Estroff (Formal analysis, Investigation, Supervision, Validation, Writing—review & editing), P. Ellen Grant (Funding acquisition, Investigation, Resources, Writing—review & editing), and Lana Vasung (Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing—original draft, Writing—review & editing)

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Data availability

The anonymized and processed data are available from the corresponding authors upon reasonable request. All the MRI analysis codes are freely available at https://imagine.med.harvard.edu/software-and-data.

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