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Spinal hemorrhages are associated with early neonatal motor function loss in human spina bifida aperta

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Abstract

Background: In spina bifida aperta (SBA), leg movements caudal to the meningomyelocele are present *in utero*, but they disappear shortly after birth. It is unclear whether leg movements disappear by impact of the neuro-developmental malformation or by superimposed traumatic damage. If superimposed traumatic damage is involved, targeted fetal intervention could improve motor outcome.
Aim: To characterize neuromuscular pathology in association with perinatal motor function loss in SBA.
Patients/methods: In fetal SBA ($n=8$; 16–40 weeks GA), the median time interval between ultrasound registrations of fetal motor behavior and post-mortem histology was 1 week. Histology was assessed cranial, at and caudal to the meningomyelocele and compared with findings in fetal controls ($n=4$).
Results: Despite fetal movements caudal to the meningomyelocele (5/6), histology indicated muscle fiber alterations (6/6) that concurred with neuro-developmental and traumatic spinal defects [*Neuro-developmental defects:* spinal ependymal denudation (3/8), reduced amount of (caspase3-negative) lower motor neurons (LMNs; 8/8), aberrant spinal vascularization (8/8). *Traumatic defects:* gliosis (7/8), acute/fresh spinal hemorrhages near LMNs (8/8)].

Abbreviations: SBA, spina bifida aperta; MMC, meningomyelocele; LMN, lower motor neuron; GA, gestational age; H&E, haematoxylin–eosin; CSF, cerebrospinal fluid.

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Conclusion: In all delivered SBA patients, recent spinal hemorrhages were superimposed upon pre-existing defects. If early therapeutic strategies can prevent these superimposed secondary spinal hemorrhages, motor outcome may improve.

1. Introduction

Spina bifida aperta (SBA) is characterized by defective fusion of the neural tube, resulting in exposure of the meningomyelocele (MMC) to the amniotic fluid. Leg movements by myotomes caudal to the meningomyelocele (MMC) are often still present *in utero*, but they disappear shortly after birth [1–3]. The early neonatal disappearance of leg movements has initiated fetal surgery to preserve these leg movements [4]. However, although fetal closure of the MMC may reduce A. Chiari and hydrocephalus [5–7], results on potential preservation of motor behavior appear less convincing [8–11]. These disappointing results could be explained by the occurrence of lower motor neuron (LMN) damage in skin-covered spinal segments caudal to the MMC [1,12]. Thus far, however, the direct cause for early neonatal movement loss remains unclear. Theoretically, early neonatal motor function loss could reflect the impact by postnatal gravity upon the pre-existing neuro-developmental malformation [2]. Alternatively, early neonatal motor function loss could also reflect acquired traumatic damage superimposed upon the neuro-developmental malformation. In this respect, histological characterization of neuromuscular damage may help to identify the direct cause of movement loss. This information could provide relevant information for optimal timing and selection of treatment options. If the underlying neuro-developmental malformation is associated with movement loss, treatment would mainly focus on rehabilitation. If traumatic damage is superimposed upon the malformation, therapy could also aim at (fetal) prevention [13]. In SBA fetuses of various gestational ages, we reasoned that histological assessment of the spinal cord and associated myotomes could provide insight in the underlying cause for early neonatal movement loss.

2. Patients

At the University Medical Center Groningen, we retrospectively investigated spinal and muscular histology in 8 SBA fetuses, which were autopsied between 1991 and 2006 (16–40 weeks GA). Parents of all included fetuses gave their informed consent. The medical ethical committee of our institute approved the investigation and analysis. The MMC was at cervical ($n=1$), thoracic ($n=5$) or lumbar ($n=2$) level. After initiation of vaginal delivery, fetuses were born the same day. Vaginal delivery was associated with *abruptio placentae* ($n=1$), misoprostol induction ($n=3$) and cephalocentesis ($n=4$). All four a-term, vaginally delivered patients

Table 1 Clinical information

Case	GA	MMC	Cause of death	Cerebral malf.	Orthopediac malf.	Other malf.
1	16	C–Th	Misoprostol induction	Cheilognato-palatoschisis Holoprosencephaly	Absent	Pancreas annulare anal atresia
2	21	Th–L	Misoprostol induction	Ventriculomegaly Bleedings	Absent	Absent
3	22	L	Misoprostol induction	Ventriculomegaly Arnold Chiari	Absent	Triplication of the central canal
4	25	Th–L	Solutio placentae	Microcephly Ventriculomegaly Arnold Chiari	Rocker bottom feet Leg muscle contractures	Cardiomegaly abnormal lobulation of lungs
5	34	Th–L	Induction, cephalocentesis	Data incomplete	Pes calcaneovalg.	Lung hypoplasia Extrophia cloacae OEIS complex
6	37	L–S	Induction, cephalocentesis	Severe HC	Absent	Absent
7	40	Th–L	Induction, cephalocentesis	Absent vermis Ventriculomegaly Arnold Chiari	Pes calcaneovalg. Fixed knee	Palatoschisis Atrial septum defect
8	41	Th–L	Induction, cephalocentesis	Dysgyration Encephalocele Arnold Chiari	Eversion feet	Dilated ureters Sacral agenesis

GA = gestational age, MMC = meningomyelocele, malf. = malformation, HC = hydrocephalus, C = cervical, Th = thoracic, L = lumbar, S = sacral, calcaneovalg. = calcaneovalgus, OEIS = omphalocele, exstrophy, imperforate anus, spinal defects.

were delivered in vertex position. Six fetuses died during delivery, two patients immediately thereafter. Clinical data are summarized in Table 1.

Spinal and muscular histology was also assessed in 4 fetal controls. The gestational age of the control fetuses [$n=4$] varied between 22 and 41 (median 35) weeks. In these fetuses, delivery occurred spontaneously [$n=2$], by caesarean section [$n=1$] or by misoprostol induction [$n=1$]. Control fetuses had died from maternal keto-acidosis (diabetes), premature rupture of the amniotic membranes, umbilical cord strangulation and complicated twin pregnancy. Spinal or cerebral malformations were absent in all four control fetuses.

3. Methods

During the prenatal period, motor behavior was assessed by means of video-taped ultrasound recordings ($n=6$). The time interval between video-recordings and delivery was 1 week (median, range 0–5 weeks). Two independent observers (D.A.S. and A.F.B.) assessed the quality of movements by Gestalt Perception [14,15]. According to previously described motor behavior characteristics in fetal SBA, movements were scored as: normal; poor repertoire (reduced variability); hardly discernible (i.e. minimal duration, small amplitude), and non-fluent (i.e. abrupt character) [2,3].

3.1. Histological data

Histology was assessed in: fused spinal segments cranial to the MMC (3/8), the cranial border of the MMC (i.e. $\leq$1 segment cranial to the MMC (7/8)), open spinal segments at the MMC (8/8), closed spinal segments caudal to the MMC (8/8) and subsequently also in corresponding myotomes (6/8). Post-mortem time before fixation ranged from 2 h to 3 days after intra-uterine fetal death. The spinal cord was immersion fixed in a solution of 4% formalin in PBS (pH 7.4). To this solution some NaCl was added to make the tissue float in order to overcome deformities of the tissue during the fixation period of 2 weeks. Transverse sections of spinal blocks were paraffin embedded and cut at 5 μm. Spinal abnormalities were subdivided into neuro-developmental defects or traumatic lesions. Histological staining of the spinal cord consisted of haematoxylin–eosin (H&E), cleaved caspase-3 (an apoptosis marker [16]), Nestin (for progenitor cells), GFAP (for gliosis) and CD68 (for macrophages and microglial cells). By H&E staining, the cellular quantity per motor neuron pool was estimated. If a spinal transverse section consisted of a motor neuron pool of less than 5 LMNs, the amount of LMNs was assessed as reduced. If a spinal transverse section consisted of a motor neuron pool of less than 3 LMNs, the amount of LMNs was assessed as severely reduced. Cleaved caspase-3 is a sensitive apoptosis marker that indicates the point at which the cell cannot return from the apoptosis cascade [17]. In this perspective, post-mortem investigation of spinal caspase-3 expression can reveal whether acute LMN cell death had been initiated recently days before, or not. We reasoned that if fetal leg movements are present despite caspase-3 positive LMNs, histological damage underlying neonatal motor function loss is initiated before birth. If fetal leg movements co-exist with caspase-3 negative LMNs, early neonatal motor function loss is initiated at a later time point (i.e. during or after delivery). For immuno-histochemistry, we applied: cleaved caspase-3 (1:1000, cell signaling technology, antigen retrieval (AR) of 1 mM EDTA pH 9 in microwave at 700 W for 8 min); nestin (1:100, Santa Cruz, AR using Tris/HCl at pH 9); and CD68 (1:100, kp1 clone, DAKO, AR using protease for 8 min). After the application of secondary and tertiary antibodies for 30 min, the slides were treated with diaminobenzidine and H_2O_2 for 10 min and counterstained with haematoxylin. Normal fetal spinal cord was used as control for the immunostaining procedures.

Muscle fiber type differentiation and type grouping (starting from the 24th week GA onwards [18]), were assessed by ATP-ase staining in all fetuses older than 24 weeks GA.

3.2. Statistical analysis

Wilcoxon signed-rank test was applied for comparison between the amount of LMNs cranial to the MMC with the amount of LMNs at or caudal to the MMC.

4. Results

4.1. Fetal motor behavior

In 6 of 8 fetuses video-recordings of motor behavior were present with a median duration of one week prior to delivery. Muscle contractions caudal to the MMC were present in 5 of 6

Table 2 Association between fetal motor behaviour and spinal histology

Case	GA	MMC	Motor behaviour	Movement quality	LMN cran. B. MMC	LMN at/caud. MMC
1	16	C–Th	L5–S1	Abrupt	N	R
2	21	Th–L	L5–S1	Normal	N	SR, aberrant
3	22	L	L5–S1	Normal	R; dystrophic	SR
4	25	Th–L	-	-	N	R; HE
5	34	Th–L	-	-	N	R
6	37	L–S	L5–S1	HD	N	R; aberrant
7	40	Th–L	Absent	Absent	-	Absent
8	41	Th–L	L5–S1	Normal	N	R

GA = gestational age, MMC = meningomyelocele, LMN = lower motor neuron, cran. B. = cranial border, caud. = caudal, C = cervical, Th = thoracic, L = lumbar, S = sacral, HD = hardly discernible,- = no data; N = normal quantity (>5 LMNs/side/section); R = reduced quantity (3–5 LMNs/side/section); SR = severely reduced (<3 LMNs/side/section); HE = hyper-eosinophilic.

fetuses (Table 2). Despite a small and short appearance, qualitative aspects were normal in 3 of 6 fetuses (case 2, 3 and 8). In two fetuses (case 1 and 6), the quality of leg movements caudal to the MMC was impaired (i.e. hardly discernible and abrupt (Table 2)). In the only fetus without movements caudal to the MMC (case 7), ankle and knee joints were immobile (Table 1) and spinal organization was severely abnormal (see next section).

4.2. Spinal histology

Histology of the spinal cord was investigated and compared between: fused segments cranial to the MMC; the cranial border of the MMC; open segments at the MMC and fused segments caudal to the MMC (in 3/8, 7/8, 8/8 and 8/8 fetuses, respectively). In two fetuses, re-epithelisation of the neurulation defect suggested a smaller morphological size of the MMC than actually present. In all SBA fetuses, spinal integrity was better preserved cranial than caudal to the MMC (Fig. 1). At and caudal to the MMC, neuro-developmental defects consisted of: (severely) reduced number of LMNs (caspase3-negative, 8/8), abnormal localization of LMNs (2/8), aberrant spinal blood vessels (8/8, Figs. 1 and 2; see also next section), abnormal transitions from epithelium to connective tissue (2/8) and abnormalities of the central canal (6/8, Fig. 2), such as: abnormal shape (2/8), localization (1/8) and di- or triplication (3/8). In three fetuses, the ependymal cell lining between the central canal and underlying neuropil was partly absent, called "ependymal denudation" [19], Fig. 2. In two fetuses, immuno-histochemistry staining (nestin, CD68 and GFAP) showed that ependymal denudation concurred with local loss of underlying neuropil (containing neural progenitor cells; Figs. 2 and 3), invasion of macrophages and subsequent astrogliosis. In all fetal histological assessments, LMNs were caspase-3 negative. Despite caspase-3 negative staining, the quantity of LMNs caudal to the MMC was $\geq$50% less than at

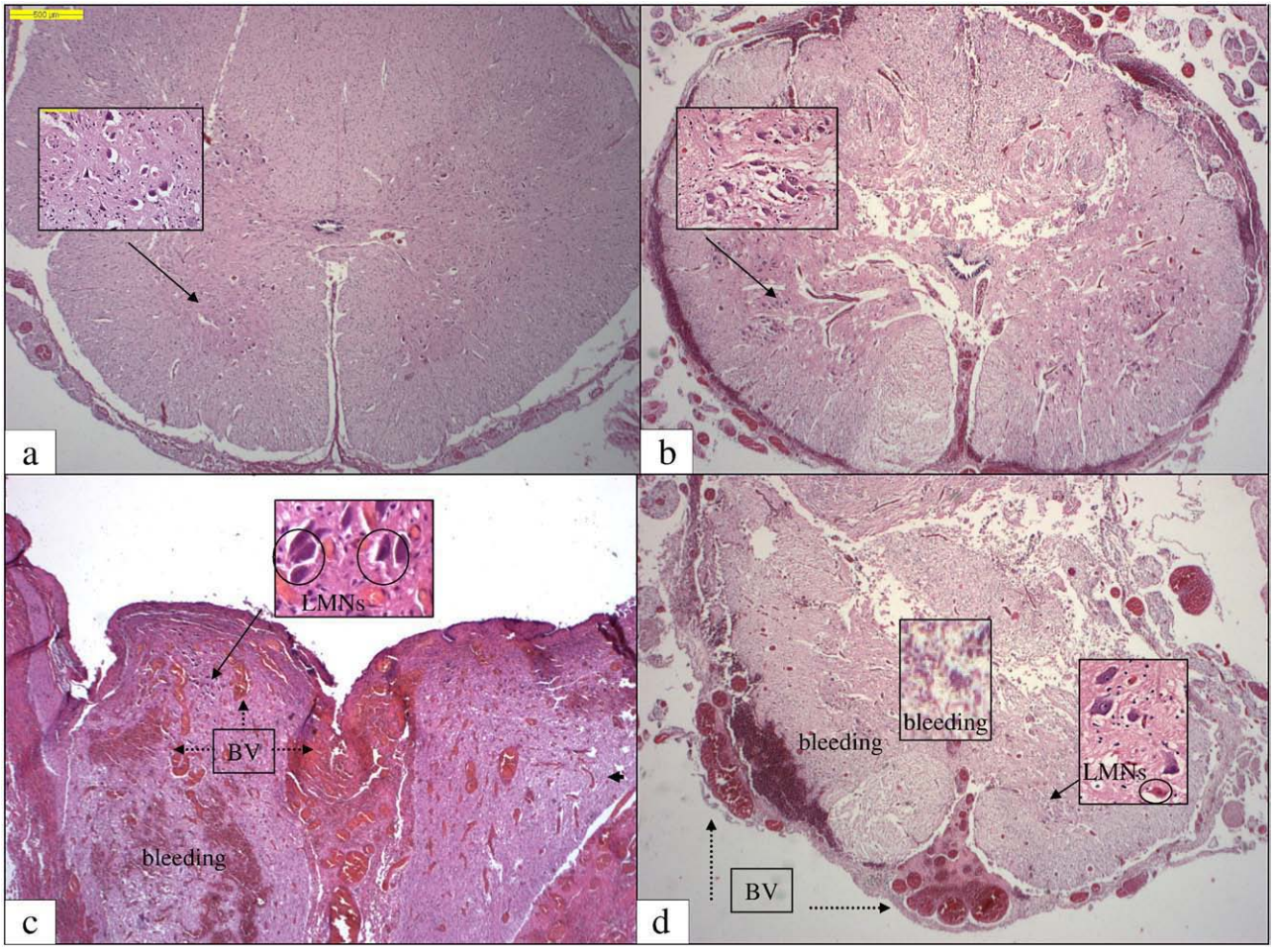

Figure 1 Cross-sectional images of the spinal cord. a: Cross-sectional image of the spinal cord in a control fetus 37 weeks GA. In the box, normal pools of LMNs are indicated. b: Cross-sectional image of the spinal cord cranial to the MMC in a SBA fetus of 25 weeks GA. In the box, normal pools of LMNs are indicated. c: Cross-sectional image of the spinal cord at the MMC in a SBA fetus of 37 weeks GA Aberrant blood vessels (indicated by dotted arrows) are present near ectopically located LMNs. An intramedullary hemorrhage is separately indicated. d: Cross-sectional image of the spinal cord caudal to the MMC in a SBA fetus of 25 weeks GA (identical fetus as in b). A small pool of LMNs with a hyper-eosinophilic LMN (encircled) is indicated. Spinal hemorrhages occur in the surroundings of LMNs. Dotted arrows indicate aberrant blood vessels (BV). BV = abundant and aberrant blood vessels; H&E staining; augmentation: 10× and 20×.

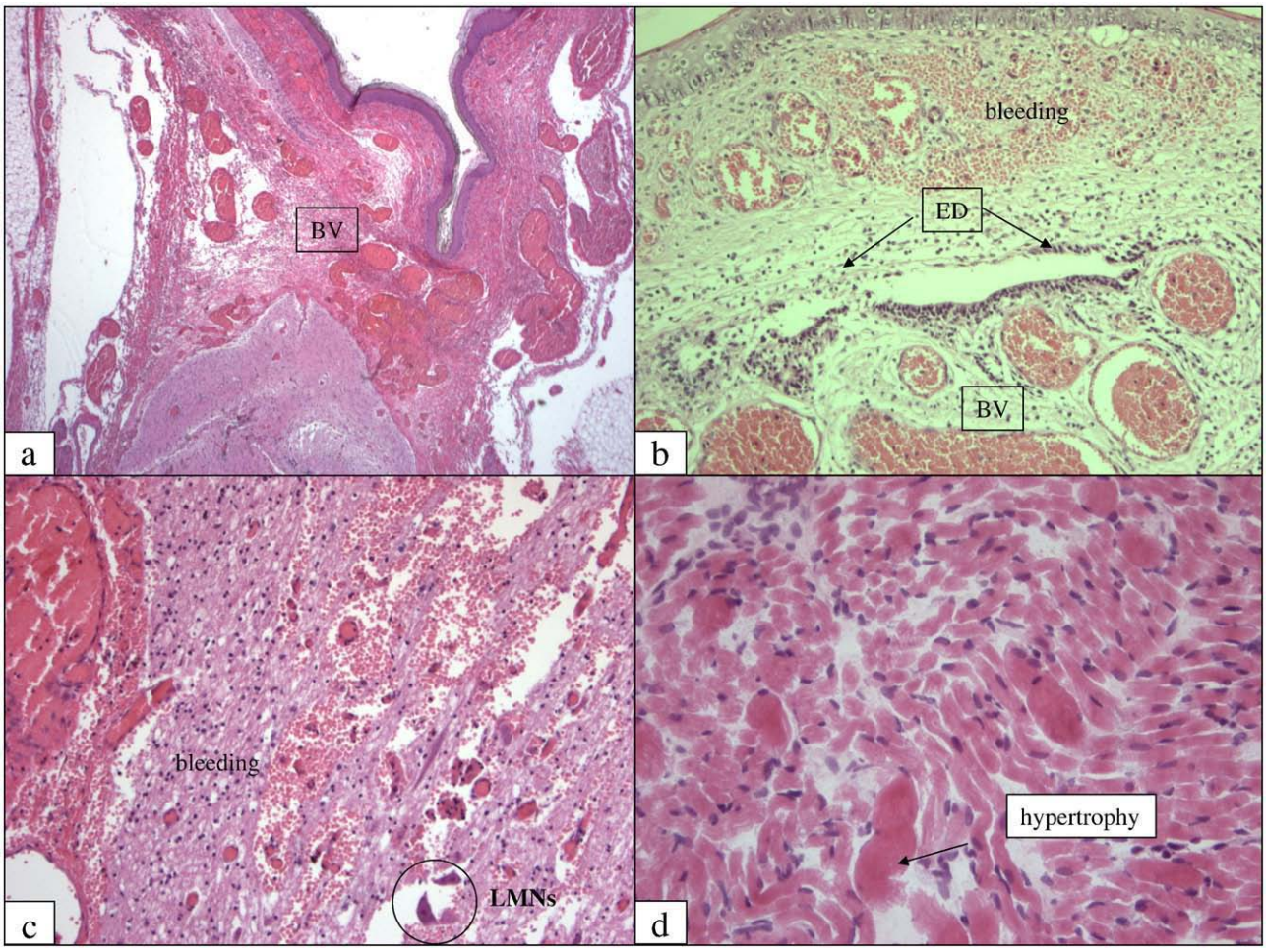

Figure 2 Spinal cross-sectional images in fetal SBA. a: *Area medullovasculosa* caudal to the MMC in a SBA fetus of 37 weeks GA. Aberrant blood vessels are indicated. b: Abnormal shape of the central canal caudal to the MMC in a SBA fetus of 40 weeks GA. At the dorsal side of the central canal, ependymal denudation and a large, recent hemorrhage is indicated. At the ventral side of the canal, aberrant blood vessels are present. c: Cross-sectional image of the spinal cord caudal to the MMC in a SBA fetus of 37 weeks GA. A recent intramedullary hemorrhage adjacent to LMNs is indicated. d: Paravertebral muscle caudal to the MMC in a SBA fetus of 25 weeks GA. Hypertrophic muscle cells are indicated. BV = abundant and aberrant blood vessels; ED = ependymal denudation; H&E staining; augmentation: 10× and 20×.

the cranial border and/or cranial to the MMC ((8/8); $p<0.05$; Table 2). Acquired, traumatic spinal damage at and caudal to the MMC was indicated by astrogliosis (7/8; Fig. 3) and by old—(2/8) or recent—(8/8) spinal hemorrhages (Figs. 2 and 3).

4.3. Vascular histology

Vascularization was normal in fetal controls ($n=4$) and in SBA fetuses at cranial distance from the MMC ($n=3$). However, spinal segments at the cranial border, segments at the MMC and segments caudal to the MMC consisted of superfluous, aberrant blood vessels (Figs. 1 and 2). Aberrant spinal blood vessels concurred with the appearance of fresh erythrocytes (i.e. recent hemorrhages) near caspase3-negative LMNs (8/8 fetuses; Figs. 1 and 2). These spinal hemorrhages were observed at closed spinal segments (at the cranial border of the MMC and caudal to the MMC) and at open spinal segments of the MMC (Figs. 1 and 2)). In addition to recent hemorrhages, macrophages containing iron pigment (Perls stain positive) were observed (2/8 fetuses; indicative for hemorrhages of at least a few days old).

4.4. Muscle histology

In six fetuses, muscle biopsies at myotomes cranial and caudal to the MMC were performed. In all six fetuses (22–40 weeks GA), muscle fibers in myotomes cranial to the MMC were normal, whereas muscle fibers in myotomes caudal to the MMC appeared a- and/or hypertrophic (Fig. 2). ATP-ase staining did not indicate type grouping in fetuses older than 24 weeks GA (5/5).

5. Discussion

In SBA, we characterized neuromuscular pathology in association with early neonatal motor function loss. Despite fetal neuro-developmental and (old) traumatic lesions, fetal leg movements caudal to the MMC persisted. Superimposed upon

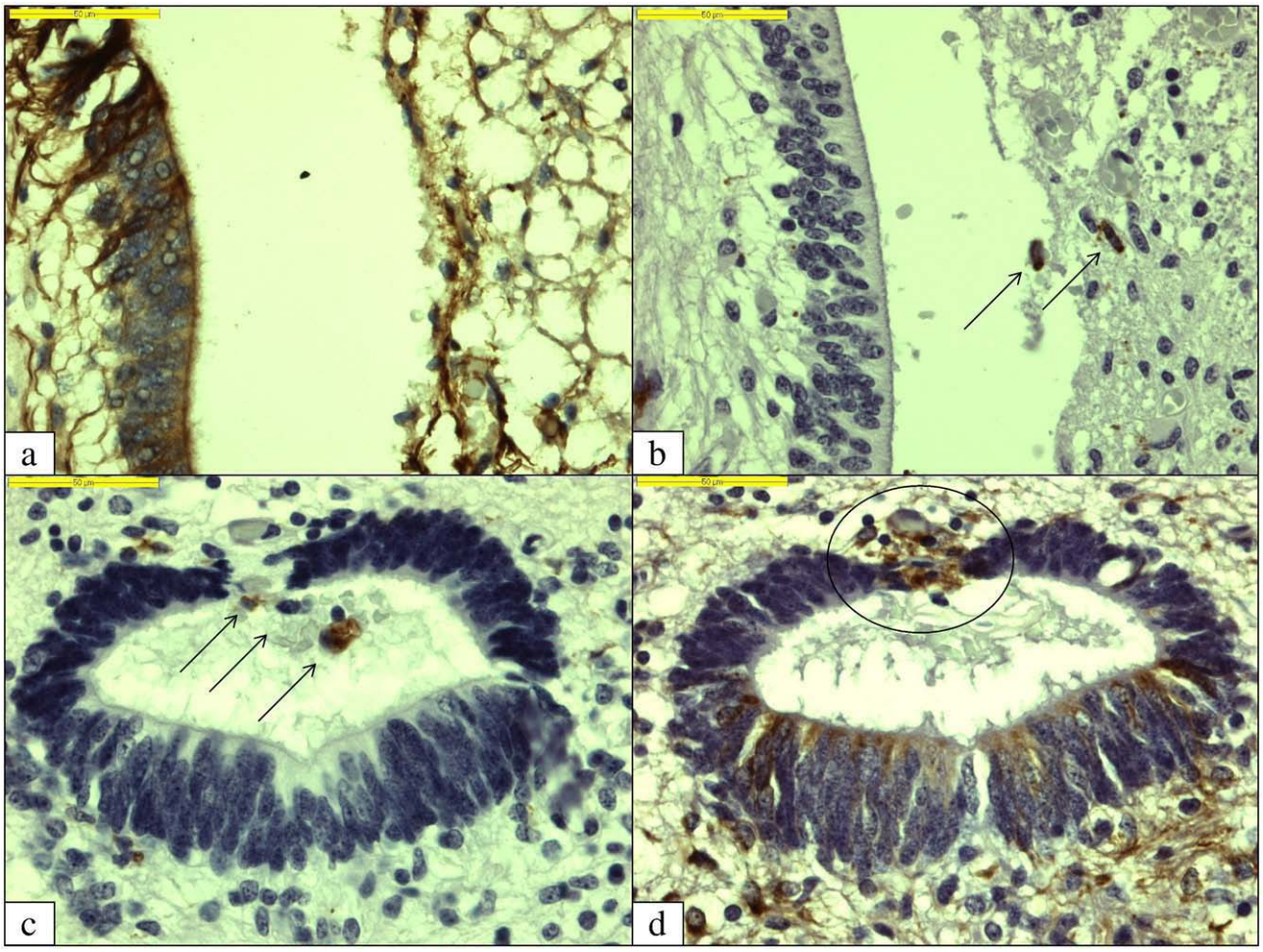

Figure 3 a: Nestin staining of a longitudinal section through the central canal in a SBA fetus of 40 weeks GA. At the right side of the central canal, the ependymal lining with underlying neuropil (containing progenitor cells) is completely lost along the length of the section. b: CD68 staining of a longitudinal section through the central canal in a SBA fetus of 40 weeks GA (same fetus as Fig. 3a). Macrophage invasion in the denuded area is indicated (arrows). c: CD68 staining of a transverse section through the central canal in a SBA fetus of 16 weeks GA. In the area with ependymal denudation, there is loss of progenitor cells and influx of macrophages (arrows). d: GFAP staining of a transverse section through the central canal in a SBA fetus of 16 weeks GA (same fetus as Fig. 3c). At the denudated area, astrogliosis is observed (encircled area).

these non-progressive fetal defects, we observed delivery-related spinal hemorrhages that precede early neonatal movement loss.

At the 24th day of gestation, primary neurulation (i.e. closure of the neural tube) is a complex process occurring at multiple fusion sites of the neural tube [20,21]. In human fetuses, primary neurulation occurs in caudal direction between the cervical and caudal fusion point [22]. After completion of primary neurulation, mesenchymal cells divide and migrate into the tail bud (i.e. secondary neurulation at the conus area) [23,24]. After primary and secondary neurulation at different areas [25,26], mesenchymal induction (formation of blood vessels and muscles) takes place. In accordance with unidirectional primary neurulation of the spinal cord, spinal organization and vascularization cranial to the MMC was essentially normal. These normal histological findings were contrasted by pathological findings observed in spinal segments at, and caudal to the MMC. Spinal pathology caudal to the MMC involved: reduced quantity of LMNs, aberrant spinal blood vessels and occurrence of ependymal denudation.

In contrast to our findings, a report of two SB fetuses with only "minor" histological abnormalities caudal to the MMC has previously been published (9 and 15 weeks GA; crown-rump lengths 4 and 11 cm, respectively) [27]. However, in the latter study, the open MMC was used as a reference and observations in closed segments cranial to the MMC were lacking. Furthermore, assessments of vascular condition, LMN quantity, ependymal integrity and underlying neuropil were absent.

Until now, ependymal denudation was described exclusively at cerebral level [19], but, to the best of our knowledge, never at spinal level. After induction of the floor plate by the notochord (at the 4th week GA), ependymal differentiation occurs in a fixed temporal and spatial pattern [28]. The process is mediated by the organizer gene *Sonic Hedgehog* [29], which is also involved in neural tube closure [30]. These fetal ependymal cells secrete important molecules that are involved in neural proliferation and migration [28]. In addition

to protein secretion, ependymal cells also function as a barrier between CSF and the underlying neuropil. When ependymal cells are lost, functional restoration is impossible and the underlying neuropil can migrate into CSF [19]. At cerebral level, ependymal denudation is followed by an invasion of macrophages into the denuded areas. This macrophage invasion will subsequently result in gliosis [19]. Accordingly, we also observed ependymal denudation at spinal level. In the spinal cord, ependymal denudation was also followed by: loss of underlying neuropil, invasion of macrophages and subsequent gliosis at the denudated area. In this respect, human ependymal denudation shows striking similarities with that in mutant Hyh mice [31–33]. In Hyh mice, ependymal denudation has been ascribed to a primary failure in the formation of cell junctions [34]. In analogy with these data, it is tempting to speculate that ependymal denudation in human SBA rather reflects a primary than secondary pathogenesis. In human fetal SBA, this would implicate that spinal damage is not only associated with exposure of the neural plate to toxic amniotic fluid [35], but also with pathological neuro-developmental processes in well covered spinal segments caudal to the MMC.

At all gestational ages, the above described spinal abnormalities corresponded with muscle fiber abnormalities (a- and/or hypertrophy) in myotomes caudal to the MMC. Nevertheless, fetal movements caudal to the MMC had still been present and recorded on videotape. The early neonatal disappearance of these movements is preceded in time by fresh (i.e. normal erythrocytes), delivery-related spinal hemorrhages near LMNs (that were still caspase-3 negative at birth). In rat models, the time window for neural apoptosis and caspase-3 activation has been studied. In rat brain, cleaved caspase-3 becomes increasingly positive between 6 and 24 h after a traumatic or hypoxic–ischemic insult [36]. In spinal cord, focal hemorrhage is associated with apoptotic motor neurons in chicken embryos [37] and rat [38]. In human, elevation of programmed cell death markers has been indicated in spinal motor fore horn diseases [39]. Accordingly, we have observed caspase-3 positive LMNs in a SBA neonate (with spinal hemorrhages) that had died two days after birth [1]. However, in the present SBA study, fetal LMNs were still caspase-3 negative (8/8). From these studies, it seems apprehensive that LMNs may become caspase-3 positive only hours after delivery-related spinal hemorrhage.

Delivery-related spinal hemorrhages were present at, and caudal to the MMC and were independent of presence or absence of asphyxia and prostaglandin induction (prostaglandins could even attenuate hemorrhages [40]). Despite traumatic influences during delivery in both control and SBA fetuses, spinal hemorrhages were absent in control fetuses (4/4) and were present in SBA fetuses (8/8). However, it is important to stress that included SBA fetuses did not represent an ad random, (relatively) favorable SBA study cohort which undergoes caesarean section (by a large, low transverse uterine incision [41]). In fact, all SBA fetuses were delivered vaginally to prevent maternal morbidity. In this perspective, we cannot exclude that traumatic influences (such as for instance cephalocentesis and protrusion of the amniotic sac [42–45]) had a negative impact on the MMC and its contents. However, spinal hemorrhages are not entirely explainable by a specific traumatic type of delivery. Firstly, hemorrhages occurred after both induction and spontaneous vaginal delivery (unpublished observation in a succumbed SBA neonate). Secondly, spinal hemorrhages were not only confined to the open location at the MMC itself, but were also observed in well covered segments caudal to the MMC. In accordance with our findings at the MMC, Meuli et al. also reported fresh hemorrhages at the MMC (i.e. aborted fetuses, 19–23 weeks GA; in absence of cephalocentesis) [46]. However, in the present study, we also observed considerably large bleedings caudal to the MMC (i.e. at unexposed, skin-covered segments (8/8)), suggesting that spinal hemorrhage is not only caused by direct exposure to amniotic fluid. If delivery trauma would be the solitary cause for spinal hemorrhages, an equal distribution between hemorrhages cranial and caudal to the MMC would be expected. However, spinal hemorrhages cranial to the MMC were absent (only at the cranial border of the MMC) and invariably present caudal to the MMC. These spinal hemorrhages were located near aberrant blood vessels. All together, spinal hemorrhages appear associated with the location of aberrant blood vessels, whereas traumatic delivery may be one of the factors that provokes them.

In histological SBA literature, the area with redundant spinal blood vessels at the MMC is called the *area medullovasculosa* [47]. In addition to presence of abnormal blood vessels, functional blood supply (blood flow and sheer wall stress) is also reported as inferior in SBA patients (compared with spinal cord injury patients) [48]. During delivery, venous stasis (reduced venous return); direct mechanical compression (at the *area medullovasculosa)* and reduced arterial blood supply (by uterine labor contractions) can all provoke acute spinal hemorrhages. In accordance with these histological data, Luthy et al. reported that caesarean section before the onset of labor contractions provides a better motor outcome than caesarean section or vaginal delivery after the onset of labor contractions [49]. Hopefully, future research (for example by application of caspase-8 and -9, or FAS and FAS-ligand staining) may allow further quantification of neural damage after these acute spinal hemorrhages.

In conclusion, our data in human fetal SBA indicate that fetal movements caudal to the meningomyelocele concur with pre-existing spinal defects. During delivery, acute spinal hemorrhages are superimposed upon these defects. If innovative fetal therapies [13] could target superimposed delivery-related spinal hemorrhages, motor outcome would be expected to improve.

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