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Artículo original

Disorders of cerebellar growth and development in preterm neonates. A neuropathological study

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RESUMEN

A fin de evaluar el desarrollo posnatal del cerebelo se analizaron 92 casos correspondientes a recién nacidos pretérmino y a término de hasta 7 días de vida (casos control) y recién nacidos pretérmino con edad posconcepcional equivalente al término. Se observó reducción del peso del cerebelo, disminución de la foliación y de la altura de las folias; disminución del espesor de la capa molecular y de la densidad de la capa de granos interna y aumento en la cantidad de células de Purkinje por segmento. Estos resultados se correlacionaron entre sí y con el peso corporal y cerebral, pero no con la edad gestacional. Las imágenes de necrosis, apoptosis en la corteza inmadura, astrocitosis reactiva y microgliosis en la sustancia blanca se correlacionaron con episodios de hipoxia-isquemia, infecciones, desnutrición y diversos tratamientos.

Los casos estudiados mostraron una suma de lesiones directas y alteraciones del desarrollo que resultaron en patrones similares a los hallados en cerebelos de 30-35 semanas de edad gestacional aun cuando estos recién nacidos pretérmino habían completado la edad posconcepcional equivalente al término. Interpretamos estos hallazgos como una consecuencia de la acción de injurias que actuaron durante la ventana de vulnerabilidad de los hemisferios cerebelosos. Las lesiones directas de la corteza y sustancia blanca del cerebelo son una importante y poco conocida causa de alteración del crecimiento del cerebelo.

Palabras clave: cerebelo, foliación, sustancia blanca, hipoxia-isquemia, pretérmino, neuropatología.

ABSTRACT

In order to evaluate postnatal development of cerebellum, we analyzed 92 cerebella from preterm and term neonates up to 7 days postnatal age (control cases) and preterm neonates with postconceptional age at term equivalent. Reduction of the cerebellar weight, diminished foliar height and foliation, diminished molecular layer thickness, diminished internal granular layer cell density, and high number of Purkinje cells per segment were observed. These results correlated with each other and with brain and body weight, but not with gestational age. Necrosis, apoptosis in the immature cortex, reactive astrocytosis and microgliosis in the white matter correlated with hypoxia-ischemia, infections, undernutrition and therapies.

The cases examined showed cerebellar lesions plus underdeveloped cerebellar structures with patterns similar to those of 30-35 weeks gestational age, although these preterm neonates had completed a postconceptional age equivalent to term. We interpreted the findings as the effect of noxa acting during the cerebellar lobes' vulnerability window. Direct injury of developing cerebellar cortex and white matter is an important though poorly recognized cause of impaired cerebellar growth.

Keywords: cerebellum, foliation, white matter, hypoxia-ischemia, preterm neonates, neuropathology.

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Whereas the cerebrum of the term neonate is frequently the target for severe injuries, the cerebellum usually shows slight to moderate cellular changes in this group of patients.[1-3] On the other hand, cerebellar compromise as a prematurity-related complication is characteristic and clinically important, and by no means a new concept.[4-11] In recent years several reports have rediscovered that the acquired lesions, in particular those associated with

periventricular leukomalacia and peri-intraventricular hemorrhage are not restricted to the cerebrum.[12-18] Premature birth apparently opens windows of vulnerability that expose the immature cerebellum to multiple external risks. The later development of the cerebellum would be subsequently damaged as a consequence of preterm birth,[12,15,19,20-23] even in cases in which the lesion is slight and perhaps would not be recognized by MRI. This situation would be worsened by the complications that frequently occur during postnatal life.

Neurological and neuropsychological studies along with clinical and imaging follow-up in this group of patients have yielded surprising results not only in the immediate postnatal period but also throughout infancy and during puberty.[8,14,21-26] This is important in light of recent findings pointing to the role of the cerebellum in cognitive function as well as the relationship between cerebellar compromise and difficulties in academic development and the pursuit of a normal social life.[25, 27-29]

Although in recent years MRI has contributed enormously to our understanding of the pathology of the CNS, the associated histologic and cellular changes can only be definitively ascertained through histopathological examination.[30]

References dealing with gross, histological and morphometric studies in cerebellar lobes in large series of autopsies of preterm neonates are very scarce.[31, 32]

This report describes the pathology of cerebella of preterm neonates who survived up to a postconceptional age equivalent to term. The aim was to analyze gross and histological aspects in order to evaluate the impairment of postnatal cerebellar development. In such a case, and as a secondary hypothesis, this study attempted to find the real developmental stage reached by those cerebella. The results were compared with controls as well as with published data, in particular those pertaining to MRI.

MATERIALS AND METHODS

Sixty-five cerebella coming from preterm neonates—gestational age 28-36 weeks, postnatal age 5-75 days—at term-postconceptional age equivalent (where postconceptional age = gestational age + postnatal age) were studied. All autopsies were performed at "Superiora Sor María Ludovica" Children's Hospital (La Plata, Argentina) between March 1977 and June 2002. The cases were selected depending only on basis of gestational age and postnatal age. PTNs with genetic, malformative and/or disruptive CNS syndromes, multimalformative syndromes with evident CNS compromise, and CNS prenatal infectious diseases were not included.

The following data were also submitted to analysis: gestational age, postnatal age, postconceptional age, birth body weight, obstetric and perinatal data, and diseases developed during the postnatal period. Low body weight was defined as birth body weight between 1000 to 1500 g, and extremely low body weight when less than 1000 g, regardless of gestational age.[33] Additionally, birth body weight was analyzed in relation to gestational age.

Control cases. Control cases were 20 cerebella coming from term newborns (gestational age: 37-42 weeks) up to 6 days postnatal age, with normal values for post-mortem body, cerebral, and cerebellar weight. Main diseases of these included congenital diaphragmatic hernia, bronchopneumonia, cardiovascular malformations of diverse complexities, adrenal hypoplasia, or bilateral renal hemorrhagic infarct. Severe neuropathological changes were not found in this group (see Results). These necropsies were performed between November 1978 and March 2006. The results were statistically matched with normal values for gross and histological measurements and no statistically significant differences were found between the gross and histological values of Control Group and already published normal data.[34]

Seven additional cerebella coming from preterm neonates (gestational age: 30-35 weeks) up to 7 days postnatal age were used as a second control for comparison with the developmental stage reached by all 65 cases. Those preterm controls had normal values for post-mortem body, cerebral and cerebellar weight as related to their gestational age. Main diseases were bronchopneumonia, hyaline membrane disease, renal infarct, necrotizing colitis and focal periventricular hemorrhage. Severe neuropathological changes were not found. Particularly, the cerebella were grossly and histologically unremarkable.

Pathology

1) Cases. Group I and Group II

The 65 cases were arranged according to increasing values of cerebellar weight: Group I, cerebellar weight ≤ 14 g (35 cases, 54%); Group II, cerebellar weight ≥ 15 g (30 cases, 46%). In the literature there is no classification of "nor-

mal" cerebellar weight in preterms at a postconceptional age equivalent to term that could serve as a guide to define the aforementioned groups. Therefore, the limit between the 2 groups was defined by taking into account some statistical characteristics of the present cohort (see below).

2) Gross

Body weight at autopsy, cerebral weight, cerebellar weight and other necropsy findings were recorded.

Ventriculomegaly was diagnosed when ventricular enlargement was clearly evident.

3) Histology

Morphometry and neuropathological changes were analyzed on 5-µm thick hematoxylin and eosin-stained sections of buffered-formalin-fixed, paraffin-embedded cerebellar tissue.

a) Morphometry

Measurements were made on slices of cerebellar lobes which included posterior (folia a) and inferior (folia b) areas and dentate nucleus,[35] as follows (Figure 1):

(a) The degree of foliation was measured on folia b by a minor modification of standard histological criteria for classifying the folia as primary, secondary and tertiary.[35]

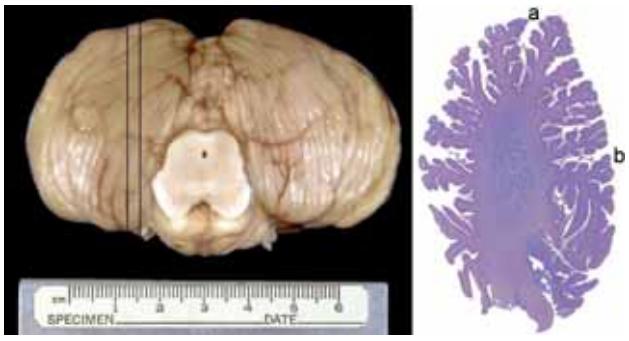

Figure 1. Measurements were performed on slices of cerebellar lobes. Left: Dorsal aspect of the cerebellum. The parallel lines represent the slice of tissue shown at the right side of the figure. At this level the slice includes *lobulus semilunaris superior*, *lobulus semilunaris inferior*, *lobulus biventer* and *lobulus gracilis*, all of them belonging to the posterior lobule of the cerebellum (with permission).[35] Right: Entire cerebellar hemispheric slice stained with luxol fast blue showing folia a, folia b and dentate nucleus as is usually used in our laboratory (with permission).[35] For color images from this paper see Annex 3.

(b) Folia a height and folia b height was measured in µm from folium base to tip using a Zeiss measurement reticule with a scale of 1/100 mounted on a Zeiss Standard 18 light microscope with a 2.5X Planapochromatic objective and 10X oculars.

(c) The thickness of the external granular layer and that of the molecular layer were measured in µm at folia b (top and lateral aspects) and expressed as the mean values of 5 determinations for each one of the layers through the use of a 25X Planapochromatic objective and 10X oculars.

(d) Cell density in the internal granular layer of folia b was scored qualitatively on a scale of one to three (+/+++) according to an already published method[35] (Figure 2).

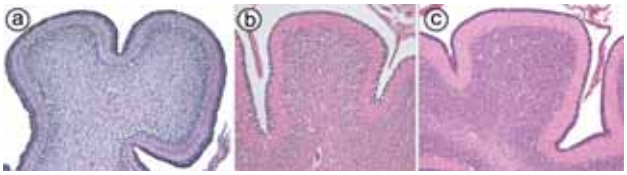

Figure 2. Nonparametric ranking of cell density was used in the internal granular layer. A: +/+++; B: ++/+++; C: +++/+++. Note that cell density has a parallel with internal granular layers' thickness (with permission)[35] H&E ×100.

(e) The number of Purkinje cells resulted from counting them in a linear segment of 980 µm in the folia b. Only those cells whose nuclei were clearly visible were scored. In each case, the data were the average of 5 determinations in different areas of the folia (top and lateral aspects).

b) Neuropathological findings

The cortex, white matter, and dentate nucleus of each specimen were examined although not quantified for the presence of apoptosis, astroglial reactions, macrophages, microgliosis, inflammatory infiltrates, necrosis, cystic change of the white matter, edema and hemorrhage. The above measurements and neuropathological findings in preterm neonates and Control Group were analyzed without knowing the clinical and gross-pathological data.

4) Cerebrum

Gross and histological findings were compiled for comparison with data coming from cerebellar tissue. No statistical correlations were performed across these cases.

5) Statistics

All values were expressed as mean +/- standard deviation. Statistical analyses were undertaken with SPSS for Windows. Calculations included comparisons of quantitative measurements across Group I and Group II and between them and Control Group through the use of the Mann-Whitney test. For comparisons of qualitative data the Chi-Square test was used. The correlation between quantitative data was evaluated through the Spearman coefficient. Scatter plots and Box plots were performed.

Control Group data were compared with those already published; the latter were taken as normal.[34,36-49]

Differences were considered statistically significant at $p < 0.05$.

RESULTS

Clinical data and gross observations

Hyaline membrane disease, periventricular leukomalacia and peri-intraventricular hemorrahge with or without ventriculomegaly were more common in cases with low cerebellar weight (Table 1) (Figure 3). Different gestational as well as post-natal ages appeared randomly distributed along the series of 65 cases. But, although gestational age was similar in both groups I and II, low and extremely low body weight at birth, low body weight for gestational age, low body weight at autopsy and low cerebral weight were seen predominantly in cases with low cerebellar weight (see Table 2 for comparison with Control Group). Extremely low cerebellar weight was found in the first 14 cases of the series of 65 cases, with values for cerebellar weight from 5 to 9 g.

Morphometry

a) Folia and foliation. Tables 3 and 4, and Figures 4 and 5 show values for foliation and for the height of folia a and b that were below the ones for Control Group.
b) Layers. In both groups the height of the external granular layer was similar, and had little difference with the Control Group (Figure 6).

The molecular layer thickness, on the contrary, showed values of 35.8, 48.4 and 68.9 μm in Group I, Group II and Control Group, respectively (Table 4) (Figure 7).

The internal granular layer displayed the lowest cellular density for Group I, higher densities for Group II, and densely packed internal granules in the Control Group (Table 5).

Table 1. Number (n) and proportion (%) of cases with perinatal complications and lesions in CNS, and in other sites in two groups of preterm infants (GI: Group I, GII: Group II)

		Clinical data			*Autopsy findings*	
Group (cases)		*Obstetrical (*)*	*IRDS*	*Others (**)*	*CNS (+)*	*Others (++)*
G I (35)	% (n)	51.4 (18)	54.3 (19)	68.6 (24)	82.8 (29)	94.3 (33)
G II (30)	% (n)	46.7 (14)	30 (9)	53.3 (16)	63.3 (19)	83.3 (25)

IRDS, idiopathic respiratory distress syndrome. (*) Premature rupture, chorioamnionitis, placenta previa, abruptio placentae, nuchal umbilical chord entanglement, gestosis, breech presentation. (**) Developed while in hospital: necrotizing enterocolitis, meconium ileus, peri-intraventricular hemorrhage (PIVH) (+) Hypoxic-ischaemic encephalopathy (HIE), sequelar HIE, leptomeningitis, meningoencephalitis, hidrocephalus. (++) Necrotizing enterocolitis, bronchoneumonia, bronchopulmonary dysplasia, necrotizing gastroenteritis, sepsis.

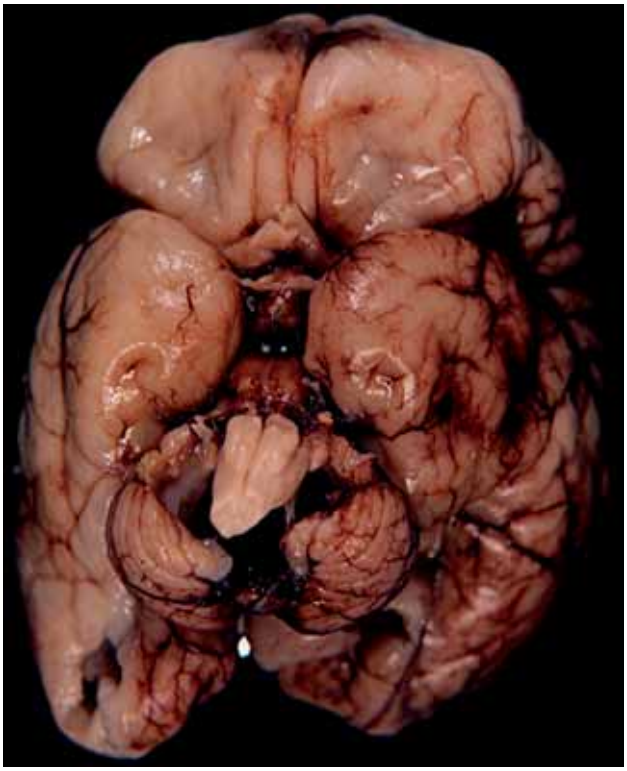

Figure 3. Supra- and infratentorial hypoxic-ischemic encephalopathy was the main disease. Case 21; GA, 32 weeks; postnatal age, 45 days; birth weight, 1100 g; body weight, 1120 g; cerebral weight, 210 g; cerebellar weight, 10 g. Enlargement of the 4th ventricle as a result of post-hemorrhagic ventriculomegaly. The cerebellar parenchyma, particularly at the lobes, is very thin.

Table 2. Mean and standard deviation of age and gross measurements for two groups of preterm infants (GI, GII) and control group (CG), and p-value for comparisons GI vs GII vs CG (Mann-Whitney test)

Group		*N*	*G*	*C*			*C*	*c*
G I (35)	Mean	33	33.7	38.5	1402	1367	204	9.8
	± sd	± 13.6	± 1.8	± 1.5	± 478	± 423	± 40	± 2.5
	(n)	(35)	(35)	(35)	(34)	(35)	(35)	(35)
	p-value		0.085	0.035	0.002	0.000	0.000	0.000
G II (30)	Mean	34	34.3	39.3	1793	1758	293	17.9
	± sd	± 17.6	± 1.9	± 1.5	± 534	± 476	± 53	± 2.5
	(n)	(30)	(30)	(30)	(30)	(29)	(29)	(30)
	p-value		0.000	0.866	0.000	0.000	0.000	0.000
C G (20)	Mean	3.1	38.9	39.2	3197	3197	395	27
	± sd	± 2	± 1.2	± 1	± 514	± 514	± 43	± 5

PNA, post-natal age [d]; GA, gestational age [w]; PCA, post-conceptional age [w]; BBW, birth body weight [g]; BW, body weight at autopsy [g]; CW, cerebral weight [g]; cw, cerebellar weight [g].

Table 3. Number (n) and proportion (%) of cases for different grades of foliation (F) in two groups of preterm infants (GI, GII) and control group (CG), and p-value for Chi-square test (p = 0.000)

Group				
G I (33)	%	39.4	60.6	0.0
	(n)	(13)	(20)	(0)
G II (27)	%	3.7	70.4	25.9
	(n)	(1)	(19)	(7)
C G (20)	%	0.0	40	60
	(n)	(0)	(8)	(12)

Chi-square p = 0.000

In Group I and Group II the number of Purkinje cells per segment was higher than the one of Control Group (p = 0.000), being in Group I higher than in Group II (p = 0.000) (Table 4) (Figure 8).

The above mentioned results fit with the concept of immature cortical layers of the cerebellar cortex.

Neuropathological findings (Figures 9I, A-F; 9II, A-D; 10 and 11, A-I)

The most severe compromise was observed in the specimens with lesser cerebellar development. Necrosis,

Table 4. Mean and standard deviation of histological measurements for two groups of preterm infants (GI, GII) and control group (CG), and p-value for comparisons GI vs. GII and GII vs CG (Mann-Whitney test)

Group		*ha*	*hb*	*ext gr*	*mol*	
G I (35)	Mean	4486	3487	28.0	35.8	33.5
	± sd	± 1263	± 946	± 7.9	± 10.2	± 9.0
	(n)	(27)	(32)	(35)	(35)	(32)
	p-value	0.000	0.000	0.357	0.000	0.000
G II (30)	Mean	6338	4764	26.2	48.4	21.5
	± sd	± 1268	± 957	± 6.9	± 16.0	± 4.4
	(n)	(22)	(26)	(30)	(30)	(30)
	p-value	0.000	0.000	0.469	0.000	0.000
C G (20)	Mean	8704	6468	25.4	68.9	13.7
	± sd	± 1704	± 1042	± 8.3	± 14.8	± 4.2

hfa, folia a height [µm]; hfb, folia b height [µm]; ext gr, external granular layer thickness [µm]; mol, molecular layer thickness [µm]; P, Purkinje cells per segment.

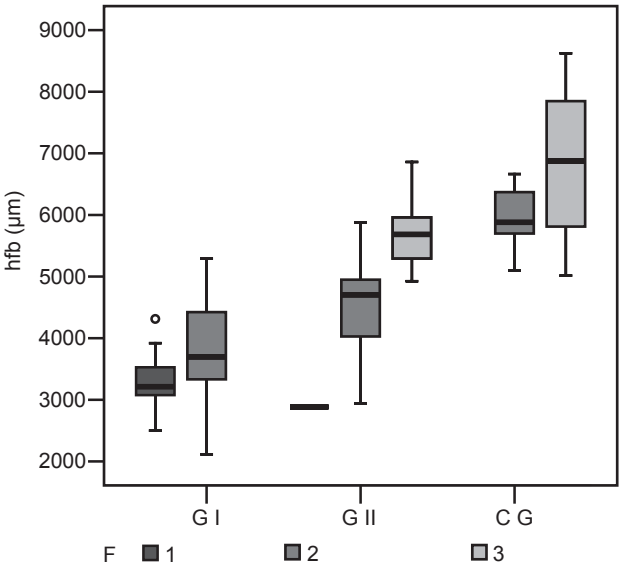

Figure 4. Box plot of folia b height for three grades of foliation within Group I, Group II and Control Group. Dark gray shading: distribution of folia b height for cases with grade 1 foliation, corresponding to Group I (GI) and Group II (GII). There are no cases with grade 1 foliation in Control Group (CG). Medium gray shading: distribution of folia b height for cases with grade 2 foliation in GI, GII and CG. Light gray shading: distribution of folia b height for cases with grade 3 foliation in GII and CG. There are no cases with grade 3 foliation in GI. F, foliation; hfb, folia b height.

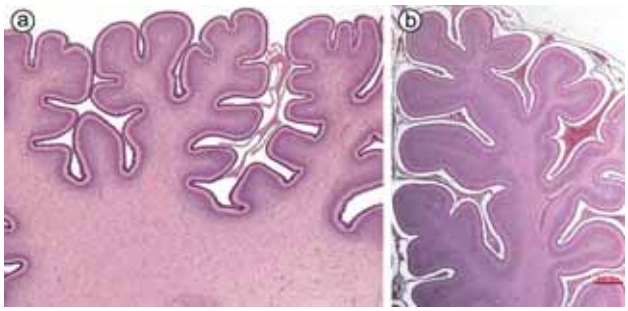

Figure 5. Cases with low cerebellar weight showed short folia and poor foliation. A**:** Same case as Figure 3. Histological section of inferior portion of cerebellar lobe (folia b). Foliar height as well as foliation is diminished compared to control. B**:** control case. GA, 40 weeks; postnatal age, 2 days; birth weight, 2500 g; cerebral weight, 400 g; cerebellar weight, 28 g. H&E. Scale bar: 500 µm (A, B).

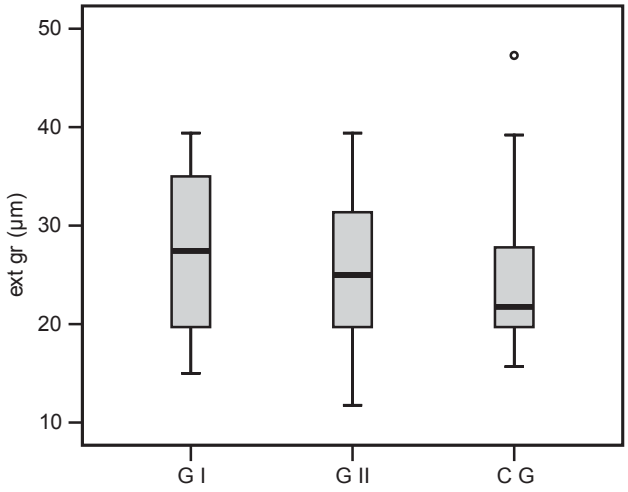

Figure 6. Box plot of external granular layer thickness, within Group I, Group II and Control Group. Ext gr, external granular layer thickness; GI, Group I; GII, Group II; CG, Control Group.

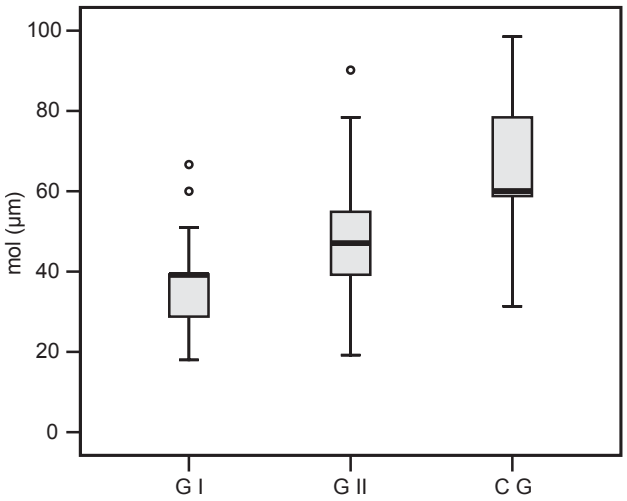

Figure 7. Box plot of molecular layer thickness, within Group I, Group II and Control Group. Mol: molecular layer thickness; GI, Group I; GII, Group II; CG, Control Group.

Table 5. Number (n) and proportion (%) of cases with different grades of cell density in the internal granular layer (Int gr) in two groups of preterm infants (GI, GII) and control group (CG), and p-value for Chi-square test (p = 0.000)

Group		*Int □r*		
		□	□	□
G I (35)	% (n)	57.1 (20)	42.9 (15)	0.0 (0)
G II (30)	% (n)	23.3 (7)	56.7 (17)	20.0 (6)
C G (20)	% (n)	0.0 (0)	25 (5)	75 (15)

Chi-square p = 0.000

hemorrhage, embolic microabscesses, microgliosis, reactive astrocytosis, and apoptosis were seen both in gray and white matter. Remarkably, although severe those findings were just recognized microscopically, and only seven cases revealed necrosis, infection or hemorrhage grossly evident.

Although not quantified, apoptosis was abundant in the external and especially in the internal granular layer, Purkinje cells and neurons of the dentate nucleus. Changes in the white matter were more intense in the center than in the peripheral area of the folia; particularly, reactive astrocytosis, edema, cystic transformation, and microglial cells with rod-shaped nuclei and/or enlarged processes were seen. Microglial response and reactive astrocytosis and gliosis were frequently seen in the hilus of the dentate nucleus.

The most common lesions were apoptosis of the Purkinje cells and internal granular layer, and cystic change of diverse magnitude in the white matter at the center of the folia.

Nineteen out of 65 cases (29%) presented extensive areas with either apoptosis, necrosis, reactive astrocytosis, gliosis and cystic changes both in gray and white matter (Group I: 16/35, 46%; Group II: 3/30, 10%); hemorrhage and/or infections were found in 20 out of 65 cases

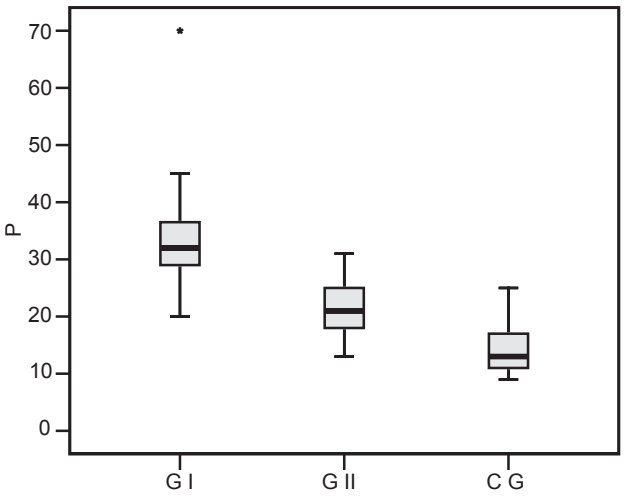

Figure 8. Box plot of Purkinje cells per segment, within Group I, Group II and Control Group. P, Purkinje cells per segment; GI, Group I; GII, Group II; CG, Control Group.

(30.8%), also predominant in Group I (Group I: 14/35, 40%; Group II: 6/30, 20%). Otherwise, the cases with late gestational age but with low body weight or extremely low body weight and a longer survival period were frequently accompanied by more serious cerebellar lesions, independently to the group they belonged (Figure 9I, A,B; Figure 11, B).

The average of weight and microscopic data in the present study failed to reach normal values, remaining arrested at figures corresponding to cerebella of gestational age 30-32 weeks (Group I) or 33-35 weeks (Group II) (Table 6) (Figure 11, A-I).

Comparison with data coming from cerebral tissue

Forty-eight out of 65 cases (74%) presented variable degrees of supra- and infratentorial hypoxic-ischemic encephalopathy as the main disease. Cases with extremely low cerebellar weight as well as very low height of folia b, and altered values for other histological measurements along with severe neuropathological changes were associated with massive cerebral necrosis, peri-intraventricular hemorrhage, periventricular leukomalacia, or diffuse gliosis of the cerebral white matter.

Control group

The control specimens consisted of 20 cases. Four cases (20 %) had congenital diaphragmatic hernia* and the

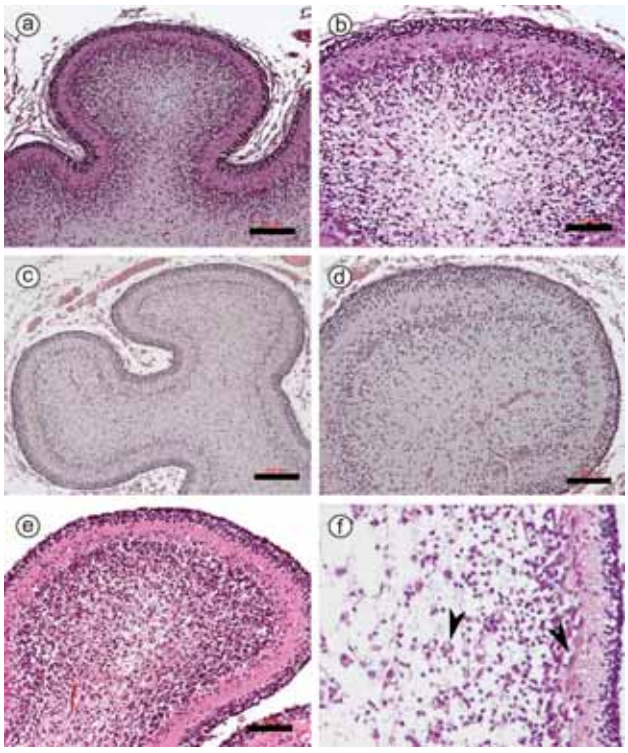

Figure 9I. Immature cortical layers and diverse cellular changes were the histological hallmark of cerebellum in most preterms along both groups. A: Case 41; Gestational age (GA), 36 weeks; postnatal age, 26 days; birth weight, 1200 g; body weight, 1200 g; cerebral weight, 310 g; cerebellar weight, 15 g. Histological section of inferior folia (folia b). Immature cortical layers as well as edema and reactive astrocytosis of the foliar white matter are seen; B: Higher magnification shows preserved external granular layer, narrow molecular layer, high number of Purkinje cells per segment, and diminished internal granular layer with sparse neurons and interspersed reactive astrocytes. Some cells look apoptotic; C: Case 50; GA, 35 weeks; postnatal age, 27 days; birth weight, 2120 g; body weight, 2120 g; cerebral weight, 280 g; cerebellar weight, 17 g. Histological section of folia b. Cortical layers look very immature and, notably, although external granule cells are greatly preserved, internal ones have nearly disappeared. *N*□□, edema, microvacuoles and reactive astrocytosis in subcortical white matter; D: At a higher magnification, molecular layer shows external granule cells migrating inwards; Purkinje cells are small, numerous, and seem to be immature. Apoptosis is seen mostly in internal granular layer; E: Case 16. GA, 34 weeks; postnatal age, 26 days; birth weight, 1120 g; body weight, 1260 g; cerebral weight, 215 g; cerebellar weight, 10 g. Histological section of folia b. White matter at the center of the folia shows edema, microvacuoles, and tiny foci of necrosis along with reactive astrocytosis. Molecular layer is thin but external granule cells seem to be preserved; F: Case 15. GA, 33 weeks; postnatal age, 33 days; birth weight, 1560 g; body weight, 1640 g; cerebral weight, 246 g; cerebellar weight, 10 g. Apoptosis is seen in small, numerous and immature Purkinje cell layer (arrow) and internal granular layer. White matter shows cystic change, cellular necrosis and foamy macrophages (arrow). H&E, ×400 (F).

Table 6. Values of body weight, cerebral weight, and cerebellar weight in groups of preterm neonates at term gestational age equivalent (GI and GII), and in preterm neonates' controls (preterm neonates at 30-32/33-35 weeks gestational age, and up to 7 days postnatal age)

	□□	*C*□	*c*□	□*C*□	*histol* □□
G I	1367 (850-3000)	204 (112-288)	9.8 (5-14)	37-42	30-32
G II	1758 (900-2780)	293 (185-421)	17.9 (15-25)	37-42	33-35
PTN (30-32 w)	1300 (1000-1600)	180 (115-306)	9.6 (6-15)	30-32	30-32
PTN (33-35 w)	1800 (1600-2000)	237 (150-342)	13.5 (9-21)	33-35	33-35

Group I (GI) and 30-32 weeks gestational age (GA) preterm neonates' mean values are similar. Mean values of Group II (GII) correlate well with those of 33-35 weeks GA preterm neonates. This equivalence was also seen histologically (see text and Figure 11). PTN, preterm neonates; w, weeks GA; BW, body weight at autopsy [g]; CW, cerebral weight [g]; cw, cerebellar weight [g]; PCA, post-conceptional age [w]; histol Eq, GA histological equivalence.

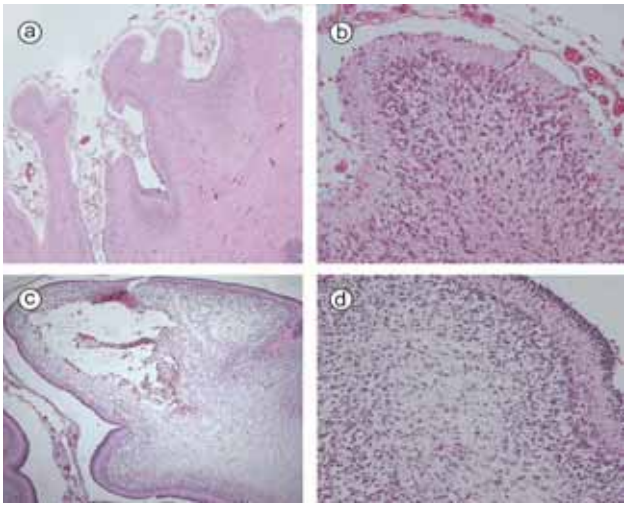

Figure 9II. Hemorrhage, necrosis, and cystic changes of white matter were specially observed in the specimens with lesser cerebellar development. A: Case 1; Gestational age (GA), 35 weeks; postnatal age, 45 days; birth weight, 1270 g; body weight, 1223 g; cerebral weight, 164 g; cerebellar weight, 5 g. Diffuse cerebellar necrosis with narrowed folia in a patient who developed hypoxic-ischemic encephalopathy and sepsis. Pallor of white matter demonstrates incomplete necrosis and gliosis. B: Higher magnification of foliar crown shows neuronal loss, microcystic change and reactive astrocytosis.
C: Case 15; GA, 33 weeks; postnatal age, 33 days; birth weight, 1560 g; body weight, 1640 g; cerebral weight, 246 g; cerebellar weight, 10 g. Cavity left by foliar hemorrhage. Severe cystic change of white matter represents necrosis. D: Higher magnification showing mild reactive astrocytosis and diffuse microgliosis. H&E, (A-D); x25 (A), ×200 (B, D), ×50 (C).

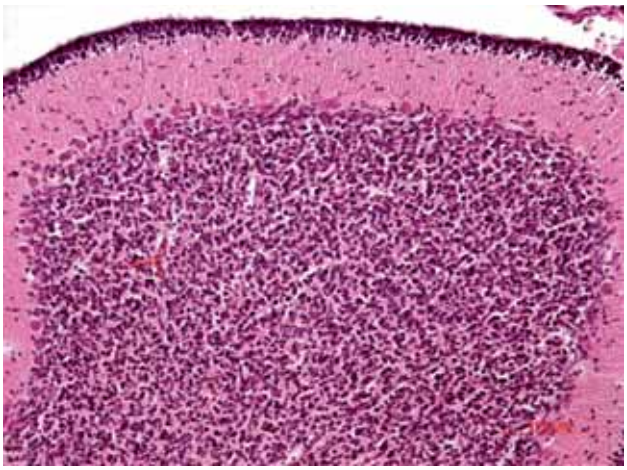

Figure 10. Histological aspect of cases with normal cerebellar weight is very similar to that of controls. Case 65. Gestational age, 30 weeks; postnatal age, 60 days; birth weight, 1800; cerebral weight, 320 g; cerebellar weight, 25 g. Histological aspect of folia b appears greatly preserved in this case, although more Purkinje cells per segment were found compared to controls. H&E, ×100.

remainder had bronchopneumonia, cardiovascular malformations, adrenal hypoplasia, or bilateral renal hemorrhagic infarct as main diseases. The main and only neuropathological finding proved to be apoptosis in 9 cases (45 %). The results of the gross and microscopical measurements (Tables 2 and 4) were comparable to those previously published[34] (*p* for all values not significant).

Correlations

Body weight at necropsy correlated positively with cerebral and cerebellar weight, folia a height, and folia b height, and negatively with Purkinje cell layer. Cerebral and cerebellar weight, folia a height, folia b height, and molecular layer correlated positively with each other. Molecular layer correlated negatively with Purkinje cell layer. Gestational age correlated positively with cerebral and cerebellar weight (the latter correlation being weak). Gestational age did not correlate with histological measurements. The thickness of the external granular layer failed to correlate with any parameter (Table 7) (Figures 12 and 13).

DISCUSSION

Along gestation the cerebellum undergoes a regulated and predictable development with a dynamic cortical growth

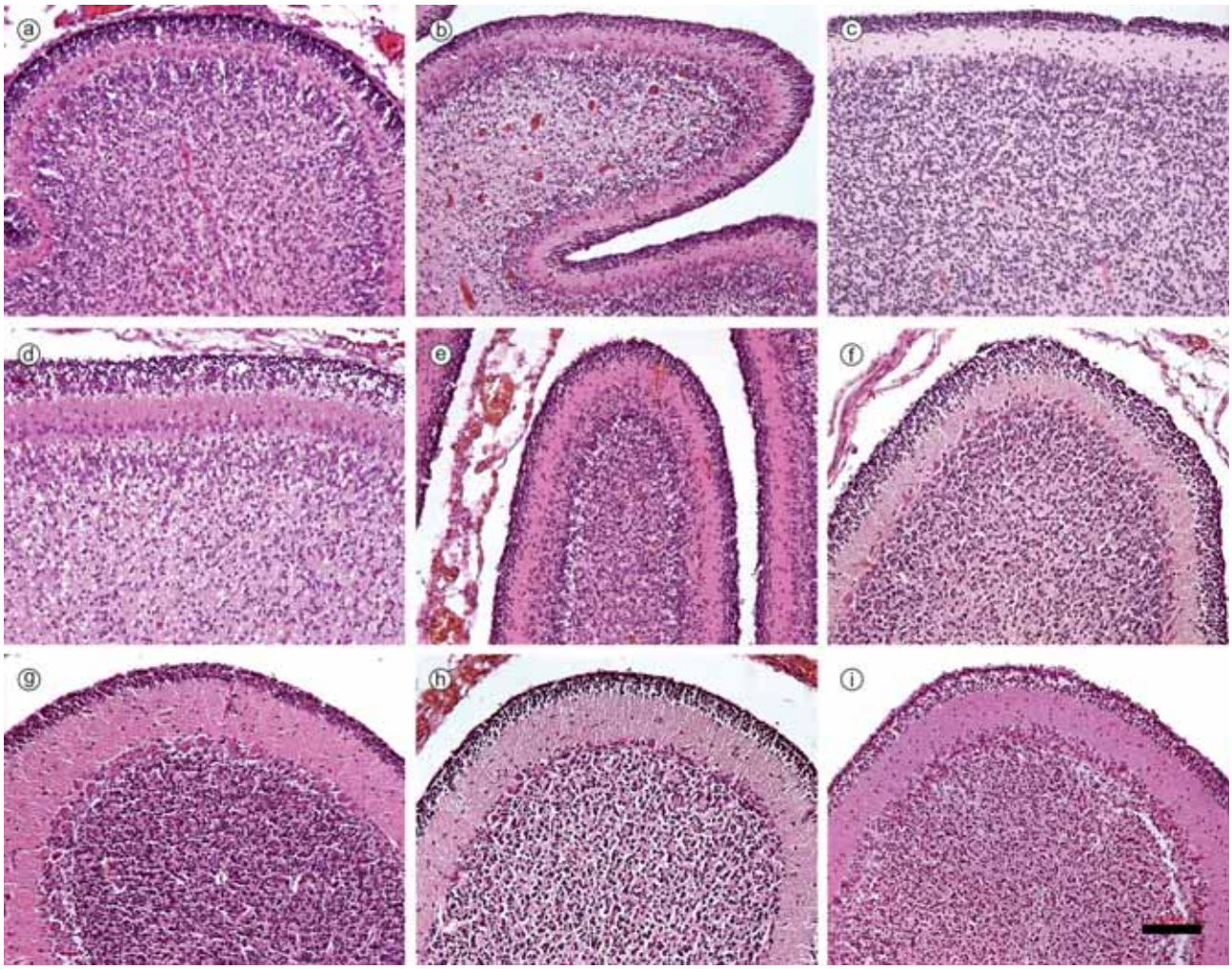

Figure 11. Comparison of histological maturation in the cerebellum of cases from Group I and II, corresponding controls of prematurity and term neonate controls. A, B, C: histological sections of cases 8, 2, and 51, respectively. The images suggest an approximate maturation of 31, 32, and 35 weeks gestationl age (GA) respectively, although these patients have completed a postconceptional age equivalent to term. D, E, F: histological controls of GA (GA, 31, 32, and 35 weeks, respectively; postnatal age up to 3 days). G, H, I belong to term neonates from CG (GA, 38 to 40 weeks; postnatal age, up to 2 days). Data of the cases: Case 8: GA, 28 weeks; postnatal age, 75 days; birth weight, 850 g; body weight, 1530 g; cerebral weight, 242 g; cerebellar weight, 8 g. Case 2: GA, 34 weeks; postnatal age, 33 days; birth weight, 1050 g; body weight, 930 g; cerebral weight, 130 g; cerebellar weight, 6 g. Case 51: GA, 35 weeks; postnatal age, 40 days; birth weight, 1850 g; body weight, 1800 g; cerebral weight, 320 g; cerebellar weight, 18 g. H&E. Scale bar: 100 µm (A-I).

and changing morphology from week to week. This process is so consistent that it is used for gross[50] as well as histological[51] index of gestational age. Nevertheless, the diverse parts of the cerebellum develop within individual time frames and possess different functions, [22] and both of these characteristics impinge on clinical pattern and histopathology.[16]

Preterm birth—of a steady incidence and on the rise in certain parts of the world[33]—implies an increment in the risk of hypoxic-ischemic encephalopathy, the main cause of perinatal morbidity and mortality.[1, 52] The implications for neurodevelopment are still unclear.[18, 53]

In neonates, both cerebrum and cerebellum suffer the consequences of preterm birth; however, the observable devastating lesions in the former seemed to overshadow subtle and sometimes overt lesions within the later. Until last decade, little attention was given to the study of the cerebellum in preterms. As new concepts relating cerebellum

Table 7. Spearman Correlation Coefficients

	□□	C□	c□	h□a	h□b	e□t □r	□ol	□
GA	.536***	.338**	.282*	.244	.177	-.016	-.081	-.102
BW	1.	.603***	.601***	.412**	.401**	-.048	.150	-.430**
CW		1.	.815***	.680***	.530***	-.117	.415**	-.632**
cw			1.	.733***	.661***	-.112	.542***	-.752***
hfa				1.	.783***	-.204	.633***	-.597***
hfb					1.	.005	.557***	-.539***
ext gr						1.	.004	.223
mol							1.	-.478***
P								1.

Entire sample (Group I plus Group II) was used. Control group was not included. GA, gestational age; BW, body weight at autopsy; CW, cerebral weight; cw, cerebellar weight; hfa, folia a height; hfb, folia b height; ext gr, external granular layer thickness; mol, molecular layer thickness; P, Purkinje cells per segment.
***p < 0.001
**p < 0.01
*p < 0.05

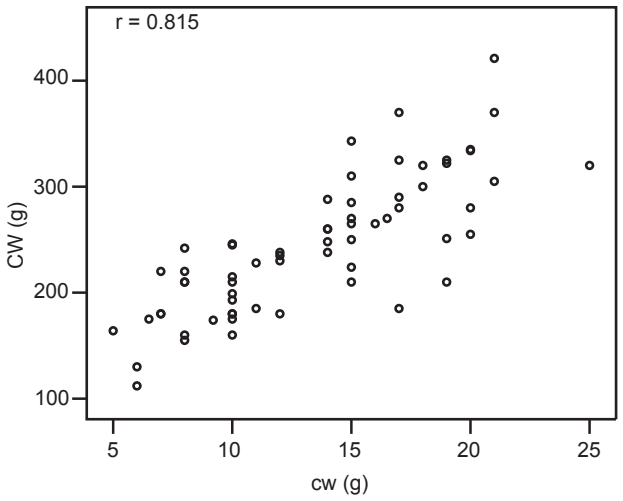

Figure 12. Scatter plot of cerebral weight against cerebellar weight. Entire sample (Group I plus Group II) was used. Control group was not included. CW, cerebral weight; cw, cerebellar weight.

and cognition began to emerge, so the number of reported infratentorial abnormalities began to grow. However, this was especially true for neurological, psychological and imaging studies. Remarkably, even nowadays, detailed histopathological and morphometric contributions in large series of human preterm cerebellum are very scarce and still in need.[31, 32] Moreover, even when the relationship

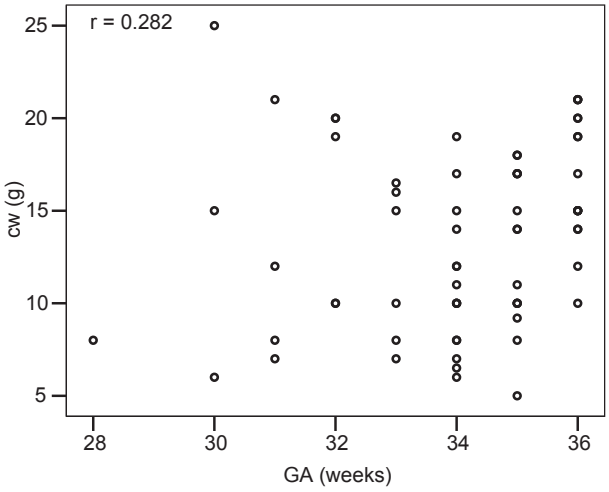

Figure 13. Scatter plot of cerebellar weight against gestational age. Entire sample (Group I plus Group II) was used. Control Group was not included. cw, cerebellar weight; GA, gestational age.

between hypoxic-ischemic encephalopathy and the immature cerebellum has been extensively documented,[4-11, 54] the cerebellar vulnerability windows are not so well understood as those in the cerebrum. The acceleration of the growth speed in the late phase of cerebellar development (the last trimester of gestation with a peak at between 28 and 34 weeks) would imply an especial weakness in this regard.[55, 56]

Clinical data, gestational age, and its relationship with body, cerebrum and cerebellar weight

More than half of the 65 cases in this study —the greatest number within Group I— presented with low body weight or extremely low body weight at birth, and low weight for gestational age. Frequently, these patients developed periventricular leukomalacia or peri-intraventricular hemorrhage either with or without ventriculomegaly. In these patients there converged severe obstetric and neonatal conditions with pronounced CNS participation[57] (Table 1). This was especially true for the 14 patients with extremely low cerebellar weight, which were epidemiologically and pathogenically related, and had the most pronounced changes in gross and histological aspects of cerebellar development.

At autopsy, nearly all cases presented low values for cerebrum and cerebellum weight, although in Group I those weights proved to be particularly low. Quite possibly, the developmental arrest resulted from the pathology

associated with premature birth itself (especially idiopathic respiratory distress syndrome), and that occurring during the subsequent hospitalization as well as from diverse treatments.[15]

Undernutrition has a clear relationship with human brain development.[58] Several reports propose a vulnerable period hypothesis of brain development, indicating that the cerebellum undergoes an especially vulnerable stage during the phase of rapid growth, in the last one half of gestation.[59,60] In the present study, more than half of the cases were both small for gestational age and low body weight or extremely low body weight at the time of birth, and nearly all of them had low body weight at autopsy. Taken together, the data suggest that intrauterine as well as postnatal undernutrition must have had an important relation with cerebellar underdevelopment in this group of patients.[58,61]

The gestational age[16,18] correlated significantly with birth body weight and with the weight of the body at autopsy, and weakly with the values of cerebral and cerebellar weight. It seems that late gestational age, low body weight at birth, and longer survival period may influence negatively on brain development.[62] In present study there was a strong tendency for the cases with the lowest values for cerebral and cerebellar weight to be accompanied by low body weight at birth, and severe complications during a prolonged post-natal life; more than half of those cases had a late gestational age (34 weeks and beyond).

Remote trans-synaptic effects and direct supra and infratentorial lesions. The cases with extremely low cerebellar weight presented with the lowest cerebral weight values, and those with the greatest cerebellar weight also had the most elevated cerebral weight.[12] The significant correlation between cerebellar weight and cerebral weight simply shows the anatomic and physiopathologic linkage between both organs. Just as the serious compromise in the cortical and subcortical gray-matter structures and in the white matter of the cerebrum must have influenced the shortfall of cerebellar development, so the pathology of the cerebellum very likely contributed to the stunting of cerebral growth (remote trans-synaptic effects).[13,24,60,63] Nevertheless, a serious supra- or infratentorial lesion along with milder changes at the supra- or infratentorial counterpart occurred only in some cases, most of them notably belonging to GII, with the most frequent abnormality (48 out of 65 cases -74%) being a lesion in overall encephalic structures from hypoxic-ischemic encephalopathy, where the cerebellum constituted simply an additional part of the spectrum. Therefore, the significant correlation that existed in the present study between the cerebral and the cerebellar weight is attributable primarily to the coexistence of direct supra- and infratentorial lesions, and probably also through diaschisis as a secondary mechanism.[13,16]

Morphometry and arrest of development: Immature folia and immature cortical layers. The histological measurements showed values very far from normal in numerous cases with a great proportion of those belonging to Group I, this suggesting an explanation identical to the one advanced for the gross observations. Under normal conditions foliation is particularly active in the last trimester of gestation and occurs in parallel with the intense proliferative activity of the external granular layer, and the widening of the internal granular and molecular layers over a relatively slowly growing white matter.[46] Thus, the very low height of the folia b (corresponding to the lower portion of the cerebellum) that was observed in cases with severe supratentorial lesions was accompanied by a striking alteration in the thickness and cell density of the cortical strata in those same folia, and was associated, in the majority of cases, with very evident diffuse cellular lesions in the cortex and white matter of the cerebellum. Moreover in these cases both, the cerebral and cerebellar weight was low compared to the values for the Control Group or for the rest of the cases in Group I and Group II. These findings would indicate that the concomitance of supra- and infratentorial lesions, both gross and microscopic, underlies the correlation between the values for cerebral and cerebellar weight. The focal lesion of the lower portion of the cerebellar hemispheres in preterm neonates with extremely low birth weight has been referred by Johnsen *et al*. through MRI.[18, 57]

The histological measurements failed to show the strong correlation with gestational age or with body weight at autopsy that is seen during normal cerebellum development.[34] Otherwise, the significant correlation resulting between some of the histological parameters themselves, as well as with cerebellar weight, reveals a certain degree of harmony in the developmental arrest of the cerebellum.

Under normal conditions from 30 weeks to 40 weeks gestational age the vertical thickness of the external granular layer remains moderately constant (35-39µ approximately);[46] its preservation within folia that otherwise

showed necrosis of the inner neuronal layers and subcortical white matter is a remarkable observation.[4] In accordance, in this study the external granular layer width showed little variation between groups (Figure 6; Tables 4 and 7). On the contrary, the thickness of the molecular layer gave a better measurement of underdevelopment.[49,55,56] In turn, as the thickness of the molecular layer depends mainly on the arborization of Purkinje cells, diminished molecular layer width was associated with high number of Purkinje cells and, conversely, the increase of the molecular layer width occurred here in parallel with the reduction in the number of those cells per segment.

As previously referred (see Results and Figure 11), the average weight and microscopic data in the present study failed to reach normal values, remaining arrested at figures corresponding to cerebella of gestational age 30-32 weeks (Group I) or 33-35 weeks (Group II).[34] Nevertheless, and particularly in Group I, some cases showed a histological image of the cerebellum that corresponded to a lower gestational age than the actual one (Figure 11, B); a low body weight or extremely low body weight at birth, as well as low body weight in relation to gestational age was found in nearly all of these patients. An early arrested maturation affecting the *in utero* development could be one possible explanation for the early stunted cerebellar development. Otherwise, the serious CNS injury found in some of these cases would have introduced an additional component such as atrophy, especially in those patients with a longer survival period. In both groups a tendency was observed for the cases with higher gestational age but low body weight or extremely low body weight at birth and a longer survival period to be accompanied by more serious cerebellar lesions, as has already been found in brains of very low birth weight infants by Golden, et al.[62]

Neuropathological findings. Apoptosis, vertical growth, horizontal growth and its relationship with foliation

Cell death in response to injury in the developing CNS is conceived nowadays as a continuous process that proceeds from apoptosis to necrosis.[33] Previous reports showed the extensive lesion of the internal granule cells as a component of disseminated cerebral necrosis during the perinatal period.[64] Apoptosis is frequent in the external and internal granule cells in preterm neonates, and so happens with other cells of greater size (Purkinje cells, neurons of the dentate nucleus), even in term newborns.[1,65-68] These concepts concur with the findings of present work. In addition, many of the patients presented here had developed sepsis or infections limited to the CNS. Immature postmitotic and recently divided neurons are especially vulnerable to apoptosis in bacterial meningitis.[69] Therefore, it is possible that a mechanism similar to the cerebral one operates in the immature cerebellum as well in response to hypoxic, ischemic, hemorrhagic and/or infectious lesions.[53,70-72] The therapeutic measures to which these patients had been subjected (mechanical ventilation, administration of oxygen, corticoid medication) could also have participated in the induction of the above-mentioned process of apoptosis.[16,73,74]

External granular layer. Apoptosis in the external and internal granular layer along with narrowness of the molecular layer was a remarkable phenomenon in the 14 cases of extreme hypoplasia presented here.[4,75,76] The implications of apoptosis as well as changes in the arborization of Purkinje cells are most important. Cellular proliferation in the external granular layer and then inward migration of these young neuroblasts through molecular and Purkinje cell layer to their final placement in the internal granular layer are essential for the development of the cerebellum. Apoptosis in the external and internal granular layer and presumably diminished neuritogenesis of Purkinje cells must have contributed to the reduction in the width of the cerebellar cortex, and in turn must have influenced negatively on the ultimate size and structure of cerebellum in these preterm neonates.[77]

Although the vertical thickness of the external granular layer was quite spared in this study, an impaired horizontal growth through reduction of proliferation, migration and neuritogenesis must be the explanation for the poor foliation and diminished foliar height recorded in the present cases.

White matter. Oligodendroglial necrosis as well as apoptosis[78,79] can diminish and even interrupt the development of white matter in the cerebrum. A mechanism similar to the cerebral one possibly operates in the cerebellum. Diffuse cerebellar white matter damage and cystic leukomalacia of the center of the folia has been reported both in humans and experimentally.[7,32,80-83]

Histological measurements on white matter were not performed in this study. However, it was obvious that a cerebellar leukoencephalopathy was present in some folia and also in the subcortical white matter (Figure 9I, E,F).

In 8 of the cases that had suffered peri-intraventricular hemorrhage, atrophy of the cerebellar parenchyma was found in addition to ventricular enlargement (*ex vacuo* ventriculomegaly) (Figure 3). In those cases, along with a cerebellar weight considerably below normal values, small folia with severe degrees of histological immaturity, a reduced amount of granule cells and a scanty white matter were observed (Figure 5 A; Figure 9I A,B and Figure 11 A). To the effect on the cerebral white matter lesions usually observed in cases with post-hemorrhagic hydrocephalus,[84,85] it must be added then the compromise of the cerebellar tissue.[18,85]

Hemorrhage. Intraparenchymatous and subarachnoid cerebellar hemorrhage as part of the lesions in hypoxic-ischemic encephalopathy,[1] such as was found in 17 of the present cases (13 belonging to Group I) (Figure 9II, C,D), is not an infrequent phenomenon in the PTN.[54] This in turn, must have contributed to the observed underdevelopment of the cerebellum.[60]

Spectrum of lesions and its relation to MRI

As we previously considered,[32] from the combined analysis of the gross, morphometric, and neuropathological data, there is a spectrum of lesions one of its ends having a highly poor outcome. This was particularly represented by the cases presenting extremely low cerebellar weight[54,57] and some cases belonging to Group II (Figure 9I, A-D, Figure 9II, A-D). Cystic change of the white matter, macrophages, necrosis, hemorrhage and/or diffuse reactive astrocytosis were the histological findings. Part of Group I and a large portion of Group II appear to be at the center of this continuum, these cases presenting mainly with cellular lesions, either focal or diffuse, along with infrequent necrosis and focal hemorrhage. The least impacted were 4 cases in Group II (cerebellar weight: 21 to 25 g) without supra- or infratentorial lesions (see Results) (Figure 10), with relative preservation of the gross and microscopic parameters, and with only isolated cellular abnormalities. The aforementioned findings (*i.e.* necrosis, hemorrhage, macrophages, astrocytosis) suggest that the principal changes associated with the developmental arrest of the cerebellum are related to a primary and direct injury with a positive relationship between the severity of the damage and the degree of such arrest.

The gross and microscopic correlation allows the establishment of a second correspondence, this time between the pathological findings and those of MRI. It is evident that the most serious cerebellar lesions (extremely low weight, necrosis, hemorrhage, diffuse cystic change) correlate with those visualized by MRI, while those situated at the middle or at the other end of the spectrum, being visible only microscopically very probably correspond to the cases in which MRI detects no lesion whatsoever. This situation was pointed to recently: the undersize cerebellum without cerebral (or cerebellar) lesion was observed by MRI in groups of preterm neonates;[21,23,25] Bodensteiner, *et al*.[17] considered the probable existence of "milder forms of injury to the cerebellum ", and Argyropoulou, *et al*.[16] suggested that "functional disconnection from the cerebral lobes is thus probably not the only cause of cerebellar atrophy". Therefore, even though deafferentation and diaschisis cannot be discarded as operative mechanisms in the hypoplasia of the cerebellum,[6,13,20,74,86] a primary and direct lesion to the cortex and white matter, though of a different intensity, constitutes a little appreciated but fundamental cause of the whole pathological process, especially in the group of preterm neonates with extremely low cerebellar weight.[32]

The findings in this work agree with the concept of "perinatal panencephalopathy"[31,87,88] adequate for the combined gray and white matter injury that is typical of perinatal neuropathology of prematurity.

Present study has a potential limitation. It seems likely that progress in therapy and other aspects of prenatal care during the period from which data were obtained could have effects upon the development of the brain. The number of cases per year in our cohort, however, is too small to draw any conclusions in that sense. Better therapies have undoubtedly increased the number of surviving preterm neonates. Presumably, those living preterms could have a lesser compromise of their CNS and even a diminished incidence of their CNS lesions because of improved therapies. Nevertheless, MRI studies in living patients frequently show a severe compromise of the cerebellum.[8,12,13,16-20] Moreover, preterm birth continues to have a steady incidence all over the world. Therefore, if cerebellar lesions exist in the way they are presently shown to by MRI, it is not obvious that those changes would have a pathological background that is very different from the lesions described in this work. Upon comparing these cases with those resulting from MRI, it is appropriate to recall that the latter studies were performed on patients

who survived a premature birth and then managed to attain a postconceptional age greater than term. For this reason, MRI studies in living patients implies, to a certain extent, a filtering out of the severe pathology found on autopsy. As we previously stated,[32] both procedures show that in preterm neonates a deficient growth, with body weight being a reliable marker, can imply deterioration of the CNS in general and of the cerebellar developmental milestones in particular.

CONCLUSIONS

In this group of preterm neonates the cerebellum maturation was arrested in late stages of development (30-35 weeks gestational age), perhaps that of the preterm birth itself, or at the most a period only shortly postnatal. Gestational age was found to have little impact in this series.

There was a spectrum of abnormalities, both developmental and destructive, with one of its ends presenting mild histological changes and nearly normal cerebellar size, and the other end with greater histological lesions found in the group of extremely low cerebellar weight.

The arrest of cerebellar growth occurred mainly in foliar height and foliation and some parts of the cortex (molecular layer, Purkinje cells and internal granular layer expressed immature cerebellar cortical layers). Although external granular layer was mostly spared in its vertical width, poor foliation and diminished foliar height represented primarily the effect of the impaired external granular layer horizontal growth.

Cellular changes were evident in external and internal granular layer (apoptosis), molecular layer (gliosis) and Purkinje cells (apoptosis). Although mild in some cases, a truly leukoencephalopathy was seen in the folia and the subcortical white matter.

Remote trans-synaptic effects could not be discarded, but the main histological changes were related to direct injuries. The cerebellar lesions comprised just one additional part of primary and direct injuries that were observed in the CNS in this group of preterm neonates. The findings may be interpreted as the result from the effect of noxa during the cerebellar lobes' vulnerability window. This pathological process, mainly cellular in its nature, was the most important and evident basis for the small cerebellum in our series.

Direct injury of cerebellar cortex and white matter is an important and poorly recognized cause of impaired cerebellar growth and development.

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