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CONGENITAL BRAIN ANOMALIES AND CHROMOSOMAL ABERRATIONS FROM THE ZAGREB COLLECTION OF HUMAN BRAINS

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Abstract

The Zagreb Collection of developing and adult human brains consists of approximately 1,300 brains of fetuses, children and adults that were collected following routine autopsies in the period from 1974 to 2014. The collection comprises brains of different normal developmental stages that may serve for investigation of normal human brain development. Previous studies on this material have led to several important contributions on human cortical development, such as the discovery of the transient fetal subplate zone. The Zagreb Collection, however, also contains approximately 100 brains with different anomalies including chromosomal aberrations such as Down syndrome. We have analyzed all the available material from the Zagreb Collection and identified 44 brains of fetuses and children with Down syndrome, 10 with Patau syndrome, 6 with Edwards syndrome as well as 7 holoprosencephalic, 7 hydrocephalic and 4 microcephalic brains. The largest part of the Collection is available for further research using modern genetic, immunocytochemical and imaging methods, especially magnetic resonance imaging. Furthermore, the histological slides from the Zagreb Collection are currently being digitally scanned and made available as virtual slides to general scientific audience. The Zagreb Collection represents unique and versatile resource for the future study of normal and abnormal human brain development.

Keywords

• Brain • Collection • Anomalies • Down syndrome • Patau syndrome • Edwards syndrome

Introduction

Congenital anomalies can be found in approximately 3% of overall newborns, 15% of autopsied newborns and 25% of fetuses and stillborns. Anomalies of the central nervous system comprise about 1/3 of all anomalies, having an incidence of 1:500 to 1:3000 of births. Etiology and pathogenesis of central nervous system (CNS) anomalies, as well as anomalies of other organ systems, are still relatively unknown (40-60%). Approximately 10-25% of all malformations are caused by gene mutation or chromosomal aberrations [1-3]. Environmental influences, such as viral infections (e.g., CMV infection, rubella, and toxoplasmosis), drugs and other chemicals, radiation or trauma, are responsible for 12% of anomalies. Around 20-25% of anomalies are caused by synergy of environmental and genetic causes [1-4]. The majority of brain malformations are neural tube defects, having an incidence of 0.4 to 1 out of 1000 births and up to 8 out of 1000 births in some countries [1-3].

Other anomalies such as holoprosencephaly, lisencephaly and polymicrogiria are much less frequent, with an incidence of 1 out of 14000 births, 1 out of 100000 births and 1 out of 2500 births, respectively [5].

Developmental malformations of cerebral cortex occur subsequently to alterations in expression of specific genes which play a key role in normal histogenesis (proliferation, migration, axonal growth, synaptogenesis, morphologic and chemical differentiation of neurons, apoptosis). Recent advances in molecular biology and neuroimaging enabled development of new diagnostic methods for the detection, as well as improved classification, of cortical disorders [6-8]. Use of magnetic resonance imaging (MRI) as a research and diagnostic tool enabled investigation and diagnosis of various cortical malformations *in vivo* that had previously been unidentified. This has led to renewed focus and added clinical importance to the study of genetic causes of aforementioned malformations. Combined with the advances in molecular biology this resulted in new methods for studying genetic

mutations and provided a wealth of data on the role of gene and protein expression in the pathogenesis of various cortical malformations [9]. These advances provided an explanation for phenotypic variations, stressed the importance of elucidating novel mutations, and resulted in improved clinical correlation between molecular phenotype and neuroimaging findings of different cortical malformations. Novel diagnostic procedures tend to recognize these cortical malformations as causes of epilepsy and other neurological deficits. To date, various genes that selectively alter key histogenetic processes have been described, for instance *HPE1-4* in holoprosencephaly, *TSC1* and *TSC2* in tuberous sclerosis or *LIS1* and *DCX* in lissencephaly [10-14]. Such anomalies are extremely rare, hence can only be investigated in specialized institutions, which hold a larger number of samples convenient for distinct histological, histochemical, immunocytochemical and genetic analysis.

In this review we analyzed the material from the Zagreb Collection for the known CNS anomalies. Our goal was to classify these

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anomalies, and provide data on the availability of MRI, genetic, morphologic, histological, and immunocytochemical data. At the end our intent was to present this valuable material in clear way to the general scientific community in hope that it will serve as a unique resource in the quest of elucidating pathogenesis of CNS anomalies.

Materials and methods

The Zagreb Collection of human brains was founded in 1974 at the University of Zagreb, School of Medicine, Department of Anatomy, and is currently housed in the Croatian Institute for Brain Research which is a part of University of Zagreb School of Medicine [15,16]. It includes fetal, newborns, stillborns and children's brains showing normal and abnormal development (Fig. 1). These brains have been collected through routine autopsies performed at pathology and forensic pathology departments, mainly in Zagreb hospitals, but also in Split and Osijek. We have implemented detailed collection protocol in order to secure the highest possible quality of the material, as follows. Upon receipt of brains, the clinical data was collected and brains were thoroughly analyzed, particularly those with cortical anomalies. Every brain was assigned identifying code of two letters and a number under which was entered in collection. Fetal brains have been labelled „ČF" (*human fetal*), newborns and children „ČD" (*human children*) and adult brains „ČO" (*human adult*). The number was assigned according to the order in which brains arrived in the collection. Following the autopsy, brains were fixed in 4% buffered paraformaldehyde (PFA) for 10-60 days depending on the age of specimen. After the grossing and photographing (Fig. 2 and Fig. 3), MR imaging was performed and the material was then cut in 1-2 cm thick slabs. Slabs were further processed for paraffin or celloidin embedding or cryoprotected and frozen. Processed slabs were then cut and routinely stained for histological quality control. Samples of poor quality were removed from the further analyses. The extent of analyses was dependent upon the type of anomaly, so in some cases, *in situ* hybridization and cytogenetic analysis were performed as

well. The samples were analyzed using three classical histological methods: Nissl staining for demonstrating cytoarchitecture (Fig. 4), Weigert of Gallyas staining for myeloarchitecture and Golgi staining for demonstrating neuronal morphology. Besides these three classical methods various other methods were used. Among more common methods applied were three histochemical methods: Acetylcholine esterase (AChE), nicotinamide adenine dinucleotide phosphatase-diaphorase (NADPH-d), and Periodic Acid - Schiff (PAS)

- Alcian (for analysis of extracellular matrix); and various immunocytochemical methods. Following routine analyses more detailed immunocytochemical analyses for the different neuronal types were performed using antibodies raised against calcium binding proteins (e.g., calbindin, calretinin and parvalbumin), and different neuropeptides (e.g., somatostatin, neuropeptide Y). Furthermore, expression patterns of many molecules in extracellular matrix, such as axon guidance molecules and synaptogenesis
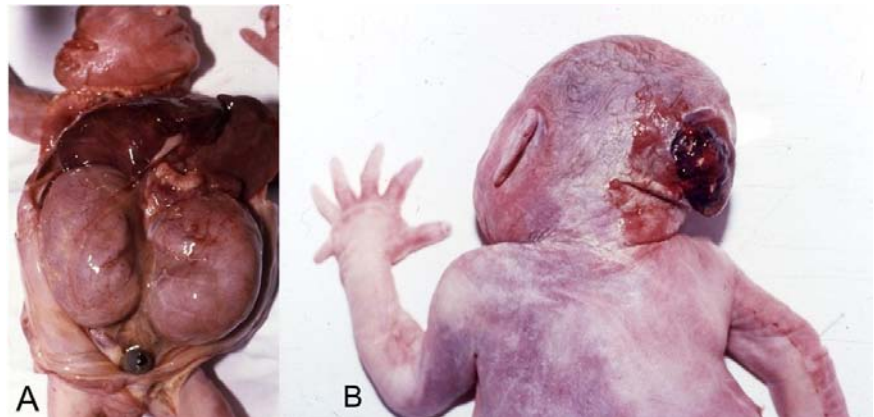

Figure 1. Fetus displaying morphology of Meckel-Gruber syndrome. (A) Abdominal cavity is opened and reveals enlarged kidneys and (B) occipital encephalocele and hexdactilia.
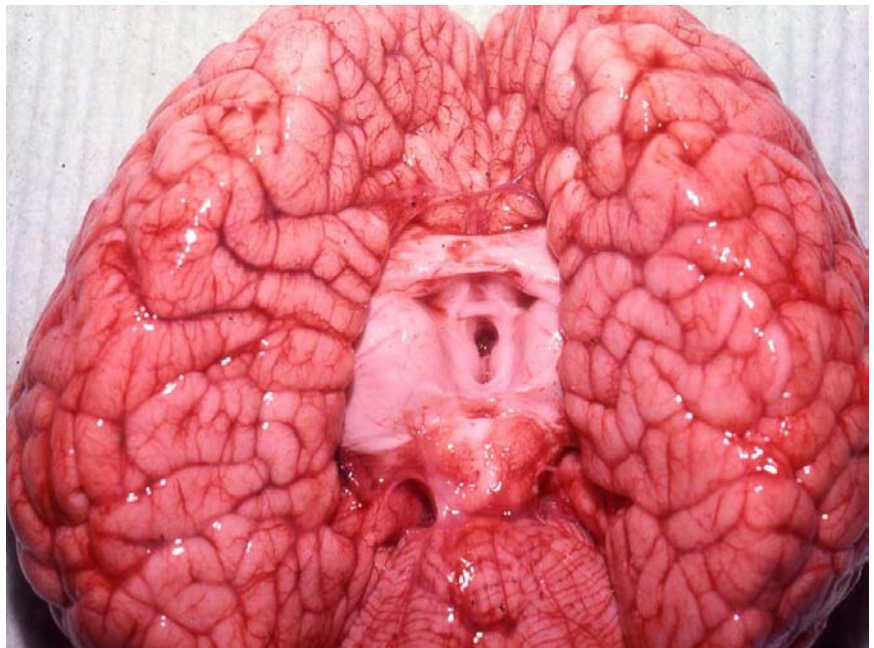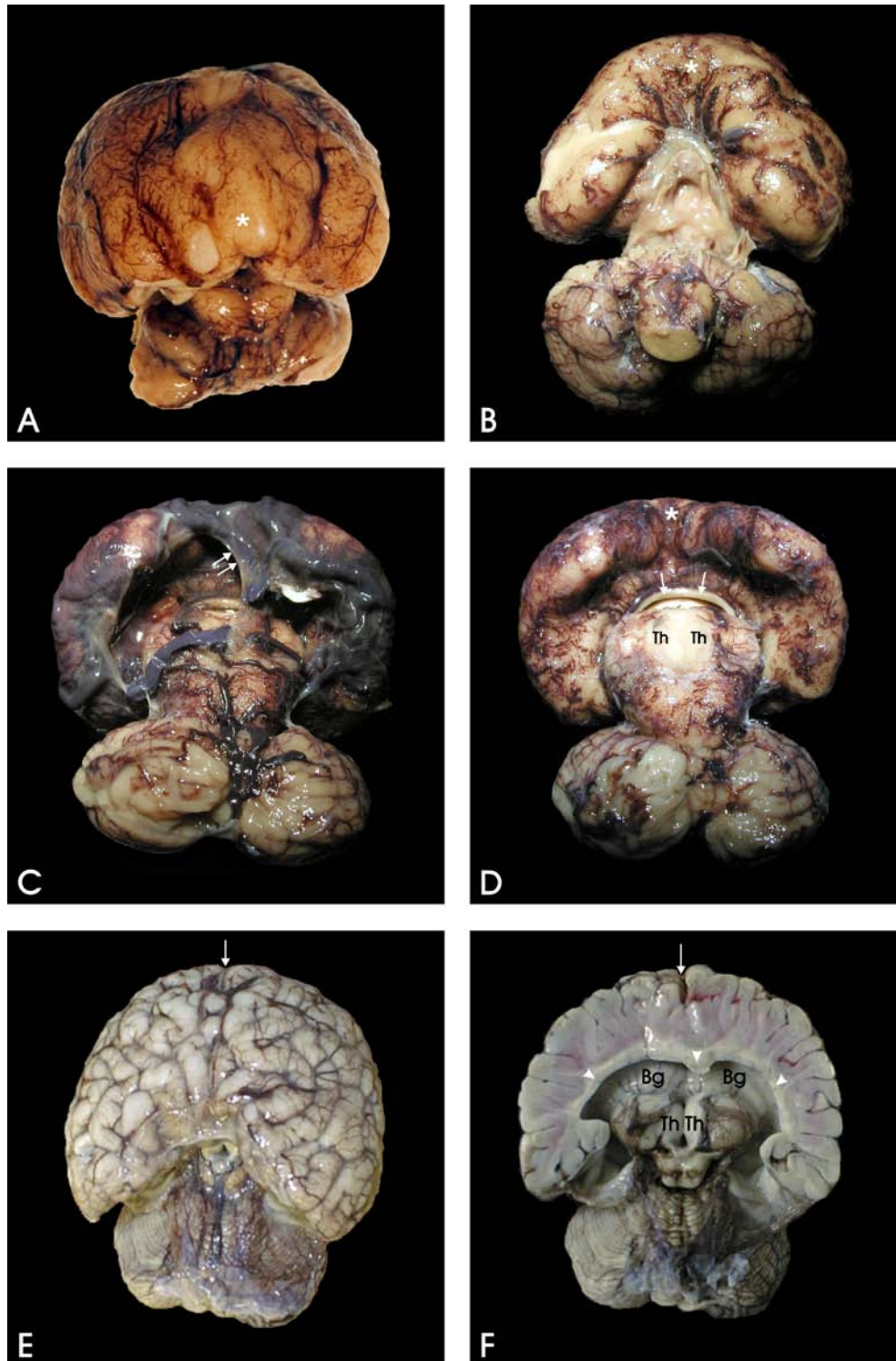

Figure 2. Image depicts brain with agenesis of corpus callosum.

Figure 3. Morphology of brain in three cases of alobar holoprosencephaly (HPE). The brain of 30-week-old premature infant with alobar HPE (A). A 39-week-old premature infant (B-D) and 4-month-old infant (E, F) with alobar HPE are shown for comparison. Note that typical features of alobar holoprosencephaly are (F): a monoventricle with fused basal ganglia (bg-bg) and thalamus (th-th), extremely thin white matter and thickened cerebral cortex with abnormal and poorly developed gyrification.
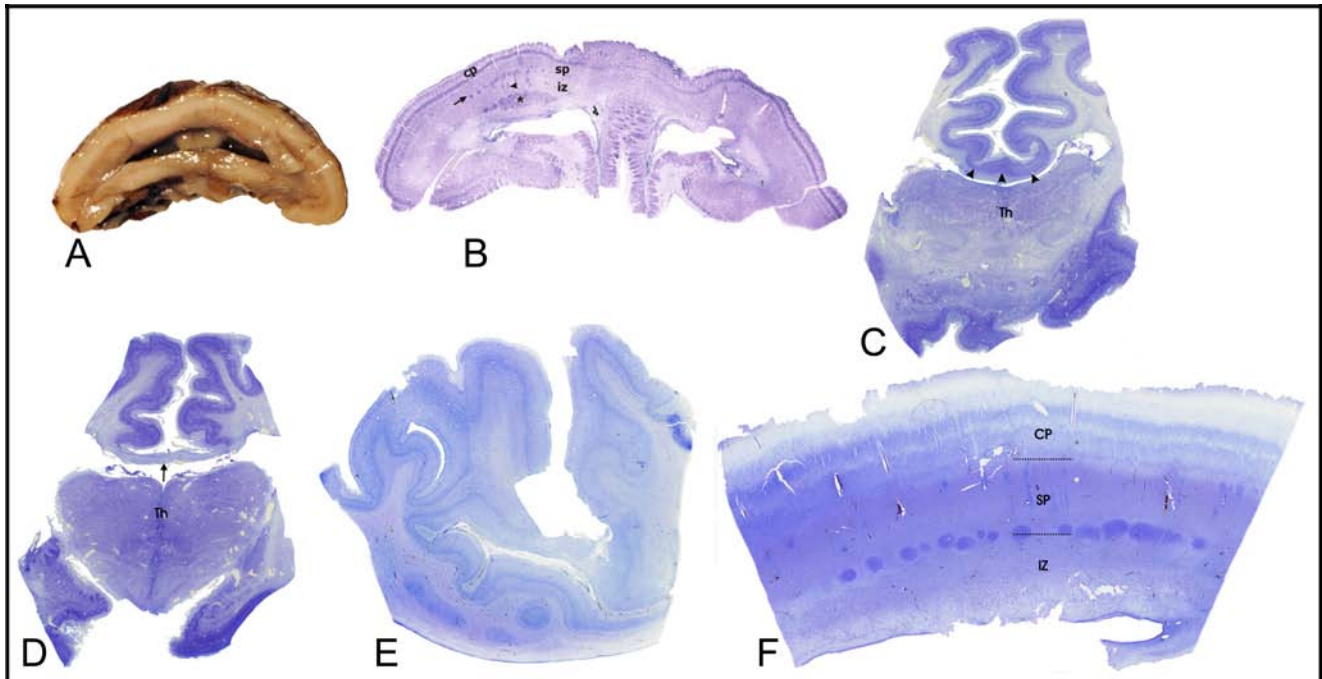
Figure 4. (A) Coronal section through a telencephalon of 30-week-old fetus with alobar holoposencephaly, and (B, F) Nissl-stained preparations from the same tissue block display abnormally laminated cortical plate (cp), narrowed subplate zone (sp) and intermediate zone (iz), with heterotopic islands of large and small neurons located at the border of sp and iz (arrow in B) and within the iz (arrowhead in B, a row of cell islands in F). The periventricular mass of heterotopic cells (asterisk in B) probably represents a mixture of heterotopic neurons from basal ganglia and cortical neurons, which failed to migrate to the cortical plate. Note that left and right thalamus are completely fused (Th in C, D) and that third ventricle is obliterated. Also note that corpus callosum (C, D, E) is reduced to a very thin band of white matter (arrow in D) and that in rostral parts of the hemisphere (C, E) the cortical plate passes from left to right hemisphere without the interruption (arrowheads in C).

markers (e.g., semaphorins and neuropilins), were investigated using immunocytochemistry. Moreover, *in situ* hybridization on frozen sections was performed to detect markers which were unable to be demonstrated by immunocytochemistry (e.g., reelin), as well as Fluorescence In Situ Hybridization (FISH) and Polymerase Chain Reaction (PCR) analysis for accurate cytogenetic diagnostics of particular disorders. The Zagreb Collection includes more than 100,000 slides stained with different, above mentioned methods, as well as more than 1000 tissue blocks embedded in paraffin or celloidin or frozen, ready to use for further analyses. Additionally, there are several hundreds of brain parts or whole brains preserved in 4% PFA.

Results

The Zagreb Collection, consisting of more than 1,300 human brains (622 fetal, 322 children and 379 adult brains), represents one of the largest collections of developing human brains [15,16]. Since its establishment in 1974, many brains with CNS abnormalities were included. Among these brains 44 were from patients with Down syndrome, among which 22 fetal (age range from 9 to 30 gestational weeks – GW) and 22 children (age range from 1 day to 12 years; Table 1). Another anomaly represented in the Collection with 10 cases is trisomy 13 (Patau syndrome; Table 2). Out of these 6 were fetal brains (age range from 24 to 42 GW) and 4 were children's brains (age range from 0 to 11 days). Trisomy 18 (Edwards syndrome) is represented with 6 brains, 4 fetal (age range from 30 to 32 GW) and 2 children's (age of 9 and 29 days; Table 3). In all these cases chromosomal aberrations were confirmed by karyotyping performed in the hospital of birth.

Apart from the classification based on genetic alterations, all anomalies were further divided according to morphological changes. Seven holoprosencephalic brains were identified, of which 3 within Patau syndrome, 1 within Edwards syndrome and 3 without defined genetic or chromosomal abnormalities (Table 4). There were also 7 hydrocephalic brains and 4 microcephalic brains. Hydrocephalus was diagnosed in 4 fetuses and 3 children, of which 4 male, 2 female and 1 of unknown sex (Table 5). Microcephaly without other identified anomalies was found in 4 children of which 2 male, 1 female and 1 of unknown sex (Table 6). Six brains with Patau and 6 with Edwards syndrome, as well as 1 without known genetic or chromosomal abnormalities are available for further immunocytochemical analyses. MR imaging was performed on 2 of aforementioned brains; 1 holoprosencephalic and 1 with Patau syndrome. All collected brains were analyzed with one of the previously mentioned methods, while those obtained after 2007 have predominantly been analyzed by MRI.

Table 1. Brains of fetuses and newborns with Down syndrome. ČF = fetal brain, ČD = newborns' and children's brains.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)	Postnatal survival
ČF 303	M		6 d
ČF 317	F	6 w	
ČF 318	M	7 w	
ČF 333	F	20 w	
ČF 335	F	24 w	
ČF 336	F	30 w	1 d
ČF 343	M	19 w	
ČF 372	M	36 w	
ČF 382	M	26 w	
ČF 390	M	29 w	
ČF 401	M	23 w	
ČF 438	M	24 w	
ČF 442	M	20 w	
ČF 456	F	22/23 w	
ČF 489			
ČF 501			
ČF 509	M	20 w	
ČF 522	M	21 w	
ČF 556	F	21 w	
ČF 607	F	20 w	
ČF 613	F	22/23 w	
ČF 614	M	21 w	
ČD 14	F	2 yrs, 10m	
ČD 73	M	1 m, 3 d	
ČD 84	M	7 d	
ČD 106	M	4 m	
ČD 117	M	2 yrs, 6 m	
ČD 127		20 d	
ČD 135	M	7 d	
ČD 141		6 d	
ČD 142		8 m	
ČD 144		newborn	
ČD 146	M	2m, 15 d	
ČD 170	F	4m, 20 d	
ČD 179	F	11 d	
ČD 191	F	15 m	
ČD 195	M	2 yrs	
ČD 209		2 m	
ČD 239	F	7 d	
ČD 240	M	34 w	
ČD 253	F	12 yrs	
ČD 260	M	6 m	
ČD 287	F	6 m	
ČD 318	F	10 yrs	

Discussion

The improvement of MRI provided new insights into discrimination of specific cortical malformations, previously classified within the same group, by recognizing subtle differences among them. A few years ago, new, revised classification was introduced based on histogenetic event that was affected (proliferation, migration, cortical development) [6-8]. These classifications are derived from the data obtained from histological (e.g., developmental stage, histogenetic process), pathologic, genetic and neuroimaging analyses. However, present MRI studies often have not been correlated to histological features of certain types of malformations, due to the lack of proper material, nor were these findings classified in the context of modern developmental neuropathology [6]. This problem could only be resolved by a multimodal approach in analyzing brain anomalies, which includes detailed morphological, MRI and cytogenetic analysis. One of the ways to tackle this problem is by analyzing available brains from various brain collections.

Possibly the largest collection of brain tissue is stored in The National Museum of Health and Medicine in the USA. This collection consists of a number of smaller collections such as Yakovlev-Haleem collection comprising more than 1200 brains gathered in the period from 1930-1994; Blackburn-Neuman collection with more than 1000 brains mostly of psychiatric patients; Lindenberg collection of traumatic brain injuries, and Rubinstein collection of tumor pathology with more than 4000 samples. Apart from human brains, this collection also contains animal brains, including mammalian brains in Harrison-Moore and Denny-Brown collections. Data from some of these collections are available online, for instance from Johnson and Welker collections, which contain 120 and 275 brains, respectively. While these collections contain large numbers of specimens collected, theirs' main weaknesses are that they consist almost exclusively of sections stained by classical histological methods (e.g., Nissl, Weigert) and that they are not being expanded. On the other hand, main features of the Zagreb Collection are great versatility of different

Table 2. Brains of fetuses and newborns with Patau syndrome.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)	Postnatal survival
ČFP 211	F	Newborn (40 w)	1 d
ČFP 360	M	42 w	
ČFP 368	F	24 w	
ČF 380	F	34 w	
ČFP 388	M		
ČFP 534	F		
ČD 19	M		8 d
ČD 275	M		11 d
ČD 295	M	36 w	3 h
ČD 298	F	37 w	1 d

Table 3. Brains of fetuses and newborns with Edwards syndrome.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)	Postnatal survival
ČFP 424	M	32 w	
ČFP 550		32 w	
ČF 563	M		
ČF 564	M	30 w	
ČD 145	F	39 w	29 d
ČD 245	F		9 d

Table 4. Holoprosencephalic brains.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)
ČFP 347	F	24 w
ČFP 360	M	37/38 w
ČF 534	F	
ČD 56	F	2,5 m
ČD 91	F	
ČD 276	F	4 m
ČD 295	M	36 w

Table 5. Brains of fetuses and children with hydrocephalus.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)
ČFP 395	M	24 w
ČFP 410	M	40 w
ČF 589	Ž	23/24 w
ČF 590	M	19 w
ČD 223	M	newborn
ČD 225	Ž	11 yrs
ČD 271	Ž	3 yrs, 6 m

Table 6. Brains of fetuses and children with microcephaly.

Sample code	Gender	Gestational age (ČF)/ age (ČD) (days, weeks, months, years)	Postnatal survival
ČD 86	M	6 m	
ČD 193	M	35 w	2 m, 6 d
ČD 200	M	3 yrs, 11 m	
ČD 288	Ž	4 yrs, 6 m	
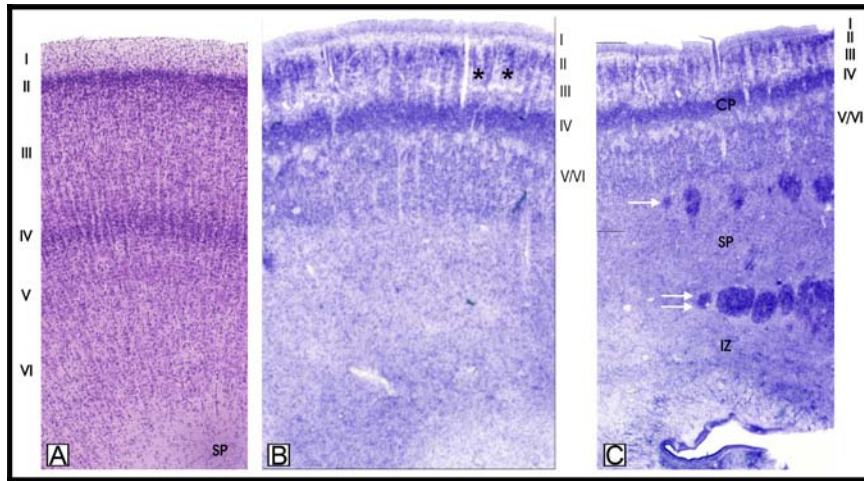
Figure 5. Nissl-stained coronal sections through a neocortex of normal newborn (A) and of a 30-week-old infant with alobar HPE (B, C). Note that basic six neocortical layers (I-VI in A) are preserved in HPE (I-VI in B, C), but the modular organization is seriously disturbed: (B) layer II has been transformed in a discontinuous band of cell islands, layer III displaying finger-like stripes of alternating cell-packing density (asterisk in B), layer IV is abnormally thick band of densely packed cells, and milder cytoarchitectural changes are present in remaining layers. There are two bands of heterotopic cell islands: the superficial one (arrow) is located at the border of the cortical plate (cp) and the subplate zone (sp), while the deep one (double arrows) is located in the intermediate zone in the vicinity of the ventricle.

classical and modern histological techniques used and the application of modern MR imaging techniques. Furthermore, new specimens are continuously added to the Zagreb Collection.

The value and quality of material from the Zagreb Collection is illustrated by the important findings in more than 100 original research articles and reviews published in leading journals in the fields of neuroscience, neuroanatomy and neuropathology [15-34].

The data obtained from the Zagreb Collections were used in two seminal discoveries in the field of developmental neurobiology: the discovery of the transient subplate zone, and of the early bilaminar synaptogenesis in the human fetal neocortex [15,16,35]. With the advent of MR imaging, the material of the Zagreb Collection was instrumental in demonstrating the potential of MRI and histological data correlation and its clinical relevance [22, 26, for detailed review see 30].

In the studies of the CNS abnormalities, material from the Zagreb Collection performed double role. It provided pathological material for the publication of several important papers on the neuronal migration, differentiation, morphology, laminar distribution and apoptosis in holoprosencephaly [24,28], Down syndrome [36,37], Walker-Warburg Syndrome [38], and spinal muscular atrophy [21,27]; as well as material for studying normal development [20,32] and the normative data used for the comparison [31]. However, samples with CNS abnormalities in the Zagreb Collection are not sufficiently researched. New disorders, for instance heterotopic islands of neurons along the border of intermediate and subplate zone and the border of subplate zone and cortical plate were identified, described for the first time and are still a focus of intense research (Fig. 4 and Fig. 5). The Collection also comprises multiple brains showing other genetic malformations which were not yet analyzed; and these could therefore be thoroughly examined using new available techniques.

The current development of Brain Banking and networking shows a tendency to make the material of various collections available to researchers around the globe. Likewise, our intention is to document and classify all samples from the Zagreb Collection and enable worldwide access to its content. The emphasis should be on detailed classification of congenital microcephalies, polymicrogyria (especially those with identified affected genes) and congenital muscular distrophies. Furthermore, we are in the process of digitally scanning already available histological slides and making them available to the general scientific community. We hope that by doing this, the unique and valuable material of the Zagreb Collection will further enhance research of normal as well as abnormal human brain development and provide basis for the collaboration of researchers form different countries and disciplines.

Acknowledgment

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