The authors present the results of a morphological study of the biological samples from patients treated with the inferior esophageal atresia with tracheoesophageal fistula, which included immunohistochemical examination of neuronal changes in the esophageal wall, predominantly in the anomalous segment, with the need surgical involvement. The results obtained in the control group shows that the period of 36–37 weeks of gestation there is persisting glial cell component. The attested features are characteristic for the morpho-functional transition period from prematurity to maturity, being a specific neuronal cytology of the norm in the course of maturation of the child. Congenital morphopathological modifications of intramural ganglio-neural structures determined in both esophageal segments in cases of esophageal atresia with inferior tracheoesophageal fistula, concomitant with fibro-muscular dysplasia, may be considered as factors with significant impact on esophageal motility regulation in children with esophageal atresia with tracheoesophageal fistula, and explains within certain limits their role in esophageal dismotility found postoperatively in this group of children.

**Key words:** Esophageal atresia, tracheoesophageal fistula, morphology, immunohistochemistry, neuronal disorders, dismotility.
Esophageal atresia is a severe problem of pediatric surgery, both by correcting this structural malformation and by managing its sequelae [27]. Dysfunction of the esophagus is considered a frequent finding in children diagnosed and treated surgically for various forms of esophageal atresia, the motility disorders being found in 75-80% of cases [29], their etiology remaining controversial.

Esophageal dysmotility in children with esophageal atresia causes development of gastroesophageal reflux, dysphagia, eating disorders, aspiration, these symptoms persisting into adulthood, negatively affecting the quality of life. Chronic exposure of the esophageal mucosa to the action of the acidic environment can lead to Barrett’s esophagus and esophageal carcinoma [9,10, 18,26,28].

Dysplasias of esophageal motility are classified as primary, secondary and tertiary. The primary motor disorders characteristic of esophageal atresia are the main cause of the abnormal development of muscular and nervous system (intrinsic and extrinsic innervation) of the esophagus, and these statements are supported in part by several histopathological studies [24]. Some authors believe that traumatic surgery can contribute to the esophageal dysmotility in cases of esophageal atresia due to a neurological defect caused by partial esophageal denervation [2], at the same time several studies found that abnormal innervation and neuromuscular defect of the esophagus are present before surgery [30]. Several authors have found the association of esophageal atresia with tracheoesophageal fistula with neural crest development malformations, where originates and innervation of the esophagus [21]. Some experimental studies have demonstrated significant changes in intramural nerve components of the esophagus in laboratory animals with esophageal atresia and tracheoesophageal fistula [23], some authors believing that the circumferential reduction of neuronal distribution in the proximal plexus of atresial segments may be an important contributing factor to the esophageal dysmotility observed in the esophageal atresia with tracheoesophageal fistula [4,5]. There are clinical studies that have concluded that significantly lower density of interstitial cells Cajal in esophageal atresia is an important factor for the pathogenesis of esophageal dysmotility observed in these patients [19]. However, there is little available information that would reflect the particularities of intramural nerve components of the human esophagus in cases of esophageal atresia with inferior tracheoesophageal fistula [4].

The purpose of the study was to analyze the morpho-pathological particularities and immunoreactivity of some neuronal markers of intrinsic nerve structures in the esophagus of neonates with esophageal atresia and inferior tracheoesophageal fistula.

Material and methods

Material studied for histological exploration was fragments drawn from the malformative esophagus from 17 newborns (10 boys and 7 girls) deceased with the diagnosis of esophageal atresia with inferior tracheoesophageal fistula. In 10 cases, babies were born 38-41 weeks of gestation, and 7 children were born within 36-37 weeks of gestation. Harvesting of the fragments was performed within 3 cm of the proximal and distal end of the fistula area, with a total of 114 samples taken, including: 43 in the upper atresial segment of the esophagus, 43 in the esophagus tracheal fistula and 51 – in the lower prefistular esophageal segment. In the quality of control, the samples taken from the normal esophagus (upper, middle and lower segment) from 10 newborns died at the same age of non-malformative pathologies from which 3 samples from each segment were taken.

Preventively, the samples were fixed in buffered Formaldehyde solution (pH 7.2-7.4) with the same 12-24 hour exposure time, then processed according to the histological standard using the «Diapath» automated histoprocessor and the coloring line «Raffello» (Italy), subsequently incorporated into the paraffin block, making histological sections at the SLEE MAINZ-CUT 6062 microtome (Germany) in a gradient of 4-5 μm. The sections obtained were stained using the hematoxylin-eosin (H & E), histochemical (HC) method with picrofuxin (van Gieson – VG) and immunohistochemical method (IHC).

In the IHC methodology, the sections were applied in silane histological blades. Antigen disassembly was performed at the «LG» microwave oven at 95°C. The time of unmasking varies between 10 and 20 minutes depending on the primary antibody used. Inhibition
of endogenous peroxidase was accomplished with 3% hydrogen peroxide. Primary were used monoclonal ready-to-use (Dako) primers: Anti-Neuron Specific Enolase (anti-NSE), Anti-Neurofilament Protein (anti-NFP), anti-Synaptophysyn (anti-SYP) and polyclonal anti-Chromogranin A (anti-CGA). Incubation with primary antibodies was performed according to the time stated by the manufacturer «Dako» and varied between 10 and 30 minutes. Visualization was performed using the LSAB2 Dako Cytomation) system. Cellular contraction was performed with hematoxylin. Negative control was achieved by excluding the primary antibody.

1. **Specific neuronal enolase** is a glycolytic enzyme present in neurons and mature neuroendocrine cells, thus representing a neuronal maturity index, also being a specific marker for neurons and neuroendocrine cells [1,13.]
2. Neurofilamentary proteins (NFPs) are intermediate filaments of neurons and their processes, believed to provide rigidity, traction resistance, and possibly guidance for intracellular transport in axons and dendrites. Given that NFP expression is determined exclusively in neurons, the NFP evaluation was used in the study to evaluate the presence of the mientericplexus in the esophageal segments to detect neuroglial structures [17,30].

3. Synaptophysin is a major transmembrane glycoprotein, isolated from neuronal presynaptic vesicles, present in such vesicles of the central and peripheral nervous system as well as in the vesicles of neuroendocrine cellulases, being responsible for neurotransmission. Immunohistochemical expression of synaptophysin is used in evaluating synaptic pathology in some nerve pathologies [15], including the gastrointestinal tract [8].

4. Chromogranin A (anti-CGA) is one of the major granines belonging to the family of acidic glycoproteins, which are a major component of the secretory granules of various endocrine and neuroendocrine cells of the classical endocrine glands and the diffuse neuroendocrine system, having multiple roles in the process of regulating neurotransmitters. The expression of chromogranin correlates with the amount of secretory vesicles in the neuroendocrine cells [12,30].

The microscopy of the tests in the usual staining, histochemical and immunohistochemical reaction was performed on the microscope «Micros» (Austria). Estimation of the expression of the reaction is performed by the recommended scale 0-3 +: 0 – no reaction, + weak reaction, ++ moderate reaction, +++ pronounced reaction.

The study was approved by the local ethical committee and informed consents were taken from all participants.

**Results and discussions**

Examinations performed in control samples by the conventional H & E and VG method revealed that, during the newborn, the esophagus is usually an intrinsic nervous system present in fascicles and Auerbach type ganglion-neural structures in the musculature and Meissner in the submucosa, well differentiated morpho-functional throughout. In the conventional coloration, the Aurbach nerve structures are more pronounced compared to Meissner. Intra-muscular myenteric ganglio-neuronal structures are presented by a number of 4-6 to 6-12 neurons, frequently with a well differentiated nucleotide localization and well-differentiated nucleotide cytoplasm (fig. 1A). In 20% [2] of concomitant cases, were observed ganglio-neuronal structures with unique neurons or reduced in number with differentiated neuronal cytology but with the presence of glial cellular elements (fig. 1B). These aspects were observed in the babies of the 36- and 37-week-old gestation. In 39-41 week-old babies, the glial component was absent or reduced to no more than 3-4 cells at the ganglioneuron level.

The results obtained in the control group shows that the period of 36-37 weeks of gestation there is persisting glial cell component. In our opinion, the attested features are characteristic for the morpho-functional transition period from prematurity to maturity, being a specific neuronal cytology of the norm in the course of maturation of the child.

Examination of the nerve component of trunk and ganglio-neuronal structures by conventional and histochemical methods in a fractional type of oesophageal malformations allowed to determine various modifications with significance of the morpho-functional structure and cytology of neurons. In the samples taken from the upper atresical segment, at the level of the third higher and medium, the presence of the nerve network, manifested by trunk and ganglio-neuronal structures well differentiated with features specific to the variant, analogous to those in the control group, was attested. In the lower third, especially at the apical portions of the upper atresical segment, within 1-5 cm, including in the areas with more pronounced fibrosis, the nervous system had a chaotic (intra- and intramuscular) truncated appearance, predominantly present the level of the musculature, more frequently being certified in the outer layers. At the mid-section of the proximal segment, the ganglio-neuronal component was characterized by a varied morphology on account of the polymorphic cell component containing mature neurons as well as the presence of the glial cell component with a much more pronounced control compared to the control group. The nervous network of that segment over 1.5 cm from the atresical apex has thin nerve trunks, more commonly seen in the discrete musculature area present. At this level, nerve trunks with non-neuronal monstrous ganglio-neuronal structural aspects, or the presence of neurons with unusual neuronal cytology through the extralaminar localization of neurons (fig. 2A), could be viewed at this level. In some sectors, compared to the internal muscular tunic, the external muscular tunic was represented by hypotrophic fibers or beams, being substituted by coagulated tissue or chaotically oriented miocytes, characterizing a dysplastic fibro-muscular process. The ganglion-neural structures of the myenteric network manifested a polymorphic hyper-cellularity with cytological aspects characterized by differenti-
Fig. 4. Anti-NFP expression in atresied esophageal segments (immunohistochemical reaction): A – expression of anti-NFP 3+ in the ganglion nerve plexus of the esophageal wall esofagian in the adjacent (0.5–0.8 cm) from the apical area of the upper bust; B – expression of anti-NFP 3+ in the fascicular and ganglio-neuronal nerve plexus in the median portion of the upper atresied esophageal segment. C – esophageal adventitious nerve trunks in the adjacent fistula area, anti-NFP 3+ expression; D – 3+ expression with anti-NFP is found in the lower atresia segment at a distance of 2.5 cm from the fistula; E – anti-NFP 2+ expression in the lower esophageal segment at a distance of 4.5 cm from the fistula.

Fig. 5. Anti-SYP expression determined in atresic esophageal segments (immunohistochemical reaction): A – anti-SYP2 + expression of the dysplastic ganglion network and neuro-muscular junctions in the middle portion of the upper segment; B – uniform 0/3 + anti-SYP expression in the mean portion of the upper segment; C – anti-SYP 3+ expression in the middle portion of the upper segment; D – expression of anti-SYP2 + in the vicinity of the fistula; E – expression of anti-SYP 2+ in nerve structures of the middle portion of the lower esophageal segment; F – expression of anti-SYP 3+ in nerve structures in the lower segment.
ated neurons but in pseudo-conglomerate aspects and abundance of glial cellular elements (fig. 2B).

These ganglio-neuronal features were also observed at distal atresia segment, where lower fibro-muscular dysplastic processes were observed in the mosaic aspect within 0.5–2.2 cm of the fistula. At this level ganglio-neural structures were more common, of various sizes and a more pronounced glial component. Neurons were more frequent with normal cytology. In 52.9% [9] cases were determined ganglio-neuronal abnormalities structures as: intumescent cell, nuclei of various sizes achromatic reflecting the reduced functionality features (fig. 2C).

Beginning with a distance of 2.0 cm distal from the fistula, a truncular and ganglionular network of this segment could be observed with a morphology corresponding to the norm. The reduction or lack of ganglio-neuronal structures, including neuronal network and neuronal cytological disorders, were found in the fistula section within 0.5–1.5 cm. In areas with fibromuscular dysplasia and fistula, along with trabecular disorders, ramifications, monstrous volume and cell dysplasia of ganglion-neural structures, some cytopathological changes of the neurons manifested as well as granular and vacuolar dystrophy were found (fig. 2D).

The immunohistochemical study of the tissue samples taken at the above mentioned levels determined the presence of various immunomorphological pictures depending on the structural and anatomic particularities of the segment as well as the antibody used. In 70.5% [12] of cases in the areas of the upper atresical segment, were appreciated intermuscular nerve strips lacking ganglionuclear structures not expressing the anti-NSE monoclonal antibody (fig. 3A), especially in the adjacent areas to and in the portion of the apical atresic segment. In areas distal to the apical bundle, could be found ganglio-neuronal structures with anti-NSE 3+ expression (14 cases) (fig. 3B, C).

In the lower esophageal segment, at 1-2 cm from the tracheoesophageal fistula, in 88.3% [15] cases, were found unique ganglio-neuronal structures with anti-NSE 0-2+ expression, in the majority of cases studied (fig. 3D, E). In the distal areas of the inferior esophageal segment, 3.5 cm from the fistula level, was found anti-NSE 3+ expression (fig. 3F).

The anti-NFP reactivity of the superior atresied segment was established in all cases. Even in some anti-NSE 0-1 + negative anti-NSE sites in the apical region of the upper segment or in the immediate vicinity it was found the anti-NFP 3+ expression of fascicular and ganglio-neuronal nerve plexes (fig. 4A) pronounced anti-NFP is also found in the distal portions of this segment (fig. 4B). In the lower esophageal segment, adjacent fistula region, were observed adventitious trunciular nerve fascicles with pronounced anti-NFP expression (fig. 4D). At the same time, in some distal fistula regions, could be observed a slight decrease in anti-NFP reactivity (fig. 4E).

The immunohistochemical reaction with anti-SYP revealed in some areas, along with the attestation of a dysplastic ganglio-neuronal network, the definite presence of neuro-muscular junctions (fig. 5), which could be found predominantly in the upper and middle pox of the upper atresied segment compared to the atresia area as well as in the inferior segment of the esophagus beginning 0.5-1.0 cm from the fistula.

At the tracheo-esophageal fistula level, impregnation with the primary anti-CGA antibody determined the 3+ expression of distributed neuroendocrine cells compared to other areas (fig. 6).

Dysfunction of motor activity of the distal esophagus of anastomosis in patients with esophageal atresia with tracheoesophageal fistula was first discussed in 1957 by Haight, with several aetiological factors including: the presence of abnormal nodes in the Auerbach plexus, abnormal muscle orientation in the fistula segment, presence of tracheobronchial cartilaginous reminiscences [7], these changes being documented and by us [3]. We note that impaired esophageal motility was also found in cases of congenital esophageal stenosis [14].

In the 70-90s of the 20th century, some experimental studies demonstrated that peristalsis of the esophagus is a complex process involving both extrinsic and intrinsic innervation, experimentally demonstrating that the myogenic control system of the esophagus is able to produce contractions with a rate of propagation similar to normal esophageal peristalsis independently. This system can be modulated by extrinsic and intrinsic nerves [20,25]. In this way, the myogenic mechanism acting in accordance with neuronal mechanisms would represent an additional level of control of esophageal motor [22].

Our study allowed the morphopathological peculiarities of the esophageal atresia with inferior tracheoesophageal fistula to be reviewed in newborns with results that characterized the structural changes of the intrinsic esophageal innervation in the atresied esophageal segments, including the fistula zone. In this context, some authors found that in patients with esophageal atresia with tracheoesophageal fistula, the plexus Meissner was well developed, whereas the Auerbach plexus was characterized by dysplastic changes, with the presence of rare or even lacking nodes structures [16].
Several immunohistochemical studies have found poor or negative immunoreactivity expression of neuronal markers, including neuron-specific enolase [16]. The anti-NSE 0 or 1+ expression found in some proximal areas of both atresied segments is characteristic of a neoglicogenesis deficiency [30].

Evaluation of anti-NF expression revealed a more dense, intrinsically fibrous esophageal fibrillation network in cases of esophageal atresia with inferior tracheoesophageal fistula, data similar to other studies [21]. At the same time some studies have described a diminished or even negative expression of this suggestive marker for diffuse neural diffusion [4,30].

Sinaptophysin is the most common membrane protein of the synaptic vesicles of the central and peripheral nervous system, being used as a marker of these synaptic vesicles as well as of the neuroendocrine cell vesicles. Eligable tool in the study of synaptic pathology of the nervous system, especially in the evaluation of axonal changes as well as in the diagnosis of neuronal and neuroendocrine tumors [11,15].

CgA has been used by several authors as immunohistochemical marker for neuroendocrine tissues, with a more pronounced positive immunoreactivity in the upper atresied segment compared to the lower one, as confirmed by our study. A reduced expression of CgA immunoreactivity in neural ganglia could be significant for a defective release of neurotransmitters [30].

Conclusions
The obtained results revealed severe morphological changes of the intrinsic ganglio-neuronal structures, especially in the region of the upper esophageal segment and in the area of the fistula segment. At the same time, the longer distance from the affected area and the fistula, the innervation has a more regular appearance, both in the upper and lower segments.

Diversification of the immunohistochemical expression of the studied neuronal markers represent selective modifications of intrinsic dysplastic esophageal innervation in the atresied segments and at distances, both in the upper and lower segments, which reflects common disabilities of the various links of the propagation of the nervous impulse.

Congenital morphopathological modifications of intramural ganglio-neural structures determined in both esophageal segments in cases of esophageal atresia with inferior tracheoesophageal fistula, concomitant with fibro-muscular dysplasia, may be considered as factors with significant impact on esophageal motility regulation in children with esophageal atresia with tracheoesophageal fistula, and explains within certain limits their role in esophageal dismotility found postoperatively in this group of children.

Conflict of Interest: No conflict of interest was declared by the authors.

References