



# Interstitial Cells of Cajal and Neural Structures in the Human Fetal Appendix

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## Background/Aims

The interstitial cells of Cajal (ICC) are located within and around the digestive tract's muscle layers. They function as intestinal muscle pacemakers and aid in the modification of enteric neurotransmission. The appendix's unique position requires an appropriate contraction pattern of its muscular wall to adequately evacuate its contents. We investigated the development and distribution of nervous structures and ICC in the human fetal appendix.

## Methods

Specimens were exposed to anti-c-kit (CD117) antibodies to investigate ICC differentiation. Enteric plexuses were examined using anti-neuron-specific enolase, and the differentiation of smooth muscle cells was studied with anti-desmin antibodies.

## Results

During weeks 13-14, numerous myenteric plexus ganglia form an almost uninterrupted sequence throughout the body and apex of the appendix. Fewer ganglia were present at the submucosal border of the circular muscle layer and within this layer. A large number of ganglia appear within the circular and longitudinal muscle layers in a later fetal period. The first ICC subtypes noted were of the myenteric plexus and the submucous plexus. In the later fetal period, the number of intramuscular ICC markedly rises, and this subtype becomes predominant.

## Conclusions

The ICC and nervous structure distribution in the human fetal appendix are significantly different from all other parts of the small and large intestine. The organization of ICC and the enteric nervous system provides the basis for the specific contraction pattern of the muscular wall of the appendix.

(J Neurogastroenterol Motil 2021;27:127-133)

## Key Words

Appendix; Cell differentiation; Enteric nervous system; Human; Interstitial cells of Cajal

Received: May 6, 2020 Revised: August 11, 2020 Accepted: August 24, 2020

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## Introduction

The vermiform appendix has long been perceived as a functionless vestigial organ. It is present in some lower mammal species yet absent in many mammals and appears as a well-defined organ in primates, including humans.<sup>1,2</sup> The function of the appendix is hypothesized to be an evolutionary adaptation tied to the immune system. The lymphatic tissue in the appendiceal wall has been proposed as the place of maturation and diversification of B-lymphocytes.<sup>1,2</sup>

The vermiform appendix is an intestinal derivative of the midgut, along with the small intestine (minus the upper duodenal section), cecum, ascending colon, and right half of the transverse colon.<sup>1,3,4</sup> The development of the vermiform appendix is thus closely related to that of the midgut. The bud-like complex of the early cecum and appendix appears during the eighth week of fetal life on the right side of the upper abdominal cavity.<sup>1,4</sup> The appendix can be seen in the eighth week of gestation, while the first accumulations of the lymphatic tissue develop during weeks 14 and 15 as a small cluster of lymphatic cells located below the epithelium.<sup>1</sup> Lymph nodules appear in the fourth and fifth fetal months and continue to grow until puberty.<sup>4</sup>

The interstitial cells of Cajal (ICC) are specialized cells present within and around the muscle layers of the digestive system from the esophagus to the anal canal, which function as intestinal muscle pacemakers and are involved in the modification of enteric neurotransmission.<sup>5-7</sup> Several ICC subtypes differ by their localization, morphology, and function.<sup>8</sup> Most ICC subtypes express the gene product of *c-kit*. This proto-oncogene encodes for the receptor tyrosine kinase, Kit.<sup>9</sup> This is used for their reliable identification at the light microscopic level. Peristaltic contractions of the digestive tract's muscle layer represent a complex mechanism responsible for propelling the digestive contents towards the anal opening. Interaction and synchronized functioning of the enteric nervous system (ENS), smooth muscle cells, and ICC are all necessary for normal peristalsis.<sup>10,11</sup> It has been demonstrated that disorders in the distribution and number of ICC lead to peristaltic abnormalities.<sup>10</sup> Cajal-like cells/tenocytes are present in the human digestive tract, but they do not express the *c-kit* instead they are transmembrane phosphoglycoprotein CD34 and platelet-derived growth factor receptor  $\alpha$  positive.<sup>12-14</sup> Previous research has shown that *c-kit* immunoreactive (IR) ICC present in the muscle layers of the human fetal bowel do not exhibit concurrent CD34 immunoreactivity.<sup>14,15</sup> The *c-kit* expression has been found in progenitors committed to the erythroid,

granulo-monocytic, and megakaryocytic cell lineages.<sup>16</sup>

The appendix is not situated along the main route of propagation of digestive tube contents and is therefore excluded from peristaltic propagation waves. This specific position of the appendix illustrates the need for an appropriate contraction pattern of its muscular wall so that any contents may be adequately evacuated, which prevents stasis, a potential cause of inflammation.<sup>17</sup>

A significant decrease in intramuscular ICC (ICC-IM) density in the appendix of diabetic patients was reported by Miller et al.<sup>18</sup> Zivanovic et al<sup>19</sup> and Richter et al,<sup>20</sup> observed a decrease in density of ICC-IM in inflamed appendices but without any statistical significance. In contrast, Bettolli et al<sup>21</sup> demonstrated a significant reduction of ICC-IM in inflamed appendices. This decrease was proportional to the severity of inflammation.<sup>21</sup>

Our study aims to investigate the development and distribution of nervous structures and ICC concerning muscle layers in the human fetal appendix.

## Materials and Methods

Fetal appendixes were obtained from the Clinic of Pathology, Clinical Center, Niš, Serbia, after spontaneous miscarriages, abortions for medical reasons, and premature births following fetal deaths. None of the fetuses included in this study had congenital malformation or any gastrointestinal disorders. The Ethics Committee of the Faculty of Medicine, the University of Nis (according to the Declaration of Helsinki) approved the study (Approval No. 12-519/7). The study material consisted of 23 human fetuses at 13-34 weeks of gestation (13 weeks, *n* = 2, 14 weeks, *n* = 2; 15 weeks, *n* = 2; 16-17 weeks, *n* = 3; 18-20 weeks, *n* = 3; 21-24 weeks, *n* = 3; 25-28 weeks, *n* = 2; 29-32 weeks, *n* = 3; and 33-34 weeks, *n* = 3). According to the Carnegie staging system, gestational ages were estimated by the anatomical criteria and the crown-rump length, head circumference, and foot length parameters.

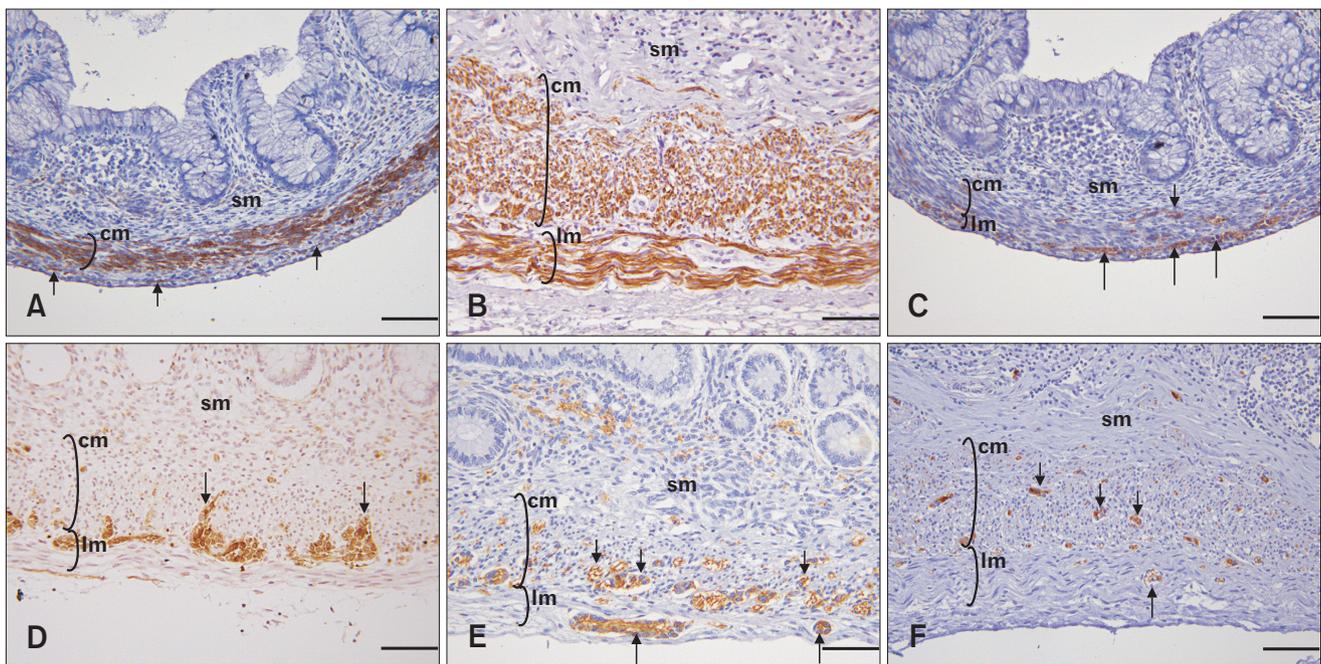
Two pieces of each fetal appendix were sampled (smaller samples were fixed as a whole), immediately fixed in 10% buffered formalin for 24 hours, routinely processed, and paraffin-embedded. The first tissue sample was taken from the appendiceal apex, always including the tip. The second sample was taken from the base of the appendix, 1-2 mm away from the cecum. The specimens were exposed to anti-*c-kit* (CD117) antibodies (monoclonal C7244, 1:300; Dako, Glostrup, Denmark) to investigate ICC differentiation. CD117 is a transmembrane protein which belongs to the class III receptor tyrosine kinase family. The differentiation of enteric neurons and smooth muscle cells was immunohistochemically ex-

ained by using anti-neuron-specific enolase (monoclonal M0873, 1:100; Dako, Glostrup, Denmark) and anti-desmin antibodies (monoclonal M0760, 1:100; Dako, Glostrup, Denmark), respectively. Two serial 4  $\mu\text{m}$ -thick sections were obtained from each paraffin block. After deparaffinization in a thermostat at 64°C in xylene and rehydration, one of these was stained with hematoxylin-eosin. At the same time, the other was pre-treated for immunohistochemical analysis, with 30 minutes heat-induced epitope retrieval in buffer pH 9.00 (EnVision FLEX target retrieval DM828 [50x], Code: K8000/K8002; Dako, Glostrup, Denmark). Endogenous peroxidase activity was blocked by a 3% solution of H<sub>2</sub>O<sub>2</sub> for 10 minutes at room temperature. The incubation of the tissue samples with the primary antibody was performed overnight at 4°C. The primary antibodies were dissolved in the Dako antibody diluent (EnVision FLEX DM830, Code: K8006; Dako, Glostrup, Denmark). After administration of secondary antibody (EnVision FLEX SM802, Code: K8000; Dako, Glostrup, Denmark) for 45 minutes at room

temperature, immune complexes were visualized using the system Dako REAL EnVision Detection System, Code: k5007 (Dako, Glostrup, Denmark). The slides were rinsed in a commercial buffer (EnVision FLEX Wash Buffer DM 831 (20x), Code: K8000/K8002; Dako, Denmark). Mayer's hematoxylin counterstained all immunolabelled sections. Microscopic analysis was performed using an Olympus BX50 light microscope equipped with a Leica DFC 295 digital camera (Leica Microsystems, Wetzlar, Germany).

### Quantitative Image Analysis

Numerical areal density of intramuscular ICC, ie, the average number of cells per  $\text{mm}^2$  of circular and longitudinal muscle layers, were determined by digital image analysis using Image J software (National Institutes of Health, Bethesda, MD, USA; available from URL: <https://imagej.nih.gov/ij/>). The developing muscle layer images were obtained on an Olympus BX 50 light microscope equipped with a digital camera (Leica Microsystems, Reuil-



**Figure 1.** Desmin immunohistochemistry (A, B) and neuron-specific enolase (NSE) immunohistochemistry (C-F). (A) Base, 15 weeks. Both circular and longitudinal muscle layers (cm and lm) are desmin immunoreactivity, but the longitudinal one is extremely thin. (B) Apex, 33 weeks. Both muscular layers are well developed, but the circular one is considerably wider than the longitudinal one. (C) Apex, 15 weeks. NSE immunoreactivity was present between the muscle layers in the form of an almost uninterrupted belt of cells (long arrows), corresponding to the myenteric ganglia. In contrast, at the submucosal border of the circular layer, there were less numerous groups of NSE-immunoreactive cells (short arrows), corresponding to submucous plexus (SMP) ganglia. Individual groups of NSE-immunoreactive cells were present within the cm as well (arrowheads). (D) Base, 18 weeks. Groups of myenteric plexus (MP) neurons extend rather deep into the cm (arrows). (E) Base, 22 weeks. Groups of neurons are rather numerous and very pleomorphic within the circular (short arrows), and slightly less numerous within the lm (long arrows). MP and SMP ganglia are also present. (F) Base, 33 weeks. Groups of neurons are present in the MP and SMP regions, and within the cm (short arrows) and lm (long arrows). sm, submucosa. Bar: A-D, F = 60  $\mu\text{m}$ ; E = 50  $\mu\text{m}$ .

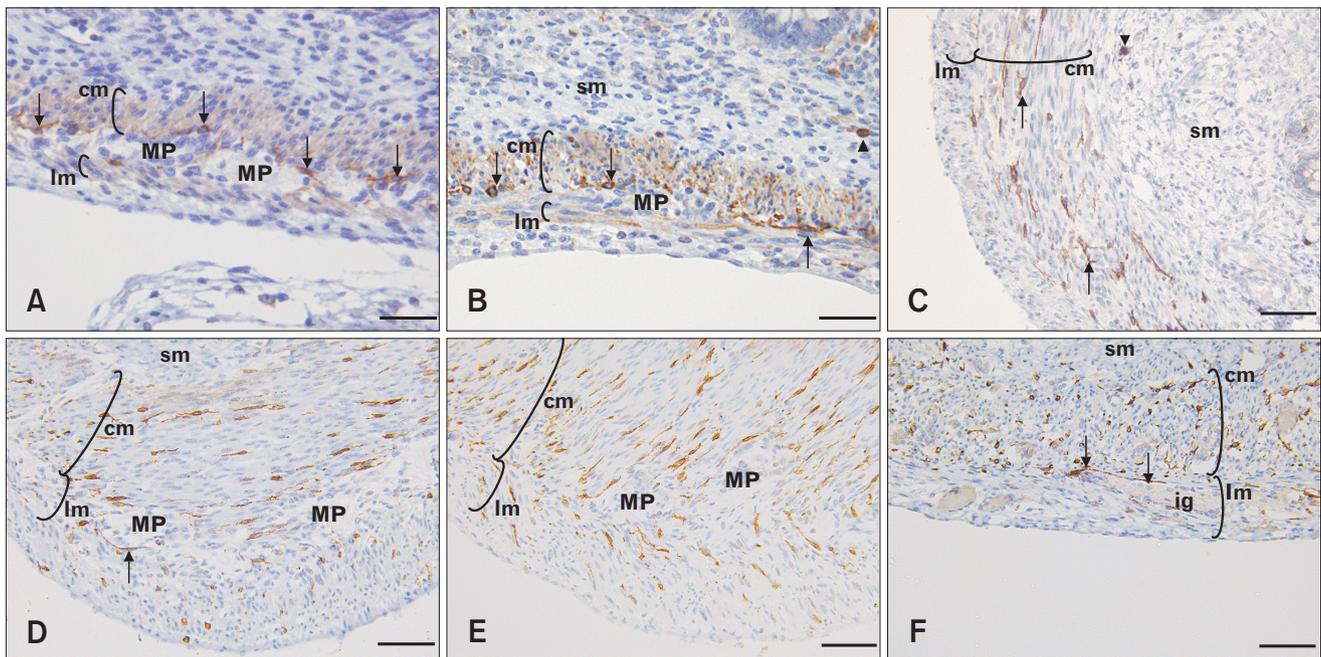
Malmaison, France) by a systematic random sampling method. Analysis of the myenteric ICC network was done by estimated ICC percentages around the myenteric plexus, ie, myenteric ICC score, the semiquantitative method proposed by Den Braber-Ymker.<sup>22</sup>

## Results

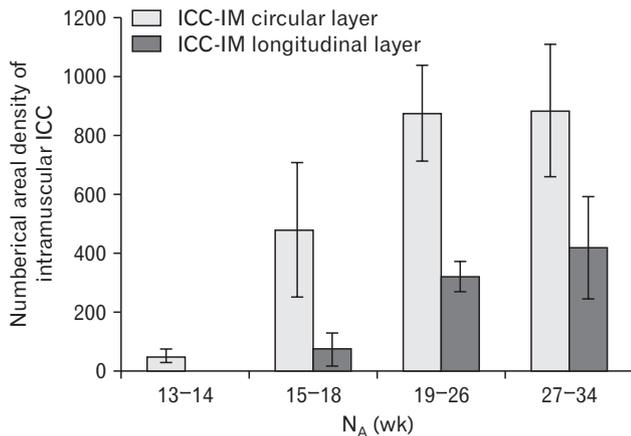
In fetuses aged 13-14 weeks, desmin immunoreactivity was present in the circular and longitudinal muscle layers of the appendiceal wall. The circular muscle layer was markedly wider than the longitudinal layer, which consisted of only 1 or 2 rows of smooth muscle cells (Fig. 1A). There were no differences in the width of the muscular layers between the apex and the base of the appendix. In older fetuses, the muscle layers were intensely labeled and visualized, but the circular layer was more extensive than any longitudinal ones (Fig. 1B).

Nervous structures were present in the appendiceal base and

apex in developmental weeks 13-14, without any differences in distribution between the base and apex. Many clustered neuron-specific enolase IR cells were situated between muscle layers at the myenteric plexus (MP) level. In contrast, markedly fewer clustered neurons were present in the circular muscle layer's submucosal border and within the circular muscle layer (Fig. 1C). During this period, clustered neurons at the MP level formed an almost uninterrupted sequence throughout the body and apex of the appendix. From weeks 15-18, clustered neurons in the MP region were less numerous and did not form an uninterrupted sequence. In contrast, the number of neuron clusters within the circular muscle layer increased significantly. Neuron clusters were present within the longitudinal muscle layer as well (Fig. 1D). The groups of neurons were different in size and shape and were distributed without any regularity within the muscle layers throughout the entire muscular wall. The neighboring groups of neurons were interconnected by nerve fibers, along which occasional individual neurons were pres-



**Figure 2.** c-kit immunohistochemistry. (A) Apex, 13 weeks (tangential cross-section). c-kit immunopositive interstitial cells of Cajal (ICC; arrows) are present at the border of myenteric plexus (MP) ganglia. (B) Base, 16 weeks. c-kit immunopositive ICC (arrows) are present around the MP ganglia and within the outer part of the circular muscle layer (cm). In the submucosa (sm), an oval c-kit-immunoreactive mast cell is seen (arrowhead). (C) Apex, 18 weeks. c-kit immunopositive ICC are present within the entire cm. Most of these cells are spindle-shaped, but some of them contain lateral branches (arrows). The c-kit-immunoreactive mast cell is seen (arrowhead). (D) Apex, 21 weeks (tangential cross-section). Numerous c-kit immunopositive ICC are present within the muscle layers. These are spindle-shaped cells oriented parallel to the longitudinal axis of the adjacent smooth muscle cells. Around the MP ganglion, there were only individual c-kit immunopositive ICC (arrow). (E) Apex, 24 weeks (tangential cross-section). c-kit immunopositive ICC are extremely numerous within the muscle layers. (F) Base, 33 weeks. c-kit immunopositive ICC are extremely numerous within the muscle layers. Two spindle-shaped c-kit immunoreactive cells (arrows) are present around the ganglion located within the longitudinal muscle layer (lm). ig, intramuscular ganglion. MoAb c-kit. Bar: A = 30  $\mu$ m, B = 40  $\mu$ m, C-E = 50  $\mu$ m, F = 80  $\mu$ m.



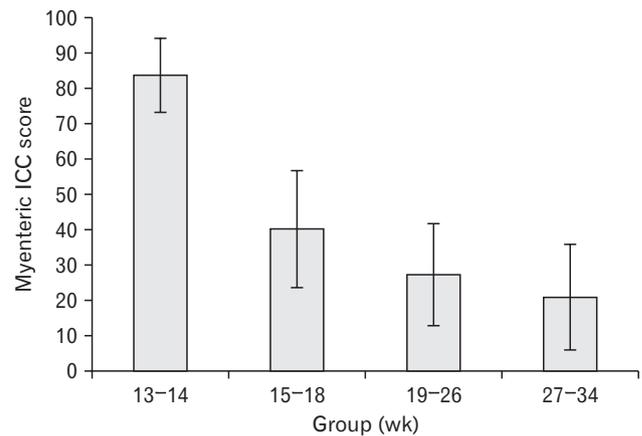
**Figure 3.** Numerical areal density of intramuscular interstitial cells of Cajal (ICC). ICC-IM, intramuscular ICC.

ent. In older fetuses (19-34 weeks of gestation), the neurons were organized similarly. The groups of neurons were numerous within muscle layers but were scarce in the MP and submucous plexus (SMP) regions (Fig. 1E and 1F).

C-kit-IR ICC were present within and around the muscle layers in the fetal appendix in all examined specimens. The distribution of c-kit-IR ICC in weeks 13-14 of development was very similar in the appendix's apex and base. ICCs were present between muscle layers and around the inception of the MP ganglia, and they corresponded to the ICC-MP. ICC-MP were most commonly multipolar, with small bodies and numerous extensions (Fig. 2A). Individual ICC were present at the submucosal border of the circular muscle layer around the inception of SMP ganglia.

In the fetuses aged 15-18 weeks, c-kit-IR ICC were present at the MP level in the form of groups of cells separated from one another. Still, many ICC were also current within muscle layers (circular and longitudinal), and they correspond to the ICC-IM (Fig. 2B and 2C). Based on their size and shape, ICC-IM were very similar to the adjacent smooth muscle cells. ICC-IMs were spindle shaped with a central elongated nucleus and were positioned in parallel with the direction of smooth muscle cells; some of them contained lateral branches (Fig. 2C). Individual c-kit-IR ICC were also present at the borders of the ganglia located within muscle layers (Fig. 2B and 2C).

In older fetuses (19-34 weeks), ICC-IM were very abundant in both muscle layers (circular and longitudinal) and were distributed following the same pattern seen in weeks 15-17 (Fig. 2D-F). In this period, a small number of ICC were present at the borders of the MP ganglia. It should be emphasized that ICC never completely encircled the MP ganglia. ICC-MP were present exclusively as individual cells, or they were instead totally absent in some



**Figure 4.** Myenteric interstitial cells of Cajal (ICC) score.

parts of this region. This specific ICC distribution was particularly visible in oblique and tangential sections involving the apex of the appendix (Fig. 2D and 2E).

In all the examined specimens, mastocytes were present, c-kit-IR as well, but clearly distinguishable by their shape, localization, and presence of granules (Fig. 2B and 2C).

The results of the numerical areal density show that there is a statistically significant difference ( $P < 0.001$ ) in the number of ICC between groups. ICC-IM are more numerous in the circular than in the longitudinal layer. In early fetuses (13-14 weeks), they are absent within the longitudinal layer because it is not yet well developed. The number of ICC-IM increases significantly with the age of the fetuses. There was no significant difference in ICC-IM between weeks 19-26 and weeks 27-34 (Fig. 3).

The myenteric ICC scores showed a decrease in the percentage of ganglion ICC-MY encirclement with increasing fetal age (Fig. 4).

## Discussion

Our results have shown significant differences in the appearance and distribution of nervous structures and ICC in the appendix related to all other parts of the gut.<sup>15,23,24</sup>

Numerous authors have demonstrated that the ENS in the appendix has a distinct pattern of organization different in comparison to other parts of the gastrointestinal tract.<sup>23,24</sup> In contrast to other small and large bowel parts, the nerves and ganglia are not concentrated between the circular and longitudinal muscle layers. Instead, they are scattered throughout both muscular layers. Hanani<sup>25</sup> reported that multiple myenteric networks exist in the muscular layer of the human appendix, but other authors have not confirmed this finding. Hanani<sup>25</sup> described the organization of MP in the form

of 3 concentric networks, with the central network placed between the muscle layers in the MP region, the internal network within the circular layer, and the external network within the longitudinal muscle layer.<sup>25</sup> Nevertheless, most authors have reported that the appendiceal ganglia were distributed both between the longitudinal and circular muscle layers and within them and were arranged in an irregular pattern.<sup>18,26</sup>

Our results showed that in an early fetal period of development during weeks 13-14, groups of neurons formed inceptions of the MP and SMP ganglia, and few groups were located within the circular muscle layer. During this development period, a similar distribution of neurons is present in the small and large intestines.<sup>15,22,24</sup> However, from 21 to 34 weeks of gestation, a large number of ganglia is evident within the circular and longitudinal muscle layers as well, distributed across the entire width of the muscular wall. The ganglia within the muscle layers differed from each other in size and shape and did not show any regular distribution. During this period of development, the ganglia in the MP region no longer formed a continuous sequence. This pattern of organization of ENS has been described in the appendix in both children and adults.<sup>18,25,26</sup>

A common characteristic of the small and large intestine is that the ICC subtypes with pacemaker function (ICC-MP in the small and ICC-MP and ICC-SMP in the large bowel) are the first to appear. These subtypes maintain their presence until the end of the fetal period and later during life, with an identical distribution pattern (they completely encircle ganglia).<sup>10,15,23,24,27</sup> ICC-IM appear in the late fetal period, and their differentiation continues after birth. ICC-IM are abundant within the sphincters, cardia, and pylorus and are less numerous in other parts of the gut.<sup>28-30</sup>

The model of distribution of ICC in the fetal appendix is distinct from other parts of the gut. The first ICC subtypes to appear are ICC-MP and ICC-SMP; with ICC-MP being very abundant in weeks 13-14, forming long sequences of cells and surrounding MP ganglions. In the period from 15 to 18 weeks, ICC-IM appear first in the circular and then within the longitudinal muscle layer, while the number of ICC-MP decreases at the same time.

In latter fetal period, from 19 to 34 weeks, the number of ICC-IM markedly rises in both muscle layers, and this subtype becomes predominant. In contrast, the number of ICC-MP and ICC-SMP significantly decreases. Individual, spindle-shaped ICC also appear in the borders of ganglia situated within the muscle layers. ICC-IM have a role in neurotransmission, although they have been proposed to function as stretch receptors as well.<sup>11,31,32</sup> Earlier studies have shown that ICC-IM are particularly numerous in the lower esophageal sphincter and stomach cardia. This distribution pattern

is established as early as the fifth month of fetal development.<sup>33,34</sup> Numerous ICC-IM have also been similarly distributed in the pyloric sphincter.<sup>28,29</sup> Previous data suggest that loss of the ICC-IM may interfere with normal motility in these sphincters.<sup>29,30</sup> The results of our study show that ICC distribution in the human fetal appendix is very similar to ICC distribution in the muscle sphincters present in the digestive tract (cardia and pylorus). Such a finding is in agreement with the fact that the appendix is excluded from the propagation of peristaltic contractions and also with the hypothesis that its muscular layer follows a different contraction pattern. Therefore, ICC-IM are probably responsible for the contraction of appendiceal muscular layers in situations when the appendix's lumen is filled with bowel contents that induce stretching of the wall. Contractions thus cause emptying of the appendiceal lumen and prevent stasis.

This particular organization of ENS and ICC enables the appendix to serve its specific function. Moreover, due to its location and length, it has been hypothesized that the appendix represents a microbiological reservoir from which the gut flora can be regenerated in situations when it becomes disturbed or disordered, such as after extended antibiotic use or colitis.<sup>35</sup>

In summary, the ICC and nervous structure distribution in the human fetal appendix are significantly different from all other parts of the small and large intestine. The particular organization of ICC and ENS provides the basis for the specific contraction pattern of the muscular wall of the appendix.

**Financial support:** This work was supported by the Internal project of Medical Faculty of Nis (Grant No. 38/20).

**Conflicts of interest:** None.

**Author contributions:** Goran Radenkovic: conception and planning the study, interpreting data, and drafting the manuscript; Vladimir Petrovic: conducting the study and interpreting data; Dragoljub Zivanovic: collecting and interpreting data; Nenad Stoiljkovic: conducting the study and drafting the manuscript; Dusan Sokolovic: conducting the study; Nikola Zivkovic: collecting data; Dina Radenkovic: planning the study and drafting the manuscript; Aleksandra Velickov: interpreting data; and Jovana Jovanovic: collecting data.

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