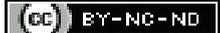


# A Histomorphometric Analysis of Interganglionic Distance in the Myenteric Plexus of the Entire Large Intestine of Aborted Human Foetuses: A Cross-sectional Study

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## ABSTRACT

**Introduction:** The Enteric Nervous System (ENS) contributes to the innervation of the gut and is considered the second brain in the gut. It is a part of the peripheral nervous system and the ganglionated plexus (GP) of enteric neurons, present in the layers of the digestive tract, is more complex than any other structure of the peripheral nervous system. The establishment of a valid standard method for quantitative analysis of the myenteric plexus has been a topic of debate among enteric neuropathologists and is essential for better characterisation of patients with gut dysmotility.

**Aim:** To determine the histomorphometric analysis of the interganglion distance of the myenteric plexus in the entire large intestine of aborted human foetuses.

**Materials and Methods:** A cross-sectional descriptive study was carried out in the Department of Anatomy at Pondicherry Institute of Medical Sciences, Pondicherry, India. The study duration was eight years and seven months, from October 2014 to May 2022. A total of 50 aborted human foetus samples were collected from Rajiv Gandhi Government Women and Children's Hospital, Puducherry, India, from the Department of Obstetrics and Gynaecology. The age of the aborted human foetuses was

determined based on Biparietal Diameter (BPD) and Crown Rump Length (CRL) and correlated with clinical history. The aborted human foetuses were assigned to two groups: group A (n=29),  $\leq 20$  Weeks of Gestation (WG), and group B (n=21),  $\geq 20$  WG. Nicotinamide Adenine Dinucleotide Phosphate (NADPH) diaphorase histochemistry, an enzyme histochemistry technique, was used to study the tissue preparations. Statistical significance was determined using Statistical Package for Social Sciences (SPSS) version 20.0, International Business Machines (IBM) corporation. The independent sample t-test was conducted for parametric analysis.

**Results:** The interganglion distance was measured from the caecum (A1 segment) to the anal canal (A7 segment) in aborted foetuses of less than 20 WG. The mean values ranged from  $78.86 \pm 38.09 \mu\text{m}$  to  $126.94 \pm 23.87 \mu\text{m}$  in foetuses of less than 20 WG. The mean values ranged from  $66.41 \pm 8.05 \mu\text{m}$  to  $107.67 \pm 30.17 \mu\text{m}$  in aborted human foetuses of more than 20 WG.

**Conclusion:** The anal canal showed a long interganglion distance. The average reference values were found to be helpful as a diagnostic tool for identifying disorders related to gut innervation and motility.

**Keywords:** Aganglionosis, Enteric neurons, Ganglionated plexus, Nervous system

## INTRODUCTION

The large intestine is derived from the midgut and hindgut, extending from the caecum to the anal canal. The mucosa is derived from the endoderm, while the muscularis externa is derived from the mesoderm. The enteric nervous system contributes to the innervation of the gut and is considered the second brain of the gut [1]. The enteric neurons of the ganglion plexus present in the layers of the digestive tract are more complex than any other structure of the peripheral nervous system [2]. The enteric nervous system originates from Neural Crest Cells (NCC) [3,4]. NCCs migrate into the Gastrointestinal (GI) tract, proliferate, and differentiate into neurons and glial cells [3]. There are two types of NCCs: Vagal NCCs and Sacral NCCs. These cell types independently innervate the gut. Vagal crest cells are highly invasive compared to sacral NCCs [3]. Neuroblast cells are responsible for the development of the intramural plexus in the gut, progressing in a craniocaudal direction [5-7]. The incorporation of neuroblast cells ceases when the process reaches the distal end of the rectum at 12 WG. If this process is interrupted or halted at any stage on or before 12 WG, the intramural plexus will disappear in that segment of the GI tract [8]. In 1954, Yntema and Hammond demonstrated that most enteric neurons were derived from rhombencephalic (vagal) NCCs [3,9]. Almost 20 years later, in 1973, Burns AJ and Douarin NM mapped

the precise location of the enteric nervous system within the NCCs, facilitated by the development of embryonic cell marking techniques in the 1960s [3,9].

The NADPH diaphorase could be a beneficial and useful method for histopathological investigations of neurogenic disorders of the digestive tract due to the clear visualisation of soma and processes of neurons [10]. The Nitric Oxide (NO) signaling pathway is a major nonadrenergic, noncholinergic inhibitory transmitter mechanism in the ENS. The enzyme responsible for NO generation is Nitric Oxide Synthase (NOS), and NADPH diaphorase is identical to NOS activity in neurons [11]. The myenteric ganglia stained strongly with NADPH diaphorase. NADPH diaphorase histochemistry not only accurately identifies myenteric ganglia from the ganglionic portions of the bowel but also demonstrates innervation abnormalities in smooth muscles. Histochemical staining of seromuscular biopsy with NADPH diaphorase provides 100% diagnostic accuracy regarding the extent of aganglionosis [10]. The NADPH diaphorase histotechnique accurately identifies sparse myenteric ganglia and hypertrophic nerve trunks in the hypoganglionic transitional zone. In early gestational weeks, enteric neurons are present in scattered form and gradually appear in ganglionated form as the gestational week advances. In the early weeks of gestation, these neurons are oval in shape and located in the serosa layer of the large intestine, while in the later stages of gestation,

they become elongated and are found in the muscular and submucosa layers of the large intestine [12]. The myenteric ganglia exhibit diversity in size, shape, structure, and number of neurons present. The available intermuscular space contributes to the appearance of different shapes, sizes, and numbers of ganglia. Myenteric ganglia typically have flat, irregular, and angulated outlined borders in longitudinal sections [13]. The number of ganglia and ganglion cells in the ENS is not uniform and varies in different locations, depending on various factors. Therefore, the location of the biopsy site and the thickness of the section are important for quantitative analysis of nerve plexuses [4]. Morphometric measurements of ENS components are valuable as a diagnostic tool for identifying disorders related to gut innervation [14, 15]. The literature reveals that many studies on the myenteric plexus have been descriptive in nature, with only a few focusing on a quantitative approach [12, 15-17]. The establishment of a valid standard method for quantitative analysis of the myenteric plexus has long been debated among enteric neuropathologists and is essential for better characterisation of patients with gut dysmotility [4, 15]. Hence, the present study aimed to determine the normal values of quantitative parameters, such as the interganglion distance of the myenteric plexus in the entire large intestine of aborted human fetuses.

## MATERIALS AND METHODS

A cross-sectional descriptive study was conducted in the Department of Anatomy at Pondicherry Institute of Medical Sciences, Pondicherry, India. The study duration was eight years and seven months, from October 2014 to May 2022. The total sample size consisted of 50 human aborted fetuses. All human fetuses were collected in accordance with the approved protocol by the Ethics committee. IEC letter number is GHIEC/37/2018 & IEC : RC/14/84.

**Inclusion criteria:** Pregnant mothers without any medical illnesses during pregnancy, and fetuses without any congenital anomalies were included in the study.

**Exclusion criteria:** Foetuses with neural tube defects were excluded from the study.

**Sample size calculation:** Sample size calculated as per the formula:  $N = Z^2 P(1-P)/d^2$ .

### Study Procedure

The fetuses were divided into two groups: group A (n=29)  $\leq 20$  WG and group B (n=21)  $> 20$  WG. Foetal age was determined using parameters such as CRL, foot length, and BPD, in relation to clinical history. Most of the fetuses used in the present study were obtained within 2-3 hours after delivery. A paramedian incision was made in the abdomen to expose and properly fix the large intestine using 4% buffered paraformaldehyde [Table/Fig-1]. The fixed specimens were preserved at 4°C to minimise postmortem changes.

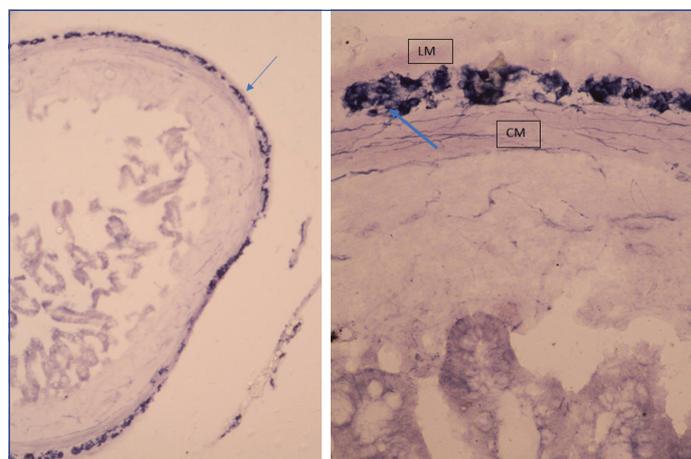


**[Table/Fig-1]:** Paramedian incision over the abdomen of the sample.

The NADPH diaphorase histochemistry, an enzyme histochemistry technique, was used to study the tissue preparations [5]. The entire large intestine was divided into seven segments: A1 - caecum, A2

- ascending colon, A3 - transverse colon, A4 - descending colon, A5 - sigmoid colon, A6 - rectum, and A7 - anal canal. The colonic tissue was fixed in fresh 4% buffered paraformaldehyde for two hours at 4°C. After fixation, the samples were thoroughly washed in chilled 0.1 M phosphate buffer. Cryoprotection was achieved by immersing the samples in 15% and 30% sucrose solution for three hours and eight hours, respectively, at 4°C. The samples were then frozen in an optimum cutting temperature compound, and 20  $\mu$ m thick sections were cut using a Leica cryostat. The frozen sections were mounted on 1% gelatine-coated slides and stored at -20°C for enzyme histochemistry. The cryostat sections on glass slides were washed multiple times with 0.1 M phosphate buffer. NADPH diaphorase activity was observed by incubating the sections in 10 mL of 0.1 M Tris hydrochloride (Tris Cl) buffer containing 10 mg NADPH, 1 mg of Nitroblue Tetrazolium (NBT), and 0.3% triton X100 at 37°C for 45 minutes to one hour in the dark.

The reaction was observed under a dissecting microscope and terminated by gently washing the tissues with chilled 0.1 M phosphate buffer when the stain was sufficiently intense. The sections were mounted in a mixture of glycerol and phosphate buffer. The stained sections were examined under a microscope, and images were captured using a camera attached to a binocular microscope. The images were saved in Joint Photographic Experts Group (JPEG) format with minimum compression and maximum quality [Table/Fig-2]. The images taken under 40x magnification [Table/Fig-3] were analysed using image J software version 1.53 t (developed at the US National Institute of Health (NIH), available at <https://imagej.nih.gov/ij/>) [Table/Fig-3] [18]. Before taking measurements, the system was calibrated using a micrometer scale for the magnification at which the images were captured. The interganglion distance in all segments of the large intestine was measured and used for analysis.



**[Table/Fig-2]:** Myenteric plexus in the muscular layer (10x) view (blue arrow).  
**[Table/Fig-3]:** Myenteric plexus (40X) (blue arrow). (Images from left to right)  
CM: Circular muscle, LM: Longitudinal muscle

## STATISTICAL ANALYSIS

Statistical significance was determined using SPSS version 20.0, developed by the International Business Machines (IBM) Corporation, Armonk, New York, United States of America. The data were expressed as mean  $\pm$  SD. The independent samples t-test was conducted for parametric analysis. A p-value of  $< 0.05$  was considered statistically significant.

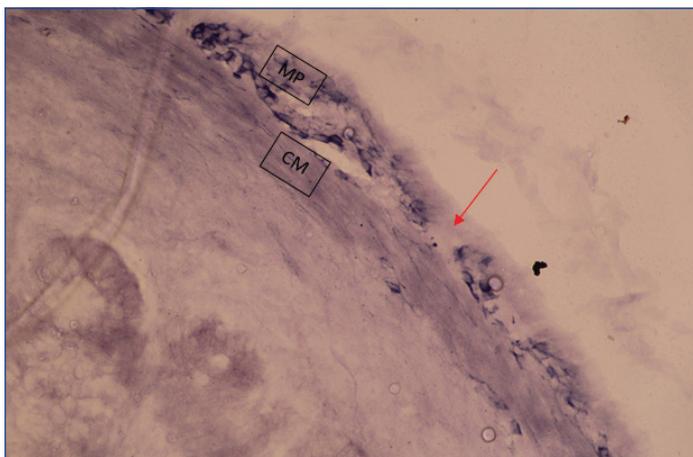
## RESULTS

The interganglion distance was measured from the caecum (A1 segment) to the anal canal (A7 segment) in aborted fetuses less than 20 WG. The mean values ranged from  $78.86 \pm 38.09 \mu$ m to  $126.94 \pm 23.87 \mu$ m. In aborted fetuses of more than 20 WG, the mean values ranged from  $66.41 \pm 8.05 \mu$ m to  $107.67 \pm 30.17 \mu$ m. The p-value was significant in the rectum part of the large intestine

Groups	Caecum/A1 segment (µm)	Ascending colon/A2 segment (µm)	Transverse colon/A3 segment (µm)	Descending colon/A4 segment (µm)	Sigmoid colon/A5 segment (µm)	Rectum/A6 segment (µm)	Anal canal/A7 segment (µm)
Group A ≤20 weeks (n=29)	85.58±29.20	87.71±32.64	87.15±10.40	96.62±8.98	93.27±31.83	78.86±38.09	126.94±23.87
Group B >20 weeks (n=21)	69.38±9.96	93.85±18.90	66.41±8.05	70.03±19.97	70.31±20.10	83.20±26.73	107.67±30.17
p-value	0.094	0.113	0.206	0.423	0.155	0.003	0.865

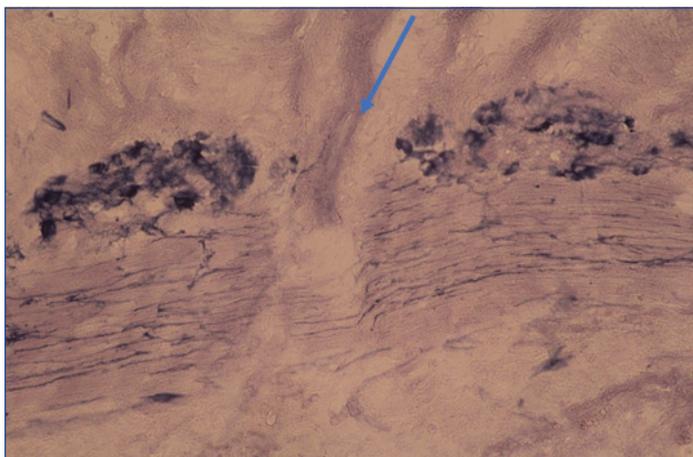
**[Table/Fig-4]:** Interganglion distance (µm) from the caecum to the anal canal of the large intestine of aborted fetuses of less than 20 weeks and more than 20 weeks of Gestation (WG) period.

[Table/Fig-4]. The interganglion distance was greater in fetuses less than 20 WG, indicating that the ganglia were not properly developed and were spaced further apart. In contrast, the interganglion distance was shorter in group B compared to group A, indicating the presence of grouped and matured ganglia in the intermuscular space. This suggests the maturity of ganglion cells. Both short and long interganglion distances were observed [Table/Fig-5,6].



**[Table/Fig-5]:** Short interganglion distance (red arrow) (40X) NADPH diaphorase staining method.

CM: Circular muscle; MP: Myenteric plexus



**[Table/Fig-6]:** Long interganglion distance in muscular layer of the large intestine (40X) (blue arrow).

## DISCUSSION

Quantitative morphometric measurements of ENS components are highly valuable as a diagnostic tool for identifying gut innervation disorders [14, 15]. The present study is one of the few studies that aimed to establish morphometric measurements of the myenteric plexus in the large intestine. Kim HK et al., conducted a study on paediatric patients with intestinal pseudo-obstruction to examine changes in the structural components of the ENS [14]. They studied 19 paediatric patients with clinical symptoms of intestinal pseudo-obstruction who underwent surgical treatment, as well as eight cases in the control group. The number, area, and distribution of myenteric ganglia, as well as the size, maturation, and number of

ganglion cells within the myenteric ganglia, were identified. They found a significant difference in plexus area/mm between the two patient groups ( $15.37 \pm 2.49$  vs  $20.35 \pm 27.35$ ,  $p=0.038$ ) and between the hypoganglionosis group and the control group ( $15.37 \pm 2.49$  vs  $25.43 \pm 7.53$ ,  $p=0.008$ ). The mean interganglion distance in the hypoganglionosis group was greater than that in the control group ( $153.01 \pm 46.96$  vs  $90.77 \pm 36.07$ ,  $p=0.035$ ) and was not significantly different between the two patient groups ( $153.01 \pm 46.96$  vs  $136.74 \pm 29.54$ ,  $p=0.914$ ). They suggested that the difference in the results of statistical analysis of findings in the myenteric plexus depends on the location of the lesion. The plexus area/mm ( $103 \times \text{mm}^2$ ), mean ganglion area ( $103 \times \text{mm}^2$ ), mean ganglion length, and mean ganglion distance (mm) were calculated. The comparative study with control sections suggested that location-matched reference values played an important role in diagnosing pathological changes in the myenteric plexus of the gut.

Bhattarai C et al., conducted a study on the histomorphology of enteric neurons and enteric ganglia in different layers of the human fetal colon [12]. They collected stillbirth and spontaneously aborted fetuses ( $n=36$ ) using the convenience sampling method. The fetuses were grouped according to Sturge's rule into eight groups: 10-14, 14-18, 18-22, 22-26, 26-30, 30-34, 34-38, and  $\geq 38$  weeks. They reported that the distance between the enteric ganglia increased from  $3.12 \mu\text{m}$  to  $49.4 \pm 26.58 \mu\text{m}$  in the serosa and from  $10.2 \pm 6.29 \mu\text{m}$  to  $96.1 \pm 18.01 \mu\text{m}$  between the muscle layers, while in the submucosa, it fluctuated as the gestational age progressed. In the present study, the mean value of the interganglion distance between the muscle layers ranged from  $78.86 \mu\text{m}$  to  $126.94 \mu\text{m}$  in aborted fetuses less than 20 WG, and the mean value ranged from  $66.41 \mu\text{m}$  to  $107.67 \mu\text{m}$  in aborted fetuses more than 20 WG.

Subramanian H et al., conducted a prospective cross-sectional comparative study on the morphometric profile of large intestinal neuronal plexuses in normal perinatal autopsies and Hirschsprung Disease (HD) [19]. The study took place in the Department of Pathology and Pediatric Surgery, JIPMER, Pondicherry, India, from October 2013 to September 2015. The study included 40 subjects in each of the following groups: i) non Hirschsprung perinatal group, consisting of neonates and stillborn babies beyond 30 WG, and ii) HD group. The samples were sectioned at seven levels: a) 1 cm from the anal verge, b) mid rectum, c) rectosigmoid junction, d) mid-sigmoid, e) descending colon, f) transverse colon, and g) ascending colon. The interganglion distance was reported as an average value of  $136.49 \pm 39.0 \mu\text{m}$ , with a statistically significant shorter distance between ganglia in the rectum, sigmoid, and descending colon compared to the rest of the intestine in the non Hirschsprung perinatal group. The rectum was the most affected site of the aganglionic zone in HD. There was an increased interganglion distance in the transition zone compared to the normal zone in the Hirschsprung disease group. The present study showed statistically significant and shorter distances in the rectum. Various studies have revealed a range of interganglion distances, with a minimum of  $10.2 \pm 6.29 \mu\text{m}$  and a maximum of  $136.49 \pm 39.0 \mu\text{m}$  [Table/Fig-7] [12,14,19].

Studies	Place and year of the study	Interganglion distance in the muscle layer ( $\mu\text{m}$ )
Kim HK et al., [14]	Department of Pathology, Ewha Medical Research Institute, Korea, 2010	93.72 $\pm$ 34.42 (51.12-151.81)
Subramanian H et al., [19]	Department of Pathology and Paediatric Surgery, JIPMER, India, 2015	136.49 $\pm$ 39.0
Bhattarai C et al., [12]	Department of Anatomy, Manipal Academy of Higher Education, Manipal, India and Pokhara, Nepal, 2021	10.2 $\pm$ 6.29 to 96.1 $\pm$ 18.01
Present study (less than 20 weeks)	Department of Anatomy and Pathology, India, 2022	78.86 $\pm$ 38.09 to 126.94 $\pm$ 23.87
Present study (more than 20 weeks)	Department of Anatomy and Pathology, India, 2022	66.41 $\pm$ 8.05 to 107.67 $\pm$ 30.17

**[Table/Fig-7]:** Comparative studies on the interganglion distance in the muscular layer of the large intestine [12,14,19].

## Limitation(s)

The sample size was inadequate, and the availability and procurement of third-trimester samples were difficult.

## CONCLUSION(S)

The anal canal exhibited a long interganglion distance. Average reference values are highly valuable as a diagnostic tool for identifying disorders related to gut innervation and motility. Location-matched reference values for different segments of the large intestine are crucial for accurately diagnosing gut motility-related disorders.

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