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RESEARCH ARTICLE OPEN ACCESS

COMPARISON OF THREE DIAGNOSIS TECHNIQUES OF HIRSCHSPRUNG DISEASE

[1]Cristianne Confessor Castilho Lopes, [2]João Vitor Bertuci, [2]Eduardo Barbosa Lopes, [3]Lucas Castilho Lopes, [2]Vanessa da Silva Barros, [2]Laisa Zanatta, [2]Daniela dos Santos, [4]Marilda Morais da Costa, [5]Tulio Gamio Dias, [6]Ana Paula de Oliveira Barbosa, [2]Werlys Araujo Ribeiro, [2]Eliana Rezende Adami, [2]Ana Julia Tibola, [2]Angelica Zardo, [2]Liamara Basso Dala Costa, [7]Fabio Kopp Vanuzzi, [2]Heliude de Quadros e Silva and [8]Youssef Elias Ammar

[1]University of the Region of Joinville - Joinville – SC; [2]High Valley University of Rio do Peixe - Caçador – SC; [3]Federal University of Santa Catarina - Florianópolis – SC; [4]Lutheran Educational Association - Faculty IELUSC - Joinville – SC; [5]USP School of Arts, Sciences and Humanities – São Paulo – SP; [6]University of São Paulo – São Paulo – SP; [7]Federal University of Rio de Janeiro - Rio de Janeiro – RJ; [8]University of the South of Santa Catarina – Tubarão – SC

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***Corresponding author:**
PASQUALOTTO, Raquel

ABSTRACT

Hirschsprung disease (HD) is a congenital disease caused by the absence of ganglion cells in the large intestine leading to functional obstruction and colonic dilatation. This work focuses on showing the scientific evidence on the evaluation of two diagnostic techniques of rectal biopsy and compares the advantages and disadvantages of each. Bibliographic research was performed using Scielo, Medline, and academic Google, related to Hirschsprung disease, Immunohistochemistry Calretinin (IHC), and Histochemistry Acetylcholinesterase. The articles were chosen for their relevance on the topic, for their advantages and disadvantages mentioned between the two techniques that have been mentioned, and related to the variants that manifest the pathology. We observed that in normal people, acetylcholinesterase (AChE) activity is minimal or nil, butin patients with HD, an increase in enzyme activity was observed in the layers of the large intestine.Furthermore, histochemical staining can produce a dark brown positioning in fibers with AChE activity on histopathological analysis (positive pattern of HD). In turn, immunohistochemistry uses calretinin. In the ganglionic parts of the intestine, there is an expression of calretinin in the submucosa, and in the aganglionic parts, there is none. Therefore, we conclude that the IHC has more advantages and greater or equivalent diagnostic accuracy than the Histochemical technique with AChE. In turn, immunohistochemistry uses calretinin. In the ganglionic parts of the intestine, there is calretinin expression in the submucosa, and in the aganglionic parts, there is none. Therefore, we conclude that the IHC has more advantages and greater or equivalent diagnostic accuracy than the Histochemical technique with AChE. In turn, immunohistochemistry uses calretinin. In the ganglionic parts of the intestine,calretinin is expressed in the submucosa, and in the aganglionic parts, there is none. Therefore, we conclude that the IHC has more advantages and greater or equivalent diagnostic accuracy than the Histochemical technique with AChE.

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INTRODUCTION

Hirschsprung's disease (HD) is a disease of the large intestine (colon) caused by the absence of parasympathetic ganglia in the intestinal wall from birth (congenital) and manifestsas problems with bowel movements. It was first described in 1888 in two babies by Copenhagen pediatrician Harald Hirschsprung. HD has an estimated annual incidence of 1/5,000 births, and in mild cases, the condition is found later in childhood.In rare cases, Hirschsprung'sdisease is first diagnosed in adulthood. Treatment consists of surgical bypass or removal of the diseased part of the colon(PAREJA et al., 2021). The

standard methodology for diagnosing the disease is a rectal biopsy, which identifies the absence of ganglion cells and an increase in the number of acetylcholinesterase positive nerve fibers (RABAH, 2010). The anatomical trajectory zone between the distal aganglionic portion and the proximal ganglionic portion admits specifying HD in three ways: 1) classic - in which the aganglionic segment elongates to the proximal sigmoid; 2) with a long segment, in which the aganglionosis affects the splenic flexure or transverse colon; 3) total colonic aganglionosis (DHACT) - in which the aganglionic segment extends from the anus to, at most, 50 cm proximal to the ileocecal valve. DHACT has clinical, histological, and genetic differences and is associated with diagnostic and management adversities (MOORE, 2015). The classic form occurs in 7%-88.8% of cases, the long-form in 3.9%-23.7%, and DHACT in up to 12.6% of patients (LAUGHLIN; FRIEDMACHER; PURI, 2012). Surgical therapy in HD reduces the complications of intestinal obstruction in cases where the aganglionic segment is completely resected. However, there is resistance to post-surgical intestinal dysmotility in some patients, most often demonstrated by chronic constipation and recurrent events of enterocolitis. Different histopathological findings can be presented in these cases, such as incomplete resection of the aganglionic portion, hypoganglionosis, and neuronal intestinal dysplasia superimposed on the aganglionic zone (MOORE, 2015). This study compares two methods that help verify the diagnosis ofHirschsprung'sdisease: histochemistry with acetylcholinesterase and immunohistochemistry with calretinin. We will revieweach of the techniques' characteristics, advantages, and disadvantages through a bibliographic review of scientific sources with less than five years of publication.

THEORETICAL FOUNDATION

Etiopathogenesis of Hirschsprung Disease: The embryological origin of HD occurs in the 4th week of development in the hindgut of the primitive bowel and is due to the absence of parasympathetic ganglia in the bowel wall. These ganglia are derived from neural crest cells that migrate from the neural folds to the intestinal wall. In addition, mutations in the Ret proto-oncogene (RET gene), a receptor tyrosine kinase involved in crest cell migration, can produce congenital megacolon. In most events, the defect extends to the midpoint of the sigmoid colon (SADLER, 2021; SCHOENWOLF et al., 2016). Hirschsprung'sdisease is a complex disease. It is a multifactorial, polygenic, autosomal dominant cause, but with incomplete penetration depending on sex, and its expressiveness varies according to the aganglionic segment. Previous studies found alterations in chromosomes 2, 10, and 13 were identified, and nine genes related to HD were described, the main ones being: RET, Glial Cell Derived Neurotrophic Factor (GDNF), Neuturin (NTN). However, we still don't have much evidence about the mechanisms of these genes in the formation of the disease (JIMÉNEZ; FERNÁNDEZ, 2018). Histologically, a congenital absence of ganglion cells is observed in the Auerbach's myenteric plexus and Meissner's submucosa, rectal wall, and other sections of the colon in the proximal direction at a variable distance and even from the small intestine.It also presents with hyperplasia of cholinergicnerve fibers lines in the circular muscle layer, mucosal muscle, and mucosa, with high acetylcholinesterase activity at the same level as the aganglionic zone (LOURENÇÃO, 2012).

Clinical Manifestations: It is a disease characterized by severe constipation accompanied by dilation of the colon, without a mechanical obstruction to justify constipation (FRYKMAN; SHORT, 2012). However, when congenital aganglionosis occurs in the intestine segment, there are changes in propulsive motility patterns mediated by neurons in the enteric nervous system, and the intestine remains paralyzed, causing a functional obstruction (HEUCKEROTH, 2018). Proliferative obstructive colitis, also called enterocolitis, can occur in 11 to 24% of newborns with Hirschsprung'sdisease (FRYKMAN; SHORT, 2012). A subgroup of patients with HD - those who do not have an abrupt change in caliber between the healthy segment and the affected segment - manifest only chronic constipation, which makes diagnosis difficult (FITZGERALD, 1998). Symptoms of Hirschsprung'sdisease include constipation, fever, diarrhea, vomiting, bloating, failure to thrive,and inability to eliminate meconium in the first week of life, which supports its congenital origin (FITZGERALD, 1998; HEUCKEROTH, 2018). In addition, the untreateddisease often causes death in childhood because bacterial bloodstream infections occur in the context of intestinal inflammation or bowel perforation (HEUCKEROTH, 2018). Patients with HD are predominantly male (84%) and usually have fecal colonic impaction duringan abdominal examination. On digital rectal examination, manualdisimpaction can be explosive with emptying in 96% of patients (FITZGERALD, 1998).

Diagnosis: Due to the evolution and distinction of diagnostic parameters, the detection phase of Hirschsprung's Disease has significantly decreased in recent times (HARICHARAN; GEORGESON, 2008). Therefore, according to the clinical history and the objective exam, most identifications are made in the neonatal phase (BUTLER TJADEN; TRAINOR, 2013) and complemented by other methods and exams. Rectal biopsy is the best diagnostic test available, both for exclusion and confirmation of the diagnosis (ALEHOSSEIN et al., 2015; DE LA TORRE; LANGER, 2010). The most regularly used methodology for the investigation of the biopsy fragment is staining with hematoxylin and eosin. However, finding ganglion cells or demonstrating their absence demands considerable histological cuts, which, in addition to being a slow methodology, are prone to failures. For this reason, new observation tools supplementing the standard histology have been inserted to contribute to the diagnosis (ANBARDAR; GERAMIZADEH; FOROUTAN, 2015). In addition, hypertrophied nerve trunks can also be found in the submucosa (ALEXANDRESCU; ROSENBERG; TATEVIAN, 2013), which correspond to extrinsic parasympathetic preganglionic nerves, whose fiber quantity is high in the aganglionic segment. Another method implemented was the immunohistochemical staining of calretinin, which shows, by the presence of this protein, the ganglion cells, and nervous plexuses, in a normal rectum (ALEXANDRESCU; ROSENBERG; TATEVIAN, 2013). This coloration in the bowel wall is a peculiarity of HD (DE LA TORRE; LANGER, 2010). Although some research shows doubtful results concerning the use of this methodology, it has been appropriately presented and valuable, with superior diagnostic values, compared to the use of hematoxylin and eosin staining (ANBARDAR; GERAMIZADEH; FOROUTAN, 2015). Although intestinal biopsy is the gold standard of investigation, initially, the complementary inquiry is the use of opaque enema (ALEHOSSEIN et al., 2015; BUTLER TJADEN; TRAINOR, 2013). It is a test performed with barium, which investigates the appearance and location of the transition zone (a zone in which there is a significant divergence in diameter between the dilated and non-dilated intestine). It may even exhibit irregular colonic contractions, irregular mucosa (suggesting enterocolitis), and an abnormal rectosigmoid sign (ratio between the largest diameter of the rectum and the largest diameter of the sigmoid, which is classified as abnormal if <1). However, severalpublications have registered that in about 10% of newborns with HD, the opaque enema does not show a transition zone, and in children who have a significantly reduced aganglionic segment (ALEHOSSEIN et al., 2015).

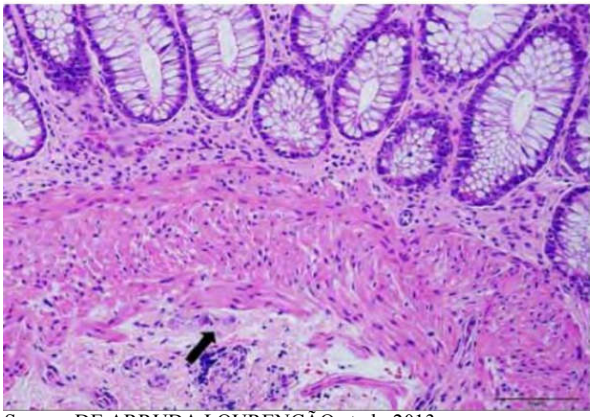
METHODOLOGY

This study is a systematic review, classified as exploratory and descriptive. The elaboration of the research was a bibliographic search in electronic databases about methods associated with RSL (Systematic Literature Review) and SMARTER (Simple Multi-Attribute Rating Technique using Exploiting Rankings) applications. The work carried out is of a quali-quantitative nature. Qualitative data analysis is performed intuitively and inductively during the survey of the theoretical framework. It is also quantitative through the use of the multicriteria method. In addition, there is also a numerical experimental study to simulate a situation of article selection based on

the observed criteria. From bibliographic research, located in the databases: *US National Library of Medicine* (Pub Med), Scientific Electronic Library Online (SCIELO), Caribbean Latin American Health Science Information System (LILACS), Science Direct (Elsevier), and Embase. The search in the databases was performed using the terminologies registered in the Health Sciences Descriptors created by the Virtual Health Library developed from the Medical Subject Headings of the US National Library of Medicine, which allows the use of common terminology in Portuguese, English, and Spanish. The keywords used in Portuguese for searching the databases were: Diagnostic Techniques, Hirschsprung Disease. As a tool to support thedecision in selecting and prioritizing articles, a set of criteria were considered essential to represent the state of the art of the subject of research. This method has the following characteristics: (i) rigorous logic allows the acceptance of the method as a decision support tool; (ii) simple to be understood and applied with results that are easy to interpret. After all, the result obtained totaled 11 (eleven) articles that contemplated the desired characteristics for the study.

RESULTS

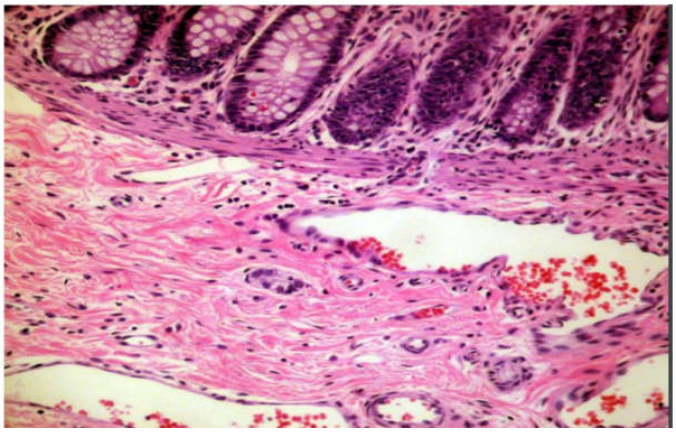
Hematoxylin & Eosin Stain: Histopathological examination using Hematoxylin & Eosin (H&E) staining is a classic method that is commonly used and is negative for Hirschsprung's Disease when it shows at least one ganglion cell in the submucosa of the segment obtained in the biopsy (SERAFINI et al., 2017). On the other hand, the diagnostic criteria for confirming the disease correspond to the absence of ganglion cells and the possible presence of hypertrophied nerve fibers in the submucosa (SZYLBERG; MARSZAŁEK, 2014). In addition, histopathological analysis with H&E staining is more complex and requires a pathologist, and can be easily misinterpreted (SERAFINI et al., 2017). A biopsy sample may be very superficial, may contain ultra-short aganglionic segments, samples without the submucosal segment, and distal regions with ganglion cells that may not be identified in the section (HOLLAND et al., 2010). There is also a difficulty with this test for identifying ganglion cells. In neonates, because they still do not have full maturity of the enteric nervous system (HOLLAND et al., 2010). Thus, the difficulties mentioned above serve as a subsidy for creating new, more straightforward diagnostic techniques that require less labor. Therefore, there are other forms of disease diagnosis such as immunohistochemical methods that use some antibodies against S100, neuron-specific enolase (NSE), microtubule-associated protein-5 (MAP5), Glucose transporter-1 (GLUT-1), protein glial fibrillary acid (GFAP), peripheral antibodies, and others(HOLLAND et al., 2010). Two other methods used and studied are Histochemistry with Acetylcholinesterase (AChE) and Immunohistochemistry using Calretinin (DE ARRUDA LOURENÇÃO et al., 2013; KAPUR et al., 2009). Below we can see the images with the positive and negative patterns of staining with H&E in the diagnosis of Hirschsprung's Disease.



Source: DE ARRUDA LOURENÇÃO et al., 2013

Figure 1.

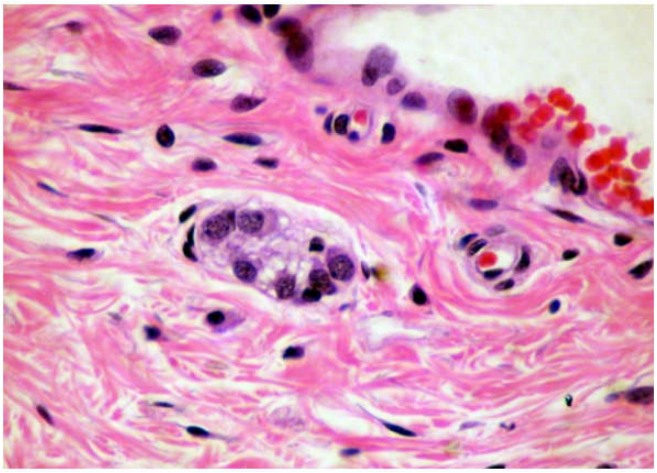
First in this H&E stained image of an individual without Hirschsprung's Disease with the presence of ganglion cells in the submucosa (arrow) excluding disease diagnosis (400X).



Source: COLMENERO, 2013

Figure 2.

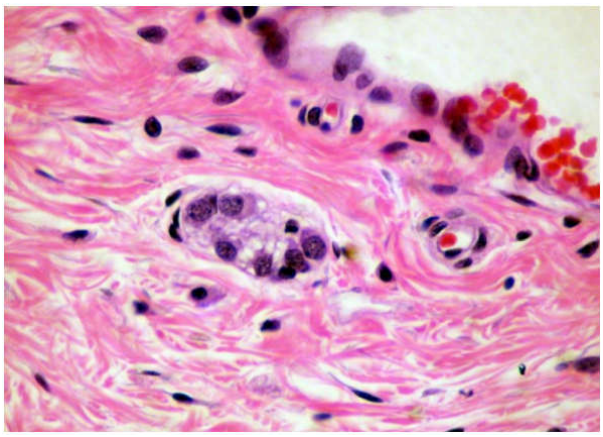
This H&E stained image shows the absence of ganglion cells (a single ganglion cell would rule out the diagnosis of Hirschsprung's Disease) and hypertrophic nerve trunks (not always present) that support the diagnosis of Hirschsprung's Disease.



Source: COLMENERO, 2013

Figure 3.

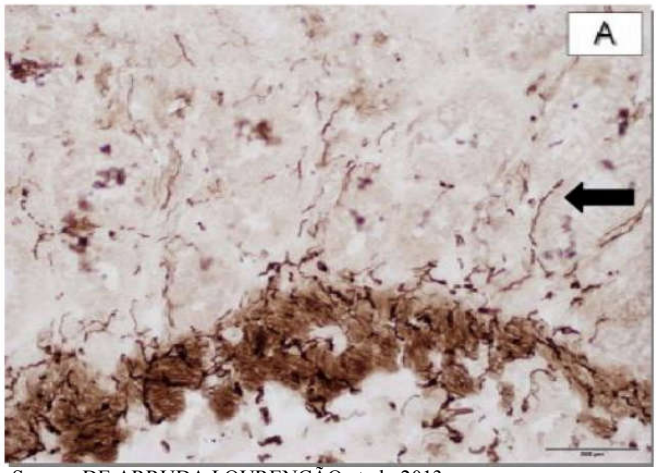
In this figure, we have the previous enlarged image with the absence of ganglion cells (a single ganglion cell excludes Hirschsprung's Disease). Hypertrophic nerve trunks support the diagnosis (not always present).



Source: COLMENERO, 2013

In this figure, we have the previous enlarged image with the absence of ganglion cells (a single ganglion cell excludes Hirschsprung's Disease). Hypertrophic nerve trunks support the diagnosis (not always present).

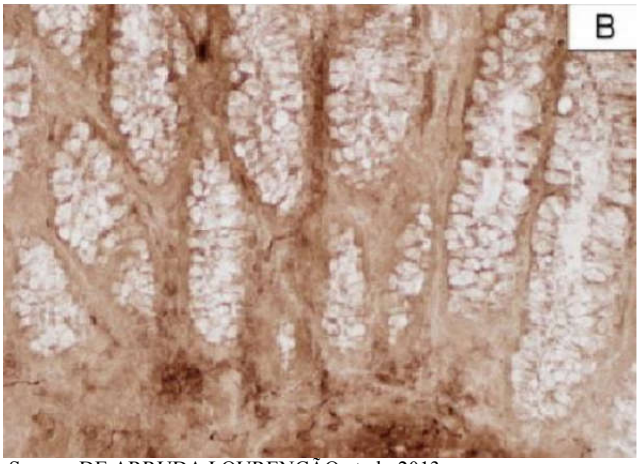
Histochemistry with AChE: AChE is an enzyme that belongs to the family of cholinesterases found in nervous tissues and red blood cells, and itsprimary function is to hydrolyze acetylcholine, a neurotransmitter. Neurotransmitter activity is to mediate the synaptic activity of the central and peripheral nervous system. In addition, acetylcholine plays a role in increased motility, gland secretion, and peristalsis in the digestive tract (KAPUR et al., 2009). According to histopathological studies from the last century, Acetylcholinesterase activity in patients with healthy bowel is minimal or absent, unlike in patients with Hirschsprung's Disease, in which biopsies showed an increase in enzymatic activity in parasympathetic nerve fibers of the lamina propria and mucosal muscle (KAPUR et al., 2009). The great advantage of this method is that the biopsy must be superficial to provide better observation of enzymatic activity in the lamina propria and mucosal muscle(SERAFINI et al., 2017). But the method of performing the biopsy must be without excessive bleeding or bleeding, as the sample can easily be contaminated with blood and generate false negatives as red blood cells also have acetylcholinesterase in their plasma membrane in the form of antigen and that can cause patterns similar in patients affected by Hirschsprung's Disease (DE ARRUDA LOURENÇÃO et al., 2013; KAPUR et al., 2009). In classic cases of Hirschsprung's Disease, Acetylcholinesterase-positive nerve fibers are seen in the mucosal muscle. However, these fibers are few and challenging to demonstrate in newborns with Hirschsprung disease and increase with age. Ideally, this technique requires a small frozen rectal fragment that contains only the mucosal and submucosal lamina(SERAFINI et al., 2017). This method is considered the gold standard for diagnosingHirschsprung's Disease in our practice and has an accuracy greater than 90% (AGRAWAL et al., 2015). AChE hyperactivity becomes pathognomonic for Hirschsprung'sdisease. Therefore, histochemical staining together with H&E staining is the gold standard in diagnosingHirschsprung's Disease. Recent studies have demonstrated the high specificity of AChE staining but with inadequate sensitivity (up to 85%)(PACHECO; BOVE, 2010). In this context, in the last two decades, other methods have been proposed to help identify ganglion cells or delineate the nature of nerve fibers in direct biopsies for the investigation of Hirschsprung'sdisease.



Source: DE ARRUDA LOURENÇÃO et al., 2013

Figure 4

The image demonstrates a slide of histochemical staining with acetylcholinesterase. Confirming the diagnosis of Hirschsprung's Disease. (A) Hirschsprung's disease - positive. Reaction showing numerous thick and irregular dark brown nerve fibers within the mucosal muscle layer and extending towards the lamina propria (arrow) at 200x magnification.

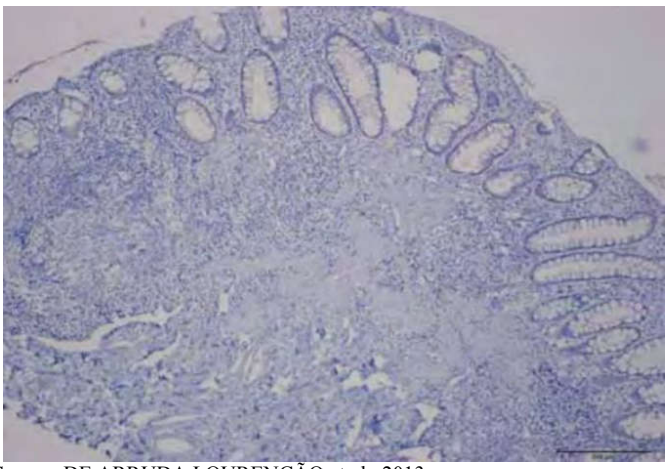


Source: DE ARRUDA LOURENÇÃO et al., 2013

Figure 4

The image demonstrates a slide of histochemical staining with acetylcholinesterase. Excluding the diagnosis of Hirschsprung's Disease. (B) normal bowel - little enzyme activity in the muscle mucosa, excluding the diagnosis of Hirschsprung'sdisease (200x).

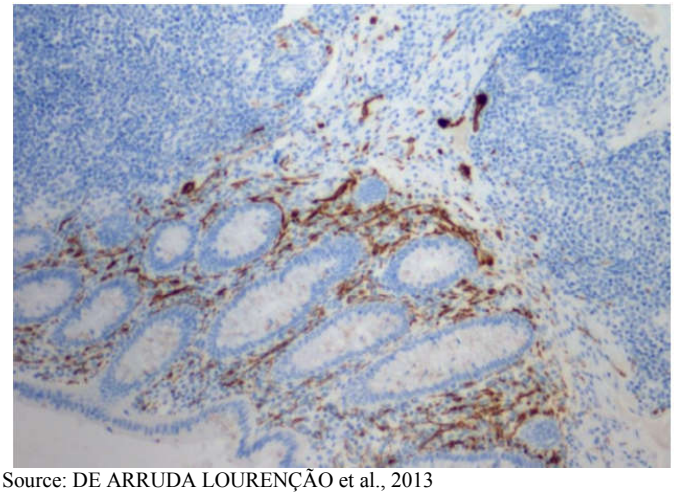
Immunohistochemistry with Calretinin: A subclass of neuron cell bodies, present in the submucosal and myenteric plexuses of the human gastrointestinal tract, demonstrate immunoreactivity to calretinin. This marker, in turn, is a 29-kDa protein, which binds to calcium, is dependent on vitamin D, and has an essential role as a sensor and modulator of calcium ions. The absence of this protein leads to the accumulation of these ions in the cytoplasm of nerve cells, promoting super-excitation and possible neurodegeneration(DE ARRUDA LOURENÇÃO et al., 2013). In pioneering work, Barshack et al., in 2004, demonstrated the expression of calretinin in ganglion cells and small intrinsic nerve fibers in the submucosal and myenteric plexuses of normal intestines and ganglionic segments of patients with Hirschsprung'sdisease. On the other hand, there was a total loss of calretinin expression in aganglionic segments. Thus, they concluded that the loss of calretinin expression could be helpful in the diagnostic investigation of colon aganglionosis. Studies have shown that immunohistochemistry using calretinin showed diagnostic accuracy greater than 90%. In addition, the method can also be performed on a small fragment of rectal and submucosal mucosa, similar to the technique with AChE. However, immunohistochemistry is more complex and expensive than staining with H&E, and it is only available in a few medical centers in Brazil (DE ARRUDA LOURENÇÃO et al., 2013; KAPUR et al., 2009). In the figures below, we will see the histopathological patterns using calretinin. For the diagnosis of Hirschsprung's Disease to be positive, calretinin must be negative and, to rule out the diagnosis, calretinin must be immunoreactive.



Source: DE ARRUDA LOURENÇÃO et al., 2013

Figure 4.

In this figure at 100X magnification, we can see negative staining (no dark brown spots) for nerve fibers and ganglion cells, supporting the diagnosis of Hirschsprung's Disease.



Source: DE ARRUDA LOURENÇÃO et al., 2013

Figure 5.

This figure shows immunoreactivity (dark brown) to calretinin, which offers a granular coloration of intrinsic neurons.

DISCUSSION

AChE versus H&E: The effectiveness of the method using H&E staining for the diagnosis of Hirschsprung'sDisease is still controversial. Two previous studies used fragments of the rectal wall stained with H&E to diagnose the disease and observed a dispersed distribution of neurons in the submucosa (AGRAWAL et al., 2015; SWENSON; SHERMAN; FISHER, 1973). This distribution could make diagnosing Hirschsprung'sdisease, thus the authors disagree with using this method for analyzingHirschsprung's Disease. In contrast to these two studies, it was shown that the limitations of the method previously described by Swenson and Agrawal could be overcome by analyzing a more significant number of sections of each fragment(KAPUR et al., 2009). However, there is no consensus in the current literature on the ideal number of sections to analyze in the histopathology of the enteric nervous plexus(BRITO; MAKSOUD, 1987; KAPUR et al., 2009). As such, it is difficult to determine the most efficient techniques. In a recent study comparing the usefulness of H&E in the diagnosis ofHirschsprung'sdisease, the authors concluded that classical staining (H&E) of small fragments of the rectal and submucosal mucosa is currently still a viable alternative for the diagnosis of the disease if at least 60 histological sections are analyzed (SERAFINI et al., 2017). As is known, many centers use the gold standard for the diagnosis of the disease, which is: staining with H&E together with histochemical assay with AChE. When hyper-reactive, it's pathognomonic for Hirschsprung's Disease (PACHECO; BOVE, 2010). Calretinin can also be used (SERAFINI et al., 2017). In another study, De Arruda Lourenção et al. (2013), 83 patients were investigated for Hirschsprung's Diseaseinitially using anorectal manometry and/or opaque enema as a screening test. Of these patients, 43 underwent rectal biopsy and were submitted to three methods: H&E, AChE, and Calretinin. According to the authors, there was a discrepancy in 11 cases, where the negative H&E result was due to the absence of ganglion cells in the tissue sample (Table 1).

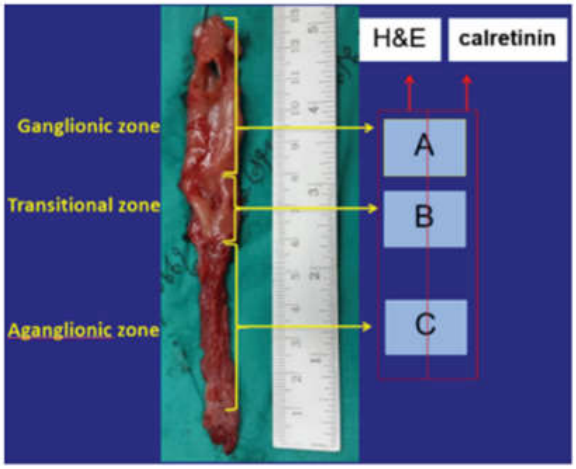
Table 1

FIND			
H&E	Positive	Negative	Total
no ganglion cells	13	11	24
Presence of ganglion cells	0	19	19
Total	13	30	43

Source: DE ARRUDA LOURENÇÃO et al., 2013

*Correlation between acetylcholinesterase (AChE) and Histopathology (H&E) pattern in rectal biopsies from children under investigation for Hirschsprung's Disease

H&E versus Calretinin: In a recent publication, a double-blind diagnostic study was carried out with 51 patients with Hirschsprung's Disease(KHORANA et al., 2020), using 153 biopsy samples from the ganglionic, transient, and aganglionic zones (Figure 6). Each sample was stained with Calretinin and H&E. Agreement, and Cohen's Kappa coefficient was 97.4% and 0.921 (95% confidence interval 0.845 to 0.997), respectively. There was disagreement in four samples. Three out of four were in the transition zone. One was in the aganglionic zone, which had no muscle layer attached (36 specimens had no muscle layer). According to the authors, immunohisto chemistry with calretinin can be used in samples that do not have the muscle layer, which is a problem with H&E staining. For example, in the suction rectal biopsy sample, calretinin is useful. Inaddition, it can be used to detect ganglions and nerve fibers in the ganglionic zone, in the transition zone, and the aganglionic zone (KHORANA et al., 2020).



Source: KHORANA et al., 2020

Figure 6

AChE versus Calretinin: In the same studyby Arruda Lourenção et al. (2013), the authors reported a divergence between the results using AChE and Calretinin in only one case, and as there were no ganglion cells in this sample, the initial Calretinin result was negative. However, in this case, the second analysis of immunostaining with calretinin revealed a mild immune response at focal intrinsic nerve fibers of the mucosal muscle and lamina propria, excluding the diagnosis of Hirschsprung's Disease (Table 2).

Table 2.

FIND			
Calretinin	Positive	Negative	Total
Negative	13	1	14
Positive	0	29	29
Total	13	30	43

Source: DE ARRUDA LOURENÇÃO et al., 2013

*Correlation between acetylcholinesterase (AChE) histochemistry and calretinin immunohistochemistry in rectal biopsies from children under investigation for Hirschsprung'sdisease

Still, on the comparison of the two techniques, a study published in 2014 (YADAV L, KINI U, DAS K, MOHANTY S, 2014), 74 rectal biopsies from 51 suspected cases of Hirschsprung's disease. As a result, a study confirmed the disease in 26 patients and non-disease in 25 patients. Calretinin results were compared with AChE with a statistically significant agreement of Kappa = 0.973 between the two. A false-positive case of Hirschsprung's Disease was noted with calretinin. According to the authors, the advantages and disadvantages of calretinin versus AChE are still under discussion. Corroborating previous studies in which precision and specificity were greater than 90% (DE ARRUDA LOURENÇÃO et al., 2013; PACHECO; BOVE, 2010).

Table 3.

Method	Ac1	St2	Sp3	PPV4	NPV5	Kappa6	P7
H&E	74.4	100.0	63.3	54.2	100.00	0.511	<0.001
calretinin	97.6	100.00	96.7	92.9	100.0	0.946	<0.001

Source:DE ARRUDA LOURENÇÃO et al., 2013
Subtitle:(1) Accuracy (2) Sensitivity (3) Specificity (4) Positive predictive value (5) Negative predictive value (6) Kappa index for static agreement (7) P-value associated with the Kappa index
*Comparison of tests with acetylcholinesterase histochemistry

AChE versus Calretinin versus H&E: According to the study by Arruda Lourenção et al. (2013), with the Kappa index analysis, the standard histology (H&E) and the immunohistochemistry with calretinin, and the histochemistry with AChE have good consistency, emphasizing that the calretinin immunostaining has an almost perfect agreement value, as shown in Table 3. Furthermore, comparing the specificity and precision values between immunohistochemistry with calretinin and H&E stained slides, using Fisher's Exact Test, it was observed that calretinin had specificity values (96.7 x 63.3; p = 0.002) and accuracy values (97.6 x 74.4; p = 0.003) significantly higher than H&E.

Final Considerations: Thus, we can fulfill that the small intestine is the final part of the digestive system and its main function is the production and storage of feces, consisting of four parts: cecum, colon, rectum and anus. The colon, in turn, is divided into the ascending colon, transverse colon, descending colon, and sigmoid colon. Furthermore, we have seen that the intestines are innervated by two enteric plexuses: the submucosal or Meissner plexus and the myenteric or Auerbach plexus. We also saw that the lack of these plexuses in the formation of the intestine, still in an embryonic process, generates an aganglionosis, that is, Hirschsprung's disease. The lack of Meissner and Auerbach plexuses means that the intestine does not perform peristaltic movement, causing the fecal bolus to stop and usually develop an intestinal obstruction and consequently a megacolon. We also analyzed three types of cell staining methods, Acetylcholinesterase Histochemistry, H&E and Calretinin Immunohistochemistry, and through the data provided throughout the review, we observed that Immunohistochemistry has some advantages over acetylcholinesterase and H&E. In the review, studies indicate that calretin can be a good tool to rule out the diagnosis of Hirschsprung's disease, showing positive stains in ganglion cells and intrinsic nerve fibers, while AChE can be useful in confirming the diagnosis of Hirschsprung's disease, revealing activity of this enzyme in hypertrophic nerve fibers. Furthermore, through these data we can interpret that an immunohistochemical staining with calretinin seems to have greater or equal efficacy to staining with acetylcholinesterase and other histochemicals as an auxiliary method in the diagnosis of HE in rectal suction biopsies. Furthermore, the technique with H&E is still viable for diagnosing the disease, if at least 60 possible histological findings are analyzed. However, immunohistochemistry with calretinin has a clearer interpretation capacity, with fewer false negatives and without the need for an experienced pathologist, as in the case of both histochemistry and staining with H&E. In summary, some of the articles discussed throughout the review suggest that H&E is still a viable technique for diagnosing the disease, when there is an association of AChE to assess the enzymatic activity of hypertrophic nerve fibers. Likewise, Calretinin is a reliable marker to exclude the diagnosis of Hirschsprung's disease and AChE remains an indispensable technique for confirming the diagnosis of the disease in fresh biopsies that, together with Calretinin, can maximize the accuracy of diagnosing the disease, especially in more complex cases.

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