Pedro Luiz Toledo de Arruda Lourenção

Desafios diagnósticos na Doença de Hirschsprung: aplicabilidade de novos métodos imunohistoquímicos e endoscópicos

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Desafios diagnósticos na Doença de Hirschsprung: aplicabilidade de novos métodos imunohistoquímicos e endoscópicos

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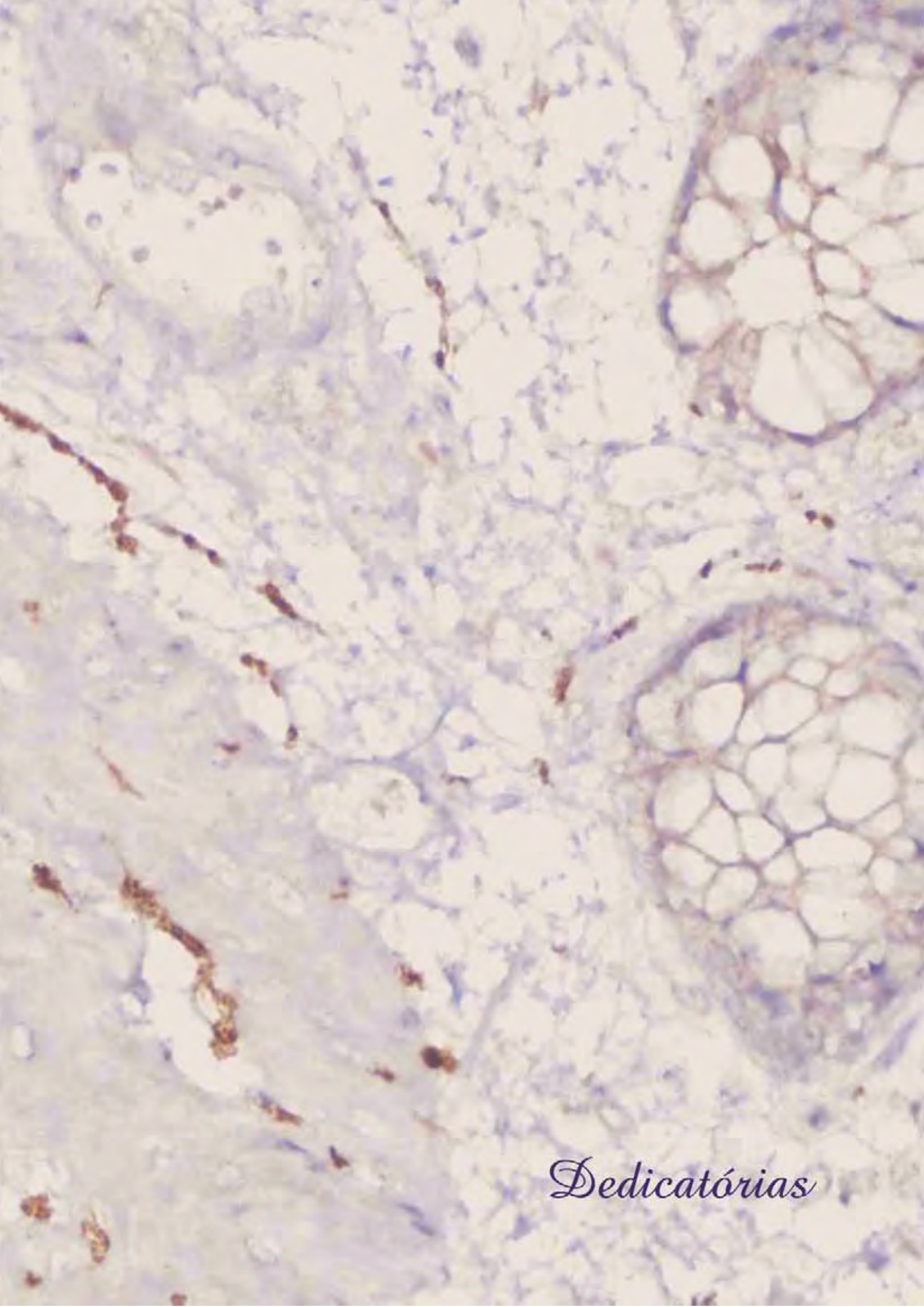

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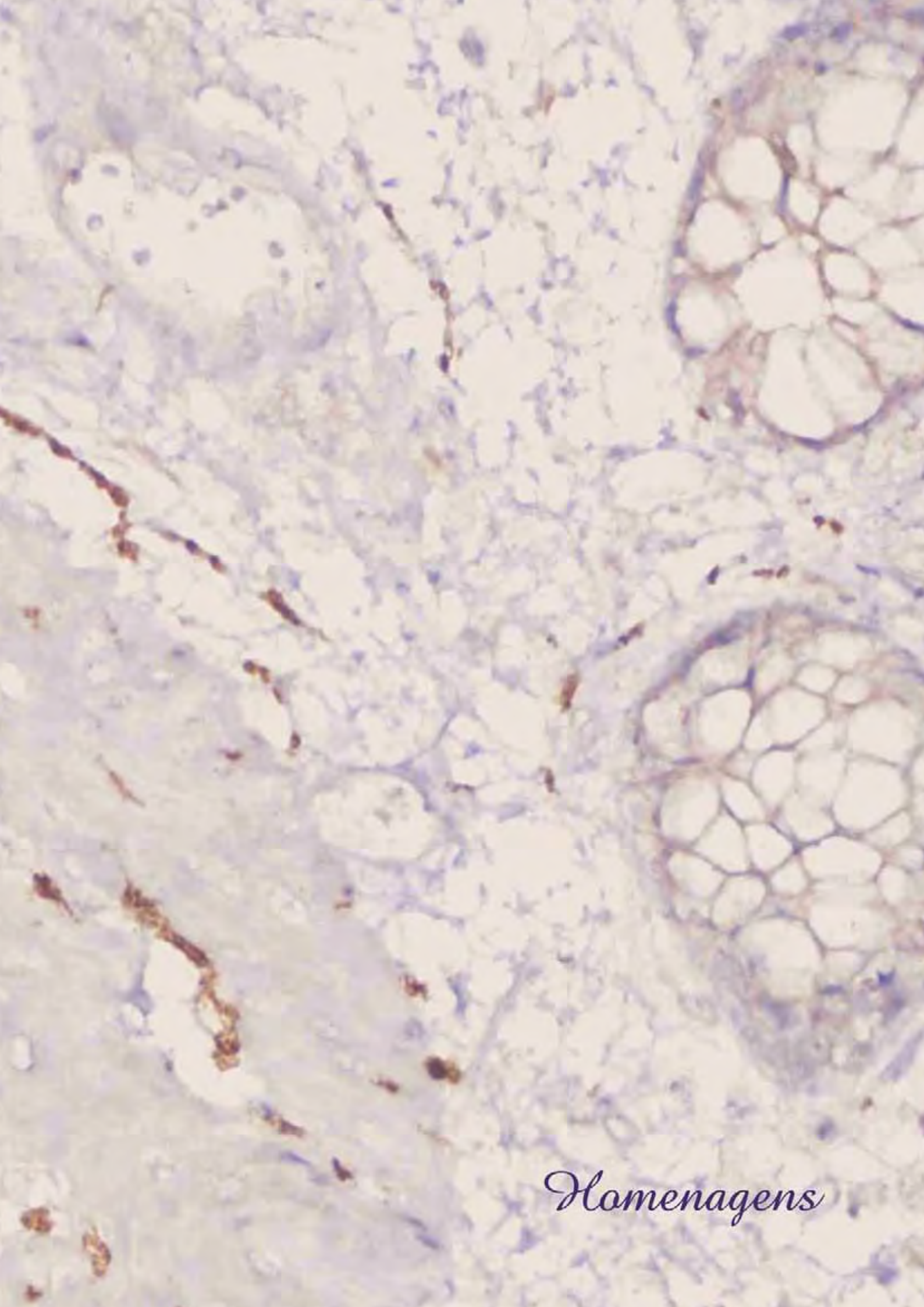
"Aos meus pais **Pedro** *e* **Maria Zilda** *e à minha irmã* **Marina**, *pelas oportunidades de uma vida, apoio e amor, sem os quais eu jamais chegaria até aqui".*

"À **Simone**, *pelo amor, compreensão e companheirismo, sempre ao meu lado".*



Homenagens

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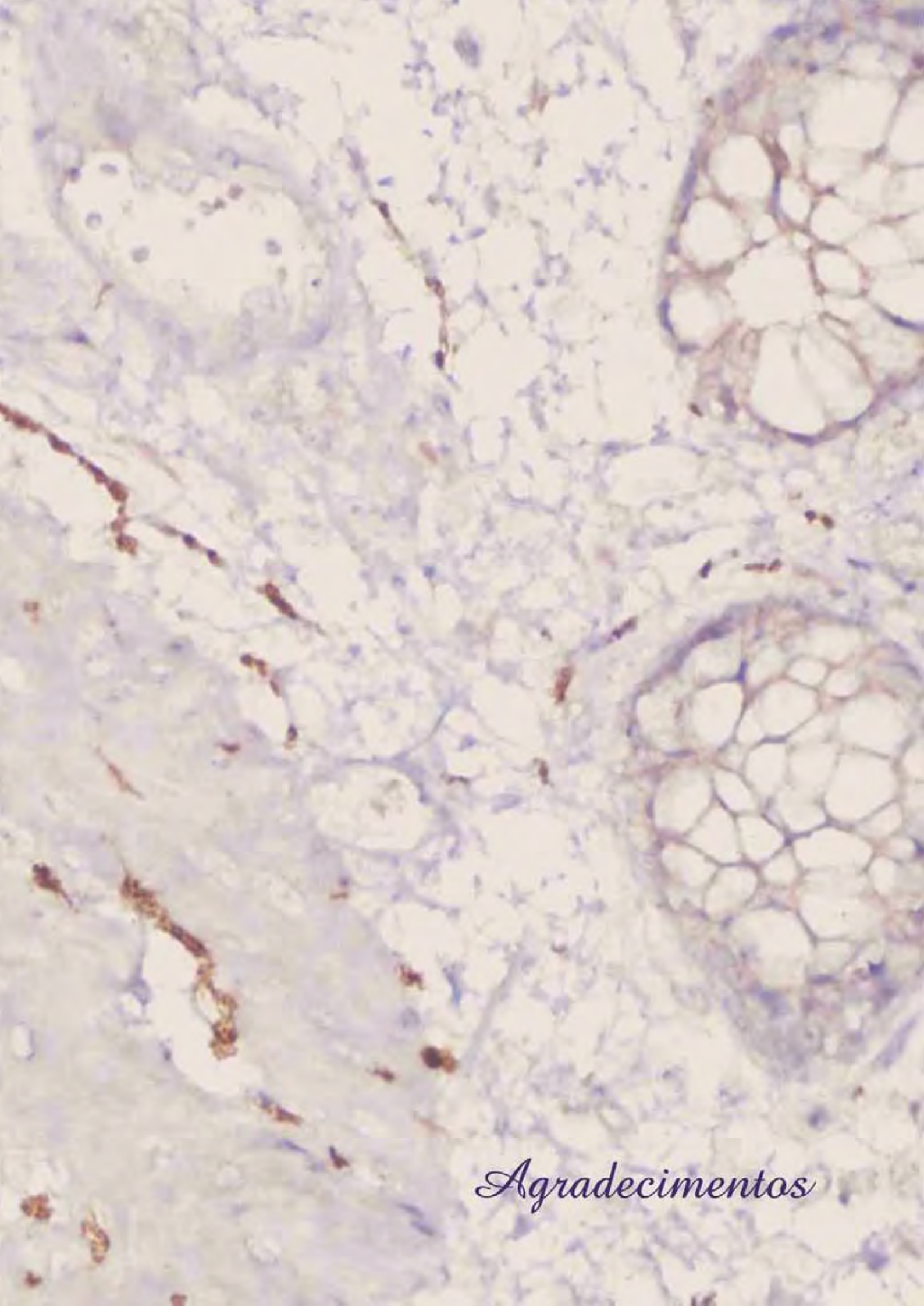

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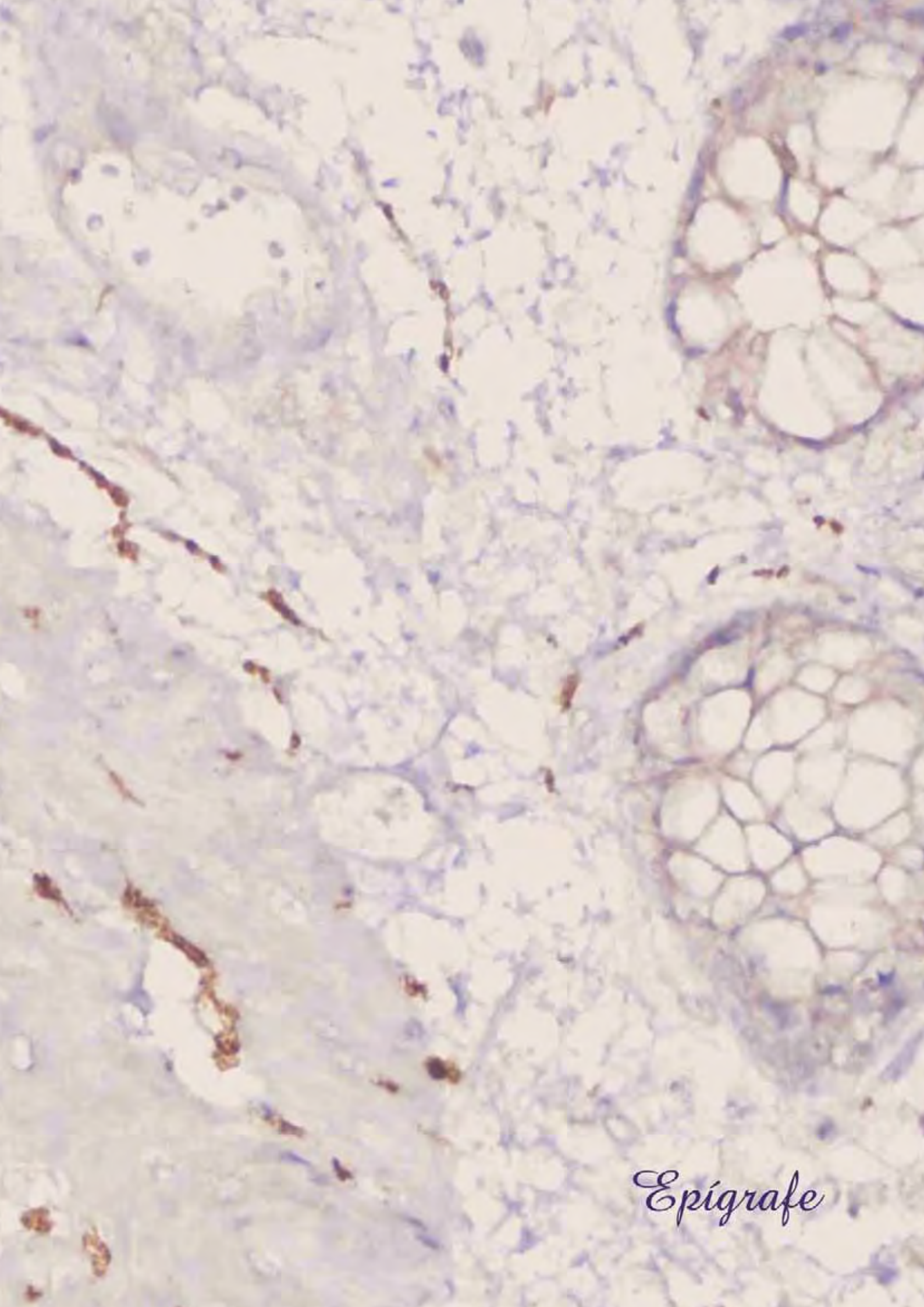
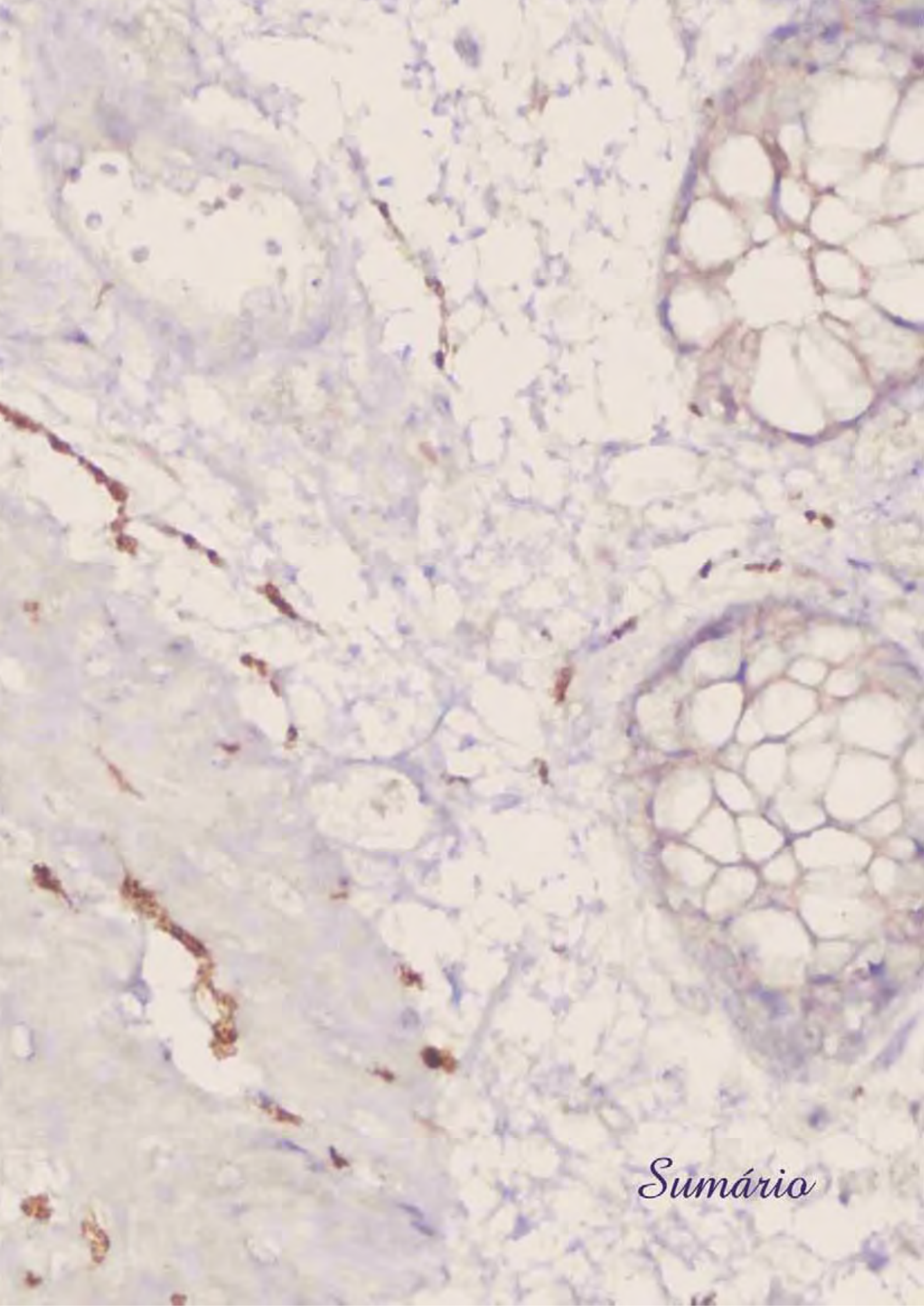


Epígrafe

"Aventure-se, pois da mais insignificante pista surgiu toda a riqueza que o homem já conheceu".

John Masefield



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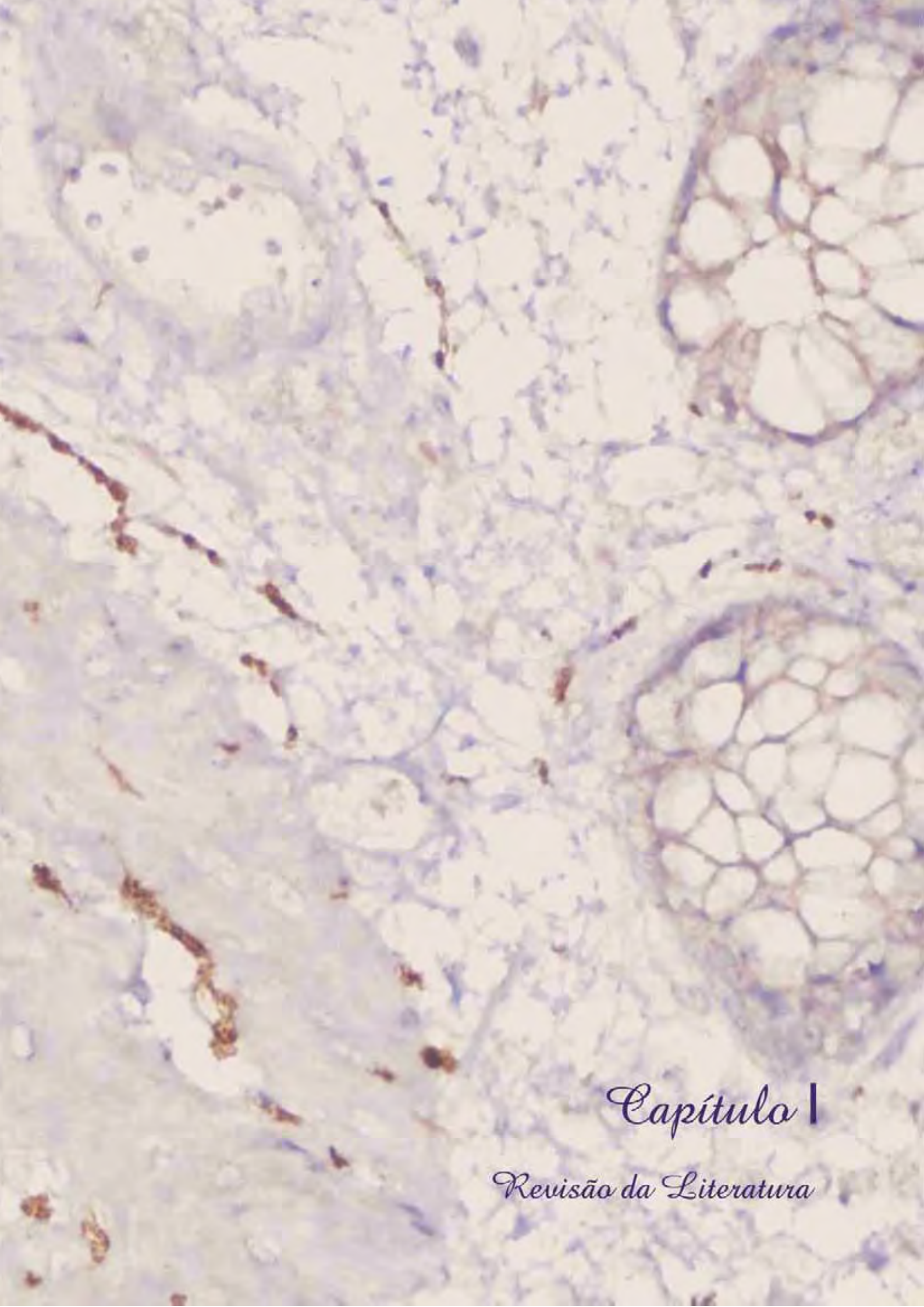

Capítulo I

Revisão da Literatura

1. Introdução

A Doença de Hirschsprung ou Aganglionose Intestinal Congênita é um processo patológico típico da faixa etária pediátrica, clinicamente expresso por obstrução intestinal no período neonatal ou constipação intestinal grave no lactente e escolar (Dasgrupta & Langer, 2004; Masiakos & Ein, 2006). É uma malformação congênita do sistema nervoso entérico caracterizada pela ausência de células ganglionares nos plexos submucosos e mioentéricos do intestino distal. Isto resulta em um segmento aperistáltico, responsável pelo quadro clínico de obstrução intestinal (Dasgrupta & Langer, 2004).

Aspectos relacionados à etiopatogenia, fisiopatologia e diagnóstico ainda são motivos de pesquisa e intensa discussão na literatura, objetivando melhorar o tratamento às crianças portadoras desta entidade patológica.

2. Aspectos Históricos

A primeira descrição das características clínicas desta doença data de 1691, quando Frederick Ruysch observou a dilatação do cólon de uma criança de 5 anos que havia falecido por obstrução intestinal (Leenders & Sieber, 1970). Em 1800, Domenico Battini relata outro caso de uma criança com estas mesmas características clínicas (Fiori, 1998). Já em 1886, o médico pediatra dinamarquês, Harald Hirschsprung, do Hospital Pediátrico de Copenhagen "Rainha Louise", apresentou, no Congresso de Berlim de Doenças da Criança, dois casos de constipação intestinal e distensão abdominal desde o nascimento, cujas autópsias revelavam cólon dilatado e hipertrofiado, sem nenhuma obstrução mecânica aparente. Ele acreditava que esta "nova doença" seria uma alteração congênita, explicada pelas alterações macroscópicas observadas no cólon (Masiakos & Ein, 2006). Seu trabalho foi publicado um ano mais tarde, em 1887, na literatura alemã, e intitulado "Constipação no recém-nascido devido à dilatação e hipertrofia do cólon" (Hirschsprung, 1887). O termo "megacolon congênito" foi introduzido por Mya (1894).

Apesar de estes trabalhos definirem uma entidade clínica, agora conhecida e estabelecida, a fisiopatologia da doença ainda era desconhecida, no início do século XX. Isto foi motivo de investigação científica intensa, revelada em alguns trabalhos publicados neste período. A ideia de que a causa da doença não estaria no cólon dilatado proximal, mas no segmento distal espástico e estreito, começou a ser descrita nos trabalhos de Fenwick (1900), Hawkins (1907) e Robertson & Kernohan (1938). A ausência de células ganglionares no cólon distal foi primeiramente descrita por Tittel (1901), no reto de uma criança de 15 meses, com constipação desde o nascimento. Até a década de 40, outros artigos foram publicados revelando alterações microscópicas na inervação colônica, com destaque para Dalla Valle (1920), que descreveu a ausência de células ganglionares no retossigmóide de dois irmãos

com sintomas típicos da doença e que apresentavam células ganglionares no segmento dilatado proximal do cólon. Em 1945, aproximadamente 60 anos após a descrição inicial de Hirschsprung, Theodor Ehrenpreis, elaborou profunda revisão da literatura e concluiu que a ausência de células ganglionares no retossigmóide teria um significado patológico e, a partir de estudos radiológicos contrastados (enemas opacos) de crianças normais e afetadas pela doença, observou que a dilatação do cólon seria um evento secundário na evolução da doença (Ehrenpreis, 1945, 1955). Em fevereiro de 1948, Zuelzer & Wilson (1948), a partir de estudo em 11 crianças falecidas com a doença, concluíram que a obstrução intestinal era funcional, e ocorria devido à alteração congênita na inervação do segmento espástico. Em julho do mesmo ano, Whitehouse & Kernohan (1948), também a partir de material de autópsias, observaram no reto espástico, ausência de células ganglionares nos plexos mioentéricos acompanhada da presença de troncos nervosos hipertrofiados. Um ano mais tarde, Bodian et al. (1949) publicaram 73 casos de constipação intestinal em crianças, divididas em dois grupos (doença de Hirschsprung e constipação idiopática), e realizaram investigação radiológica (enema opaco) e patológica (peças cirúrgicas e autópsias). Os pacientes com a doença apresentavam padrão radiológico peculiar, com zona espástica e dilatada, além da ausência de células ganglionares no segmento doente.

A consolidação destes conceitos sobre a fisiopatologia da doença, no meio de século passado, abriu novas perspectivas para o diagnóstico e tratamento das crianças acometidas. Estes aspectos serão discutidos mais adiante.

3. Epidemiologia

A incidência da doença é estimada em 1: 5.000 nascidos vivos (Dasgrupta & Langer, 2004). Puri & Montedonico (2008) citam 12 estudos epidemiológicos, realizados entre 1962 e 2005, revelando variações desde 1: 2.000 (Bodian & Carter, 1963), até 1:12.000 (Althoff, 1962). A casuística mais recente é a de Suita et al. (2005), realizada no Japão, que demonstra incidência de 1: 5.343 nascidos vivos.

Com relação ao gênero, é bem definida a prevalência do sexo masculino, em proporção de 4:1. Esta razão varia na dependência da extensão da doença, apresentando taxas de 1:1 a 2:1 nas formas longas, e até mesmo, pode ser revertida para 0,8: 1 na aganglionose colônica total. O motivo desta variação não é esclarecido (Puri & Montedonico, 2008).

Não há consenso sobre a prevalência da doença em alguma raça. Um completo programa de monitoramento de doenças congênitas na Califórnia, realizado entre 1983 e 1997, revelou diferenças raciais na distribuição da doença de Hirschsprung. As incidências encontradas foram de 1,5: 10.000 em brancos, 2,1: 10.000 em negros, 1:10.000 em hispânicos e 2,8:10.000 em asiáticos (Torfs, 1998). Os estudos de Kleinhaus et al. (1979) e de Sherman et al. (1989) demonstraram diferenças na incidência da doença entre brancos e negros norte-americanos, apenas em função das formas da doença, sendo a forma longa mais frequente nos brancos.

4. Embriologia e Etiopatogenia

O sistema nervoso entérico é o ramo mais complexo do sistema nervoso periférico (Laranjeira & Pachinis, 2009). Os cerca de 80 a 100 milhões de neurônios, números maiores que os da medula espinhal, têm capacidade de realizar atividade reflexa na ausência do sistema nervoso central. Desempenha papel fundamental na motilidade intestinal (Puri & Rolle, 2008) e o entendimento dos aspectos de sua embriologia são importantes no estudo da etiopatogenia da doença de Hirschsprung.

Durante a 3ª semana de desenvolvimento embrionário, no processo chamado neurulação, ocorre a formação da crista neural a partir da ectoderme (Moore & Persaud, 2000). Suas células pluripotentes darão origem aos melanócitos, células da medular adrenal, dentina e aos neurônios do sistema nervoso autônomo, incluindo os do sistema entérico (Puri & Rolle, 2008). Assim, as células da crista neural são as responsáveis pelo desenvolvimento das células nervosas do tubo digestório. Isto ocorre através de um processo de migração, no sentido craniocaudal, atingindo o reto, até o final da 12ª semana do desenvolvimento (Okamoto, 1967; Webster, 1973). Após migrarem, já entre a 12ª e 16ª semanas, os neuroblastos invadem a parede intestinal, originando primeiramente os plexos mioentéricos de Auerbach nas camadas mais externas, e posteriormente os plexos de Meissner na submucosa. Isto também segue uma sequencia craniocaudal (Puri & Rolle, 2008).

Desta forma, a ausência de células ganglionares tem sido atribuída à falha na migração das células da crista neural, durante a embriogênese. De acordo com esta teoria, a altura da zona aganglionar é determinada pelo momento em que esta falha ocorreu. Bolande (1974) utilizou o termo "neurocristopatia" para descrever as anomalias derivadas de falhas no desenvolvimento da crista neural. Nesse contexto, a aganglionose é classificada como uma neurocristopatia simples, já que envolve um único processo patológico.

Outra teoria explica a patogenia de maneira diferente. A proposta é que as células da crista neural atingem seu destino de migração, povoando todo o tubo digestivo. Entretanto, estas células não se proliferam nem se diferenciam, possivelmente por alterações no microambiente das regiões distais do cólon, dando origem aos segmentos aganglionares da doença de Hirschsprung. O papel da fibronectina e da laminina, como glicoproteínas que facilitam a migração e o desenvolvimento das células da crista neural, é discutido por Dasgrupta & Langer (2004). Estes autores citam alterações das moléculas de adesão das células neurais (NCAM) e ausência de fatores neurotróficos, como possibilidades etiológicas para a falta de proliferação local dos neuroblastos.

5. Aspectos Genéticos

O risco aumentado para doença de Hirschsprung entre irmãos de indivíduos afetados, a desproporção na sua distribuição entre os sexos e a associação com outras malformações e síndromes cromossômicas constituíram as evidências iniciais para a pesquisa de fatores genéticos nesta entidade patológica (Kenny et al., 2010). Os fatores genéticos são heterogêneos e exibem interações complexas que influenciam na penetrância e severidade da doença (Kapur, 2009). Foram descritos mais de 10 diferentes genes que podem estar alterados em pacientes portadores de aganglionose colônica. As alterações genéticas mais comuns incluem mutações no proto-oncogene RET, identificada em 7 a 35% dos casos, no gene EDNRB em 7% e no gene END3 em menos que 5% (Kenny et al., 2010).

A forma esporádica da doença de Hirschsprung ocorre em 70% dos pacientes. Anomalias congênitas e síndromes associadas estão presentes em 5 a 32% dos casos (Amiel et al., 2008). Devem ser destacadas algumas neurocristopatias que estão comumente associadas, como a Síndrome da Hipoventilação Central Congênita, a Síndrome de Waardenburg e a Síndrome de di Georgi (Kapur, 2009; Kenny et al., 2010). A aganglionose colônica também está associadas à neoplasia endócrina múltipla tipo 2 e a neurofibromatose (Haricharan & Georgeson, 2008; Amiel et al., 2008). Das anomalias cromossômicas a mais frequentemente associada é a trissomia do 21, relatada em aproximadamente 7% das crianças com aganglionose congênita. Os pacientes com Síndrome de Down têm risco 100 vezes maior de apresentar a doença (Haricharan & Georgeson, 2008; Kenny et al., 2010).

Estudos recentes têm investigado o papel dos comandos genéticos e moleculares na migração e desenvolvimento das células do sistema nervoso entérico (Amiel et al., 2008). O proto-oncogene RET e a proteína RET atuam na migração e proliferação dos neuroblastos. Aproximadamente 50% dos pacientes com doença de Hirschsprung familiar apresentam

mutações no proto-oncogene RET, o que destaca a importância das alterações deste gene na patogênese da doença de Hirschsprung (Amiel et al., 2008). Mais de 20 diferentes mutações foram descritas neste proto-oncogene e alguns de seus polimorfismos estão associados com fenótipos particulares, expressos pela extensão da zona aganglionar (Haricharan & Georgeson, 2008). Alterações genéticas também têm sido observadas nas células tronco da crista neural nos pacientes com aganglionose colônica (Iwashita et al., 2003).

6. Fisiopatologia

O conhecimento da patogenia da doença, fundamentado na ausência de células ganglionares nos plexos submucosos e mioentéricos do intestino distal, constitui a base para o entendimento de sua fisiopatologia. A região aganglionar é sabidamente aperistáltica, constituindo um obstáculo ao trânsito intestinal. Em consequência, as regiões proximais ganglionares dilatam-se, apresentando progressiva hipertrofia muscular e paredes espessadas, caracterizando o segmento conhecido como megacolon. Entre a área espástica aganglionar e o segmento dilatado ganglionar há uma zona de transição, cônica e de extensão variável, na maioria das vezes localizada em nível de retossigmóide (Maksoud, 2003), conforme observado na Figura 1.

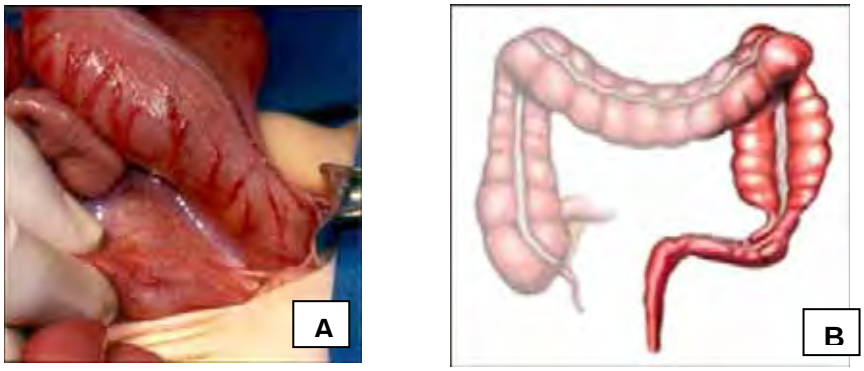

Figura 1 – (A) Imagem do intra-operatório da zona de transição; (B) Esquema da região espástica distal, zona de transição e cólon proximal dilatado na doença de Hirschsprung

A principal responsável pela fisiopatologia da doença é, portanto, esta região aperistáltica distal, caracteristicamente espástica, que leva à obstrução intestinal funcional, conforme descrito por Swenson & Bill (1948). Entretanto, ainda não há, na literatura recente, explicação completa sobre os mecanismos que levam a esta espasticidade (Dasgrupta & Langer, 2004; Puri & Montendonico, 2008).

Em recente revisão, Puri & Montedonico (2008) citam as possíveis explicações fisiopatológicas para a ocorrência desta espasticidade. Segundo estes autores, a motilidade do sistema gastrointestinal depende da interação de elementos nervosos e musculares. Uma das hipóteses propõe que a hiperinervação colinérgica seria responsável pela espasticidade da região distal. Haveria um aumento do número de fibras nervosas colinérgicas na zona aganglionar, representadas por troncos nervosos grossos e hipertrofiados na submucosa, muscular da mucosa e ao nível do plexo intermuscular. São fibras nervosas pré-ganglionares dos neurônios extrínsecos do sistema nervoso parassimpático que, não encontrando as células ganglionares para a sinapse, sofrem hipertrofia, dicotomizam-se e espalham-se pelas várias camadas da parede intestinal. Há liberação contínua de acetilcolina, neurotransmissor excitatório, que seria responsável pelo estímulo de contração. Entretanto, modelos experimentais demonstram a possibilidade de ocorrência da espasticidade na ausência de hiperatividade colinérgica (Imamura et al., 1975). Outra hipótese advoga o papel da inervação adrenérgica. Ocorreria um aumento do número e uma distribuição caótica de fibras adrenérgicas no segmento agangliônico. Entretanto, a sensibilidade desta região à epinefrina não está aumentada, apesar do aumento do número de fibras adrenérgicas. Além disso, estas fibras atuam no relaxamento da musculatura intestinal sendo, portanto, bastante improvável que sejam as responsáveis pelo aumento do tônus (Puri & Montedonico, 2008). Outro fator, de descoberta mais recente, é o papel do óxido nítrico. Trata-se de um dos neurotransmissores mais importantes no relaxamento da musculatura lisa da parede intestinal. Há indícios de que a síntese deste neurotransmissor no segmento intestinal aganglionico está prejudicada e tal deficiência poderia impedir seu relaxamento (Rolle et al., 2002).

Outros elementos também vem sendo investigados. As células intersticiais de Cajal, as células enteroendócrinas, o músculo liso entérico, a matriz extracelular e alterações

no segmento proximal ganglionar podem colaborar, de diferentes formas, para a formação do segmento espástico (Puri & Montedonico, 2008).

Em síntese, acredita-se que o segmento agangliônico receba dois fluxos de estímulos nervosos: um proximal, a partir do segmento ganglionar, que é um estímulo intrínseco inibitório, e outro extrínseco e excitatório, a partir da extremidade inferior da zona aganglionar. A estagnação da propagação de estímulos nervosos na região da zona de transição poderia explicar a diminuição dos estímulos inibitórios, levando à espasticidade do segmento distal agangliônico, conforme observado na Figura 2 (Puri & Montedonico, 2008).

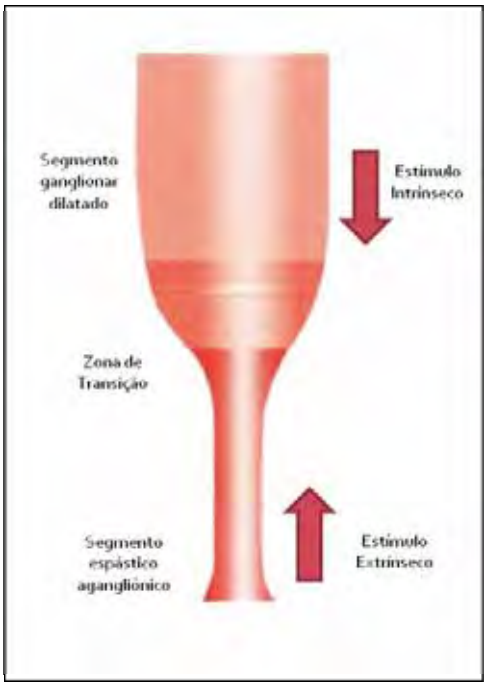

Figura 2 - Esquema demonstrando o papel dos estímulos nervosos na fisiopatologia da Doença de Hirschsprung (Adaptado de Puri & Montedonico, 2008).

7. Classificação

A doença de Hirschsprung é classificada com base na extensão da zona aganglionar. Entretanto, há divergências sobre as divisões utilizadas.

Puri & Montedonico (2008), consideram o esfíncter anal interno como o limite inferior da zona aganglionar e, a partir daí, classificam a doença nas formas clássica, quando o segmento espástico não se estende além do sigmóide; longa, quando a região agangliônica vai além do ângulo esplênico e aganglionose colônica total, quando se estende por todo o colon e pequena extensão do íleo terminal. Há também referência a uma forma rara, denominada aganglionose intestinal total, caracterizada pela ausência de células ganglionares do duodeno ao reto. A forma clássica é responsável pela grande maioria dos casos, com incidência variável de 72 a 88,8%, seguida pelas formas longa, em 3,9 a 23,7% dos casos e aganglionose colônica total, em até 12,6% dos pacientes.

Maksoud (2003) considera, além destas formas de aganglionose, a existência da forma curta da doença, na qual a região agangliônica estende-se pouco além do esfíncter interno e que é responsável por cerca de 5% dos casos.

Por outro lado, pela classificação proposta por Kaiser & Bettex (1982), a forma clássica corresponde ao envolvimento do reto, sigmóide e colon descendente, até a flexura esplênica, a forma ultra-curta é caracterizada pela aganglionose restrita às porções mais distais do reto e a forma ultra-longa, que ocorre quando mais da metade do colon é representanda pelo segmento agangliônico. Holschneider & Kunst (2008) descrevem a forma ultra-curta com mais detalhes. Trata-se de forma rara da doença, cujos sintomas comumente iniciam-se a partir dos seis meses de idade. O segmento agangliônico estende-se de 3 a 4 cm a partir da linha pectínea podendo, entretanto, estar limititada ao canal anal (Meier-Ruge & Bruder,

2008). O diagnóstico desta forma de aganglionose depende fundamentalmente de biopsias retais na altura da linha pectínea, que revelam aumento da atividade da acetilcolinesterase (Meier-Ruge et al., 2004; Holschneider & Kunst, 2008).

8. Quadro Clínico

A doença de Hirschsprung pode se apresentar sob diferentes formas e gravidades, na dependência da extensão e do grau de espasticidade do segmento agangliônico (Maksoud, 2003). Os sintomas podem aparecer já nos primeiros dias de vida. Até 90% dos casos se apresentam no período neonatal, caracteristicamente como obstrução intestinal (Maksoud, 2003; Martucciello, 2008). Desta forma, representa 20 a 25% dos casos de obstrução intestinal neste período (Loening-Baucke & Kimura, 1999). O atraso na eliminação do mecônio nas primeiras 24 horas de vida é relatado em até 90% dos pacientes com a doença (Puri & Montedonico, 2008). Entretanto, outros estudos demonstram que até 40% dos pacientes podem eliminar mecônio dentro das primeiras horas de vida, revelando que este não é um sinal obrigatório para o diagnóstico (Singh et al., 2003). Habitualmente, trata-se de recem-nascido a termo, com distensão abdominal progressiva (63 a 91% dos casos), intolerância às mamadas e vômitos biliosos (19 a 37% dos casos) (Puri & Montedonico, 2008; Haricharan & Georgeson, 2008). O toque retal pode demonstrar reto estreito, com ou sem eliminação explosiva de fezes (Maksoud, 2003). Entretanto, algumas crianças não apresentam obstrução intestinal neonatal. Manifestam clinicamente constipação intestinal grave na infância, que pode evoluir com distensão abdominal crônica, peristaltismo visível, déficit de crescimento e desnutrição (Dasgrupta & Langer, 2004; Puri & Montedonico, 2008). Há pouca resposta ao tratamento clínico, sendo comum a dependência de enemas (Dasgrupta & Langer, 2004).

O diagnóstico deve ser suspeitado em qualquer criança com história de constipação intestinal desde o período neonatal (Puri & Montedonico, 2008). A idade média de diagnóstico tem diminuído progressivamente nas últimas décadas, evidenciando maior conscientização sobre a doença (Dasgrupta & Langer, 2004; Puri & Montedonico, 2008). Um

levantamento de 1979, da seção cirúrgica da Associação Americana de Pediatria, demonstrou o diagnóstico no período neonatal em apenas 8% dos casos. Até os 3 meses de idade, este valor atingia 30% dos casos (Kleinhaus et al., 1979). Por outro lado, estudo australiano do fim da década de 90, demonstrou taxas de até 90% de diagnóstico no período neonatal (Singh et al., 2003). Levantamentos nacionais revelam taxas de 47,2% de diagnóstico neonatal (Villar et al., 2009) e mediana de 8,8 meses para a idade de diagnóstico (Bigélli et al., 2002).

Diarréia, febre e distensão abdominal na criança com aganglionose devem levantar a suspeita diagnóstica de enterocolite, a mais grave complicação clínica relacionada à doença, podendo evoluir com desidratação e sepse (Puri & Montedonico, 2008). A incidência desta complicação varia de 12 a 58% dos pacientes, e pode ocorrer antes ou após o tratamento cirúrgico, conforme as descrições clínicas iniciais de Bill & Chapman (1962). Apresenta infiltrado inflamatório no exame histopatológico da mucosa colônica. Isto pode levar a inflamação transmural e eventual perfuração intestinal (Teitelbaum et al., 1989). Sua etiologia ainda é incerta, mas a estase fecal e o crescimento bacteriano causados pela obstrução intestinal da região agangliônica são fatores predisponentes fundamentais (Bill & Chapman, 1962). *Clostridium difficile* e *rotavirus* são descritos como patógenos associados (Dasgrupta & Langer, 2004). Há evidências de alterações na produção intestinal de mucina e imunoglobulinas nestas crianças, o que estaria associado a alterações na barreira mucosa intestinal, favorecendo a invasão bacteriana (Teitebaum et al., 1989). Como o quadro clínico da doença de Hirschsprung é caraterizado por constipação intestinal, a presença de diarréia nos episódios de enterocolite pode levar a erros diagnósticos (Dasgrupta & Langer, 2004). A incidência de enterocolite pré-operatória é mais alta em pacientes com diagnóstico pós-neonatal, revelando a importância do diagnóstico precoce da doença (Puri & Montedonico, 2008). O tratamento adequado com estabilização hemodinâmica, irrigações retais e

antibioticoterapia são fundamentais para diminuir o risco de óbito (Haricharan & Georgeson, 2008).

O diagnóstico de agangliose colônica raramente é considerado em adultos, porém pode representar até 2% dos pacientes com constipação crônica refratária nesta faixa etária (Chatelain et al., 2006). A forma adulta da doença de Hirschsprung é considerada quando o diagnóstico é estabelecido a partir dos 10 anos (Doodnath & Puri, 2010). Em revisão sistemática da literatura, que levantou dados dos últimos 50 anos, Doodnath & Puri (2010) encontraram relatos de 490 casos diagnosticados nesta faixa etária. A idade de diagnóstico variou dos 11 aos 74 anos, sendo mais comum na terceira década de vida. A maioria dos pacientes apresentava constipação intestinal desde a primeira infância, com pequena melhora após uso de laxativos ou enemas, mantendo este quadro clínico ao longo da vida. Apenas alguns casos apresentaram início dos sintomas na idade adulta. A forma mais comumente encontrada foi a doença confinada ao reto, em 79,8% dos casos, compatível com quadro clínico menos exuberante.

9. Diagnóstico

Frente a um paciente com quadro clínico suspeito para doença de Hirschsprung, evidenciado a partir de anamnese e exame físico completo, inicia-se a investigação diagnóstica.

A radiografia simples do abdome pode revelar distensão gasosa de alças colônicas e o nível da obstrução pode ser inferido pela presença de ar no colon ou reto não-dilatados. Esta imagem pode ser observada na incidência em decúbito dorsal e facilitada pela incidência lateral, com o paciente em decúbito ventral e com as nádegas elevadas. Além disso, tem importante papel na investigação de perfuração intestinal, que constitui grave complicação da aganglionose colônica (Kelleher & Blake, 2008).

Swenson et al. (1949) publicaram técnica para realização de enema opaco, com bário, utilizada até os dias atuais, e que permite a identificação da zona de transição entre o segmento espástico agangliônico e o colon dilatado (Figura 3). Este exame permite avaliar a extensão da zona agangliônica. Apesar de 75% dos neonatos com doença de Hirschsprung apresentarem o cone de transição, a ausência deste sinal não exclui a possibilidade de aganglionose (Dasgrupta & Langer, 2004). Em crianças mais velhas, a ausência da zona de transição ao enema opaco é menos comum, mas pode ocorrer devido a um segmento doente muito curto (Dasgrupta & Langer, 2004). Além disso, até 75% das crianças com aganglionose colônica total apresentam colon de calibre normal (De Lorink et al., 2006). Lavagens e toques retais prévios são fatores que também aumentam a taxa de falsos negativos no enema opaco (De Lorink et al., 2006). Radiografias de retardo, após 24 a 48 horas do exame radiológico inicial podem colaborar para o diagnóstico. Permitem acesso ao bario residual e podem facilitar a identificação da zona de transição (Kelleher & Blake, 2008).

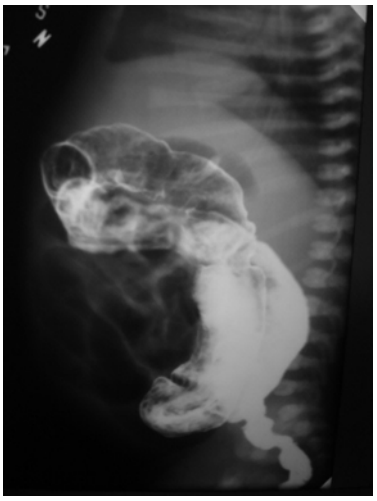

Figura 3 - Enema opaco pela técnica de Neuhauser, evidenciando zona de transição no reto-sigmóide

Outro dado, descrito como sugestivo da doença, é o índice retossigmóide menor que 1,0 (Siegel et al., 1981; Garcia et al., 2007). Este índice mede a razão entre o maior diâmetro do reto e o maior diâmetro do sigmóide. Fornece uma medida objetiva que deve ser utilizada em conjunto com a identificação da zona de transição na investigação radiológica da doença (Garcia et al., 2007).

A manometria anorretal vem sendo utilizada na investigação diagnóstica da doença de Hirschsprung desde a metade do século passado. As descrições iniciais da ausência do reflexo inibitório retoanal nos pacientes portadores de aganglionose colônica (Hiatt, 1951; Callaghan & Nixon, 1964) permitiram a caracterização da manometria como método válido na investigação diagnóstica desta entidade patológica (Lawson & Nixon, 1967).

A demonstração do relaxamento do esfíncter interno após a insuflação de balão com ar no reto simula o reflexo evacuatório. Este processo fisiológico depende da presença de células ganglionares nos plexos nervosos do intestino distal (Holschneider & Steinwegs, 2008). Desta forma, a evidência manométrica da existência deste reflexo exclui a

possibilidade de doença de Hirschsprung. Entretanto, sua ausência não confirma o diagnóstico de aganglionose colônica. A taxa de falsos positivos é muito alta para que este método possa ser utilizado isoladamente para o diagnóstico desta entidade patológica (Martuciello, 2008).

O reflexo inibitório reto-anal pode ser fisiologicamente rudimentar devido à imaturidade neuronal na criança com até 14 semanas de vida (Holschneider & Steinwegs, 2008). Entretanto, estudos recentes demonstraram que a sensibilidade e a especificidade da manometria anorretal são adequadas para a investigação diagnóstica no período neonatal (Huang et al., 2009; Zarabozo et al., 2010). Desta forma, a eletromanometria deve ser considerada como um método de triagem inicial na investigação da doença de Hirschsprung (de Lorijn et al., 2006; Holschneider & Steinwegs, 2008).

O diagnóstico definitivo da doença de Hirschsprung deve ser estabelecido pelo exame histopatológico, que permite visualizar as células ganglionares do sistema nervoso entérico em amostras de tecido obtido por biópsias retais (Dasgupta & Langer, 2004; Kapur, 2009).

Swenson et al. (1955) descreveram a biópsia retal como método preciso, superior ao enema opaco, para o diagnóstico da aganglionose. Estes autores propuseram a realização de biópsias da parede posterior do reto, a 3 cm da linha pectínea, envolvendo a espessura total da parede intestinal. Este procedimento deveria ser conduzido sob anestesia geral e necessitaria de sutura da ferida operatória.

A análise histopatológica deveria compreender a busca por células ganglionares nos plexos intermusculares e a presença destas células excluiria o diagnóstico de doença de Hirschsprung (Swenson et al., 1955). Bodian (1960), concluiu que amostras de tecido menores, envolvendo mucosa e submucosa, seriam suficientes para a pesquisa de células ganglionares nesta entidade patológica. As células ganglionares estariam invariavelmente

ausentes na submucosa do reto distal nos pacientes portadores de aganglionose e as características da submucosa corresponderiam também à ausência de células ganglionares nos plexos mioentéricos.

Este conhecimento foi fundamental para a evolução das técnicas de biópsia retal, com o surgimento de novos métodos, minimamente invasivos, sem necessidade de anestesia geral e realizados em caráter ambulatorial (Shandling, 1961; Dobbins & Bill, 1965). Merecem destaque os trabalhos de Helen Noblett, com a descrição de uma nova pinça de biópsia retal por sucção, que se tornou o instrumento mais utilizado para a investigação diagnóstica da doença de Hirschsprung (Campbell & Noblett, 1969; Noblett, 1969), demonstrado na Figura 4 (A).

Em sua descrição original, Noblett (1969) salientava a importância de alguns aspectos técnicos. Suas biópsias foram realizadas sob pressão negativa de 20 a 25 polegadas de mercúrio, sem lavagem intestinal prévia e com afiamento periódico da lâmina da pinça. As amostras eram consideradas adequadas quando apresentavam ao menos 3,5mm de diâmetro e contemplavam a submucosa, como demonstrado na Figura 4 (B). Todos os 116 espécimes analisados pela autora, em seu trabalho original, foram considerados adequados.

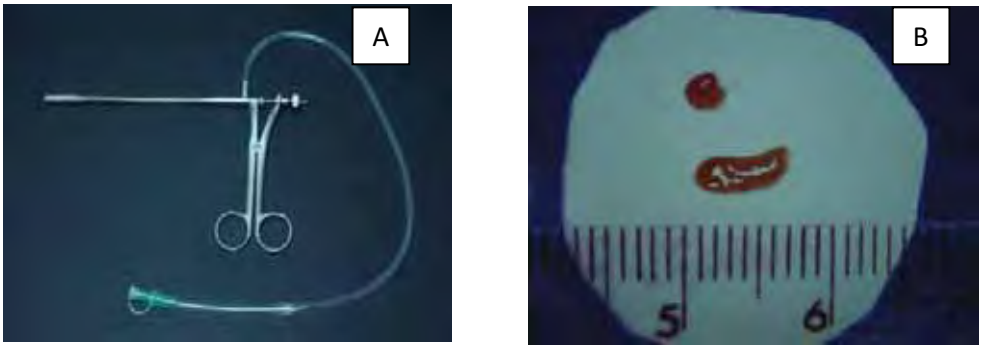

Figura 4 – (A) Pinça de biópsia retal de sucção desenvolvida por Noblett;

(B) Fragmentos adequados obtidos com este método

Entretanto, este não pode ser considerado um método perfeito (Kobayashi et al., 2002). Trata-se de um procedimento realizado "às cegas", o que dificulta a determinação do sítio exato da biópsia e da espessura do fragmento obtido. São descritas possíveis complicações como sangramento massivo (Pini-Prato et al., 2011), perfurações de reto, fibrose peri-retal, celulite em pelve e sepse (Lewis et al., 2003; Hirsch et al., 2011).

Além disso, taxas de insucesso na obtenção de espécimes adequados com este método são relatadas na literatura, e correspondem a 13% (Alizai et al., 1998), 26% (Kobayashi et al., 2002) e 28% (Athow et al., 1990). Nestes casos, os fragmentos não envolvem a submucosa, sendo impossível a exclusão da doença de Hirschsprung (Kobayashi et al., 2002; Ali et al., 2006). Isto leva à necessidade da realização de nova biópsia, habitualmente sob anestesia geral, para a obtenção de fragmentos mais profundos da parede do reto (Gil-Vernet et al., 2006; Hall et al., 2009). Há desta forma, além dos riscos inerentes deste novo procedimento, atraso no diagnóstico e no tratamento, maior ansiedade aos pais e considerável aumento de custos (Ali et al., 2006).

Por tudo isso, há intensa pesquisa por novos métodos de biópsias. A adequação do espécime, contemplando a camada submucosa, constitui o ponto crítico para a análise histopatológica adequada (Alizai et al., 1998). Resultados satisfatórios são descritos com o uso de fórceps de biópsias (Alizai et al., 1998; Kobayashi et al., 2002), com a realização de sucção por máquina de aspiração (Ali et al., 2006), com nova pinça de sucção (Hall et al., 2008) e com auxílio de visão endoscópica e biópsia por fórceps jumbo (Hirsch et al., 2011).

As biópsias retais de sucção geralmente fornecem amostras adequadas de submucosa, quando realizadas em neonatos e crianças mais jovens (Kapur 2009), com limites de idade de 6 meses a 3 anos (Alizai et al., 1998; Croffie et al., 2007). Para crianças mais velhas, há uma maior tendência deste método resultar em espécimes inadequados. Isto pode

ser explicado pela própria evolução da constipação intestinal, que leva ao espessamento fibroso da submucosa devido ao mega-reto (Croffie et al., 2007; Kapur, 2009).

Não existe consenso quanto à padronização do número de fragmentos necessários e dos locais para realização das biópsias retais (Kapur, 2009). Sabe-se que os 2 cm distais do reto são, fisiologicamente, hipogangliônicos (Aldridge & Campbell, 1968). Isto pode levar a falsos diagnósticos de aganglionose e exigem, na prática, análise de múltiplos cortes histológicos na busca por células ganglionares (Kapur, 2009). Desta forma, classicamente, propõe-se a realização de ao menos duas biópsias, que devem obtidas a 2 e 3 cm acima da linha pectínea (Aldridge & Campbell, 1968).

Durante a realização da biópsia retal, a determinação exata do local de biópsia é difícil, em decorrência da manipulação "às cegas" da pinça. Assim, não é incomum, na análise histopatológica, a observação de fragmentos da junção escamo-colunar (Kapur et al., 2009). Presumivelmente, deve ocorrer também desvio no sentido proximal, o que pode dificultar o diagnóstico da forma ultracurta da doença. Por estas razões, recomenda-se a realização de biópsias a 1, 2 e 3 cm da linha pectínea (Kapur, 2009). Meier-Ruge & Bruder (2008) preconizam a obtenção de quatro fragmentos: na linha pectínea (0cm), e a 1, 3 e 6cm acima.

O exame histopatológico pela coloração da Hematoxilina e Eosina (H&E) constitui o método padrão, mais comumente utilizado, e exclui o diagnóstico de doença de Hirschsprung ao evidenciar ao menos uma célula ganglionar na submucosa do reto (Kapur, 2009). Por outro lado, os critérios para o diagnóstico da doença correspondem à ausência de células ganglionares e à presença de hipertrofia de troncos nervosos na submucosa (Knowles et al., 2010). Estas estruturas são fibras nervosas do sistema nervoso extrínseco que estão presentes na submucosa e muscular própria do segmento aganglionar. São aumentadas em

número e possuem diâmetro superior a 40 μm. No entanto, a hipertrofia das fibras nervosas não está presente em alguns dos pacientes portadores da doença sendo, portanto, considerada um marcador com sensibilidade incompleta (Kapur, 2006; Kapur et al., 2009).

Apesar da aparente simplicidade do quadro morfológico, esta análise histopatológica pode se tornar uma experiência estressante, principalmente para patologistas não-especialistas (Kapur, 2006). Algumas situações são críticas nesta análise morfológica, tais como a realização de biópsias retais em regiões muito distais, com pobreza de células ganglionares ou a obtenção de fragmentos de biópsia superficiais, sem envolver a submucosa. Há também dificuldades na identificação precisa das células ganglionares, especialmente em neonatos que apresentam imaturidade do sistema nervoso entérico (Kapur et al., 2009; Guinard-Samuel et al., 2009).

Para garantir especificidade, os fragmentos devem medir cerca de 3 mm de diâmetro, possuir ao menos um terço de submucosa e serem adequadamente orientados no processo de inclusão em parafina, para obtenção de cortes histológicos perpendiculares à mucosa. Devem ser feitos cortes seriados, cuja recomendação de número varia entre os diferentes serviços (Kapur, 2009). A habilidade e a experiência do examinador são fundamentais no diagnóstico da doença de Hirschsprung com este método (Kapur, 2009).

Ao longo de décadas, pesquisadores buscam um método simples, factível e específico para facilitar o diagnóstico da doença de Hirschsprung por meio da análise de biópsias retais de sucção (Kapur, 2006). A atividade da enzima acetilcolinesterase, por método histoquímico, foi demonstrada como evidência indireta da presença de fibras nervosas colinérgicas no segmento agangliônico (Meier-Ruge, 1968). Para isto, fragmentos de biópsias retais a fresco são submetidos a cortes histológicos em criostato (-20ºC) e passam por reações químicas para detectar a atividade da desta enzima (Karnovsky & Roots, 1964; Hanker et al.,

1964; Feichter et al., 2009). Modificações técnicas, objetivando diminuição do tempo para a coloração, foram descritas com resultados adequados (Kobayashi et al., 1994; Martuciello et al., 2001).

O papel desta técnica histoquímica como adjuvante à análise histopatológica convencional foi determinado por Meier-Ruge et al. (1972). Desta forma, a atividade da acetilcolinesterase nos indivíduos normais está praticamente ausente ou é mínima. Por outro lado, as biópsias de pacientes portadores da doença de Hirschsprung demonstram aumento da atividade da enzima nas fibras nervosas parassimpáticas da lâmina própria da mucosa, da muscular da mucosa e na camada circular da muscular própria (Meier-Ruge & Bruder, 2008).

A grande vantagem deste método é que biópsias mais superficiais são adequadas para revelar a atividade da enzima na lâmina própria e na muscular da mucosa, confirmando o diagnóstico de doença de Hirschsprung (Kapur, 2009). Entretanto, a atividade da enzima na lâmina própria da mucosa é fraca em recém-nascidos e em pacientes portadores da forma ultracurta da doença, sendo, portanto, necessário que as biópsias contemplem ao menos a camada muscular da mucosa (Meier-Ruge & Bruder, 2008; Kapur, 2009).

Alguns estudos revelam índices de acurácia diagnóstica de 99 a 100% para a doença de Hirschsprung, tendo como critério o aumento de atividade da acetilcolinesterase nas fibras nervosas da lâmina própria e da muscular da mucosa (Moore & Johnson, 2005). Por outro lado, há relatos com resultados variáveis e de problemas na interpretação deste método (Kapur, 2006). Resultados falsos positivos podem ser justificados pela presença de acetilcolinesterase na membrana celular de hemácias, em casos de espécimes com hemorragia (Moore & Johnson, 2005). Os resultados falsos negativos, no entanto, são os mais amplamente discutidos, atingindo taxas de até 40% (Kapur et al., 2009). A intensidade da atividade da enzima parece aumentar com a idade. Neste sentido, a ausência de reação

positiva para acetilcolinesterase em recém-nascidos, com menos de 3 semanas de idade, não exclui o diagnóstico. Além disso, são descritos padrões de distribuição dos troncos nervosos, evidenciados pela distribuição da reação enzimática nas diferentes camadas da parede retal. Estes padrões apresentam variação direta com a idade. Crianças mais novas, com até 3 meses de idade, apresentam troncos nervosos espessos, que não alcançam a lâmina própria da mucosa, enquanto crianças mais velhas, acima de 1 ano de idade, comumente apresentam troncos nervosos mais finos na lâmina própria da mucosa (Chow et al., 1977; Brito & Maksoud, 1987). Padrões incomuns podem também ser encontrados em aganglionose colônica total e em casos de displasia neuronal intestinal (Moore & Johnson, 2005).

De forma geral, o método histoquímico da acetilcolinesterase é considerado útil no diagnóstico da aganglionose colônica e pode superar as dificuldades relacionadas com amostras superficiais da mucosa retal (Moore & Johnson, 2005; Martucciello et al., 2005; Knowles et al., 2010). Por outro lado, há necessidade de fragmentos a fresco para a obtenção de cortes em criostato e todo um aparato técnico histoquímico para que a investigação da presença da enzima seja realizada (Feichter et al., 2009). Além disso, a existência de diferentes padrões de atividade enzimática exige análise quantitativa e qualitativa, dificultando sua interpretação mesmo para patologistas experientes (Guinard-Samuel et al., 2009; Kapur et al., 2009). Por estas razões, este método não tem sido amplamente utilizado (Kapur et al., 2009).

Por tudo isso, a pesquisa de novos métodos complementares para análise destas biópsias retais teve continuidade (Kapur, 2006). O advento da imunohistoquímica, na segunda metade do século XX, abriu nova perspectiva para a busca de marcadores que facilitariam o diagnóstico da doença de Hirschsprung (Kapur, 2006; Leong et al., 2010). O marcador imunohistoquímico ideal deveria ser de fácil execução e interpretação, a partir de material de

biópsias retais incluídas em parafina e apresentar alta sensibilidade e especificidade, evitando a análise de múltiplos cortes histológicos e a necessidade de biópsias repetidas (Kapur, 2006).

Neste contexto, nas duas últimas décadas, uma série de marcadores histoquímicos e imunohistoquímicos vêm sendo propostos para auxiliar a identificação das células ganglionares ou delinear a natureza das fibras nervosas nas biópsias retais para investigação da doença de Hirschsprung (Holland et al., 2010). Podemos destacar o uso da enolase neurônio-específica (NSE) e da periferina facilitando a identificação das células ganglionares. A proteína S-100 é um marcador que evidencia as fibras nervosas extrínsecas hipertrofiadas no segmento agangliônico (Robey et al., 1998; Kapur, 2006; Holland et al., 2010).

Kapur (2006) analisou os estudos publicados sobre diversos marcadores imunohistoquímicos e concluiu que nenhum deles demonstrou vantagem significativa sobre o estudo histopatológico convencional analisado por examinador experiente. Entretanto, a calretinina, marcador imunohistoquímico utilizado pela primeira vez, neste contexto, por Barshack et al. (2004) pode ser uma exceção (Kapur et al., 2009).

Uma subclasse de corpos celulares de neurônios, presentes nos plexos submucosos e mioentéricos do trato gastrointestinal humano, demonstra imunopositividade para calretinina (Walkers et al., 1993; Wattchow et al., 1997). Este marcador, por sua vez, é uma proteína de 29-kDA, que se liga ao cálcio, dependente de vitamina D, e que possui importante papel como sensor e modulador dos íons cálcio. A ausência desta proteína leva ao acúmulo destes íons no citoplasma das células nervosas, promovendo hiper-excitabilidade e consequente neurodegeneração (Barshack, 2004; Kapur et al., 2009). A calretinina foi identificada na retina de galinhas (Rogers, 1987) e tem sido utilizada por método imunohistoquímico na investigação de neoplasias, principalmente na diferenciação de alguns

tipos de câncer (Gotzos et al., 1996; Doglioni et al., 1996; Papka et al., 1998; Zhang et al., 2003; Fine et al., 2004; Cathro & Stoler, 2005; Portugal & Oliva, 2009).

Em trabalho pioneiro, Barshack et al. (2004) demonstraram a expressão da calretinina nas células ganglionares e em pequenas fibras nervosas intrínsecas nos plexos submucosos e mioentéricos de intestinos normais e em segmentos ganglionares de pacientes portadores da doença de Hirschsprung. Por outro lado, havia perda total da expressão da calretinina em segmentos agangliônicos. Deste modo, concluíram que a perda da expressão da calretinina poderia ser útil na investigação diagnóstica da aganglionose do cólon.

Kapur et al. (2009) publicaram estudo retrospectivo testando o uso da calretinina em comparação com o método histoquímico da acetilcolinesterase na avaliação de biópsias retais para o diagnóstico da doença de Hirschsprung. Os autores observaram expressão da calretinina em segmentos gangliônicos na lâmina própria, muscular da mucosa e nos plexos submucosos e mioentéricos. Muitas vezes as células ganglionares não eram reconhecidas, mas havia a identificação da imuno-reatividade em filetes nervosos da submucosa superficial e da muscular da mucosa, apresentando padrão granular ou "em contas". Os autores concluíram que a identificação da expressão da calretinina nestas fibras nervosas intrínsecas seria a chave para a interpretação imunohistoquímica deste marcador na investigação diagnóstica da doença de Hirschsprung. Além disso, observaram que este novo método possuía acurácia diagnóstica equivalente ou superior à reação da acetilcolinesterase e apresentava um papel informativo na avaliação de biópsias antes consideradas inadequadas, superficiais ou muito distais, próximas da transição ano-retal (Kapur et al., 2009).

Guinard-Samuel et al. (2009), na série envolvendo o maior número de casos de doença de Hirschsprung investigadas pelo método imunohistoquímico da calretinina, analisaram retrospectivamente 131 biópsias retais de sucção, comparando este método

imunohistoquímico com a reação da acetilcolinesterase. Além da acurácia diagnóstica superior, o método da calretinina apresentou como vantagem permitir análise mais simples e clara, inclusive para patologistas com menor experiência na área. Destacaram, também, o fato deste método utilizar o mesmo material da biópsia retal já incluída em parafina, não sendo necessários novos fragmentos à fresco para congelação. Assim, concluíram que a expressão imunohistoquímica da calretinina constitui método valioso para ser utilizado em conjunto com a análise histopatológica padrão (Guinard-Samuel et al., 2009).

Em trabalho mais recente, Holland et al. (2011) novamente caracterizaram o método imunohistoquímico da calretinina como importante na avaliação diagnóstica complementar das biópsias retais de sucção, podendo acrescentar informações principalmente em casos de biópsias superficiais e próximas à transição ano-retal.

10. Tratamento

O tratamento da doença de Hirschsprung é cirúrgico. Entretanto, em algumas situações a estabilização clínica pré-operatória é fundamental, principalmente nos casos de complicações tais como enterocolite e sepse (Dasgupta & Langer, 2004). Neste sentido, alguns pacientes necessitam de reposição hidroeletrolítica, sondagem nasogástrica e irrigações retais antes da realização do procedimento cirúrgico (Maksoud, 2003; Dasgrupta & Langer, 2004).

A evolução das técnicas cirúrgicas apresentou grande avanço nas últimas décadas, permitindo a realização do tratamento cirúrgico em único tempo, mesmo em crianças mais novas (So et al., 1980), sem a necessidade da realização de colostomia primária. Isto diminuiu a morbidade relacionada à colostomia nas crianças, além de demonstrar redução dos custos (Dasgrupta & Langer, 2004). Atualmente a realização de colostomia primária no tratamento destas crianças possui indicações restritas como enterocolite severa, perfuração intestinal, desnutrição grave ou grande dilatação do cólon proximal (Dasgrupta & Langer, 2004).

Todas as técnicas cirúrgicas descritas obedecem como princípios a remoção do segmento agangliônico e a reconstrução do trânsito intestinal, trazendo o segmento ganglionar até o ânus, com preservação da função esfincteriana (Dasgrupta & Langer, 2004).

A primeira técnica cirúrgica foi descrita por Swenson & Bill (1948) e possui como particularidade a inversão e exteriorização do reto, seguida do abaixamento do cólon através do reto invertido, realizando-se a ressecção do segmento agangliônico e anastomose colo-retal término-terminal. Apresentou bons resultados e foi a técnica utilizada em muitos serviços, durante anos (Sherman et al., 1989). Entretanto, dificuldades técnicas foram descritas, principalmente para a realização da dissecção pélvica (Maksoud, 2003).

Duhamel (1956) descreveu a técnica cirúrgica que levou seu nome e logo se tornou o procedimento operacional padrão, em todo o mundo, com excelentes resultados. A técnica consiste no abaixamento do cólon através do espaço retro-retal, sem dissecção das paredes anterior e laterais do reto. O reto é aberto em sua parede posterior, sendo realizado abaixamento do cólon por este orifício com posterior anastomose término-lateral (Duhamel, 1956, 1963). Algumas modificações técnicas foram descritas, entretanto os princípios da cirurgia foram mantidos (Vrsansky et al., 1998).

Outra técnica cirúrgica bastante utilizada para o tratamento da doença de Hirschsprung é a proposta por Soave (1964), e modificada por Boley (1964). Este procedimento objetiva evitar lesões a vasos e nervos da pelve, protegendo o esfíncter interno. Pela via abdominal realiza-se mucosectomia do reto com preservação de seu manguito muscular, e o abaixamento do cólon é realizado por via endo-retal, com anasotomose primária do segmento ganglionar logo acima da linha pectinea.

O abaixamento primário do cólon via endoretal, assistido por videolaparoscopia, desenvolvido por Georgeson et al. (1999), constituiu importante marco na evolução das técnicas cirúrgicas para o tratamento da doença de Hirschsprung. Este procedimento propõe a mobilização do cólon com o auxílio videolaparoscópico e seu abaixamento por dissecção endoanal e com anastomose do segmento gangliônico realizada primariamente. Esta técnica demonstrou resultados adequados, com menor tempo de recuperação pós-operatória (Georgeson et al., 1999; Haricharan & Georgeson, 2008). Desta forma, consolidou novos princípios para o tratamento cirúrgico da aganglionose colônica, como tratamento em tempo único para lactentes e recém-nascidos, através de procedimento cirúrgico minimamente invasivo (Maksoud 2003; Dasgrupta & Langer, 2004).

A ressecção do segmento agangliônico exclusivamente pela via transanal, sem mobilização intra-abdominal foi inicialmente descrita por de La Torre & Ortega-Salgado (1998), e desde então, tornou-se o procedimento de eleição para o tratamento da doença de Hirschsprung. Apresenta inúmeras vantagens que incluem a recuperação pós-operatória mais rápida, com menor tempo de internação hospitalar e melhores resultados estéticos (de La Torre & Langer, 2010).

Estes avanços no tratamento cirúrgico da doença de Hirschsprung, atualmente realizado em tempo único, exigiram precisão diagnóstica ainda mais crucial. O diagnóstico pré-operatório correto por meio da análise das biópsias retais, amplamente discutido, constitui a base para a indicação do tratamento cirúrgico (Barshack et al., 2004). Além disso, no intra-operatório, a identificação da presença de células ganglionares, pela análise histológica imediata de tecido, tornou-se passo fundamental no tratamento cirúrgico, determinando o local seguro para o término da ressecção e realização anastomose endoanal (Shayan et al., 2004; De La Torre & Langer, 2010).

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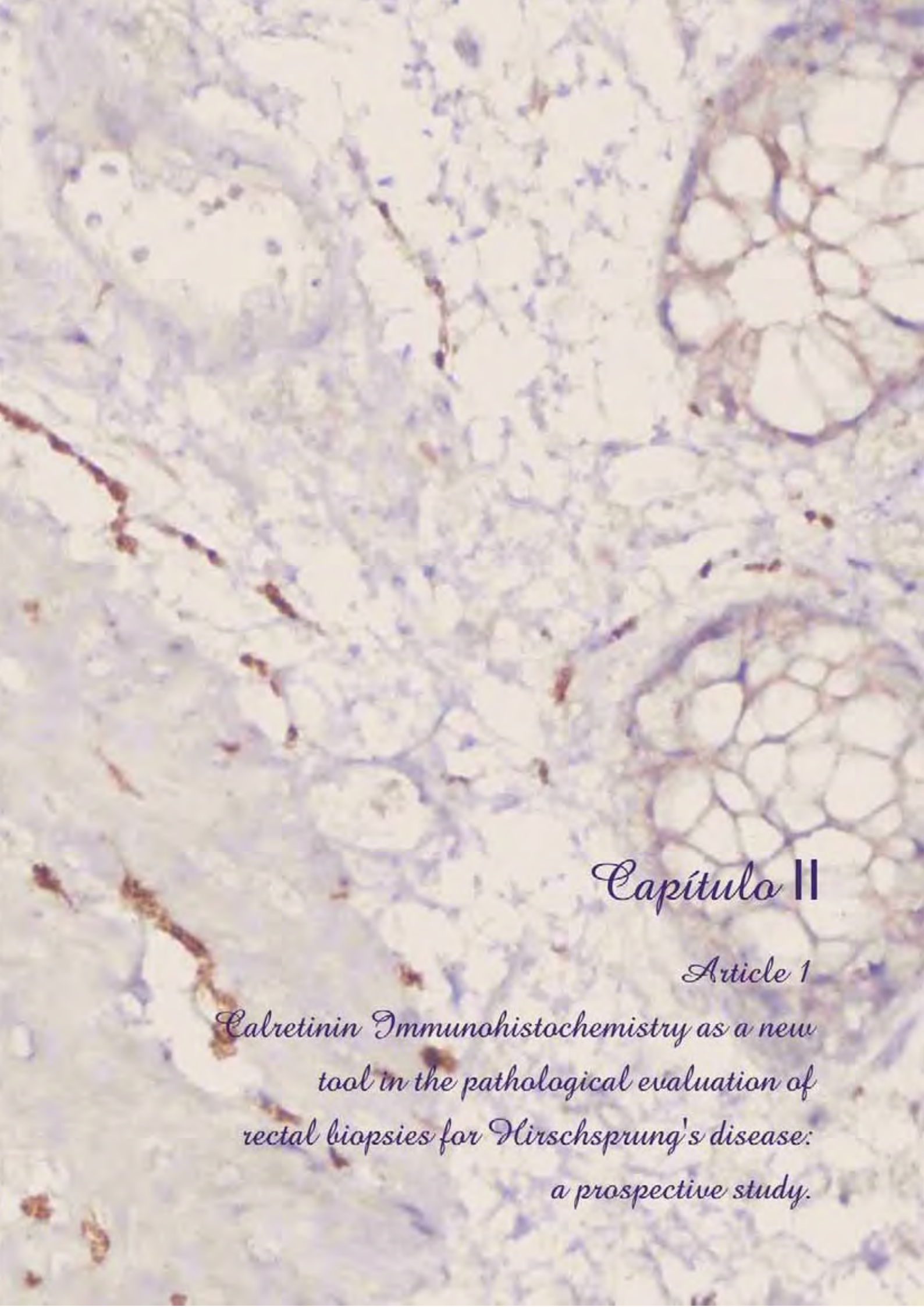

Capítulo II

Article 1

Calretinin Immunohistochemistry as a new tool in the pathological evaluation of rectal biopsies for Hirschsprung's disease: a prospective study.

Abstract

Introduction: Hirschsprung's disease (HD) is a congenital malformation characterized by absence of ganglions cells that coordinate intestinal motility. The gold standard for diagnosis is the histolopathological analysis obtained from rectal suction biopsies. Histochemical and immunohistochemical markers have been proposed to assist the identification of ganglion cells. Calretinin immunoexpression was recently described as new tool to diagnose Hirschsprung's disease. We aimed to investigate, for the first time by a prospective study, the diagnostic accuracy and the applicability of calretinin immunostaining, in the evaluation of rectal biopsies from patients under investigation for Hirschsprung's disease**. Patients and Methods:** We conducted a prospective study from the monitoring of children under investigation for HD, submitted to rectal biopsies. The samples were processed by standard staining Hematoxylin & Eosin (H&E), acetylcholinesterase histochemical staining (Ache) and calretinin immunohistochemistry. When the diagnosis of HD was confirmed, the patients were submitted to surgical treatment. The results obtained with H&E, Ache and calretinin immunohistochemistry were statistically compared, taking the acetylcholinesterase histochemistry as the gold standard. **Results:** In total, 43 patients were submitted to rectal biopsies. Of these, 13 were diagnosed with HD and underwent surgical treatment. The disagreement between the acetylcholinesterase and calretinin methods occurred in only one case, in which the negative result for calretinin was initially performed due to the absence of ganglion cells in the sample. According to the Kappa index, the standard histology (H&E) and the calretinin immunohistochemistry were in good agreement with Ache histochemistry, with statistically significant results. Furthermore, we observed that calretinin presents significant higher specificity and accuracy values than H&E stained slides. The 13 patients, who had received the diagnosis of HD by this biopsy protocol, received the same diagnosis after histopathological analysis of their surgical specimens. **Conclusion:** Calretinin immunohistochemistry is a reproducible modality and may be a useful ancillary technique.

Resumo

Introdução: A doença de Hirschsprung (DH) é uma malformação congênita caracterizada pela ausência de células ganglionares que coordenam a motilidade intestinal. O exame padrão-ouro para o diagnostico é a análise histopatológica de biópsias do reto. Marcadores histoquímicos e imunohistoquímicos vêm sendo propostos para auxiliar na identificação das células ganglionares. Nós decidimos investigar, pela primeira vez por um estudo prospectivo, a aplicabilidade do método imunohistoquímico da calretinina em biópsias do reto de crianças em investigação para DH. Em condições normais, a expressão imunohistoquímica da calretinina ocorre nas células ganglionares e nas fibras nervosas da muscular da mucosa. A ausência de imunoreatividade para calretinina é considerada fator diagnóstico para DH. **Pacientes e métodos:** As amostras foram submetidas à análise histopatológica convencional pela Hematoxilina & Eosina (H&E), à reação histoquímica da acetilcolinesterase (Ache) e ao método imunohistoquímico da calretinina. Quando o diagnostico de DH foi confirmado, os pacientes foram submetidos ao tratamento cirúrgico. Os resultados obtidos pelas três técnicas foram comparados, considerando-se a reação da acetilcolinesterase como padrão-ouro. **Resultados:** No total, 43 pacientes foram submetidos a biópsias retais. Destes, 13 foram diagnosticados com DH e submetidos ao tratamento cirúrgico. A análise pelos métodos da acetilcolinesterase e da calretinina foi discordante em um caso, que apresentou resultado inicialmente negativo para a calretinina, devido à ausência de expressão imunohistoquímica nas células ganglionares na amostra. De acordo com o Índice Kappa, os resultados da análise histopatológica (H&E) e da imunohistoquímica da calretinina concordaram com os resultados da reação da acetilcolinesterase, com adequada significância estatística. Além disso, a análise imunohistoquímica da calretinina apresentou valores significativamente maiores de especificidade e acurácia do que a análise isolada pelo H&E. Os 13 pacientes com diagnóstico

de DH, a partir deste protocolo de análise de biópsias retais, obtiveram o mesmo diagnóstico após a análise histopatológica das peças cirúrgicas. **Conclusão:** O método imunohistoquímico da calretinina é uma modalidade reprodutível e útil como método complementar na avaliação histopatológica de biópsias de reto para investigação da doença de Hirschsprung.

1. Introduction

Hirschsprung's disease (HD) is a congenital malformation characterized by absence of the ganglion cells that coordinate intestinal motility, involving the distal rectum and a variable length of contiguous bowel (Hirschsprung, 1887; Tittel, 1901; Ehrenpreis, 1946; Zuelzer & Wilson; 1948; Bodian et al., 1949). It is an uncommon childhood pathological process (Suita et al., 2005; Puri & Montedonico, 2008), clinically expressed by neonatal intestinal obstruction or severe constipation in toddlers and schoolers (Dasgrupta & Langer, 2004; Masiakos & Ein, 2006).

The diagnosis of HD usually involves a combination of clinical features, anorectal manometry and barium enema (Hiatt, 1951; Swenson et al., 1949; Bodian, 1960; Callaghan & Nixon, 1964; Lawson & Nixon, 1967; Campbell & Noblett, 1969; Dasgupta & Langer, 2004; De Lorink et al., 2006; Holschneider & Steinwegs, 2008). However, a definitive diagnosis is quite entirely based on histological analysis obtained from rectal suction biopsies that sample mucosa and underlying submucosa (Dasgupta & Langer, 2004; Guinard-Samuel et al.; 2009; Kapur, 2009).

Typical histological features of HD include the absence of ganglion cells and increased number of hypertrophic nerves in the submucosal and myenteric nerve plexuses (Whitehouse & Kernohan, 1948). However, the assessment of serial hematoxylin-eosin (H&E) stained slides is complicated with interpretative difficulties including superficial samples without enough submucosa, cases with too distal site of biopsy, where there is physiological paucity of ganglion cells and even problems to identify ganglion cells, particularly in neonates, due to the immaturity of the enteric nervous system (Kapur et al., 2009; Guinard-Samuel et al., 2009).

For these reasons, standard histology is frequently supplemented with acetylcholinesterase histochemical staining (Ache) which demonstrates the presence of this enzyme in cholinergic nerve fibers located at the mucosa and muscularis mucosa of the aganglionic segment (Meier-Ruge, 1968, 1972; Meier-Ruge & Bruder, 2008). The great advantage of this method is that superficial biopsies are adequate to reveal the activity of the enzyme, confirming the diagnosis of HD (Kapur, 2009). However, this technique requires frozen tissue and a complex methodological sequence. Furthermore, the results of Ache assay can be very difficult to interpret, leading to false positive and false negative results (Hamoudi et al., 1982; de Brito & Maksoud, 1987; Athow et al., 1990; Gugelmin & Torres; 2005; Moore & Johnson, 2005; Santos et al., 2008). In spite of this, Ache has been used as an ancillary method in the evaluation of rectal biopsies for diagnosis of HD (Qualman et al., 1999; Martuciello et al., 2005; Knowles et al., 2010).

In this context, over the past two decades, a series of histochemical and immunohistochemical markers have been proposed to assist the identification of ganglion cells or to delineate the nature of nerve fibers in rectal biopsies to diagnosis HD (Holland et al., 2010; Granström et al, 2011; Perurena & Donner, 2012). However, none of these have shown significant advantage over conventional histopathology reviewed by an experienced examiner (Kapur, 2006).

Calretinin, an immunohistochemical marker, can be an exception. A subclass of ganglion cells, present in the submucosal and myenteric plexuses of the human gastrointestinal tract, shows immunopositivity for calretinin (Walkers et al., 1993; Wattchow et al., 1997). This marker is a 29-kDA calcium binding protein, which plays an important role in the organization and functioning of the central nervous system. Its use is already established in the immunohistochemical panel to differentiate some types of cancer (Gotzos et

al., 1996; Doglioni et al., 1996; Papka et al., 1998; Zhang et al., 2003; Fine et al., 2004; Cathro & Stoler, 2005; Portugal & Oliva, 2009).

Barshack et al. (2004) discovered that calretinin was not expressed in aganglionic segments of HD cases, whereas, in normal colon, both ganglion cells and nerve fibers were immunostained by calretinin. Thus, the loss of calretinin expression has been considered a diagnostic criterion for HD (Barshack et al., 2004). Since then, other retrospective studies were published, showing encouraging results for this immunohistochemical marker and suggesting its use as an ancillary method in the evaluation of rectal biopsies from patients under investigation for HD (Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011). Therefore, we decided to investigate, for the first time by a prospective study, the diagnostic accuracy and the applicability of calretinin immunostaining, in association with standard histology (H&E) and acetylcholinesterase histochemistry, during the evaluation of rectal biopsies from patients under investigation for HD.

2. Patients and Methods

2.1. Patients

We proposed to carry out a prospective study conducted from January 2010 to January 2012, from the monitoring of children under investigation for HD, at Botucatu Medical School Hospital, São Paulo State University - Botucatu, São Paulo, Brazil. A total of 83 patients were initially investigated with anorectal manometry and/or barium enema. Of them, 43 patients had results suggestive of HD and were submitted to rectal biopsies. Samples were processed by standard staining with hematoxylin-eosin (H&E), acetylcholinesterase (Ache) histochemical staining and calretinin immunohistochemistry. When the diagnosis of HD was confirmed in rectal biopsy, the patients were submitted to surgical treatment and the colectomy specimens were sent to histopathological analysis. The cases in which rectal biopsies excluded the diagnosis of HD were followed clinically. The study was approved by Botucatu Medical School Hospital Research Ethics Committee (protocol number 3917-2011).

2.2. Rectal Biopsies

Two different techniques were used to perform rectal biopsies. Rectal suction biopsy was the most commonly used method (Noblett, 1969). Full-thickness rectal biopsy (Swenson et al., 1955) was restricted to patients who did not cooperate to rectal suction biopsy (2 cases) or when it provided inadequate samples (4 cases).

Rectal suction biopsies were performed using the apparatus and methods described by Nobllett (1969), as an outpatient procedure, without the need for general anesthesia (Campbell & Noblett, 1969; Noblett, 1969). At least two samples were obtained, between 2 and 3 cm above the dentate line (Aldridge & Campbell, 1968; Kapur, 2009). Full-

thickness rectal biopsies were obtained under general anesthesia, in the operating room, from a surgical excision of the posterior wall, 3cm above the pectinate line (Swenson et al., 1955).

2.3. Sample processing

Fresh samples were kept moistened over a saline soaked filter paper and sent immediately to the Department of Pathology. At this time, they were divided into two fragments: one was fixed in 10% formalin and paraffin embedded for H&E stained slides and calretinin immunohistochemistry and the other was frozen in liquid nitrogen for Ache histochemical reaction.

2.4. Standard Histology Analysis (H&E)

One fragment was placed into 10% buffered formalin and embedded in paraffin, cut and stained with hematoxylin-eosin (H&E) for standard histological observation. Serial H&E sections were analyzed to screen for the presence or absence of ganglion cells, which was considered the diagnostic criteria for this method. Additional sections were requested if no ganglion cells were found (Qualman et al., 1999).

2.5. Acetylcholinesterase Histochemistry (Ache)

Frozen samples were submitted to cryostat sectioning in a plane perpendicular to the mucosal surface. Each block was trimmed until submucosa was visible in the sections and six 12-μm thick sections were collected in three different slides for Ache histochemistry (Meier-Ruge et al., 1972; Moore & Johnson, 2005; Gugelmin & Torres, 2005; Feichter et al., 2009). The Ache was analyzed according to the modified reaction of Karnovsky and Roots (Hanker, 1964; Karnovsky & Roots, 1964).

An increase in Ache activity could be observed in HD. It was expressed by dense brown color in the parasympathetic nerve fibers of lamina propria and muscularis mucosa (Meier-Ruge et al., 1972; Feichter et al., 2009).

2.6. Calretinin Immunohistochemistry

Calretinin immunolocalization was performed on paraffin wax-embedded 4 μm-thick sections, using a rabbit monoclonal antibody (Dako Carpenteria, California, clone DAK Calret 1 –M7245) at 1:100 dilution, for 30 minutes. Antigen retrieval was accomplished with pretreatment of the sections with target retrieval solution Trilogy (cell marquee) using a pressure cooker (Pascal –Dako) at 117 ºC for 30minutes. The slides were successively incubated with biotinylated secondary antibody horse anti-rabbit (Vector Laboratories, Inc., Burlingame, CA, USA) at a 1:200 dilution for 60 minutes and avidin-biotin-horseradish peroxidase solution (Vector Laboratories, Inc., Burlingame, CA, USA) at 1:1:50 dilution for 45 minutes at room temperature. Chromogen color development was accomplished with 3,3´-diaminobenzidine tetrahydrochroride (DAB, Sigma-Aldrich Co., St. Louis Mo, USA) as the substrate to demonstrate the sites of peroxidase binding. The slides were counterstained with Harris´s hematoxylin.

Calretinin immunohistochemistry was considered positive if a brown staining was present in ganglion cells and intrinsic nerve fibers, in the submucosal, muscularis mucosa or lamina propria. The absence of immunoreactivity for calretinin was considered diagnostic for HD (Barshack et al., 2004; Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011).

2.7. Diagnostic conclusion and Surgical Procedure

The histopathological analysis was performed by at least two experienced pathologists. The interpretation for H&E and calretinin immunostaining was performed first, followed by the Ache histochemistry analysis. Surgical decision was based on the analysis of this panel of histopathological results. Patients diagnosed with HD were submitted to surgical treatment. The Transanal Endorectal Pull-through Technique (de La Torre-Mondragon & Ortega-Salgado, 1998) was performed in 12 cases and the Martin surgical technique (Martin, 1972) was used in a patient with total colonic aganglionosis. All procedures were carried out by the same group of pediatric surgeons.

2.8. Processing and analysis of the surgical specimens

Immediately after surgery, the specimen was sent fresh to the Department of Pathology, opened along the antimesenteric border, washed with water, placed on a hard surface to keep its shape, and immersed in 10% formalin**.** After 24 hours, a longitudinal strip was cut along the specimen. This strip was cut into small pieces, about 2 cm each, and placed on numbered cassettes. The material was processed, embedded in paraffin and stained with H&E. Histopathological analysis was performed by at least two experienced pathologists and the entire length of the resected bowel was analyzed. The extent of the aganglionic segment, the transition zone and the dilated proximal colon were determined.

2.9. Statistical Analyses

Statistical analysis was performed using the software SPSSv15.0. Kappa index was applied to assess the agreement between H&E and calretinin immunohistochemistry with Ache histochemistry, considered as the gold standard. The value for accuracy, specificity, sensibility, positive predictive value and negative predictive value were also estimated and compared (Fisher's exact test).

3. Results

3.1 Patients

A total of 83 patients were initially investigated with anorectal manometry and/or barium enema as screening tests. Of them, 43 patients were submitted to rectal biopsies. They were 27 boys (62.8%) and 16 girls (37.2%). According to the age distribution, they presented a median of 35 (1/195) months. The distribution of patients, according to the age groups, is shown in Figure 1.

HD was diagnosed in 13 (30.2%) of these 43 patients. They were 9 boys (69.2%) and 4 girls (30.8%). The diagnosis of HD was performed during the neonatal period in only 4 cases (30.7%).

3.2 Histopathological Analysis of Rectal Biopsies

3.2.1 Standard Histology (H&E)

The analysis of serial H&E stained slides did not reveal the presence of ganglion cells in 24 of 43 cases (55.8%). Ganglion cells, as seen in Figure 2, were identified in the submucosal of rectal biopsies in 19 cases (44.2%), thus excluding the diagnosis of HD.

3.2.2 Ache Histochemistry

In 13 of 43 cases (30.2%) rectal biopsies stained with Ache histochemistry were diagnostic for HD. In all of these cases, the positive reaction for increased Ache activity was demonstrated in nerve fibers strongly stained in dark brown to black, within the muscularis mucosa and extending up to the lamina propria, as shown in Figure 3(A). In another 30 cases (69.8%) the negative reaction for Ache activity was considered due to the absence or still little enzyme activity within the muscularis mucosa, as seen in Figure 3(B).

3.2.3 Calretinin Immunohistochemistry

Absence of immunoreactivity for calretinin was found in 14 of 43 cases (32.6%), which was considered diagnostic for HD, as shown in Figure 4. The other 29 cases (67.4%) presented calretinin immunostaining, thus excluding the diagnosis of HD. Typical positive staining in non-HD patients consisted of intense granular staining of intrinsic nerve fibers in the upper submucosa and the lamina propria or muscularis mucosa (Figure 5). Calretinin immunostaining allowed the identification of ganglion cells in the submucous plexus, as seen in Figure 6.

3.3 Correlation between the histopathological methods

The disagreement between standard histopathology (H&E) and acetylcholinesterase (Ache) occurred in 11 cases, in which the negative results for H&E were due to the absence of ganglion cells in tissue samples (Table 1). The disagreement between the Ache and calretinin methods occurred in only one case, in which the negative result for calretinin was initially performed due to the absence of ganglion cells in these samples. The second analysis of calretinin immunostaining, in this case, revealed the presence of a slightly immunoreativity in focal intrinsic nerve fibers within the muscularis mucosa and lamina propria, thus excluding the diagnosis of HD (Table 2).

According to the Kappa index analysis, standard histology (H&E) and calretinin immunohistochemistry were in good agreement with Ache histochemistry, emphasizing that calretinin immunostaining presents an almost perfect agreement value ($k=0.946$; $p<0.001$; Landis & Koch, 1977), as shown in Table 3. Furthermore, comparing the values of specificity and accuracy between calretinin immunohistochemistry and H&E stained slides, by the Fisher's Exact Test, we observed that calretinin presented significantly higher specificity (96.7 x 63.3; $p=0.002$) and accuracy (97.6 x 74.4; $p=0.003$) values than H&E.

3.4 Colectomy analysis

The histopathological analysis of the surgical specimens confirmed the diagnosis of HD in all the 13 patients. The presence and length of the aganglionic segment, the transition zone and the dilated proximal colon was determined and HD cases were classified: seven patients (53.8%) presented the rectosigmoid-segment disease, 4 patients (30.7%) had the aganglionic segment restricted to the distal rectum, characterizing the short-form of HD, 1 patient (7.6%) had a long form of HD, with the transition zone at the colonic splenic flexure and one patient (7.6%) presented a total colonic aganglionosis (Haricharam & Georgeson, 2008; Puri & Montedonico, 2008).

4. Discussion

In the present study, the diagnosis of HD was investigated during a period of 24 months. The patients were first submitted to anorectal manometry and/or barium enema, as screening tests (De Lorink et al., 2006). Those who had results suggestive for HD were submitted to rectal biopsies. In total, 43 patients were investigated by rectal biopsies, and 13 of these were diagnosed with HD and underwent surgical treatment. The ratio of neonatal diagnostic was 30.7%, which is markedly lower than other published studies, such as 48.7% (Ikeda & Goto, 1984) and 90.5% (Singh et al., 2003). We have also noted that only 8 of the 43 patients investigated (18.6%) were younger than a month, thus showing that patients come late for the investigation of this organic disease. Furthermore, 4 of the 13 patients with HD (30.7%) were older than 60 months (Figure 1). Two of these can be classified as HD adult cases (Doodnath & Puri, 2010). They were two girls, with 142 and 192 months of age, who presented the short form of HD, the most common in this age group (Doodnath & Puri, 2010). These findings lead us to conclude that, in the setting of our study, in a developing country like Brazil, there is still little awareness about HD, which causes delay in its diagnosis and treatment.

Histopathological examination by H&E staining is the most commonly used method, and excludes the diagnosis of HD by showing at least one ganglion cell in the submucosa of the rectum (Whitehouse & Kernohan, 1948; Martuciello et al., 2005). Despite the apparent simplicity of the morphologic features, this histopathological method presents some difficulties, leading to the need for analysis of multiple serial sections, whose number varies between different protocols (Qualman et al., 1999; Kapur, 2009). The skill and

experience of the examiner are considered as key factors for the pathological diagnosis of HD with the standard H&E method (Kapur, 2009).

Ache histochemistry has been established as an ancillary method for the diagnosis of HD (Meier-Ruge, 1972). It demonstrates the enzyme activity in parasympathetic nerve fibers on the lamina propria and muscularis mucosa in the aganglionic segment (Meier-Ruge & Bruder, 2008). In this way, the main advantage of this method is the ability to analyze superficial fragments without the presence of ganglion cells (Moore & Johnson, 2005; Martucciello et al., 2005; Knowles et al., 2010). However, technical difficulties in its processing and interpretation can lead to high rates of false-negative results, thus explaining why Ache histochemistry, despite its well-established use, has not been considered as an ideal method for the diagnosis of HD (Hamoudi et al., 1982; Athow et al., 1990; Gugelmin & Torres; 2005; Moore & Johnson, 2005; Santos et al., 2008; Guinard-Samuel et al., 2009; Kapur et al., 2009).

All these methodological limitations are responsible for the high rates of repeated rectal biopsies, leading to increased costs and risks, and delay in diagnosis and treatment of HD (Ali et al., 2006; Kapur, 2006; Guinard-Samuel et al., 2009; Pini-Prato et al., 2011). In this context, new ancillary methods for the pathological diagnosis of HD are currently being researched. Barshack et al. (2004) described their experience with calretinin, a useful neural immunostaining method. They concluded that the loss of calretinin expression indicates aganglionosis in HD. This method has been considered as a new diagnostic tool for HD and was submitted to investigation in other retrospective studies (Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011).

We investigated, for the first time by a prospective series, the applicability and diagnostic accuracy of calretinin immunostaining, in association with the standard

histopathology (H&E) and the Ache histochemistry, in the histopathological evaluation of rectal biopsies for the diagnosis of HD. One advantage of this immunohistochemical method is that it is applied to sections from the same paraffin-embedded tissue used for standard H&E sections, which, unlike Ache histochemistry, does not require a frozen specimen. This represents an important technical facility that can provide a greater acceptance of this method. Furthermore, the results obtained with the standard histology and the calretinin immunostaining can be directly correlated.

Calretinin immunostaining facilitates the identification of ganglion cells in the submucosa and reveals the presence of intrinsic nerve fibers, showing a granular pattern in the lamina propria, muscularis mucosa and submucosa, which allows that superficial samples can be useful to exclude the diagnosis of HD (Kapur et al., 2009; Holland et al., 2011; Melendez et al., 2012). In the present study we have found calretinin immunoreactivity in 29 of the 43 cases analyzed. Of these, just in 19 cases, H&E stained sections demonstrated the presence of ganglion cells, which explains the higher values of specificity (96.7% x 63.3%, p=0.002) and accuracy (97.6% x 74.4%, p=0.003) of the calretinin immunohistochemistry when compared with the standard histopathological analysis by H&E. These 29 cases also presented absence of activity for Ache, allowing to rule out for the diagnosis of HD.

We observed 14 cases with loss of the calretinin expression. Of these, 13 patients presented a positive Acetylcholinesterase histochemistry reaction. Acetylcholinesterase assay is an excellent method to confirm the diagnosis of HD, by showing the presence of this enzyme in hypertrophied nerve fibers in the lamina propria. The case with disagreement between these two methods was considered as a false-positive for the calretinin immunohistochemistry. This case was from a 3 months old girl who presented intestinal constipation since birth. The barium enema showed a mega-rectum and rectoanal inhibitory

reflex was not detected by anorectal manometry. The sample which had been obtained from a rectal suction biopsy was considered appropriate, involving one third of the submucosa, and the histopathological standard analysis (H&E) did not reveal the presence of ganglion cells. Calretinin immunostaining was initially considered as negative, since ganglion cells were absent in the sample. However, Ache histochemistry did not show activity for this enzyme. In the review of the calretinin immunohistochemistry slides, we noted the presence of a slight granular staining in the lamina propria, which had been missed in the first analysis. Thus, the diagnosis of HD was excluded and clinical follow up was performed with adequate results, in a period of 14 months.

Therefore, the interpretation of calretinin immunohistochemistry should be performed carefully. Several reports already emphasized the possibility of false-negative results due to confounding factors, such as calretinin immunoreactivity present in mast cells and in deeper hypertrophic extrinsic nerves, typically found in HD (Barshack et al., 2004; Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011). This was not observed in our series of cases. However, as described, we had a false-positive result, attributed to the absence of immunostaining in ganglion cells in the submucosa. In this case, Ache histochemistry was very important since its negative reaction was in disagreement with a calretinin negative result, leading us to review the slides, avoiding a false positive case. Thereby, we learned that the interpretation of calretinin immunohistochemistry is not so easy and simple, as described in previous reports (Barshack et al., 2004; Guinard-Samuel et al., 2009). Thus, we believe that the association between these two ancillary methods, adding up their information, should constitute a useful panel for the histopathological diagnosis of HD in rectal biopsies.

It is well known that the Ache histochemistry requires frozen samples and depends on a complex technical apparatus which limits its use for only a few specialized laboratories (Qualman et al., 1999; Holland et al., 2011). In face of it, H&E stained slides remains the most widely used technique during the analysis of rectal biopsies for HD worldwide. In our series, we observed that Calretinin immunostaining presented an almost perfect agreement with Ache histochemistry ($k=0.946$; $p<0.001$). Furthermore, calretinin immunostaining presented higher specificity and accuracy values than H&E stained slides. Therefore, we also believe that this ancillary method may be considered as an alternative to assist the histopathological evaluation of rectal biopsies, in those services that do not perform Ache histochemistry.

In conclusion, the results of the present study reinforced the usefulness of calretinin immunohistochemistry in the diagnostic work up for HD in rectal biopsies.

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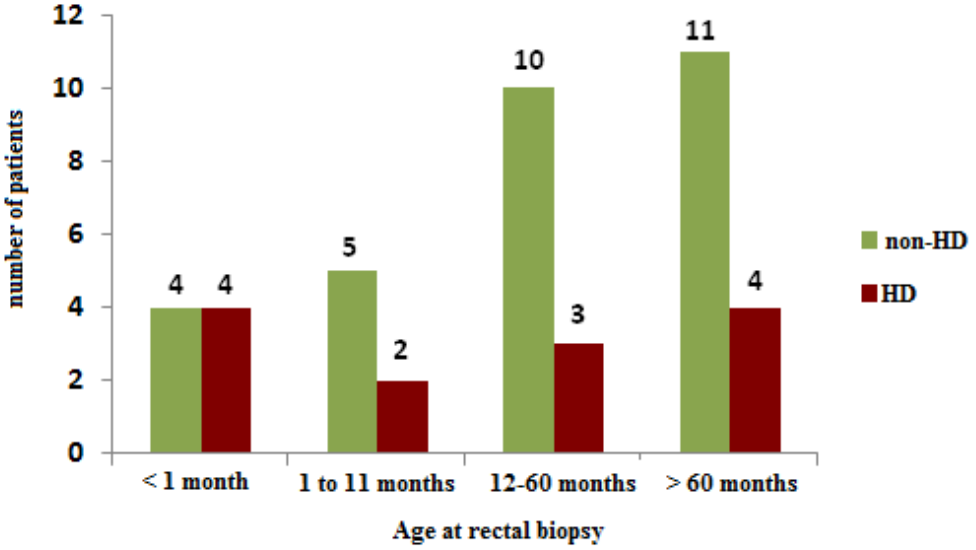
6. Figures and Tables



Non-HD = non- Hirschsprung's disease, HD= Hirschsprung's disease, n= number of patients

Figure 1 – Age groups (in months) of the 43 patients submitted to rectal biopsies to investigate Hirschsprung's disease.

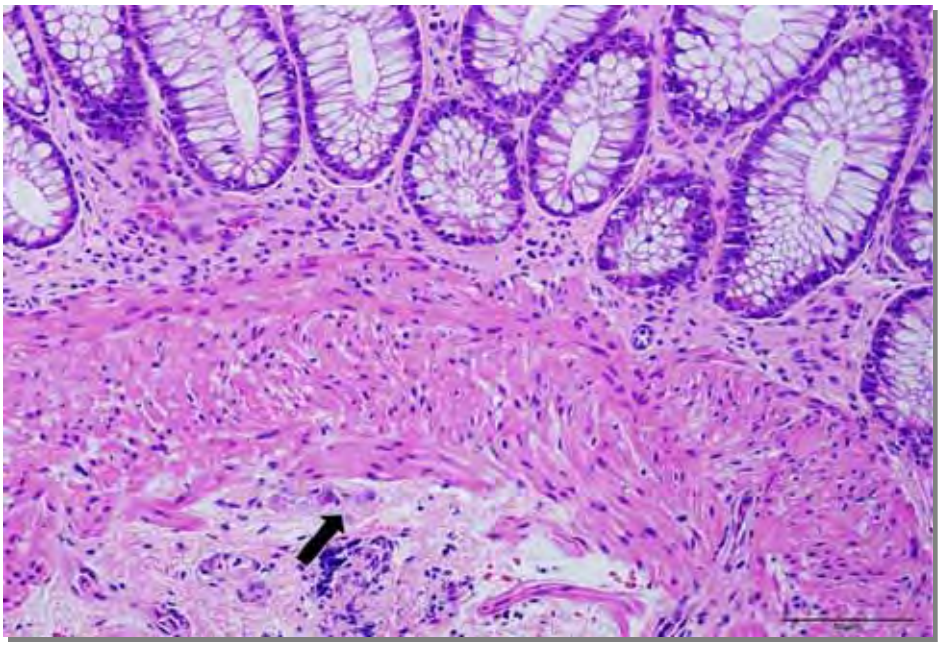


Figure 2 – H&E stained slide revealing the presence of ganglions cells in the submucosa (arrow), excluding the diagnosis of Hirschsprung's disease (400x).

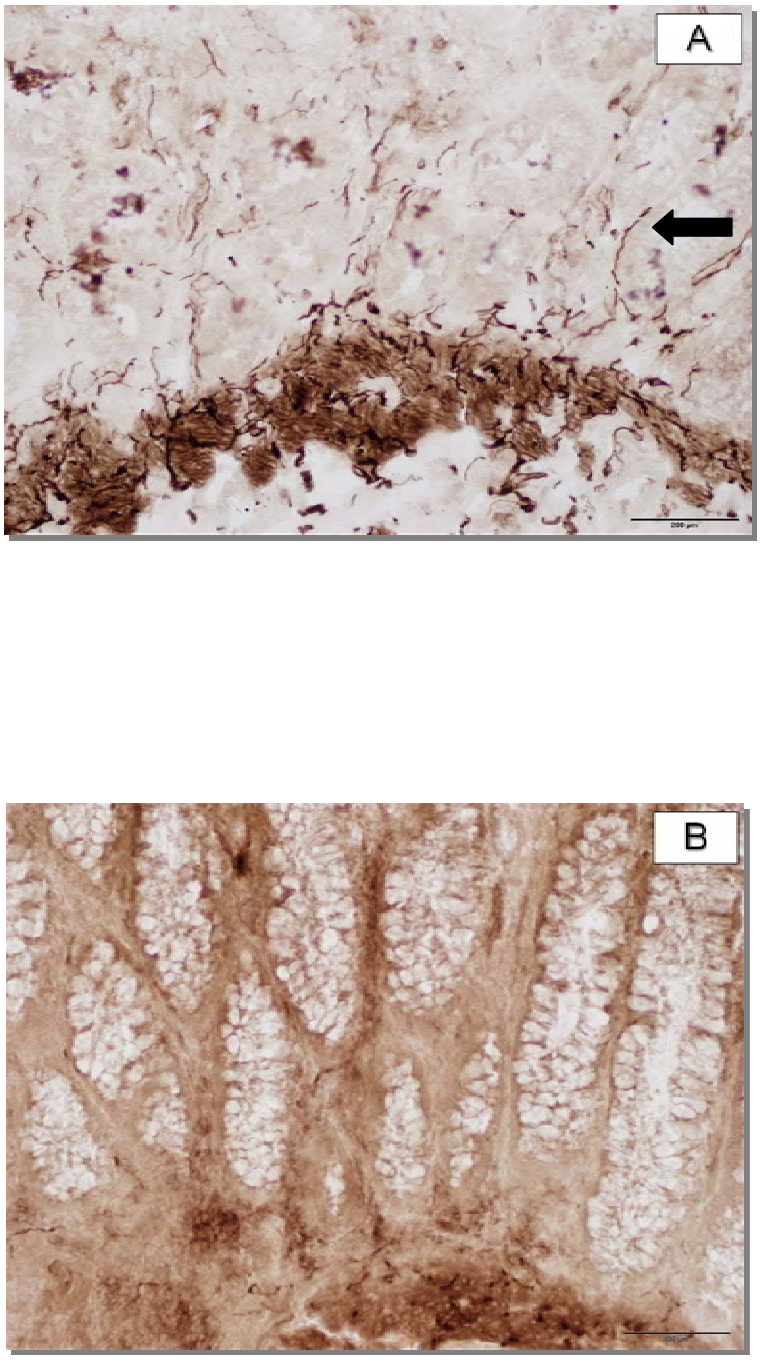


Figure 3 – Acetylcholinesterase histochemical staining (A) Hirschsprung's disease – positive reaction showing numerous thick and irregular dark brown nerve fibers within muscularis mucosa and extending up into the lamina propria (arrow) (200x); (B) Normal bowel - little enzyme activity within the muscularis mucosa, excluding the diagnosis of Hirschsprung's disease (200x).

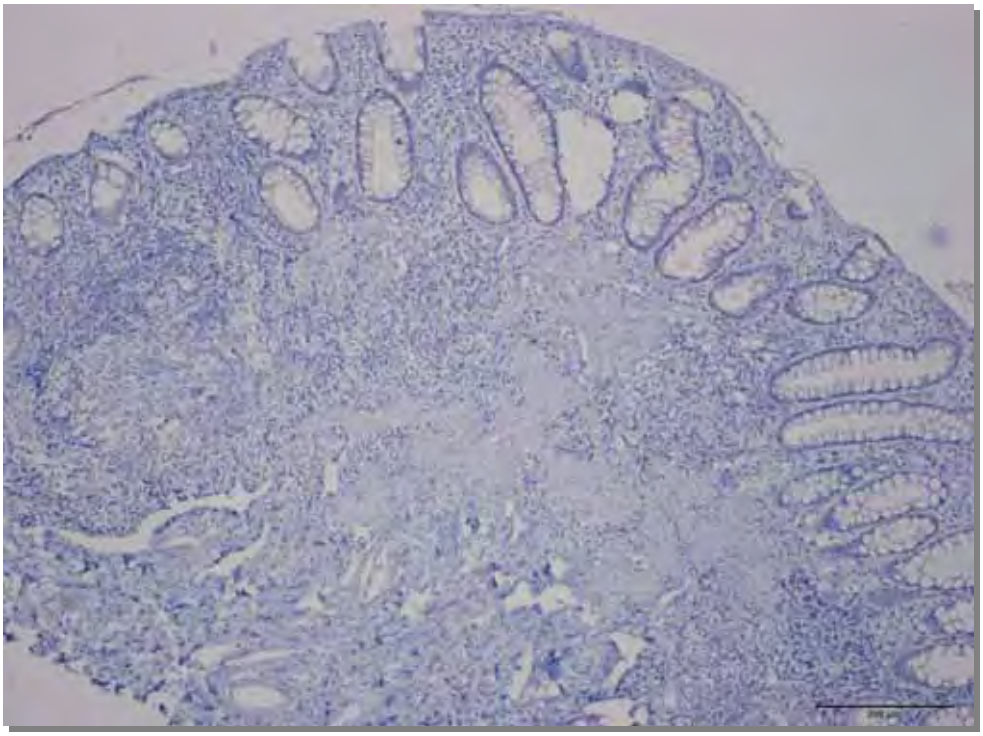


Figure 4 – Calretinin immunohistochemistry in Hirschsprung's disease: negative staining for intrinsic nerve fibers and ganglion cells (100x).

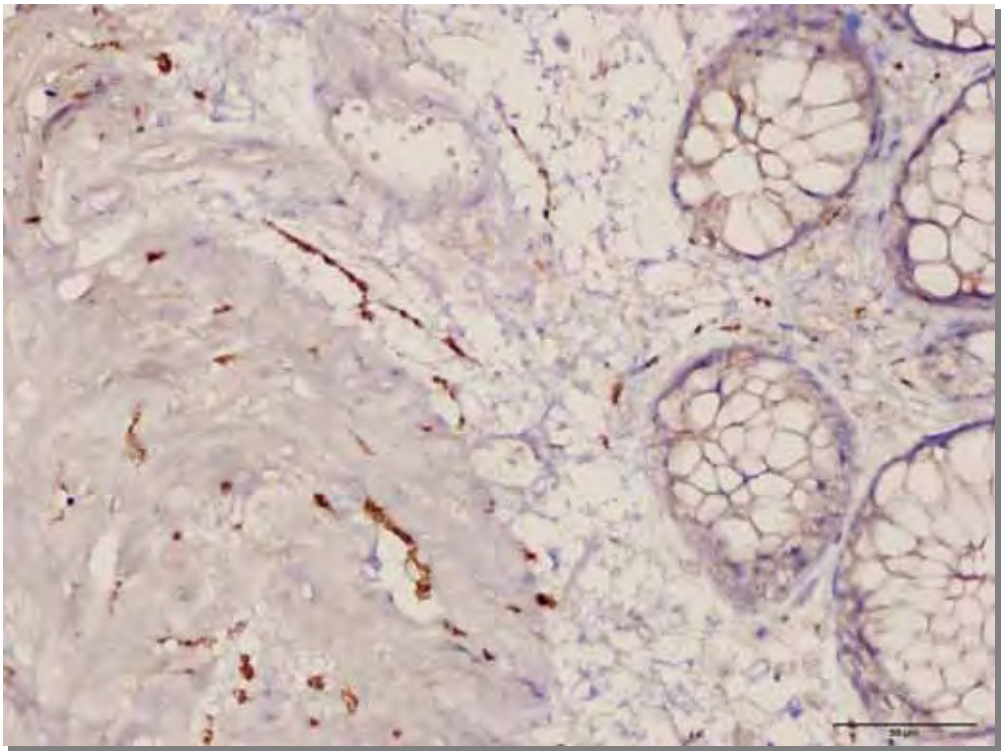


Figure 5 – Calretinin immunoreactivity showing intense granular staining of intrinsic nerve fibers in the muscularis mucosa and lamina propria, excluding the diagnosis of Hirschsprung's disease (400x).

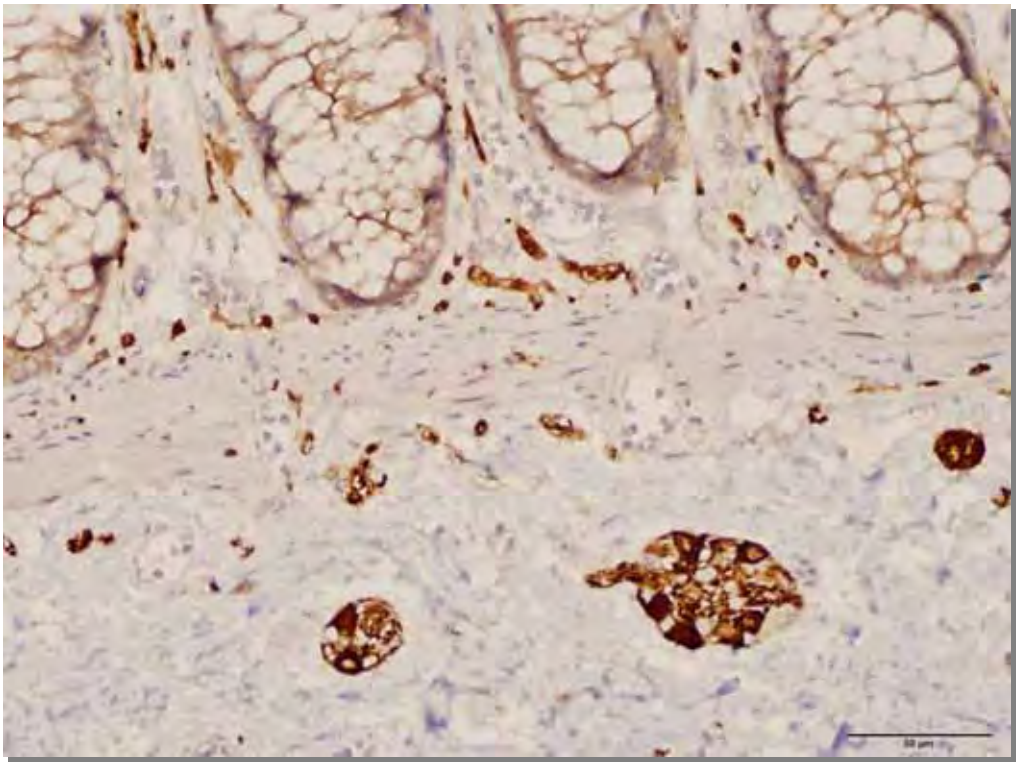


Figure 6 – Calretinin immunoreactivity in submucosal ganglion cells and intrinsic nerve fibers, excluding the diagnosis of Hirschsprung's disease (400x).

Table 1 – Correlation between acetylcholinesterase histochemistry (Ache) and Standard Histopathology (H&E) in rectal biopsies of children under investigation for Hirschsprung's disease

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

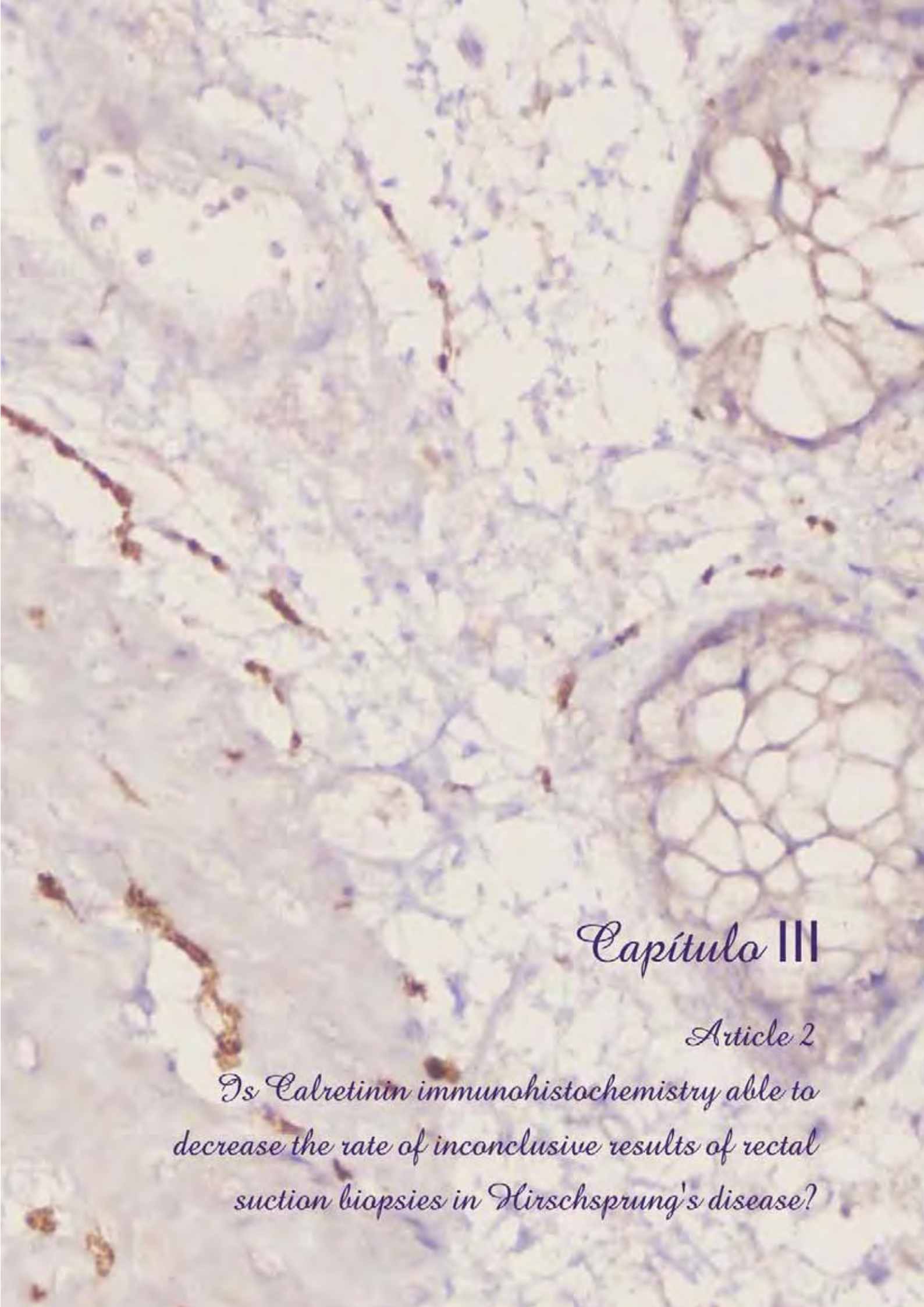
Table 2 - Correlation between acetylcholinesterase histochemistry (Ache) and Calretinin immunohistochemistry in rectal biopsies of children under investigation for Hirschsprung's disease

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

Table 3 – Comparison of the tests against acetylcholinesterase histochemistry

Method	Ac [1]	St[2]	Sp[3]	PPV[4]	NPV[5]	Kappa[6]	p[7]
H&E	74.4	100.0	63.3	54.2	100.0	0.511	<0.001
Calretinin	97.6	100.0	96.7	92.9	100.0	0.946	<0.001

(1) Accuracy (2) Sensitivity (3) Specificity (4) Positive predictive value (5) Negative predictive value (6) Kappa Index for agreement statistics (7) p-value associated with Kappa Index



Capítulo III

Article 2

Is Calretinin immunohistochemistry able to decrease the rate of inconclusive results of rectal suction biopsies in Hirschsprung's disease?

Abstract

Introduction: Hirschsprung's disease (HD) is a developmental disorder of the enteric nervous system, characterized by absence of the ganglion cells along the distal rectum and contiguous bowel. The gold standard for diagnosis is the histopathological analysis obtained from rectal suction biopsies. However, many difficulties occur during this evaluation. Inadequate biopsy samples and interpretative details and pitfalls in the histopathological analysis are associated with inconclusive results. We decided to investigate whether the introduction of a new ancillary method, the calretinin immunohistochemistry, in the diagnostic panel composed by Hematoxylin & Eosin (H&E) and Acetylcholinesterase histochemistry (Ache) is able to decrease the rate of inconclusive results. **Patients and Methods:** We analyzed data from patients undergoing rectal suction biopsies, before and after the introduction of calretinin immunohistochemistry in the diagnostic work up for HD. The rate of biopsies without a conclusive diagnosis was determined. **Results:** Data from 82 patients were analyzed, 41 in each series. The failure rate obtained in the second series of cases, after the introduction of calretinin immunohistochemistry was 11.9%, significantly lower than that observed in the first series of cases of 37.8%, using only H&E and Ache histochemistry. **Conclusion:** The introduction of calretinin immunohistochemistry in the diagnostic work up for the histopathological analysis proved to be able to decrease the rate of inconclusive diagnosis in rectal suction biopsies for HD.

Resumo

Introdução: A doença de Hirschsprung (DH) é uma malformação congênita do sistema nervoso entérico, caracterizada pela ausência de células ganglionares a partir da região distal do reto. O padrão-ouro para seu diagnóstico é a análise histopatológica de amostras obtidas por biópsias de sucção do reto. Entretanto, muitas dificuldades podem ocorrer durante esta avaliação. Amostras inadequadas, assim como detalhes e armadilhas próprias da análise histopatológica estão associadas a altas taxas de resultados inconclusivos. Nós decidimos investigar se a introdução de um novo método auxiliar, a análise imunohistoquímica da calretinina, no painel de diagnóstico histopatológico destas biópsias retais, composto pela coloração padrão da Hematoxilina & Eosina (H&E) e pela pesquisa da reação histoquímica da acetilcolinesterase (Ache), é capaz de diminuir o percentual de resultados inconclusivos. **Pacientes e métodos:** Foram analisados dados de pacientes submetidos a biópsias de sucção do reto, antes e após a introdução da imunohistoquímica da calretinina no painel diagnóstico para DH. A taxa de biópsias sem resultado conclusivo foi determinada. **Resultados:** Os dados de 82 pacientes foram analisados, 41 em cada série de casos. A taxa de falha obtida na segunda série de casos, após a introdução da calretinina no painel de análise foi de 11,9%, significativamente menor do que a observada na primeira série de casos de 37,8% que utilizou apenas H&E e Ache. **Conclusão:** A introdução do método imunohistoquímico da calretinina no painel de investigação histopatológica foi capaz de diminuir a taxa de resultados inconclusivos das biópsias de sucção do reto para o diagnóstico da DH.

1. Introduction

Hirschsprung's disease (HD) is a developmental disorder of the enteric nervous system, characterized by absence of the ganglion cells in the myenteric and submucosal plexuses along the distal rectum and contiguous bowel (Hirschsprung, 1887; Tittel, 1901; Ehrenpreis, 1946; Zuelzer & Wilson, 1848, Bodian et al., 1949). Since the 1950s, rectal suction biopsy and its histopathological analysis were established as the methods of choice for definitive diagnosis of HD (Swenson et al., 1955; Bodian, 1960, Dobbins & Bill, 1965; Campbell & Noblett, 1969).

Typical histological features of serial Hematoxilin-Eosin (H&E) stained slides include the absence of ganglion cells and increased number of hypertrophic nerves in the rectal submucosa (Whitehouse & Kernohan, 1948). Meier-Ruge et al. (1972) reported the use of Acetylcholinesterase histochemistry (Ache) as an adjuvant method for the histopathological diagnosis of HD, by revealing the presence of this enzyme in cholinergic nerve fibers in the lamina propria and muscularis mucosa of the aganglionic segment. Nowadays, the association between H&E and Ache histochemistry has been considered the gold standard histopathological method for the diagnosis of HD (Qualman et al., 1999; Martuciello et al., 2005; Feichter et al., 2009).

However, in practice, many difficulties occur during the histopathological evaluation (Kapur et al., 2009). Inadequate biopsy samples, involving too small and superficial specimens, or distal biopsies, too close to the pectinate line, are associated with inconclusive results (Ghrosh & Griffiths, 1998; Qualman et al., 1999; Guinard-Samuel et al; 2009). Other possible factors, such as biopsy forceps model and the age of the patient at time of biopsy, can exert some influence on the failure rates of HD diagnosis (Alizai et al., 1998;

Croffie et al., 2007). Moreover, the histopathological analysis involves interpretative details and pitfalls which are responsible for false-positives and false-negatives diagnosis (Athow et al., 1990; Qualman et al., 1999).

Several modifications for rectal biopsies techniques have been proposed revealing highly variable results (Shandling & Auldist, 1972; Pease et al., 1976; Weintraub et al., 1977; Hirose et al., 1993; Scmittenbecher et al, 1995; Alizai et al, 1998; Kobayashi et al, 2002; Ali et al, 2006; Pini-Prato et al, 2006; Croffie et al, 2007; Hall et al, 2009; Campeotto et al, 2011; Hirsh et al, 2011). On the other hand, a serial of histochemical and immunohistochemical markers have been tested as new ancillary methods for the histopathological diagnosis of HD (Kapur, 2006; Holland et al, 2010). In recent retrospective series, promising results have been reported using the immunohistochemical expression of calretinin in the search for ganglion cells and intrinsic nerve fibers in the diagnostic investigation for HD (Barshack et al., 2004; Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011).

We have recently introduced calretinin immunohistochemistry in the diagnostic panel composed by H&E and Ache histochemistry, in the histopathological evaluation of rectal suction biopsies for HD (Knowles et al, 2010). So, we decided to investigate whether the introduction of this new immunohistochemical method is able to decrease the rate of inconclusive results.

2. Patients and Methods

Patients were selected from the Pediatric Surgery Outpatient and Pathology Department records at Botucatu Medical School Hospital, São Paulo State University - Botucatu, São Paulo, Brazil. They were children presenting with bowel obstruction or severe chronic constipation, initially investigated with anorectal manometry and/or barium enema as screening methods for the diagnosis of Hirschsprung's disease (HD) (de Lorijn et al., 2006). Those patients who presented results suggestive of HD were submitted to rectal biopsies.

We have analyzed data from patients undergoing rectal suction biopsies (Noblett, 1969), before and after the introduction of calretinin immunohistochemistry in the diagnostic work up for HD. From March 2004 to June 2007, histopathological diagnosis was performed taking into account H&E and Acetylcholinesterase histochemistry (Ache) analysis. Since January 2010 to January 2012, calretinin immunohistochemistry was added to this panel for the histopathological evaluation of HD. The rate of biopsies without a conclusive diagnosis was determined, as well as the causes for these diagnostic failures. Inconclusive cases led to repeated rectal biopsies, which were performed by the same suction method or by open rectal surgical full-thickness procedure (Swenson et al., 1955). Only patients with rectal suction biopsies were included in the present investigation.

It was also taken into account the age of the patient at biopsy and the frequency of complications. For further characterization, the patients were allocated into 3 age-related groups: group A, younger than 12 months-old; group B, patients aged 12 to 36 months-old and group C, older than 36 months-old.

Rectal suction biopsies were performed using the apparatus and method described by Noblett (1969). They were carried out as an outpatient procedure, without the need of

general anesthesia. All biopsies were performed by the same pediatric surgeon. No bowel wash-out or stool removal was previously performed, avoiding edema of rectal mucosa. At least two specimens were taken nearly 2 cm above the dentate line, at the posterior or lateral rectal walls, in each episode of rectal suction biopsy. A 60 ml syringe was used to provide suction of 20 to 25 inches of mercury. The blade on the forceps was sharpened whenever it was felt necessary (Aldridge & Campbell, 1968; Noblett 1969; Campbell & Noblett, 1969; Hall et al, 2009; Kapur, 2009).

Specimens were collected fresh onto a piece of filter paper and immediately sent to the Pathology Department. At this time, they were divided into two fragments: one was fixed in 10% formalin and paraffin embedded, cut and stained with H&E for standard histological observation; the other fragment was frozen in liquid nitrogen, submitted to cryostat sectioning (12 μm) and stained for Ache histochemistry, according to the modified reaction of Karnovsky and Roots (Karnovsky & Roots, 1964; Hanker 1964; Feichter et al, 2009). Calretinin immunolocalization was performed on the same paraffin wax-embedded sections, using a rabbit monoclonal antibody (DAKO, Carpenteria, California, clone DAK Calret 1- M7245), 1:100), by the standard avidin-biotin complex method (Barshack et al, 2004; Kapur et al, 2009; Guinard-Samuel et al, 2009; Holland et al, 2011).

The histopathological analysis was conducted by at least two experienced pathologists. The diagnostic criteria used for the diagnosis of HD were: absence of ganglion cells in H&E sections, positive reaction in parasympathetic nerve fibers of the lamina propria and muscularis mucosa in Ache histochemistry method and the absence of reactivity for Calretinin in the immunohistochemical evaluation (Knowles et al, 2010).

Patients with diagnosis of HD by rectal suction biopsies underwent surgical treatment. Data from the histopathological evaluation of colectomy specimens were adopted to compare with the preoperative diagnosis of rectal suction biopsies.

Statistical analysis was performed using the software SPSS v15.0 by comparing the results between the two studied series: the Chi-squared test was used to compare the rate of inconclusive results, the Mann-Whitney test to study age differences and the Odds Ratio to estimate relationships between age groups and failure rates. The study was approved by the Botucatu Medical School Hospital Research Ethics Committee (protocol number 3917-2011).

3. Results

In total, data from 82 patients were analyzed, 41 in each series. The median age of patients was 25 months (1-287 months) in the first series and 19 months (1-195 months) in the second series. There was no statistically difference according to the age between these two studied sets, as shown in Table 1.

HD was diagnosed in 7 of 41 patients (17%) in the first series of cases and in 11 of 41 patients (26.8%) in the second series of cases. No complications were observed after rectal suction biopsies. All patients with the final diagnosis of HD underwent surgical treatment. The histopathological evaluation of the surgical specimens confirmed aganglionosis in all cases.

In the first series of cases, 41 patients were submitted to rectal suction biopsies, during a period of 38 months. In total, there was 45 episodes of rectal suction biopsies, of which 17 (37.8%) presented inconclusive results (16 patients). These cases required repeated biopsies, which were performed by the same suction method in 2 patients and by open rectal surgical procedure in 14 patients. One patient who had repeated rectal suction biopsy once again, had an inconclusive result. This patient was submitted to a third rectal suction biopsy which, at this time, allowed a conclusive result. The causes of these failure results were also determined: 13 cases (76.5%) presented superficial specimens, without or minimal submucosal tissue (Figure 1); 3 cases (17.6%) were too distal samples, with only stratified squamous epithelium (Figure 2) and 1 case (5.9%) presented the submucosa occupied by a lymphoid follicle, which did not allow an adequate histopathological analysis.

The second series of cases was performed during a period of 24 months, from January 2010 to January 2012, when calretinin immunohistochemistry was added to the histopathological evaluation for HD. Forty one patients were submitted to 42 rectal suction

biopsies episodes. Five patients (11.9%) presented inconclusive results: 4 of these underwent full-thickness rectal surgical biopsies and 1 patient repeated the rectal suction procedure which allowed a conclusive diagnosis. The causes of these failure results were determined: 3 cases (60%) presented too superficial specimens, with a lack of muscularis mucosa and submucosal tissue and 2 cases (40%) were distal fragments, close to the pectinate line.

The failure rate obtained in the second series of cases, after the introduction of calretinin immunohistochemistry (11.9%) was significantly lower than that observed in the first series of cases (37.8%), using only H&E and Ache histochemistry ($p= 0.006$, chi-squared test). The potential influence of age on the failure rate was also investigated. For ease of analysis, the subjects were grouped, in each series, into 3 aged categories. No significant relationship between the increase of age, in months, and the failure rates of rectal suction biopsies in the diagnosis of HD was detected, as shown on Table 2.

4. Discussion

The definitive diagnosis of Hirschsprung's Disease (HD) depends on the histopathological evaluation of rectal biopsies (Swenson et al., 1955, Bodian, 1960, Dobbins & Bill, 1965, Campbell & Noblett, 1969). However, in many times, this analysis generates a real diagnostic challenge (Kapur et al, 2009). Frequently problems include the inadequacy of biopsy specimens, some small and superficial, without enough submucosal tissue, and other samples too distal, near to the dentate line, which is from a physiologically hipoganglionic zone (Aldridge & Campbell, 1968; Grosh et al., 1998; Qualman et al., 1999; Kapur et al., 2009; Guinard-Samuel et al; 2009). Furthermore, the histopathological analysis may have some pitfalls, such as subjective interpretation, leading to false-positive and false-negative results (Brito & Maksoud, 1987; Athow et al., 1990; Qualman et al., 1999; Kapur, 2009).

A variety of different forceps models, technical modifications and new biopsy methods have been proposed to provide deeper and larger specimens (Shandling & Audist, 1972; Pease et al., 1976; Weintraub et al., 1977; Hirose et al., 1993; Schmittenbecher et al., 1995; Alizai et al., 1998; Kobayashi et al., 2002; Ali et al., 2006; Pini-Prato et al., 2006; Croffie et al., 2007; Hall et al., 2009; Hirsh et al., 2011). However, none of them have demonstrated clearly advantages when compared to Noblett's suction biopsies (Campbell & Noblett, 1969). Beside this, the risk of complications increases directly with the size of the biopsy specimen (Shandling & Audist, 1972; Pease et al., 1976; Melendez et al., 2012.)

We have been dealing with the diagnosis and management of HD in our Service of Pediatric Surgery in the last 2 decades (Takegawa, 1997, 2010; Takegawa et al., 2005; Toledo de Arruda Lourenção et al., 2012). As the failure rates of rectal suction biopsies in the diagnosis of HD are a challenging issue, we decided to investigate whether the introduction of a new ancillary immunohistochemical method, in the histopathological evaluation of HD, was

able to decrease the rate of inconclusive results of rectal suction biopsies. Data from the histopathological analysis was reviewed into two series, before and after the introduction of calretinin immunohistochemistry in the diagnostic work up for HD. Only patients with rectal suctions biopsies, performed by the same apparatus and method (Noblett, 1969) were included in this study.

The results of the present study have demonstrated that the introduction of calretinin immunohistochemistry led to a significant lower rate of inconclusive results (11.9%) when compared to the first series of cases (37.8%) which used only H&E and Ache histochemistry in the work up for the histopathological diagnosis of HD (p=0.006). This significant difference may be explained by a better diagnostic capability due to the association among H&E staining, Ache histochemistry and calretinin immunohistochemistry in the histopathological analysis of rectal suction biopsies. In practice, most of the inconclusive results from the first series of cases was related to superficial specimens (76.5%). There was paucity of submucosa in these samples, which explains why ganglion cells could not be identified by H&E stained slides. Furthermore, in all these cases, Ache histochemistry did not reveal the presence of hypertrophied parasympathetic nerve trunks. Due to the high rates of Ache histochemistry false negative results (Hamoudi et al., 1982; Athow et al., 1990; Moore & Johnson, 2005; Guinard-Samuel et al., 2009), we could not exclude the diagnosis of HD in these cases, leading to repeated rectal biopsies. During our second series of rectal suction biopsies, similar superficial cases were also observed. However, calretinin immunohistochemistry proved to be an excellent method to exclude HD, by showing the presence of immunohistochemical expression for calretinin in intrinsic nerve fibers in the lamina propria and muscularis mucosa (Figure 3), which allowed to rule out the diagnosis of HD (Barshack et al., 2004; Kapur et al., 2009; Guinard-Samuel et al., 2009; Holland et al., 2011; Melendez et al., 2012). Beside this, some of distal biopsies, involving samples from the

physiologic hipoganglionic zone (Aldridge & Campbell, 1968), have also demonstrated the presence of intrinsic nerve fibers immunostained by calretinin, thus allowing to exclude the diagnosis of HD (Kapur et al., 2009).

In the present study, no significant difference on the age of the patients was observed between the 2 series of cases, which could have some influence on the results of the failure rates (Table 1). On the other hand, in each series of cases analyzed, we could not find a significant relationship between the increase of age and the failure rates of rectal suction biopsies (Table 2). We observed, however, in the second series of cases, a trend of to increase failure rates in the older age groups. Indeed, the lack of cooperation and the presence of a thickened megarrectum, common in older children, can influence the failure rates, but there is no evidence of a limit of age to perform rectal suction biopsies (Alizai et al., 1998; Croffie et al., 2007; Hirsh et al., 2011).

Most of the published studies have investigated changes in biopsy techniques and forceps models to improve the rate of conclusive biopsies in the investigation of HD A wide range of results have been observed (Shandling & Auldist, 1972; Pease et al., 1976; Weintraub et al., 1977; Hirose et al., 1993; Schmittenbecher et al., 1995; Alizai et al., 1998; Kobayashi et al., 2002; Ali et al., 2006; Pini-Prato et al., 2006; Croffie et al., 2007; Hall et al., 2009; Hirsh et al., 2011). In the present study, we decided to investigate another approach to reduce the rate of failure results of rectal suction biopsies. We have added calretinin immunohistochemistry in the diagnostic work up for the histopathological analysis and we have obtained a low rate of inconclusive results. Therefore, calretinin immunohistochemistry has proved to be a useful ancillary method and its inclusion in the histopathological panel for HD biopsies is advisable to improve the diagnostic accuracy of this challenging disease.

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6. Figures and Tables

Table 1 – Comparison between age values of the patients in the two series of cases

	Series of Rectal Suction Biopsies		p [2]
	Series 1	Series 2	
Age (in months)[1]	25.0 (1 – 287)	19.5 (1 – 195)	1.000

(1) Median, minimum and maximum age values.
(2) Mann-Whitney test.
Series 1: histopathological analysis composed by H&E stained slides and Ache histochemistry.
Series 2: histopathological analysis composed by H&E stained slides, Ache histochemistry and Calretinin immunohistochemistry.

Table 2 – Relationship between age groups and the failure rates of rectal suction biopsies, in the two series of cases

Age group	Number of inconclusive rectal suction biopsies	Number of rectal suction biopsies	Failure Rates (%)	Odds Ratio	IC (OR; 95%)		p
Series 1	≤ 12 months-old	7	17	41.20			
	13 to 36 months-old	4	10	40.00	0.998	(0.986 – 1.013)	0.977
	≥ 36 months-old	6	18	33.33			
Series 2	≤ 12 months-old	1	16	6.33			
	13 to 36 months-old	0	8	0.00	1.016	(0.999 – 1.034)	0.067
	≥ 36 months-old	4	18	22.2			

Series 1: histopathological analysis composed by H&E stained slides and Ache histochemistry.
Series 2: histopathological analysis composed by H&E stained slides, Ache histochemistry and Calretinin immunohistochemistry.

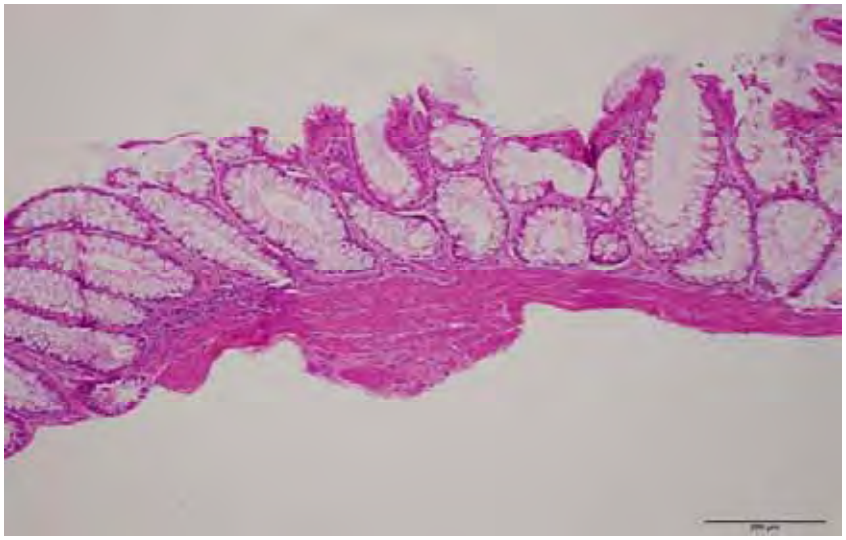


Figure 1 – H&E stained slide revealing a superficial specimen, without submucosal tissue (200x)

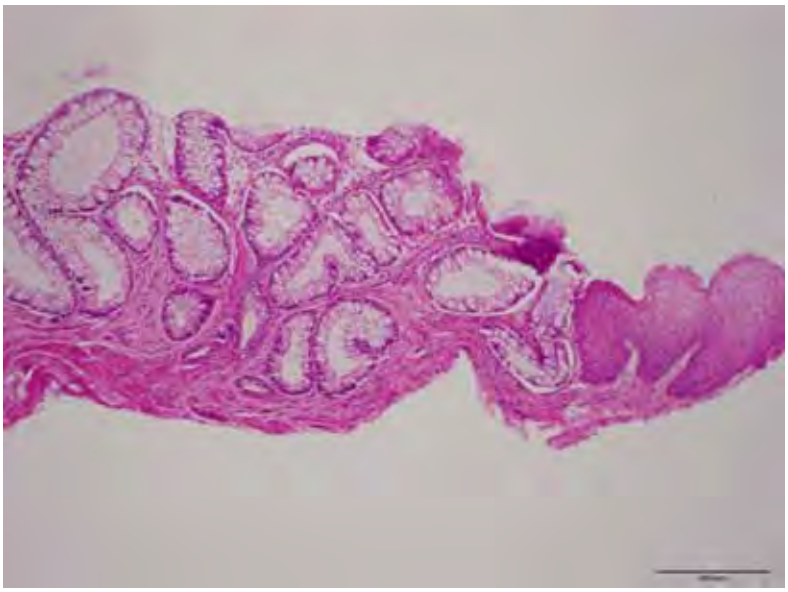


Figure 2 – H&E stained slide of distal inadequate specimen. Note the presence of the stratified squamous epitellium (200x)

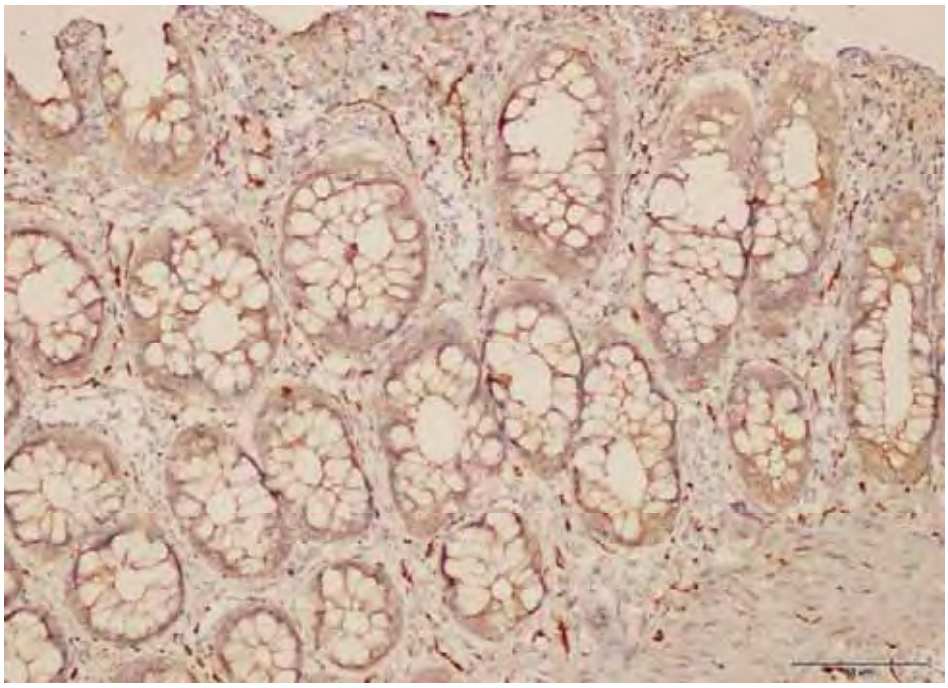
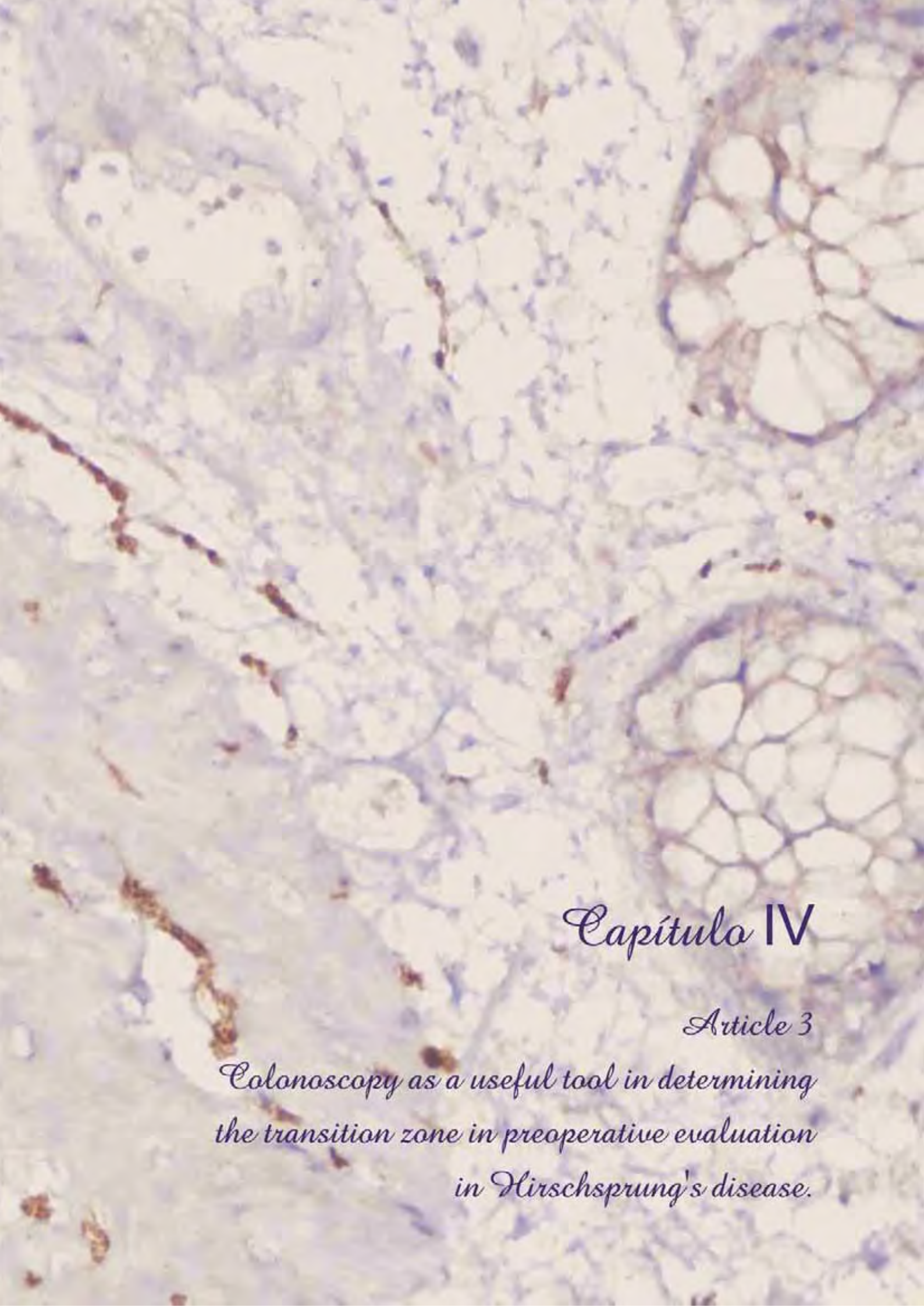


Figure 3 – Calretinin immunohistochemistry revealing the presence of intrinsic nerve fibers in the muscularis mucosa and lamina propria, thus allowing to exclude diagnosis of Hirschsprung's disease (400x)



Capítulo IV

Article 3

Colonoscopy as a useful tool in determining the transition zone in preoperative evaluation in Hirschsprung's disease.

Abstract

Introduction: Preoperative identification of the transition zone in Hirschsprung's disease (HD) has become an essential issue for surgical planning, especially for Transanal Endorectal Pull-Through (TEPT) procedure. The present study aimed to investigate prospectively, the value of endoscopic marking of the transition zone between normal and aganglionic bowel, as a landmark of the location of pull-through procedure for treatment of HD. Patients and Methods: Colonoscopy was performed on twelve patients with HD diagnosis, previously confirmed by anorectal manometry, contrast enema and rectal suction biopsies. Endoscopically, the first site with absence of motility was identified as the beginning of the aganglionic area. Just above this point, the transition zone was marked with an Indian Ink tattooing. During the TEPT, a full-thickness biopsy for frozen section analysis was performed just above this mark. The results of colonoscopic making were compared with contrast enema. Results: Colonoscopy allowed the identification and tattooing of the junction between normal bowel with peristalsis and aganglionic bowel without peristalsis in all 12 cases (100%). Barium enema revealed the transition zone in 7 patients (58.3%). Frozen samples, obtained just above the marked areas revealed the presence of ganglion cells in all cases and the histopathological analysis of surgical specimens confirmed the diagnosis of HD in all cases and checked the location of the transition zone at the same site previously identified by colonoscopy. Conclusion: Colonoscopic marking of the transition zone may be a useful tool to set the location of pull-through procedure for treatment of HD.

Resumo

Introdução: A identificação pré-operatória da zona de transição na Doença de Hirschsprung (DH) tornou-se um passo fundamental para o planejamento cirúrgico, especialmente para a técnica de abaixamento endorretal transanal. O presente estudo tem como objetivo investigar prospectivamente o valor da determinação colonoscópica da zona de transição na avaliação pré-operatória dos pacientes com DH. **Pacientes e Métodos:** A colonoscopia foi realizada em doze pacientes com diagnóstico de DH previamente confirmado pela manometria anorretal, enema opaco e biópsia de sucção do reto. Endoscopicamente, o primeiro local com ausência de peristaltismo foi identificado como o início da zona agangliônica. Pouco acima deste ponto, a zona de transição foi marcada através de uma tatuagem com tinta nanquim. Durante o abaixamento endorretal transanal, uma biópsia de congelação envolvendo a espessura total da parede foi sempre realizada. Os resultados da determinação colonoscópica da zona de transição foram comparados com os obtidos pelo enema opaco. **Resultados:** A colonoscopia permitiu a identificação da zona de transição em todos os 12 casos (100%). O enema opaco revelou a presença da zona de transição em apenas 7 pacientes (58,3%). A análise das amostras de congelação, obtidas pouco acima das áreas endoscopicamente marcadas, revelou a presença de células ganglionares em todos os casos. A análise histopatológica das peças cirúrgicas confirmou o diagnóstico de DH em todos os casos, assim como a localização da zona de transição no mesmo local previamente tatuado endoscopicamente. **Conclusões:** O exame colonoscópico pré-operatório demonstrou ser uma ferramenta útil para determinar a localização da zona de transição em pacientes com DH.

1. Introduction

The surgical treatment of Hirschsprung's disease (HD) has changed in recent decades, to reduce the need for extensive surgical dissections. Independent of the technique used, surgery should remove the aganglionic region beyond the transition zone (Dasgrupta & Langer, 2004; Gunnarsdóttir & Wester, 2011). The presence of ganglion cells in the intraoperative frozen sections has been used to determine the extension of the colonic segment to be resected (de La Torre & Langer, 2010; Coe et al, 2012).

Since the first description of the Transanal Endorectal Pull-through Technique (TEPT) in 1998, this approach has become the method of choice for the treatment of HD (de La Torre & Ortega-Salgado, 1998; Langer et al., 1999; Gunnarsdóttir & Wester, 2011). In long-aganglionic segment cases, proximal to the descending colon, the TEPT technique may require laparoscopic or laparotomy assistance (de La Torre & Langer, 2010).

Thus, the preoperative identification of the transition zone is an essential issue for surgical planning, even more important during the TEPT procedure, because it indicates the location from which biopsies should be taken for frozen pathological evaluation (de La Torre & Langer, 2010). Recognizing the transition zone during the TEPT procedure can be difficult and may require several frozen section biopsies (Kohno et al., 2005). The contrast enema, which is classically used to determine the transition zone, may be inaccurate in up to 25% of cases (Dasgrupta & Langer, 2004; de Lorijn et al., 2006).

The endoscopic identification of the aganglionic bowel, without the normal peristaltic movements was described by Kitatani (1989). He has called the transition zone as the "shorebreak" area, because it resembled "waves breaking on a beach" (Kitatani, 1989). In 2005, Kohno et al. proposed the use of this endoscopic evaluation in determining the

transition zone in HD. In a retrospective study, he compared the use of this technique with the findings of preoperative contrast enema and obtained encouraging results (Kohno et al., 2005).

We tested, prospectively, the usefulness and accuracy of colonoscopic determination of the transition zone, during the preoperative evaluation in HD.

2. Patients and Methods

From April 2009 to January 2012, 12 patients with HD confirmed by rectal suction biopsies were studied. The histopathological diagnosis was performed with acetylcholinesterase histochemistry and calretinin immunostaining analysis (Barshack et al., 2004; Guinard-Samuel et al., 2009; Kapur et al., 2009; Feichter et al. 2009; Knowles et al., 2010). All patients had been previously screened with anorectal manometry and barium enema (Swenson et al., 1949, Lawson & Nixon, 1967; Dasgrupta & Langer, 2004; de Lorink et al., 2006).

A colonoscopy was performed just prior to the TEPT, using the same anesthetic and bowel preparation procedure. The first site with absence of motility was identified as the beginning of the aganglionic area (Figure 1). Just above this point, the transition zone was marked. An endoscopic tattooing was performed by the injection of physiological saline (1.0 ml) in the submucosa, to raise the mucosa, followed by India Ink injection (0.1- 0.2ml) (Figure 2). All of the colonoscopies were performed by the same pediatric surgery team member, who was unaware of the contrast enema results.

During the TEPT procedure, a full-thickness biopsy for frozen section analysis was performed just above the endoscopic tattooing (Figure 3). The presence of ganglion cells in this sample allowed the location to perform TEPT anastomosis (Shayan et al., 2004; de La Torre & Langer, 2010; Coe et al., 2012). Histopathological analysis of the surgical specimens was performed to determine the aganglionic segment, the transition zone and the proximal normoganglionic colon.

The results of the colonoscopic marking were compared with those of the contrast enema, using the histopathological analysis of the resected colon as the gold standard.

Informed consent to perform the exam was obtained from the parents of each patient, and the study was approved by Botucatu Medical Scholl Hospital Research Ethics Committee (protocol number 3917-2011)

3. Results

Twelve patients with HD confirmed by histopathological analysis of rectal suction biopsies were included in the study. There were 8 boys and 4 girls. The median age was 24 months, ranging from 1 to 192 months. Anorectal manometry revealed absence of inhibitory rectoanal reflex in all patients. Colonoscopy allowed the identification and tattooing of the junction between normal bowel with peristalsis and aganglionic bowel without peristalsis in all 12 cases (100%). Barium enema identified the transition zone in 7 patients (58.3%). In 6 cases, it was located in the sigmoid colon, which is characteristic of the classic-form of HD (Puri & Montedonico, 2008). In 1 case, it was located in the descending colon, which is characteristic of the long-form of HD (Puri & Montedonico, 2008). In the other 5 cases (41.7%), the contrast enema was unable to identify the transition zone, revealing only mega-rectum.

The "shorebreak" area was endoscopically found in the sigmoid colon in the same 6 cases with a sigmoid transition zone previously identified by contrast enema. In one case, the "shorebreak" area was located slightly distal to the splenic flexure, in the descending colon. This was the patient who presented the long-form of HD according to the barium enema. In the other 5 cases, without a transition zone in contrast enema, the "shorebreak" area could be identified in the rectum by colonoscopy (Table1). The statistical comparison between these two methods revealed higher values of sensitivity and accuracy for colonoscopy (100%) than for barium enema (58.3%).

There was no need for laparoscopy or laparotomy assistance in any of the patients submitted to TEPT procedure. The frozen section samples, obtained from biopsies slightly above the colonoscopic marked areas, revealed the presence of ganglion cells in all cases.

These morphological results were used to determine the location to perform the TEPT anastomosis.

The histopathological analysis of the colectomy specimens confirmed the diagnosis of HD in all cases. It also checked the location of the transition zone at the site previously marked with India ink and the status of the proximal surgical margin, revealing the presence of ganglion cell in all cases (Table 1).

4. Discussion

The determination of the transition zone has become more crucial after the introduction of the new surgical techniques for the treatment of HD (Kohno et al., 2005; de La Torre & Langer, 2010; Feichter et al., 2009; Lawal et al., 2011). Abnormalities on the innervation of the bowel after the pull-through procedure is one of the most common causes of poor outcomes following surgical therapy for HD (de La Torre & Langer, 2010; Lawal et al., 2011; Coe et al., 2012). Thus, the intraoperative evaluation by frozen section biopsies, performed above the transition zone has been considered an essential step in the surgical treatment of HD (Shayan et al., 2004; Coe et al., 2012). Contrast enema is known to be ineffective for the precise identification of the transition zone in some cases like in the rectum, typically showing a mega-rectum, impossible to be distinguished from other forms of constipation (Dasgrupta & Langer, 2004; Kohno et al., 2005; de Lorink et al., 2006). Moreover, during the TEPT procedure, macroscopic localization of the transition zone can be very difficult, requiring several frozen section biopsies, which increases the costs and the duration of the surgical procedure (Kohno et al., 2005). So, we decided to investigate a new approach to the preoperative determination of the transition zone in HD. Our study is the first prospective trial to compare colonoscopic results with the contrast enema, frozen biopsies and the histopathological analysis of the resected colon.

In the present study, colonoscopic examination was performed in the operating room, just prior to the TEPT procedure, using the same anesthetic and bowel preparation. The "shorebreak" area was easily identified at the end of the peristaltic movements, as “waves breaking on a beach” (Kitatani, 1989). There were no complications during colonoscopic evaluations.

The preoperative barium enema was unable to locate the transition zone in 5 patients. In these cases, the "shorebreak" area was identified by colonoscopy in the rectum, characterizing the short-form of HD (Puri & Montedonico, 2008). The results of sensitivity and accuracy obtained for barium enema (58.3%) were lower than those achieved by colonoscopy (100%).

The prior colonoscopic determination of the transition zone and its tattooing with India ink facilitated the intraoperative collection of frozen sections biopsies. None of the cases required additional biopsy samples. The histopathological examination of the colectomy specimens confirmed the presence of normal ganglion cells in proximal borders and the location of the transition zone at the previously colonoscopic marked areas (Table 1).

In conclusion, we believe that the colonoscopic determination of the transition zone may be considered a useful tool in the preoperative evaluation in HD. It is promising to determine precisely the transition zone, allowing to perform intra-operative frozen section biopsies. However, this preliminary study represented only a small number of patients. Further investigation, with larger series of cases, are necessary to better evaluate the precise value of this endoscopic approach in the preoperative planning of the surgical treatment of Hirschsprung’s disease.

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6. Figures and Tables

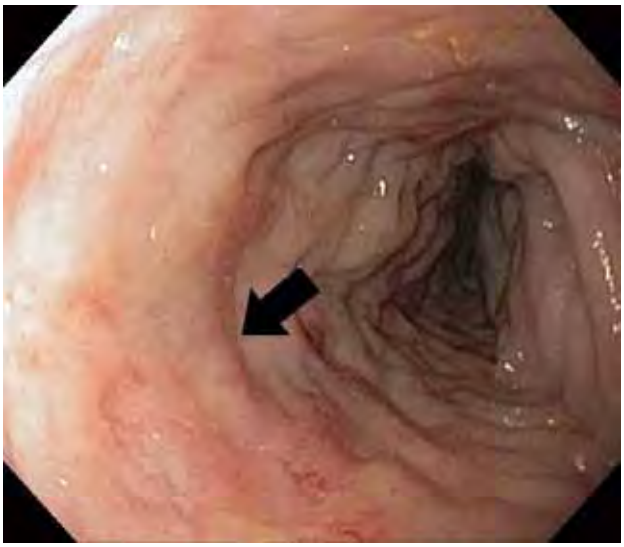

Fig. 1 Colonoscopic view in Hirschsprung disease. The arrow indicates the endpoint of peristaltic movements, which resembles waves breaking on a beach (the "shorebreak" area)

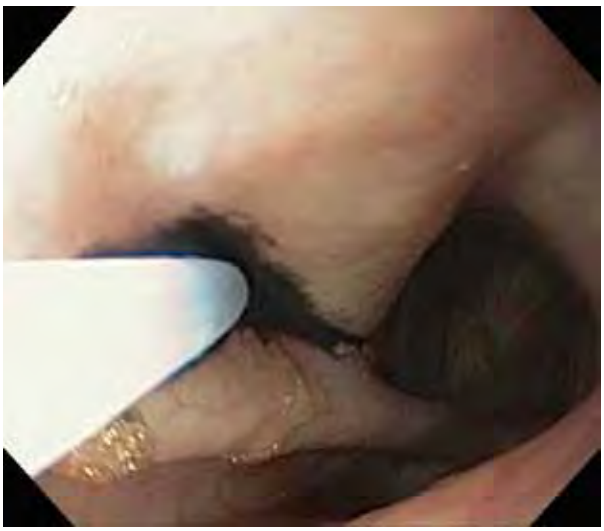

Fig. 2 Colonoscopic view showing the tattooing with India ink at the colonoscopic transition zone

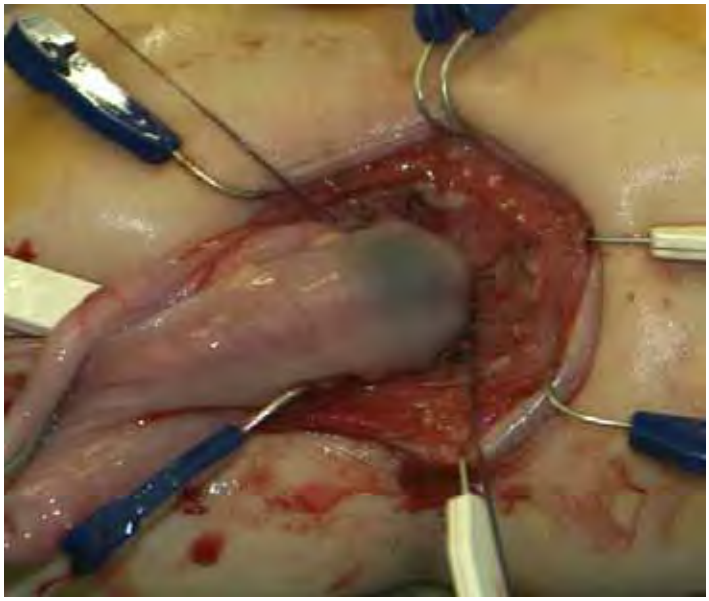

Fig. 3 Colonoscopic marked area easily identified during the TEPT procedure, determining the location for frozen section biopsies.

Table 1: Correlation among the radiographic transition zone, the endoscopic marking (the "shorebreak" finding) and the results of the histopathological analysis of surgical specimens.

Radiographic Transition Zone (n)	Endoscopic Marking (n)	Histopathology of surgical specimens (n)
Sigmoid colon (6)	Sigmoid colon (6)	Classic-HD (5)
Descending colon (1)	Descending colon (1)	Long-HD (1)
Absent (5)	Rectum (5)	Short-HD (5)

(n): number of cases