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DOCTORAL DISSERTATION

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Abstract:

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Results: Study I: Diagnostic efficacy of RSB was significantly higher in children < 9 kg and lower in specimens from aganglionic tissue. **Study II**: Orientation improved tissue quality, reduced diagnostic turnaround time, and decreased histopathological workload, particularly enhancing diagnostics in aganglionic specimens. **Study III**: The modified contrast enema showed high accuracy and strong interobserver agreement. A visible rectosigmoid caliber change accurately predicted the extent of rectosigmoid aganglionosis. **Study IV**: Ganglionic tissue showed a thicker muscularis interna and a higher ratio of muscularis interna to externa, distinguishing all cases of aganglionosis in a proposed diagnostic algorithm.

Conclusions: This thesis identifies key factors and introduces innovations that enhance the diagnostic process for HD, contributing to safer, faster, and more reliable diagnostics, while enabling the exclusion of the disease through less invasive methods. The findings also provide new structural insights of bowel wall that may support future diagnostic innovations.

Key words: Hirschsprung's disease, diagnosis, rectal suction biopsy, orientation, specimen handling, modified contrast enema, rectoanal inhibitory reflex, frozen biopsy, ultra-high frequency ultrasonography.

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The important thing is not to stop questioning
Albert Einstein

To the children undergoing evaluation for Hirschsprung's disease and their families, whose trust made this thesis possible

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I. **Fransson, E**.; Granéli, C.; Hagelsteen, K.; Tofft, L.; Hambraeus, M.; Munoz Mitev, R.U.; Gisselsson, D.; Stenström, P. Diagnostic Efficacy of Rectal Suction Biopsy with Regard to Weight in Children Investigated for Hirschsprung's Disease. Children 2022, 9, 124. doi: 10.3390/children9020124

II. **Fransson, E.**; Gottberg, E.; Munoz Mitev, R.; Gisselsson, D.; Hagelsteen, K.; Tofft, L.; Stenström, P. and Graneli, C. Systematic orientation of fresh rectal suction biopsies improves histopathological diagnostics in hirschsprung's disease - a method description and preliminary report. BMC Pediatrics 2023, 23, 242. doi: 10.1186/s12887-023-04048-4

III. **Fransson, E.**; Götestrand, S.; Mc Michael, N.; Vult von Steyern, K.; Andersson, G.; Hagelsteen, K.; Graneli, C.; Stenström, P. Modified Contrast Enema for Hirschsprung's Disease: Accuracy and Prediction of Aganglionic Segment. Under review in Scientific Reports, submitted 2025-05-12.

IV. **Fransson, E.**; Evertsson, M.; Lundberg, T.; Hawez, T.; Andersson, G.; Granéli, C.; Cinthio, M.; Erlöv, T.; Stenström, P. Histoanatomic Features Distinguishing Aganglionosis in Hirschsprung's Disease: Toward a Diagnostic Algorithm. Diseases 2025, 13, 264. doi: 10.3390/diseases13080264

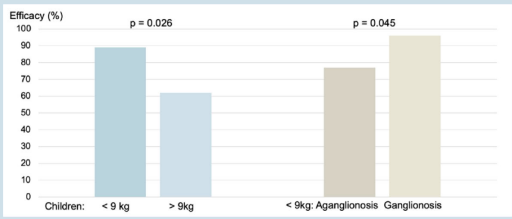
Abbreviations

G-series	Ganglion cell series
HD	Hirschsprung's disease
H&E	Hematoxylin and Eosin
RAIR	Rectoanal inhibitory reflex
RSB	Rectal suction biopsy

Thesis at a glance

	Aims	Methods	Key Results
I:	To assess the impact of weight on diagnostic efficacy of rectal suction biopsy, and to compare efficacy between aganglionic and ganglionic tissue.	Observational retrospective study of children weighing < 15 kg undergoing first-time rectal suction biopsy. Non-parametric statistical analysis.	Diagnostic efficacy was significantly higher in children weighing < 9 kg. Among children < 9 kg, diagnostic efficacy was significantly higher in ganglionic than in aganglionic specimens.
II:	To evaluate if an in-house developed method for systematic orientation of rectal suction biopsy specimens improved tissue quality, diagnostic efficacy, turnaround time and histopathological workload.	Observational case-control study comparing systematically oriented and non-oriented rectal suction biopsy specimens across two time periods. Non-parametric statistical analysis.	Orientation improved tissue quality, and reduced diagnostic turnaround time as well as need for additional sectioning. In aganglionic tissue, orientation improved diagnostic efficacy, tissue quality and reduced the need for additional sectioning.
III:	To evaluate the diagnostic accuracy, interobserver reliability, and predictive value for disease extension of an in-house developed radiological method based on modified contrast enema using cold contrast in the assessment of Hirschsprung's disease.	Prospectively collected patient cohort of children < 1 year of age. Histopathology served as the reference standard for disease. Interobserver analyses. Non-parametric statistical analysis.	The modified contrast enema demonstrated 100% sensitivity and 87% specificity for diagnosing Hirschsprung's disease. Interobserver agreement was strong. A caliber change at the rectosigmoid level accurately predicted disease extension.
IV:	To compare histoanatomic morphometrics of the bowel wall between aganglionic and ganglionic segments in Hirschsprung's disease and to explore a diagnostic algorithm based on these findings. To investigate potential correlations between full bowel wall thickness and patient weight or age.	Histopathological images of resected specimens from children with Hirschsprung's disease were analyzed. An in-house designed MATLAB® program was used for manual delineation of bowel wall layers and automated calculations. Paired parametric statistical tests.	Ganglionic bowel showed a thicker muscularis interna and a greater muscularis interna to muscularis externa ratio. An algorithm based on these features achieved 100% accuracy in distinguishing aganglionic from ganglionic segments. Full bowel wall thickness did not correlate with either weight or age.

Conclusions and implementation



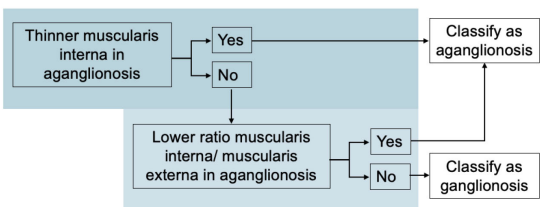
For clinical and diagnostic efficacy purposes, rectal suction biopsy may be best reserved for children weighing < 9 kg. This cut-off is applied in routine diagnostic practice.

	Effect in the oriented group among all specimens	Effect in the oriented group among aganglionic specimens
Efficacy	-	↑
Diagnostic turnaround time	↓	-
Tissue quality	↑	↑
Workload	↓	↓

Systematic orientation of fresh rectal suction biopsy specimens improves diagnostic performance. The major benefits are observed in aganglionic specimens. The method is implemented in routine clinical practice.

	Hirschsprung's disease (n=31)	No Hirschsprung's disease (n=129)	p-value
Overall evaluation consistent with Hirschsprung's disease	100%	13%	<0.001
Rectoanal inhibitory reflex absent	100%	21%	<0.001
Statically contracted rectal/rectosigmoid segment	94%	9%	<0.001
Caliber change identified	77%	0%	<0.001

The modified contrast enema provides high diagnostic accuracy and strong interobserver reliability. It serves as a reliable selection tool for rectal biopsy in suspected Hirschsprung's disease. Visualization of a rectosigmoid caliber change shows high sensitivity for assessing disease extension, supporting surgical planning.This examination is a part of the clinical diagnostic workflow.



Distinct histoanatomic differences between aganglionic and ganglionic bowel segments support further development of novel diagnostic imaging techniques. Full bowel wall thickness appears independent of both weight and age in children below one year. These data have been incorporated into an algorithm used in research on ultrahigh-frequency ultrasound.

Abstract

Background: Hirschsprung disease (HD) is a congenital disorder characterized by absence of ganglion cells extending proximally from the distal rectum, causing functional obstruction. Accurate and timely diagnosis is crucial to prevent morbidity and distress, but remains challenging. This thesis aimed to optimize HD diagnostics by refining existing methods, minimizing invasiveness, and evaluating novel diagnostic approaches.

Methods and aims: Four studies were conducted between 2011 and 2024 at Lund University Hospital, Sweden, a national referral center for HD. **Study I**: Retrospective analysis of first-time rectal suction biopsy (RSB) assessing diagnostic efficacy in relation to weight. **Study II**: Evaluation of a systematic orientation technique for RSB and its effect on diagnostic efficacy. **Study III**: Prospectively collected children, using a modified contrast enema technique, assessing diagnostic accuracy and interobserver agreement. **Study IV**: Morphometric analysis of bowel wall layers of histopathologically stained bowel specimens in surgically treated children with HD, to identify structural differences between ganglionic and aganglionic segments.

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Conclusions: This thesis identifies key factors and introduces innovations that enhance the diagnostic process for HD, contributing to safer, faster, and more reliable diagnostics, while enabling the exclusion of the disease through less invasive methods. The findings also provide new structural insights of bowel wall that may support future diagnostic innovations.

Populärvetenskaplig sammanfattning

En tryggare och skonsammare diagnos för barn med misstänkt Hirschsprungs sjukdom.

Att utreda Hirschsprungs sjukdom hos barn kan vara både stressande och påfrestande, inte minst för barnen själva, men också för deras familjer. Varje undersökning och provtagning väcker oro. Den här avhandlingen handlar om hur vi kan göra diagnostiken säkrare och mer effektiv, så att färre barn behöver genomgå onödiga ingrepp.

Vad är Hirschsprungs sjukdom?

Hirschsprungs sjukdom är en medfödd tarmsjukdom där en del av tarmen saknar nervceller. Utan dessa nervceller fungerar inte tarmen som den ska, den blir stel och trång, vilket hindrar avföringen från att passera. Det kan leda till allvarliga komplikationer, och i värsta fall död om barnet inte får behandling. Sjukdomen drabbar ungefär 1 av 5000 nyfödda, vilket motsvarar cirka 20 barn per år i Sverige. Fyra gånger fler pojkar än flickor får diagnosen.

Hur märks sjukdomen?

Hos nyfödda märks det ofta genom att barnet inte bajsar inom de första två dygnen efter födsel, får uppsvälld mage och kräks. Hos äldre barn kan det visa sig som långvarig förstoppning, magont eller dålig tillväxt. Eftersom förstoppning är vanligt även hos friska barn, är det viktigt att kunna avgöra vilka som behöver utredas vidare.

Hur ställs diagnosen?

För att ställa diagnosen krävs ett vävnadsprov (biopsi) från ändtarmen. Om man i mikroskop ser att nervceller saknas, bekräftas sjukdomen. Provet kan tas med ett vakuuminstrument (sugbiopsi) utan att barnet behöver sövas, eller som ett större vävnadsprov under narkos. Ibland behöver provet tas om, vilket innebär ytterligare belastning för barnet.

Ofta görs också en kontraströntgen, där kontrastvätska sprutas in i ändtarmen för att visa tarmens form. Vid Skånes universitetssjukhus har en unik metod utvecklats där man även använder kall kontrast för att testa tarmens tömningsreflex. Om reflexen fungerar, talar det starkt för att barnet inte har sjukdomen, och då kan man ibland undvika biopsi helt.

Vad visar avhandlingen?

Avhandlingen består av fyra studier som alla syftar till att förbättra diagnostiken vid Hirschsprungs sjukdom:

1. **Viktgräns för sugbiopsi.** Studien visade att sugbiopsier är tillförlitliga hos barn som väger under 9 kg. Barn som väger mer än 9 kg sövs, för att kunna ta ett större vävnadsprov direkt.
2. **Orientering av vävnadsprov.** Ett vävnadsprov från tarmen består av flera lager. Genom att barnkirurgen markerar vilken sida som är slemhinna, förbättras provets kvalitet och bedömningen underlättas hos patologen. Detta leder till snabbare diagnos, mindre arbete för patologer och färre omtagningar, särskilt hos barn som har sjukdomen.
3. **Modifierad kontraströntgen.** Metoden med kall kontrast som utvecklats vid Skånes universitetssjukhus visade sig vara mycket tillförlitlig för att utesluta sjukdomen. Undersökningen kan även ge en indikation på hur långt upp i tarmen sjukdomen sträcker sig, vilket vägleder kirurgen i att planera operationen.
4. **Tarmväggens vävnad.** I denna studie undersöktes om det är möjligt att skilja mellan frisk och sjuk tarm genom analys av tarmväggens olika lager. Vi fann att vissa lager skiljer sig i tjocklek beroende på om nervceller finns eller saknas. Utifrån detta utvecklades en algoritm som med 100 % säkerhet kan särskilja frisk och sjuk tarmvägg, ett viktigt steg mot framtidens diagnostiska metoder.

Vad betyder detta för barnen?

Flera av resultaten har redan införts i vården vid Skånes universitetsjukhus. Barn under 9 kg genomgår sugbiopsi, medan äldre barn och de över 9 kg sövs för att ta ett större vävnadsprov. Vävnadsprover orienteras som standard, och modifierad kontraströntgen används för att avgöra vilka barn som behöver gå vidare till biopsi.

Sammanfattningsvis visar avhandlingen hur ny kunskap och förbättrade metoder kan göra diagnostiken av Hirschsprungs sjukdom tryggare, skonsammare och mer tillförlitlig, till stor nytta för barnen och deras familjer.

Short definitions

Diagnostic terms:	
Diagnostic efficacy	The percentage of sufficient rectal biopsy specimens to determine the presence or absence of ganglion cells.
Inconclusive biopsy	A biopsy in which the presence of ganglion cells cannot be determined due to low tissue quality or insufficient depth.
Positive biopsy	Adequate sample showing absence of ganglion cells and presence of nerve hypertrophy.
Negative biopsy	Adequate sample showing presence of ganglion cells with no evidence of nerve hypertrophy.
HD diagnosis	Absence of ganglion cells and presence of S-100 positive, calretinin-negative nerve fibers at the level of the muscularis mucosa.
Specimen quality:	
High	Includes mucosa, muscularis mucosa, and submucosa, without cross-sectioning in the mucosal plane.
Acceptable	Includes sparse submucosa and/or cross-sectioning, but still allows assessment of ganglion cells.
Low	Contains only mucosa or lymphoid tissue, without submucosa, and cannot be assessed for ganglion cells due to cross-sectioning.
Diagnostic process:	
G-series	Automatically performed after the initial hematoxylin and eosin staining, including additional hematoxylin and eosin staining and immunohistochemistry for S-100 and calretinin.
First answer	Preliminary diagnosis issued by the pathologist, if the presence or absence of ganglion cells is already evident. May be provided after: 1. Hematoxylin and eosin staining (with G-series planned), or 2. Complete G-series with further examinations planned (reorientation and/or sectioning).
Final answer	Conclusive diagnosis given after: 1. Single answers after the mandatory hematoxylin and eosin staining

	and complete G-series (with no further examinations planned), or 2. Confirming a first answer after additional planned investigations.
Histopathological workload	Any additional work beyond standard hematoxylin and eosin and G-series, including specimen reorientation and/or deeper sectioning, requiring extra time and resources.
Ready-to-use antibodies	Pre-prepared antibodies supplied at optimal concentration, ready for direct use in immunohistochemical staining without dilution.
Bowel wall layer:	
Muscularis externa	The outer longitudinal muscle layer of the bowel wall (muscularis propria externa).
Myenteric tissue layer	The tissue layer located between the muscularis propria externa and muscularis propria interna.
Muscularis interna	The inner circular muscle layer of the bowel wall (muscularis propria interna).

Introduction

Hirschsprung disease (HD) is a rare congenital disorder of the intestine, characterized by the absence of ganglion cells in the distal bowel wall, aganglionosis. This leads to a tonic contraction of the affected segment, causing functional bowel obstruction. The definitive treatment is surgical, involving resection of the aganglionic segment.

Diagnosing HD can be particularly challenging, as it often requires confirming the absence, rather than the presence, of pathognomonic findings. The diagnostic process itself, and the selection of which children should undergo it, can also be difficult, since many children without HD experience constipation and impaired bowel emptying, accounting for a large number of pediatric consultations each year. While the need for diagnostic evaluation is usually evident in newborns presenting with the classic symptoms from birth, the clinical picture is often less clear in children who develop symptoms later, weeks or even months after birth, especially when symptoms are severe but not entirely typical. In these cases, the decision to proceed with further investigation is more complex.

This thesis aims to validate and improve current diagnostic methods for HD, in order to better understand their diagnostic accuracy. The ultimate goal is to ensure that children receive a timely and reliable diagnosis through the least invasive procedures possible.

Hirschsprung's disease

Embryology

HD is a rare congenital disorder of the enteric nervous system, characterized by the absence of ganglion cells in the myenteric and submucosal plexuses of the bowel wall [1, 2]. These enteric neurons coordinate normal peristalsis, and their absence results in tonic contraction of the affected intestinal segment, leading to functional obstruction and progressive dilation of the proximal bowel [1].

HD arises from disruptions in the developmental processes of the enteric nervous system during embryogenesis. Normally, vagal neural crest cells migrate from the head along the gastrointestinal tract, beginning around the fourth week of gestation and reaching the colon by the seventh week [3]. The myenteric plexus of the esophagus forms first, while that of the colon develops approximately three to four weeks later [3, 4]. Failure in migration, proliferation, differentiation, or survival of these cells leads to aganglionosis, which typically starts in the rectum and extends proximally to varying lengths [5].

Epidemiology

HD has a birth prevalence of 1.91 per 10,000 live births in Sweden [6], though it varies globally [7, 8]. The incidence is highest in Asian populations (2.8 per 10,000), intermediate in African Americans (2.1 per 10,000) and Caucasians (1.5 per 10,000), and lowest in Hispanics (1 per 10,000) [9]. A slight increase in reported cases has been observed in registry-based studies between 1980 and 2009, which is thought to reflect improved diagnostic awareness and reporting [7].

HD is typically classified by the length of the aganglionic segment (**Figure 1**) [10]:

1. Short-segment HD (72%): Aganglionosis up to the sigmoid-descending colon junction.
2. Long-segment HD (15%): Aganglionosis proximal to the sigmoid-descending colon junction, with ganglion cells present in part of the colon.
3. Total colonic aganglionosis (8%): Aganglionosis throughout the colon and < 5 cm of the terminal ileum.
4. Small intestinal HD (5%): Aganglionosis extending > 5 cm beyond the terminal ileum.
5. Total intestinal HD (< 1%): Less than 20 cm of ganglionated intestine beyond the ligament of Treitz.

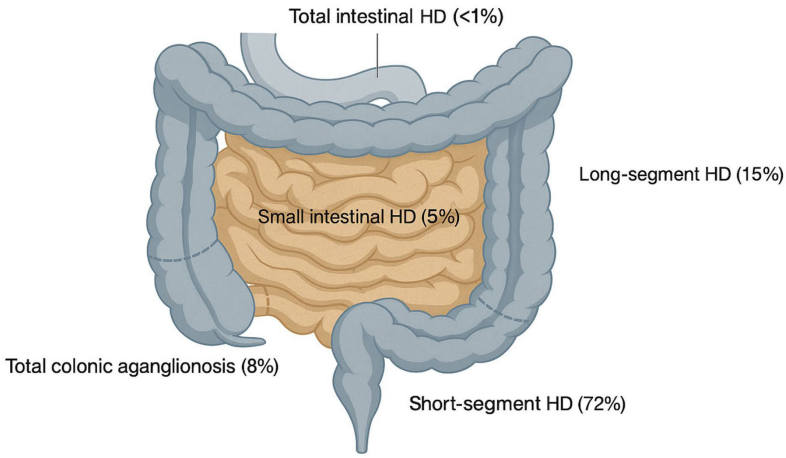

Figure 1. Classification based on the length of the aganglionic segment and the relative frequency of each type of Hirschsprung's disease. Illustration created with AI assistance (ChatGPT, OpenAI, 2025).

There is a clear male predominance, with a male-to-female ratio of approximately 3.7:1 [6]. However, this ratio approaches 1:1 in total colonic aganglionosis and syndromic HD [11]. Only about 7% of children with HD are born preterm, suggesting a lower incidence among premature infants [12].

Approximately 70% of HD cases are isolated, while the remainder are associated with familial clustering, genetic syndromes, or chromosomal anomalies [13, 14]. The recurrence risk among siblings is approximately 4%, representing a 200-fold increase compared to the general population [9]. Sibling recurrence ranges from 1% to 33%, depending on the gender and length of aganglionosis of the proband and the gender of the sibling, with the highest recurrence risk in a male sibling of a female proband affected with long-segment HD [9].

Short-segment HD is often inherited in an autosomal recessive pattern or result from the combined effects of multiple genes, whereas long-segment HD typically show dominant inheritance with incomplete penetrance [14]. Mutations in the RET gene are the most common, followed by EDNRB, though many other mutations have been identified [14, 15].

Syndromic HD occurs in about 12% of patients, with trisomy 21 being the most frequent (> 90% of syndromic HD) [13]. Other associated syndromes include Waardenburg-Shah syndrome, Haddad syndrome and Mowat-Wilson syndrome [16].

Associated anomalies are reported in 20–32% of cases [6, 8]. In a prospective study of systematic screening for associated anomalies in children with HD, the following frequencies were reported: visual impairment and ophthalmologic abnormalities (43%), renal anomalies (21%), gastrointestinal tract anomalies (3%), cardiac defects (5%), hearing impairment (5%), and genital, tumor-related, or central nervous system abnormalities (each 2%) [17]. Urinary tract ultrasound and audiometric testing are generally recommended for patients with HD [17, 18]. Cardiologic evaluation is also common and is typically performed at Skånes University hospital where these studies were conducted. Genetic screening is part of the routine protocol at the center in question. Cerebral ultrasound and ophthalmologic assessment should be guided by clinical findings and standard care practices, but are specifically recommended for children with suspected syndromes or chromosomal abnormalities [17].

Bowel layers

The bowel wall consists of several functionally specialized histoanatomical layers supporting nutrient absorption, fluid and electrolyte regulation, and peristaltic propulsion. The innermost layer, the mucosa, lines the intestinal lumen and contains villi and crypts for absorption. Beneath it lies the muscularis mucosae, a thin smooth muscle layer, followed by the submucosa, which contains connective tissue and the submucosal (Meissner's) plexus, a key component of the enteric nervous system that regulates secretory functions [19].

The next layer, the muscularis propria, is responsible for peristalsis and consists of two smooth muscle layers: an inner circular layer (muscularis interna) and an outer longitudinal layer (muscularis externa). Between these two layers lies the myenteric tissue layer (Auerbach's plexus), which coordinates bowel wall contractions and gut motility [19]. The outermost layer, the serosa, provides structural integrity and houses extracellular matrix components like collagen and elastin, contributing to the mechanical properties of the bowel. **Figure 2** illustrates the different layers of the intestinal wall.

Peristaltic movement is driven by coordinated smooth muscle contractions under the control of the enteric nervous system, which operates autonomously via the myenteric and submucosal plexuses, ensuring effective motility and secretion along the gastrointestinal tract [15].

There is scarce literature on whether and how the bowel wall differs between ganglionic and aganglionic segments. A better understanding of these differences may have implications for the development of future diagnostic methods.

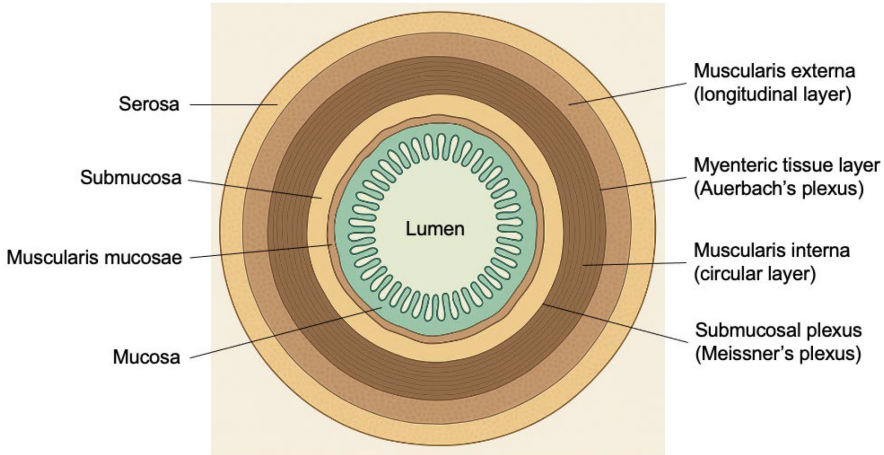

Figure 2. Illustration showing the different layers of the intestinal wall. Created with AI assistance (ChatGPT, OpenAI, 2025).

Symptomatology

Early diagnosis of HD is warranted to avoid serious, potentially fatal complications such as Hirschsprung-associated enterocolitis [20]. In Western countries, approximately 80–90% of children with HD are diagnosed in the neonatal period, typically within the first four weeks of life [7, 8].

The classic triad of HD symptoms, delayed passage of meconium (> 48 hours), abdominal distension, and bilious vomiting, is present in about 18% of cases, while one or more of these symptoms occur in 98% of diagnosed children [21]. Other symptoms include chronic constipation, failure to thrive, explosive stools after rectal examination, enterocolitis or signs of bowel obstruction [15]. Normal meconium passage does not exclude HD, as it is reported in 35% of children with HD who develop constipation before 1 year of age [21].

In Sweden, functional constipation affects approximately 15–20% of otherwise healthy children [22]. The prevalence varies worldwide from 0.5% to 32.2%, depending on geographical location, lifestyle factors, stressful life events, and study design [23]. Investigation for HD is warranted in refractory cases or in children who are dependent on washouts.

A delayed diagnosis increases the risk of Hirschsprung-associated enterocolitis, a potentially life-threatening inflammatory condition characterized by diarrhea, explosive stools, abdominal distension, lethargy, fever and sometimes rectal bleeding [24]. Mild cases may mimic viral gastroenteritis, presenting with fever,

mild distension, and diarrhea, common in young children [25]. Enterocolitis occurs preoperatively in approximately 18% of children and may represent the first clinical manifestation of HD, particularly in infants with Down syndrome or long-segment disease [25, 26].

Initial management for HD consists of bowel decompression by rectal washouts one to three times daily [27]. In cases with more extensive disease or severe symptoms, a diverting stoma in normally innervated bowel may be required [8, 27].

Diagnostic evaluation

A definitive diagnosis of HD requires histopathological confirmation of aganglionosis [27]. Two main techniques for rectal biopsy are commonly used worldwide: rectal suction biopsy (RSB) and full-thickness biopsy.

The decision to perform a rectal biopsy is based on a combination of clinical history, typical symptoms of HD, and often findings from diagnostic methods such as contrast enema, modified contrast enema, and anorectal manometry. In many cases, children presenting with symptoms suggestive of HD initially undergo abdominal radiography, which typically shows dilated bowel loops throughout the abdomen, indicating distal bowel obstruction with little or no air in the lower part of the pelvis [16]. At the Department of Pediatric Surgery, Skåne University Hospital, the modified contrast enema has been evaluated [28] and is used as a selection tool prior to rectal biopsy, while anorectal manometry is not performed. Nevertheless, rectal biopsy remains the gold standard for establishing a definitive diagnosis. **Figure 3** illustrates the diagnostic workflow for suspected HD at the Department of Pediatric Surgery at Skåne University Hospital.

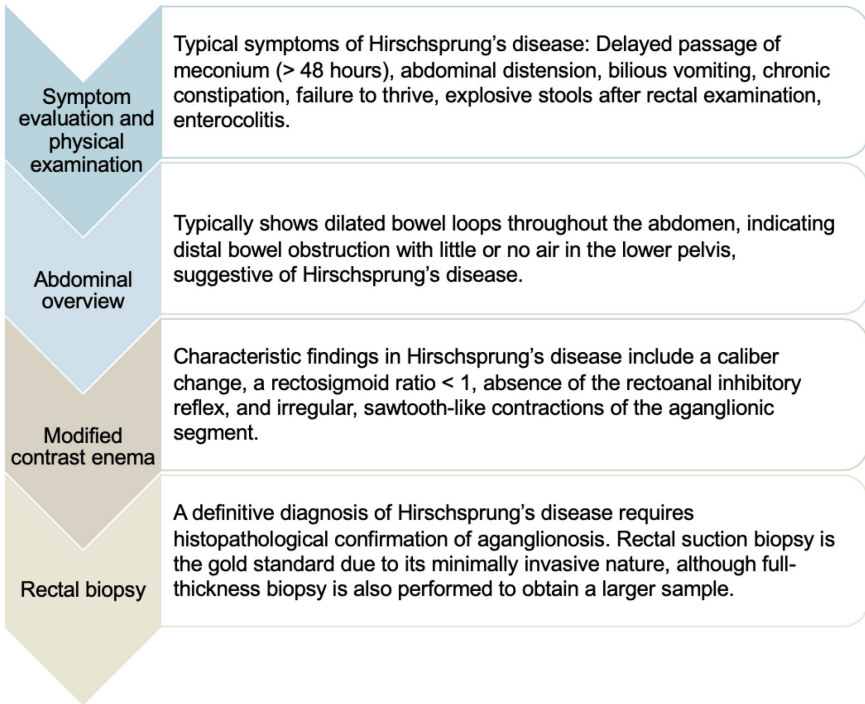

Figure 3. Diagnostic workflow for the evaluation of Hirschsprung's disease, including descriptions of typical findings.

Rectal biopsy

Due to its minimally invasive nature, RSB has become the preferred biopsy technique in most centers [29]. It can be performed at the bedside without anesthesia or suturing, and the parents may be present during the procedure. Despite these advantages, the diagnostic reliability of RSB may be compromised by the risk of obtaining too superficial specimens lacking sufficient submucosa, which is essential for identifying ganglion cells [30, 31].

A key clinical challenge with RSB is the frequent need for repeated biopsies. According to a systematic review, repeated RSB was required in 10% of cases [32], but also higher rates have been reported [33-35]. Repeat biopsies contribute to diagnostic delays, increased healthcare costs, and additional psychological distress for patients and their families [36]. Several factors may influence the diagnostic yield of RSB, including the age of the child, biopsy site, number of biopsies taken per occasion, staining techniques, the proportion of children with HD included, and the experience of the pathologist [30, 31, 37-41]. Different cut-off ages for when RSB should be replaced have been proposed [34, 38, 42], however, findings regarding the influence of age remain inconsistent across studies [33, 35, 43-45].

A systematic review of 58 studies reported an overall complication rate of 0.65% for RSB, with persistent bleeding requiring blood transfusion being the most common [32]. Complications were significantly more frequent in newborns and infants than in older children [32], although other studies have reported no complications among preterm infants and children younger than six months [37, 46].

Full-thickness biopsy, although more invasive, remains widely used. It provides larger tissue samples but requires general anesthesia in an operating room setting. Samples are taken 1 cm above the dentate line, under direct visualization of the anorectal canal and rectum [16]. Even with the full-thickness biopsies, inconclusive results are reported in 5.9% to 13.6% of cases, most often because the specimens are too superficial or collected too distally [44, 47]. The complication rate associated with full-thickness biopsy is reported to be approximately 6.6%, most commonly fever, and with 1% of patients requiring reoperation or examination under general anesthesia [47].

Histopathology and staining

In HD, histopathological examination of aganglionic bowel typically reveals a complete absence of ganglion cells in both submucosal and myenteric plexuses, accompanied by hypertrophy of submucosal nerve fibers [40].

Biopsies are routinely formalin-fixed, paraffin-embedded, and stained with hematoxylin and eosin (H&E) for microscopic examination. While H&E remains the sole diagnostic method in some countries, most centers use additional staining techniques to enhance diagnostic precision [41].

In recent years, calretinin immunohistochemistry has become a preferred alternative to acetylcholinesterase histochemistry in the diagnosis of HD [16, 48-50]. It can be performed on paraffin-embedded, formalin-fixed biopsies using methods available in most pathology laboratories [31]. Calretinin is a vitamin D-dependent calcium-binding protein involved in neuronal calcium signaling and the modulation of neuronal excitability [51]. In healthy bowel, calretinin-immunoreactive nerve fibers are consistently present in the submucosa, muscularis mucosae, and lamina propria, with nuclear staining observed in ganglion cells of the myenteric and submucosal plexuses [49]. In contrast, complete absence of calretinin-positive mucosal nerves strongly supports a diagnosis of aganglionosis [52]. However, calretinin-positive fibers may still be visible in the submucosa in some patients with very short-segment HD, which can complicate interpretation [53].

Another common staining method is acetylcholinesterase, which demonstrates increased and prominent cholinergic nerve fibers in aganglionic bowel [54]. A disadvantage is that this method requires frozen sections, which are incompatible with traditional formalin fixation and paraffin embedding, limiting its use in some

settings [41, 48]. Moreover, in total colonic aganglionosis where nerve hypertrophy may be absent, the acetylcholinesterase pattern can appear normal despite aganglionosis [54].

Several other immunohistochemical markers have been proposed, including S-100, which highlights nerve fibers and is useful for assessing number, distribution and thickness in the submucosa and mucosa [55]. In aganglionosis S-100 staining typically reveals prominent, thickened submucosal nerve trunks, reflecting nerve fiber hypertrophy in the absence of ganglion cells [55]. Combining markers for both ganglion cells and hypertrophic nerve fibers may provide a valuable complementary diagnostic approach in HD [55].

A new marker, PHOX2B, labels both immature and mature ganglion cells with nuclear staining and may therefore be particularly helpful in ambiguous cases to facilitate an accurate diagnosis of HD [54].

If ganglion cells are identified in a rectal biopsy, HD can almost certainly be ruled out. Conversely, if no ganglion cells are detected, the diagnostic reliability depends on the adequacy and the presence of sufficient submucosa [16]. In such cases, additional sectioning, repeat staining, or re-biopsy may be required to establish a definitive diagnosis.

Contrast enema

Contrast enema is commonly performed prior to rectal biopsy [56], both to support suspicion of HD and to help exclude differential diagnoses such as meconium plug syndrome, meconium ileus, or intestinal atresia [15]. Characteristic radiographic findings in HD include a caliber change, a rectosigmoid ratio < 1, and irregular, sawtooth-like contractions of the aganglionic segment [57-59].

The reported sensitivity and specificity of contrast enema are approximately 75% and 85%, respectively [60, 61]. A visible caliber change, suggesting a transition zone between aganglionic and ganglionic bowel, typically a narrow rectum with a dilated proximal segment, has been observed in 55% to 89% of HD cases [57, 58, 61-64]. **Figure 4** illustrates two children with HD showing rectosigmoid caliber changes. In contrast enemas, both false-positive and false-negative findings related to the transition zone have been reported [58, 60, 61, 65]. The concordance between histopathologically determined levels of aganglionosis is high when the transition zone is located in the rectosigmoid region, but it decreases for more distal or proximal aganglionosis [61, 63, 66-68]. Several studies suggest that contrast enema often underestimates the length of the aganglionic segment compared to histopathology [63, 67], although overestimation has also been reported in rectal aganglionosis [68]. The rectosigmoid ratio, defined as the ratio of the widest diameter of the rectum and that of the sigmoid colon, is variably reported as a useful or unreliable diagnostic indicator, with values < 1 suggesting HD [58, 62].

In addition to its diagnostic role, contrast enema may assist in surgical planning if a visible caliber change is present [61, 64, 69]. Some authors have suggested that contrast enema should be deferred until after HD has been confirmed by rectal biopsy, only to predict and delineate the level of aganglionosis for surgical planning [61].

A normal contrast enema cannot reliably exclude HD, especially in cases of total colonic aganglionosis, where radiological appearances may remain deceptively normal despite aganglionosis [64]. Thus, rectal biopsy remains essential in all clinically suspicious cases, regardless of contrast enema results.

Modified contrast enema

The modified contrast enema, developed at Skåne University Hospital, Sweden, builds upon the traditional contrast enema technique by using cold contrast medium to evaluate the rectoanal inhibitory reflex (RAIR). The RAIR is typically absent in HD and serves as an important physiological marker. In an initial study, the sensitivity and specificity of the modified contrast enema were reported as 100% and 98%, respectively [28]. However, the study included children of all ages and only six cases of HD, along with 95 children without the disease. Therefore, validation in a larger cohort of clinically relevant age (0-1 year) is warranted. Furthermore, its interobserver agreement has also not yet been evaluated. This technique was introduced to improve diagnostic precision and to guide selection of patients for rectal biopsy.

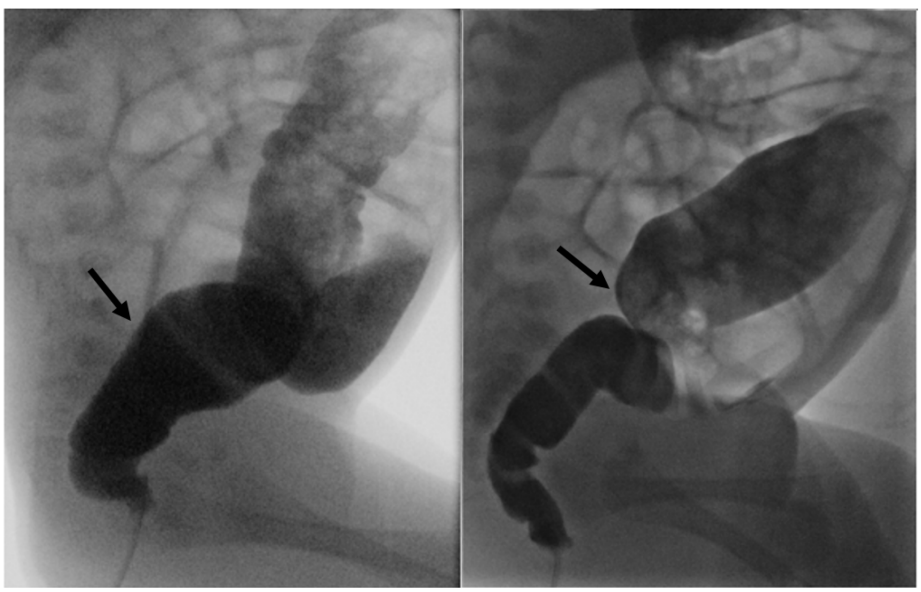

Figure 4. Images from modified contrast enemas in two children with Hirschsprung's disease, demonstrating a suspected rectosigmoid caliber change (black arrows).

Anorectal manometry

Anorectal manometry is a diagnostic method used to evaluate anorectal function by assessing the presence or absence of the RAIR. During the procedure, a catheter equipped with pressure sensors is inserted into the rectum, and a balloon at its tip is gently inflated [41]. The procedure is typically performed without anesthesia. In healthy children, balloon inflation triggers relaxation of the internal anal sphincter, indicating a normal RAIR. The catheter is connected to a computer system that records pressure changes and muscle activity in real time. However, absence of RAIR alone is not sufficient for a definitive diagnosis of HD, as the positive predictive value is relatively low [70]. Although there is general consensus that anorectal manometry is not necessary for HD diagnosis, some centers continue to use it as an adjunctive tool [56, 69]. A systematic review reported a sensitivity and specificity of anorectal manometry for diagnosing HD to be 91% and 93%, respectively [39], although lower accuracy has been observed [70]. The equipment is expensive, and both performance and interpretation are susceptible to technical errors and require clinical experience to minimize false-positive and false-negative results [60, 71]. Minor complications are rare but may include mucosal injury with bleeding or accidental balloon retention. Recently, high-resolution anorectal manometry has been introduced. This technique employs catheters with densely spaced sensors that provide detailed, three-dimensional topographic maps of pressure distribution within the rectum and anal canal [71], allowing for more precise anatomical and functional assessment.

Frozen biopsy

A critical intraoperative step is determining the level of healthy bowel. This is guided by intraoperative frozen section biopsies, which are routinely used to confirm the presence of ganglion cells at resection margins [27, 69]. Biopsies obtained during HD surgery are quickly frozen before sectioning and staining. While this method remains the clinical standard, it has several inherent limitations. First, accurate selection of biopsy sites is inherently challenging, as the transition zone between aganglionic and ganglionic bowel often displays a variable paucity of ganglion cells, and its location can be difficult to determine due to both length and circumferential variability [72, 73]. Visual estimation by the surgeon is therefore imprecise and carries the potential risk of either incomplete resection or unnecessary removal of healthy bowel [74]. To ensure the complete removal of the transition zone, analysis of the entire bowel circumference (“donut section”) should be performed at the proximal end of the resection or stoma site [75]. Second, assessment of frozen sections introduces intraoperative delays due to the time required for pathological analysis, leading to prolonged anesthesia. Frozen section evaluation is technically demanding and requires an experienced pathologist, occasionally necessitating repeated biopsies [74].

Surgical treatment

The primary goal of surgery is to remove the aganglionic bowel and the adjacent transition zone, and to restore bowel continuity through a colorectal anastomosis. Worldwide, three main surgical techniques are primarily used: Swenson, Soave, and Duhamel (see **Figure 5**). All major reconstructive techniques for HD can be performed with laparoscopic assistance, which has been reported to offer clinical advantages such as shorter recovery times and improved cosmetic outcomes [76-78]. In all of them, it is important to place the anastomosis above the dentate line to preserve the sphincter mechanism and maintain continence.

The most commonly employed surgical technique for rectosigmoid HD is the transanal endorectal pull-through [27]. In patients with rectosigmoid HD, a single-stage pull-through procedure (without stoma) is generally preferred. For long-segment disease, particularly when rectal washouts are ineffective, a diverting stoma in normally innervated bowel is often required. In some cases, a three-stage procedure may be necessary, involving a protective ileostomy after pull-through to safeguard the anastomosis, and a later, separate ileostomy closure. The colon should, according to recommendations, be transected at least 5 cm proximal to the first normal biopsy to minimize the risk of a transition zone pull-through [27]. Transition zone pull-through is associated with obstructive defecation problems, often requiring redo pull-through [79]. There is variation in timing of the pull-through. A European survey reported that 33% of surgeons perform the pull-through at the time of diagnosis, whereas the majority (67%) delay surgery until the infant is a few months old [56]. There are insufficient data to establish the optimal timing of pull-through surgery, but it should be performed when the patient is stable, growing well, and the bowel has been sufficiently decompressed, usually within 2–3 months after diagnosis [27]. Postoperative complications include enterocolitis, which occurs in approximately 18% of cases [26], as well as stool leakage, anastomotic stricture, anastomotic leak with abscess formation and chronic constipation [15].

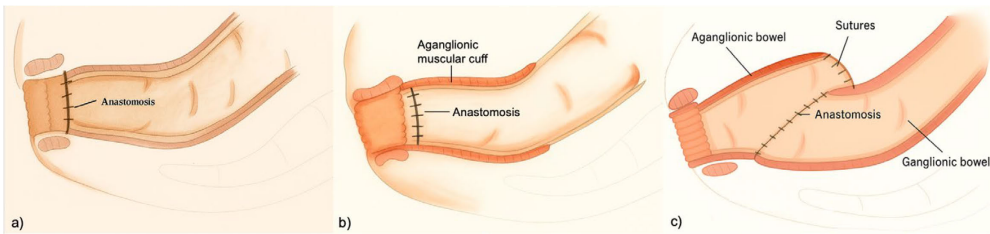

Figure 5. The three main surgical techniques for Hirschsprung's disease:
a) Swenson technique with full thickness dissection and resection of the aganglionic bowel.
b) Soave technique with pull-through of the ganglionic bowel through the aganglionic muscular cuff.
c) Duhamel technique with end to side anastomosis between the ganglionic and aganglionic segments.
Illustration created with AI assistance (ChatGPT, OpenAI, 2025).

Emerging diagnostics in Hirschsprung's disease

Other imaging modalities discussed in the literature for potential use in the diagnosis of HD include computed tomography and magnetic resonance imaging, including their potential role in prenatal diagnosis [16, 80]. Furthermore, preliminary studies on the application of artificial intelligence suggest that it may enhance diagnostic accuracy in both radiological and histopathological interpretation [81, 82].

One novel diagnostic technology for HD is ultra-high frequency ultrasonography. This detailed imaging modality has demonstrated significant potential in various medical fields including dermatology, rheumatology, neurology and oral medicine [83-86]. Despite its increasing use in other medical fields, its application in pediatric gastrointestinal diagnostics remains limited but promising [87]. Recent studies have indicated its potential in distinguishing between aganglionic and ganglionic bowel segments [88, 89]. This distinction is based on differences in histoanatomical dimensions, such as variations in the thickness of bowel wall layers, that can be captured with ultra-high frequency ultrasonography [90, 91]. In this thesis, histoanatomical differences were analyzed based on histopathology, in order to lay ground for further development.

Aims

The overall aim of this thesis was to improve the diagnostic process for HD by making it safer, more reliable, and, when possible, less invasive. Another aim was to validate current diagnostic methods to ensure their accuracy and clinical reliability. The specific aims of each individual study were:

I: To explore whether the child's weight influenced the diagnostic efficacy of RSB and to evaluate whether the efficacy differed between aganglionic and ganglionic tissue.

II: To describe an in-house developed method for orienting and handling fresh RSB specimens immediately after collection. To assess whether this orientation technique improves tissue quality, diagnostic efficacy, time to diagnosis, and histopathological workload, particularly for aganglionic biopsies.

III: To evaluate the sensitivity and specificity of the modified contrast enema for HD. To determine interobserver agreement between two pediatric radiologists, and to assess the method's ability to preoperatively predict the extent of aganglionosis in children with confirmed HD.

IV: To investigate whether histoanatomic layer dimensions differ between aganglionic and ganglionic bowel segments in histopathological specimens using an in-house developed software tool. To assess whether these measurements could be incorporated into a diagnostic algorithm to distinguish aganglionic from ganglionic segments, and to evaluate whether bowel wall thickness correlates with patient weight or age.

Methods

Setting

All studies were conducted at Skåne University Hospital, Lund, Sweden. Since 2018, the hospital has served as a national specialized medical care center for HD, covering a population of approximately five million. Prior to 2018, it functioned as a tertiary center serving 1.8 million residents.

Patients and study design

Study I

All children weighing 0–15 kg who underwent RSB for suspected HD between July 2011 and June 2019 were retrospectively identified from a local HD and biopsy register. The registry is maintained by the Department of Pediatric Surgery at Skåne University Hospital, Lund, Sweden. Background and biopsy data were collected from medical charts. Procedure-related complications were classified using the Clavien-Dindo system [92]. Outcomes included diagnostic efficacy and re-biopsy rate. Follow-up was defined as the period from biopsy to the study end date. Only the first RSB per patient was included in the analysis of diagnostic efficacy and complications.

Study II

This observational case-control study included all children examined for suspected HD. The diagnostic outcomes of oriented RSB specimens (February 2019–January 2022) were compared with non-oriented specimens (January 2015–January 2019). Orientation was performed using an in-house method for positioning fresh biopsy samples. Data were obtained from the local HD registry and pathology department records, including biopsy leveling and recoloring. Outcomes included diagnostic efficacy, need for repeat biopsy, diagnostic turnaround times, and histopathology workload.

Study III

All children aged ≤ 1 year evaluated for suspected HD between 2013 and 2023 were identified through a prospective procedure registry. Demographic and clinical data were collected from the local HD registry, medical records, and radiology reports. Modified contrast enema was performed in cases with persistent HD-specific symptoms where emergency surgery was not required. Referrals for modified contrast enema were made by pediatric surgeons or pediatricians.

Study IV

This observational study included children undergoing primary surgery for rectosigmoid HD between April 2018 and January 2024. Inclusion criteria were: age < 1 year at surgery, no prior stoma, and aganglionosis limited to ≤ 25 cm. Data on patient demographics and clinical characteristics were retrieved from the local HD registry.

Study timeline

There was a partial overlap in patient inclusion across the studies. Although the inclusion criteria varied, some patients were included in more than one study. This overlap primarily resulted from the studies being conducted during overlapping time periods and within the same clinical setting. The inclusion periods for Studies I–IV are illustrated in **Figure 6**.

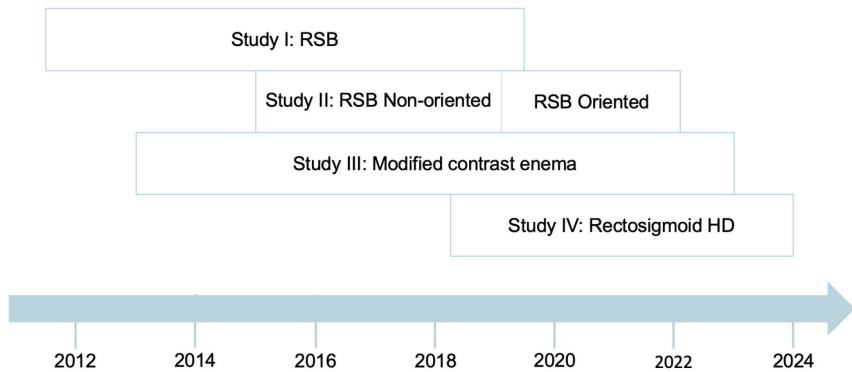

Figure 6. Overview of the patient inclusion periods for Studies I–IV.

Rectal suction biopsy (Studies I and II)

Techniques for collecting rectal suction biopsy

RSB was introduced as the first-line diagnostic method at the department in 2011 [93]. The procedure was performed in an outpatient setting using the Rbi2® suction instrument (Aus Systems, Australia) calibrated to a vacuum of 300 mm H_2O. Three biopsies were routinely obtained from the posterior rectal wall at 1, 2 and 3 cm above the dentate line, using a suction pressure of 200–250 mm H_2O. Specimens were extracted from the capsule by saline flushing or, if necessary, gently removed with a BD Microlance 3 needle. Until February 2019, all specimens were placed without orientation in a single container with 10% neutral buffered formalin and sent to the Department of Clinical Genetics and Pathology.

Oriented RSB (Study II): From February 2019 onward, biopsies were oriented by the operating pediatric surgeon immediately after collection, using loupe magnification and a BD Microlance 3 needle. Each biopsy was positioned in a foam cushion notch to align mucosa and submucosa in predefined directions and prevent distortion. The oriented biopsies were placed in separate cassettes, fixed in formalin, and labeled according to biopsy level (1-3 cm above the dentate line). Orientation details were documented and communicated to the pathologist. The orientation technique is illustrated in **Figure 7**.

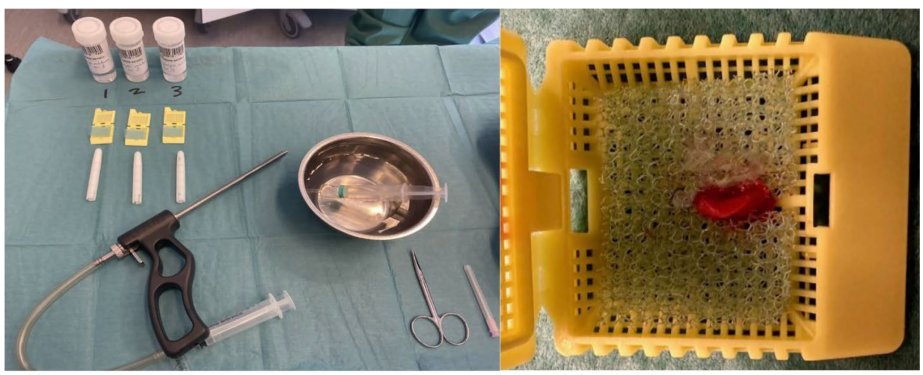

Figure 7. Rectal suction biopsy orientation method.
Left: The Rbi2® suction instrument and materials used for specimen retrieval, including saline for flushing and a needle for manual extraction.
Right: Each biopsy is carefully positioned in a precut notch of a foam cushion, with the mucosal and submucosal surfaces aligned in predefined directions. Each foam cushion is then placed in an individual cassette, which is placed in a labeled container prefilled with formalin (top left).

Histopathological processing and assessment

Upon arrival at the pathology department, RSB specimens were routinely formalin-fixed, paraffin-embedded, and serially sectioned onto microscopy slides. Initial H&E staining was followed by an automatically ordered ganglion cell staining series (G-series), including H&E and immunohistochemistry with S-100 and calretinin. H&E-stained sections were evaluated for the presence or absence of ganglion cells in the submucosa. To support identification of ganglion cells and nerve fibers, S-100 and calretinin immunostaining were performed using standard automated methods with ready-to-use antibodies. Biopsies were classified into three categories: ganglion cells present, ganglion cells absent, or inconclusive. Aganglionosis was diagnosed based on absence of ganglion cells and presence of S-100 positive, calretinin negative nerve fibers at the level of the muscularis mucosa.

In Study II, tissue quality was assessed by a pathologist following completion of the G-series and categorized as high, acceptable, or low. Diagnostic responses were dichotomized into first and final answers, as illustrated in **Figure 8**. Histopathological workload was defined as any additional procedures beyond the standard H&E and G-series, such as specimen reorientation and/or deeper sectioning, which required extra time and resources.

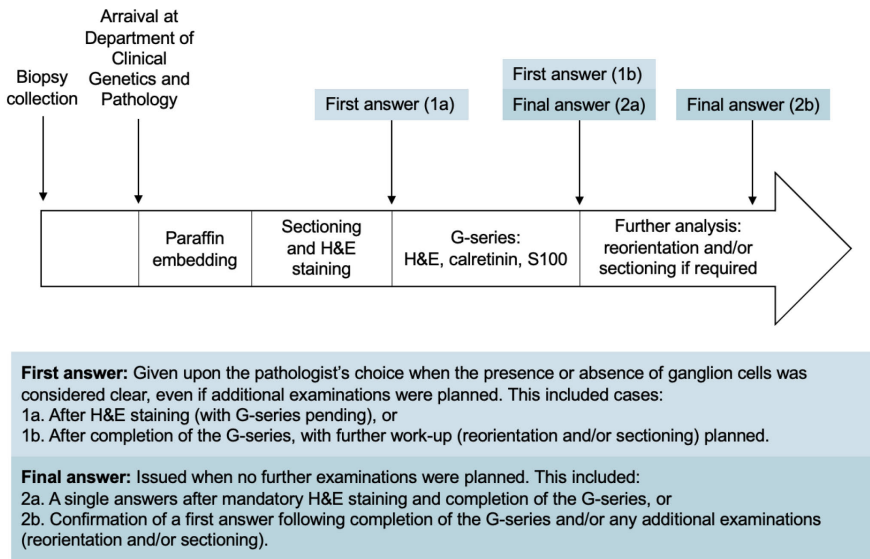

Figure 8. The rectal suction biopsy workflow from collection to the first and final answer at the Department of Clinical Genetics and Pathology. Definitions of the first and final answers are provided in the text box.

Modified contrast enema procedure (Study III)

The modified contrast enema technique was developed in 2007 and evaluated in 2013 [28], based on previous work assessing the RAIR using cold water and sonography [94]. All examinations were performed by pediatric radiologists and radiology nurses.

Procedure overview

Preparation and positioning: Rectal catheterization was avoided for 24 hours prior to the examination. The child was positioned on their left side with knees flexed and stacked on top of each other, while an assistant (parent, nurse or doctor) stood by their side and held them firmly. A 6-Fr feeding tube with a single end-hole was inserted into the anal canal, as superficially as possible.

Warm contrast phase: A slow injection of water-soluble isotonic contrast medium (Omnipaque 140 mg/ml at 37°C) was administered under low-pulse fluoroscopy until the rectum and distal sigmoid colon were filled.

Cold contrast phase: The catheter was advanced into the rectal ampulla, and 20 ml of cold contrast medium was rapidly injected (< 5 seconds). Fluoroscopy sequences were recorded for approximately 40-60 seconds to visualize the RAIR. If the RAIR was not clearly observed, the cold contrast injection was repeated once.

Full contrast evaluation: Warm contrast was reintroduced to prevent hypothermia and to assess colonic appearance and visualize a potential transition zone. The contrast medium was administered until at least the left colon was filled, and in cases of pathological findings, until normal colon was visualized.

Post-procedure imaging and recommendations: After 12-24 hours, a supine abdominal image and a lateral rectal view were obtained to evaluate rectal and colonic emptying. Rectal catheterization was avoided during this period.

Diagnostic assessment

All examinations were reviewed by a pediatric radiologist. In uncertain cases, evaluations were discussed by a team of pediatric radiologists. Each examination was assessed using a standardized clinical protocol, including the presence of RAIR, rectal or rectocolonic contraction, rectosigmoid ratio, caliber change, wall irregularities, and contrast retention after 12-24 hours. A normal RAIR was classified into three phases displayed in **Figure 9**. RAIR was considered present if phases one and two, or all three phases, were observed. Any caliber change in the rectum, rectosigmoid, or colon was noted in the radiology report and subsequently categorized as either rectosigmoid or more proximal (i.e., extended aganglionosis).

A summary radiology report was generated for each examination, evaluating all findings and concluding whether HD was suspected or not. In the accuracy analysis, any ambiguous results were counted as consistent with HD. Aganglionosis was confirmed preoperatively through histopathology and immunohistochemistry. Based on pathology reports from resected specimens, rectosigmoid aganglionosis was defined as a segment of 0-25 cm, and extended aganglionosis as > 25 cm.

To validate the modified contrast enema, an interobserver analysis was conducted. Two pediatric radiologists independently and blindly reviewed all examinations, including fluoroscopy and images. Their results were compared, including verification of the RAIR, assessment of caliber change, and an overall evaluation of whether HD was suspected.

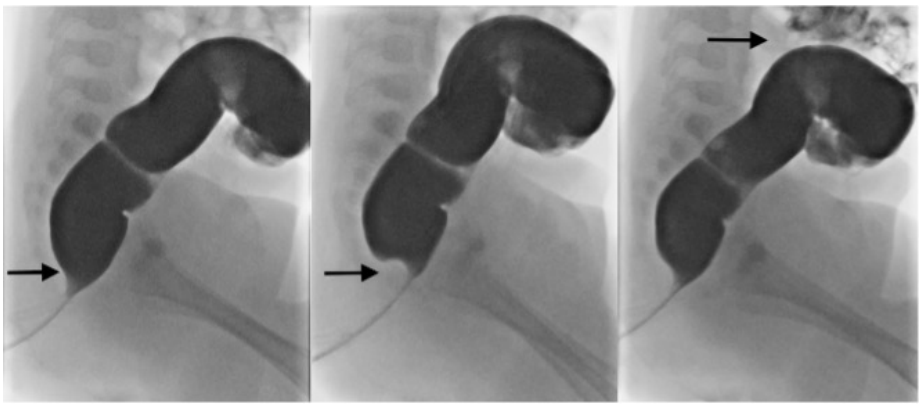

Figure 9. Illustration of the three phases of a normal rectoanal inhibitory reflex as visualized on modified contrast enema.
Phase 1: Relaxation of the internal anal sphincter with entry of contrast medium and dilatation of the anal canal. Sometimes emptying of rectal contents.
Phase 2: Contraction of the sphincter and the distal rectum, with closure of the anal canal.
Phase 3: Retrograde movement of contrast back into the sigmoid colon.

Specimen treatment, staining and histological layers (Study IV)

During surgery, the aganglionic part of the colon was resected. The freshly resected specimen was pinned onto a cork mat, with aganglionic and ganglionic segments positioned in opposite directions. Histopathological processing was conducted at the Department of Clinical Genetics and Pathology. Specimens were formalin-fixed and sectioned to assess the aganglionic segment, the transition zone, and the ganglionated proximal margin. Paraffin-embedded tissue was stained with H&E and further evaluated using immunohistochemistry with calretinin and S-100 to confirm

the presence of ganglion cells and nerve structures. All slides were reviewed by a specialist in pediatric pathology. For each patient, H&E-stained slides representing aganglionic and ganglionic bowel segments were selected by a pathologist. The digitized slides, stored in Sectra PACS/IDS7 system, were transferred to MATLAB (MathWorks, USA), a software customized by engineers at the university's Faculty of Engineering. This software has previously been used for histoanatomic measurements in correlation analyses, but has only been validated for ultra-high frequency ultrasonography applications [90, 95]. The software enabled manual delineation of the bowel wall layers (muscularis externa, myenteric layer, muscularis interna, submucosa, mucosa, and full wall) and semi-automatic calculation of their thicknesses at 14 µm measurement intervals, as illustrated in **Figure 10**. In cases where artifacts were present, only intact portions of the section were analyzed. For each layer, the following parameters were computed: mean thickness, standard deviation, area, and inner and outer lengths. The ratio of muscularis interna to externa was calculated, as well as a submucosal folding ratio (inner submucosal length divided by outer submucosal length) to enable comparison between aganglionic and ganglionic tissue.

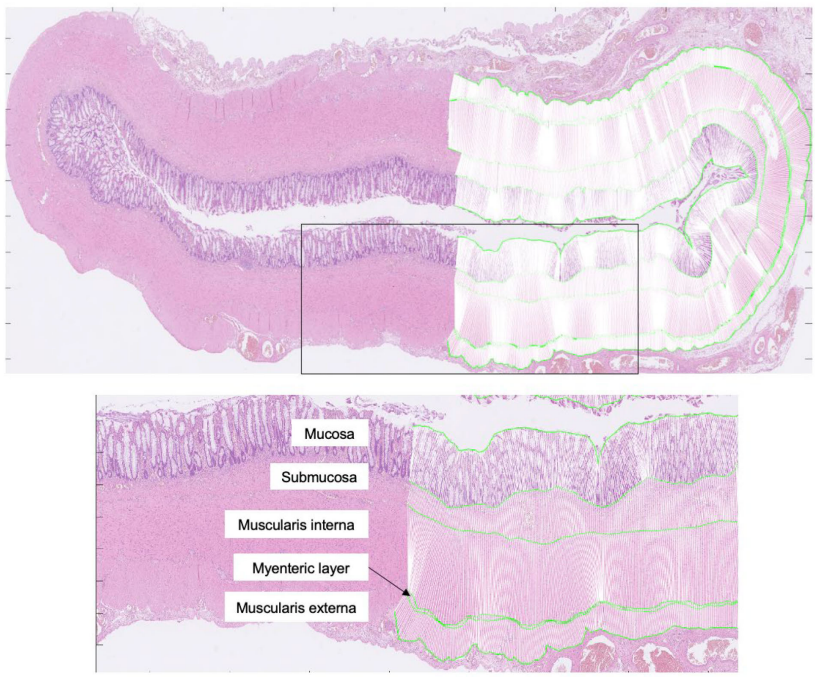

Figure 10. Images showing a full circular cross-section of a ganglionic segment from a child who underwent surgery for Hirschsprung's disease. The green line indicates the manual delineation of individual histoanatomical layers, performed using the analysis software. White lines represent measurement points used for the automated calculation of layer thickness. For illustrative purposes, a half-circumferential delineation was shown.

Statistical analysis

All statistical analyses were performed using SPSS (IBM Corp., USA), with statistical significance set at $p < 0.05$. Statisticians at the Department of Clinical Studies, Statistics, Forum South, provided guidance for the statistical analyses in Studies III and IV. **Table 1** provides an overview of the statistical tests applied in the four studies.

Studies I-III

Fisher's exact and Pearson's chi-square test were used for dichotomous and categorical comparisons, respectively. The Mann-Whitney U test was applied for continuous non-parametric data.

In Study I, Spearman's correlation was used to evaluate associations between age, weight, and RSB diagnostic efficacy due to the non-parametric distribution of the data. A weight cut-off was defined based on significant group differences.

In Study III, diagnostic accuracy measures, including sensitivity, specificity, positive and negative predictive values for HD and aganglionic length, were calculated based on concordance between radiology reports, rectal biopsy findings, and the aganglionosis length in resected specimens. Interobserver agreement was calculated using Cohen's kappa (κ), interpreted according to the following grading scale: 0.00–0.20 very low, 0.21–0.4 low, 0.41–0.6 moderate, 0.61–0.80 strong, 0.81–1.00 almost perfect agreement.

Study IV

The normality of data distribution was verified by statisticians at the Department of Clinical Studies, Statistics, Forum South, using distributional analysis. Paired t-test were used to compare histoanatomical layer thicknesses between aganglionic and ganglionic bowel segments, with each patient serving as their own control. Pearson's correlation was applied to evaluate associations between full bowel wall thickness and patient age and weight at the time of surgery. A correlation coefficient of ± 1 indicated strong correlation, whereas 0 indicated no correlation.

Table 1. Overview of the statistical tests used in the four studies.

Statistical test	Study I	Study II	Study III	Study IV
Median	X	X	X	-
Mean	-	-	-	X
Fisher's Exact Test	X	X	X	-
Pearson's Chi-Square Test	X	X	X	-
Mann-Whitney U Test	X	X	X	-
Spearman's Correlation	X	-	-	-
Sensitivity, Specificity, Positive and Negative Predictive values	-	-	X	-
Cohen's Kappa	-	-	X	-
Paired t-test	-	-	-	X
Pearson's Correlation	-	-	-	X

Ethical considerations

All studies were conducted in accordance with the Declaration of Helsinki, as revised by the World Medical Association in 2013. Ethical approval was obtained from the Regional Ethical Review Board (DNR 2017/191), and for Studies III and IV, also from the Swedish Ethical Review Authority (DNR 2023-01833-01).

Funding

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Results

Study I

A total of 84 children weighing between 0 and 15.0 kg who underwent first-time RSB for suspected HD were included. All presented with HD-typical symptoms, and in 80% of cases, symptom onset occurred within the first month of life. A contrast enema had been performed prior to biopsy in 90% of children. All who were later diagnosed with HD showed abnormal findings on radiology.

Two children experienced complications within 24 hours after biopsy, both requiring surgery and blood transfusion (Clavien–Dindo grade IIIb). Neither of these children was diagnosed with HD. No further complications were reported within 4 weeks.

The diagnostic efficacy of first-time RSB was 85% **(Table 2)**. Children with inconclusive results underwent either repeat RSB or full-thickness biopsy to reach a definitive diagnosis. In the weight-based analysis 9.0 kg was identified as a significant diagnostic cut-off. Higher efficacy was observed in children weighing less than 9.0 kg (**Figure 11**). No child weighing more than 12.0 kg (n = 4) had a conclusive biopsy result. Age and weight correlated positively (Spearman's rho = 0.763).

Table 2. Comparison of diagnostic efficacy within and between study groups.

	Diagnostic efficacy	p-value
First-time RSB, overall n=84	85%	
Aganglionosis vs ganglionosis	74% vs 89%	0.104
Children <9 kg vs >9 kg	89% vs 62%	0.026
Aganglionosis vs ganglionosis in children <9 kg	77% vs 96%	0.045

HD was diagnosed in 32% of the children. Among those with aganglionosis, 93% weighed between 2.0 and 4.0 kg at first-time biopsy. The diagnostic efficacy of first-time RSB was 74% in children with aganglionosis and 89% in those with ganglionosis (p = 0.104). In children weighing less than 9.0 kg (the cut-off weight), diagnostic efficacy was significantly lower for aganglionosis (77%) than for ganglionosis (96%) (p = 0.045).

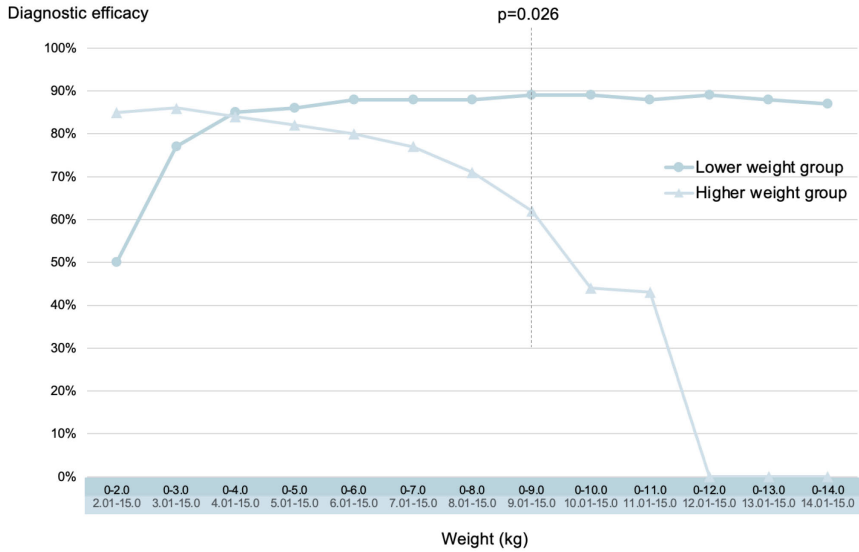

Figure 11. Diagnostic efficacy of rectal suction biopsy compared across weight groups. The cut-off point corresponds to the weight at which the difference between groups reached statistical significance.

Study II

A total of 78 children were included, comprising 37 (46%) oriented and 44 (54%) non-oriented biopsy occasions. **Figure 12** illustrates the included children, biopsy occasions, and biopsy results. The diagnostic efficacy of RSB did not differ significantly between the oriented and non-oriented biopsy groups (97% vs. 86%; p = 0.119, **Table 3**). A first diagnostic answer after H&E staining or after completion of the G-series (with further examinations planned) was significantly more frequent in the oriented group compared with the non-oriented group (54% vs. 20%; p = 0.002). The median time to both the first and final diagnostic answer (after completion of the G-series with or without further examinations) was one working day shorter in the oriented group (p = 0.015 and p = 0.002, respectively). No incorrect diagnoses were reported in either group.

In total, 242 biopsies were analyzed, comprising 106 oriented and 136 non-oriented specimens, with a median of three biopsies per occasion in both groups. High-quality RSB specimens were more frequently obtained in the oriented group (40% vs. 25%; p = 0.018). Fewer additional sections beyond the automatically ordered G-series were required for oriented biopsies (median 8 vs. 16; p = 0.012).

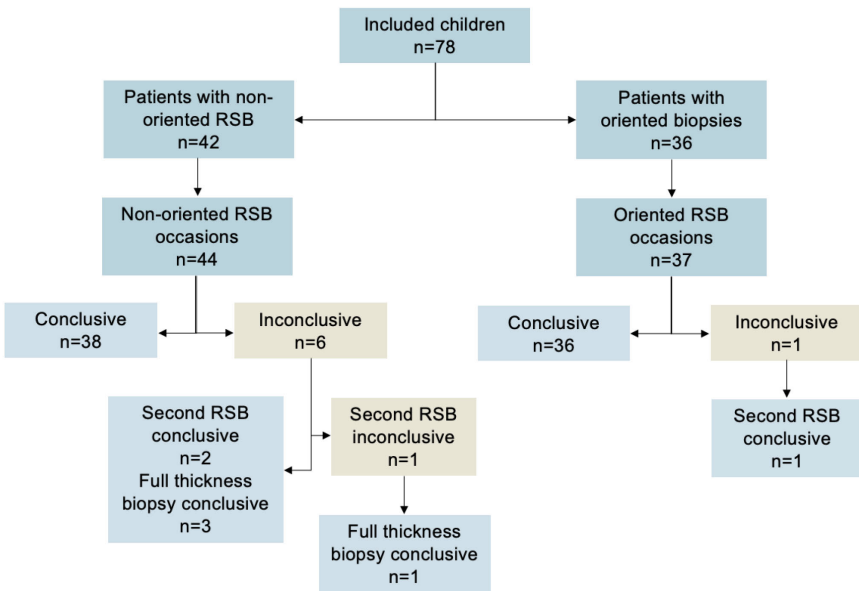

Figure 12. Overview of included children and biopsy occasions in the non-oriented and oriented biopsy groups. The flowchart illustrates conclusive and inconclusive biopsy results, as well as the number of repeated biopsy procedures.

Table 3. Comparison of outcomes between non-oriented and oriented biopsy occasions.

	Non-oriented rectal suction biopsy occasions (n=44)	Oriented rectal suction biopsy occasions (n=37)	p-value
Efficacy	86%	97%	0.119
First answer given	20%	54%	0.002
Median days to first answer	3	2	0.015
Median days to final answer	5	4	0.002
Diagnosed with aganglionosis	31%	53%	
Total number of biopsies	136	106	
High quality specimens	25%	40%	0.018
Additional sectioning	16	8	0.012

Aganglionosis

Aganglionosis was diagnosed in 41% of the children overall; 53% in the oriented group and 31% in the non-oriented group. All 19 cases of aganglionosis in the oriented group were diagnosed by RSB alone, compared with 9 of 13 cases in the non-oriented group ($p = 0.020$). Consequently, the diagnostic efficacy per biopsy occasion was significantly higher in the oriented group (95% vs. 60%; $p = 0.027$).

The median time to both the first and final diagnostic answer (after completion of the G-series with or without further examinations) was one working day shorter in the oriented group ($p = 0.027$ and $p = 0.169$, respectively). High-quality RSB specimens were more frequently observed in the oriented aganglionic group (47% vs. 14%; $p < 0.001$), and fewer additional sections were required (median 9 vs. 22; $p = 0.030$).

Study III

A total of 160 children underwent modified contrast enema during the time period and all were included in the study. Of these, 80 (50%) proceeded to rectal biopsy. Among them, 31 children were diagnosed with HD, representing 19% of those examined with modified contrast enema and 39% of those who underwent rectal biopsy. All children with HD subsequently underwent surgery.

Table 4 presents a comparison of radiology report findings after modified contrast enema in children with and without HD. The modified contrast enema demonstrated a sensitivity of 100% and a specificity of 87%. The positive predictive value was 65%, while the negative predictive value was 100%.

Table 4. Comparison of radiology report findings from modified contrast enemas between children with and without Hirschsprung's disease. Ambiguous results were classified as consistent with Hirschsprung's disease.

	Hirschsprung's disease (n=31)	No Hirschsprung's disease (n=129)	p-value
Overall evaluation consistent with Hirschsprung's disease	100%	13%	<0.001
Rectoanal inhibitory reflex absent	100%	21%	<0.001
Statically contracted rectal/rectosigmoid segment	94%	9%	<0.001
Caliber change identified	77%	0%	<0.001

Follow-up imaging within 12–24 hours, conducted without any rectal manipulation in accordance with the study protocol, was feasible in 29% of children with HD and in 79% of non-HD cases ($p < 0.001$). Among children with HD, the only limitation was the inability to abstain from rectal washouts, whereas in the non-HD group, limitations were primarily related to other clinical interventions or logistical constraints. At follow-up imaging, contrast retention in the colon was observed in 67% of HD cases compared with 10% of non-HD cases ($p < 0.001$).

Interobserver agreement was strong for RAIR absence ($\kappa = 0.697$), overall HD diagnosis ($\kappa = 0.769$), and identification of a caliber change ($\kappa = 0.688$), all with $p < 0.001$.

Histopathology confirmed rectosigmoid aganglionosis (< 25 cm) in 28 children and extended aganglionosis (> 25 cm) in three. A caliber change was identified in 71% of rectosigmoid cases and 33% of extended cases (**Figure 13**). In all 21 cases with a visible caliber change, the radiological and histopathological levels corresponded, yielding a positive predictive value of 100%. Caliber change was absent in 10 (32%) children with HD, yielding a negative predictive value of 94% for rectosigmoid HD and 93% overall.

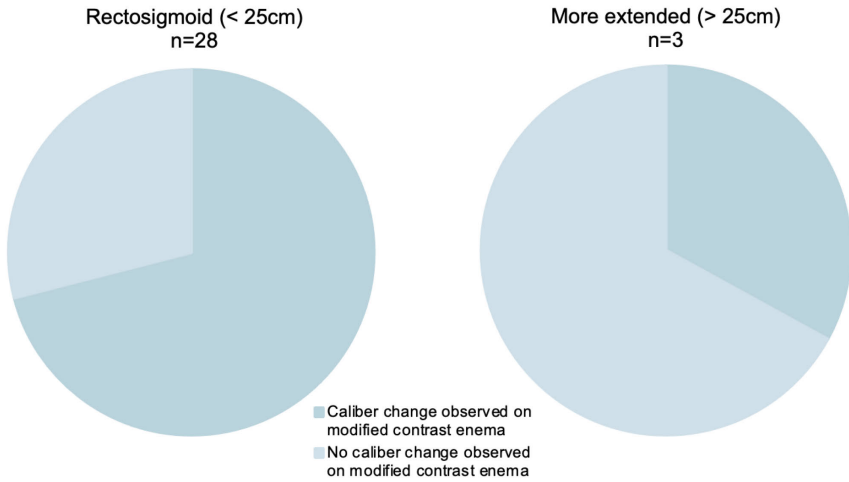

Figure 13. Histopathological length of aganglionosis categorized as rectosigmoid (< 25 cm) or more extensive (> 25 cm), and the presence of a caliber change on modified contrast enema. In all cases where a caliber change was observed, the radiological level corresponded to the histopathological transition zone, resulting in a positive predictive value of 100%.

Study IV

A total of 30 patients were included in the study. Among these, five (17%) had genetic syndromes (three with trisomy 21, one with trisomy X, and one with Mowat-Wilson syndrome).

Histological analysis of the bowel layers revealed that the muscularis interna was significantly thicker in ganglionic segments (mean 0.666 mm) compared with aganglionic segments (mean 0.461 mm; $p < 0.001$). The pattern of a thicker muscularis interna in ganglionosis was observed in 28 of 30 patients. The ratio of muscularis interna to muscularis externa was also significantly higher in ganglionic segments (mean 2.047) than in aganglionic segments (mean 1.287; $p < 0.001$). This greater ratio in ganglionosis was present in 29 out of 30 patients. No statistically significant differences were found between ganglionic and aganglionic segments in the thickness of other histoanatomic layers, including the muscularis externa, myenteric tissue layer, submucosa, mucosa, full bowel wall, or in the degree of submucosal folding.

Based on these findings, a diagnostic algorithm incorporating the two most discriminatory histological parameters was proposed (**Figure 14**). This algorithm correctly classified all 30 patients, including three with borderline measurements. Two aganglionic specimens showed a thicker muscularis interna, but both still had a lower muscularis interna to externa ratio compared with the ganglionic segment. In one specimen the muscularis interna to externa ratio was higher in the aganglionic segment, but the muscularis interna remained thinner than in the ganglionic bowel.

No significant correlations were observed between full bowel wall thickness and patient age or weight at time of surgery, in either aganglionic or ganglionic segments.

Additionally, a subgroup of six children with a diverting stoma prior to definitive HD surgery, excluded from the main statistical analysis, was analyzed separately. Similar trends were observed in this group: all had a higher muscularis interna to muscularis externa ratio in ganglionic segments, and five of six showed a thicker muscularis interna in the ganglionic compared with aganglionic bowel.

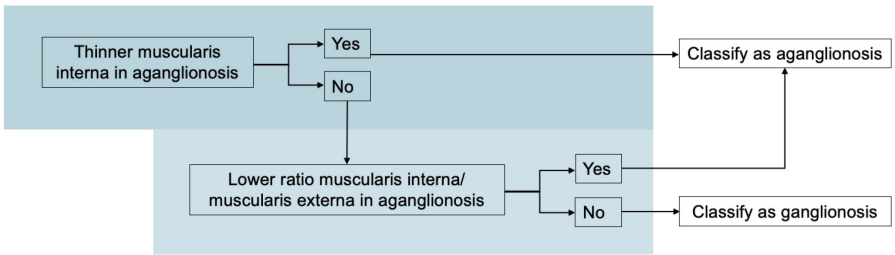

Figure 14. Diagnostic algorithm for distinguishing between aganglionic and ganglionic bowel based on histomorphometric measurements. All specimens in the study (n = 30) were correctly classified using this algorithm. Two aganglionic specimens exhibited a thicker muscularis interna in the aganglionic segment, but both still showed a lower muscularis interna to externa ratio compared with the ganglionic segment. One specimen demonstrated a higher ratio in the aganglionic segment, but the muscularis interna remained thinner than in the ganglionic bowel.

Discussion

Main findings

The main findings of this thesis were that:

- Diagnostic efficacy of RSB was higher in children weighing less than 9 kg compared with those of higher weight. Among children below 9 kg, diagnostic efficacy was significantly greater in ganglionic than aganglionic specimens.
- Specimen orientation of fresh RSB improved sample quality, reduced diagnostic turnaround time, and minimized histopathological workload. Furthermore, orientation increased diagnostic efficacy specifically in aganglionic tissue.
- The modified contrast enema achieved a sensitivity of 100% and a specificity of 87% for the diagnosis of HD. In cases with a visible rectosigmoid caliber change, the positive predictive value for disease extension was 100%.
- Quantitative histopathological differences in bowel wall morphology between ganglionic and aganglionic segments enabled development of a diagnostic algorithm that correctly identified all aganglionic cases in the study.

General discussion of findings

The diagnostic challenges of HD are considered to reflect the inherent difficulty of proving the absence of ganglion cells, rather than confirming their presence. Demonstrating the absence of a structure places particularly high demands on tissue quality, as illustrated in both Studies I and II.

Diagnostic challenges with rectal biopsy in aganglionosis

In Study I, the diagnostic efficacy of RSB was lower in aganglionic specimens compared to ganglionic ones, and this was particularly among children weighing less than 9 kg. This suggests that obtaining a conclusive diagnostic result is more challenging in aganglionic tissue and underscores the critical importance of meticulous specimen handling, particularly in the subgroup of children with HD. In Study II, the proportion of high-quality specimens among aganglionic samples was only 14% prior to the introduction of standardized orientation, increasing to 47% after. This led to an increase in diagnostic efficacy among aganglionic specimens from 60% to 95% after orientation started.

The tendency for aganglionic biopsies to be more frequently inconclusive has previously been reported in studies for RSB, with predictive values for eventually confirming HD of 79% and 83%, respectively [37, 45]. Also, with full-thickness biopsy inconclusive biopsies are reported more frequent among aganglionic biopsies [47]. One plausible explanation for the problem is a proposed increased rigidity of aganglionic bowel tissue, suggested to be caused by intestinal fibrosis [96]. Increased intestinal stiffness with age has also been the suggested explanation for more frequent inconclusive results of rectal biopsies in older children [31]. Speculating, in patients with chronic constipation and a megarectum with reduced elasticity [97], it may be more difficult to approximate the suction device to the rectal mucosa [34].

A clinical observation made by the pediatric surgeons performing RSBs at Skåne University hospital is the absence of bleeding at the biopsy site in children with HD, in contrast to children with normally ganglionated bowel, where bleeding is often observed. This subjective impression aligns with the findings in Study I, where both children who experienced complications following RSB, specifically persistent bleeding, were later found not to have HD, while no children with HD experienced any complications. The underlying mechanism may also be related to structural tissue characteristics of the aganglionic bowel wall.

Orientation and specimen handling

In Study II, an improved diagnostic outcome for HD was achieved through the implementation of the reported standardized biopsy handling and orientation method for fresh RSB. Although the importance of careful specimen handling has been emphasized previously in the literature [16, 31], and specimen orientation has

been shown to improve diagnostic yield in other fields [98], no orientation studies specific to HD, nor detailed methodological descriptions comparable to the present one, have been published to date. Both the study design and its findings were novel. The orientation increased the proportion of high-quality specimens from 25% to 40% overall. Notably, among aganglionic samples, the proportion of high-quality biopsies rose from 14% to 47%, significantly improving diagnostic efficacy. These findings underscore the impact that systematic handling and orientation can have on both specimen quality and diagnostic efficacy.

During the study period of Studies I-III, an increased number of patient evaluations for HD were referred to the department, and team-based care was implemented. This was because the Department of Pediatric Surgery at Skåne University Hospital was assigned by the Swedish National board of Health and Welfare as a national specialized medical care center for HD. Therefore, also the organization and workload of examinations may have influenced the diagnostic success. A notable reduction in the number of pediatric surgeons performing RSB procedures was observed; in Study I, 13 pediatric surgeons carried out the biopsies, whereas in Study II, only seven performed the procedures, and just four conducted the oriented RSB. Although we did not specifically assess whether the number of operators affected diagnostic efficacy, previous studies have reported that RSB outcomes are not user-dependent when performed by a surgeon [34-36]. This suggests that the true reason for the reduction in repeated biopsies and improved efficacy, was the procedural standardization rather than individual operators.

Diagnostic difficulties with modified contrast enema specifically for aganglionosis

The modified contrast enema procedure also presents specific difficulties in patients with aganglionosis, but for other reasons. In Study III, six patients were excluded due to their inability to avoid rectal catheterization within 24 hours prior to the examination, a prerequisite for optimal image interpretation. Additionally, most children who required rectal catheterization before follow-up imaging (12–24 hours post-procedure) were later diagnosed with HD. This suggests that maintaining optimal preparation conditions for the contrast enema is particularly difficult in this patient group. Consistent with previous studies, the majority of children with aganglionosis who performed follow-up imaging presented with retained contrast [58, 65].

Similarly to identifying the absence of ganglion cells, assessing the absence of RAIR presents distinct methodological challenges. Several non-HD conditions, such as motility disorders, colitis, and severe functional constipation, have been reported to be able to impair RAIR [28, 97]. This may also relate to stiffer tissue, increasing the risk of false-positive results and thereby reducing specificity.

To date, the effect of using a thin rectal catheter prior to RAIR assessment within the modified contrast enema method has not been systematically studied. This

represents an area of potential interest, as a better understanding could enable and facilitate the examination process. In anorectal manometry rectal wash-outs have in some cases been performed a few hours prior to the examination without implications on examination results [71]. One concern is that prior colonic evacuation through regular washouts, performed to relieve symptoms and prevent enterocolitis, may obscure a potential transition zone [99], which in theory could lead to false-negative imaging results. However, in Study III, all children with HD underwent rectal washouts prior to the examination (though not within 24 hours before). This timing likely did not affect the visibility of any caliber change since only 32% had a lack of it on the imaging. The modified contrast enemas were performed at a median of 17 days, and washouts were initiated when HD was first suspected, which was in close temporal proximity to the examination.

It is important to remember that both the modified contrast enema and RAIR assessment are intended as screening tools, and not definitive diagnostics. Their primary role is to guide the decision of whether or not to proceed with biopsy. In this context, high sensitivity is more critical than perfect specificity. As long as the method reliably identifies all children with HD (i.e., no false negatives), the presence of some false positives is acceptable, as these cases will undergo confirmatory testing via rectal biopsy.

The role of bowel wall thickness in RSB-efficacy

In Study I, we hypothesized that body weight might serve as a more accurate predictor of RSB diagnostic efficacy than age. This assumption was based on the premise that body weight may more directly reflect inter-individual differences in bowel wall thickness and histoanatomical layers. These anatomical variations could in turn, influence the likelihood of obtaining an adequate sample of submucosa during biopsy. However, in Study IV, full bowel wall thickness at the time of surgery did not significantly correlate with either age or weight. However, the thickness of the mucosa was not examined separately in relation to weight or age. The generally low age of patients included in Studies I, II, and IV, where children were between one and two months old at the time of diagnosis and surgery, limits the ability to examine broader weight and age ranges, which could be relevant for assessing bowel wall thickness throughout childhood and across all patients with HD.

Our findings in Study IV, showing no significant increase in colonic wall thickness with age, align with a previous study reporting no significant increase in colonic wall thickness among healthy individuals from 1 month to 39 years of age [100], but contradicts another study reporting a gradual age-related increase, reaching a mean maximum of 2.0 mm in individuals aged 20–29 years [101]. Notably, in our cohort of children with a mean age of 45 days, the bowel wall thickness was already 2.0 mm. This can imply that bowel wall thickness reaches its greatest thickness already early in life. The lower diagnostic efficacy of RSB observed in older

children or those with higher weight may theoretically be attributed to factors such as altered tissue texture and increased rigidity of the mucosa and submucosa, or to technical aspects of the biopsy procedure, all of which play a critical role in obtaining tissue of sufficient depth during RSB.

Tightened selection criteria for rectal biopsy

Although we did not specifically investigate changes in the number of children undergoing RSB at the Department of Pediatric Surgery at Skåne University Hospital since the introduction of the method 2007. Data from a first study on RSB between 2007-2012 revealed that 80% (16/20) of children who underwent rectal biopsy did not receive a diagnosis of HD [93]. In comparison, Study II, covering the period from February 2019 to January 2022, the proportion of children undergoing RSB, turning out negative for HD, had decreased to 47% (17/36). Although the total number of children referred for HD evaluation was not systematically recorded, the department's designation as a national specialized medical care center in 2018 led to a higher inflow of children diagnosed and treated for HD. Despite this increase, the number of rectal biopsy procedures, including both RSB and full-thickness biopsies, remained relatively stable. This suggests a shift in diagnostic practice and the trend may reflect a more selective and targeted approach to biopsy indications, potentially indicating improved clinical assessment and triage prior to RSB.

One important reflection is that since most inconclusive biopsies in previous studies were later confirmed as HD [37, 45], and since diagnostic efficacy in Study I was lower for aganglionic specimens, a more selective approach could potentially and paradoxically reduce overall diagnostic efficacy.

Diagnostic criteria for selection to rectal biopsy

In clinical practice, following established criteria for which children should undergo rectal biopsy seems to be challenging. In the United Kingdom, the National Institute for Health and Care Excellence has proposed the following indications for rectal biopsy [102]: 1. Passage of meconium > 48 hours after birth 2. Constipation since the first weeks of life 3. Chronic abdominal distension with vomiting 4. Family history of HD 5. Altering growth in addition to any of the previous features. A study evaluating adherence to these guidelines found that 68.1% of children underwent biopsy based on any of these indications, and of these, 84% had ganglion cells (i.e., no HD) [103]. Only 80% of children with HD actually met the criteria for biopsy [103], suggesting that cases would likely have been missed if the guidelines had been applied too rigidly. There was no report on how many children were missed in the diagnosis of HD. In our setting, selection for radiological examination and biopsy was based on the surgeon's discretion and not guided by any established criteria. During the study periods covered by the thesis, one child with HD was initially missed, since this child was not selected for either radiology or rectal biopsy during the first consultation. The diagnosis was made later, and the child

subsequently underwent surgery for HD. Hypothetically, the use of selection criteria might have prevented the delay in this particular case; however, it could also have led to more children undergoing unnecessary diagnostic procedures as described in the British setting.

As with most diagnoses, the application of guidelines and selection criteria requires not only a thorough understanding of the disease and clinical experience but also, critically, nuanced clinical judgment. While reducing unnecessary biopsies is a valid and important clinical objective, such decisions must be grounded in sound clinical reasoning. Diagnostic strategies should aim to streamline care pathways without compromising the timely identification and treatment of children with HD. The potential risk of missed or delayed diagnoses must be carefully balanced against the benefits of minimizing invasive procedures. Consequently, ongoing clinical follow-up and reassessment are essential, particularly in cases where symptoms persist, evolve, or worsen over time.

Strength and limitations

My position as a pediatric resident has allowed me to approach the research questions surrounding HD, a condition managed within pediatric surgery, from a more objective standpoint. Being somewhat external to the field has helped me avoid influence from established clinical routines, institutional habits, or personal preferences. This external perspective may offer valuable insights by questioning assumptions and maintaining analytical distance.

In Study I, which involved retrospective review of clinical and pathological data, an external analytical approach minimized the risk of confirmation bias when interpreting inconclusive or borderline biopsy results. My neutrality allowed for a critical assessment of the RSB method without influence from prior clinical expectations. In Study II, my independent position enabled objective evaluation of histopathological quality and turnaround times, free from bias associated with those who developed or performed the orientation method. This strengthened the credibility of the observed improvements in diagnostic efficiency. In Study III, my position outside the radiology and pediatric surgery teams allowed for a balanced interpretation of diagnostic accuracy, interobserver variability, and methodological limitations, reducing the risk of overly favorable conclusions regarding the technique. In Study IV, objectivity contributed to methodological rigor during digital measurements and statistical interpretation. The analysis was performed largely blinded to clinical data, minimizing observer bias and enhancing reliability of the proposed diagnostic algorithm.

However, not being within the field of pediatric surgery may also imply clinical limitations. My limited direct contact with this patient group means a lack of

informal, experience-based insights that emerge from daily clinical care, multidisciplinary discussions, and operative decision-making. Greater first-hand diagnostic and surgical experience might have provided a deeper understanding of practical challenges and the lived experiences of affected children and their families. On the other hand, my pediatric background may facilitate broader clinical application of these findings, particularly in improving the selection and referral of patients to pediatric surgical specialists, thereby contributing to safer and more efficient diagnostic pathways for HD.

Another strength of this thesis is the multidisciplinary setting employed in all studies involving collaboration with professionals from other clinical and scientific disciplines. Studies I, II, and IV were conducted in close collaboration with pathologists, Study IV also included a translational study with engineering researchers from the Faculty of Engineering. Study III involved pediatric radiologists. These collaborations broadened the scope of the research and facilitated comprehensive discussions, thereby increasing the scientific depth and relevance of the work. Importantly, the multidisciplinary foundation of the project facilitated the implementation of the results. In addition, the doctoral project has been conducted in collaboration with the National Hirschsprung's Disease Patient Organization in Sweden, which has helped to maintain a patient-centered focus throughout the research process.

Nevertheless, several limitations should be acknowledged. All studies were conducted at a single center, which may limit the generalizability of the results. Moreover, due to the rarity of HD and the limited patient population, some children were included in more than one study. This could be considered a potential limitation, as it may introduce bias and reduce the independence of the results. However, we carefully considered this issue and found no reason for such overlap to influence the outcomes in our studies. Each study had distinct aims, inclusion criteria, and methodologies, and none analyzed the exact same endpoints. From an ethical standpoint, one might argue that some children were enrolled in several studies, but all received standard clinical care identical to what they would have otherwise undergone. Based on clinical experience, parents generally welcome the opportunity to contribute to research that may improve diagnostics and care for future patients.

HD is a rare condition, and only a small number of children are diagnosed and evaluated each year. As a result, inclusion periods were long, and some studies had small sample sizes. While this is a limitation, it also reflects the broader challenges of conducting clinical research in rare pediatric diseases. These challenges highlight the need for national and international collaboration in future studies to ensure larger, more diverse cohorts and improved generalizability of findings. However, in the context of HD, it is important to recognize that diagnostic protocols and patient selection criteria differ considerably across institutions, which poses challenges for direct multicenter comparisons.

Clinical implications

The findings of this thesis have already been integrated into clinical care and influenced clinical practice in diagnostic workup of children evaluated for HD. In current clinical routines at the center, children weighing less than 9 kg are routinely evaluated using RSB, whereas older or bigger children are assessed using full-thickness biopsy. The specimen orientation technique and modified contrast enema method have been fully implemented in daily clinical practice.

Findings from Study IV, demonstrating structural differences between histological layers of ganglionic and aganglionic bowel, have provided essential background information for interpretations and analyses in the ongoing novel research in ultra-high frequency ultrasound. The proposed diagnostic algorithm in Study IV represents a theoretical framework behind the ultrasound studies. Although it still requires further validation in larger, independent cohorts to establish clinically relevant cut-off values, it already serves as a starting point.

Notably, the modified contrast enema technique has already been adopted at two additional departments in Sweden and one in Iceland. A future multicenter study would help validate its generalizability across different settings. It remains to be determined whether the modified contrast enema protocol would be effective in clinical settings outside Sweden, where resources, workflows, and healthcare structures differ.

In Sweden, surgical care for HD has been centralized to two national specialized medical care centers, with the aim of improving surgical outcomes. However, the centralization of HD surgery has also had implications for the diagnostic processes, and referrals for diagnostics have shifted to the national centers. This shift has occurred despite the fact that the designation as a national specialized medical care center for HD does not formally include responsibility for diagnostics. Over time, however, diagnostic procedures have been subject to a gradual centralization, resulting in a depletion of diagnostic expertise at other pediatric units. Given the rarity of HD and the limited annual case volume in Sweden, maintaining clinical expertise across multiple centers can be challenging. While many hospitals already perform standard contrast enemas, implementing cold contrast enemas (as used in the modified protocol) could be a feasible next step. However, interpreting findings, particularly the RAIR, requires experience and training, which may support the case for more centralized diagnostic workflows, resulting in an increased inflow of patients undergoing diagnostic evaluation for suspected HD.

From a clinical perspective, the centralization has been an important step toward ensuring that children with HD are managed by experienced multidisciplinary teams. Initial national evaluations suggest that centralization has not adversely impacted the preoperative phase, diagnostic efficiency, preoperative management,

or risk of complications [104]. However, centralization has not yet been shown to translate into a significant reduction in major postoperative complications, unplanned reoperations, or readmissions within three months after pull-through surgery [105]. In our experience, centralization has facilitated closer collaboration between pediatric surgeons, radiologists, and pathologists, which likely benefits complex diagnostic cases. Still, the full potential of this system may only become apparent over time and need continuous evaluations.

Conclusions

Study I

This novel study evaluating the diagnostic efficacy of RSB in relation to weight, shows a significantly higher efficacy in children weighing 0–9 kg compared with heavier children. Diagnostic efficacy is lower in aganglionic tissue. These findings inform both clinical expectations and procedural decision-making regarding first-time RSB, suggesting that RSB is more reliable in children with lower weight.

Study II

This novel report on the orientation of RSB specimens demonstrates that a systematic and meticulous in-house method for orienting fresh RSB samples offers several advantages. Orientation increases the proportion of high-quality specimens, shortens diagnostic turnaround time, and reduces histopathological workload in terms of re-orientation and further sectioning of the tissue. Of particular importance for children with HD, specimen orientation significantly improves diagnostic efficacy in cases of aganglionosis.

Study III

The modified contrast enema with supplementary injections of cold contrast demonstrates high diagnostic accuracy for HD in children under one year of age, with a good interobserver agreement. The findings indicate that this method has high reliability in ruling out HD and may serve as a valuable tool for identifying children who do not require rectal biopsy. Furthermore, sensitivity for determining disease extension is high when a rectosigmoid caliber change is visualized, making this examination valuable for surgical planning.

Study IV

The muscularis interna is significantly thicker, and the ratio of muscularis interna to muscularis externa is significantly higher, in ganglionic compared to aganglionic bowel. These findings provide valuable insight into the histoanatomical morphometric changes associated with HD. Based on these parameters, a diagnostic algorithm is proposed, demonstrating 100% accuracy in distinguishing aganglionosis from ganglionosis.

Future research questions

The studies included in this thesis have generated new questions that could be explored in future research. Examples of such questions include:

To what extent can the number of rectal biopsies be safely reduced without increasing the risk of missed HD cases?

At what distance above the dentate line should a rectal biopsy be obtained to optimize diagnostic yield and minimize inconclusive results?

How does rectal catheterization within 24 hours before RAIR assessment in modified contrast enema affect diagnostic accuracy?

What has been the measurable impact of implementing the modified contrast enema protocol on reducing biopsy rates?

Could new staining techniques, such as Phox2b, further improve the efficacy of RSB?

Can clearer, symptom-based criteria be established to identify which children should be investigated for HD?

Does the current rectosigmoid index threshold (> 1) require re-evaluation or adjustment to improve diagnostic performance?

What role could other imaging modalities, such as computed tomography or magnetic resonance imaging, play in the diagnosis of HD?

How can artificial intelligence assist in the interpretation of both histological and radiological images in diagnosing HD?

How do parents of a child with suspected HD experience the diagnostic process? Which aspects are most distressing, and how can the pathway be improved?

What is the future potential for prenatal detection of HD?

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