Determining the correct resection level in patients with Hirschsprung disease using contrast enema and full thickness biopsies: Can the diagnostic accuracy be improved by examining submucosal nerve fiber thickness?

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\textbf{A B S T R A C T}

\textbf{Background:} Intraoperative resection level in patients with Hirschsprung disease (HD) is determined by contrast enema, surgeon’s intraoperative judgement and full thickness biopsy (FTB) identifying ganglia. This study aims to evaluate diagnostic accuracy of contrast enema and FTB in determination of resection level and whether this can be improved by measuring submucosal nerve fiber diameter.

\textbf{Methods:} We retrospectively analyzed contrast enema and intraoperative FTBs obtained in our center, determining diagnostic accuracy for level of resection. Gold standard was pathological examination of resection specimen. Secondly, we matched transition zone pull-through (TZPT) patients with non-TZPT patients, based on age and length of resected bowel, to blindly compare nerve fibers diameters between two groups using group comparison.

\textbf{Results:} From 2000–2021, 209 patients underwent HD surgery of whom 180 patients (138 males; median age at surgery: 13 weeks) with 18 TZPTs (10%) were included. Positive predictive value of contrast enema was 65.1%. No caliber change was found in patients with total colon aganglionosis (TCA). Negative predictive value of surgeon’s intraoperative judgement and FTB in determining resection level was 79.0% and 90.0% (91.2% single-stage, 84.4% two-stage surgery) respectively. Mean nerve fiber diameter in TZPT was 25.01 μm (SD = 5.63) and in non-TZPT 24.35 μm (SD = 6.75) \((p = 0.813).\)

\textbf{Conclusion:} Determination of resection level with combination of contrast enema, surgeon’s intraoperative judgement and FTB results in sufficient diagnostic accuracy in patients with HD. If no caliber change is seen with contrast enema, TCA should be considered. Resection level or transition zone cannot be determined by assessment of submucosal nerve fiber diameter in FTB.

\textbf{Type of Study:} clinical research paper.

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Abbreviations: HD, Hirschsprung disease; FTB, Full thickness biopsy; TCA, Total colon aganglionosis; TZ, Transition zone; TZPT, Transition zone pull-through.

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1. Introduction

Hirschsprung disease (HD) is a congenital disorder characterized by a dysfunctional distal bowel of various lengths [1,2]. As a result, patients with HD suffer from fecal stasis, causing intestinal obstruction [2]. HD is caused by failure of neural crest cell migration, which results in the absence of ganglia and presence of hypertrophic nerve fibers in the (distal) bowel.

After the diagnosis of HD, surgery is planned, aimed at resecting the diseased bowel section. Surgery can take place in single-stage or in two-stages, with the latter being preferred in patients with a long diseased bowel (diseased bowel extending from the rectosigmoid) [4]. To determine the type of surgery, preoperative contrast enema is usually performed, providing information about the length of diseased bowel [5]. However, due to the low diagnostic accuracy of the contrast enema, intraoperative biopsies are often taken to determine the resection level [6]. Full thickness biopsy (FTB) is obtained at the level where healthy bowel is expected, based on caliber change observed on the contrast enema and by visual examination of the surgeon. If ganglia are present in the biopsy, the accompanying bowel location is considered healthy bowel and can be used for the anastomosis following resection. However, despite the use of both contrast enema and FTBs, about 50% of patients are found to suffer from persistent symptoms [7,8] and it is estimated that in about 25–50% of these patients an incomplete removal of the diseased bowel has taken place [9]: a transition zone pull-through (TZPT) [10–12]. The transition zone (TZ) is located between the diseased and healthy bowel and has restricted functionality. The TZ is mainly characterized by circumferential hypoganglionosis, a characteristic that cannot be assessed using FTB [13]. All in all, it is still not feasible to correctly determine the right resection level in approximately 10% of patients during surgery despite the use of preoperative contrast enema and intraoperative FTB [10–12].

In the search for an alternative marker to recognize the TZ without the need for circumferential examination, the assessment of nerve fiber diameter has been suggested [14]. Several studies have reported on submucosal hypertrophic nerve fibers in rectal suction biopsies taken from patients with postoperative persistent complaints [10,14,15]. Hence, if nerve fiber diameter in FTB is found distinctive for the TZ, diagnostic accuracy of FTB may substantially be improved, resulting in less incomplete bowel removal and improved postoperative outcome.

The primary aim of the current study was to evaluate the diagnostic accuracy of contrast enema, the surgeon’s intraoperative judgement and presence of ganglia in FTB, in determination of the correct level of resection, using the resection specimen as gold standard. The secondary aim was to investigate whether examination of submucosal nerve fiber diameter may improve diagnostic accuracy of FTB.

2. Methods

2.1. Patient population

This study included all eligible patients in whom definitive surgery for HD was performed between 2000 and 2021 at the Amsterdam university medical centers (Academic Medical Center and VU Medical Center). Patients were excluded if they had no histopathological confirmation of HD, received no intraoperative FTBs, or caretaker’s objection to participate in this study.

2.2. Clinical procedure

A rectal suction biopsy was obtained if a patient was suspected of HD based on clinical symptoms and radiological findings. Biopsies were stained using acetylcholine-esterase staining until the end of 2011, whereafter calretinin staining was used [16,17]. Rectal suction biopsies were examined by senior pathologists with experience in pediatric gastroenterology. In case of absent ganglia on hematoxylin–eosin staining combined with increased acetylcholine-esterase reactivity of nerve fibers or negativity on calretinin, the biopsy was positive for HD [17]. After diagnosis, the suspected length of disease was determined by location of caliber change (i.e. the radiological TZ) on routine contrast enema which was examined by a senior radiologist experienced in pediatric gastroenterology [5,18]. If (clinically) possible, no rectal irrigation was performed 24-hours before the contrast enema [3,19].

In case of a suspected short segment disease (defined as aganglionosis extending to the rectosigmoid) single-stage definitive surgery was performed using transanal endorectal pull-through technique. During single-stage surgery, pathological examination of FTBs was performed during the same procedure as the resection of diseased bowel and performing the anastomosis. The location of the first FTB was determined by the surgeon based on a combination of the expected level of the caliber change presented on the contrast enema and the intraoperative visible level of the caliber change [6,20]. The obtained FTB was sent for examination by one senior pathologist with experience in pediatric gastroenterology. When no ganglia were visible, the biopsy level was considered unsuitable for the anastomosis and a more proximal FTB was obtained approximately five centimeter more proximal than the biopsy found to show no ganglia [21]. This procedure was continued until ganglia were found, considering the biopsy location as healthy bowel. When the FTB was found non-assessable, a new FTB from the same origin was obtained.

Two-stage definitive surgery was performed in case of a suspected long-segment disease (defined as aganglionosis extending to the proximal colon), total colonic aganglionosis (TCA), in case of disease requiring acute surgery or previous complication requiring acute surgery. During two-stage surgery, the first operation included colon mapping, stoma placement or both. Colon mapping consisted of obtaining FTBs from the rectosigmoid, descending colon, transverse colon, and terminal ileum. The extent of the mapping was determined based on the level of the caliber change on the contrast enema and the intraoperative visible level of the caliber change. The FTBs were sent for examination by the pathologist. During the second surgery, resection of diseased bowel took place performing Duhamel’s technique, based on the most distal FTB location containing ganglia.

In both single-stage and two-stage surgery, surgery was performed by 10 different pediatric surgeons specialized in gastrointestinal surgery with ample experience in HD. In both single-stage and two-stage surgery, the resection was performed at least five cm proximal of the most distal healthy biopsy location, ensuring a margin for the TZ [21]. Larger margins were taken in case of a longer expected length of disease and higher age at resection. TZPT patients were selected by retrospectively reviewing pathology reports, selecting patients in whom the proximal resection plane contained myenteric or submucosal aganglionosis of >1/8th circumference or myenteric presence of ectopic or hypocellular ganglia [13,24]. In case of a TZPT the standard treatment involved redo surgery until 2012. Hereafter, the clinical progress of the TZPT patient was monitored and discussed in the multidisciplinary consultation meeting, deciding for conservative or surgical treatment.

2.3. Preparation FTB

FTBs were taken by the surgeon through open or laparoscopic assisted excision of small fragment bowel wall and aimed to include all layers of the bowel. The FTB was sent to pathology
department where frozen and paraffin sections were made. For intraoperative examination during one-stage surgery, all FTBs were
stained equally using frozen sections with hematoxylin-eosin. Regarding postoperative examination of the FTB obtained during
two-stage surgery, staining was done with both hematoxylin-eosin and acetylcholinesterase on frozen sections until the end of 2011.
From 2012 onwards, hematoxylin-eosin and calretinin staining’s were performed on paraffin sections.

Tissue for frozen sections was embedded in Tissue-Tek and flash
frozen in liquid nitrogen, after which 5 μm sections were cut and
stained with hematoxylin-eosin in accordance with standard pro-
cedures using a Tissue-Tek Prisma Automated Slide Stainer. Tissue for paraffin sections was fixed overnight in buffered formalin, em-
bedded in paraffin and 5 μm sections were cut and stained with
hematoxylin-eosin in accordance with standard procedures. Gelatin
glycerol was used for mounting the slides [22].

2.4. Data extraction

Medical records of all eligible patients were extracted by mul-
tiple authors (LB, IS and MB) and stored in a Castor database. Data
validation was done by checking 10% of the records by one of the
researchers not involved in the initial extraction of data. In case
there were inconsistencies, the complete record was checked by
the other author not involved in the initial extraction of data. The following patient characteristics were extracted: sex (male/female),
contrast enema (yes/no), visible caliber change on contrast en-
ema (yes/no), location of caliber change on contrast enema (recto-
sigmoid defined as proximal to linea-dentata), sigmoid, sigmoid-
descendens, colon descendens, flexura lienalis, colon transversum,
flexura hepatica, colon ascendens, cecum, or ileum), age at time
of biopsy (weeks), type of surgery (single-stage, two-stage), to-
total number of FTBs, pathological results of FTBs (ganglionated,
aganglionated, or non-assessable), length of resected bowel spec-
imen measured by the pathologist (cm), length of diseased seg-
ment (short-segment was defined as aganglionosis extending to
the rectosigmoid, long-segment as aganglionosis extending to the
proximal colon or TCA) [4], location of TZ (recto-sigmoid defined as
proximal to linea-dentata), sigmoid, sigmoid-descendens, colon
descendens, flexura lienalis, colon transversum, flexura hepatica,
colon ascendence, cecum, or ileum) and TZPT (yes/no). In patients
in which redo surgery was performed, the following procedure
characteristics were collected: intraoperative FTB (yes/no), num-
ber of intraoperative FTB, pathological result of FTB (ganglionated,
aganglionated, or non-assessable) and length of resected segment
measured by pathologist (cm) and TZPT (yes/no). For all variables
obtained from the medical records, the percentage of missing data
was less than 10%.

2.5. Outcomes and definitions

Diagnostic accuracy was determined in accordance with the
STARD guidelines, using the resection specimen as gold standard
[23]. The diagnostic accuracy for determining the correct resection
level was calculated for: (1) contrast enema, (2) surgeon’s intraop-
erative judgment and (3) intraoperative FTBs. The surgeon’s intra-
operative judgment was defined as the (first) biopsy level, which
is considered a combination of the findings of the contrast enema
together with the visual intrareoperative level of the caliber change.
Consequently, this could only be determined in patients who un-
derwent single-stage surgery. Regarding contrast enema, only the
positive predictive value (PPV) could be calculated, because in con-
trast enemas without caliber change no estimated location of TZ
could be determined. Regarding surgeon’s intraoperative judgment
and FTBs, only the negative predictive value (NPV) could be cal-
culated as all obtained FTBs that were compared to the resection
specimen were expected to be healthy. The NPV of FTB was deter-
mined for all most proximally obtained FTB and was split up for
two-stage surgery in FTB obtained before 2012 (frozen sections)
and FTB obtained from 2012 onwards (paraffin sections) because
of the use of different staining techniques.

2.6. Nerve fiber diameter in FTB

To assess whether measurement of the nerve fiber diameter
could increase diagnostic accuracy of the FTB, we identified TZPT
patients and control patients from the total cohort. Control pa-
patients were selected from the remaining cohort, based on match-
ing with the TZPT patients. Per TZPT patient, two control patients
were selected based on staining technique of FTBs (frozen or paraf-
fin), where after was matched based on age at time of surgery and
length of resection in the TZPT patient, using the following ranges:
a) age at FTBs < 1 year: range age at time of surgery <2 months
and range length of resection <10 cm b) age at time of FTBs >1
year: range age at time of FTBs <1.5 years and range length of re-
section <10 cm c) patients with TCA: range age >4 years matched
with patients with TCA. Patients who had undergone redo surgery
for any reason other than transition zone pull-through (TZPT)
were excluded from the control group.

Slides of the most proximal FTB from both TZPT patients and
control patients were digitized using an automated slide scanner
with 20x microscope objective (Slide, Olympus, Tokyo Japan), and
a Philips pathology scanner combined with Philips Digital Pathol-
yogy Solutions software. Examination of the slides was done in a
blinded fashion by one examiner (IS), who was trained for exam-
ining the slides for both staining methods. Training comprised of
examination under supervision of an experienced and dedicated
pediatric GI pathologist until no interobserver variability occurred.
First, the quality of the slide was examined and when found in-
sufficient, new deeper cuts were made. If the new slide pertained
low in quality, the slide was excluded from the analysis. Second,
the surface of the submucosa was measured in square millime-
ters (mm²) by measuring the length and width of submucosa, to
test if there were differences in the surface of the obtained tis-
sue. Third, nerve fiber diameter was measured at the widest point
on the transverse axis in micrometers (μm) from all visible nerve
fibers. Fourth, the 10 thickest nerve fibers were selected for analy-
sis, to correct for potential bias caused by differences in nerve fiber
number. We calculated the minimal, maximal, and average nerve
fiber diameter per FTB. In addition, we counted the amount of hy-
pertrophic nerve fibers which were defined as diameter >40μm
[25].

2.7. Statistical analysis

Extracted data were stored in a Castor database [26]. IBM SPSS
Statistics for Windows, version 28 (IBM Corp., Armonk, N.Y., USA)
was used for statistical analysis. Descriptive statistics of the TZPT
and control patients were compared using Mann Whitney U test or
unpaired T-test for continuous data and chi-square test or Fisher’s
exact test for categorical data. The diagnostic accuracy of con-
trast enema, surgeon’s intraoperative judgment and FTB both dur-
ing one-stage and two-stage surgery was calculated using STARD
guidelines [23]. For comparison of nerve fiber diameter in TZPT
and control patients unpaired t-test or Mann Whitney U test were
used. P-values lower than 0.05 was considered statistically signif-
ificant. Receiver operating characteristic curves (ROC) were used
to determine the optimal cut-off values for nerve fiber diameter in
discriminating TZ from ganglionated bowel. The original cut-off
value (40μm) and the derived cut-off value were tested by assess-
ing the sensitivity, specificity and area under the curve (AUC). An
AUC of ≥0.8 was considered distinctive between TZ and ganglionated bowel [27].

2.8. Ethics

This study has been approved by the Ethics Review Committee of the Amsterdam UMC (W18_160 # 18.198) and is compliant with the 1964 Helsinki declaration and its later amendments.

3. Results

3.1. Population characteristics

We analyzed 209 patients whom 29 were excluded. In eight patients surgery was performed elsewhere, in 17 patients no intraoperative FTB was obtained and four caretakers did not provide informed consent. Subsequently, 180 patients were included in this study, of whom 138 were male (76.7%). A total of 143 patients suffered from a short-segment disease (79%), 19 from a long-segment disease (11%) and 18 from TCA (10%). Surgery was performed at a median age of 13 weeks (range 2–498 weeks). In 148 patients single-stage surgery was performed (82.2%) in whom a median of 17 cm bowel was resected (range 4–110 cm) and in 32 patients two-stage surgery (17.8%) was performed with a median of 45 cm resection (range 6–85 cm). In total, 18 patients turned out to have a TZPT (10%) of whom 13 during one-stage surgery (8.8%) and five during two-stage surgery (15.6%). From all patients with a TZPT, 11 patients (61.1%) were treated with redo surgery with the following indications: no persistent symptoms without applying additional treatment (n = 2, 28.6%), no persistent symptoms with applying laxatives (n = 2, 28.6%) and no persistent symptoms after botulin toxin injections (n = 3, 42.9%). Population characteristics of the included patients are described in Table 1.

3.2. Contrast enema

In 169 patients (93.9%) a contrast enema was conducted before definitive surgery of whom 144 patients (85.2%) had one contrast enema. In the remainder 11 patients, no contrast enema was performed (6.5%) of which in seven patients as a result from complications necessitating acute surgery (4.1%) and in four patients the reason for not conducting contrast enema is unknown (2.4%). A total of 23 patients (12.8%) had two contrast enemas and two patients (1.1%) had three contrast enemas (Table 1). From all patients with a contrast enema, in 93 patients (55.0%) a caliber change was found on the first contrast enema and in 12 patients (71%) on the second contrast enema. Caliber change was observed in four patients at the level of the rectum (2.4%) in 63 patients at recto-sigmoid level (37.3%), in 18 patients at sigmoid level (10.7%), in 10 patients at sigmoid-descendens level (5.9%), in three patients at colon descendens level (1.8%), in six patients at flexura lienalis level (3.6%) and in one patient at the level of the flexura hepatica (0.6%).

In 63 patients both the location of caliber change on contrast enema and location of TZ in the resection specimen was known and could therefore be compared. In 13 patients (59.1%) the caliber change observed on contrast enema was located below the location of TZ in the resection specimen leading to a TZPT in two patients (16.7%). In nine patients (40.9%) the location of caliber change on the contrast enema was higher than in the resection specimen. No patients within this group had a TZPT. The PPV of the total group was 65.1%, in patients with a short-segment disease 67.9%, and in patients with a long-segment disease 55.6%. The PPV of patients
with a TCA was 0% because no caliber changes were found on contrast enema in this group.

3.3. One-stage FTB

One-stage surgery was performed in 148 patients whereby the first obtained FTB, based on the surgeon’s intraoperative judgement, was ganglioneuromatous in 123 patients (83.1%), ganglioneuromatous in 20 patients (13.5%) and non-assessable in five patients (3.4%). Ten out of 123 ganglioneuromatous first FTBs (8.1%) resulted in a T2PT. Therefore, NPV for determining of resection level based on surgeon’s intraoperative judgement of correct resection level in single stage definitive surgery was 79%.

From the 148 patients undergoing one-stage surgery, the median obtained number of FTBs obtained was one (range 1–7) with 13 patients (8.8%) showing T2PT, of whom eight patients underwent redo-surgery (5.4%) whereby a median of one FTB was obtained (range 0–6). The NPV of FTB during one-stage surgery was 91.2%.

3.4. Two-stage FTB

Two-stage surgery was performed in 32 patients, with 11 patients (34%) being operated before 2012 (frozen sections) and 21 patients (66%) being operated from 2012 onwards (paraffin sections). The median obtained FTBs during two-stage surgery was five (range 1–8) with five patients (16%) showing T2PT, resulting in a NPV of FTB of 84.4% for two-stage surgery.

From the 11 patients undergoing two-stage surgery before 2012, the median obtained number of FTBs was three (range 1–6) with two patients (18%) showing T2PT, of whom one patient underwent redo surgery (5%) whereby one FTB was obtained. Therefore the NPV of FTB during two-stage surgery using frozen sections was 81.8%.

From the 21 patients undergoing two-stage surgery from 2012 onwards, the median obtained number of FTBs was five (range 2–8) with three patients (14.2%) showing T2PT of whom two patients underwent redo surgery (9.5%) whereby a median of one FTB was obtained (range 1–1), resulting in a NPV of FTB of 85.7%.

3.5. Assessment of nerve fibers in FTB

To assess the nerve fiber diameter in FTB, first the frozen and paraffin FTB slides of 18 TZPT patients were retrieved from the archive. In eight TZPT patients the frozen sections were available (44.4%) and paraffin sections in four TZPT patients (22.2%). In six TZPT patients FTB sections were not available or did not contain submucosa and were thus excluded from the analysis. All 12 TZPT patients for whom slides were available for analysis were successfully matched with control patients using the predefined criteria. Thus, in total 36 slides were assessed including 12 TZPT slides (eight frozen slides, four paraffin slides) matched with 24 control slides (16 frozen slides, four paraffin slides) (Table 2). Mean nerve fiber diameter of the TZPT slides was 23.99 µm (SD = 5.67) and 23.93 µm (SD = 5.96) in the control slides. We found hypertrophic nerve fibers (> 40 µm) in eight slides (72.7%) of which three were derived from TZPT patients (37.5%). No significant differences were found between TZPT slides and control slides, both in the frozen slides and paraffin slides, when comparing the average, maximal and minimal nerve diameter, and the number of hypertrophic nerve fibers (Table 3). In frozen sections a cut-off value of 35.56 µm for maximal nerve fiber diameter was determined to optimally distinguish TZPT slides and control slides, with an AUC of 0.406, sensitivity of 50.0% and specificity of 50.0%.

4. Discussion

The aim of this retrospective study was to evaluate the diagnostic accuracy using STARD guidelines of contrast enema, the surgeon’s intraoperative judgement and the intraoperative FTB results, in determining the correct level of resection in surgery for HD. We report a PPV of 61.1% for contrast enema, a NPV of 79.0% of surgeon’s intraoperative judgement and of 90.0% for FTB result.

Contrast enema was found to have a low diagnostic accuracy for indicating the correct level of resection. The diagnostic accuracy of contrast enema has been studied before, varying widely between 62.5%–99.4% in determining the correct length of disease [6,18,28–30]. The studies that report higher diagnostic accuracy have only included patients with short-segment disease which is in line with our findings, reporting higher PPV of 69.4% in patients with a short-segment disease. However, in the total group and in subgroups, divided per segment of disease, we report lower diagnostic accuracy than other studies. This could be a result of differences in study methods: excluding patients with persistent postoperative symptoms [6], using FTB as gold standard [28] or not stating how the pathology as gold standard was examined [18]. Also, all relevant studies did not report if rectal irrigation was performed 24-hours before the contrast enema. This could be of influence because irrigation of fecal stasis could result in less colonic dilatation causing the caliber change to be less visible [19]. Another explanation may be related to differences in age at time of contrast enema, reporting a higher diagnostic accuracy in younger children. However, the mean age at time of contrast enema is not reported in all studies investigating the diagnostic accuracy [6,18,28,29].

We found that a contrast enema is not sufficient in patients with a long-segment disease and TCA, which is in line with results of other studies [31]. Next, our findings emphasize the importance of creating new diagnostic tools to preoperatively estimate the correct length of disease for these patients [4]. Ultra-high frequency ultrasound can be the future solution for this problem, which is recently tested for intraoperative ex vivo determination of the resection level [32].

The diagnostic accuracy of the surgeon’s intraoperative judgement showed a substantially lower diagnostic accuracy compared to FTB. This can be explained by the composition of the TZ, the main characteristic being non-circumferential ganglionosis, which increases in severity distally [13]. As a result, finding a ganglioneuromatous FTB is more likely in the proximal TZ. This is in contrast to the caliber change, which is expected to be present in the distal TZ, because the restricted functionality of the TZ increases distally, causing fecal stasis. Therefore, the intraoperative FTB adds diagnostic value to the surgeon’s intraoperative judgement.

Regarding intraoperative FTB, we found a high diagnostic accuracy for determining the correct resection level of 90%. The diagnostic accuracy we found is higher than studies including only seromuscular tissue, containing less tissue, expecting to give less information about the correct resection level [10,12,33–35]. The high diagnostic accuracy found in this study is also reflected in the low prevalence of TZPT that we reported (10%), being lower than other studies that report a ranging prevalence of 13–19% [12,33–35]. This can firstly be explained by the variety of diagnostic techniques we used for determining the correct resection level where other studies did not additionally perform a contrast enema. A second explanation might be related to the resection margin we use in our center. We standardly apply a resection margin of minimal 5 cm proximal to the positive FTB, which is not mentioned to be applied in the comparative studies. Unfortunately, we could not determine the exact used margin due to the retrospective study.
Table 2

Submucosal nerve fibers in TZPT patients compared with two matched control patients (n = 36).

| Matched pairs | Patients | Age (weeks) | FTB level (cm) | Mean (range) diameter (μm) | Number nerves >40 μm | Total number nerves | Surface submucosa (mm²) |
|---------------|----------|-------------|----------------|----------------------------|----------------------|---------------------|------------------------|
| Frozen sections | 1. | TZPT 15 20 | 26.55 (43.20–17.33) | 1 | 23 | 2.63 |
| Control 17 20 | 29.99 (37.12–26.77) | – | 65 | 4.50 |
| Control 9 19 | 29.66 (36.47–21.60) | – | 34 | 4.82 |
| 2. | TZPT 17 24 | 22.21 (35.61–12.66) | – | 20 | 2.15 |
| Control 17 24 | 30.19 (42.18–25.84) | 1 | 37 | 3.92 |
| Control 13 21 | 24.55 (35.32–16.83) | – | 19 | 1.27 |
| 3. | TZPT 56 14 | 24.25 (35.05–17.46) | – | 19 | 1.96 |
| Control 52 20 | 22.35 (34.04–17.62) | – | 43 | 13.74 |
| Control 56 18 | 23.16 (30.98–18.30) | – | 14 | 1.92 |
| 4. | TZPT 21 26 | 20.62 (31.64–6.11) | – | 10 | 3.36 |
| Control 21 28 | 24.52 (38.53–18.93) | 1 | 21 | 2.79 |
| Control 15 32 | 19.91 (25.45–15.91) | – | 24 | 1.87 |
| 5. | TZPT 4 14 | 32.52 (36.07–28.71) | – | 51 | 6.29 |
| Control 4 15 | 21.02 (26.3–14.81) | – | 23 | 1.02 |
| Control 7 15 | 14.67 (26.56–7.00) | – | 14 | 0.84 |
| 6. | TZPT 34 8 | 24.02 (38.47–16.40) | – | 12 | 1.20 |
| Control 39 8 | 21.35 (48.09–12.40) | 1 | 23 | 1.99 |
| Control 26 10 | 16.84 (35.51–6.51) | – | 9 | 2.57 |
| 7. | TZPT 304 12 | 33.21 (46.74–39.71) | 2 | 15 | 3.24 |
| Control 295 16 | 43.48 (63.25–33.81) | 5 | 81 | 47.40 |
| Control 256 20 | 24.15 (42.28–18.12) | 1 | 28 | 1.41 |
| 8. | TZPT 17 26 | 16.77 (32.95–8.82) | – | 11 | 0.45 |
| Control 16 25 | 18.99 (27.00–12.56) | – | 12 | 2.44 |
| Control 17 28 | 24.80 (34.27–19.16) | – | 20 | 3.05 |
| Paraffin sections | 9. | TZPT 17 24 | 13.26 (17.28–10.89) | – | 17 | 1.78 |
| Control 17 24 | 31.41 (38.65–27.96) | – | 42 | 2.84 |
| Control 13 21 | 22.69 (33.01–17.17) | – | 18 | 1.22 |
| 10. | TZPT 19 20 | 24.17 (34.25–16.80) | – | 16 | 1.90 |
| Control 21 20 | 22.04 (34.80–15.36) | – | 30 | 1.94 |
| Control 21 18 | 24.13 (34.04–18.23) | – | 24 | 17.11 |
| 11. | TZPT 17 8 | 23.58 (41.31–8.25) | 1 | 10 | 0.72 |
| Control 15 8 | 22.61 (46.73–13.87) | 1 | 13 | 0.93 |
| Control 17 7 | 17.89 (30.07–7.38) | – | 10 | 0.90 |
| 12. | TZPT 304 TCA | 26.70 (32.79–20.22) | – | 28 | 13.22 |
| Control 304 TCA | 25.40 (34.42–18.34) | – | 22 | 6.09 |
| Control 161 TCA | 18.61 (26.18–15.08) | – | 17 | 6.05 |

* Based on 10 thickest nerves.

Table 3

Submucosal nerve fiber characteristics in TZPT patients and their matched controls.

| Frozen sections (n = 24) | Control group (n = 24) | p-value |
|-------------------------|------------------------|---------|
| Nerve diameter in μm, mean (SD) | 25.01 (5.63) | 24.35 (6.75) | 0.813 |
| Maximal nerve diameter in μm, median (range)¹ | 35.84 (31.64–46.74) | 35.41 (25.45–63.25) | 0.540 |
| Minimal nerve diameter in μm, median (SD) | 18.40 (20.97) | 17.66 (7.02) | 0.842 |
| Number of nerves >40 μm, median (range)² | 0 (0–2) | 0 (0–5) | 0.968 |
| Total number of nerves fibers, median (range)³ | 17 (10–51) | 23 (9–81) | 0.133 |
| Surface area of submucosa in mm², median (range)⁴ | 2.39 (0.45–6.29) | 2.50 (0.84–47.40) | 0.854 |

| Paraffin sections (n = 12) | Control group (n = 12) | p-value |
|---------------------------|------------------------|---------|
| Nerve diameter in μm, mean (SD) | 21.93 (5.93) | 23.09 (4.21) | 0.699 |
| Maximal nerve diameter in μm, mean (SD) | 31.40 (24.03) | 34.74 (6.07) | 0.486 |
| Minimal nerve diameter in μm, mean (SD) | 18.04 (5.46) | 16.67 (5.75) | 0.465 |
| Number of nerves >40 μm, median (range)² | 0 (0–1) | 0 (0–1) | 0.600 |
| Total number of nerves fibers, mean (SD) | 18.70 (5) | 22.10 (2.24) | 0.482 |
| Surface area of submucosa in mm², median (range)⁴ | 1.84 (0.72–13.22) | 2.39 (0.90–17.11) | 0.610 |

¹ p<0.05 ² p<0.001.
³ In case of skewed data Mann Whitney U test was performed.

design. Despite this, we do advise to apply wide operative margins, taking the composition of the TZ into account. Thirdly, due to the small number of patients in all studies, the composition of the included patients is heterogeneous in and between studies. This could be of influence on the outcomes, as certain characteristics as female gender, suffering from syndrome and familial HD may be correlated with a longer diseased bowel [36]. These patients might have a higher change of incorrect identification of the resection level resulting in a TZPT. This is also found in our results: FTB have a lower diagnostic accuracy in two-stage surgery, despite obtaining more FTBs during this type of surgery. This is supported by the findings of Coyle et al. reporting that TZ of long segment HD patients can measure up to 22 cm while TZ of short segment HD patients measures up to 13 cm [37]. This underlines why assessing FTB on ganglia alone is not sufficient in identifying the TZ which has particularly adverse consequences for the patient with a longer diseased bowel.

Therefore, the second aim of our study was to investigate whether the diagnostic accuracy of FTB could be improved when examining submucosal nerve fiber diameter in FTB. We found no
significant difference in average, maximal and minimal nerve diameter between TZPT patients and patients with successful definitive surgery. In addition, we found an equal prevalence of hypertrophic nerve fibers in patients with a TZPT and patients with no TZPT. This is not in line with previous findings reporting hypertrophic nerve fibers in patients with a TZPT [10]. However, the included studies in this review did not compare the results with matched control patients and did not take into account factors as staining technique, level of resection and age at resection that may affect the diameter of nerve fibers. Age at time of resection is a factor that is known to influence the diameter of nerve fibers, independent of aganglionated or ganglionated tissue [38]. The median age of patients in this review is 7.5 years, which could be the cause of the thicker nerve fibers found in this study. This is also substantiated by our findings: all patients who were older than five years at time of surgery showed hypertrophic nerve fibers, except one patient with a TCA, independent of the outcome of the surgery. Another limitation of the review is that the nerve diameter is not corrected for origin location. This is of importance because we know that hypertrophic nerve fibers are more frequently found at rectal level of the rectum [38,39]. Also, we know that different types of staining can be of influence on the nerve diameter, which is also not taken into account.

We also assessed whether an alternative cut-off value derived from ROC, was distinctive for the TZ and healthy tissue, finding a diameter of 35.56 μm for frozen section and of 34.15 μm for paraffin section. Both cut-off values were not found distinctive for the TZ. We therefore conclude that assessment of nerve fiber diameter should not be incorporated in examination of intraoperative FTB.

4.1. Limitations

This study has several limitations which are mainly related to the retrospective study design. Due to this, the findings of the contrast enema were subject to both inter-observer variability between radiologists and variability caused by technical differences. Although all radiologists and technicians followed the hospital protocol, this could still have influenced the results. Additionally, we depended on retrospective findings of pathologists in patient selection of a TZPT, with a possible interobserver variability in pathological judgment [40]. However, in case of inadequate or unclear pathology reports, we reexamined the proximal slide to prevent unjustified inclusion of TZPT patients. Also, the quality of the available FTB was variable: some FTB sections were lost or did not contain submucosa while others were old causing fading of the staining. However, in case of low quality slides we made new deeper cross-sections to limit the influence of the quality of the slides and in case of pertained low quality we excluded the slides. In addition, the surface of the submucosa did not differ between the two groups and we corrected for the difference in nerve fibers by only analyzing the 10 thickest nerve fibers. Furthermore, the pathology slides were analyzed by one researcher and have not been reviewed by a second researcher, preventing interobserver variability but thereby resulting in less generalizability.

4.2. Future perspectives

Our results show that preoperative estimation of the length of disease remains troublesome. Early recognition of patients with TCA can prevent severe complications to occur [4]. Therefore, future studies should focus on development of diagnostic tools to recognize the length of diseased bowel. The ex vivo used ultra-high frequency ultrasound shows promising results for determination of the resection level in vivo [32]. Also, we were not able to increase the diagnostic accuracy of FTB by measurement of nerve fiber diameter and therefore we are still not able to intraoperatively recognize the TZ. We therefore recommend the use of a full circumferential tissue sample instead of FTB. In addition, we recommend future studies to investigate the added value of intraoperative ultra-high frequency ultrasonography to facilitate the time consuming process to recognize the TZ without the use of pathology [41]. So far, only ex-vivo results are available and therefore testing the application of this technique intraoperatively in determining the correct resection level is recommended.

5. Conclusion

Our findings show that determination of the resection level with a combination of contrast enema, visual examination of the caliber change and FTB result in a sufficient diagnostic accuracy in patients with HD. If no caliber change is seen on the contrast enema TCA should be considered. Determination of the correct resection level cannot be done by the assessment of submucosal nerve fiber diameter in FTB.

Level of evidence

level III

Declarations of competing interest

None of the authors have any conflicts-of-interest to disclose.

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