Rectal biopsy for diagnosis of intestinal neuronal dysplasia in children: a prospective multicentre study on interobserver variation and clinical outcome

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Abstract

Background—Intestinal neuronal dysplasia (IND) of the colonic submucous plexus is considered to be a congenital malformation of the enteric nervous system causing symptoms resembling those of Hirschsprung's disease. In contrast with the established diagnosis of aganglionosis using enzyme histochemistry, controversy exists over the diagnostic criteria of IND on rectal biopsies previously defined by a consensus report and the causal relation between morphological findings and clinical symptoms.

Aims—The interobserver variability was prospectively investigated with respect to final diagnoses and several histological features in rectal biopsy specimens from children suspected of having colonic motility disturbances.

Methods—377 biopsy specimens from 108 children aged 4 days to 15 years were independently coded without knowledge of clinical symptoms by three experienced pathologists for 20 histological features, and a final diagnosis was given for every case. Interobserver variation for the different items and the final diagnosis were analysed using Cohen's κ statistic. Clinical data at biopsy and outcome after 12 months were related to morphological findings.

Results—The three pathologists agreed completely with respect to the diagnosis Hirschsprung's disease ($\kappa = 1$), but in only 14% of the children without aganglionosis. In 15 (17%) of the 87 children without aganglionosis, at least one pathologist judged the case as normal, while another diagnosed IND. κ values were close to the zero value expected by chance for the diagnoses normal and IND. Young age was related to the presence of several morphological features—for example, acetylcholine esterase staining and presence of giant ganglia. Children with chronic constipation diagnosed as having IND, given no other specific diagnosis by any of the pathologists, were significantly younger (median 8.8 months) and had a higher cure rate after one year (60%) than constipated patients considered by all observers to have no histological abnormalities (median 6.1 years, cure rate 23%).

Conclusions—In contrast with Hirschsprung's disease, there is a high interobserver variation with regard to the different morphological features and final diagnosis of IND, based on the criteria and conditions of the previous consensus report. The high frequency of histological "abnormalities" in young infants suggests that some of the features may represent a normal variant of postnatal development rather than a pathological process. Investigations using more refined and morphometric methods in rectal specimens from infants and children without bowel disease are needed to define the normal range of morphological appearance at different ages. These preliminary data indicate that, with current knowledge, rectal biopsy for diagnostic purposes should only be performed in constipated children for diagnosis of Hirschsprung's disease.

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Keywords: intestinal neuronal dysplasia; Hirschsprung's disease; constipation; enzyme histochemistry

There is general agreement that Hirschsprung's disease can be diagnosed from rectal suction biopsy specimens, but controversy exists as to whether innervation disturbances other than aganglionosis can be identified with certainty from these superficial samples,^{1,2} which only contain parts of the plexus submucosus internus (Meissner's plexus). In 1971, Meier-Ruge³ described hyperganglionosis with a total increase in the number of ganglia and ganglion cells per ganglia in the plexus myentericus and submucosus in the colon of three patients with severe motility disturbances. These morphological features, called neuronal colonic dysplasia and later renamed intestinal neuronal dysplasia (IND), have been considered to be a developmental defect of the submucous plexus.^{4–6} IND has been diagnosed from suction and forceps biopsy specimens and reported as an isolated form, with frequencies equal to or exceeding those of Hirschsprung's diseases^{5,7} and proximal to an aganglionic segment.^{8–11} Although doubts have been raised about the existence of a distinct clinical entity,^{4,12–17} the term IND was used synonymously.

Abbreviations used in this paper: IND, intestinal neuronal dysplasia; AChE, acetylcholinesterase.

mously with a clinical diagnosis. Sphincteromyectomy and resection of the affected bowel segments were recommended and performed not only in patients with aganglionosis and proximal IND, but also in severely constipated children with histological features of IND who did not respond to conventional therapy.^{5 7 8 10 18–20} IND had been exclusively reported in paediatric cases until 1990, when Stoss²¹ described it in rectal biopsy specimens from 16/18 adults with longstanding constipation, but in none of 11 controls with normal stool pattern. Since then, hemi- and subtotal colectomies have been performed in constipated adolescents and adults with IND in rectal biopsy samples.^{5 22}

The causal relation between histological findings of IND and clinical symptoms has been questioned repeatedly, as none of the several retrospective^{23–25} and prospective^{13 26} studies performed showed a correlation between morphological features and symptoms. It has been suggested that some of the histological findings may be secondary to functional or mechanical obstruction^{15 25} or reflect normal age related phenomena of the maturing enteric nervous system,^{13 16 23 24 27–29} as the diagnostic criteria for IND have never been validated in comparison with healthy age matched children. Another controversy was brought up by the varying frequencies of IND in different series ranging from 0.3%²⁷ up to 62%.⁵ Among the reasons for these discrepancies may be the selection of populations studied, as well as differences in the techniques used by the investigators, as those reporting low prevalence rates of IND^{23 24 27} used neither specific nerve cell staining—for example, lactate dehydrogenase reaction—nor high biopsy specimens (>5 cm above the pectinate line), both considered to be essential for interpretation of the specimens.^{30–32} Another explanation may be variation in the interpretation of histological findings.

The present study was designed to investigate prospectively and in a blinded fashion the interobserver agreement with respect to final diagnosis of innervation disturbances and single morphological features in rectal biopsy specimens. In addition, we studied the relation of histological findings on rectal biopsy specimens to age, clinical symptoms, and outcome after 12 months in this paediatric population.

Patients and methods

STUDY DESIGN AND CLINICAL DATA

Clinicians from seven tertiary care centres (four paediatric surgeries and three paediatric gastroenterological departments) were asked to send rectal biopsy specimens from prospectively enrolled children less than 18 years of age suspected of having innervation disturbances of the colon. The specimens were forwarded to any of three experienced reference pathologists (J B, W M-R, H M); all three had participated in a consensus meeting on diagnostic guidelines and agreed on the handling of rectal biopsy specimens and the criteria for the different diagnoses.³⁰ The indication for the biopsies was made exclusively on clinical grounds by the caring physician, who also

decided on the number and location of specimens. No clinical information was given to the pathologists except the patient's age and sex. Samples were either delivered within an hour of biopsy or sent frozen on dry ice (frozen CO₂) at –80°C. They were immediately worked up and a pathology report was sent to the caring physician in order to avoid any delay in the diagnostic process. The study protocol was approved by the medical ethics committee of the university hospital of Düsseldorf.

The slides of the biopsy specimens were coded, giving information on patient's age and sex, location (distance from the pectinate line), clinical centre (1–7), and pathologist (A, B or C) whose laboratory did the first work up. All coded slides rotated between the three pathologists who were blinded to the evaluations of the others. All pathologists reviewed the complete set of slides prepared from each child at one time point. If a child had repeated biopsies or a bowel resection later on during the course of the disease, these specimens did not enter the study and therefore were not considered for the final judgment of the observers.

On the day of the biopsy, clinical data were provided via a structured questionnaire to the study coordinator (SK). Data included family history, prematurity, perinatal events, clinical symptoms, stool pattern, physical findings, previous diagnostic procedures, and therapeutic interventions. Six and 12 months later, the child was reassessed, and information was given with regard to additional diagnostic results, continuing symptoms, medical therapy during the last six months and any surgical intervention.

PATHOLOGICAL WORK UP AND REPORT

The work up of the biopsy samples was performed according to the previous consensus report,³⁰ and has been described previously.^{31 33} In brief, the native tissue was briefly thawed if previously frozen, oriented rectangularly, and mounted in cryogen on a cryostat. Serial sections of 15 µm were cut vertically to the surface of the mucosa at –10°C to –15°C and distributed on to slides. One slide each with at least six to ten sections from every biopsy was used for enzyme histochemistry with acetylcholinesterase (AChE) (reaction time 90 minutes at 37°C) for staining of cholinergic nerves and ganglia, lactate dehydrogenase (reaction time 10–13 minutes at 37°C) for selective staining of nerve cells, and succinate dehydrogenase (reaction time 90 minutes at 37°C) for differentiation between mature and immature nerve cells. Haemalum counterstaining was used for the AChE reaction and covered with a water soluble polyacryl resin to prevent fading.

In every biopsy specimen, the pathologist first decided whether submucous tissue was present or not. Thereafter he reported on 20 histological features on a structured form. All pathologists had agreed on the items and the design of the structured questionnaire. Four of these features reflected the AChE activity in different locations (lamina muscularis mucosae, lamina propria mucosae, adventitia around submucous blood vessels, and afferent

Table 1 Diagnoses given by the three pathologists (A, B and C)

Diagnosis	A (n=105)	B (n=102)	C (n=104)
Hirschsprung's disease	13	13	15
Hirschsprung's disease with IND	5	5	4
Normal	34	7	49
IND	33	17	12
Heterotopic ganglion cells only	1	7	0
Other abnormalities	16	17	21
No diagnosis because of poor quality	3	35	2
No diagnosis for other reasons	0	1	1

IND, intestinal neuronal dysplasia.

submucous fibres). Eight items related to the ganglia in the submucous plexus (number and size of ganglia, bud-like nerve cell groups along or around thick afferent fibres, presence of a ganglion with more than eight nerve cells, ganglia with hypogenetic cells, ganglia with normal sized cells, ganglia with signs of immaturity, and the maximal number of cells/ganglion). Six features were related to the nerve cells (isomorph large or small, anisomorph, lactate dehydrogenase and succinate dehydrogenase activity, and single cells), and the last two items referred to the presence of heterotopic nerve cells in the lamina propria or lamina muscularis mucosae. Eleven of these items required a "yes" or "no" decision on presence or absence, the question about the maximal number of nerve cells per ganglion required a numerical value, and the remaining nine items were to be answered in a graded fashion ranging from 1 to 6 (absent, decreased, normal, slightly increased, moderately increased, and severely increased). In addition, for every item there was the option "judgment not possible", as in the absence of submucous tissue or in cases of aganglionosis some questions could not be answered.

For every case, each pathologist gave a final histological diagnosis based on all available biopsy specimens; the choices included: "normal", "aganglionosis", "aganglionosis with proximal IND", "IND", "isolated heterotopic ganglion cells in the muscularis mucosae and/or lamina propria mucosae", and "other abnormalities". The last had to be specified. The definitions of the histological diagnoses were those of the consensus report.³⁰ Obligatory diagnostic criteria for aganglionosis in the rectal biopsy specimens included (a) a dense network of nerve fibres with increased AChE activity in the mucosa and (b) absence of ganglion cells from the submucous plexus. Obligatory diagnostic criteria for IND included (a) hyperplasia of the submucous plexus, characterised by increases in the frequency and size of ganglia, "giant ganglia" with more than eight nerve cells per ganglion (hyperganglionosis)

with bud-like nerve cell groups along or around thick afferent fibres and (b) increased number of AChE-positive nerve fibres around submucous blood vessels. Facultative criteria were an increase in AChE-positive fibres in the lamina propria mucosae or heterotopic ganglion cells within the mucosa. "Other abnormalities" included mostly immaturity of ganglion cells or the presence of some signs of IND not fulfilling the above diagnostic criteria for IND. The term normal was used when no morphological abnormalities with regard to innervation were found. In addition, the pathologist had the option to code "no judgment or specific diagnosis due to poor quality of specimen".

RATING OF CLINICAL OUTCOME

Clinical outcome after 12 months was defined as excellent, when there were no symptoms and a normal stool pattern with more than three stools/week in the absence of any therapy. A moderate outcome was considered in children with persistent constipation still requiring medical therapy—for example, laxatives, enemas. Outcome was called poor in cases with more severe symptoms like pseudo-obstructive episodes or conditions requiring parenteral nutrition or enterostomy.

STATISTICAL ANALYSIS

Interobserver agreement on final diagnoses and different histological features was evaluated for the three observer pairs (A and B, A and C, B and C). For each pair of observers the judgments were cross classified in a contingency table. As a certain amount of agreement could have occurred by chance alone, the data were analysed using Cohen's κ statistic,³⁴ which measures observed agreement relative to agreement expected by chance. Values ranging from -1 to +1 can occur. Positive values indicate a better than chance agreement, with 1 for a perfect agreement; zero represents the agreement expected by chance, and negative values mean more discrepancies than expected by chance. Ordinary κ values were applied for binary and other nominal variables with few categories. For graded measurements, weighted κ values with a quadratic weighting scheme were computed.³⁵ In contrast with the ordinary κ , which treats all discrepancies equally, weighted κ penalises larger differences over proportionally higher than smaller differences. Confidence intervals for κ were computed using approximate asymptotic variance formulae for ordinary κ ³⁶ and for weighted κ .³⁵

For analysing agreement on the single morphological findings, biopsy specimens were

Table 2 κ values with 95% confidence intervals for the three pairs of observers for the final diagnosis in the whole study population (n=108)

	κ values		
	A v B	A v C	B v C
Final diagnosis (including the category "diagnosis not possible because of poor quality")	0.26 (0.16 to 0.36) n=99	0.41 (0.29 to 0.54) n=103	0.26 (0.16 to 0.35) n=98
Final diagnosis possible/not possible because of poor quality	0.11 (-0.01 to 0.22) n=98	0.39 (-0.16 to 0.94) n=102	0.07 (-0.02 to 0.17) n=96
Final diagnosis (excluding cases without a definite diagnosis because of poor quality)	0.39 (0.24 to 0.54) n=63	0.43 (0.30 to 0.56) n=98	0.40 (0.26 to 0.54) n=62

Results are given for all children seen by both observers. After exclusion of cases in which at least one of the pair felt the quality too poor to make a definite diagnosis, the difference between the three pairs vanished.

Table 3 Interobserver agreement for the diagnoses Hirschsprung's disease (HD), intestinal neuronal dysplasia (IND) and normal

	All cases (n=108)			Workup in laboratory B (n=32)			Workup in laboratory A or C (n=76)		
	A v B (n=99)		A v C (n=103)	B v C (n=98)		A v B (n=28)	A v C (n=29)		B v C (n=29)
	A	v B	A	B	v C	A	A	v C	B
HD yes/no	1 (n=63)	0.20 (0.01 to 0.39)	1 (n=98)	1 (n=62)	0.11 (-0.06 to 0.29)	1 (n=27)	1 (n=29)	1 (n=28)	1 (n=28)
IND yes/no	0.20 (0.01 to 0.39)	(n=47)	0.26 (0.11 to 0.40)	0.11 (-0.06 to 0.29)	(n=45)	0.41 (0.12 to 0.71)	0.27 (-0.02 to 0.57)	0.20 (0.02 to 0.39)	0.20 (0.02 to 0.39)
(HD excluded)						(n=21)	(n=23)	(n=21)	(n=21)
Normal yes/no	0.13 (-0.13 to 0.38)	(n=47)	0.33 (0.14 to 0.51)	0.05 (-0.12 to 0.23)	(n=45)	0.36 (0.03 to 0.70)	0.26 (-0.05 to 0.58)	0.10 (-0.03 to 0.23)	0.10 (-0.03 to 0.23)
(HD excluded)						(n=21)	(n=23)	(n=21)	(n=21)

Results are expressed as κ values with 95% confidence intervals between the three pairs of observers for all cases and stratified by the laboratory of initial workup. Cases with no definite diagnosis because of poor quality are excluded. Negative values in the confidence interval indicate that observed agreement is not significantly different from chance agreement.

taken as observational units, ignoring the possibility of stochastic dependencies resulting from the fact that several (between 1 and 10) tissue samples were obtained from each child. This seemed justified because determinants of rating variability are supposed not to be influenced by child characteristics. Histological diagnoses given after inspection of the various specimens from every child are child-specific; therefore interobserver variation of diagnoses was analysed with cases rather than specimens as observational units.

Observer B felt unable to give a definite diagnosis in almost half of the cases worked up by observers A and C. This could possibly be due to different standards of work up in the laboratories. To adjust for these possible differences, all results were analysed (a) before and after exclusion of biopsy samples with no definite answer because of poor quality and (b) stratified by the laboratory of primary work up.

Results

CHARACTERISTICS OF PATIENTS

Within 20 months, 117 children from seven centres were recruited. Nine patients had to be excluded because their biopsy samples arrived damaged or thawed and were insufficient for further work up. Of the remaining 108 children, 62 were male and 46 female. About half of the patients (n = 56) were recruited by paediatric surgeons, the remaining 52 children by paediatric gastroenterologists. Ages at the time of biopsy ranged from 4 days to 15.3 years, with a median of 2.1 years.

The presenting symptom was constipation in 69% of the patients, followed by enterocolitis (11%), ileus (10%), faecal incontinence (2%), intestinal pseudo-obstruction (1%), and other diagnoses (7%) including rectal prolapse, cloacale extrophy, recurrent vomiting, intractable diarrhoea, perianal bleeding, and severe bloating. The age at onset of symptoms ranged from 1 day to 14 years with a median of 4 weeks. A total of 14 children were known to have bowel malformations (anal stenosis or atresia, duplication or atresia of the colon, gastroschisis). At the time of biopsy, 24 patients had an enterostomy.

SAMPLING AND ROTATION OF BIOPSY SPECIMENS

In 60 children, biopsy specimens were taken with a suction instrument, in 24 with forceps, and in a further 24 with scissors, the latter all being performed by paediatric surgeons. No complications from the procedure were reported. A total of 377 biopsy specimens from 108 children were available, ranging from one to ten per patient. In the majority (73%), three or four specimens were taken. All children had specimens taken 1–2 cm above the pectinate line producing a total of 113 specimens; 90 specimens were taken at 3–4 cm, 134 between 5–15 cm, and 38 from the proximal colon or small bowel during surgical procedures. No information on location was available for two biopsy specimens. In six children the most proximal specimen was taken at a level below 5 cm, and in 22 patients at 5 cm above the pectinate line; the remaining 80 children had specimens taken at higher levels.

Ninety seven cases (318 samples) were seen by all three pathologists, and nine (48 samples) were judged by two observers only. The remaining 11 specimens from two children were not rotated at all and therefore could not be included in the interobserver analysis. Owing to the incomplete rotation, the total number on which every analysis is based is given with the results.

DIAGNOSES PROVIDED BY THE PATHOLOGIST WHO DID THE INITIAL WORK UP

Seven of the 108 cases were worked up initially in laboratory A, 32 in laboratory B, and 69 in laboratory C. Only the diagnosis of the first pathologists, but not the judgments of the two other observers, was reported to the clinician and could potentially influence further clinical management. In 20 children (19%) a diagnosis of Hirschsprung's disease was made, six of them with IND proximal to the aganglionosis. In 35% of the cases, the histology was reported to be normal, in 14% IND, in 5% isolated heterotopic ganglion cells, and in 24% of all cases the pathologist saw "other abnormalities". In 4% of the children, no final conclusion could be reached.

DIAGNOSES GIVEN BY THE THREE OBSERVERS

Table 1 gives the number of cases seen by the three pathologists and the frequency of the final diagnoses given by each of them. Three findings are noteworthy. (a) Observer B gave a judgment of normal in only 7/102 (6.9%) of cases, in contrast with observers A and C who gave this diagnosis for 32% and 47% of the children respectively. (b) Observer A diagnosed IND more often (33 cases *v* 17 and 12 cases found by the other pathologists). (c) Observer B more often judged the biopsy specimens to be of such poor quality that in 35 cases he felt unable to make a definite diagnosis; all 35 cases were initially worked up by A or C. The pathologists A and C made this statement in only three and two cases respectively, including one which could not be judged by any of the three pathologists.

AGREEMENT WITH RESPECT TO DIAGNOSIS IN CASES SEEN BY ALL THREE OBSERVERS

Of the 97 cases seen by all three observers, there was complete agreement on the diagnosis of Hirschsprung's disease in 14 children, two of them combined with proximal IND. In two other children the pathologists agreed on the

diagnosis of aganglionoses, but disagreed on the presence of proximal IND. Of the remaining 81 children without Hirschsprung's disease seen by all observers, there was complete agreement on the final diagnosis in only 11 (14%): three diagnoses of normal, six of IND, one case of "other diagnosis", and in one case they agreed that the specimens were not adequate to make a diagnosis. In 15/81 (19%) cases seen by all three pathologists, at least one observer concluded IND was the diagnosis whereas another judged the identical specimens from the child to be normal. The combination of these two extreme judgments occurred four times between observers A and B, nine times between observers A and C, and six times between observers B and C. Thus, on sending the same slide of a specimen from a child without aganglionosis to three experienced pathologists, completely discordant diagnoses (normal innervation *v* IND) appeared more often than identical judgments.

INTEROBSERVER VARIATION BETWEEN OBSERVER PAIRS WITH RESPECT TO FINAL DIAGNOSIS

Table 2 gives the κ values for the comparisons within the three pairs of observers (A *v* B, A *v* C, and B *v* C) with respect to the final diagnoses for all children. The κ values are lower when observer B is involved (0.26 *v* 0.41). This is because he concluded in more cases "no diagnosis possible due to poor quality", expressed by very low κ values with respect to this question (κ 0.11 and 0.07 when B was involved). After exclusion of these cases, the κ values for the three observer pairs were very similar (0.39, 0.43, and 0.40).

The κ values given in table 3 for the diagnoses Hirschsprung's disease, IND, and normal were calculated after excluding cases without definite diagnosis, and therefore they are in favour of a better agreement. There was 100% agreement between the observers with respect to the diagnosis of Hirschsprung's disease indicated by a κ of 1. In contrast, the κ values were low and close to values expected by chance for the judgments normal and IND. When results were stratified by the laboratory of primary work up, this hardly influenced the κ values at all for the observer pair A and C. However, the agreement differed markedly when observer B was part of the pair, with better agreement when specimens were initially worked up in his own laboratory. The number

Table 4 Interobserver agreement expressed as ordinary κ values with 95% confidence interval for binary morphological features (presence/absence) in specimens. Negative values in the confidence interval indicate that the observed agreement is not significantly different from chance agreement

	A <i>v</i> B (n=263)	A <i>v</i> C (n=279)	A <i>v</i> C (n=254)
Ganglion with >8 nerve cells	0.57 (0.43 to 0.72)	0.52 (0.38 to 0.66)	0.60 (0.46 to 0.73)
Ganglion with hypogenetic cells	0.14 (−0.01 to 0.29)	0.05 (−0.05 to 0.16)	0.37 (0.17 to 0.56)
Ganglion with normal sized cells	0.35 (0.26 to 0.45)	0.46 (0.37 to 0.56)	0.40 (0.28 to 0.52)
Ganglion with signs of immaturity	0.08 (−0.06 to 0.22)	0.00 (−0.10 to 0.10)	0.27 (0.09 to 0.44)
Large isomorph-like nerve cells	0.20 (0.08 to 0.32)	—	—
Small isomorph-like nerve cells	0.01 (−0.10 to 0.12)	−0.01 (−0.12 to 0.10)	0.26 (0.13 to 0.40)
Anisomorph-like nerve cells	0.19 (0.04 to 0.34)	0.13 (0.01 to 0.26)	0.30 (0.13 to 0.48)
Single nerve cells	0.09 (−0.02 to 0.19)	0.19 (0.09 to 0.29)	0.53 (0.41 to 0.64)
Heterotopic nerve cells within the lamina propria	0.48 (0.21 to 0.75)	0.25 (−0.15 to 0.64)	0.14 (−0.10 to 0.38)
Heterotopic nerve cells within the muscularis mucosae	0.17 (0.03 to 0.32)	0.06 (−0.09 to 0.21)	0.06 (−0.03 to 0.15)

Table 5 Interobserver agreement for graded morphological items expressed as weighted κ values with 95% confidence interval

	A v B (n=263)	A v C (n=279)	B v C (n=254)
AChE activity in the lamina muscularis mucosae (all patients)	0.55 (0.47 to 0.63)	0.64 (0.57 to 0.70)	0.73 (0.68 to 0.77)
AChE activity in the lamina muscularis mucosae (HD excluded)	0.15 (−0.01 to 0.31) (n=209)	0.20 (0.04 to 0.35) (n=210)	0.29 (0.17 to 0.42) (n=194)
AChE activity in the lamina propria mucosae (all patients)	0.36 (0.30 to 0.42)	0.56 (0.49 to 0.63)	0.56 (0.50 to 0.62)
AChE activity in the lamina propria mucosae (HD excluded)	0.13 (0.06 to 0.20) (n=209)	0.34 (0.23 to 0.45) (n=210)	0.23 (0.13 to 0.33) (n=194)
AChE activity in the adventitia around submucous blood vessels	0.11 (0.03 to 0.26)	0.12 (−0.04 to 0.28)	0.26 (0.15 to 0.37)
AChE activity in afferent submucous fibres	0.32 (0.22 to 0.42)	0.33 (0.22 to 0.44)	0.59 (0.51 to 0.66)
Number of ganglia	0.67 (0.59 to 0.75)	0.61 (0.54 to 0.69)	0.76 (0.68 to 0.83)
Size of ganglia	0.69 (0.60 to 0.77)	0.71 (0.65 to 0.77)	0.76 (0.67 to 0.84)
Bud-like nerve cell groups along afferent fibres	0.10 (−0.21 to 0.40)	−0.06 (−0.34 to 0.22)	0.09 (−0.20 to 0.38)
Maximal number of nerve cells/ganglion	0.76 (0.67 to 0.84)	0.77 (0.72 to 0.83)	0.76 (0.67 to 0.85)
LDH activity in nerve cells	0.69 (0.60 to 0.78)	0.67 (0.59 to 0.75)	0.78 (0.70 to 0.86)
SDH activity in nerve cells	0.64 (0.55 to 0.73)	0.63 (0.54 to 0.72)	0.79 (0.72 to 0.87)

Results are given for all items of the whole study population and also for the first two features after excluding specimens of patients with Hirschsprung's disease (HD). Negative values in the confidence interval indicate that the observed agreement is not significantly different from chance agreement. AChE, acetylcholinesterase; LDH, lactate dehydrogenase; SDH, succinate dehydrogenase.

of biopsy samples examined per patient did not correlate with the interobserver agreement.

cluding these from the analysis, κ values decreased considerably.

INTEROBSERVER VARIATION BETWEEN OBSERVER PAIRS WITH RESPECT TO MORPHOLOGICAL FEATURES

Interobserver variation in different morphological features was assessed for the specimens as observational units. In contrast with results of final diagnoses, there was no clear trend towards a better interobserver agreement for the different histological features when biopsy specimens were worked up by observer B. Therefore stratified data are not reported.

Table 4 gives the κ values for the non-graded features. The highest κ value was reached for "presence of ganglia with more than eight ganglion cells". Considering that this is an objective feature and an obligatory criterion for the diagnosis of IND, a discordance rate of 13% as calculated from each of the original contingency tables seems high. More subjective features like "ganglia with signs of immaturity" showed a low agreement, not significantly different from chance agreement in two of the observer pairs.

Table 5 gives the weighted κ values for the histological items, which had to be graded from 1 to 6. Remarkably, the agreement was close to chance agreement for the obligatory criterion for the diagnosis of IND "AChE stained fibres around submucous vessels" and another characteristic feature for IND "bud-like nerve cell groups along or around thick afferent fibres". The high κ values for the items "AChE staining in the lamina muscularis mucosae" and "AChE staining in the lamina propria mucosae" resulted mostly from the good agreement in cases with Hirschsprung's disease. After ex-

RELATION BETWEEN HISTOLOGICAL DIAGNOSIS AND AGE

The age at biopsy was much lower in children with Hirschsprung's disease (median 2.8 months, range 4 days to 3.0 years) than in the remaining patients without aganglionosis (median 2.9 years, range 13 days to 15 years).

The high percentage of discrepant diagnoses in children without Hirschsprung's disease made it difficult to evaluate a relation between clinical data (age, presenting symptoms, outcome) and histological diagnosis. Therefore, we defined the following diagnostic classifications depending on the combinations of diagnoses given by the three observers:

"Consistently judged as normal": all judged "normal", including combinations with "no diagnosis due to poor quality";

"Contradictory judgments with $\geq 1 \times$ normal": at least one observer judged "normal" and another said "IND" or "other abnormalities";

"Consistently judged as not normal": none of the observers judged the case as "normal";

"Consistently judged as IND": all said "IND", including combinations with "no diagnosis due to poor quality";

"Contradictory judgments with $\geq 1 \times$ IND": at least one observer said "IND" and another judged "normal" or "other abnormalities";

"Consistently judged as not IND": none of the observers judged the case as "IND".

The age quantiles differ markedly among children with these different diagnostic classifications (table 6). Children "consistently judged as normal" or "consistently judged as

Table 6 Median age and range for the different combinations of diagnosis given by the three observers for all children excluding patients with Hirschsprung's disease and for the subgroup of children with chronic constipation without complication

	All children without HD (n=88)			Children with constipation only (n=57)			Cure rate (%)	n
	Median	Range	n	Median	Range			
Consistently judged as normal	4.3 y	13 d–15.3 y	19	6.1 y	3.3 m–15.3 y	23		13
Contradictory judgments with $\geq 1 \times$ normal	3.4 y	45 d–14.8 y	41	3.7 y	1.5 m–13.4 y	31		29
Consistently judged as not normal	7.1 m	35 d–10.9 y	27	1.3 y	1.1 m–10.9 y	40		10
Consistently judged as not IND	3.7 y	13 d–15.3 y	47	4.3 y	3.3 m–15.3 y	27		33
Contradictory judgments with $\geq 1 \times$ IND	2.5 y	45 d–14.8 y	28	3.1 y	1.5 m–10.9 y	29		14
Consistently judged as IND	10.9 m	35 d–6.0 y	12	8.8 m	1.1 m–2.9 y	60		5

Pathological diagnoses are related to young age, but a better cure rate after one year in the constipated children. IND, intestinal neuronal dysplasia; y, years; m, months; d, days.

Table 7 Duration of symptoms, diagnostic and therapeutic procedures, and cure rate in the subgroup of 57 children with chronic constipation but no other complications

	Children treated by surgeons (n=30)	Children treated by paediatricians (n=27)
Median duration (months) of constipation	21	29
Encopresis (children >3 years) (%)	62	64
Barium enema (%)	90	15
Rectoscopy (%)	54	15
Myectomy of internal sphincter (%)	43	0
Resection of sigmoid colon (%)	3	0
Cure rate after 12 months (%)	28	35

Patients from surgical departments did not have more severe constipation, but underwent a more invasive diagnostic and therapeutic approach.

not IND" are significantly older than children "consistently judged as not normal" or "consistently judged as IND" ($p < 0.01$, Wilcoxon test for each of the comparisons). All three observers found ganglia with more than eight ganglion cells in a higher percentage of biopsy samples from infants 12 months or younger compared with the older children (observer A 24% *v* 16%; observer B 41% *v* 22%; observer C 53% *v* 20%).

RELATION BETWEEN HISTOLOGICAL DIAGNOSIS AND OUTCOME

To study the relation between histological diagnosis and clinical outcome, 51 of 108 patients with conditions known to have major effects on prognosis and stool pattern were excluded from the analysis. These conditions included aganglionosis, colonic or anorectal malformations, gastroschisis, enterostomy, enterocolitis, and previous bowel resections. The remaining 57 patients represented quite a homogeneous subgroup of children with solitary chronic constipation and none of the above mentioned complications. Again, there was a strong relation between age and diagnostic classification, with more abnormal diagnoses in young patients (table 6).

In 52/57 children with uncomplicated constipation, complete follow up data after 12 months were available. Outcome was considered "excellent" in 16/52 giving a cure rate of 31% after one year. A "moderate" outcome was reported in 36 children, and none of these children had a "poor" outcome. The cure rate was higher in children "consistently judged as IND" (60%) or "consistently judged as not normal" (40%) than in patients "consistently judged as normal" (23%) or "consistently judged as not IND" (27%) (table 6).

RELATION BETWEEN CLINICAL MANAGEMENT AND OUTCOME

The 57 children with uncomplicated constipation were recruited in almost equal portions from surgical ($n = 30$) and paediatric ($n = 27$) departments. Between the two subgroups, there were neither differences regarding the severity of constipation measured as duration of symptoms and presence of encopresis nor regarding the histological classification (table 7). However, patients from surgical departments underwent a more invasive diagnostic and therapeutic intervention. A total of 13 of 30 children underwent a sphinctermyectomy during the one year follow up, and one child had a resection of the sigmoid colon. None of

the paediatric patients required operation. The cure rate (off therapy with normal stool pattern) after one year in the operated children was only 17% compared with 31% in the non-operated group of children with uncomplicated constipation.

Discussion

This prospective study confirms that aganglionosis can be diagnosed with high reliability from rectal biopsy specimens using the criteria given in the consensus report.³⁰ After exclusion of patients with aganglionosis, interobserver agreement was poor with respect to most of the single morphological features and the final diagnoses. This high degree of interobserver variability with regard to innervation abnormalities other than aganglionosis reflects the experience of many clinicians, who received discordant diagnoses when sending rectal biopsy specimens from a child to more than one pathologist. In these anecdotal individual cases it remained unclear whether the different diagnoses obtained were due to variation of sampling conditions or a maturational process of the enteric nervous system if samples were obtained at two time points or if they were due to different qualifications of the pathologists with regard to work up and interpretation of the biopsy samples or to problems with the diagnostic criteria and definitions themselves. The first two factors could not influence the results in this study, because identical slides were examined by the three pathologists.

The work up of the biopsy specimens (cutting and fixing of specimens on the slide, and the type of staining and counting methods) has been the subject of controversy in the past and blamed, at least in part, for the wide range of frequencies of IND reported from different laboratories.^{12 13 32} In this study, all specimens were handled and stained with AChE, lactate dehydrogenase, and succinate dehydrogenase according to the consensus report.³⁰ However, as it turned out, there were obvious differences between the three laboratories with regard to the number of sections cut from each specimen, with up to 160 sections/specimen in laboratory B, compared with 20–30 sections in the laboratories of the other two pathologists. As observer B felt that a reliable diagnosis of IND can only be made when at least 30 sections are viewed,³⁷ he did not make a final judgment in a high proportion of cases worked up by pathologists A and C. This affected the percentage agreement and the κ values negatively when observer B was a member of the observer pair. After exclusion of items and cases with no definite diagnosis, the observer agreement improved with respect to final diagnosis. However, there was no such trend with regard to the different morphological items.

Coding different histological features and making a diagnosis seem to be two different issues. The first can certainly be improved by clearly defined requirements for cutting and staining of biopsy samples and application of morphometric methods.^{37 38} Making a histological diagnosis from different single features is a somewhat subjective integrating process

which is influenced by an individual's expertise.³⁹ The decision making process for a diagnosis may also be influenced by the clinical data known to the pathologist. In our study the pathologists were not aware of any clinical features except for the patients' sex and age and the distance of the biopsy specimen from the pectinate line. The latter two features are regarded as essential for interpretation with respect to innervation disturbances. The blinding to other clinical findings seemed crucial for both of our main investigations, the interobserver variation of histological diagnoses and the correlation of the pathological data with clinical symptoms and outcome.

The interobserver variability may have been negatively influenced by the fact that the biopsy specimens were worked up in three different laboratories and the number of sections to be viewed was not fixed. Despite this, there was complete agreement on the diagnosis of aganglionosis. In contrast, the κ values for the judgment "normal" were not significantly different from chance agreement in two of the observer pairs even when biopsy specimens underwent the more extensive work up in laboratory B. In addition, incompatible diagnoses were given in 15 cases, with one pathologist judging them "normal" and another diagnosing IND. If such high rates of severe discrepancies occur even under controlled study conditions and between very experienced pathologists, an even higher interobserver variability may be expected under normal clinical conditions and with less specialised pathologists.

This high degree of discordance with regard to the judgment of "normal innervation" points to a main problem, the lack of morphological data for comparison from non-diseased children of different ages.¹² As enzyme histochemistry requires fresh biopsy specimens, autopsy studies appear to be inappropriate, and interpretation may be particularly difficult in cases of ischaemia, shock, or sepsis before death. For ethical reasons and because of the potential risk of major bleeding or perforation, suction or deep forceps biopsy specimens cannot be taken from healthy children, particularly preterm and young infants. "Controls" used in previous pathological studies using enzyme histochemistry were mostly from symptomatic children investigated because of colitis or to rule out Hirschsprung's disease.^{37 38 40} In two studies using morphometric methods, children with rectal biopsy specimens that were considered to look histologically normal served as healthy controls and were compared with children with morphological indications of IND.^{37 38} No clinical data were given for the controls. The design itself introduces a strong selection bias and makes the controls inappropriate for defining normal features.

We found a strong correlation of age with more abnormal morphological features and giant ganglia in infants compared with older children. Children "consistently judged as IND" or "consistently judged as not normal" were significantly younger than those "consistently judged as normal" or "consistently judged as not IND". This was also true in the

subgroup of 57 children with constipation but no serious complications. This age dependence, with a higher rate of diagnosis of IND in young children, particularly premature infants and neonates, was also reported in two previous pathological series applying the same diagnostic criteria.^{23 24} This relation to young age may reflect the fact that children with IND become symptomatic earlier in life. However, in the absence of morphological data from age matched healthy controls, it can also be speculated that some of the morphological features described are normal age related phenomena rather than pathological findings indicating a motility disorder. This hypothesis is supported by the findings of two autopsy studies that reported a higher density of ganglia and ganglion cells in the submucous plexus in infants compared with older children⁴¹ and in fetuses of less than 28 weeks gestation compared with fetuses of higher gestational age and infants.⁴² However, the investigators used haematoxylin and eosin staining^{41 42} and immunohistochemistry,⁴² but no dehydrogenase reactions, which can clearly differentiate between nerve cells and glial cells in immature ganglia.³⁷

Except for the age dependence, we found no relation between clinical presentation and morphological findings. In the subgroup of children with uncomplicated chronic constipation, there was no positive correlation between severity of constipation, outcome after one year, and pathological findings on biopsy. On the contrary, poor outcome was related to normal histology. However, this may have been confounded by age, as uncured children were older with a longer duration of their constipation compared with children cured after one year (median 39.0 v 15.5 months). In fact, in a logistic model for the probability of poor outcome, long duration of symptoms turned out to be a strong predictor, while each of the variables "early onset of symptoms" and the diagnostic classification "consistently judged as IND", which correlate with each other, showed a slight effect in the opposite direction. These findings confirm the experience of investigators from other tertiary centres for constipated children: patients transferred to a specialist and treated efficiently early on have a better outcome, and long duration of constipation is a predictor of poor outcome after one year.^{43 44}

We found a low cure rate (17%) after one year in children with uncomplicated constipation treated with a sphincteromyectomy. Although the numbers were small and a strong bias existed with operations performed only in children cared for by surgeons, the role of this procedure in constipated children without Hirschsprung's disease should be questioned. Psychological and physical traumatisations⁴⁵ may occur. As there is progressive weakening of the anal sphincter with increasing age, earlier muscle injury may become apparent as incontinence later in life.

Our results do not question the existence of IND of the enteric nervous system as a cause of severe constipation and chronic intestinal pseudo-obstruction.² However, we conclude

from our results that application of the published methods and diagnostic criteria for IND³⁰ on suction or forceps biopsy specimens results in too high an interobserver variability and lacks correlation with prognosis, and therefore is not useful for clinical decision making. Our results question the recommendations and the practice in some countries with regard to surgical interventions in patients with morphological signs of IND as defined above. This is true for both the isolated form of IND and IND proximal to aganglionosis. The high rate of disagreement also challenges the results of all previous studies using rectal biopsy specimens and applying the published diagnostic criteria³⁰ regardless of whether histological findings were associated with clinical symptoms, outcome, manometric findings, or, recently, even mutations in the RET proto-oncogene.⁴⁶ Further investigations are urgently needed, and a study of the age dependent normal variation of the morphology of the enteric nervous system should be encouraged using these and other more refined methods and defined counting techniques. Any new criteria for pathological diagnoses made on rectal biopsy specimens should be validated against these normal values and investigated in well performed clinical investigations for their clinical usefulness. Until we have a better understanding and knowledge, rectal biopsy specimens for clinical purposes should be taken from constipated children only to diagnose or rule out Hirschsprung's disease.

Some of this work has been presented at the annual meeting of the European Society of Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) and the annual meeting of the Gesellschaft für Pädiatrische Gastroenterologie und Ernährung, and published in abstract form (*J Pediatr Gastroenterol Nutr* 1997;24:477 and *Monatsschr Kinderheilkd* 1997;145:558 respectively).

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