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ORIGINAL ARTICLE

Aganglionosis with the absence of hypertrophied nerve fibres predicts disease proximal to rectosigmoid colon

Sarath Kumar Narayanan[1] · Soundappan S. Soundappan[1] · Edwin Kwan[1] · Ralph C. Cohen[1] · Amanda Charlton[1] · Daniel T. Cass[1]

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Abstract
Purpose The gold standard for the diagnosis of Hirschsprung's disease (HSCR) is the pathologic evaluation of a rectal biopsy that demonstrates the absence of ganglion cells and nerve fibre hypertrophy. However, it has been frequently reported that hypertrophic nerves may not be present in some variants like long-segment HSCR, total colonic aganglionosis, premature and very young infants. The aim of this study was to determine this association.
Methods We performed a retrospective review of the HSCR database at our tertiary care children's hospital from 2000 to 2013. In order to analyse the relationship between the diameter of the nerve fibres and the level of aganglionosis, we classified the patient sample into two groups—fibres ≤40 and >40 μm. The groups were statistically compared with $P < 0.05$ being significant.
Results Rectal biopsies of 92 patients confirmed as HSCR with definitive operation performed at the same institution were reviewed. The mean nerve diameter was 50.1 μm (range 20–87.5 μm). Nerve fibre diameter ≤40 μm was predictive of transition zone above the sigmoid colon. A specificity of 77.3 % and a likelihood ratio of 2.03 supported this perception. No correlation was noted between nerve fibre diameter and gestational age at birth, birth weight or age at biopsy.
Conclusion The absence of nerve fibre hypertrophy in the presence of aganglionosis on rectal biopsy specimens is predictive of long-segment HSCR.

Keywords Aganglionosis · Hirschsprung's disease · Rectal biopsy · Hypertrophied nerve fibres

Introduction

Hirschsprung's disease (HSCR) is an intestinal disorder characterised by the failure of migration of the neural crest cells during foetal development that results in an absence of ganglion cells [1]. In most centres, the initial diagnosis of the Hirschsprung's disease is based on histo-pathological study of suction rectal biopsies (SRB). The gold standard for the diagnosis of HSCR is the pathologic evaluation of a rectal biopsy that demonstrates the absence of ganglion cells and nerve fibre hypertrophy [2].

Although most cases with classic HSCR show several prominent nerves (>40 μm diameter) in the submucosa, nerve hypertrophy alone is not sufficient to establish the diagnosis of HSCR [3]. Ultra-short, long-segment HSCR (LS-HSCR) and total colonic aganglionosis (TCA) are infrequent forms of HSCR with clinical, histological and genetic changes which are even more challenging to diagnose and treat. It has been frequently reported that hypertrophic nerves may not be present in these variants and also in young infants [3–5]. However, this relationship has not been established thus far. Hence, the aim of this study was to assess the diagnostic implications of the absence of hypertrophied nerve fibres in SRB or open rectal biopsies (ORB) during the work-up for suspected HSCR. In this study, we sought to begin testing our

✉ Sarath Kumar Narayanan
drsharat77@gmail.com

[1] The Children's Hospital at Westmead, Westmead, New South Wales, Australia

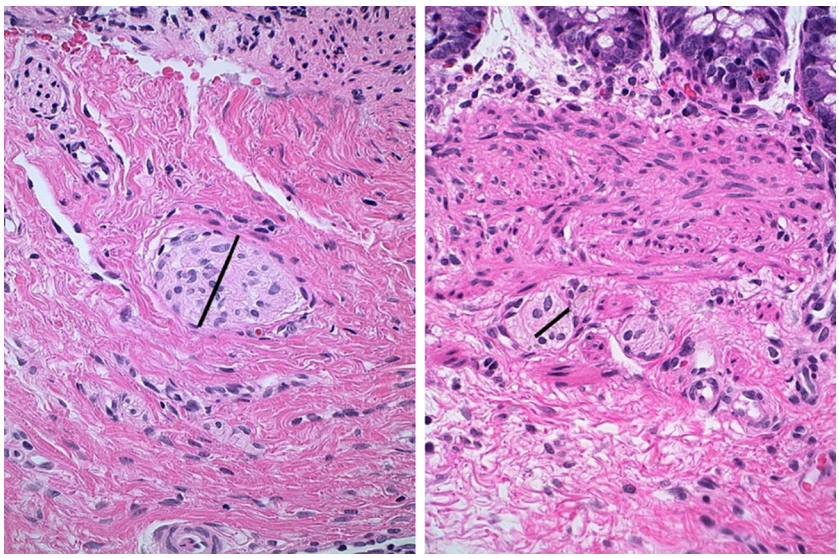

Fig. 1 Submucosa of suction rectal biopsy from a newborns with aganglionosis, *bar* is 40 μm, haematoxylin and eosin stain. *Left panel* shows a submucosal hypertrophied nerve. The *right panel* shows a hardly noticeable non-hypertrophied nerve

hypothesis that the lack of nerve fibre hypertrophy suggests longer segment of aganglionosis.

Materials and methods

The study was approved by The Children's Hospital at Westmead ethics committee. Between 2000 and 2013, 242 rectal biopsies (ORB/SRB) were performed in our institution as part of the work-up for symptoms of HSCR. After exclusion of the normal biopsies, insufficient samples and inconclusive or unsuitable biopsies, we were left with 101 biopsies that were diagnostic for HSCR. We had to further exclude ($n = 9$) for lack of complete data (including two deaths before the definitive surgery) or faded H&E stains. All the remaining patients ($n = 92$) subsequently underwent the definitive surgery and were established as HSCR.

Staining methods and slide review

The biopsies at our institution were reported based on analysis of haematoxylin-eosin (H&E)-stained paraffin sections with frozen sections for AChE-enzyme histochemistry. Calretinin immunohistochemistry was used in addition in the last few years. The biopsies were obtained at least 1–1.5 cm above the anorectal squamo-columnar junction. Typically, each patient had an initial sample of one or two SRB each up to 2 mm in maximum dimension. From each biopsy, 50–75 serial H&E-stained sections were examined. If ganglion cells were not identified, additional H&E-stained serial sections (up to 100) were examined. All these slides were reviewed under supervision of an experienced paediatric histopathologist (AC) and quantified by one author (SKN). Every H&E-stained paraffin section was reviewed to determine the largest hypertrophied nerve dimension. All slides were screened with an Olympus BX53 microscope at 100× (objective × ocular) magnification. Then, the largest nerve fibre was measured at 400× magnification (objective × ocular). Diameter larger than 40 μm was categorised as hypertrophied [3–6]. Only clearly defined nerve fibres on H&E-stained sections were evaluated, and the diameter was taken perpendicular to the long axis of the nerve fibre at its widest portion using ocular micrometre. Figure 1 demonstrates H&E-stained submucosa of suction rectal biopsy with aganglionosis. The left panel shows a submucosal hypertrophied nerve in comparison with a hardly noticeable non-hypertrophied nerve on the right.

Data collection

The database of the hospital was searched for the clinical data (Powerchart 2012, Cerner Corporation, USA). The collected variables included age, sex, birth weight, gestational age, co-morbidities, associations, results of contrast study, transition zones on contrast study, age at biopsy, types of biopsy, age at definitive surgery, length of aganglionic segment, definitive biopsy point and the type of pull through procedure. In order to analyse the relationship between the diameter of the nerve fibres and the length of aganglionosis, we classified the patient sample into two groups—those with nerves ≤40 μm and those with at least one nerve above 40 μm (henceforth referred to as '>40 μm'). The HSCR was classified on the basis of the definitive resection specimen into six subsets as rectal, rectosigmoid, sigmoid, descending colon, transverse colon, ascending colon and TCA. No effort was made to consider the ultra-short segment HSCR separately as this subset is

controversial, variably defined by authors and often does not require a pull through surgery.

Proportions of the diameters of the largest nerve dimensions were compared statistically according to each type of HSCR. In addition, comparisons were also made with gestational age, birth weight and age at biopsy.

For statistical analyses, descriptive statistics were used where appropriate. Associations between nerve size (categorised as ≤40 and >40 μm) and extent of disease ("short segment" and "long segment") were tested using χ^2 test. Association between nerve fibre diameter in μm and "long segment" disease was tested using logistic regression. Associations between nerve fibre diameter, gestational age and birth weight were tested using Pearson correlation coefficients. All analyses were performed using SPSS V 17.0 (SPSS, Chicago, IL) and *P* value less than 0.05 was accepted as indicating statistical significance.

Results

Demographics

Ninety-two patients who had a rectal biopsy (SRB/ORB) and who underwent definitive surgery (pull through procedure) for HSCR at our centre were included in this analysis. Patient demographics and clinical characteristics are summarised in Table 1. The most common underlying association was Down's syndrome (8/92). The ages ranged between 4 days and 11.1 years at the time of biopsy and 33 days and 11.5 years at the time of definitive surgery with a male:female ratio of 3.39:1. Patients presented with a variety of comorbid conditions, with prevalences enumerated in Table 2. At definitive surgery, the ganglion cells were present (the site of identifiable ganglion cells above the aganglionic zone) in the rectum in 13 % ($n = 12$), rectosigmoid in 44.5 % ($n = 41$), sigmoid in 27.2 % ($n = 25$), descending colon in 5.4 % ($n = 5$), transverse colon in 2.1 % ($n = 2$) and total colonic in 7.6 % ($n = 7$). The mean nerve diameter was 50.1 μ (range 20–87.5 μ).

Table 1 Patient demographics and clinical characteristics

Characteristics	No. of patients ($n = 92$)
Sex (M/F)	71/21
Biopsy type (open/suction)	20/72
Age at biopsy (mean ± SD) in months	4.69 ± 16.52
Age at definitive surgery (mean ± SD) in months	5.68 ± 17.12
Birth weight in kg (mean ± SD)	3.33 ± 0.78
Gestational age at birth in months (median and range)	39.5 (31–42)
Prior colostomies (yes/no)	28/64
Contrast study (yes/no)	39/53
Definitive surgery	
Open	
Soave	29
Duhamel	26
Endorectal Soave	13
Permanent Ileostomy	06
Hartmann procedure	01
Ileo-anal Soave	01
Laparoscopic	
Soave	15
Swenson	01

Table 2 Major associations and co-morbidities with Hirschsprung's disease

Conditions	No. of patients	
	LS-HSCR	Classical HSCR
Down's syndrome	1	7
Hypothyroidism	1	2
Anorectal malformation	0	1
Hypospadias	1	0
Cardiac anomalies	1	2
Mowat Wilson syndrome	1	0
Upper airway obstruction	1	0
Syndactyly/club foot	2	0

Correlation between submucosal nerve fibre diameter and extent of aganglionosis on definitive surgery

We analysed the results in more detail by classifying the patients into six groups and then plotting them against the diameter of the nerve fibres (Table 3). Overall, the mean fibre diameter did not differ significantly with the level of aganglionosis ($P = 0.209$) using logistic regression. As evident from Fig. 2, the nerve fibres ≤40 μm does not suggest a specific pattern of distribution with the levels of transition. However, the numbers in the transverse colon, descending colon and total colonic groups were minimal to come to a statistically significant conclusion. The definition of a 'long segment' in HSCR is variable in the literature. Most authors consider rectal and rectosigmoid types to be a classical HSCR, while sigmoid and above are considered 'long segment'. We divided the patient groups similarly into classical HSCR (rectosigmoid and rectum) and the LS-HSCR (sigmoid and above). When a subset analysis was performed with these groups, the nerve fibre diameter

Table 3 The extent of aganglionosis and the diameter of nerve fibres

Level of aganglionosis	Mean nerve fibre diameter ± SD in μm	Number of patients with nerve diameter ≤40 μm ($n = 30$)	Number of patients with nerve diameter >40 μm ($n = 62$)
Rectum	49.7 ± 15.1	3 (10 %)	9 (14.5 %)
Rectosigmoid	54.5 ± 16.3	9 (30 %)	32 (51.6 %)
Sigmoid	46.1 ± 18.1	12 (40 %)	13 (21 %)
Descending colon	41.0 ± 15.2	3 (10 %)	2 (3.2 %)
Transverse colon	42.5 ± 10.6	1 (3.3 %)	1 (1.6 %)
Total colonic aganglionosis	47.1 ± 17.9	2 (6.7 %)	5 (8.1 %)

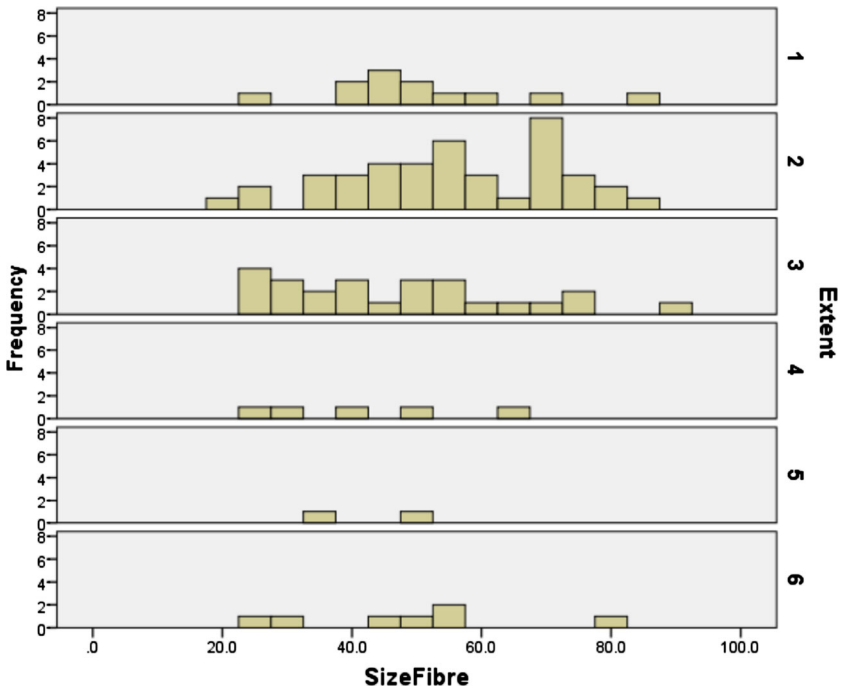

Fig. 2 Distribution of diameter of nerve fibres for the level of aganglionosis (*1* rectum, *2* rectosigmoid, *3* sigmoid, *4* descending colon, *5* transverse colon, *6* total colonic; nerve fibre diameter is in μm). Frequency indicates the number of cases of HSCR

≤40 μm was associated with a transition zone above the rectosigmoid junction ($n = 53$ and $n = 39$; 53.4 ± 16.1 vs. 45.4 ± 17; $P = 0.017$). We analysed this relation further, which demonstrated that the presence of nerve fibres ≤40 μm in diameter was specific (77.3 %) for a level of aganglionosis at sigmoid colon or above. The positive predictive value was 60 %, while the negative predictive value is 66 % at the same level of transition. A likelihood ratio of 2.03 supported the perception that the absence of hypertrophied nerve fibres is associated with a LS- HSCR.

Correlation between nerve fibre diameters and gestational age, birth weight and age at biopsy

On the scatter plots (Fig. 3), gestational age at birth showed no correlation with diameter of the fibres ($r = 0.133$, $P = 0.240$). Similarly, there was also no correlation between birth weight and size of nerve fibres ($r = 0.064$, $P = 0.574$). When analysed with the age at which biopsy was performed, the diameter of fibres again showed no correlation ($r = -0.0168$).

Discussion

The diagnosis of HSCR is classically made by a rectal biopsy. It is known that the hypertrophic nerve fibres that exist in most patients with HSCR arise from extrinsic autonomic and sensory fibres, which enter along with vessels from the perirectal region and project for a finite distance proximally. The number and diameter of these fibres increase in HSCR, giving rise to the hypertrophic

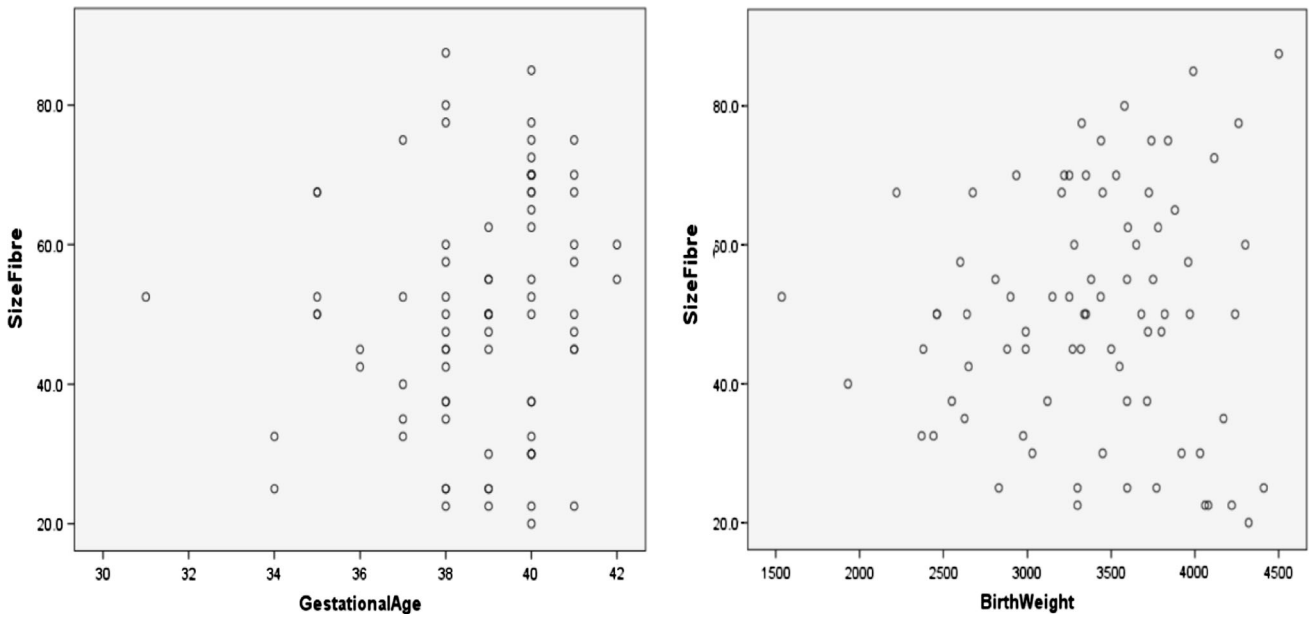

Fig. 3 Scatter plots for all patients correlating nerve fibre diameters with gestational age (size of nerve fibre in μm and gestational age at birth in weeks) and birth weight (size of nerve fibre in μm and birth weight in grams)

nerves that are frequently, but not always, observed in the myenteric plexus, submucosa and mucosa of HSCR patients [5]. The nerves are considered as 'hypertrophied' if the diameter is above 40 μm [5, 6]. Although some authors (including the Gastro 2009 International Working Group) believe that the lack of hypertrophied nerves shows an increased prospect of LS-HSCR, there is no study that has examined the relationship between the absence of hypertrophied nerve fibres and the extent of aganglionosis [3]. To our knowledge, this is the first study to address this issue.

Predicting patients likely to have LS-HSCR is clinically relevant because it may help surgeons identify which patients might cause technical difficulty during the definitive operation. It is also of relevance to the surgical pathologists who can use the absence of hypertrophied nerves in the absence of ganglion cells to raise the possibility of LS-HSCR. Hypertrophied submucosal nerves in the context of aganglionosis are supportive evidence that the lack of ganglion cells is not due to insufficient sampling or lack of detection.

The notion of scantiness of nerve fibres in LS-HSCR was perhaps first put forth by Meier-Ruge et al. as early as 1972 [7] which was subsequently reiterated in his monograph [8]. It was shown that the thickness and density of the extrinsic nerves were the greatest in the distal rectum and exponentially decrease proximally. This phenomenon was thought to occur more rapidly in a LS-HSCR than the classic type, leading to a shorter compressed zone of hypertrophied nerves in the distal rectum (Raj P Kapur, personal communication, November 2013). The perception was further strengthened by Solari et al. and Doi et al. who established that there was a marked reduction in the number of nerve trunks in the submucosal and myenteric plexuses of the aganglionic bowel of the TCA (a severe form seen in 2–14 % of HSCR) and LS-HSCR, respectively [9, 10]. Various other authors have also cited this relationship with TCA which is often based on nerve density and not the diameter of the fibre [4, 11]. Despite these observations, not much is known about the situation of aganglionosis with a lack of hypertrophied nerve submucosal fibres, based on a rectal biopsy which is obtained from the distal-most rectum. In contrast, Monforte-Muñoz et al. have had examples of TCA with distinct nerve hypertrophy in their series [6]. The apparent contradiction is partly because it is not clear whether the scarcity of thickened nerve fibres meant decreases in density on sections or a decrease in the actual diameter. The objective of this study was to gain some clarity on this issue.

Our morphometric observations on rectal biopsies from HSCR showed that the absence of hypertrophied submucosal nerves had a high specificity of 77.3 % for transition zones proximal to the sigmoid colon. The sensitivity, however, was only 46.1 %. However, the numbers of the patients in this subset are not large enough for good statistical significance. Contrary to what has been believed hitherto, our study did not show the gestational age, low birth weight or the age at which the biopsy is associated with nerve fibre diameter $\leq$40 μm. These findings give credence to the fact that the aetiology of LS- HSCR and

TCA may be different from the classic type due to different innervation profiles. Additional detailed studies from other institutions with larger number of cohorts, particularly in the LS-HSCR group, could be of value in refining this objective parameter.

Conclusions

We found that nerve fibres $\leq$40 μm in the presence of aganglionosis on rectal biopsy specimens are specific but not sensitive for predicting LS-HSCR. The nerve fibres $\leq$40 μm in diameter have no relation to birth weight, gestational age at birth and the age at which the biopsy is performed. These findings help to assist the clinicians in interpreting such a report.

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