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ESPS PEER-REVIEW REPORT

Name of journal: World Journal of Gastrointestinal Pathophysiology
ESPS manuscript NO: 31448
Title: Multiple endocrine neoplasia 2B: Differential increase in enteric nerve subgroups in muscle and mucosa.

General comments to reviewers:
We thank the reviewers for their comments and hope we have addressed all issues. Please note this is a case report and limited in length and numbers of figures by the journal.

General changes: We had control tissue from a 12 year old FAP patient AND a 4 year old HSCR (normal margin) in the original manuscript but did not include the 4 yr old HSCR in the abstract. We have added the 4 yr old HSCR to the abstract, emphasized the 4 year old control in the revised manuscript and reorganized figures to bring quantitation together into figure 2 and used the 4 year old HSCR control in all images.

Reviewer's code: 00000652

COMMENTS TO AUTHORS

In this descriptive case report, the authors investigate the morphology by IHC (confocal IF) in MEN2B-intestinal ganglioneuromatosis of a 4yr-old child.
As control, tissues from a 12yr-old child are used. --> I strongly recommend to include age-matched control tissue instead. *Both reviewers missed in the original manuscript that we did use tissue from a 4 yr old HSCR patient (normal margin) and from a 12 year old with FAP. We compared the results of the 2 'controls' and found they were the same, so we combined them for the analysis. We have added this statement to page 9.*

It would be also helpful to provide some quantative data (= measurements) regarding the observed "massive increases" (see conclusion p. 10). *Quantitative data is provided in figure 2 showing ganglia increase in size from 50um to 500 um and nerve fibre density increased 2-3 fold for TUJ1, NOS and SP containing fibres. We have changed wording from 'massive' to 'large' in the abstract and page 8.*

All images (patient vs. control) should include scale bars. *Done*

Page 22: Images A,C appear to be blurry? *While not as sharp as the others, we hope the information is still clear. These images are comparable to most immunohistochemistry images.*

MM: I recomend to include a table of all antibodies used. *We have added the table (Table 1).*

Please also indicate that appropiate negative controls have been used for all antibodies (no primary and isotype IgG). *We have added the controls that were included – see page 7.*

Reviewer's code: 00092753
COMMENTS TO AUTHORS
In view of the paucity of data on MEN 2B-induced intestinal ganglioneuromatosis, it would be great if the authors would kindly clarify the following points, which would greatly enhance the clinical relevance of their manuscript:
1. The natural comparator for MEN 2B-induced intestinal ganglioneuromatosis, causing constipation (also dubbed pseudo-Hirschsprung's disease), is Hirschsprung's disease (in which only the second, but not the first, efferent cholinergic neuron is affected, and which progresses in severity from proximally to distally). Hirschsprung's disease also may affect a subset of MEN 2A patients, i.e., carriers of RET germline mutations in exon 10, codons 609, 611, 618, or 620, which may be worthwhile mentioning. *We did include bowel from a 4 year old HD taking the normal margin of colon as well as the 12 yr old FAP patient. We have tried to make this more obvious in the manuscript (abstract and page 8).*

2. The present manuscript would be clinically more meaningful if the authors provided quantitative information on whether the increases in ganglionic growth in number and thickness and immunohistochemical subgroups of ganglia differed: By location (terminal ileum vs. proximal/ascending colon vs. distal/descending colon): - in total - broken down by submucosal vs. myenteric ganglia - broken down by longitudinal vs. circular myenteric layers. *We agree this would be nice but the tissues from different regions were not collected. The patient is now 14, these samples were collected as part of his anatomical pathology when he was 4 and had a colcetomy. The numbers of neurons in myenteric and submucosal ganglia is given page 9 paragraph 2.*

3. To better convey that point, box and whisker diagrams, detailing the number of counted neurons per stratum with a legend clarifying all abbreviations used, would be greatly appreciated. These box and whisker diagrams could replace most, if not all, of the present numerous black and white immunostains which are much less informative. *There are no images published of the different labels in MEN2B and we feel this makes it worthwhile to include them. As we only have one patient and two controls, we are wary of pushing the quantitation too far. Rather our aim is to examine if there maybe differential increases in nerve fibre density and raise the issue for others to examine this in the future. Although thyroidectomy is now routine, this child had thyroidectomy at 9 months and has still had progression of the nerve growth with visible nerve fibres in the cornea, neuromas on the tongue and as shown here, neuronal proliferation in the intestine.*

4. A rationale is necessary for the (convenience?) antibody panel the authors chose to use in their investigation, specifically for not using VAChT (vesicular acetyl choline) or high-affinity CHT

(choline transporter) antibodies (Discussion, p. 9). *The nerves labelled with these markers are explained in the introduction (page 6 para 2) and page 7 end para 1. We agree it would be good to do the VAChT and CHT labelling. VAChT labelling requires the tissue to be incubated (cultured) for 24 hours prior to labelling to get clean results. We used archived tissue that was fixed immediately. We did do VAChT staining but it gave non specific labelling. We have previously shown that CHT labels human cholinergic nerve fibres* (Harrington AM, Lee M, Ong SY, Yong E, Farmer P, Peck CJ, et al. Immunoreactivity for high-affinity choline transporter colocalises with VAChT in human enteric nervous system. Cell Tissue Res. 2010;341(1):33-48) . *Unfortunately our antibody stock was finished and we were not able to get the antibody again. We did label with ChAT that labels the cholinergic cell bodies.*

For example, the choice of NOS (nitric oxide synthase) antibodies is intuitive giving the known dilative properties of nitric oxide on smooth muscle. Yet the authors do not discuss how more and larger, intrinsic NOS-synthetizing nerve fibers in the myenteric and submucosal ganglia may give rise to constipation, the leading symptom of MEN 2B-associated intestinal ganglioneuromatosis, and muscular hypertrophy/thickening (reactive?). *NOS is present in inhibitory motor neurons in the ganglia, forming connections within the ganglia and sending process into the muscle layers. We have added to the discussion (page 12) that more NOS synthesizing fibres in the myenteric layer would increase the inhibitory pathways within the ganglia and more in the muscle layer might inhibit contraction or lead to relaxation. NOS is normally sparse in the submucosal ganglia and these fibres innervate the inner muscle layer. NOS is NOT present in the muscosa.*

5. The clinical references are dated and do not include relevant clinical literature on MEN 2B, specifically Brauckhoff et al., Ann Surg 2014; Brauckhoff et al., Surgery 2008. *We have added updated references (4, 22).*

a. In MEN 2B patients, intestinal ganglioneuromatosis develops in an age-dependent fashion (not mentioned by the authors), not all of whom develop severe constipation necessitating gastrointestinal surgery. Hence, the wisdom to use tissue from a 12-year-old child as a control for tissue from a 4-year-old MEN 2B carrier is debatable and requires a comment. *We used tissue from a 12 year old FAP and from a 4 year old HD (normal margin). We have added- MEN2B develops progressively. This MEN2B child was first identified due to enlarged ganglia in rectal biopsies performed to exclude HD when 4 months old. Pages 5, 7 and 13*

As detailed by Brauckhoff (Ann Surg 2014), the most important clinical consequence of a positive RET gene test (also not mentioned by the authors) is pre-emptive thyroidectomy, with or without central node dissection depending on the child's serum calcitonin level. Pre-emptive neck surgery in children 4 years and younger in expert hands clears most, if not all, malignant C-cell disease before it spreads beyond the thyroid gland, resulting in biochemical cure (postoperative normalization of serum calcitonin.

We debated among ourselves adding the detail on thyroidectomy to the original manuscript and decided it was a well-established standard procedure and therefore we did not include much detail of it in the original manuscript. We have added that the patient underwent thyroidectomy at 9 months (page 5 and 13) and is alive and well at 14 yrs (page 14) and last seen by author JMH in May 2017.

The RET gene test was performed after finding the enlarged ganglia in the rectal biopsies at 4 months old and was positive resulting in a pre-emptive thryroidectomy at 9 months (page 5). The samples studied were collected at 4 years during a partial colectomy. The child has 6 monthly screening for thyroid with annual ultrasound, annual review of corneal nerves and bowel function. He last had removal of tongue ganglia in 2015 and was seen 3 months ago with no new nodules needing treatment. He is now 14 and doing well. But this study indicates that the neuronal growth continues due to the RET activation, independent of thyroid presence. So although removing the thyroid does prevent the MTC as detailed nicely by Brauckhoff (Ann Surg 2014), the children require ongoing monitoring and maintenance and are not problem free. Added page 13-14.

Reviewer's code: 03084420
acceptable