Response to Brosens *et al*

To the Editor: We appreciate this opportunity to respond to the letter "Do *RET* Somatic Mutations Play a Role in Hirschsprung Disease?," by Brosens et al.,[1] regarding our article[2].

First, we thank Brosens and colleagues for pointing out that "routine genetic testing on DNA derived from blood or saliva would not find these ENCC-specific mutations, nor would it easily detect low mosaic variants" because this is exactly why we say *RET* somatic mutations are underrecognized in Hirschsprung disease. This is also why we suggest that "deep sequencing (with enough sensitivity) of parental blood for pathogenic DNMs [de novo mutations] seen in children would be highly recommended, and should enable meaningful stratification of families into a substantial majority with a $<1\%$ recurrence risk and a small minority with a recurrence risk that could be at least an order of magnitude higher." Second, we agree with the authors that "HSCR is a complex inherited disorder" and the "missing heritability seen in HSCR is a common feature of many complex disorders, and explaining it remains challenging." We would like to emphasize that we did not discuss missing heritability in our paper, even though amplicon-based deep sequencing (ADS) indeed revealed high-frequency (75%) *RET* mosaicism among our cases with deleterious variants. While explaining the property of these variants, we seriously considered the usage of our terms and added "All of the six mosaic mutations we identified showed strong evidence of pathogenicity. Four are predicted to be null alleles, one has been reported previously, and one inserts an amino acid at a highly conserved site, is absent from the unaffected sibling, and has never been reported previously by the public National Heart, Lung, and Blood Institute or Exome Aggregation Consortium exome sequencing projects." We believe that this description is objective and completely in line with American College of Medical Genetics and Genomics guidelines.

It should be noted that among the eight cases with deleterious *RET* variants, only two (families 7 and 8) were demonstrated to be true germ-line DNM carriers. Of the remaining six, four (families 3–6) of the parents have some mosaicism, including in the germ line because the mutant allele was transmitted to the child. This simply says that there is *RET* mosaicism and the deleterious allele may not always be recognized when present. Besides, it's not uncommon to use the term "somatic mosaicism" when apparently healthy parents can potentially have multiple affected children.[3] With respect to the patients in families 1 and 2, we think the variants represent a postzygotic instead of germ-line origin for the following reasons:

1. The mutation showed a significant deviation from the expected ratio for true germ-line heterozygosity ($P = 0.04$, Mann–Whitney U test). By contrast, the average mutant allelic ratio of all germ-line heterozygous probands in families 3–9 was 49.5% (ranging from 45 to 52%, **Supplementary Table S3** in the original article), except for HSCR0146 in family 4.
2. We collected multiple tissue samples (blood, saliva, and colon) for both patients to eliminate the possible influence of DNA quality on the quality of the reads/coverage/biallelic ratio.
3. The same primer pair was used for amplifying the polymerase chain reaction product covering the mutant site in families 1–3 (**Supplementary Table S2** in the article), and it is only HSCR0129 in family 3 that showed a mutant frequency within the normal range of detection (heterozygous variant at 52%).
4. We used ADS to test the fidelity of the results. As stated in the manuscript, by comparing four different sequencing techniques (whole-genome sequencing, ADS, Sanger sequencing, and single-molecule molecular inversion probes), Acuna-Hidalgo et al.[4] showed that ADS is the most precise and sensitive technique for identifying true heterozygosity, with an allelic ratio of $48.2 \pm 4.4\%$ (average $\pm$ SD). In contrast, Sanger sequencing had a broader allelic ratio of $51.4 \pm 8.7\%$. On the basis of the obtained distributions for the allelic ratio, they determined that de novo mutations with an allelic ratio below 39.3% for ADS had a statistically significant deviation from the expected ratio for true heterozygous mutations and might, as such, reflect mosaic mutations. In the current study, ADS on 2/3 tissue samples for HSCR0116 (in family 1) and 3/3 tissue samples for HSCR0127 (in family 2) fall below the lower boundary.
5. Both the in-built software in Ion Torrent (Torrent Variant Caller 4.6), Integrative Genomics Viewer, and the Bayesian-based mosaic genotyper analysis suggested the variants identified in families 1 and 2 were mosaic.

As Biesecker[5] has indicated, for CLAPO syndrome, it is currently unjustifiable to judge whether (and how) *RET* somatic mutations play a role in HSCR. One challenge is the countless known and undiscovered modifiers—these do not fit well into frameworks that are designed for single-gene disorders and can somehow modify or even determine the presence and nature of the phenotype. Another challenge comes from the mosaicism itself, which may directly "blur" the carrier's clinical manifestation. There is no rational lower boundary that can be established for the mosaicism level. Patients with recognizable HSCR phenotypes could have variant allele frequencies at any percentage lower than 50% in the affected tissue of the body, depending on the nature of the variant and the mutation load it exerts on the tissue.

In summary, we conducted an in-depth study in which we applied both ADS and TA cloning and sequencing on multiple

tissue samples to determine the frequency of *RET* mosaicism in HSCR. After surveying eight de novo families with deleterious variants, somatic mosaicism was detected in 75% of cases, in either the patient or an asymptomatic parent. This suggested that many more of the de novo families may carry genetic mosaicism for the disease allele that largely goes undetected using the standard diagnostic techniques. Close attention should be given to this issue for better genetic counseling.

DISCLOSURE

The authors declare no conflict of interest.

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