

Clinical Genetics Group

MRC-WIMM

Dr Robert Steiner,

Editor in Chief, *Genetics in Medicine*

15 June 2021

Dear Dr Steiner,

**Re: Evaluating the performance of a clinical genome sequencing programme for diagnosis of rare genetic disease, seen through the lens of craniosynostosis,** by Zerin Hyder *et al*.

Thank you for returning this manuscript. We were very pleased to receive your summary comment that “*your manuscript was favorably reviewed and if you respond satisfactorily to the reviewers the outcome should be positive*.”

We have revised the manuscript taking the reviewers’ comments into account, in the summary below the reviewers’ comments are reproduced verbatim (upright black text); our responses are in *italics* (with existing quotes from the manuscript underlined), and new text is in blue.

**Reviewer Comments:**

**Reviewer #1:** Overall, this is an excellent manuscript describing the diagnostic utility of clinical genome sequencing in the specific case of craniosynostosis. It appears that the main takehome of this manuscript is that Tier 3 variants need to be analyzed by someone expert in the field; Tier 1 and Tier 2 genes are likely included in targeted sequencing approaches that largely would have been performed before enrolling a patient for clinical genome sequencing, thus those patients that proceed to CGS / research sequencing efforts are more likely to harbor Tier 3 variants, the interpretation of which is more nuanced. The identification of novel mutations in genes associated with developmental disorders is somewhat expected, as these genes are usually pleiotropic and highly intolerant to loss of function mutation. CS can often be missed in cases with syndromic intellectual disability.

*Response: thank you for the positive comments and summary of some of the conclusions.*

It would be good if the authors could include some mention of whether matchmaker platforms (Decipher, genematcher, matchmaker exchange, etc) have been used to connect with others with mutations in these genes, and whether these efforts have identified other probands with CS to bolster evidence that CS should be considered a phenotypic expansion of the known gene.

*Response: The key question that is that this work addresses is whether the NHS diagnostic process has missed* ***routine*** *diagnoses. Such diagnoses should not depend on taking additional steps such as this reviewer recommends, because these represent the interrogation of what are essentially research resources. Therefore, we have deliberately not routinely used such resources for the assessment of pathogenicity.*

*In terms of whether any of our findings may represent a “phenotypic expansion of the known gene”, we had addressed this in the Discussion (p.18) in the paragraph starting: “The large number of researcher-based diagnoses that involve variants in genes not Green-listed on the CRS panel (n=11) is not surprising.” Lower down this paragraph, we note that for 7 genes, evidence already exists that craniosynostosis represents a rare associated complication “Four of the genes identified (GPC3, PTCH1, SOX6, TRAF7) are now Amber or Red-listed in PanelApp, and mutations in ARID1B, CDK13, FBXO11 and HNRNPK have also been associated with craniosynostosis in a small number of cases (Table S5).” To be more explicit about the remaining genes, we have added two additional sentences at the end of this paragraph. We hope the reviewer will agree that this amendment acknowledges the issue that they have raised, without muddying the waters by bringing in research-based criteria into the diagnostic process.*

We are not aware of previous descriptions of CRS associated with variants in the genes *BRWD3* or *MMP21*, but the other clinical features in these cases, and associated variants identified, were considered sufficient to assign pathogenic or likely pathogenic status. Craniosynostosis may represent an extension of previously described phenotypes, the frequency of which will become evident as each pathological entity is better delineated.

Another important aspect of this manuscript is the power of performing trio-based sequencing to identify de novo mutations, which is highlighted in table S1. As noted in the manuscript, fewer than two coding DNMs are expected per proband, thus de novo mutations in an intolerant gene with a plausible role in CS make these highly relevant candidates. De novo mutations in tier 3 genes appear to have been almost uniformly classified as the likely disease allele in cases studied. Similar approaches in syndromic craniosynostosis have identified new bona fide disease genes and genes of interest requiring further investigation (ie Tonne et al 2020, Timberlake et al 2019).

*Response: Thank you for this comment. Indeed we already made reference to the paper by Tonne et al (ref. 21). The 2019 paper by Timberlake et al studied a different population (non-syndromic midline craniosynostosis) with a much lower expected load of monogenic pathological variants, so we think it is less relevant in this context (please note, Genetics In Medicine limits references to 40 and we have already reached this limit).*

Highlighting the complexity of consent for research analysis in clinical genome sequencing efforts is important, particularly as many institutes have mandated data sharing of sequencing data as a condition for funding sequencing projects.

Finally, could the authors comment on their proposed "order of operations" in syndromic craniosynostosis, given the knowledge that they have from analyzing this dataset? With rapidly declining costs of genome sequencing, are we approaching a point where its use might come sooner in what many call the diagnostic odyssey? For example, should targeted sequencing be applied, and then straight to genome sequencing? Should probands be sequenced first to identify CNVs, SVs, etc, before sequencing the trio? These are topics that are of course subject to debate, but given the diagnostic yields from each scenario, the opinions of these authors merit inclusion.

*Response: Thank you for this comment. These are indeed important questions, but this study wasn’t designed to answer them, and in addition, there is a danger that a discussion of these issues could divert from the more general messages that we are trying to convey in this paper. For those interested, Tables S1 and S2 do already provide detail on the case mix and comparative success rates. For example from Table S1, in answer to the reviewer’s question “*Should probands be sequenced first to identify CNVs, SVs, etc, before sequencing the trio*?”, it is evident that the success rate for sequencing sporadic cases as singletons (5/11, 45.5%) was - counterintuitively - higher than for sequencing as trios (24/72, 33.3%). However the number of singletons analysed was relatively small, so the lower 95% confidence limit for the 45% figure is only 17%, and the hidden extra bioinformatics workload of analysing a singleton has not been quantified as part of this work, so these limited data do not really provide a clear answer either way. Given these inconclusive findings, we don’t think it would be helpful to devote space to discussing this particular question. However, we do think it worth drawing the readers’ attention to the significantly higher success rate in achieving a diagnosis in syndromic compared to non-syndromic cases, so we have added to the end of the sentence on p.15 “*The final rate of diagnoses for CRS from the 100kGP was 28.9% (33/114)…”

…, with a much higher success rate for syndromic (39.0%) than non-syndromic (6.25%) presentations (Table S2; Fisher’s Exact test 1-tailed *P*=0.0003).

By referring back to Table S2, our aim is to remind the interested reader of the details of our case mix for genome sequencing in craniosynostosis, without losing the attention of what we intend to be a wider readership who may not necessarily be interested in this issue.

**Reviewer #2:** In this publication, Hyder et al describe the differences in diagnostic yield between two approaches of genome sequencing analysis and pipelines, using craniosynostosis as an example phenotype. Overall this paper is very interesting because finding the correct boundary between being overly thorough and interpretation burden is something all laboratories think about and wrestle with. While I think the authors have great points and data to show, the manuscript feels bogged down by discussion of the processes (RIPDs, GECIPS, CGG, GMC, etc) as opposed to the reasons for the different diagnostic yields (which really is the part people are going to be interested in!).

It was surprising that so many of the missed diagnosis were due to missed de novo variants. I would think that analysis of the de novo variants by the clinical laboratories would be standard?

*Response: We entirely agree, but unfortunately this was not standard practice at the time that the 100kGP was underway, as we already highlighted on p.9: “Importantly, GMCs were only mandated to examine all Tier 1 and 2 variants, whereas examination of the longer list of Tier 3 variants and Exomiser hits was discretionary, with effort varying between GMCs (****Box 1****).” As noted in the Discussion (p.16), this deficiency has now been corrected: “This approach (combining panels with DNMs) harmonises with draft NHSE reporting guidance for enhanced analysis of GS data;34”. If the failure to identidy DNMs was the only reason that the GEL/GMC pipelijne fell short it might be considered that the conclusions of our study were trivial, but as we state in the Discussion (p.16), we identified two other key factors eroding overall diagnostic efficiency, which remain important challenges to address.*

I think a bit more about PanelApp and Exomiser in the introduction would be helpful. Since both tools seemed to have a big impact, further explanation about the tools would be helpful.

Response: We are mindful of strict word limitations here. We believe that the description of PanelApp on p.6, with a key supporting reference, provides adequate introduction: “*An automated pipeline, centred on the use of updateable, crowd-sourced and disease-focused panels (PanelApp)9 was created by GE for processing, calling and prioritising genome sequence variants*,”. However, as further explanation, since Box 1 explains the prioritisation functions of PanelApp in detail, we have added “by PanelApp” to the end of the title of this Box. For Exomiser, we have added the following descriptive clause on p.9:

(comprising a suite of algorithms using random-walk analysis of protein interaction networks, clinical relevance and cross-species phenotype comparisons)

This may just be personal preference, but the amount of acronyms is excessive. Sentences like "Twenty-two RIPDs were submitted for the CRS cohort by the CGG…" and "...reported as pathogenic by the GMC before the RIPD was returned by GE to the GMC…" and "...variant by the CGG, a RIPD form was submitted to GE; in some instances, the case was still undergoing review by the GMC…" are very difficult to follow as a reader because I am constantly having to jump back to see what that acronym stands for. I've read the paper multiple times now and I still can't remember what half of the acronyms stand for. I would reconsider the use of so many acronyms. For example, is using "DNM" for "de novo mutation" really necessary? (I think if you need to save space in a table or figure using that acronym is fine, but in the manuscript I would just use "de novo"). Or "CVA" is only used twice in the manuscript - is using the acronym beneficial?

*Response: We entirely sympathise with this reviewer! Unfortunately the strict word limit of 4000 words in the text set by Genetics in Medicine, combined with (i) the wish to make the presentation and analysis as rich as possible and (ii) further additions to the text in response to the requests above, do necessitate the use of several acronyms/abbreviations.*

*To make the analysis objective, we have quantified the use of acronyms/abbreviations (not including commonly used genetic ones such as SNV, CNV and VUS) in the main text:*

*GS (genome sequencing): 18 (saves 17 words)*

*100kGP (100,000 Genomes Project), 24 (saves 47 words)*

*NHSE (National Health Service England), 2 (saves 2 words)*

*GMC (Genomic Medicine Centre), 37 (saves 73 words)*

*GE (Genomics England), 23 (saves 22 words)*

*GECIP (Genomics England Clinical Interpretation Partnership): 2 (saves 3 words)*

*RIPD (Researcher-identified Potential Diagnosis): 20 (saves 59 words)*

*REC (Research Ethics Committee):3 (saves 3 words)*

*CGG (Clinical Genetics Group): 9 (saves 17 words)*

*CVA (Clinical Variant Ark): 2 (saves 3 words)*

*HPO (Human Phenotype Ontology): 4 (saves 7 words)*

*DNM (De novo mutation): 8 (saves 15 words)*

*GMS (Genomic Medicine Service): 1 (uses one extra word)*

*To (only partially no doubt!) assuage the reviewer’s irritation we have got rid of the NHSE, GECIP, REC, CVA and GMS acronyms (although we have had to define “NHS” instead), for a net loss of 4 acronyms. Unfortunately the other acronyms save too many words to be dispensable in the context of the 4000 word limit. We hope this will be viewed as an acceptable compromise.*

*In this context it may be worth highlighting that the term “CRS” as an abbreviation for craniosynostosis appears 29 times in the text. This saves no words at all, but “craniosynostosis” is a bit of a mouthful and we judged that readers might tire of reading the fully spelt-out term so many times. But this is a stylistic matter and we would be happy to spell it out if preferred.*

*The initial net effect of incorporating the above changes in response to reviewers’ requests was to increase the number of words in the main text to 4103, 103 above the word limit. Aside from edits of “throat-clearing” phrases, we have addressed the need to make cuts elsewhere in the main text primarily by deleting mentions of cases dually identified by both the GE/GMC pipeline and researchers (on p.12 we had written that “these cases are not discussed further”, but had in fact gone on to discuss them, so these omissions should not materially detract from the new manuscript).*

**Editorial Office Requirements:**

GIM has preferred usage for certain terms (detailed here <https://www.nature.com/gim/authors-and-referees/preparation-of-submissions#terminology>). We ask you to check your article to ensure those terms are used correctly.

• Variant instead of mutation

• pathogenic variant to denote a disease-causing variant

*Response: This has been checked and “mutation” substituted with “pathogenic variant” except in the context where the variant is known to have arisen de novo (making it a mutation by definition)*

When referring to Richards et al <https://www.nature.com/articles/gim201530> please be sure to refer to them as ACMG/AMP guidelines.

*Response: Changed to “ACMG/AMG” (p.10)*

**Additional changes made:**

1. Whilst the manuscript was under review, an additional variant classified as pathogenic was reported by one of the Genomic Medicine Centres (designated case 33). All numbers have been adjusted to incorporate this additional diagnosis, which does not materially affect any of the conclusions of the manuscript.

2. Although all data are presented anonymously, Genomics England has now stipulated the requirement that every referring clinician consents to the inclusion of the individual–level data, and furthermore confirms that the family has also provided consent. From the 36 cases presented, we have obtained such consent for 28. For the remaining 8 cases for which such consent has not been obtained, Genomics England has required us to redact the following information: (WE ARE STILL AWAITING CONFIRMATION OF WHAT INFORMATION WILL HAVE TO BE REDACTED). Given the small number of cases for which redaction has been necessary, we do not believe that this materially influences the quality of the data presented, or the ability to justify the conclusions made from this work.

3. If not already authors, all clinicians involved in taking such consent are now mentioned in the acknowledgements. For the two clinicians who submitted cases classified as Variants of Unknown Significance we have offered full authorship, as these cases remain of potential research interest. The addition of these two authors (Andrew G. L. Douglas and Ruth McGowan) has been approved by all existing authors, as confirmed in a separate cover document attached to this submission.

We trust that these changes will be acceptable to the reviewers and the journal, and hope that what we believe is an important piece of work can now proceed to publication.

Yours sincerely

Richard H Scott Andrew O M Wilkie