**Evaluating the performance of a clinical genome sequencing programme for diagnosis of rare genetic disease, seen through the lens of craniosynostosis**

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**Conflict of interest**

The authors declare no conflicts of interest.

**Abstract**

**Purpose:** Genome sequencing (GS) for diagnosis of rare genetic disease is being introduced into the clinic, but the complexity of the data poses challenges for developing pipelines with high diagnostic sensitivity. We evaluated the performance of the Genomics England 100,000 Genomes Project (100kGP) panel-based pipelines, using craniosynostosis as a test disease.

**Methods:** GS data from 114 probands with craniosynostosis and their relatives (314 samples), negative on routine genetic testing, were scrutinised by a specialized research team, and diagnoses compared with those made by 100kGP.

**Results:** Sixteen likely pathogenic/pathogenic variants were identified by 100kGP. Eighteen additional likely pathogenic/pathogenic variants were identified by the research team, indicating that for craniosynostosis, 100kGP panels had a diagnostic sensitivity of only 47%. Measures that could have augmented diagnoses were improved scrutiny of existing panel genes (+18% sensitivity), review of updated panels (+12%), comprehensive analysis of *de novo* small variants (+29%) and copy number/structural variants (+9%). Recent NHS England recommendations that partially incorporate these measures would achieve 82% overall sensitivity (+35%).

**Conclusions:** GS identified likely pathogenic/pathogenic variants in 29.8% of previously undiagnosed patients with craniosynostosis. This demonstrates the value of research analysis and the importance of continually improving algorithms to maximise the potential of clinical GS.

**INTRODUCTION**

Early evaluations of genome sequencing (GS) of rare disorders in a research setting showed that it could provide diagnostic enhancement of 21-42%, according to clinical context.1-3 This has led to initiatives to introduce GS into clinical diagnostics. In the UK, the 100,000 Genomes Project (100kGP), delivered by National Health Service (NHS) England through 16 NHS Genomic Medicine Centres (GMCs) together with Genomics England (GE), was inspired by the potential for GS to provide patient benefit in the NHS, offering prompter diagnoses and improving prediction and prevention.4-6 Genome sequencing is particularly valuable in conditions presenting with variable phenotypes or nonspecific clinical features, where the number of contributory genes may be extensive, and can identify non-coding variants and unravel new pathogeneses of disease.7,8

Recruitment of participants with rare disease and cancer into the 100kGP was carried out by GMCs across the UK between 2015-2018. 71,682 participants in 36,063 families have had GS in the rare disease programme. 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, and the results were returned to the recruiting GMC to evaluate and potentially validate.4 The rate of diagnoses achieved by the GE/GMC pipeline for rare diseases is currently 20.0%.

The 100kGP allowed access to de-identified clinical and genomic data in the Research Environment to academic researchers accredited as members of one of 49 GE Clinical Interpretation Partnerships, investigating a wide range of diseases and applications.4 “Diagnostic Discovery” describes the process by which potential diagnoses identified by academic researchers but not flagged by the automated GE/GMC pipeline can be returned to GMCs, using an online Researcher Identified Potential Diagnosis (RIPD) form. This would prompt the GMC to reanalyse the case on the updated pipeline with the researcher-identified variant, embedding researcher discovery into the diagnostic process (Fig.S1).

Given the substantial investment in sequencing and data storage required for clinical GS, assurance that the clinical pipeline can efficiently identify clinical-grade molecular diagnoses is critical. This task is challenging in the context of diverse diseases, given the extensive and complex nature of human genome variation (encompassing single nucleotide variants (SNVs), small indels, copy number variants (CNVs) and structural variants (SVs)).10,11 Here, we have used craniosynostosis (CRS), the premature fusion of one or more cranial sutures,12 as a model disorder to examine the performance of the 100kGP pipeline, by comparison with findings from intensive scrutiny of the data in the Research Environment aimed at generating a “truth dataset”.

Several characteristics make CRS a suitable phenotype for this approach. First, CRS is relatively common (~1 in 2,000 live births),13 constituting a primary rare disease recruitment category in 100kGP. Second, CRS is clinically and etiologically heterogeneous, with environmental,14 polygenic,15,16 and monogenic/chromosomal factors all contributing. In the Oxford birth cohort of 666 individuals with CRS requiring surgery,17 24% had an identifiable genetic cause, either monogenic (22%) or chromosomal (2%); 63% of patients with fusion of more than one cranial suture and/or associated syndromic features (including a positive family history) had an identified genetic cause, indicating that these clinical categories merit prioritization for genetic investigation. Third, 84% of the monogenic component could be screened out by testing just six17 (now seven)18,19 commonly implicated genes; this testing was already widely available in the NHS, so that most facile molecular diagnoses had already been made prior to GS. Fourth, a previous study of CRS with suspected genetic cause but negative on routine genetic testing found that exome or genome sequencing yielded a substantial (37.5%) uplift in genetic diagnoses.20

Importantly in this context, CRS is characterized by a long “tail” of rare genetic diagnoses. In the Oxford survey,17 pathogenic variants in 20 rarely involved genes accounted for 23/666 (3.5%) of all cases, and in the exome/genome sequencing study,20 the 15 new diagnoses were identified in 14 different genes. A recent study from Norway reported similar findings.21 As we expect the patients enrolled into 100kGP to be enriched for rare genetic causes, this heterogeneity presents a substantial challenge for pipeline-based diagnosis. We considered that CRS could represent a stringent test of how well the GE/GMC pipeline worked. This work demonstrates the substantial benefit of exploiting specialist research expertise to augment the overall diagnostic rate in 100kGP, and indicates ways in which the diagnostic pipeline could be improved.

**MATERIALS AND METHODS**

**Craniosynostosis disease cohort**

The clinical protocol for 100kGP was approved by East of England–Cambridge South Research Ethics Committee (14/EE/1112). Written informed consent to obtain samples for genetics research was obtained. Patient recruitment for CRS required (1) the presence of multiple suture fusions and/or (2) additional clinical features or positive family history, indicating a syndrome; previous genetic testing for common causes of CRS and, if syndromic, normal chromosomal microarray, were also required (see Box S1 for details). Peripheral blood samples were obtained by venepuncture and DNA extracted for sequencing on Illumina instruments. Whenever possible, sporadically affected cases were sequenced as trios with their unaffected parents.

In 51 of the 114 families recruited, written informed consent had previously been obtained by researchers in the Clinical Genetics Group, Oxford (CGG) to investigate genetic causes of CRS (Oxfordshire Research Ethics Committee B (C02.143) and London–Riverside Research Ethics Committee (09/H0706/20)). This enabled independent molecular confirmation of some diagnoses by the CGG.

**Tiering pipeline**

The pipeline used by GE/GMC to prioritize small variants (SNVs and indels <50 base pairs) into Tiers is summarized in **Box 1**.22 Genomes were interrogated as family units; algorithms including frequency in control populations, mode of inheritance, appropriate segregation, effect on protein coding and genotype-phenotype association were used to assign variants into four categories (Tiers 1-3, with Tier 1 the highest ranked, and “Tier null” for the remainder), using complete or incomplete penetrance modes according to clinical indication.22 This information was intersected with curated gene panels in PanelApp (applied depending on the clinical indication and phenotype data for each participant), to prioritise variants occurring in diagnostic-grade (“Green”) genes (**Box 1**).9 Part-way through the program (Data Release V7, 25/07/19), Exomiser (comprising a suite of algorithms using random-walk analysis of protein interaction networks, clinical relevance and cross-species phenotype comparisons)23 was incorporated as an additional tool to rank potentially pathogenic variants based on frequency, predicted pathogenic impact, inheritance and phenotype match. Genomic Medicine Centres validated the prioritized results experimentally (usually by dideoxy-sequencing), and closed the case once assessment was complete. 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 variable effort (**Box 1**).5 Addition of new genes to the Green category in PanelApp did not automatically trigger reassessment of closed cases.

Copy number variant (CNV) calls produced by Canvas software24 were introduced into the pipeline in January 2019, but were not implemented on closed cases. The pipeline reported CNV calls >10 kb with a call quality score >10, and annotated and displayed CNV calls from the proband without considering mode of inheritance. Calls were assigned Tier A if the CNV overlapped with a pathogenic region in a Green gene in a panel applied to the patient (**Box 1**). In contrast to small variant tiering, a heterozygous CNV encompassing a biallelic gene would be tiered. Tier null CNVs were those that did not meet the criteria for Tier A reporting.

**Audit of GE/GMC-reported variants**

Probands were identified by searching the Clinical Variant Ark (a restricted-access database detailing all cases, variants, and phenotypes reported from 100kGP) for participants recruited with the clinical indications “CRS syndromes” or “CRS syndromes phenotypes”. Phenotype data, applied gene panels, their iterations, and case status information were collected for each participant. Cases lacking CRS-related terms in the associated Human Phenotype Ontology (HPO) data25 were excluded. For each case we determined whether the GMC had established a pathogenic or likely pathogenic variant, according to ACMG/AMP criteria,26 which we considered established a *molecular diagnosis*.

**Researcher-identified potential diagnoses (RIPDs)**

The research-based analysis was performed by the CGG, through membership of the musculoskeletal GE Clinical Interpretation Partnership (Research Registry projects 65 and 365). Data were accessed within the GE Research Environment. The CGG considered reasons why variant(s) may not have been prioritized by the GE/GMC pipeline, and interrogated the data accordingly. The reasons identified were classified into four categories (1-4), as summarized in **Box 2**. To reduce the search space, variants were usually required to exhibit segregation concordant with the phenotype in the family (complete penetrance). The inheritance of each variant was separately annotated into one of five categories (A-E; **Box 2**), so that each RIPD could be classified with a number-letter combination. Detailed methods used to interrogate the data are provided in the Supplementary Information.

Following the detection of a putatively pathogenic variant by the CGG, a RIPD form was submitted to GE; in some instances, the case was still undergoing review by the GMC, whereas in others, it had already been closed with no primary findings. Genomics England then re-identified the patient and returned the variant to the recruiting GMC for review and reanalysis on the current, updated pipeline. The outcome of each GMC review of the RIPD was recorded in Clinical Variant Ark (Fig.S1). In four additional instances judged by the CGG to be of research interest but likely falling short of the threshold for clinical diagnosis, a “contact clinician” request was submitted instead of the RIPD; these cases are not discussed, as our focus here is on the diagnostic pipeline rather than novel findings.

**RESULTS**

**Patient composition and diagnostic summary**

In total, 127 families primarily classified with CRS were recruited to 100kGP (**Fig.1**). We excluded seven families from the Pilot phase,27 as their data were not available in the Research Environment; in an additional six families, no CRS phenotypes were annotated in the associated HPO terms. Hence, we focused on 114 *bona fide* CRS families in the main programme, including 15 families with more than one affected individual, and 72 sporadically affected probands analysed as parent-child trios (Table S1). Eighty-two of the probands (72%) were classified as having a syndromic clinical presentation and 53 (46%) had fusion of multiple cranial sutures (Table S2). To date, GMCs have autonomously confirmed molecular diagnoses in 16 cases (14.0%), RIPDs have independently provided diagnoses in 16 cases, and two diagnoses came from other sources (one pathogenic variant identified before 100kGP recruitment, and one unpublished research finding (**Fig.1**, **Table 1**, Table S3, Table S4)), yielding an overall diagnosis rate of 34/114 (29.8%).

**GMC-identified variants**

Sixteen variants (in cases 1-3 and 19 in **Table 1** and 23-34 in Table S3) were classified by GMCs as likely pathogenic or pathogenic. In 13/16 cases, the causative variants were identified from Tier 1/2 or Tier A data (**Box 1**). Of the remaining three variants, the *KMT5B* *de novo* mutation (DNM) (case 3) was found in Tier 3 data, whilst the X-linked *OGT* variant in case 19 and the *de novo ZBTB20* variant in case 34 were untiered but were identified because the respective GMC had searched the Exomiser23 data.

**Researcher-identified potential diagnoses (RIPDs)**

Twenty-two RIPDs were submitted by the CGG (**Fig.1**), of which 20 (comprising 22 variants; 18 monoallelic and 2 biallelic) were either Tier 3 or untiered. The outcome of assessment and validation by the GMC is summarized in **Table 1**. In four cases (1-3 and 19), the variant was independently reported as pathogenic by the GMC; these cases are not discussed further. From the remaining 18 “researcher-only” RIPDs, 16 cases (comprising 18 variants) were classified as pathogenic/likely pathogenic and two were reported as VUS.

**Monoallelic Tier 3 variants**

Ten of 18 researcher-only RIPDs (cases 4-13) were monoallelic Tier 3 variants that were not Tier 1/2 because the gene was not diagnostic-grade (Green) on the panel(s) applied at the time of analysis. Whilst for three cases (5, 7, 8) the genes are now diagnostic-grade on at least one relevant panel, no process currently exists for GMCs routinely to reanalyse cases on updated panels. The remaining seven Tier 3 RIPDs are variants in genes that are still not rated diagnostic on the panels applied to the patient. However, most are still likely to be contributing fully or partially to the patient’s phenotype. All genes except *SOX6* (which we distinguish as a “research gene” because the two cases [9, 10] contributed to the original discovery cohort)28 were already known to harbor pathogenic variants contributing to developmental disorders.19,29 Notably 9/10 monoallelic Tier 3 variants (excepting case 10, for whom parental GS was not available) were DNMs in sporadically affected cases analysed as parent-child trios; these nine were all ranked within the top five candidates by Exomiser. Combining all available evidence, two variants were classified as VUS, four as likely pathogenic and four as pathogenic (**Table 1**, Table S4).

**Untiered small variants**

Five researcher-only RIPDs (cases 14-18) were submitted for cases including an untiered SNV or indel (**Table 1**, Table S4). Cases 14 and 15 both harbored biallelic variants in diagnostic grade genes (*MEGF8*, *MMP21*) on one of the panels applied, but, one of the variants was a heterozygous deletion (of 3 or 14 nucleotides, respectively) that had been filtered out based on quality settings. In each case the second variant, a heterozygous missense, was not specifically flagged, even though the patient had a very characteristic phenotype (*MEGF8* - Carpenter syndrome; *MMP21* - heterotaxy) associated with a limited number of known disease-causing genes. Case 16 harbors a *de novo* indel in *ARID1B* (deletion of 1 nucleotide and insertion of 6 nucleotides) that was also filtered out during variant quality control. In case 17, a DNM in *TRAF7* (ranked 3 by Exomiser) was filtered out from tiering because 1 of 32 reads in the mother appeared to match the child’s mutation; inspection in the Integrative Genomics Viewer (IGV)30 suggested this was caused by a low quality read, as a nucleotide two residues away was also mis-called. The family in case 18 comprises three affected male siblings with differing cranial phenotypes; in one sibling with bicoronal synostosis, a DNM in *TCF12* was reported as an RIPD. This variant had in fact been identified prior to submission to 100kGP in a panel screen, and had been classified as pathogenic; however within 100kGP, it had been missed both in tiering and by Exomiser, because the analyses assumed that the three siblings must share the same genetic pathology.

**Copy number and structural variants**

Three researcher-only RIPDs (Cases 20-22) were untiered SV/CNV, comprising a complex inversion involving *TWIST1* (case 20), deletion including *ERF* (case 21) and duplication involving the *HOXC* gene cluster (case 22), each of which was detected by the CGG using overlapping Canvas24 and Manta31 calls (**Table 1**, Table S4, and Supplementary Information). As *TWIST1* and *ERF* are diagnostic grade genes for CRS, the rearrangements were retrospectively analysed on the updated GE pipeline. Although the *ERF* deletion was called as Tier A, the *TWIST1* inversion was still missed because the breakpoints flanked the gene. The *HOXC* duplication was associated with a distinctive craniofacial phenotype resembling a published mouse mutant32 and classified as a research finding.

**Additional d**ia**gnoses**

Two diagnoses that were neither found by the GE/GMC pipeline nor submitted as RIPDs are summarized in Table S3. A child (case 35) with the clinical features of Simpson-Golabi-Behmel syndrome previously had targeted testing of *GPC3*, and a deletion of exons 7 and 8 was reported. The patient was referred to 100kGP by a clinician unaware of the molecular diagnosis; this case was analysed with CNVs on the 100kGP pipeline, however as *GPC3* was not a diagnostic-grade gene in the panels applied, the CNV was not called and a negative report issued. In case 36, an affected mother and child, members of a 4-generation family affected by CRS, had GS by 100kGP. Independent investigation by the CGG had previously revealed a segregating 11.5 kb duplication in a non-coding region of chromosome 1p31.3, which was not tiered by GE. This was shown to be causative based on mouse modelling (unpublished).

**DISCUSSION**

Using CRS patients recruited to the 100kGP as an example, we sought to measure the added value from scrutiny of GS data by a research team, compared to the clinical pipeline. From 22 submitted RIPDs, 16 additional researcher-only diagnoses were confirmed by GMCs as likely pathogenic or pathogenic, doubling the number of diagnoses from 16 to 32. An additional two diagnoses were made outside the GMC/RIPD reporting systems; hence the diagnostic sensitivity of the GE/GMC pipeline for CRS was only 47% (16/34), considerably lower than the overall 77% figure suggested by the 100kGP Pilot.27 The final rate of diagnoses for CRS from the 100kGP was 29.8% (34/114), 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). In the context of CRS, this work demonstrates the substantial uplift that expert researcher-led examination of GS data can contribute to clinical-grade molecular diagnoses.

A major goal of this study was to use the insights from researcher-identified diagnoses to highlight ways to improve the clinical pipelines. We summarize in **Fig.2** the major features of the missed diagnoses, to signpost which approaches would have detected them.

In evaluating how this information could be implemented in diagnostic GS, we recognize that the search effort in a clinical setting needs to be substantially less intensive than might be feasible in a research laboratory. This requires balancing the conflicting demands of high sensitivity (recall), which minimizes false negative calls, and high precision (positive predictive value), which minimizes false positive calls. It is evident that exclusive use of a panel-based approach (PanelApp) with the aim of maximizing precision was inadequate, because, even with optimal application (incorporating recent updates to PanelApp, adding 4 diagnoses; and optimizing variant calling, adding 6 diagnoses; see **Fig. 2**), the sensitivity achieved would still only be 76% (26/34), with 4 additional clinical diagnoses (variants in *BRWD3*, *CDK13*, *GPC3* and *PTCH1*) continuing to be missed. A comprehensive approach would be to consider as candidates *all* validated genes mutated in developmental disorders (for example, from the G2PDD [DDG2P] list);29 whilst this would overall add 14 diagnoses (sensitivity 88%), the workflow would be very laborious owing to the large number of genes to scrutinise (currently 2149 in G2PDD), which would generate many false positive calls hence reducing precision.

An approach that balances the joint requirements of high sensitivity and precision is suggested by the observation (**Fig. 2**) that 10 of the additional clinical diagnoses are SNV or indel-associated DNMs; systematic scrutiny of DNMs would have increased sensitivity by 29% to 76% (26/34) with modest additional analysis burden, because fewer than two protein-altering DNMs are expected per genome.33 This approach (combining panels with DNMs) harmonises with draft NHS England reporting guidance for enhanced analysis of GS data;34 scrutiny of the Top 3 Exomiser hits, which is also mandated by this guidance, would yield substantially overlapping information (**Fig.2**). In the 100kGP Pilot analysis, Exomiser-based prioritization was shown to yield a 19% enhancement over panels.27

We identified two further key factors eroding the overall diagnostic sensitivity for CRS in the 100kG programme: incorrect filtering out of SNVs/indels (4 cases), and difficulties with prioritizing causative SV/CNVs (4 cases). In combination, this led to a loss of 8/34 (24%) of all diagnoses (**Fig.2**). We observed three instances (cases 14, 15, 16, **Table 1**, Table S4) in which multinucleotide indel calls were mistakenly filtered out. Other dropouts were caused by poor quality parental variant reads (case 17), and forcing a specific segregation model on a multiply affected sibship (case 18). Four probands (cases 20, 22, 35 and 36) had pathogenic CNVs/SVs that would be missed, even by the updated GE/GMC pipeline that intersects Canvas-based calling with green PanelApp genes (however two of these cases can be considered to be research rather than diagnostic findings). Of note, the Manta output, which both complements and augments Canvas data, was not utilized for clinical CNV/SV calling. Given the structural complexity of the human genome and the inbuilt limitations of short-read sequencing technology (which yields CNV/SV calls of poor specificity and unpredictable sensitivity),35 optimized clinical CNV/SV calling represents a key target for methodological improvements in GS, essential for leveraging the added value from sequencing genomes compared to exomes.

Whilst the use of HPO terms for clinical classification has major benefits, reliance to the exclusion of clinical acumen has drawbacks. Case 14 had a clinical diagnosis of Carpenter syndrome, an autosomal recessive disorder with a very restricted spectrum of disease-associated genes. However, this diagnosis was not recorded in 100kGP data and neither of the two contributing variants in *MEGF8* was tiered. Flagging of previously reported pathogenic alleles in recessive disorders relevant to the phenotype36 would have triggered intensive search for a second damaging variant. Along similar lines, the *GPC3* deletion (case 35) was missed because PanelApp interrogation was based on HPO terms, rather than on the information that the clinical diagnosis was Simpson-Golabi-Behmel syndrome.

Our findings show that to optimize molecular diagnosis from GS data, the active engagement of research laboratories is essential. Unfortunately this cannot be relied upon, owing to multiple factors including (1) the patchiness of research efforts across different clinical disorders, (2) potential lack of perceived priority in research laboratories to identify and/or communicate clinical diagnoses, and (3) reluctance of research-funding bodies to invest monies into what appears to be diagnostic, rather than research activity. For GS-based diagnostics in the UK, this work has important implications for the new NHS Genomic Medicine Service,37 in which subjects can choose to opt in or out of additional research being performed on their data. The precise means by which the “research question” is presented to the patient/family, in terms of the written information and consenting process, will have material effect on the proportion of patients/families in which further diagnostic discovery would be feasible from their GS data.

The large number of researcher-only diagnoses that involve variants in genes (n=10) not Green-listed on the CRS panel is not surprising.17,21 This wide genetic spectrum likely reflects the pathogenesis of cranial suture fusion, whereby some genes that are recurrently mutated directly perturb the intrinsic functions of the sutures,38 whereas for more rarely mutated genes, the mechanism may be more non-specific, for example through predisposition to macrocephaly (which may trigger CRS in a restricted intrauterine environment), or by perturbation of the poorly understood interactions between brain enlargement and growth at the cranial sutures.38,39 Four of the genes identified (*GPC3*, *PTCH1*, *SOX6*, *TRAF7*) are now Amber or Red-listed in PanelApp, and pathogenic variants in *ARID1B*, *CDK13, FBXO11* and *HNRNPK* have also been associated with craniosynostosis in a small number of cases (Table S5). 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, in combination with the 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.

Identification of several of the variants has led to new molecular diagnostic insights, as illustrated by publications on *SMAD6*18 and *SOX6*;28 additionally, the duplication of the *HOXC* cluster (case 22) gives rise to an apparently novel combination of phenotypes. Many other discoveries from the combined clinical-research approach have been reported in other disease domains of 100kGP.27,40

Our analysis of CRS may not be representative of 100kG data as a whole. CRS likely represents a stringent test of the GS pipeline, given the extensive prior molecular and phenotypic screening undertaken before case recruitment (Box S1), and because CRS is known to be associated with a long tail of rare genetic diagnoses.17,21 The reliance of GE/GMC on a panel-based diagnostic approach was evidently not well suited to this scenario. Nevertheless this “truth” dataset provides test cases to evaluate future improvements to the NHS pipelines, as well as valuable insights into ways to optimise implementation of clinical GS pipelines more generally.

**Data availability**

Primary data from 100kGP, which are held in a secure Research Environment, are available to registered users. Please see <https://www.genomicsengland.co.uk/about-gecip/for-gecip-members/data-and-data-access/> for further information.

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**Ethics Declaration**

The clinical protocol for 100kGP was approved by East of England–Cambridge South Research Ethics Committee (REC) (14/EE/1112). Written informed consent to obtain samples for genetics research was given by each child’s parent or guardian. In a subset of the individuals recruited, written informed consent was also obtained by researchers in Oxford to investigate genetic causes of craniosynostosis (Oxfordshire REC B (C02.143) and London–Riverside REC (09/H0706/20)).

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**Figure legends**

**Fig. 1 Summary of craniosynostosis cases and outcomes.** 127 cases with CRS were identified from the Clinical Variant Ark search, shortened to 114 after exclusion of participants recruited to the 100kGP Pilot project, and participants with no definite CRS-related phenotype terms. Potentially diagnostic variants have been identified in 36 cases thus far. 78 remaining cases have either been closed with no primary findings or are awaiting GMC review.

**Fig. 2 Improved approaches to identifying diagnostic variants in craniosynostosis.** Venn diagram classifying each of 16 RIPD considered diagnostic (excluding VUS, and those independently found by GMC) and 2 additional cases, according to methods that would have identified them.

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