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Systematic evaluation of genome sequencing as a first-tier diagnostic test for prenatal and pediatric disorders

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ABSTRACT

Current prenatal and pediatric genetic evaluation requires three tests to capture balanced chromosomal abnormalities (karyotype), copy number variants (microarray), and coding variants (whole exome sequencing [WES] or targeted gene panels). Here, we explored the sensitivity, specificity, and added value of whole genome sequencing (WGS) to displace all three conventional approaches. We analyzed single nucleotide variants, small insertions and deletions, and structural variants from WGS in 1,612 autism spectrum disorder (ASD) quartet families (n=6,448 individuals) to benchmark the diagnostic performance of WGS against microarray and WES. We then applied these WGS variant discovery and interpretation pipelines to 175 trios (n=525 individuals) with a fetal structural anomaly (FSA) detected on ultrasound and pre-screened by karyotype and microarray. Analyses of WGS in ASD quartets identified a diagnostic variant in 7.5% of ASD probands compared to 1.1% of unaffected siblings (odds ratio=7.5; 95% confidence interval=4.5-13.6; $P=2.8x10^{-21}$). We found that WGS captured all diagnostic variants detected by microarray and WES as well as five additional diagnoses, reflecting a 0.3% added yield over WES and microarray when combined. The WGS diagnostic yield was also inversely correlated with ASD proband IQ. Implementation in FSA trios identified a diagnostic variant not captured by karyotype or microarray in 12.0% of fetuses. Based on these data and prior studies, we estimate that WGS could provide an overall diagnostic yield of 47.6% in unscreened FSA referrals. We observed that WGS was sensitive to the detection of all classes of pathogenic variation captured by three conventional tests. Moreover, diagnostic yields from WGS were superior to any individual genetic test, warranting further evaluation as a first-tier diagnostic approach.

INTRODUCTION

Over the last decade, improvements in the standardization and scalability of DNA sequencing, together with substantial decreases in cost, have impacted all areas of clinical genetic testing.[1] This is particularly true for neonatal and pediatric diagnostics, where individuals with multiple congenital anomalies (MCA) and developmental disorders (DDs) of unknown etiology routinely undergo chromosomal microarray analysis (CMA) as a first-tier diagnostic test,[2] and are often followed-up with targeted gene panels and/or whole exome sequencing (WES) when CMA is negative.[3-5] The combination of CMA and WES can provide a molecular diagnosis in 25-45% of such clinically referred cases.[2,6,7] However, CMA and WES are unable to detect certain classes of pathogenic variation, including balanced structural variants (SVs) that are accessible to routine karyotyping, small copy number variants (CNVs), and non-coding single nucleotide variants (SNVs) and small insertions/deletions (indels). Furthermore, sequential testing is time-consuming and expensive, particularly in the prenatal setting where rapid diagnosis is critical.

Whole genome sequencing (WGS) has the potential to ascertain almost all pathogenic variation captured by existing technologies in a single test.[8,9] This technology also holds the promise of discovering novel diagnostic variants that remain cryptic to conventional tests, such as karyotype, CMA, and WES. To date, evaluation of WGS as a first-tier diagnostic test has only been performed in highly specific clinical situations (*e.g.*, for critically ill infants with a suspected genetic disorder)[10] or in small pediatric cohorts with variable phenotypes that have not been systematically screened with conventional tests.[11-14] As a consequence, the sensitivity of WGS to capture diagnostic variants accessible to current standard-of-care tests, and the additional diagnostic yield of WGS for variants intractable to these technologies, has not been systematically demonstrated in large cohorts with matched technologies to facilitate direct comparisons.

The largest evaluations of pathogenic or likely pathogenic (P/LP) variation from WES in fetal structural anomalies

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(FSAs) to date have included 234 consecutive referrals from Columbia University that were pre-screened for CMA and karyotype abnormalities, as well as 610 trios from the Prenatal Assessment of Genomes and Exomes (PAGE) Consortium in the UK.[15,16] These two studies identified remarkably similar diagnostic yields, with a P/LP variant being detected in 10.0% of the cases from the Columbia study and in 8.5% of the cases from the PAGE study.[15,16] There have been no comparable analyses of the utility of WGS using consecutive referrals in the prenatal setting, and guidelines for the assessment of FSAs advocate for sequential testing of targeted genes when a specific genetic etiology is suspected.[3] However, the challenges of prenatal phenotyping, including the difficulty in recognizing the fetal presentation of syndromes that are well-characterized postnatally, can lead to missed molecular diagnoses due to a failure to investigate clinically relevant genes through panel testing.[17] By contrast, WGS can survey almost all genes and classes of variation in a single test,[8,9] though it increases the complexity of interpretation and requires efficient computational strategies to identify diagnostic variants. In this study, we sought to determine the utility of WGS compared to conventional diagnostic tests (karyotype, CMA, and WES) in prenatal and pediatric phenotypes commonly referred for genetic testing.

METHODS

Subject ascertainment and phenotyping

We analyzed WGS data from 2,385 ASD quartet families from the Simons Simplex Collection (SSC).[8,18,19] Each quartet included one proband diagnosed with ASD, one unaffected sibling, and two unaffected parents. To benchmark our WGS methods, we restricted analyses to a subset of 1,612 ASD families (n=6,448 total individuals) where every individual in the family had CMA, WES, and WGS data available (**Table S1**).[20] To determine the extent to which WGS diagnostic yield was impacted by IQ, we divided the ASD probands with full-scale IQ (FSIQ) available (n=1,608) into five groups: intellectual disability (FSIQ ≤70), borderline IQ (FSIQ 71-85), average IQ (FSIQ 86-115), above average IQ (FSIQ 116-130), and gifted (FSIQ >130). We further considered non-verbal learning disorder (NVLD; non-verbal IQ at least 15 points lower than verbal IQ) as a sixth group.[21]

We also analyzed WGS data from 175 prenatal parent-child trios (525 total individuals) with an FSA detected by ultrasound (**Table S2**). The FSA trios came from two sources: 1) 135 trios were prospectively recruited from Columbia University (88 trios were directly derived from previously published CMA and WES studies from this site[15,22] and 47 were unique to this study), and 2) 40 trios were prospectively recruited from the University of North Carolina (UNC) Chapel Hill Prenatal Diagnosis Program.[23,24] When possible, FSA cases were pre-screened for absence of a P/LP variant from CMA (n=171; 97.7%) and karyotype (n=155; 88.6%). Each FSA was reviewed by a board-certified perinatologist and assigned to one of eleven phenotype categories (**Table S2**) to assist in classifying cases as having a single congenital anomaly or MCAs. This study was approved by the IRBs at Mass General Brigham, Columbia University, and UNC Chapel Hill and all participants provided written informed consent.

WGS data processing and variant calling

As previously described,[8,19] whole-blood-derived DNA from the ASD families was sequenced at the New York Genome Center on the Illumina HiSeq platform following standard library protocols (150-bp paired-end sequence reads) to a mean genome coverage of 35.5 (**Table S1**). For the FSA families, parental DNA was obtained from whole-blood and fetal DNA was obtained from chorionic villi, amniocytes, umbilical cord blood, or products of conception and sent to the Broad Institute Genomics Platform for WGS. All 525 samples from FSA probands and their unaffected parents were sequenced on the Illumina HiSeq X Ten or NovaSeq machines to a mean genome coverage of 35.8 (**Table S2**). Samples within each cohort were jointly processed in batches following the Genome Analysis Toolkit (GATK) Best Practices Workflows (https://software.broadinstitute.org/gatk/best-practices/) for short variant (SNV and indel) discovery,[25] as described in the Supplementary Appendix. SV discovery and genotyping was performed using GATK-SV (https://github.com/broadinstitute/gatk-sv), an ensemble method that leverages data from multiple SV algorithms to boost sensitivity and improve specificity.[8,26] In this study, we ran seven SV detection algorithms on all individuals from the ASD and FSA families, and provided these data as inputs to GATK-SV (described in detail in the Supplementary Appendix).[26] Sample relatedness and sex were confirmed for all individuals using KING,[27] PLINK,[28] and GATK-SV (**Figures S1-3**).

WGS variant analysis pipeline

We developed a bioinformatic pipeline for filtering SNVs, indels, and SVs identified from WGS (**Figure 1**) aimed at retaining as many P/LP variants as possible while reducing the total number of variants requiring manual review (described in detail in the Methods of the Supplementary Appendix). This included annotating all variants for genic location and functional consequence against GENCODE v.26 gene annotations using ANNOVAR (for short variants) and svtk (for SVs).[8,26,29] Variants that passed our quality control filters, inheritance-specific genotype filters, and allele frequency thresholds were retained if they were predicted to alter the protein product of a known disease gene. We derived disease gene lists from multiple sources that were broadly associated with neurodevelopmental disorders (NDDs; n=907 genes) and DDs/FSAs (n=2,535 genes) to filter variants in the ASD and FSA cohorts, respectively (**Tables S3-4**). Additional SV-specific filters were applied, including overlap with known genomic disorder loci (**Table S5**) and genes intolerant to loss-of-function (LoF) mutations.[30,31]

Determining WGS diagnostic yield

All SNVs, indels, and SVs output by our analysis pipeline were reviewed for gene-phenotype association on a case-specific basis. If a reliable match was determined for the

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case in question, the variant(s) in that gene were reviewed following the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines for sequence variation and CNV interpretation.[32,33] We also incorporated published[34-37] and unpublished recommendations (https://clinicalgenome.org/working-groups/sequence-variant-interpretation/) on individual evidence codes from the Clinical Genome (ClinGen) Sequence Variant Interpretation Working Group. Gene-level and variant-level manual review for children in the ASD cohort was performed blind to affected status (*e.g.*, all variants identified in unaffected siblings were reviewed as if the child were diagnosed with ASD) to determine the specificity of these guidelines to identify P/LP variants in affected cases. Candidate P/LP variants were assessed by a variant review panel consisting of board-certified clinical geneticists, obstetricians and gynecologists, maternal-fetal specialists, cytogeneticists and molecular geneticists, population geneticists, bioinformaticians, and genetic counselors. Variant interpretation was performed blind to prior karyotype, CMA, and WES results. All variants classified as P/LP in a gene robustly associated with the case's phenotype were considered a 'molecular' diagnosis and were counted towards the diagnostic yield of WGS. Raw read-level evidence was manually visualized to confirm all P/LP variants using the Integrative Genomics Viewer (http://software.broadinstitute.org/software/igv/).[38]

Benchmarking WGS against conventional clinical genetic tests

To uniformly compare the diagnostic yield of WGS to CMA and WES in the ASD cohort, we included all unfiltered CNVs and short variants identified from CMA and WES, respectively,[8,20,39] and filtered them using our WGS analysis pipeline (**Figure 1**) with minor modifications as needed for each data type (**Figure S4**). All CMA and WES variants were manually reviewed following the same guidelines described for WGS, allowing for direct comparisons across technologies. In contrast, the vast majority of the FSA samples were pre-screened with CMA and karyotype, and given that our WGS analysis did not identify any large P/LP CNVs or balanced chromosomal abnormalities (BCAs) in the unscreened FSA cases, we estimate that the yield of WGS in this cohort represents the added diagnostic yield of WGS beyond these two tests.

RESULTS

We analyzed WGS data from 1,612 ASD quartet families that had CMA and WES data available for all four family members.[8,18-20] Overall, GATK applied to WGS data generated 3.7M high-quality short variants (3.4M SNVs and 0.3M indels),[8] and GATK-SV identified 8,814 SVs per genome (**Figure 1 and Figure S5**). Our variant analysis pipeline produced a total of 1,743 variant observations (1,102 unique variants) in 907 NDD genes for manual

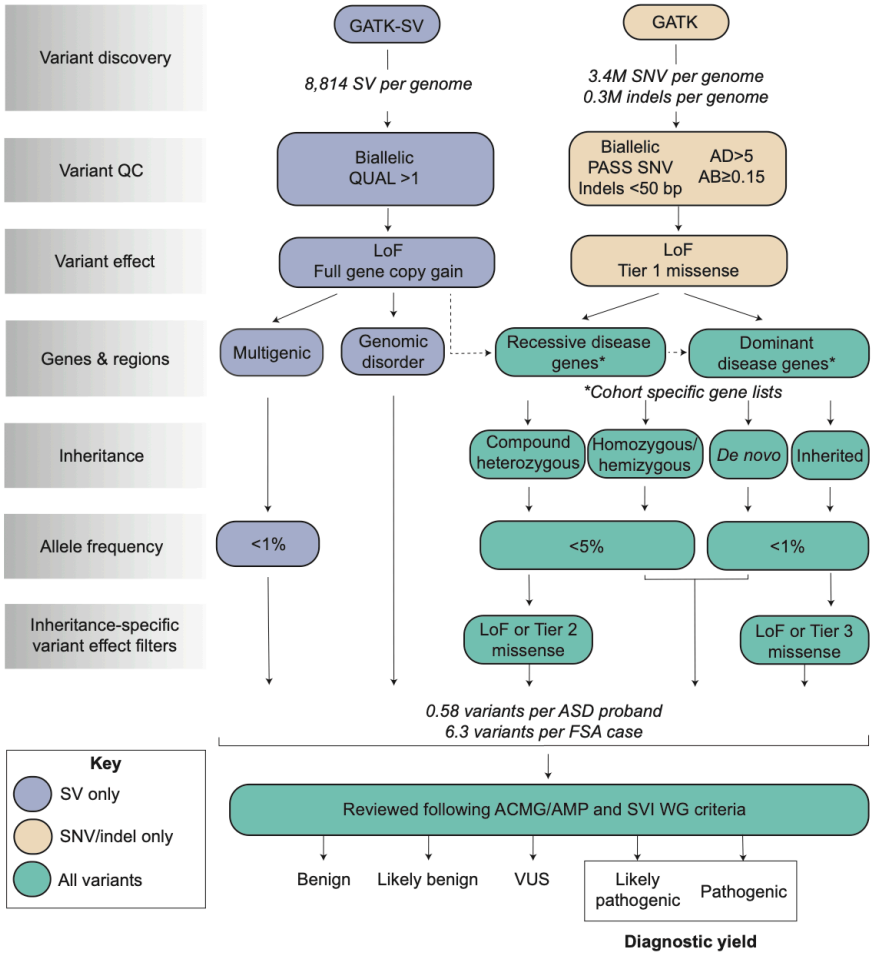

Figure 1. WGS variant filtering pipeline

All variants (SNVs, indels, SV) discovered by GATK and GATK-SV from WGS data were filtered using the analysis pipeline depicted to the left (see the Supplementary Appendix for complete details). Unless otherwise noted, the same analysis pipeline was applied to both the ASD and FSA cohorts. All variants that remained after filtering were manually reviewed following clinical guidelines from the ACMG/AMP and the ClinGen SVI Working Group (described in the Methods).[32-37] The diganostic yield of WGS was comprised of P/LP variants identified in a gene that has been previously associated with the case phenotype.

GATK: Genome Analysis Toolkit; SV: structural variant; QC: quality control; QUAL: SV quality metric; SNV: single nucleotide variant; indel: small insertion or deletion; AD: allelic depth; AB: allele balance; LoF: loss-of-function; ACMG: American College of Medical Genetics and Genomics; AMP: Association for Molecular Pathologists; SVI: Clinical Genome Resource Sequence Variant Interpretation Working Group; VUS: variant of uncertain significance.

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review across both proband and unaffected siblings, corresponding to an average of 0.49 variants per child (range=0-9). While interpretation was performed blind to affected status, retrospective analyses found that the ASD probands had significantly more variants requiring manual review than their unaffected siblings (0.58 mean variants per ASD proband compared to 0.39 in siblings; $P=4.12x10^{-14}$; two-sided Wilcoxon test). Manual review from 1,612 ASD quartets identified 98 unique P/LP variants in 121 (7.5%) ASD probands and 17 (1.1%) unaffected siblings (odds ratio [OR]=7.5; 95% confidence interval [CI]=4.5-13.6; $P=2.8x10^{-21}$; Fisher's exact test) (**Figure 2 and Tables S6-7**). After removing P/LP variants, there remained a significant excess of probands with a variant output by our filtering pipeline (OR=1.46; $P=5.2x10^{-7}$; Fisher's exact test), suggesting that there is additional diagnostic yield to be gained from WGS beyond what was captured using existing interpretation guidelines (**Figure S6**). As expected, we observed an inverse relationship between IQ and WGS diagnostic yield (**Figure 2**), with the highest yield occurring in probands with ASD and a NVLD (n=21/182; 11.5%) or comorbid ID (n=53/465; 11.4%).

We benchmarked the WGS diagnostic yield against conventional tests by applying the WGS analysis and interpretation pipelines to CMA and WES data, with minor modifications as needed for each data type (**Figure S4**). Overall, WGS identified almost two-fold more probands with a diagnostic variant than CMA (n=69; 4.3%; OR=1.8; 95% CI 1.3-2.5; $P=1.2x10^{-4}$) and an almost three-fold higher yield than WES (n=47; 2.9%; OR=2.7; 95% CI 1.9-3.9; $P=4.2x10^{-9}$). Our WGS analysis recapitulated 100% of the P/LP variants identified from CMA and WES that could be evaluated in probands (**Figure 2**). There was one pathogenic coding variant from WES that was captured by WGS but excluded from the filtering pipeline as the inheritance status could not be inferred due to a missing genotype in the father (**Figure S7**).

In addition to capturing all P/LP variants from conventional technologies, WGS identified a diagnostic variant in five additional ASD probands, reflecting a modest increase in diagnostic yield (0.3%) above CMA and WES when combined (**Figure 2**). These WGS-unique variants included

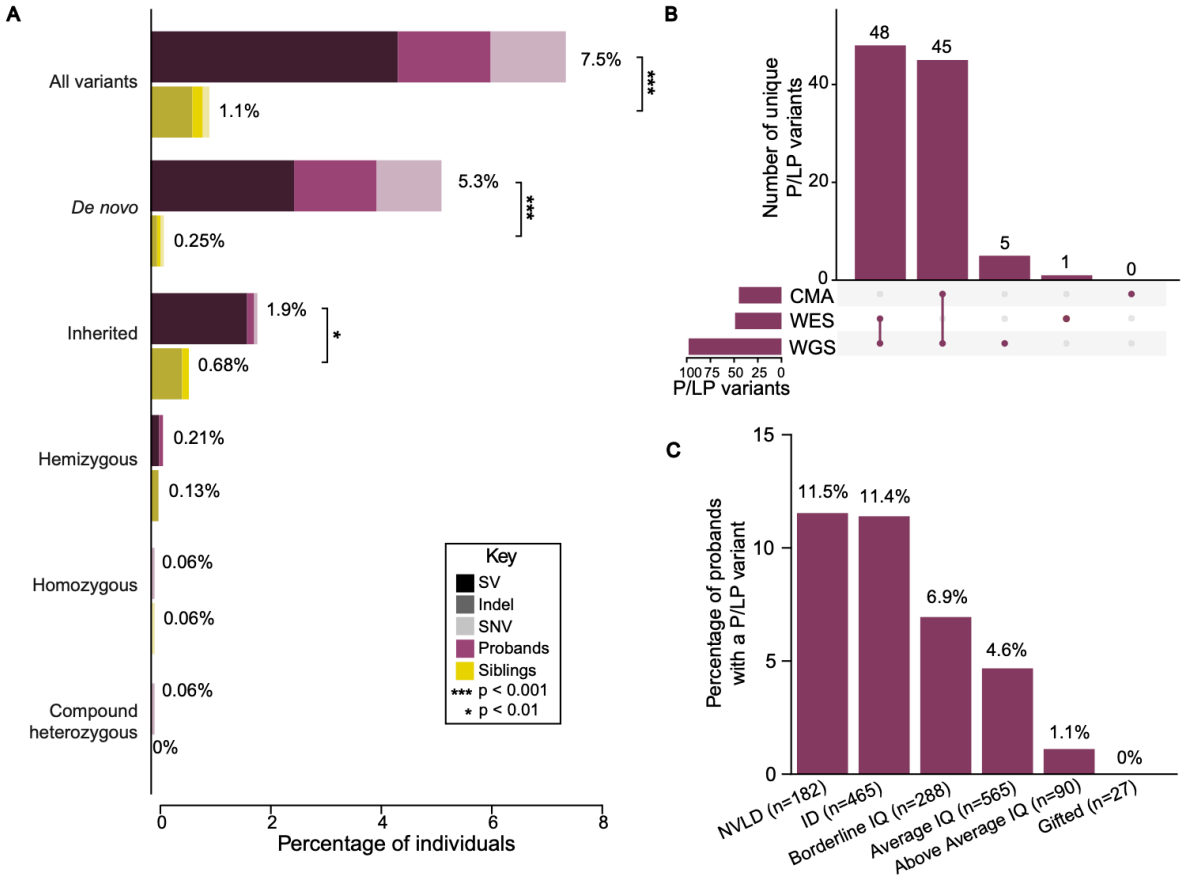

Figure 2. WGS diagnostic yield in ASD probands and unaffected siblings

(**A**) The fraction of probands and unaffected siblings with a P/LP variant broken down by inheritance and variant type. The denominator used for all categories was 1,612 except for hemizygous variants where only males were considered (n=1,440 male probands and 755 male siblings). (**B**) The total number of unique P/LP variants detected across WGS, WES, and CMA. (**C**) The fraction of ASD probands (n=1,608 with available IQ scores) with a P/LP variant displayed by IQ group. SV: structural variant; SNV: single nucleotide variant; indel: small insertion and deletion; P/LP: pathogenic or likely pathogenic variant; CMA: chromosomal microarray; WES: whole exome sequencing; WGS: whole genome sequencing; NVLD: non-verbal learning disorder; ID: intellectual disability (FSIQ≤70); borderline IQ (FSIQ 71-85); average IQ (FSIQ 86-115); above average IQ (FSIQ 116-130); gifted (FSIQ>130).

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a small (14.0 kb) *de novo* deletion overlapping exons 12-16 of *ARID1B*, two balanced SVs (a hemizygous SVA insertion in *DMD* and a *de novo* translocation disrupting *GRIN2B*), a *de novo* stopgain in *ANKRD11*, and a *de novo* frameshift insertion in *SMARCA4* (**Figure S8**). WGS also uniquely identified two small in-frame single exon deletions in *RERE* (5.6kb) and *RORA* (505 bp) that were each classified as a variant of uncertain significance (VUS) due to a lack of additional affected cases to support classification (**Table S9**). We anticipate that deletions like these have a high likelihood of being escalated to P/LP over time as the widespread adoption of clinical WGS will be powered to routinely detect small CNVs to help inform clinical interpretation.

In addition to the interpretation challenges of small, single exon CNVs, WGS also discovered a *de novo* complex SV in an ASD proband that involved six breakpoints and four deletions (size range: 581kb to 3.0Mb, total: 6.4 Mb) that disrupted 42 protein-coding genes in total, none of which have been associated with ASD or are supported by case evidence in the literature (**Figure 3 and Table S9**). Current ACMG/AMP guidelines require separate assessment of individual CNVs and breakpoints in a complex event to arrive at an overall classification,[33] which would result in a VUS classification for this variant because the largest deletion doesn't meet the gene count threshold (35 genes) to be classified as LP (*e.g.*, it disrupts 18 genes). Given that complex SVs were not included in the analyses used for determining ACMG/AMP gene-number cut-offs,[33] future clinical guidelines could consider including more specific guidance on the classification of complex events, particularly those comprising multiple CNVs, that are only accessible to WGS and long-read sequencing technologies.

Application of WGS in diagnostic testing of FSAs

To evaluate WGS in a prenatal cohort, we performed WGS on 175 FSA trios with structural defects spanning eleven phenotypic categories, with 44.6% (n=78/175) of cases presenting with MCAs (**Table S2**). The WGS variant detection pipelines described above identified 988 unique variants in 2,535 developmental disorder genes for manual review, or 6.3 variants per FSA case on average (median=5.0,

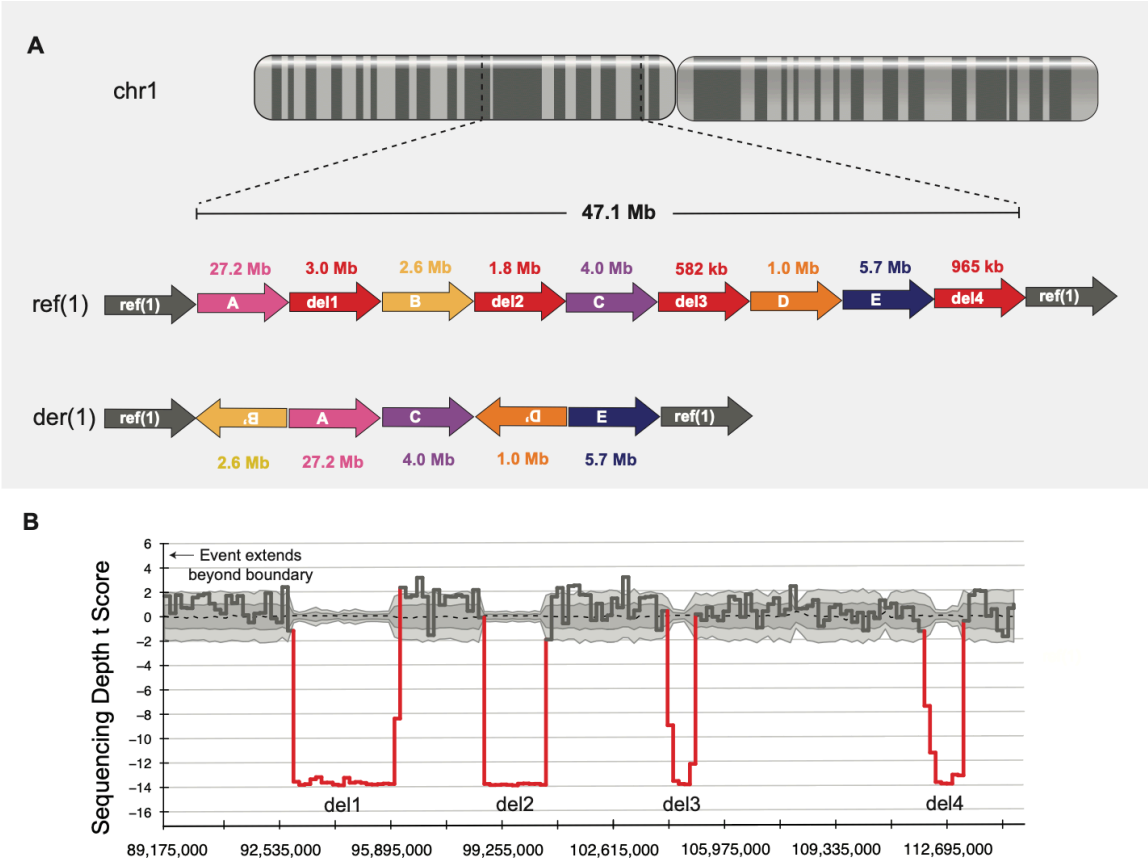

Figure 3. Complex SV resolved by WGS highlights interpretation complexity

(**A**) Linear representation of a *de novo* complex SV occurring on chromosome 1 (gray) and classified as a VUS in an ASD proband. Each rearranged segment of DNA in the derivative chromosome is depicted by a unique color and letter (A-E), while the four deleted segments of DNA are colored in red and sequentially numbered del1-del4 (size range=0.582 Mb to 3 Mb; total deleted sequence in variant=6.3 Mb). Arrows are not drawn to scale, and inverted segments are denoted by a reverse orientation of arrows. The genomic coordinates for this rearrangement are provided in **Table S9**. (**B**) Sequencing depth t-scores for deleted segments (chr1:89,175,000-112,695,000). Red bins indicate a statistically significant read-depth change compared to background that exceeds a Bonferroni-corrected threshold. Shading represents one (dark gray) or two (light gray) binwise median absolute deviations across all samples. Chr: chromosome; Mb: megabase; kb: kilobase; ref: reference; der: derivative.

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range=0-18). We identified 25 unique P/LP variants (17 single variants and 4 pairs of compound heterozygous variants) in 21 cases, suggesting that the added diagnostic yield of WGS beyond karyotype and CMA is 12.0% for fetuses in this cohort (**Figure 4**). Overall, we observed a non-significant increase in P/LP variants in cases with MCAs (11/78; 14.1%) compared to those with a single anomaly (10/97; 10.3%) (P=0.48; Fisher's exact test), though this comparison is underpowered due to the relatively small sample size.

The 25 P/LP variants identified by WGS included 20 SNVs (14 missense, 5 nonsense, and 1 splice donor), 3 frameshift deletions, and two SVs, including a 67kb *de novo* deletion overlapping exons 5-8 of *MED13L* and a maternal uniparental disomy event involving chromosome 20 with evidence of isodisomy and heterodisomy (**Table S8**). In addition to these P/LP variants, WGS also identified a 143kb intragenic exonic duplication (IED) in *DYNC2H1* that was confirmed to be in *trans* with a pathogenic missense variant in an FSA case with short-rib thoracic dysplasia and polydactyly (MIM 613091). Interestingly, the missense variant in this compound heterozygous pair was originally identified by WES, and due to the specificity of the gene-disease association, the laboratory manually reviewed the WES read depth profile across this gene and identified the duplication, which was later confirmed with fluorescence *in situ* hybridization.[24] While WGS discovered both variants in a single test, the challenges of predicting the functional impact of in-frame IEDs resulted in a classification of VUS for the *DYNC2H1* exonic duplication, despite the strong likelihood that these variants represent the molecular diagnosis for this case based on the specificity of the phenotype, the robust gene-disease assocation, and the limited number of genes associated with short-rib thoracic dysplasia.[40-43] Similar to the sequence variant guidelines,[32] future CNV recommendations could consider including additional mechanisms to increase classifications of CNVs, particularly IEDs, that are proven to be in *trans* with a P/LP variant.

Given that the majority of the FSA trios were pre-screened for aneuploidies, BCAs, and large CNVs from karyotype and CMA, we estimated the likely overall diagnostic yield from WGS in consecutive referrals of unscreened FSA trios using previously reported diagnostic yields from karyotype and CMA in FSAs.[22] The largest published study of prenatal FSAs with CMA and karyotype testing previously identified a diagnostic variant from karyotype in 31.9% of FSAs (28.3% aneuploidy, 3.6% BCAs) and noted that an additional 6.0% of karyotype-negative FSAs harbored a P/LP variant from CMA. In this study, we demonstrated in the ASD cases that WGS captured all P/LP CNVs and aneuploidies from CMA and our previous WGS studies have shown that WGS can identify breakpoints in at least 92.4% of BCAs defined by karyotypes.[44-46] The majority of missed BCAs are caused by repetitive sequences at the breakpoints (*e.g.*, translocation into acrocentric arms), which cannot be detected with short-read WGS using existing algorithms. Given that our analyses revealed an additional 12.0% diagnostic yield from WGS, we estimate that WGS is likely to discover a diagnostic variant in approximately 47.6% of consecutive FSA referrals at a major medical center (**Figure 4**).

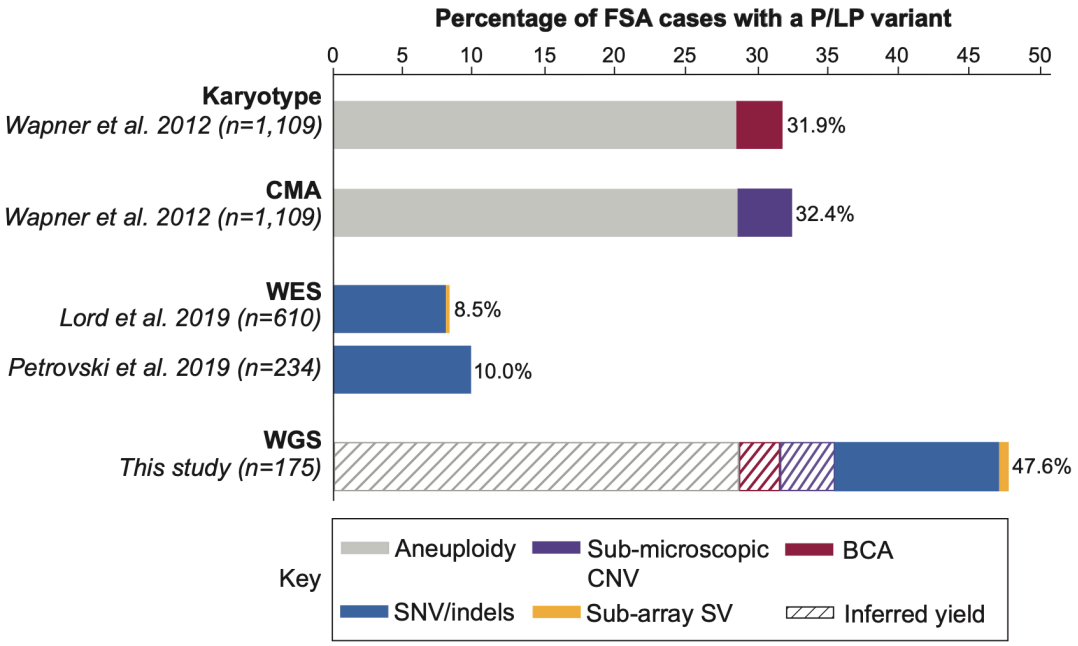

Figure 4. FSA diagnostic yield across technologies

Comparison of the diagnostic yields from karyotype, CMA, WES, and WGS in FSAs. Each variant class is represented by a different color. The diagonal lines indicate the estimated yield of WGS if applied to unscreened FSA cases. FSA: fetal structural anomaly; P/LP: pathogenic or likely pathogenic variant; CMA: chromosomal microarray; WES: whole exome sequencing; WGS: whole genome sequencing; BCA: balanced chromosomal abnormality; CNV: copy number variant; SNV: single nucleotide variant; indel: small insertion and deletion; SV, structural variant.

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DISCUSSION

Technological advances have historically driven the adoption of new clinical testing capabilities. For several decades, karyotype was the mainstay of cytogenetics to assess FSAs until CMA superseded it when incremental gains in diagnostic yield were demonstrated by a large-scale, unbiased study.[22] Last year, two WES studies identified novel diagnostic variants in 8.5% and 10.0% of FSA cases that were unexplained following both karyotype and CMA.[15,16] In the current study, we take the next technical leap in routine diagnostics by performing an unbiased assessment of the utility of WGS in ASD and FSAs. We demonstrate that WGS captures virtually all P/LP variation identified by the combination of karyotype, CMA, and WES in a single test. We then apply these carefully benchmarked methods to demonstrate that the diagnostic yield of WGS is markedly superior to each individual technology. Remarkably, WGS also yielded novel molecular diagnoses that were cryptic to the combination of all conventional tests. These analyses suggest that WGS warrants further evaluation as a first-tier genomic test to displace the combination of karyotype, CMA, and WES currently used in prenatal and pediatric diagnostics.

These analyses identified a P/LP variant in 7.5% of 1,612 ASD probands using WGS and our interpretation framework. This represented an almost two-fold higher yield than either WES (2.9%) or CMA (4.3%) on the same samples. In addition to demonstrating high sensitivity of WGS against conventional tests, our interpretation framework also demonstrated good specificity by classifying very few (1.1%) P/LP variants in ASD siblings that were unaffected at the time of their assessment. Notably, these siblings have not been re-evaluated for later onset phenotypes that could be related to these variants, and 64.7% of the variants observed in siblings have been previously associated with reduced penetrance (**Tables S6-7**), which are known to pose challenges for variant interpretation and genetic counseling. These data suggest that the sensitivity and specificity of WGS are converging on a pending technological shift in standard-of-care, while also highlighting some of the technical and interpretation challenges that this transition will introduce. Our analyses of affected probands and their unaffected siblings also reassure that, while ambiguous interpretations will inevitably arise, these rapidly evolving annotation and interpretation pipelines are already sufficiently sophisticated to preferentially identify P/LP variation in affected children. Importantly, these data also suggest that these methods avoid an excess of false-positive interpretations that might have previously contributed to the historical reluctance to introduce genome-wide analyses into routine diagnostics.

The added value of WGS was reinforced in the implementation of these methods in 175 FSA trios that were pre-screened with karyotype and CMA. These analyses identified a P/LP variant in 12.0% of cases above the routine yield from conventional testing. We note that the application of karyotype, CMA, and WES together could theoretically capture most of the diagnostic yield provided by WGS based on current annotation and interpretation guidelines. However, the potential for these tests to miss cryptic diagnostic variants that are uniquely captured by WGS and the likelihood of significant future advances in noncoding variant interpretation renders this combination of serially conducted tests ill-suited for routine diagnostics. A compelling example of a potentially novel WGS molecular diagnosis included the compound heterozygous missense SNV and IED in *DYNC2H1* in a case with short-rib thoracic dysplasia with polydactyly (MIM 613091). The combination of these variants demonstrates the need to establish uniform guidelines for interpretation of variants with ambiguous functional consequences such as in-frame IEDs. We previously demonstrated in population genetic studies from the genome aggregation database (gnomAD) that IEDs strongly correlate with patterns of LoF constraint, suggesting that LoF may be a common mechanism for IEDs.[26] As the joint discovery of SNVs, indels, and SVs becomes more common, comprehensive interpretation methods to account for IEDs and increased synergy across variant classes will be critical, especially for cases with non-specific phenotypes where predictive power is lower for targeted gene interpretation.

Despite the improved sensitivity of WGS, its diagnostic value is hindered by classes of variation that are either difficult to discover (*e.g.*, short tandem repeats, such as in fragile X syndrome),[47] involve complex mechanisms or pleiotropic outcomes (*e.g.*, oligogenic mechanisms such as the combination of an *SMCHD1* mutation and repeat expansion in *D4Z4* in facioscapulohumeral muscular dystrophy type 2),[47,48] or that lack clinical-grade interpretation frameworks (*e.g.*, non-coding variation) in genetic diagnostics. While prior studies suggest the contribution of these variant classes may be relatively minor in severe pediatric phenotypes,[49,50] their exclusion nonetheless limits the overall diagnostic yield of WGS reported here. Further, current CNV interpretation guidelines have largely excluded specific guidance on interpreting complex SV that include multiple CNVs, which we expect to be increasingly identified with routine use of clinical WGS. We anticipate that re-analysis of existing WGS data will lead to an increasing number of molecular diagnoses over time due to the inclusion of novel disease genes and functional elements,[51] the accumulation of classified variants with supporting evidence deposited into open-access databases such as ClinVar,[52] continued improvements in methods for comprehensive variant discovery, and refinement of interpretation guidelines for overlooked classes of genetic variation.

In conclusion, we report the largest clinical WGS study in the field to date and compare diagnostic yields in pediatric ASD cases and their unaffected siblings and in prenatal FSA cases. We developed a framework to harmonize methods for detecting pathogenic SNVs, indels, CNVs, balanced SVs, and complex SVs at base pair resolution from WGS and benchmark its utility in both prenatal and FSA cases. The relatively modest increase in diagnostic yield should

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temper enthusiasm regarding immediate significant increases in interpretable pathogenic variation from WGS, absent improvements in noncoding variant annotation. Nonetheless, we demonstrate here, and in prior studies,[44-46] that WGS can effectively capture all pathogenic variation detected by three conventional methods in a single test. We also propose that WGS will provide a modest but important increase in diagnostic yield above the combination of all conventional technologies using current interpretation tools, thus warranting further evaluation as a first-tier genetic diagnostic test.

METHODS & SUPPLEMENTARY INFO

There are detailed methods provided in a separate supplementary document, together with additional figures and tables, which will be linked directly from *bioRxiv*.

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