P-536 Pre-selected for an award: Validation of a Next Generation Sequencing (NGS) workflow integrating simultaneous analysis of ploidy, microdeletions and de novo monogenic diseases for expanded preimplantation genetic testing (PGT).

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Study question: Can major *de novo* genetic and chromosomal abnormalities (i.e., ploidy, microdeletions) be effectively tested on a single embryo biopsy specimen using an integrated NGS approach?

Summary answer: The integrated NGS workflow provided high accuracy for multilevel chromosome and genetic abnormalities analysis based on single biopsies expanding PGT informativity to *de novo* conditions.

What is known already: Current NGS-based methodologies employed in PGT for aneuploidy (PGT-A) do not detect embryo ploidy level nor frequent pathogenic *de novo* microdeletions below resolution limits. Moreover, despite their considerable incidence and adverse pregnancy outcomes, *de novo* mutations causing severe dominant monogenic fetal structural defects (FSD) are not investigated during PGT. The development of a single biopsy specimen-based PGT-A sequencing strategy that integrates ploidy and *de novo* microdeletions/mutations assessment would significantly widen PGT-A diagnostic scope and technical capabilities. This comprehensive approach would provide additional valuable genetic information of unquestionable clinical utility to further refine embryo selection process among those showing euploid profiles.

Study design, size, duration: Chromosomal conditions were validated using 24 embryo rebiopsies and 5 cell lines with both known ploidy level and known microdeletions (-4p; -8q; -1p; -22q; -5p; -15q; -11q). Genotyping for monogenic conditions was validated using 5 genomic DNA samples (33pg/µl) carrying known pathogenic Single Nucleotide Variants (SNVs) in COL1A1, SOS1, PTPN11, TSC2 and FGFR2 genes. To assess technical performance across identified SNPs, genotyping accuracy was evaluated on 17 samples from 5 embryos and 2 cell lines.

Participants/materials, setting, methods: Thirty-two *de novo* dominant monogenic conditions with FSD and strong gene-disease relationship were tested using a multiplex PCR panel with sequencing for the genes' whole coding region. Eight common microdeletions (<10Mb) syndromes (Wolf-Hirshorn, Langer-Geidion, 1p36 deletion, De George, Cri-du-Chat, Prader-Willy/Angelman, Jacobsen) were tested using B-allelic frequency (BAF) of 356 highly polymorphic Single Nucleotide Polymorphisms (SNPs). These SNPs were also used for ploidy assessment. Library preparation and sequencing were performed on the IonTorrent S5 (ThermoFisher).

Main results and the role of chance: Blinded NGS data analysis confirmed the ploidy status in all (19) samples with known constitution (8 diploids, 7 polyploids, 4 haploids). Specifically, the proportion of heterozygote calls (BAF 40%-60%) was 60.9% (95%CI:47.6-72.8) for diploid samples and <1% for haploid samples($P<0.001$). All polyploid samples showed a typical splitting of BAF among 3 experimental ranges (20-40%,40%-60%,60-80%): 34.1%,18.2% and 47.7%, respectively. For microdeletions, all interstitial SNPs genotyped showed a loss of heterozygosity (LOH) as expected. The analysis of positive controls consisting of 20 blastocyst rebiopsies and 3 cell lines (-4p: n=3; -8q: n=4; -1p: n=5; -22q: n=3; -5p: n=2; -15q: n=4; -11q: n=2), allowed to accurately characterize 6 out of the 7 microdeletions (18/23 samples). In particular, all interstitial SNPs genotyped showed a LOH, while diploid controls showed an overall heterozygosity of 30.9% (average number of hetSNP x deletion=9/28). Only the very small telomeric 1p36 region failed to properly amplify. For monogenic conditions, sequencing analysis of 5 positive gDNA controls confirmed the presence of 4 known SNVs, whilst only 1 did not achieve the minimum coverage for variant calling. Moreover, 4 additional *de novo* SNVs detected by sequencing analysis in the gene panel on 8 blastocyst rebiopsies were all confirmed by qPCR/Taqman assays.

Limitations, reasons for caution: Positive controls were not available for all genes and microdeletions included in the panel. Moreover, inefficient amplification has affected some target regions and further optimization will be required. However, analytical performance on technical and biological replicates were highly promising for the tested conditions both cell lines and trophectoderm biopsies.

Wider implications of the findings: This study demonstrates that the integration of genotyping and chromosomal analyses can be efficiently achieved in the same NGS workflow. This approach can be employed to expand PGT diagnostic scope to conditions undetectable in parents due to their *de novo* onset, or that are below the standard PGT-A resolution.

Trial registration number: N/A