Chromosomal microarrays: next-generation karyotyping assays for detecting inherited chromosomal anomalies

In this white paper, we describe:

- The development and use of chromosomal microarrays for analyzing chromosomal anomalies
- The benefits of using chromosomal microarrays over traditional G-band karyotyping
- Case studies of instances where chromosomal microarrays detected anomalies that were missed by G-banding

Introduction

First-trimester miscarriage is one of the major causes of pregnancy failure, occurring in 10–15% of successful embryo implantations [1,2]. In about half of the cases, detailed examination of miscarriage tissue retrieved in the first trimester can detect whole-chromosome abnormalities, including autosomal trisomies, polyploidies, monosomy X, and chromosomal mosaicism [1-8]. It is therefore critical to evaluate the chromosomal complement of products of conception to evaluate the immediate and long-term health of the fetus.

The analysis of genetic composition of fetal cells was pioneered in 1966 by Steele and Breg. They collected cells obtained from invasive prenatal testing (amniotic fluid from amniocentesis) to evaluate the chromosomal complement of a fetus [9]. Subsequently, chorionic villus sampling and fetal blood from cordocentesis provided other means of collecting fetal cells. Typically, these cells are cultured *ex vivo* and stained with Giemsa stain to reveal distinct banding patterns (G-banding). As a result of more than 60 years of use, fetal karyotyping by culturing and G-band analysis is a well-established method for prenatal chromosome analysis.

In spite of the beneficial information provided by conventional karyotyping, there are drawbacks. Conventional karyotyping by G-banding has a resolution of between 5 and 10 Mb, so smaller pathogenic variations could be missed. It requires culturing the cells collected, and stochastic changes that occur during the culturing period could confound results. The cells have to be in a particular phase of the cell cycle when stained, and the chromosomes have to exhibit good morphology. Performing and interpreting the method requires a skilled cytogenetic analyst.

The Human Genome Project (HGP) opened up tremendous vistas for understanding human variation. Chromosomal microarrays (CMAs), which combined microscale manufacturing techniques with well-understood nucleic acid hybridization chemistries, were one of the technologies arising from the HGP and significantly altered the landscape of medical genetics research in the mid- to late 2000s. These microarrays were optimized for detecting genetic variations, including variations in the number of times a specific sequence was present (copy number variations, or CNVs) and variations in sequence at a single base pair (single-nucleotide polymorphisms, or SNPs). Since thousands to millions of sequences can be queried at a time, a single microarray experiment has the potential to give novel information about a subject's genome, highlighting the nucleotide variants present at each of the

many loci assayed by the array. These CMAs have been successfully adapted to be used for prenatal molecular karyotyping, providing an alternative to G-banding that is rapidly becoming the clinical research standard. In fact, CMA screening is recommended as a first-tier test by numerous societies, including the American College of Obstetricians and Gynecologists (ACOG) and the Society for Maternal-Fetal Medicine (SMFM) [10].

Types of DNA microarrays

Copy number variation analyzed on chromosomal microarrays historically has made use of many different kinds of immobilized nucleotide probes, including bacterial artificial chromosomes (BACs), cDNAs, and oligonucleotides. Screening on microarrays in clinical contexts was first done using BAC arrays around 2003. These microarrays consisted of between 2,000 to 30,000 BAC probes and provided a rough idea of chromosomal microdeletions in mental retardation and developmental dysmorphisms (for example, see [11]). BAC arrays were relatively easy to manufacture at the time and were useful because they could identify CNVs in discrete regions of the human genome known to play roles in specific genetic diseases. However, resolution was limited due to the large size of the probes (Figure 1).

Once oligonucleotide deposition techniques were perfected, however, oligo arrays became the platform of choice for CNV analysis. Oligonucleotide arrays consist of up to 1 million or more single-stranded 25–85 bp oligonucleotides immobilized in discrete areas of the array. Many studies have shown that oligonucleotide arrays offer higher resolution for the detection of smaller CNVs compared to BAC arrays, from about 1 Mb resolution with BAC arrays to around 100 kb with oligonucleotide arrays [12,13]. Because they can accommodate a very large number of sequences, a subset of oligonucleotide arrays are produced that contain large numbers of SNP probes. Each SNP is assayed by a set of partially overlapping probes that can distinguish single-nucleotide differences. These SNPs are scattered throughout the genome, and therefore a single microarray experiment can yield a profile of a donor's genome, including CNVs and SNPs at defined loci. Furthermore, because of their high density, oligonucleotide and SNP arrays can query regions of the genome with resolution down to 100 nucleotides.

There are two basic strategies for using oligonucleotide-based arrays for CNV measurements (Figure 2). One of these, array comparative genomic hybridization (aCGH), measures the amount of each sequence present in test and normal samples. For this method, the genomic DNA of a test sample is labeled with one fluorescent dye, and genomic DNA of a normal sample is labeled with a different fluorescent dye (Figure 2A). The labeled DNAs are mixed together in equal proportions and hybridized to the array. The test and control DNA competitively hybridize to the complementary sequences on the array.

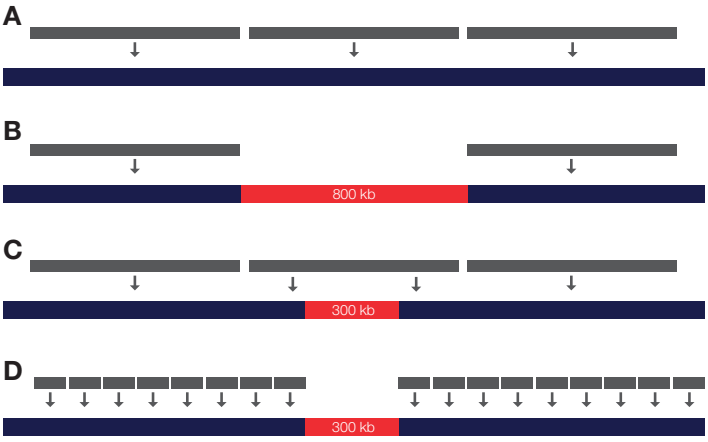


Figure 1. Smaller probe size improves microarray resolution. (A) DNA probes (gray) hybridize normally to sample DNA (dark blue), indicating a normal copy number in this region. **(B)** The middle probe does not hybridize to an 800 kb deletion (red), resulting in accurate detection of the deletion during data analysis. **(C)** The middle probe has sufficient homology to hybridize on each side of a 300 kb deletion. This deletion is likely to be missed during data analysis. **(D)** Smaller probes have sufficient resolution to hybridize on each side of the 300 kb deletion but not span it, resulting in accurate detection.

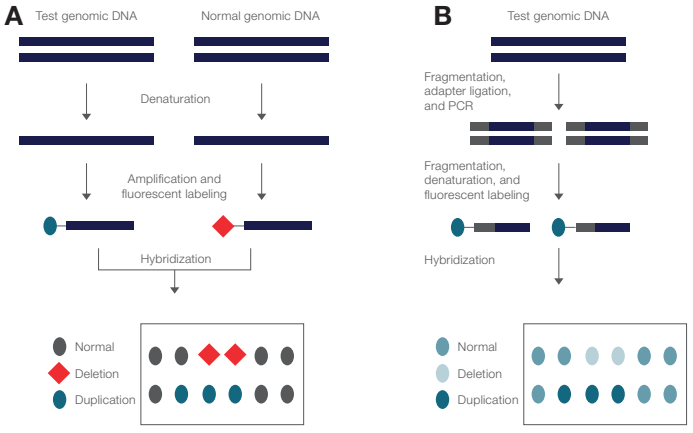


Figure 2. Example workflows for oligonucleotide-based arrays. (A) In aCGH, test and normal genomic DNA samples are labeled with different fluorophores and hybridized to the same array. Deletions and duplications are indicated by an abundance of one signal over the other. **(B)** In other microarrays, the test genomic DNA sample alone is hybridized to the array. Copy number is determined by comparing the signal intensity to a reference data set. Deletions are indicated by a decrease in signal relative to the reference, while duplications are indicated by an increase in signal relative to the reference.

The amount of fluorescence is directly proportional to the abundance of sequence in the sample. The fluorescence signal of both colors is measured at every position on the array. Various manipulations of the signal measurements are used to convert the fluorescent signals to a ratio of test to control intensities, and thus to a sequence copy number. A typical array used in clinical settings contains from a few hundred thousand to millions of unique probes.

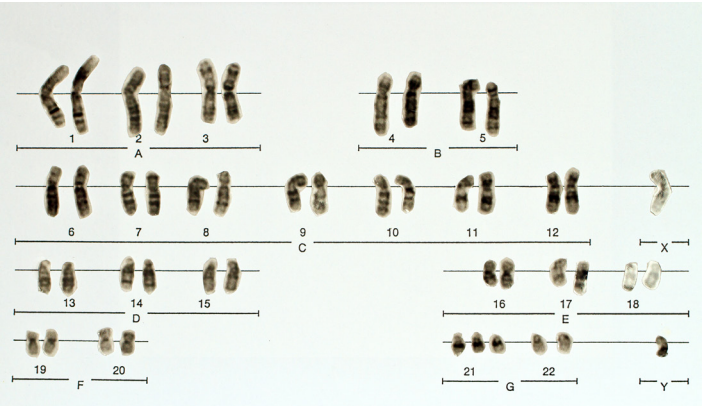
The other strategy makes use of oligonucleotides immobilized on arrays, but the abundance levels are not measured by competitive hybridization (Figure 2B). In these microarrays, genomic DNA from only a test sample is needed, eliminating the need for a separate control sample. The genomic DNA to be tested is labeled with a fluorescent dye, and after hybridization to the microarray and washing, the absolute fluorescence at each immobilized oligonucleotide position is measured. To convert the measured signal to a CNV, the intensities are compared to a standard reference data set where numerous normal control samples were run independently and combined. The resulting ratio between the test and the normal reference is calculated *in silico*. As with aCGH, the ratio of the test to reference signals is converted to a CNV measurement.

Advantages of karyotyping by CMA over traditional G-banding

Molecular karyotyping using CMAs provides several advantages over traditional G-banding. First, although the same methods are used to collect fetal cells, there is no need to culture the cells before analysis, reducing the time required to obtain an answer. Second, because genomic DNA from a large number of cells is analyzed, it can detect mosaicism in the fetus. Third, because live cells are not needed for analysis, genomic DNA extracted from preserved or archived formalin-fixed, paraffin-embedded (FFPE) material can be analyzed. Fourth, analysis software that can collect and interpret the molecular results has been developed, reducing the reliance on trained specialists. And finally, the spacing between the probes on a CMA can provide much better resolution, facilitating the detection of microdeletions and microduplications that can be pathogenic (Figure 3). For these reasons, karyotyping by CMA has become the preferred first-line choice for karyotyping at-risk pregnancies.

A specialized type of CMA is known as a SNP array. In this array type, oligonucleotide-based DNA probes, based on regions in the genome that show single-nucleotide diversity among individuals, are immobilized on the array. Because there are multiple SNPs present at each locus, other specialized types of chromosomal anomalies can be detected. These can include copy-neutral loss of heterozygosity (cnLOH), uniparental disomy (UPD), long contiguous stretches of homozygosity (LCSH), trisomies, and other whole-chromosome polyploidies (Figure 4). These anomalies can be pathogenic and therefore should be considered in a screening strategy.

A



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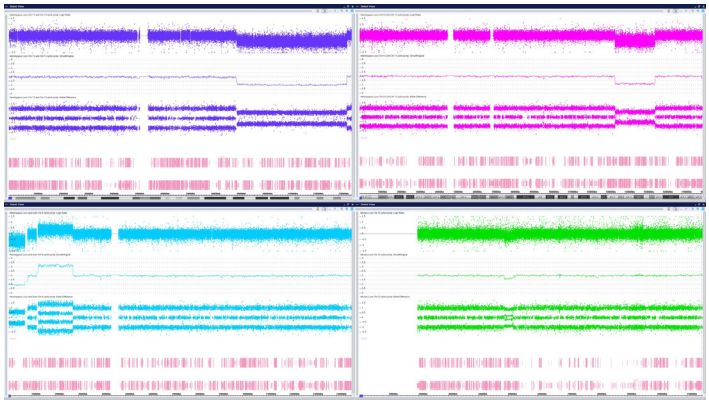

Figure 3. Resolution of karyotyping technologies. (A) Traditional karyotyping offers a resolution of 5–10 Mb for detection of genomic variations. **(B)** CMA analysis offers a resolution of <500 kb, which enables the detection of much smaller deletions and duplications.

Most contemporary arrays used for clinical research purposes are hybrid arrays, containing a combination of a large number of probes for SNPs and copy number probes that lack SNP information. The density of the probes on the array is also a factor in determining the resolution of the assay. Although low-density arrays with 30,000 or fewer unique copy number and SNP probes were suitable for detecting UPD, a study by Mason-Suares et al. [14] found that these arrays miscall absence-of-heterozygosity (AOH) regions arising by identity by descent. Higher-density arrays give greater confidence in the detection and positive identification of copy-neutral abnormalities. However, higher resolution can also result in finding more variants of unknown significance (VOUS). Examination of the VOUS inheritance pattern in a family can provide "living proof" that the variant is nonpathogenic. For example, if a child with a congenital abnormality carries a VOUS that is also present in the phenotypically normal parents, the VOUS is not likely to be the causative variant. The need for parental testing in the future could decrease with the sharing of the variant findings across labs and would improve the interpretation of VOUS results. In the meantime, many providers will omit VOUS in their reports, since it may lead to higher anxiety for the parents.

It is important to note that neither aCGH nor SNP arrays can easily detect balanced chromosomal rearrangements, such as translocations with no change in copy number. Making these determinations still requires conventional karyotyping or FISH analysis. Since no single platform is able to fulfill all the needs of prenatal testing, complementary approaches, such as SNP array and molecular karyotyping followed by next-generation sequencing to identify pathogenic variants, might be necessary. Some examples of multiplatform use are described in the following sections. Therefore, choosing an appropriate platform or platforms for clinical research analyses requires balancing many factors, including likelihood of successful outcomes, cost- or time-effectiveness, and minimizing uncertainty in interpretation of results.

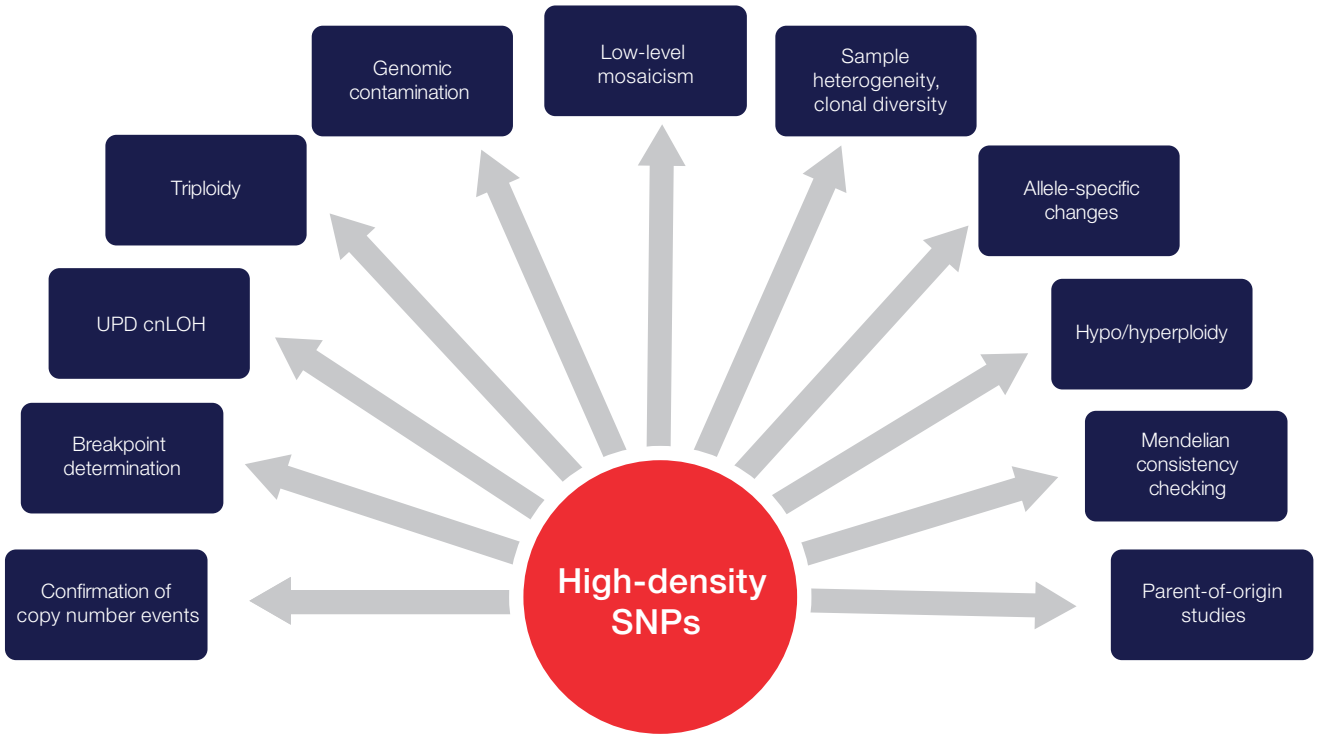

Figure 4. The power of SNP arrays. High-density SNP arrays with high genotyping accuracy enable a range of applications, including confident breakpoint determination and copy number change confirmation.

CMAs improve information yields for pre- and postnatal analyses

Because of the increased density and superior resolution relative to traditional karyotypes, CMA analysis is rapidly becoming the established method for evaluating products of conception. Many studies have documented the benefits of analysis using SNP arrays. For example, in 2013 Liao et al. [15] summarized the results from their laboratory analysis of 446 fetuses with structural malformations. CMA analysis was used to reveal a clinically relevant genomic imbalance in 51 of these fetuses (51/446; 11.4%). Of these, pathogenic CNVs in 16 fetuses were less than 1 Mb in size; these would have been missed by low-resolution BAC arrays or conventional karyotyping. There was little difference in the number of pathogenic CNVs and VOUS detected in this study. They conclude that because high-resolution SNP arrays identify a higher proportion of pathogenic CNVs that would otherwise be undetected by standard chromosome analysis (with an acceptable proportion of VOUS), whole-genome high-resolution SNP array analysis provides valuable data in prenatal investigation of fetuses with structural malformations.

Nuchal translucency

Nuchal translucency (NT) analysis is an ultrasonographic prenatal scan to assess the quantity of fluid present within the nape of the fetal neck. It is typically used to predict chromosomal abnormalities and can also detect cardiovascular abnormalities in a fetus [16]. NT measurements have been used as a stand-alone test for aneuploidy screening in the absence of other karyotypic analysis. To compare results of NT analysis relative to karyotyping by G-banding and CMA analysis, Cicatiello et al. [17] performed a retrospective study of 249 fetuses between 11 and 18 weeks of gestation with increased NT. Their results showed that CMA analysis had a 5–8% incremental yield of CNV detection in fetuses with increased NT without other ultrasound anomalies and normal karyotype.

Similarly, Su et al. [18] performed a prospective study to compare the efficacy of CMA analysis relative to conventional karyotyping. Out of 192 women enrolled in the study carrying fetuses with increased NT, 174 were found to have a normal karyotype by G-banding. Using CMA analysis, they found that among fetuses with NT >3.5 mm (n = 43), 5 had CNVs, 3 of which were clinically significant. Interestingly, even in fetuses with smaller NT (2.5–3.4 mm, n = 104), 6 were found to have CNVs, 2 of which were clinically significant. From these results, Su et al. concluded that CMA analysis improved the data utility of chromosomal aberrations for fetuses with NT and apparently normal karyotypes, regardless of whether other ultrasonic abnormalities were observed.

Intellectual disability

Edwards syndrome (OMIM 300484) is a genetic disorder caused by a complete or partial trisomy of chromosome 18. Eighty percent of cases of Edwards syndrome present full trisomy, and the other 20% present mosaic or partial 18 trisomy [19]. Partial trisomy causes less severe but highly variable penetration of the symptoms, depending on the degree of trisomy and the cells and tissues affected [20]. One feature of Edwards syndrome is intellectual disability and developmental delay. Pinto et al. in 2014 [21] described a case of a 4-year-old child that had several of the features, both developmental and intellectual, of Edwards syndrome. However, G-band karyotyping found that the proband had a normal female karyotype (46, XX), without any suggestion of chromosome alteration. Her parents also presented normal karyotypes. To gain more information, Pinto performed CMA analysis of the affected child. These results found 4 genomic imbalances in the patient's genome: a *de novo* 1.23 Mb microdeletion at 18p11.32 with 30% mosaicism, an 18q partial trisomy with 40% mosaicism, an inherited 386.73 kb microdeletion at 7q31.1, and a *de novo* 25.72 Mb microduplication at Xp22.33p21.3. CMA analysis of the parents confirmed that these genomic imbalances arose *de novo* in their child. None of these novel changes were detected by G-banding. Because CMAs excel at detecting aberrations that might be missed by conventional karyotyping, the American College of Medical Genetics and Genomics (ACMG) [22], the International Collaboration for Clinical Genomics (ICCG) [23], and the American Academy of Neurology (AAN) [24] recommend chromosomal microarray analysis as the first-line test in patients with unexplained developmental delay (DD), intellectual disability (ID), multiple congenital abnormalities (MCA), or autism spectrum disorder (ASD).

Heart defects

Congenital heart defects (CHDs) are fetal developmental defects commonly observed by ultrasound during prenatal diagnosis. Although the pathways leading to cardiological defects are many, chromosomal aberrations and single-gene defects are likely to be involved in CHDs [25,26]. Studies have shown that there are pathogenic CNVs associated with tetralogy of Fallot, thoracic aortic aneurysms, and congenital left-sided heart disease, among others [27,28]. Song et al. [29] in 2018 published a study

of 190 fetuses with normal karyotypes but demonstrated CHD by fetal ultrasound. CMA analysis detected pathogenic copy number variants (pCNVs) in 13/190 (6.8%) fetuses, likely pCNVs in 5/190 (2.6%) fetuses, and VOUS in 14/190 (7.4%) fetuses. These variants affected the viability of the fetuses: among those with pCNVs, none yielded a normal live birth; of those with likely pCNVs, 2 out of 5 yielded a live birth; and of those with VOUS, 10 out of 14 yielded a live birth. Song et al. concluded that, because normal karyotyping failed to detect these variants, fetal genotyping by CMA analysis is extremely useful for prenatal genetic clinical research of fetuses with CHDs.

Fetal growth restriction

Fetal growth restriction (FGR) refers to poor embryonic growth during pregnancy. Up to 19% of fetuses have FGR that is thought to be caused by chromosomal anomalies, and triploidy and trisomy 18 are thought to be the most common associations [30]. However, the potential contributions of microdeletions, microduplications, and single-gene disorders in FGR with normal karyotype are not well established. In a comprehensive metadata study, Borrell et al. [31] collected and analyzed published data from 2009 to 2016 to estimate the incremental yield of CMA analysis over conventional karyotyping in FGR. They found that there was a 4% incremental yield with CMA analysis compared to karyotyping in nonmalformed growth-restricted fetuses, and a 10% incremental yield in FGR when associated with fetal malformations. The most frequently found pathogenic CNVs were 22q11.2 duplication, Xp22.3 deletion, and 7q11.23 deletion (Williams-Beuren syndrome), particularly in isolated FGR. They point out that these results will allow fetal medicine specialists to improve antenatal counseling in cases of isolated FGR.

Aberrations scored as balanced translocations by G-banding

CMA analysis can sometimes be used to detect other microdeletions and microduplications in a karyotypically abnormal fetus. Lallar et al. [32] performed a study of prenatal amniotic fluid in 128 cases over the course of 5 years. They note that over that time, there was a gradual increase in the number of amniocenteses as well as CMA analysis (CMA analysis opted by 2.2% of women undergoing amniocentesis in 2013, versus 11.4% of women in 2017), indicating the increased acceptance of the method in the expecting mothers and clinicians. Interestingly, in their studies they encountered a case with ventricular septal defects by prenatal ultrasonographic evaluation, and a fetal karyotype displaying a balanced translocation between chromosomes X and 22 [46,t(X;22)(q11.2;q28)]. However, CMA analysis of this same fetus identified multiple other CNVs: heterozygous duplication on 20q13.2, duplication on Xp22.33, and deletion on Xq28, all pathogenic. This case highlights the utility of CMA in identifying small CNVs in apparently balanced fetal karyotypes. Overall, Lallar et al. observed that the acceptance rate of prenatal CMA analysis increased 5-fold over a period of 5 years, and concluded that CMA analysis provides greater data utility in structurally abnormal cases.

Cost-effectiveness and data utility

As the costs of health care increase, there is an increased emphasis on balancing health care quality with expenditures. It is therefore critical that any test utilized provide the maximum benefit to the clinicians and patients.

As described above and in other examples, the data utility of CMAs relative to conventional karyotyping in analyzing products of conception (POC) has been repeatedly demonstrated. CMA analysis can identify pathogenic microaberrations where karyotyping displays a normal or nonpathogenic pattern. Furthermore, CMAs have been highly successful when analyzing difficult samples, such as archived FFPE POC samples [33]. This is particularly beneficial for cases of recurrent pregnancy loss, and provides options for women who have experienced a pregnancy loss but did not have a fresh tissue sample analyzed at the time of the loss. CMA analysis methods therefore present opportunities to increase the amount of information obtained from POC analyses.

Although CMA analysis might entail higher initial costs than traditional karyotyping methods, the benefit of increased data utility has been shown to make them cost-effective. Analysis as recent as 2018 using models that incorporated actual reimbursement rates set by governmental agencies, and the reliable estimates of test outcomes derived from studies with tens of thousands of patients, clearly showed that prenatal CMA testing is cost-effective [34]. In the same study, parental CMA testing that is used to aid in the interpretation of VOUS detected in patients was explored and found to be cost-effective; however, the authors noted that the need for parental testing in the future could decrease with the sharing of the variant findings in genetic databases, which would improve the interpretation of VOUS results. A separate analysis led by Sinkey et al. [35] in 2016 found that CMA testing was the preferred strategy for analyzing sonographically detected fetal anomalies,

based on the incremental cost-effectiveness ratio between CMA analysis and conventional karyotyping. They showed that CMA alone led to an additional 17 determinations per 1,000 fetuses, further illustrating the advantages of CMA. The authors conclude that in spite of the increased cost, CMA analysis is superior for providing actionable information, and thus is cost-effective when used for prenatal evaluation of an anomalous fetus.

Conclusions

Establishing a causative evaluation reduces or eliminates prolonged medical evaluation and testing, thus reducing both short- and long-term health-care costs. The results of studies like those described can help guide health-care providers, organizations, professional societies, and policymakers to determine how and to whom particular health-care services are provided. Given its much greater data utility over conventional karyotyping, CMA analysis is recommended by a number of medical societies as the first-tier test in the clinical research of unexplained global developmental delay (GDD) or intellectual disability (ID) [22-24,36]. Taken together, these findings provide a strong cost-effectiveness rationale for health-care systems to use CMA as the first-tier test for the genetic diagnosis of unexplained GDD or ID. About a third of positive CMA findings are clinically actionable, with some of the actions being specific pharmacological treatments [37-40].

Glossary

ASD: Autism spectrum disorder, a condition related to brain development that impacts how a person perceives and socializes with others, causing problems in social interaction and communication.

LOH: Loss of heterozygosity, referring to a locus that no longer has two different alleles. This can involve deletion of one of the normally diploid copies, leaving only one.

BAC: Bacterial artificial chromosome, an *E. coli* replication vector that can accommodate and maintain large inserts such as segments of human DNA.

CMA: Chromosomal microarray, a method to analyze genomic content by hybridization to hundreds of thousands to millions of immobilized probes.

cnLOH: Copy-neutral loss of heterozygosity, referring to when a locus that should be heterozygous based on inheritance is diploid but homozygous for a variant.

CNV: Copy number variant, a sequence that is present at more or less than the normal diploid number.

DD: Developmental Delay

FFPE: Formalin-fixed, paraffin-embedded, a method for preserving dissected tissue by infiltration with formalin and storage in wax blocks.

GDD: Global developmental delay, a significant delay in cognitive or physical development.

Haplotype: A set of DNA variations, or polymorphisms, that are contiguous and tend to be inherited together.

ID: Intellectual disability, significant limitations in both intellectual functioning and adaptive behavior.

LCSH: Long contiguous stretches of homozygosity, large stretches of the diploid genome that have identical sequence.

MCA: Multiple congenital abnormalities, two or more unrelated major structural malformations that cannot be explained by an underlying syndrome or sequence.

SNP: Single-nucleotide polymorphism, a difference in nucleotide sequence involving only a single base change.

UPD: Uniparental disomy, a chromosome or region that has the genotype of only one parent, but in two copies.

VOUS: Variant of unknown significance, a difference in nucleotide sequence that is not correlated with a known phenotype, disease, or change in protein function.

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