Diagnosis and clinical management of duplications and deletions

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Chromosome deletions and duplications—copy number variations (CNVs)—are a major contribution to the genome variability and can be either pathogenic or not. A particular class, the microdeletions and microduplications, which alter <5 Mb, have been extensively associated with developmental delay and intellectual disability. Although their prevalence in pregnancies and newborn is relatively low, their estimates in preimplantation embryos are poorly defined. The introduction of novel technologies for preimplantation genetic diagnosis of aneuploidies (PGD-A) caused new possibilities and challenges associated with diagnosis of subchromosomal CNVs. Both technical aspects of performing genomewide microarray or next generation sequencing analysis on single cells and interpretation issues are subject of debate. The latter include the reliability of detection of CNVs from embryonic biopsies, their clinical classification based on reproductive outcomes, as well as how before and after test counseling should be organized. It is also important to consider that the current resolution of these technologies from single cells is usually >10 Mb, thus ruling out the possibility to diagnose the most important recurrent microdeletion and microduplication syndromes. Furthermore, at present we face with a lack of well-designed studies addressing the actual resolution and accuracy of CNVs detection in PGD-A and no reference databases is available to evaluate their pathogenicity. Accordingly, it seems reasonable at the moment to avoid the reporting of subchromosomal CNVs in PGD-A. However, although these issues require proper handling, they should not lead us away from providing an improved preimplantation genetic diagnosis. (Fertil Steril® 2017;107:12–8. ©2016 by American Society for Reproductive Medicine.)

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Chromosome abnormalities accounts for approximately 15% of the major congenital anomalies diagnosed before the age of 1 year and are associated with 25% of perinatal deaths (1). Aneuploidies have also been largely associated with infertility and reproductive complications, where they represent the single most important causative factor for implantation failure and miscarriages (2). Fetoplacental aneuploidies account for >50% of sporadic first trimester miscarriages (3), and this rate increases significantly with advancing female age.

In the prenatal setting, chromosome abnormalities have a wide range of genomic imbalances, from polyploidy, to whole chromosome aneuploidy, to submicroscopic deletions and duplications that can only be detected by DNA-based copy number methods, such as fluorescence in situ hybridization or chromosomal microarray (CMA). Of chromosome aneuploidies in spontaneous miscarriages 85%–90% involve whole chromosome copy number alterations (3). Although for these abnormalities the clinical consequences are very well defined and range from embryonic lethality to a few viable autosomal trisomies and sex chromosome copy number aneuploidies, the impact of subchromosomal variations is still a subject of investigation in reproductive genetics. Partial chromosomal deletions and duplications—collectively termed copy number variations (CNVs)—are a major contribution to the genome variability among individuals (4–6) and can be either pathogenic or without clinical consequences. Furthermore, CNVs seems not to be related to female age and originate as an error in the male and female meiosis at apparently similar rates.

The DNA sequence along human chromosomes is constantly changing, and this process enables humans to evolve and adapt (7). About 10 years ago, scientists began to recognize abundant variation of an intermediate size class known as structural variation (8, 9). At present, within this class, CNVs accounts for the largest component. We now typically define the size of CNVs as >50 bp (10), whereas smaller elements are known as insertions or deletions (indels). These structural variations encompass more polymorphic base pairs than SNPs by an order of magnitude (11, 12).

There is a continuous spectrum of phenotypic effects of CNVs, from adaptive traits, to underlining cause of

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disease, to embryonic lethality (13). As technologies have improved to detect smaller and smaller CNVs across the genome, we are learning the very high frequency and important role that this type of genomic variation plays in human diseases. Chromosomal microdeletions and microduplications (MMs) have been associated with syndromic forms of intellectual disability and developmental delay since the 1980s and in the past decade, >200 recurrent MM syndromes have been identified (14).

At present, by evaluating the entire genome at once, previously described syndromes and novel etiologies can be identified faster. The CNV assessment by CMA is becoming recognized as a first-tier test for individuals with intellectual disability and developmental delay in human genetic investigation (15) and represented a paradigm shift in the diagnosis of genetic disorders from ''phenotype-first,'' where clinicians used the patient's phenotype to guide decisions about which genetic tests to consider, to ''genotype-first,'' where clinicians used the patient's genotype to guide their clinical evaluation and management. Given that CNVs are now appreciated as one of the most frequent causes of a broad spectrum of human disorders, early diagnosis and accurate interpretation is important to implement timely interventions and targeted clinical management. The analysis of genomic syndromes using CMA is becoming a common test now also in prenatal diagnosis (PND), especially when amniocentesis or villocentesis is indicated after an abnormal ultrasound result (16). This application became an effective option after the development of large databases incorporating the classification of thousands of normal and pathogenic CNVs that are commonly identified in human pregnancies, although there are still many controversies related to its routine application in the prenatal setting (17, 18). Similar interest has also been recently raised in preimplantation genetics, where the introduction and systematic application of CMA and next-generation sequencing (NGS) technologies hold the potential to improve the detection of subchromosomal abnormalities. The rate limiting step for these high resolution chromosome testing technologies in preimplantation genetics has been, for years, the paucity of starting material that usually consist of a single or few cells collected either at the cleavage or the blastocyst stage. The introduction of whole genome amplification (WGA) protocols has allowed CMAs and NGS protocols to be applied at the embryo biopsy level as micrograms of DNA can be obtained from single cells after the amplification. In the past decade of systematic application of CMA protocols in preimplantation genetic diagnosis for aneuploidies (PGD-A), partial aneuploidies have been reported together with whole chromosome aneuploidies. In this review, a comprehensive evaluation of existing data on the incidence and clinical management of deletions and duplications in the preimplantation setting will be provided in comparison with the prenatal and postnatal setting. There will also be a focus on the strength of evidences for the diagnosis of these subchromosomal abnormalities in embryonic biopsies, as well as a consideration on how these data should be appropriately handled in the clinical setting of an IVF cycle in relation to available scientific evidence.

ORIGIN AND TYPE OF CHROMOSOME DELETIONS AND DUPLICATIONS

There are two major classes of CNVs: recurrent and nonrecurrent. Recurrent CNVs generally arise by nonallelic homologous recombination during meiosis, with breakpoints in the large duplicated blocks of sequence flanking the CNV event. Because the breakpoints cluster within defined regions, the extent of recurrent CNVs is fundamentally identical even in unrelated individuals (19). In contrast, nonrecurrent CNVs have breakpoints that generally lie within unique sequence and do not result from a predisposing genomic architecture. Nonrecurrent CNVs can arise by several mechanisms, including nonhomologous end joining and fork stalling and template switching (20, 21). As a result, although two unrelated individuals may have overlapping nonrecurrent CNVs, they are unlikely to share the same breakpoints. It is possible to estimate that 4.8%–9.5% of the genome contributes to CNV and occasionally it has been reported as up to 100 nondosage sensitive genes can be completely deleted without producing apparent phenotypic consequences.

PREVALENCE OF CHROMOSOME DELETIONS AND DUPLICATION IN THE POSTNATAL, PRENATAL, AND PREIMPLANTATION PERIODS

Postnatal and Prenatal Period

The estimates of prevalence for large chromosomal deletions and duplications are relatively low in the neonatal population. The reported prevalence of chromosomal deletions from congenital anomaly register data ranges from 0.3–2 per 10,000 births (22, 23), with newborn investigation suggesting a similar rate of 0.5–1 per 10,000 (24, 25). A more recent study showed 4.7% of all chromosome abnormalities reported were deletions, including microdeletions, giving a prevalence of 1.99 per 10,000 births (3). Duplications are even less common, showing a prevalence of 0.7 per 10,000 births and representing the 1.6% of all reported chromosome abnormalities (3).

Although large deletions and duplications are rare events, chromosomal MMs make up the most significant fraction of subchromosomal CNVs and a particular class of them have been clearly defined as pathogenic. This special class of recurrent CNVs is termed genomic disorders; mechanistically and phenotypically these are the best-characterized imbalances in the genome (14). Genomic disorder refers to a class of syndromes caused by a partial chromosomal deletion or duplication, usually <5 Mb. spanning several genes that is too small to be detected by conventional cytogenetic methods or high-resolution karyotyping (2–5 Mb). The health and developmental effects associated with MM syndromes can vary tremendously and depend on where in the genome the deletion/duplication is and how many genes it involves. Essentially, these pathogenic CNVs continue to be described in different classes of disease (26). The systematic application of CMAs helped to underline how causative submicroscopic chromosomal imbalances can be found in 10%–15% of patients with DD, multiple congenital abnormalities, or autism,

thus increasing the diagnostic yield of karyotype analysis (27, 28). Classic examples are the 17p12 duplications and deletions, which result in the development of Charcot-Marie-Tooth disease type 1A and hereditary neuropathy with liability to pressure palsies, owing to contrasting dosage effects of the PMP22 gene (29). These recurrent, reciprocal, disease-causing CNVs were two of the first genomic disorders described. More examples soon followed, including Prader-Willi and Angelman syndromes (15q11–q13) (30) and Smith-Magenis syndrome (17p11.2), the 22q11 deletions associated with velocardiofacial syndrome, and the 16p11.2 recurrent microdeletion characterized by developmental delay, intellectual disability, and/or autism spectrum disorder. At present >200 microdeletions/duplications syndromes have been described and catalogued (14). An essential step for their classification and clinical management in prenatal and postnatal genetic investigation was the development of repository databases, such as Decipher (http://decipher.sanger.ac.uk/), Ecaruca (http://www.ecaruca.net/), and ISCA (https://www.iscaconsortium.org/) where pathogenic CNVs are reported or the Database of Genomic Variants (DGV, http://projects.tcag.ca/variation/) where CNVs detected in apparently healthy controls are assembled (4). This intensive work has allowed assigning a pathogenic or benign connotation to many recurrent MMs occurring in our genome by the observation of their prevalence in affected individuals with respect to the control healthy population.

In pregnancies, pathogenic MMs detectable by CMAs have a considerably high prevalence. Using CMAs, Wapner and colleagues (16) reported one of the largest investigations about the prevalence and type of MMs in the common population undergoing PND. Overall, 96 of the 3,822 fetal samples with normal karyotypes (2.5%) had a microdeletion or duplication of clinical significance not detected on karyotyping. Uncertain findings occurred in 3.4% of all karyotypically normal cases analyzed. Most of these cases had findings that were not easily dismissed as likely to be benign and therefore required expert adjudication for clinical relevance. They also examined the results from microarray analysis in subgroups of women categorized based on the indication for PND: pregnancies with abnormal ultrasound result and from women presenting with advanced female age but normal ultrasound results. In samples from fetuses with suspected growth or structural anomalies, 2.8% had clinically relevant findings on microarray that were not found on karyotyping. On the contrary, only 0.5% of the women without ultrasonography identified anomalies who were tested because of advanced maternal age had a normal karyotype and a clinically relevant finding on microarray. Collectively these data suggest that large CNVs are very rare in pregnancies and newborns, whereas MMs present with a relatively high frequency, particularly in the presence of ultrasound defects.

Preimplantation Period

The situation in preimplantation embryo development is still poorly defined, as very few data have been reported on the prevalence and type of subchromosomal CNVs in the earliest window of embryo development. This paucity of knowledge is mainly due to two factors. First, is the later application of high-resolution chromosome testing technologies in PGD-A compared with prenatal or postnatal genetics where the amount of DNA to be processed on microarrays does not represent a limiting factor. The use of CMAs on embryo biopsies had to wait until the development of whole genome amplification protocols, WGA, that were introduced after the year 2000. This has lowered the acquisition of knowledge on the prevalence and type of pathogenic and potentially lethal CNVs that can be found in preimplantation embryos. Second, is the limited chromosome resolution that can be achieved when WGA is used as a DNA enrichment step before CMA. The WGAs provide the amplification of only a portion of the genome (40%–60%) and occasionally amplification bias can be introduced with some region of the genome being over or less represented as a consequence of a technical artifact (31). Furthermore, current methodology does not take into account the phase of the cell cycle, despite the variable copy number status of different genomic regions in S phase. Accordingly, the accuracy to detect segmental chromosomal imbalances is reduced in S-phase cells, which could be a source of misdiagnosis in PGD-A cycles (32). The difficulty to distinguish between technical and genuine biological variation makes it challenging to accurately assess and catalogue the presence of small imbalances from embryonic biopsies. The average resolution for de novo subchromosomal aneuploidies of commercially available microarray protocols in PGD-A is 5–10 Mb. As a consequence, segmental imbalances, other than the ones suspected based on parental chromosome rearrangement, have rarely been reported and characterized in PGD-A studies. Their reported prevalence in preimplantation embryos ranged from 2% to as high as 70% (33–36) and the few data available relate only to large deletions and duplications. Some of the possible explanations of the discrepancy include the use of different CMA platforms, algorithms, and validation levels, making it difficult to obtain reliable and comprehensive data on the prevalence and type of CNVs in the preimplantation period of development. Furthermore, the criteria used in the research laboratory for detection of CNVs can be very different than those used by a state-of-the-art clinical genetics laboratory. It should be also acknowledged that none of the studies reporting CNVs in PGD-A have reported a validation for the criteria used to define partial aneuploidies on embryonic biopsies as well as none of them has used an independent genetic technology to confirm the original diagnosis. Finally, at present, none of the studies has attempted to characterize and catalogue the pathogenicity of such abnormalities including the analysis of parental DNA and reporting the breakpoints and the gene content of the involved genomic regions (Table 1). Generally speaking, a frequency of 70% implies that there is some problem with analysis, as that is never observed in pregnancies or newborns.

In summary, the picture at the moment is that there is a significant lack of knowledge. It can be speculated that as these earliest phases of embryo development are not subjected to a stringent negative selection against aneuploidies, partial imbalances can be more prevalent compared with what was

TABLE 1

Current state of limitation for the detection and interpretation of deletion and duplication encountered in preimplantation genetic diagnosis of common aneuploidies (PGD-A) in comparison to prenatal diagnosis (PND).

Characteristic	PND	PGD
Genotype/phenotype correlation	Some abnormal ultrasound results relates with specific pathogenic CNVs	Total absence of genotype-phenotype correlation
Resolution	Usually 100 kb	Undetermined; usually >5 Mb (most common recurrent pathogenic microdeletions/ duplications goes undetected)
Breakpoints mapping	Possible to be mapped to the single base	Impossible to be determined with precision
Accuracy	>99%	Undetermined
Catalogues	Many repository databases present to investigate for pathogenicity (e.g., Decipher, Ecaruca, ISCA, DGV)	None present

Capalbo. Management of duplications and deletions. Fertil Steril 2016.

observed in pregnancies and newborns. As for whole chromosome aneuploidies, it is possible that some lethal segmental aneuploidies are selected against at the time of implantation or soon after being missed in PND or in postnatal genetic investigation. Accordingly, we might expect a slightly higher incidence of partial aneuploidies in preimplantation embryos although this still needs to be defined and properly addressed in future well-designed studies.

CLINICAL MANAGEMENT OF DELETION AND DUPLICATION

Postnatal and Prenatal Period

The overwhelming presence of benign CNVs in the genome of healthy controls requires the careful interpretation of CNVs detected in test samples (37). Guidelines addressing clinical and technical aspects of CMA application, including interpretation, in the prenatal and postnatal period are now available (38, 39), whereas these are missing for PGD-A.

In general, CNVs are classified as likely benign, likely pathogenic, and variants of unknown significance. Many criteria can be used to help interpreting the clinical relevance of a CNV, including inheritance, size, type, and genetic content (15, 39).

The inheritance pattern of a CNV, when accompanied by clinical and family history information, can be useful. De novo CNVs are more likely than inherited CNVs to be pathogenic, although there are certainly exceptions (40). Conversely, as illustrated by the example of the 1q21.1 syndrome and others, inherited CNVs may cause a range of severity and presentation of neurodevelopmental disorders and can evidently be pathogenic, even when present in a phenotypically normal parent (41–43). The factors underlying such extreme clinical variability are still poorly understood, although several possible explanations for different clinical presentations in carriers of the same deletion or duplication have been proposed (44). Differences in genetic background—that is, the milieu of sequence and CNVs present within the genomes of specific individuals—could contribute, and there is now evidence showing that common and rare variants can play a role in modifying phenotypic outcome. Epigenetic differences are another possible factor. Finally, environmental or sporadic effects might interact to alter the risk of abnormalities associated with a specific CNV.

The CNV size and type (deletion or duplication) are often used as guides to interpret pathogenicity. Large CNVs are more likely than small CNVs to cause disease (28). In part, this is because larger CNVs generally encompass more genes, with a concomitant increase in the probability of altering a dosage-sensitive element. Likewise, deletions result in haploinsufficiency, the consequences of which are known for some genes. Duplications are more difficult to interpret, and in the clinical setting, a larger minimum size threshold is often used for duplications than for deletions. Gene content is also a consideration. The CNVs that contain many genes or known disease genes are more likely to be pathogenic than those that contain few genes or genes of uncertain function. The CNVs within "gene deserts" are particularly difficult to interpret, although as we learn more about regulatory regions within noncoding DNA (45), interpretation will become easier. All of these criteria are probabilistic in nature, and there are documented instances in which, for example, small noncoding duplications cause genetic syndromes (46).

As discussed, the availability of large repository databases is probably one of the most effective ways to evaluate the pathogenicity of a CNV. However, one of the biggest concerns for the systematic application of CMA or NGS technologies in reproductive genetics still relates with the detection of variants of unknown significance and CNVs showing incomplete penetrance and variable expressivity and how to handle with these cases in the clinical practice. This is a particularly relevant situation in PND where the couple might decide to terminate a pregnancy based on this information. Although in PND and postnatal genetics high degree of experience has been accumulated with cataloguing and management of CNVs, these issues are still quite common and deserve careful investigation (16). Specific national and international guidelines and reporting recommendations have been produced to manage the amount of data obtained in CMA testing to balance the need for the information with the risk for findings of uncertain clinical significance.

Preimplantation Period

The clinical management of deletions and duplications in PGD-A cycles faces several unique challenges, in particular the lack of repository databases for the classification of CNVs, the complete absence of a phenotype to evaluate pathogenicity, the technical limitation related with single cell analysis, and the need for the use of WGA (Table 1). Furthermore, in the clinical setting of a PGD-A cycle, it may be difficult to test at the same time both parents for financial or other reasons, therefore other criteria for genetic evaluation and clinical management must be considered.

As discussed, an important resource for the clinical management of CNVs is the availability of repository databases where pathogenic and benign variants are classified. In contrast to postnatal and PND, one of the main limitations in PGD-A is the lack of CNV data in appropriate controls and embryos with defined reproductive outcomes. A database of CNVs in reproductively competent embryos is missing, as well as a database of CNVs detected in embryos resulting in implantation failures or miscarriages. The absence of such references makes the clinical interpretation of the reproductive meaning of any CNVs detected at the preimplantation stage more complicated. It is therefore extremely important in the future to develop specific repository databases where to catalogue normal and pathogenic CNVs that are detected in preimplantation embryos to assist in their clinical interpretation, management, and the counselling with patients. However, it should be highlighted that the building of reference databases will be a demanding task, particularly for very rare CNVs, which require large sample sizes to detect recurrence and for pathogenic CNVs that show incomplete penetrance and/or variable expressivity. Furthermore, most of the patients will refuse the transfer of embryos showing CNVs, limiting the possibility to obtained information on their reproductive outcomes.

Another singularity of the preimplantation analysis of CNVs is the complete absence of phenotype to evaluate possible genotype-phenotype associations (Table 1). In pregnancies some fetal structural defects can be linked to a higher probability for some chromosomal abnormality. In patients affected by ID or DD it is easier to relate a genomic imbalances to a specific class of phenotypes. Preimplantation embryos do not show any morphological or dynamic change, even when bearing a whole chromosome aneuploidy (47, 48), further complicating the evaluation of the pathogenicity of a CNV at the preimplantation stage.

Finally, it is of extreme importance to underline that at present in PGD-A the analysis of few cells with the use of WGA protocols results in a low resolution for the de novo partial aneuploidies compared with prenatal or postnatal genetic analysis and faces many challenges for the accurate profiling of partial aneuploidies. All methods for single-cell genomics face the difficulty to detect with accuracy (de novo) DNA subchromosomal copy number and/or single-nucleotide variants in a cell, and thus far, none of the methods has proven the ability to unravel the genomic structure of detected DNA copy number variants. Multiple displacement amplification (MDA)- and polymerase chain reaction (PCR)-based WGA methods create many chimeric DNA molecules that distort the structure of a cell's genome in a specific manner. It has been showed how WGA protocols significantly impact the resolution and reliability of chromosome analysis in PGD-A cycles (31, 35) and how at least 100 times more putative DNA rearrangements are detected stochastically in the cells with the WGA-based chromosome analysis when compared with the structure of the reference genome (49). These artifacts can thus create false-positive CNV diagnosis. Due to this resolution limits, it is also close to impossible in PGD-A to map breakpoints of deletions and duplications to establish the exact genomic extend of a deletion or duplication. Taking into account the empirical resolution levels described by the manufacturer, only segmental imbalances >10 Mb can be considered for the analysis. With this level of resolution most CNVs cannot be detected in PGD-A cycles as well as the most significant MMs that are the far more prevalent subchromosomal pathogenic abnormalities encountered in pregnancies and newborn population. If on one hand this low chromosomal resolution limits our ability to accurately diagnose CNVs in embryos, on the other hand the clinical management of large deletions and duplications encompassing >10 Mb poses less challenges as these large partial aneuploidies can be likely be defined as pathogenic. However, it should be stressed that although the theoretical resolution of microarray and low-pass NGS protocols for PGD-A has been reported down to 10 Mb, so far no study has attempt to properly validate the reliability for partial aneuploidies diagnosis from embryo biopsies with the use of independent genetic technologies. Accordingly, at present we still urgently need well-designed prospective studies to assess the reliability of contemporary PGD-A technologies to diagnose CNVs and evaluate their potential impact on reproductive outcomes. Studies using a nonselection design can be useful to evaluate the positive (PVP) and negative predictive values (PVN) of partial aneuploidies diagnosis in PGD-A cycles. Until such evidences will be showed, it seems reasonable to avoid the reporting of subchromosomal CNVs in PGD-A cycles, minimizing the risk of discarding potentially viable embryos as a consequence of false-positive diagnosis or incorrect clinical interpretation.

In conclusion, the scope of knowledge regarding the nature of structural variation and its relationship to human health has expanded considerably during the past several decades. The introduction of improved genetic technologies in a routine PGD-A setting brought novel challenges. The technical aspects of performing a genomewide microarray and NGS technologies—such as which type of platform and resolution should be used—and ethical issues are a subject of debate. The latter include the indications for deletion and duplication clinical management and reporting, the guidelines on how to deal with variants of unknown significance, as well as how before and after test counseling should be organized. In general, due to the low-resolution limits that we face when working on single cells, only large deletion and duplication are detectable. Although it is reasonable to define such imbalances as pathogenic, at present there is a lack of well-designed studies showing the reliability of

detection for deletions and duplications from embryonic biopsies. Likewise no study has attempted to characterize the clinical predictive value when these abnormalities are detected in embryonic biopsies. According to the available evidences, it thus seems cautious at present to avoid the reporting of subchromosomal aneuploidies to minimize the potential for false-positive detections and to not compromise the cumulative chance of live-birth per IVF cycle. An alternative possibility is to include the reporting of CNVs but confirming the partial aneuploidy on a second biopsy to allow the patients to make a more educated reproductive decision. In any case, this diagnostic limit and the few data available have to be acknowledged in consent forms and carefully discussed with patients during the genetic and reproductive counselling as well as the significant and residual risk for MMs when a pregnancy after PGD-A is obtained.

Although these issues require proper handling and further discussion, they should not lead us away from the primary goal (i.e., providing an improved preimplantation genetic diagnosis). Future studies are thus urgently warranted to evaluate the application of improved genetic tests for PGD-A cycles. Increased data sharing and collaborative works will lead to substantial progress in understanding the relationships between variants and embryonic reproductive competence, making possible to provide improved genetic testing for infertile couples.

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