Karyotyping and Chromosomal Microarray Analysis in Women Requesting Amniocentesis for Isolated Sonographic Soft Markers or Advanced Maternal Age

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1. BACKGROUND

Prenatal screening is offered to all women during pregnancy to determine the risk of the fetus to be born with a genetic condition or the risk of a pregnancy complication. Thus, it is useful in determining different pregnancy options or management plans for the pregnancy and delivery, so as to improve the maternal and fetal outcome (1). Several types of prenatal screening are available, depending on the gestational trimester and the type of condition in question (1).

Concerning fetal chromosomal abnormalities, the gold standard is the first trimester combined test (2). However, ultrasound screening in the second trimester of pregnancy can detect congenital fetal anomalies that may partially reflect fetal chromosomal aberrations. The value of the second trimester ultrasound screening has been established, especially in recent decades that its detection capacity has been significantly improved following the technological advances in the field of obstetric ultrasound imaging. There are limitations, however, as ultrasound can exclude fetal structural anomalies by less than 70% in general and in some anatomical systems, such as the cardiovascular system, this percentage is significantly lower (3).

Even greater evolution has been
achieved in the field of genetic analysis techniques. The most common fetal aneuploidy is Trisomy 21 (Down syndrome) and the definite prenatal diagnosis is made through invasive testing, such as chorionic villus sampling or amniocentesis (4). The standard karyotype illustrates the characteristic number and morphology of chromosomes and may detect those aberrations that are visible under the microscope. Quantitative Fluorescent Polymerase Chain Reaction (QF-PCR) has recently entered the field of prenatal diagnosis to allow rapid examination results (5).

Chromosomal microarray analysis (CMA) is an emerging molecular genetic technology that has enabled the accurate detection of small chromosomal alterations, such as microdeletions and microduplications, which may be associated with serious clinical phenotypes, such as DiGeorge, Prader Willi, Angelman and other syndromes; these abnormalities cannot be detected with standard karyotype (6). CMA has the advantage of high resolution analysis, relatively quick and objective results (6). The array-CGH analysis can lead to the elucidation of genetic predisposition and prenatal diagnosis of common clinical diseases, such as cardiovascular disease, schizophrenia and autism (6).

Invasive procedures, however, are associated with an increased risk of miscarriage and other complications (7, 8), making couples counseling a demanding challenge for health care providers. On behalf of the parents, making an informed final decision/choice concerning undergoing a prenatal diagnosis is also a difficult and painful process. However, many women proceed to invasive prenatal testing based on their advanced age (>35 years old) or personal desire and not due to medical indication (9). In recent years, efforts have been made to develop non-invasive screening tests in order to reduce the number of unnecessary invasive procedures. However, the depth of analysis of these techniques cannot currently replace the one of invasive tests (10). Thus, standard techniques are required for cases expected to hide smaller hypochromosomal abnormalities, which may not always be clinically irrelevant (11, 12).

Finding a serious abnormality or multiple abnormalities of the fetal anatomy in the second trimester usually leads to pregnancy termination, the consultation challenge, however, remains when minor isolated findings, small structural abnormalities or deviations of normal, called “soft markers”, are detected. These may be of little or even no pathological significance, but potentially semiology of a heavier genotype (13). Using such isolated ultrasound markers to redefine the risk of chromosomal abnormality is debatable (14). In such cases the question for detailed chromosomal evaluation, through invasive testing, raises.

2. OBJECTIVE

The aim of the present study was to investigate the prevalence of sub-chromosomal abnormalities in fetuses with isolated ultrasound findings during the routine anatomy scan in the second trimester after maternal request. In addition, the study investigated advanced maternal age (older than 35 years) as an indication for CMA in an otherwise normal pregnancy.

3. PATIENTS AND METHODS

This is a retrospective study, as the rarity of abnormal ultrasound findings in the second trimester of a pregnancy with normal (low-risk) screening in the first trimester would not allow a sufficient sample collection in a logical time period to draw safe conclusions. The study sample was derived from a specific fetal medicine center in a time period of 5 years (between 2015-2020) and consisted of two different groups. The first group of 84 cases consisted of all cases with an isolated soft marker during the fetal anatomy scan of the second trimester that consented after maternal request, whereas the second group of 42 cases included those pregnant women that undergone amniocentesis for karyotyping and CMA after their request due to advanced maternal age, although their first trimester screening returned normal results.

Isolated soft markers included: fetal brain abnormalities, such as ventriculomegaly and choroid plexus cysts, facial abnormalities, such as hypoplastic nasal bone, cardiovascular abnormalities, such as intracardiac echogenic focus and aberrant right subclavian artery (ARSA), kidney abnormalities, such as renal pelvic dilatation, gastrointestinal abnormalities, such as echogenic bowel, musculoskeletal abnormalities, such as shortened long bones, and other abnormalities, such as single umbilical artery. Though the sample was relatively small, an attempt was made to assess the value of detailed karyotyping per fetal anatomical system.

The study included single and multiple pregnancies, regardless maternal age. For the second group maternal age should be over 35 years, as already stated. Pregnancies with multiple fetal abnormalities or those with high risk results from the first trimester screening were excluded as an invasive testing for thorough chromosomal evaluation is routinely indicated, in these cases.

The amniocentesis was performed between 18-23 weeks of gestation. The genetic analysis included QF-PCR followed by CMA, using the SurePrint G3 CGH ISCA v2 Microarray Kit (G5955A, Agilent Technologies). The analytical capacity of this particular microarray platform is approximately 500 kb along the genome and approximately 50 kb in more than 240 regions of known clinical significance associated with more than 512 genetic diseases according to the International Standards for Cytogenomic Arrays (ISCA) Consortium. This method detects numerical chromosomal abnormalities, syndromes associated with deficits and/or duplications, as well as all unbalanced chromosomal shifts within the analytical ability of the method. The CNVs (copy number variations) were interpreted according to the most used appropriate databases of pathogenic and benign variants, including Online Mendelian Inheritance in Man (OMIM) and the Database of Chromosomal Imbalance and Phenotype in Humans Using Ensemble Resource (DECIPHER). Possible complications that were recorded included miscarriage, premature rupture of membranes, infection and fetal deformities.
Statistical analysis was performed with the IBM SPSS statistical package (IBM SPSS Statistics for Windows, Version 26. Armonk, NY: IBM Corp.).

All participating women gave their consent after they were explicitly informed that the results could be used and published anonymously for medical research purposes.

4. RESULTS

The total number of cases that underwent chromosomal micro-array analysis (CMA) was 126. The first group with the indication of isolated sonographic soft markers included 84 fetuses with a median maternal age of 32 years. Fetuses were respectively assigned in 7 subgroups by affected anatomical system, including central nervous system (n=10 cases), craniofacial system (n=20 cases), cardiovascular system (n=15 cases), renal system (n=6 cases), gastrointestinal system (n=6 cases), musculoskeletal system (n=18 cases) and other systems (n=9 cases).

| Anatomical system | Subgroup | Cases | Significant Findings |
|-------------------|----------|-------|----------------------|
| Central nervous system | 10 | 3 | 30% |
| Craniofacial system | 20 | 3 | 15% |
| Cardiovascular system | 15 | 1 | 6.7% |
| Musculoskeletal system | 10 | 3 | 16.7% |
| Intestinal system | 6 | 0 | 0% |
| Renal system | 6 | 0 | 0% |
| Other systems | 9 | 0 | 0% |

Table 1. Diagnostic yield of standard karyotyping and CMA* for different referral indications. *CMA=Chromosomal Micro-array Analysis, N=Number of cases, AMA=Advanced Maternal Age, IUM=Isolated Ultrasound Markers

Clinically significant genetic abnormalities were detected in 10 fetuses (n=10, 11.9%), including 4 cases with Trisomy 21 (40% of abnormal cases), 2 cases with Klinefelter syndrome (47XXY) (20% of abnormal cases) and 4 cases with CMA findings (40% of abnormal cases), namely 2 microdeletion syndromes and 2 microduplication syndromes (Table 1). More specifically, genetic abnormalities were detected in 3 out of 18 fetuses (16.7%) with musculoskeletal findings, in 1 out of 15 fetuses (6.7%) with cardiovascular findings, in 3 out of 20 fetuses (15%) with craniofacial findings, and in 3 out of 10 fetuses (30%) with central nervous system findings. Thus, the subgroup of fetal central nervous system defects carried the highest risk of an underlying chromosomal aberration. In our study’s population all fetuses with mild isolated gastrointestinal or renal abnormalities returned a normal CMA. Trisomy 21 was the most common chromosomal abnormality in fetuses with craniofacial abnormalities, especially those with hypoplastic nasal bone. Interestingly, both fetuses of our study with mild ventriculomegaly were found to be Klinefelter syndrome. Submicroscopic chromosomal aberrations, identified only after CMA, were found in 3 fetuses with short long bones and in 1 fetus with ventriculomegaly.

The second group with advanced maternal age being the indication for invasive testing included 42 fetuses with a median maternal age of 38 years. Significant genetic abnormalities were detected in 3 fetuses (7.1%), including 1 case with Klinefelter syndrome and 2 cases with segmental genomic microdeletions smaller than 10 Mb (Table 1).

Thus, in total, clinically significant genomic alterations were identified in 13 cases (10.3%) of our study, with almost half of them (n=6, 46.2%) identified only by CMA (Table 2). These cases would have missed diagnosis with standard karyotype or QF-PCR alone.

There were no complications after the invasive procedures performed to our center concerning these cases.

5. DISCUSSION

According recent literature data, even in low-risk pregnancies, the likelihood of clinically significant CMA aberrations is substantial. Given that the number of women requesting amniocentesis for advanced maternal age or other minor indications is increased this risk should be discussed with all pregnant women seeking advice.

The present study aimed to go a step further investigating the anatomical systems of the fetus that are more commonly associated with CMA abnormal findings. The
number of referrals of some sub-groups was too small to derive proper conclusions or recommendations. However, some interesting findings can be highlighted.

Specifically, fetal central nervous system abnormalities were most commonly associated with a genetic component. Facial abnormalities were also associated with a high rate of abnormal karyotyping and this can be mainly attributed to the significance of hypoplastic nasal bone. We believe that short nasal bone in the second trimester should be considered as a significant marker for genetic evaluation even in cases with a low risk first trimester screening. Musculoskeletal and cardiovascular abnormalities were also associated with CMA abnormalities, though less commonly. It is known that severe abnormalities in these anatomical systems are commonly associated with a genetic component, but soft markers, such as intracardiac echogenic focus and aberrant right subclavian artery, which were included in our study, are less commonly associated with chromosomal abnormalities. Mild shortening of long bones is also rarely associated with CMA aberrations and other causes are more common, such as growth restriction or constitutional smallness. As expected, mild abnormalities of renal and intestinal systems and single umbilical artery were not associated with CMA abnormal findings.

The increased resolution of CMA over routine karyotyping results in an increased rate of diagnosis of chromosomal abnormalities, many of which are sub-microscopic. There is, however, a risk of producing findings of unknown significance or of highly variable and unpredictable outcome. In the context of a pregnancy, these findings may be difficult to interpret. There is also a small risk of finding pathogenic variants irrelevant to the initial indication, although important for the fetal health and potentially for the family.

According to the Joint Committee on Genomics in Medicine (JCGM) CMA testing is recommended if one or more structural anomalies are identified on an ultrasound scan, though that does not include soft markers which at present do not justify conventional karyotyping. It is stated, however, that testing indications must be updated as further evidence becomes available (15). It is also clear that in case an invasive procedure is finally decided, detailed chromosomal analysis is of benefit.

Taking into consideration the latest data available on the safety of invasive procedures it is expected that couples request for chromosomal micro-array analysis will be augmented (16). The American College of Obstetricians and Gynecologists and the Society for Maternal-Fetal Medicine have stated that fetuses with one or more major structural defects should be assessed by CMA (17).

The Joint Committee on Genomics in Medicine also refers to the CMA variants that should always be reported; any variant that could add information to the management of the pregnancy should be reported regardless the size of imbalance (15).

A study similar to our study including 283 fetuses with structural anomalies detected by ultrasound after a low risk first trimester screening showed a CMA positive rate of 12.7% (36 out of 283 fetuses) and 19 of those cases were sub-microscopic (6.7%) (18). The same study also included 328 cases of advanced maternal age (over 35 years) without other indications. Invasive CMA testing detected clinically significant genetic imbalances in 7 fetuses (2%), including 4 aneuploidies, 2 microdeletions and 1 duplication (18). Our study had similar findings concerning CMA positive rate (11.9%) and sub-microscopic aberrations (4.8%). Slight difference in detection rates might be associated with patient selection bias and number of cases. Even more, in some studies, cases with abnormal standard karyotyping are excluded. Concerning advanced maternal age, our rate of abnormal karyotyping was higher (7.1%), but abnormalities detected only by CMA were noted in only 4.7% of this group of women.

In a study of 5541 low risk pregnancies, clinically significant CMA aberrations were detected in 78 cases (1.4%) with normal ultrasound and from those 47 (60%) were sub-microscopic, so they could be detected only through chromosomal micro-array analysis (19). The study concluded that CMA may be recommended as a first-tier test in pregnancies with normal ultrasound. Considering the fact that the possibility of CMA aberrations is even higher in fetuses with structural abnormalities or in case of advanced maternal age as shown in our study and the existing literature, CMA testing could be an option for such cases.

A similar very recent study including 6431 pregnancies with normal ultrasound at the time of genetic testing evaluated the yield and utility of CMA routine use for prenatal genetic diagnosis, compared with pregnancies with abnormal sonographic findings. The prevalence of a clinically significant CNV related to early-onset disease was 1.1% (72/6431), which was significantly lower than the 4.9% prevalence in high-risk pregnancies. Interestingly, in almost 17% of these low-risk pregnancies, an ultrasound abnormality was discovered later on in gestation (20). Thus, even in low risk pregnancies, the risk of identifying a clinically significant early-onset abnormal CMA result is substantial and should be conveyed to all pregnant women.

It could be argued that CMA testing may lead to detection of variations of unknown significance (called VOUS) but actually this happens only in 1% of the cases (21). This is in compliance to our findings.

We recognize the limitation of our study concerning the number of cases included, however larger studies with strict inclusion criteria are challenging to perform. Nevertheless, our findings are in compliance with the existing literature and future studies are expected to confirm our conclusions and clarify the value of chromosomal microarray analysis in women requesting amniocentesis for isolated sonographic soft markers or advanced maternal age.

6. CONCLUSION

This study presents the risk of underlying chromosomal aberrations in fetuses with minor indications namely isolated structural abnormalities found in routine anatomy scan or pregnancies of advanced-aged women, after maternal request. The study suggests that the incidence of
clinically significant CMA findings is greater than the current procedure-related risk of miscarriage. Therefore, CMA could be considered as a first-tier test in such cases. Appropriate pre- and post-test genetic counseling, as well as personal interpretation of risks and benefits of an invasive procedure, is mandatory. Nevertheless, every case should be individualized and the couple should consent before any intervention.

- **Patient Consent Form:** All participants gave informed consent prior participation.
- **Author’s Contribution:** P.T. and K.G. contributed to the conception and design of the study. P.T. performed data acquisition and statistical analysis. P.T. and K.G. were involved in article drafting. N.A. contributed in manuscript revision and interpretation of data. P.A. contributed in samples collection. All authors have approved the final version of the manuscript.
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