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Re: Microarray application in prenatal diagnosis: a position statement from the cytogenetics working group of the Italian Society of Human Genetics (SIGU), November 2011

We read with interest the paper by Novelli *et al.*¹, concerning the use of chromosomal microarray analysis (CMA) in prenatal diagnosis. The authors recommended that it should be offered only after standard karyotyping, as a second-line test for selected groups of high-risk pregnancies.

Recently, numerous independent prospective studies^{2–7}, involving the use of various strategies and validated with several different array platforms, have demonstrated the effectiveness and usefulness of CMA in clinical prenatal diagnosis. In a large-scale prospective study headed by our center⁶, the average improvement in detection rate using CMA compared with traditional karyotyping was 0.9%. Other similar large-cohort prospective studies have been published^{4,5,7}, all reporting results concordant with our and previous findings. The combined experience from the prospective analysis of a cohort of over 10 000 prenatal samples^{2–7} (12 000 including the updated results from our study), with parallel processing for both CMA and conventional cytogenetic analysis, indicates that the use of CMA in prenatal diagnosis produces a substantial improvement, of ~1–3%, in the detection rate of pathogenic chromosomal abnormalities compared with conventional

karyotyping. In our opinion, there is now no doubt that CMA markedly enhances the detection of fetal chromosomal aberrations, both when it is performed for any clinical indication and when it is performed because congenital malformations are noted on ultrasound investigation^{2–7}.

Novelli *et al.*, in their review of the recent literature, omitted to cite three important large-scale prospective studies^{4–6} that were listed in PubMed and other similar databases at the time of submission of their manuscript. A recent prospective study⁷ also provides a considerable amount of additional clinical data. The conclusions reached by Novelli *et al.* should therefore be considered flawed. These missing data, totaling over 9000 prenatal samples, represent nine times the amount of combined data included in their paper. Had the authors also considered the above papers, they would have found an answer to most of their questions and concerns, especially regarding the diagnostic yield of CMA in different categories of indications.

Novelli *et al.* stated that their position was based on review of the recent literature and the knowledge and experiences of the members of the SIGU Committee¹. Considering the number of papers which provide substantial evidence for the feasibility of introducing CMA into routine prenatal practice as a first-line diagnostic test^{5–11}, we would be interested to learn more about the Committee's knowledge and experience particularly concerning large-scale prospective clinical trials which might contradict the findings of the recent literature. It is also curious that one of the authors of the position statement¹ has recently expressed herself as being strongly in favor of substituting traditional karyotyping with CMA¹¹.

Our view in this discussion is firmly that CMA should be used as a first-line test, since offering CMA only as a second-line test in high-risk pregnancies may substantially limit the diagnostic potential of this assay, missing pathological copy number variations (CNV). The most relevant point in relation to this argument relates to the nine (0.9%) fetal conditions detected in the preliminary results of our still ongoing large-scale prospective trial⁶ that would have remained undiagnosed if only conventional karyotyping had been performed. More importantly, four (0.4%) of these findings would have been overlooked following the diagnostic strategy of using CMA as a second-line test only after standard karyotyping, as suggested by Novelli *et al.*¹. The latest data from our trial continue to show a similar outcome, with 22/2500 (0.9%) submicroscopic chromosomal abnormalities that would have been missed, 15 (0.6%) of which would still have been missed using the above proposed strategy. Similar results have also been reported by other independent large-scale prospective studies^{4,5,7}.

In our opinion, this technology should also be available to all pregnant women undergoing invasive prenatal testing, regardless of risk factors. The updated results of our study speak clearly in favor of such a testing paradigm, showing that the use of aCGH gives an

increased detection rate regardless of the indication for analysis. This became especially evident when examining the data from high-risk groups, in which the detection rate was elevated to 6.5% (7/107), but also in groups with *a priori* low risk for detection of submicroscopic chromosomal abnormalities, such as advanced maternal age (7/958, 0.7%) and parental anxiety (8/1355, 0.6%) groups (unpubl. data). Lee *et al.*⁷ found the average improvement in detection rate in advanced maternal age and parental anxiety groups to be 0.5% (11/1911) and 0.7% (7/973), respectively. Armengol *et al.*⁵ recently reported similar results.

Novelli *et al.*¹ also raised concerns about the potential detection by CMA of mild or unpredictable phenotypes and variations of unknown clinical significance (VOUS). We agree with them that the current challenge in the application of CMA in routine prenatal diagnostic practice is minimizing the potential to detect VOUS whilst maximizing the detection of pathogenic CNVs. However, it is well known that the differences in proportions of VOUS detected is related mainly to the array platform used and its resolution. In our study⁶, we carefully selected a platform specifically developed for prenatal application, with a balance between increased resolution in locations of known constitutional disorders and less coverage in polymorphic regions. This platform allowed us to detect a single VOUS occurrence out of 1037 samples. In a recent systematic review², VOUS were reported to occur in ~1% of prenatal samples. Combining the above data, the average probability of detecting such findings in prenatal samples can be estimated at around 0.3%, similar to the value (5/3171, 0.2%) reported by Lee *et al.*⁷ in their large-scale prospective study. It is also well known that the frequency of detection of VOUS increases if parental samples are not available. This was the case in several papers included in the manuscript of Novelli *et al.* for calculation of the incidence of VOUS.

The point to debate is whether we are able or willing to deal with such a level of VOUS detection. We believe this ~0.3% rate of finding a VOUS does not differ dramatically from that observed with cytogenetic karyotype analysis, with which findings of unclear significance or with unclear clinical consequences are occasionally encountered. Genetic counselors are also familiar with the problem related to CNVs of incomplete penetrance and variable expressivity, and preliminary guidelines are already available¹². VOUS identified by prenatal CMA might be approached in a similar manner and managed by providing patients with thorough pre- and post-test counseling¹³, keeping the family fully informed and remaining respectful of patients' autonomy.

Although detection of VOUS cannot be excluded completely with CMA, the growing clinical experience with genome-wide arrays and the increasing availability, sophistication and size of CNV databases for both healthy and affected individuals, together with parental analysis, allow classification and interpretation of most alterations, thus minimizing the number of VOUS detected and

making CMA increasingly applicable to clinical prenatal diagnostic practice.

We now move on to other aspects mentioned in the position statement of Novelli *et al.*¹, in which a strategy is recommended for prenatal diagnosis involving conventional karyotyping with CMA as a second-line test in high-risk pregnancies. It is evident that by using such a strategy patients would obtain their results more than 3 weeks after initial testing. The rapid confirmation of prenatal findings is essential for purposes of best-practice clinical management. The logic of this approach is therefore questionable since, if CMA is performed and reported to patients only after results from conventional karyotyping are available, instead of simply offering CMA as first-line test, patient anxiety is prolonged.

We should also bear in mind the further disadvantages of conventional karyotyping in routine prenatal diagnosis: the resolution limits of conventional cytogenetic analysis, the requirement for cell culture and the possibility of culture failure or contamination; the variable quality of chromosomal preparations; the need for subjective interpretation which requires skilled and experienced cytogeneticists to carry out the analysis; the labor-intensive nature of karyotyping which limits the possibility for high-throughput automated analysis.

Novelli *et al.*¹ also stated that CMA is unable to detect mosaicism with rates of lower than 30% (low-level mosaicism, LLM). On the contrary, several studies performed in postnatal samples have demonstrated that CMA may detect mosaicism with rates as low as 8–10%^{14–16}. In our study⁶, we were able to detect LLM at rates of 10% in prenatal samples.

Novelli *et al.*¹ also focused on the risk of phenotypic consequence related to the impossibility of CMA detecting *de novo* balanced chromosomal rearrangements. Although we agree with this, in a recent paper this risk has been estimated to be very low (0.0001%)¹⁷.

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Re: Microarray application in prenatal diagnosis: a position statement from the cytogenetics working group of the Italian Society of Human Genetics (SIGU), November 2011

We have read the recommendations proposed by the Italian Society of Human Genetics (SIGU) Committee¹ for the use of chromosomal array (CMA) in prenatal testing. The SIGU Committee recommends that CMA in prenatal testing should never be used as a substitute for conventional karyotyping. Its application is strictly for specific diagnostic purposes in selected pregnancies, not for general prenatal screening. The reason for this is mainly concerns regarding: (1) additional parental anxiety resulting from the detection of variations of uncertain significance (VOUS); (2) the low rate of detection of known, disability-causing pathogenic copy number variations (CNV) by CMA in all pregnant women (0.16–0.3% as quoted in the SIGU recommendation); (3) the impossibility to detect balanced rearrangements with CMA; and (4) the inability to detect polyploidy and low-rate mosaicism.

Since 2011, more and more studies with large prenatal cohorts have been published supporting prenatal screening by CMA. However, these studies were not reviewed in the SIGU article^{2–4}. These new published data may provide us with answers to the four major concerns proposed by Novelli *et al.*¹. We would like to clarify the newly reported evidence in favor of substituting traditional karyotyping with CMA. First of all, the rate of detection of VOUS was 5/3171 (0.3%) in our study⁴ and 1/1037 (0.1%) in that of Fiorentino *et al.*². Secondly, the reported detection rates of pathologic microdeletions or microduplications in all pregnant women were 34/3171 (1.1%)⁴, 9/1037 (0.9%)² and 15/4073 (0.4%)³. These differences between the detection rates of VOUS and pathogenic CNVs further justify the use of CMA technology in the prenatal setting without a specific clinical indication. Thirdly, CMA is limited in the detection of balanced translocation; however, the risk of *de-novo* balanced translocation is very low and translocations in phenotypically abnormal patients are more likely to be associated with genomic imbalances at the breakpoints or elsewhere and with chromosomal complexity⁵. In our prospective survey, array comparative genomic hybridization (CGH) identified genomic losses in two of 17 (11.8%) fetuses carrying *de-novo* balanced translocations, while the remaining 15 newborns had normal phenotype. In clinical scenarios, when *de-novo* balanced translocation is identified by karyotyping and CMA shows no genetic dosage gain or loss, further ultrasonographic follow-up is suggested, rather than surveying for disruption or modulation of the expression of genes located at the breakpoint, inactivation (position effect) of genes or imprinting syndromes. As for the concern of triploidy, array CGH would only miss cases with 69,XXX mixed with female reference DNA (46,XX). Taking this into consideration, we recommend using a

male reference to avoid misdiagnosis. Last but not least, whilst we agree that CMA is hampered by low rates of mosaicism, this applies to rates lower than 10%, not 30% as Novelli *et al.* stated.

Nevertheless, we are not stating that CMA should be used as the only prenatal diagnostic technology. The primary disadvantage of karyotyping is its requirement of cell culture, because of possible culture failure or contamination. Additionally, we have studied two cases with inconsistent results from uncultured and cultured amniotic fluid; one was a case of trisomy 2 and the other trisomy 12p^{6,7}. Conventional karyotyping requires culturing of amniocytes, but abnormal cells may disappear after long-term culturing. Array CGH analysis of DNA from uncultured cells thus offers an advantage over karyotyping in this situation. Besides CMA, regular prenatal examinations and ultrasonographic survey for fetal structural anomalies are still necessary. Novelli *et al.* recommended a strategy for prenatal diagnosis involving conventional karyotyping and CMA as a second-line test in high-risk pregnancies. This strategy may lead to a prolonged wait, need for repeat amniocentesis and involves the drawbacks of cell culture. Due to its high diagnostic rate and short turnaround time, the role of CMA in prenatal diagnosis should be far more than as an adjunct tool preserved for specific purposes. However, it is still worthwhile considering karyotyping as back-up technology to rule out low-rate mosaicism or balanced translocations.

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Reply

We read with interest the comments on our position statement from Drs Fiorentino and Baldi and from Dr Lee and colleagues. Their letters focus on several controversial issues regarding the use of chromosomal microarray analysis (CMA) in prenatal diagnosis.

First of all we would like to highlight that our report does not represent the opinion of any one individual but is a collaborative statement of the cytogenetic working group of the Italian Society of Human Genetics (SIGU). The recommendations were framed after several months of debate among SIGU members, including clinical and laboratory geneticists, and the literature quoted in the position statement was up-to-date at the time of official approval of the document by the working group. In order to keep this as a scientific debate rather than reduce it to a personal one, we will briefly discuss three main points regarding CMA in prenatal diagnosis, in response to the comments made by Fiorentino and Baldi and by Lee and colleagues and referring to the papers they cited to support their opinions.

Firstly, regarding the detection rate of CMA in low-risk prenatal populations (such as advanced maternal age and parental anxiety groups), Fiorentino and Baldi stated that the use of CMA in prenatal diagnosis would allow a substantial improvement of 1–3% in the detection rate of pathogenic chromosomal abnormalities. They then claimed that 0.4% (4/1000) of these findings would have been overlooked following our recommendations regarding the use of CMA. Park *et al.*¹ found 14 cases out of 4073 prenatal samples (0.3%) that would have been missed using conventional cytogenetic diagnosis. Using CMA, Lee *et al.*² identified 15 fetuses with an apparently normal karyotype and cryptic pathogenic

imbalances, among 2900 (0.4%) prenatal samples with an indication for advanced maternal age or parental concern. Finally, the report by Armengol *et al.*³, comparing the currently available assays for detection of chromosomal abnormalities in women referred for advanced maternal age or anxiety, found 12 aberrations in 333 analyzed cases (their Table 1) of which four were not detectable by cytogenetic analysis (their Table 3). We would like to emphasize that in all these cases the pregnancies were continued, delivering healthy babies. Taking into consideration all these findings, and reviewing the data reported in all these prospective studies, the additional detection rate of CMA in the advanced maternal age/parental anxiety population is 33/7973 (0.4%), thus confirming the data of Ogilvie *et al.*⁴ that we cited in our position statement.

Secondly, the frequency of variations of unclear significance (VOUS) depends on which platform is used for prenatal diagnosis; a range of 0–4% has been found for targeted/low-resolution (0.5–1.0 Mb) platforms. This is clearly stated in the SIGU position paper. The study by Armengol *et al.*³ provides further evidence of the frequencies of VOUS using different techniques. These authors analyzed a total of 900 pregnant women with four different techniques (karyotyping, quantitative fluorescent polymerase chain reaction, multiplex ligation-dependent probe amplification, CMA) and a total of 22 VOUS were identified. Using a targeted microarray, 17 VOUS were detected by CMA (1.9%) and only three (0.3%) by karyotyping. In the studies cited by Fiorentino and Baldi, the reported VOUS frequencies were significantly lower: zero in the study of Fiorentino *et al.*⁵, 0.1% in that of Lee *et al.*² and the frequency was not specified by Park *et al.*¹. In all these studies targeted/low-resolution CMA platforms were used, making it difficult to compare results with those of the genome-wide platform recommended in our position statement.

Moreover, our extensive experience in prenatal testing and genetic counseling leads us to emphasize that simplifying the discussion about the best management of prenatal diagnostic genetic testing to a sequence of percentages and technical performances is misleading.

It appears that the incidence of VOUS is also strictly dependent on the criteria of interpretation of CMA results and on the level of expertise involved in database consultation. An accurate review of the cases reported in the above papers supports this consideration and, more generally, the importance of genetic counseling. In pregnant women undergoing CMA for advanced maternal age or anxiety, one of four cases (Table 3, Fiorentino *et al.*⁵), six of 15 cases (Table 3, Lee *et al.*²) and five of five cases (Table 3, Armengol *et al.*³) with inherited VOUS and/or associated with variable, hard-to-predict, phenotype, decided to continue the pregnancy. Surprisingly, Case 9 of Fiorentino *et al.*⁵ opted to terminate the pregnancy in the presence of a 22q11.2 duplication inherited from the normal mother; while in Case 11 of Armengol *et al.*³ the decision was to continue the pregnancy in the presence of the same

duplication, despite the fact that it occurred *de novo*. This duplication is associated with inter- and intrafamilial phenotype variability, and a reliable phenotype–genotype correlation cannot be done. In addition, the two cases with PMP22 deletion and duplication identified by CMA in the study of Fiorentino *et al.* (their Cases 1 and 4), both inherited from affected mothers, should have been suspected during pretest genetic counseling. Finally in 3/3 cases of VOUS reported by Lee *et al.*² (their Table 3) the women opted to terminate the pregnancy. All these data strongly support, according to SIGU guidelines, the necessity, when performing CMA, of pre- and post-test genetic counseling by trained geneticists. Finally, Fiorentino and Baldi pointed out the issue of the best diagnostic strategy. It is obvious that the translation into clinical practice of a diagnostic test is a critical point in the prenatal setting. In the SIGU position statement, CMA has been recommended as a second-level analysis after karyotyping in cases with single/multiple ultrasound fetal malformations and an apparently normal karyotype, and in cases with supernumerary chromosomal markers or *de novo* chromosomal aberrations. A different strategy, using ‘first-tier’ CMA analysis in all pregnant women, would consistently anticipate the cytogenetic diagnosis, providing a better time frame for genetic counseling and the parental decision-making process. However, while respecting the legitimate commercial interests of many operators involved in prenatal diagnosis, we still believe that the analytical sensitivity or the earliness of the test should not be the sole basis for introducing a genetic test into clinical practice. We would like to underline the fact that in the recent European Cytogeneticists Association (ECA) newsletter⁶ (29 January 2012), the Permanent Working Group for Cytogenetics and Society reached, independently, similar conclusions to those reported in our document and included in the Clinical Indications For Investigation Of Array-CGH in the prenatal setting only a few selected conditions (Appendix, page 23). The ECA conclusions, as did ours, take into consideration the whole benefit of the test rather than single features such as sensitivity or earliness. Neither association has any conflict of interest and both evaluated the indication of CMA in terms of performance, quality control, effectiveness and usefulness. Starting from 2000, a model was developed by the CDC’s (Centers for Disease Control and Prevention) Office of Public Health Genomics (OPHG) as a process for assessing the safety and effectiveness of DNA-based genetic tests⁷. This model, the ACCE model, took its name from the terms Analytical validity, Clinical validity, Clinical utility and associated Ethical, legal and social implications. We still believe that, for the good of our patients, this approach is mandatory.

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