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Limited additional value of karyotyping cultured amniotic fluid cell colonies in addition to microarray on uncultured cells for confirmation of abnormal non-invasive prenatal testing results

Stephany H. Donze[1] | Malgorzata I. Srebniak[1] | Karin E. M. Diderich[1] | Myrthe van den Born[1] | Robert-Jan Galjaard[1] | Lutgarde C. P. Govaerts[1] | Vyne van der Schoot[1] | Maarten F. C. M. Knapen[2] | Marieke Joosten[1] | Diane Van Opstal[1]

[1]Department of Clinical Genetics, ErasmusMC, University Medical Center, Rotterdam, The Netherlands

[2]Department of Obstetrics and Prenatal Medicine, ErasmusMC, University Medical Center, Rotterdam, The Netherlands

Correspondence
Diane Van Opstal, Wytemaweg 80, Rotterdam 3015 CN, The Netherlands.
Email: a.vanopstal@erasmusmc.nl

Abstract

Objectives: Non-invasive prenatal testing (NIPT) allows the detection of placental chromosome aberrations. To verify whether the fetus also has the chromosome aberration, diagnostic follow-up testing is required. The aim of this retrospective study was to assess the added value of analyzing amniotic fluid (AF) cell cultures in addition to uncultured AF cells for the detection of fetal mosaicism.

Method: NIPT was performed as part of the Dutch TRIDENT study. Cytogenetic studies in uncultured AF were performed using single nucleotide polymorphism (SNP)-array. Cultured AF cell colonies (in situ method) were investigated with fluorescent in situ hybridization and/or karyotyping. Clinical outcome data were collected in cases with discordant results.

Results: Between April 2014 and December 2021, 368 amniocenteses were performed after a chromosomal aberration was detected with NIPT. Excluding 134 cases of common aneuploidies (confirmed by quantitative fluorescence polymerase chain reaction), 29 cases with investigation of uncultured cells only and 1 case without informed consent, 204 cases were eligible for this study. In 196 (96%) cases, the results in uncultured and cultured cells were concordant normal, abnormal or mosaic. Five cases (2%) showed mosaicism in cultured AF cells, whereas uncultured AF cells were normal. Two (1%) of these, one mosaic trisomy 13 and one mosaic trisomy 16, were considered true fetal mosaics.

Conclusion: The added value of investigating AF cell cultures in addition to uncultured cells is limited to two of 204 (1%) cases in which true fetal mosaicsm would otherwise be missed. The clinical relevance of one (trisomy 13) remained unknown

Stephany H. Donze and Malgorzata I. Srebniak contributed equally.

and the other case also showed ultrasound anomalies, which determined pregnancy management. This seems to justify limiting prenatal cytogenetic confirmatory testing to SNP arrays on uncultured AF cells, considerably shortening the reporting time.

Key points

What's already known about this topic?

- Abnormal non-invasive prenatal testing requires diagnostic testing to verify whether the chromosome anomaly is present in the fetus as well.
- Investigation of both uncultured and cultured amniotic fluid (AF) cells is necessary for accurate detection of true fetal mosaicism.

What does this study add?

- The added value of investigating AF cell cultures in addition to uncultured cells is limited to two of 204 (1%) cases. In these cases, true fetal mosaicsm would otherwise be missed.

1 | INTRODUCTION

Non-invasive prenatal testing (NIPT) is a screening method that allows the early detection of chromosome aberrations in cell-free DNA in maternal blood. NIPT is offered to all pregnant women as part of a nationwide screening program in the Netherlands.[1] It is a reliable method to screen for fetal trisomy 13, 18 and 21, but may also reveal other chromosome abnormalities in the placenta, the fetus and/or the mother.[2,3] Following an aberrant NIPT result, diagnostic testing in chorionic villi (CV) or amniotic fluid (AF) is required to verify whether the chromosome anomaly is present in the fetus as well.[1,4,5] If amniocentesis is performed, our standard procedure is to perform single nucleotide polymorphism (SNP) array on uncultured AF cells and, depending on the chromosomal aberration, also on the blood of the mother. If both results were normal or if mosaicism in AF was found, this was complemented with karyotyping or fluorescent in situ hybridization (FISH) investigations of cultured AF cell colonies (in situ method). In case the SNP array revealed a non-mosaic abnormal result, only a few cells were karyotyped. In cases where NIPT indicated an increased risk of trisomy 13, 18 or 21, SNP array and karyotyping are preceded by rapid aneuploidy detection (RAD) with quantitative fluorescence polymerase chain reaction (QF-PCR) and if positive for trisomy 13 or 21, followed by karyotyping a few cells in order to differentiate between a trisomy or an unbalanced robertsonian translocation. If negative or mosaicism is assumed, the standard procedure of investigating both uncultured and cultured AF cell colonies is applied.

This protocol is based on previous research in which we showed that investigation of both uncultured and cultured AF cells is necessary for accurate detection of true fetal mosaicism (TFM), including an exceptional case of tissue specific chromosomal mosaicism.[6] Based on karyotyping studies, chromosomal mosaicism is estimated to occur in 0.1%–0.3% of amniocenteses and is defined by two or more cell lines with different karyotypes derived from one zygote in a single individual.[7,8] In cultured AF, one or more cells with an identical chromosome abnormality in at least two independent primary cultures are required to diagnose true chromosomal mosaicism, while pseudomosaicism is defined as one or more cells with a chromosomal abnormality in one primary culture.[9]

Karyotyping or FISH investigations of cultured AF cell colonies are costly and time-consuming, resulting in an extended reporting time.[10] With the availability of genome-wide NIPT to all pregnant women in the Netherlands, the absolute number at risk of fetal mosaicism, eligible for this protocol, increased.[11] Therefore, it would be beneficial for patients and laboratories to abolish the analysis of AF cell cultures and only investigate uncultured AF cells. Moreover, cytogenetic techniques have changed since 2001 with FISH on uncultured AF cells being replaced by SNP arrays since 2010.[12] This motivated us to reevaluate our current protocol and assess whether or not analysis of cell cultures in addition to SNP array investigation of uncultured AF cells adds to the prenatal diagnosis of fetal mosaicism. To answer this question, we retrospectively evaluated all cases in which cytogenetic analysis of both uncultured and cultured AF cell colonies was performed for confirmation of an abnormal NIPT result and we collected all cytogenetic and clinical follow-up data of the discordant cases.

2 | METHODS

2.1 | Study design

This study is a retrospective observational study evaluating the results of cytogenetic follow-up testing in uncultured and cultured AF cells after an aberrant NIPT result. Patients who underwent amniocentesis between April 2014 and December 2021 at the Erasmus Medical Center, Rotterdam, The Netherlands were included in the retrospective analysis. Ultrasound, cytogenetic, and clinical follow-up data of the pregnancies were collected in cases where chromosomal

mosaicism was detected in cultured AF cells but not in uncultured AF cells and vice versa.

2.2 | Non-invasive prenatal testing

NIPT was performed as part of the Dutch TRIDENT studies.[2,11,13] Shortly, during the time period 2014–2018, genome-wide shallow sequencing (0.2×; 51bp single-end) was performed on the Illumina HiSeq4000 or the NextSeq500 sequencer (Illumina) as described previously.[2,11,13] From 2018 on, the VeriSeq NIPT Solution was used, initially version 1 until the beginning of 2021,[13] after which version 2 was used. It involves a 36-bp paired-end sequencing on a NextSeq500, according to the specifications of the supplier (Illumina). For the entire time period, bioinformatic analysis was performed using the WISECONDOR algorithm under standard settings to call aneuploidy and other unbalanced chromosomal aberrations.[14] The resolution of the test was approximately 10–15 Mb at the sequencing depth used. In case NIPT indicated T13, T18 or T21 counseling was performed by a consultant obstetrician at a center for prenatal diagnosis. All other cases were counseled by a clinical geneticist.

2.3 | Cytogenetic analysis

Follow-up diagnostic testing of uncultured AF was performed using SNP-array (Illumina Infinium_CytoSNP_850K genotyping array or Illumina Infinium global screening array + MD-24 v3 BeadChip) with a resolution of 0.5 Mb.[12] The sensitivity for mosaicism detection is about 5%–10% depending on the origin of a chromosome aberration.[15] In case of one of the common aneuploidies, SNP array was preceded by QF-PCR (Devyser Compact V3 kit, Devyser). Cultured AF in situ cell colonies were investigated by karyotyping and/or FISH. When SNP array results were normal, at least 24 cell colonies were investigated, excluding a mosaic of 12% or more with 95% confidence.[16] Fetal samples, for example, cord blood or buccal smear, were investigated using the same SNP array and/or FISH. If possible, cytogenetic testing of biopsies from two to four different quadrants of the placenta was performed. The cytotrophoblast (CTB) and mesenchymal core (MC) were separated according to standard techniques and investigated separately.[17]

3 | RESULTS

Between April 2014 and December 2021, 368 amniocenteses were performed after a chromosomal aberration was detected with NIPT (Figure 1). One case was excluded as the couple did not provide consent for scientific evaluations. In 134 of 368 cases (36%), a fetal trisomy 13, 18 or 21 was diagnosed with QF-PCR and only two metaphases were investigated. Cytogenetic testing of uncultured AF cells was performed in only 29 cases, mainly because the SNP array showed a non-mosaic chromosome abnormality or the chromosome

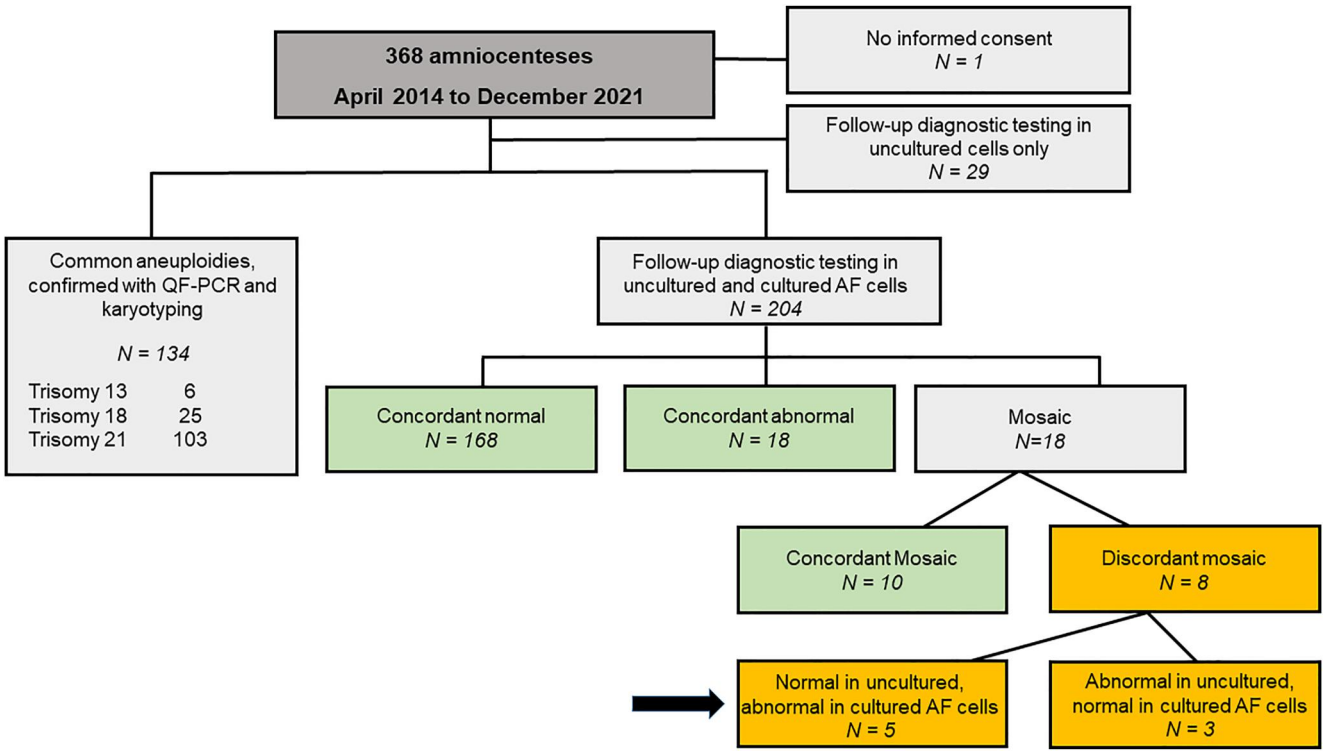

FIGURE 1 Results of cytogenetic analysis of 368 amniocenteses after non-invasive prenatal testing showed a chromosome aberration. In 204 cases, both uncultured and cultured cells were investigated with discordant mosaic results in eight cases (4%): five (3%) showed mosaicism in cultured but not in uncultured amniotic fluid cells and three (1%) vice versa.

aberration detected with NIPT had a maternal origin, both taking away the risk of fetal mosaicism. Therefore, in 204 cases, follow-up cytogenetic studies were completed in both uncultured cells and cultured AF cell colonies. In 186 of 204 cases (91%), results were concordant normal or abnormal. In 18 of 204 cases (9%), chromosomal mosaicism was detected: 10 showed mosaicism in uncultured *and* cultured AF cells (5%), five showed mosaicism in cultured but not in uncultured AF cells (3%) and three vice versa (1%). Of the eight discordant cases, cytogenetic and clinical details are shown in Table 1 and Table S1, respectively.

There were five cases in which only cell cultures revealed chromosomal mosaicism, whereas the uncultured cells were normal. These mosaics would have been missed if karyotyping of cultured AF cells was not performed. These involved (low level) mosaicism for trisomy 8, 13, 16 and 22 and an isodicentric chromosome 9 (Table 1). In three out of these five cases (cases 1, 2 and 5), the chromosome aberration was restricted to one cell colony, which does not comply with the definition of TFM.[9] However, due to previous detection with NIPT and CV in one case, and the possibility of (very) low-level mosaicism in the fetus, an

TABLE 1 Overview of cases with abnormal cytogenetic testing results in cultured but normal results in uncultured AF cells.

	NIPT Result	Prenatal cytogenetics	Postnatal cytogenetics	Pregnancy outcome
1	Trisomy 8	Chorionic villus sampling (GA 13 weeks) CTB: Gain chromosome 8 MC: Trisomy 8 (~70%) Amniocentesis (GA 15 + 3 weeks): SNP array: Normal FISH: Trisomy 8 in 1/19 clones Cordocentesis (GA 20 weeks): SNP array: Normal FISH: Normal	FISH buccal smear: Normal Array umbilical cord biopsy: Normal Array amnion and chorion biopsy: Trisomy 8	Ultrasound: No congenital abnormalities GA 40 + 1 week Birth weight: 2755 g (−1.7 SDS) No congenital abnormalities
2	Gain 9p	Amniocentesis (GA 15 + 5 weeks): SNP array: Normal Karyo: Isodicentric chromosome 9 in 1/37 clones[a]	4 placental biopsies: - CTB: Terminal loss 9p (max 3.3 Mb), gain 9p (max 38.9 Mb) all biopsies, 4q gain (97 Mb) in 1 biopsy, 10q gain (82 Mb) in 1 biopsy - MC: Terminal loss 9p and mosaic trisomy 9 all biopsies, gain 1q (15 Mb) in 1 biopsy	Intrauterine growth restriction GA 39 + 6 weeks Birth weight: 2464 g (−2.3 SDS) No congenital abnormalities Age 8 months: Healthy growth and development
3	Trisomy 13	Amniocentesis (GA 15 + 2 weeks): SNP array: Normal Karyo: Trisomy 13 in 3/31 clones	No postnatal cytogenetics	Ultrasound: No congenital abnormalities Termination of pregnancy GA 19 + 3 weeks Normal physical examination
4	Trisomy 16	Amniocentesis (GA 21 weeks): SNP array: mUPD16 Karyo: Trisomy 16 in 12/32 clones	No postnatal cytogenetics	Ultrasound: VSD and fetal growth restriction Termination of pregnancy GA 23 + 2 weeks
5	Trisomy 22	Amniocentesis (GA 16 + 3 weeks): SNP-array: Normal FISH: Trisomy 22 in 1/38 clones	Fetal samples: Array umbilical cord blood: Normal FISH and array buccal smear: Normal Placental biopsy: Placental villous immaturity Distal villous immaturity Insufficient DNA for further testing	IVF pregnancy Ultrasound: No congenital abnormalities Stillborn GA: 40 + 6 weeks Birth weight: 2739 g (−1.9 SDS)

Abbreviations: AF, amniotic fluid; CTB, cytotrophoblast of chorionic villi; FISH, fluorescent in situ hybridization; GA, gestational age; IVF, in vitro fertilization; MC, mesenchymal core of chorionic villi; mUPD16, maternal uniparental disomy of chromosome 16; SDS, standard deviation score; SNP, single nucleotide polymorphism; VSD, ventricular septum defect.

[a]Not reported during pregnancy.

abnormal result was reported. Two of these pregnancies ended with the birth of a healthy child, one in a stillbirth (Table 1). In two cases (cases 3 and 4 in Table 1), the chromosome aberration was present in multiple cell colonies from different culture flasks, which fits the definition of TFM and would have been missed if AF cell cultures were not investigated. Cases 2, 3 and 4 are presented in more detail below.

3.1 | Case 2—Isodicentric chromosome 9

The NIPT result of case 2 showed a gain of the short arm of chromosome 9 and while the SNP array on uncultured AF cells was normal, an isodicentric chromosome 9 was found in 1 out of 37-cell colonies (~3%). As confined placental mosaicism (CPM) seemed most likely, expert ultrasound at a gestational age (GA) of 20 weeks and additional growth scans every 4 weeks after a GA of 26 weeks were advised. Growth scans revealed fetal growth restriction. A baby girl was born at a GA of 39 weeks and 6 days with a birth weight of 2464 g (−2.3 standard deviation score). At 8 months of age, the child was discharged from pediatric follow-up showing normal growth and development. The gain of chromosome 9p in the CTB of placental biopsies after birth confirmed the aberrant NIPT result. The chromosome aberrations in the MC as detected with SNP array fit the presence of an isodicentric chromosome 9 that was also found in 1 out of 37-cell colonies in cultured AF cells. Retrospectively, a related small deletion of 192 kb on chromosome 9 (chr9:133,828-326,767), involving the *DOCK8* gene, was present in uncultured AF cells. This deletion was not detected during pregnancy due to the 0.5 Mb analysis filter and is associated with carriership of the autosomal recessive disease "Hyper-IgE recurrent infection syndrome" (OMIM #243700).

3.2 | Case 3—Trisomy 13

In case 3, NIPT showed trisomy 13. SNP array on uncultured AF cells was normal, while karyotyping of cultured AF cells showed trisomy 13 in three out of 13 clones (~23%) in two different culture flasks. Ultrasound in early pregnancy did not show fetal anomalies. After genetic counseling, the parents decided to terminate the pregnancy at a GA of 19 weeks and 3 days. Expert ultrasound was not performed, as the presence or absence of congenital abnormalities would not have changed their decision. Although cytogenetic follow-up studies were offered, the parents did not want any further investigation, except for physical examination of the fetus, which did not reveal any anomaly.

3.3 | Case 4—Trisomy 16

The fourth case concerned a NIPT showing trisomy 16. SNP array on uncultured AF cells showed a maternal uniparental disomy of chromosome 16, but no trisomy 16. FISH on cultured AF cells showed trisomy 16 in 12 out of 32 cell colonies (~38%). An expert fetal ultrasound scan at a GA of 20 weeks showed a fetal growth restriction and a ventricular septum defect. The pregnancy was terminated at a GA of 23 weeks and 4 days. Except for slightly low set ears, no dysmorphic features were detected on physical examination. Follow-up investigations in different fetal tissues were offered. The parents, however, declined any further investigation.

4 | DISCUSSION

The aim of this study was to investigate whether cytogenetic analysis of cultured AF cell colonies in addition to SNP array on uncultured cells is still mandatory for accurate detection of TFM in cases of an abnormal NIPT result. We show that the added value of investigating AF cell cultures is restricted to two of 204 (1%) cases in which TFM would have been missed if karyotyping of cultured AF cells was not performed. The clinical relevance of one case (case 3—trisomy 13) remains unknown because of the absence of clinical follow up. The other case (case 4—trisomy 16) also showed structural fetal anomalies, which determined pregnancy management.

Based on these results, it seems justified to limit prenatal cytogenetic confirmatory testing after the detection of a chromosome aberration with NIPT to SNP arrays on uncultured AF cells. This is beneficial for the pregnant women because the results of follow-up cytogenetic testing can be disclosed earlier (in our setting within four to five working days instead of two-three weeks) and waiting time and distress can be reduced. Only performing cytogenetic testing of uncultured AF cells is also of benefit for the laboratory that can abolish the labor intensive karyotyping/FISH on individual cell colonies.

When TFM is detected, it may impair physical and intellectual development depending on the tissue involved, the proportion of cells affected, and the involved chromosome aberration. The phenotypic spectrum of mosaics is therefore very broad, complicating the genetic counseling of pregnant women where TFM is diagnosed.[18–20] Of the detected rare autosomal trisomies during pregnancy, T16 is one of the most common,[21–23] with mosaic T16 mostly resulting from postzygotic trisomic rescue and with an associated risk of uniparental disomy (UPD), as had occurred in our fourth case. The discrepancy between the uncultured and cultured cells is striking, and can possibly be explained by tissue specific fetal mosaicism.[6] Unfortunately, we could not confirm this as the parents refrained from any further investigation after the termination of pregnancy. Fetal mosaicism for T16 is associated with structural fetal anomalies and adverse obstetric outcomes, including fetal growth restriction, preeclampsia, intrauterine fetal demise and preterm delivery.[24–26] This is, however, also seen in cases where CPM for T16 is suspected based on normal results in AF.[24] A possible explanation for the latter is a so-called occult

fetal mosaicism.[27,28] In this study, analysis of cultured AF cells would probably not have changed pregnancy management as SNP array analysis of uncultured cells showed UPD and ultrasound abnormalities were present. Therefore, a fetal mosaic trisomy 16 can never be excluded. In the mosaic T13 case (case 3), concluding whether there was TFM was more difficult and predicting clinical consequences for the fetus was almost impossible. Identifying low-level mosaicism is very challenging if there are no clinical features, that is, ultrasound abnormalities, to relate to.

Besides the two cases with TFM, there were three cases with one abnormal colony (cases 1, 2 and 5). These were considered to be cases of potential low-level fetal mosaicism instead of pseudomosaicism because of the initial detection of the chromosome aberration with NIPT showing that at least the CTB of CV was affected.[9,29] Cytogenetic follow-up investigations in two of three cases, however, make the diagnosis of CPM more likely, which is supported by the clinical outcome of all three cases and confirms previous studies reporting on an association between CPM and an increased risk of adverse pregnancy outcomes.[30–32] TFM can, however, never be excluded based on the analysis of AF cells and a few fetal tissues like cord blood or buccal cells.[2,8,20,28] Therefore, post-test counseling in all cases with normal AF results after abnormal NIPT needs to include information on the risks of potential CPM and occult TFM. Therefore, we conclude that even if cell cultures were not investigated, close monitoring of the pregnancy was indicated anyway.[1,13,23,31,33]

Case 2 is of special interest. Firstly, it illustrates that the abnormal AF cell colony most probably originated from extra-embryonic mesoderm (EEM) since the isodicentric chromosome was seen only in the MC of placental CV that originates from the EEM.[34] It is well known that AF consists of a very heterogenous cell population derived from fetal tissues that are in contact with the AF. This case illustrates that cultured cells may also be derived from the EEM instead of fetal tissues. Secondly, it illustrates that the clinically irrelevant terminal deletion on 9p in the fetus originated from an isodicentric chromosome, only present in the MC of CV, and with the reciprocal product, an inv dup del, present in the CTB as described by Zuffardi et al.[35]

Our results show that to detect all cases of fetal mosaicism, investigation of both uncultured and cultured AF cells is necessary. An exceptional case of tissue specific mosaicism, such as the trisomy 16 and trisomy 13 cases here, could remain undetected if the analysis is restricted to uncultured cells. However, this phenomenon is rare and restricted to two cases in 7 years in our laboratory. Moreover, the detection of low-level mosaicism in AF cell cultures may be very challenging since the clinical consequences are difficult to predict in case of mild or no ultrasound anomalies. Moreover, we show that of five discordant cases, in three cases the chromosome aberration was restricted to just one cell colony that probably had an EEM origin, complicating the differentiation between generalized and CPM. The interpretation of such results is very challenging and may lead to the termination of unaffected fetuses. On the other hand, it has been shown that the level of mosaicism in AF is often not related to the severity of the abnormal phenotype. Therefore, non-detection of fetal mosaicism due to restricting the analysis to uncultured cells may result in the birth of an affected child. This limitation should therefore always be mentioned in the pretest counseling. Moreover, cases with normal AF after abnormal NIPT or chorionic villus sampling also have to be assisted by appropriate post-test counseling on residual risks for occult fetal mosaicism and the likely presence of at least CPM that is associated with adverse pregnancy outcome. Therefore, close prenatal monitoring of the pregnancy is indicated in all cases of confirmed or suspected CPM.[13]

In conclusion, this study shows that when amniocentesis is performed for the indication of an abnormal NIPT, cytogenetic results of uncultured AF cell colonies are in the vast majority of cases (96%) in concordance with those of cultured AF cells. In 7 years, only two true fetal mosaic trisomies, most probably tissue specific mosaics, would not have been identified if the analysis of uncultured AF cells with SNP array was not complemented with the analysis of AF cell cultures. However, the clinical relevance of one (trisomy 13) is unknown and missing the mosaic T16 in cell cultures would not have changed the pregnancy management. Based on these results, we conclude that limiting confirmatory testing by SNP array on uncultured AF cells seems to be justified in cases of abnormal NIPT. This is beneficial for the pregnant woman because of a shorter reporting time (four to five days instead of two to three weeks) as well as for the laboratory that can abolish the labor intensive culturing and karyotyping of individual AF cell colonies.

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CONFLICT OF INTEREST STATEMENT

None declared.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the paper and supplementary material of this article.

ORCID

Stephany H. Donze https://orcid.org/0000-0002-9249-4284
Malgorzata I. Srebniak https://orcid.org/0000-0003-3429-6156
Karin E. M. Diderich https://orcid.org/0000-0003-2637-9998
Diane Van Opstal https://orcid.org/0000-0002-3895-6775

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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