An Improved Method to Extract DNA from 1 ml of Uncultured Amniotic Fluid from Patients at Less than 16 Weeks’ Gestation

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Abstract

The aim of this study was to develop an improved technique for DNA extraction from 1 ml of uncultured AF from patients with a gestational age less than 16 weeks and to allow the use of array-CGH without DNA amplification. The DNA extraction protocol was tested in a series of 90 samples including 41 of uncultured AF at less than 16 weeks of gestation. Statistical analyses were performed using linear regression. To evaluate the sensitivity and the specificity of array-CGH on 1 ml of uncultured AF, five samples with an abnormal karyotype (three with aneuploidy, two with structural abnormalities) and five with a normal karyotype were studied. This protocol was reproducible and we were able to show a great improvement with higher yield of DNA obtained from all patients, including those with a gestational age less than 16 weeks (p = 0.003). All chromosomal abnormalities were detected and characterized by array-CGH and normal samples showed normal profiles. This new DNA extraction protocol associated with array-CGH analysis could be used in prenatal testing even when gestational age is less than 16 weeks, especially in cases with abnormal ultrasound findings.

Introduction

The majority of unbalanced chromosomal rearrangements diagnosed prenatally have been identified by prenatal karyotyping and the prevalence was estimated at 4% by the French Biomedicine Agency (http://www.agence-biomedecine.fr). Prenatal karyotyping is limited by resolution (10 Mb), requires cell culture and results are generally available within 2 weeks. In an attempt to overcome these limitations, alternative detection methods based on comparative genomic hybridization microarray (array-CGH) have been applied to prenatal diagnoses using higher resolution microarrays [1–16].

Historically, amniotic fluid (AF) samples were not considered for use in array-CGH analyses because of insufficient quantities of DNA. In prenatal diagnosis, it was shown that fetal cells extracted from amniotic fluid could be analyzed by array-CGH. Fetal DNA is present in large quantities in amniotic fluid, and Rebello et al. [17] showed that it could be extracted and analyzed by PCR from uncultured amniocytes. Bianchi et al. [18] showed that fetal DNA could be extracted from amniotic fluid supernatant (AF cffDNA) and used for genetic research and clinical applications. It has since been discovered that with an appropriate protocol, it is possible to release microgram amounts of DNA from amniotic fluid and perform array-CGH analysis [8]. The minimum volume of uncultured amniotic fluid was 1 to 4 ml with or without Whole Genome Amplification (WGA) [2,3,7–9]. However, in many publications, WGA was used because it has been shown to amplify the original DNA when not enough DNA is available [3,7–9,14]. Cell culture was not required in some of these published methods [1,2,4,5]. However, fetal DNA was reliably extracted from cell-free fetal DNA [5] or uncultured amniotic fluid (AF) [2,5,8,15] and DNA yields have been reported to be influenced by gestational ages [8].

Here, we describe an improved technique for DNA extraction from 1 ml of uncultured amniotic fluid from patients with a gestational age less than 16 weeks. The technique allows array-CGH analysis to detect chromosomal copy number imbalances prenatally.

Materials and Methods

DNA Extraction Protocol

In the first step, we developed a new DNA extraction protocol, which was tested on 90 samples of uncultured amniotic fluid from a bank of 1400 samples of amniotic fluid collected in our laboratory since 2002 (average of 1 ml) and stored at –80°C. The indications for testing included advanced maternal age, abnormal ultrasound findings, a history of chromosomal abnormalities and convenience. Among the ninety samples of uncultured amniotic fluid (gestation age ranging from 13.1 to 34 weeks), 41 samples

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Concentration with the DNA Clean & Concentrator Kit were according to the protocol provided by Qiagen. Purification and on 10 samples of uncultured amniotic fluid samples of less than 16 weeks of gestation. Approximately 10 μl of purified concentrated DNA was obtained and were estimated using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). The experiments were only performed once in each case, due to the limited quantity of amniotic fluid (1 ml).

Array-CGH

In the second step, we performed microarray Agilent 4×44 K on 10 samples of uncultured amniotic fluid samples of less than 16 weeks of gestation to evaluate the sensitivity of the protocol for the detection of abnormalities using five chromosomal abnormalities and the specificity using five normal karyotypes (Table 1). Furthermore, 12 samples of uncultured amniotic fluid of more than 16 weeks of gestation were analyzed by array-CGH (Table 2).

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Table 1. Summary of karyotype, array-CGH results, indication and DNA isolated from 1 ml of uncultured amniotic fluid at less than 16 weeks' gestation.

| Normal | Samples | Karyotype | Array-CGH (size) | Indication | Gestation (wks) | DNA yield per 1 ml of AF (μg) | DLRS |
|--------|---------|-----------|------------------|------------|----------------|-----------------------------|------|
| Aneuploidy | 1 | 45,X | arr Xx1 | Cystic hygroma | 14 | 78 | 0.26 |
| | 2 | 47,XY,r21 | arr 21X3 | AMA | 15 | 116.2 | 0.25 |
| | 3 | 47,XY,r21 | arr 21X3 | Cystic hygroma | 15 | 50.4 | 0.31 |
| Structural abnormalities | 4 | 46,XY,iarr del(5)(p15.3) | arr 5p15.3 | Convenience | 15.1 | 140 | 0.09 |
| | 5 | 46,XY,iarr del(5)(q22q27) | arr 6q22.q27 | Cystic hygroma | 15.1 | 190 | 0.25 |
| Normal | 6 | 46,XX | arr(1-22)x2 | R>1/100 | 15 | 28.3 | 0.60 |
| | 7 | 46,XX | arr(1-22)x2 | R>1/100 | 15 | 60 | 0.48 |
| | 8 | 46,XX | arr(1-22)x2 | R>1/212 | 15 | 55.1 | 0.41 |
| | 9 | 46,XY | arr(1-22)x2, ΔYx1 | Cystic hygroma | 15 | 41.5 | 0.36 |
| | 10 | 46,XX | arr(1-22)x2 | AMA | 15.4 | 97 | 0.26 |

AMA, Advanced maternal age; IUGR, intrauterine growth retardation; AF, amniotic fluid; R, risk evaluation after triple test screening.
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Aneuploidy 1 45,X arr Xx1 Cystic hygroma 14 78 0.26
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In order to compare our improved technique with previously published techniques. Data reported by Bi et al. [8] were recaptured in order to compare them with ours, under the assumption that the populations studied shared the same baseline characteristics. Amounts of DNA and gestational age were described using their means, standard deviations, medians, and 95% confidence intervals. The normal distribution of DNA quantities and gestational age was checked using polynomial transformations. The logarithmic transformation was the most appropriate in this task and also stabilized their variances. The relationship between DNA quantities and gestational age was tested using a linear regression model, with a robust estimator of weeks of gestation to evaluate the sensitivity of the protocol for the detection of abnormalities using five chromosomal abnormalities and the specificity using five normal karyotypes (Table 1). Furthermore, 12 samples of uncultured amniotic fluid of more than 16 weeks of gestation were analyzed by array-CGH (Table 2). Array-CGH was performed using the Agilent Human Genome CGH Microarray Kit 44 K (Agilent Technologies, Santa Clara, CA). This platform is a high-resolution 60-mer oligonucleotide-based microarray representing 44,000 probes (44 K) that allow a genome-wide survey and molecular profiling of genomic aberrations with an average resolution of ~35 kb [19]. Array-CGH experiments were performed with the maximum amount of DNA available. Array-CGH analysis was performed according to the Agilent protocol with minor protocol modifications: DNA was labeled by direct incorporation of Cy3 and Cy5 using a CGH labeling kit for Oligo-array (Enzo Life Sciences, COVALAB, France) for 4 hr, and labeled products were purified by QIAamp DNA Mini kit (Qiagen, Valencia, CA). A graphical overview was obtained using CGH analytics software (v4.0), and the statistical algorithms ADM-2 according to a sensitivity threshold at 6.0 and a moving average window of 0.5 Mb [20]. Mapping data were analyzed on the human genome sequence build hg18 using ensemble (www.ensemble.org), Copy Number Variations (CNV) were assessed in the Database of Genomic Variants (http://projects.tcag.ca/variation/).

Statistical Method

In order to compare our improved technique with previously published techniques. Data reported by Bi et al. [8] were recaptured in order to compare them with ours, under the assumption that the populations studied shared the same baseline characteristics. Amounts of DNA and gestational age were described using their means, standard deviations, medians, and 95% confidence intervals. The normal distribution of DNA quantities and gestational age was checked using polynomial transformations. The logarithmic transformation was the most appropriate in this task and also stabilized their variances. The relationship between DNA quantities and gestational age was tested using a linear regression model, with a robust estimator of
Table 2. Summary of karyotype, array-CGH results, indication and DNA isolated from 1 ml of uncultured amniotic fluid with chromosomal rearrangement at more than 16 weeks of gestation.

| Chromosomal rearrangement | Samples | Karyotype                          | Array-CGH (size) | Indication                 | Gestation (wks) | DNA yield per 1 ml of AF (ng) | DLRS |
|---------------------------|---------|------------------------------------|------------------|----------------------------|----------------|------------------------------|------|
| Aneuploidy                | 11      | 47,XX,+18                          | arr 18X3         | Multiple anomalies         | 21             | 230                          | 0.29 |
|                           | 12      | 47,XX,+21                          | arr 21X3         | AMA                        | 20             | 330                          | 0.28 |
| Structural abnormalities  | 13      | 47,XX,der(22)(p1terq12)            | arr 22q11.21 X3  | AMA                        | 17             | 395                          | 0.16 |
|                           | 14      | 46,XY,del(2)(q24q31)               | arr 2q24.3q31.1 X1 | Cystic hygroma             | 17.7           | 260                          | 0.18 |
|                           | 15      | arr 3p13 X1 (3 Mb)                 | arr 3p14.1p13 X1 | Ventricular Kyste          | 33             | 550                          | 0.19 |
|                           | 16      | 46,XX,del(7)(q33)                  | arr 7q33q37 X1   | IUGR, heart defect         | 22.8           | 430                          | 0.17 |
|                           | 17      | arr 9q33.3 X1 (3.5 Mb)             | arr 9q33.3 X1    | IUGR                       | 25             | 500                          | 0.16 |
|                           | 18      | 46,XX,dup(11)(pter-q25::q25-q22.2)| arr 11q22.3q24.3 | Convenience                | 20.6           | 840                          | 0.14 |
|                           |         | arr 11q24.3q25 X1 (4 Mb)           |                  |                            |                |                              |      |
|                           | 19      | 46,XX,r(13)                        | arr 13q31.2q34 X1| IUGR, holoprosencephaly    | 24             | 640                          | 0.15 |
|                           | 20      | 46,XY,dup(17)(p12pter)             | arr 17p13.3p13.2 | Multiple anomalies         | 26             | 230                          | 0.18 |
|                           | 21      | 45,XY,der(15;18)(q10q10)           | arr 18p12p10 X1  | Holoprosencephaly          | 26.7           | 570                          | 0.16 |
|                           | 22      | 46,XY,del(22)(q11.2q11.2)          | arr 22q11.21 X1  | Heart defect               | 30             | 180                          | 0.25 |

AMA, Advanced maternal age; IUGR, intrauterine growth retardation; AF, amniotic fluid.
Detected by *postnatal karyotype or by bpostnatal array-CGH.
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Table 3. Summary of DNA yield, purity (260/280 and 260/230 reading) extract by this optimized protocol of the 90 uncultured amniotic fluid.

| Weeks gestational age | DNA yield per 1 ml of AF (ng) | A260/A280 ratios | A260/230 ratios | Samples |
|-----------------------|-------------------------------|------------------|----------------|---------|
| 13.1                  | 90.1                          | 1.59             | 1.4            |         |
| 14                    | 188                           | 1.9              | 1.43           |         |
| 14                    | 78                            | 2.1              | 0.74           | 1       |
| 14.7                  | 67                            | 1.58             | 0.95           |         |
| 15                    | 116.2                         | 2.5              | 1              | 2       |
| 15                    | 115.5                         | 1.93             | 0.71           |         |
| 15                    | 50.4                          | 2.04             | 1.15           | 3       |
| 15                    | 76.5                          | 1.83             | 0.91           |         |
| 15                    | 132.6                         | 1.44             | 0.69           |         |
| 15                    | 238.7                         | 1.75             | 0.67           |         |
| 15                    | 66.3                          | 2.66             | 1              |         |
| 15                    | 76.8                          | 2.53             | 0.34           |         |
| 15                    | 55.1                          | 2.55             | 0.4            | 8       |
| 15                    | 84.7                          | 2.31             | 0.58           |         |
| 15                    | 28.3                          | 2.63             | 0.36           | 6       |
| 15                    | 92.4                          | 2.06             | 0.63           |         |
| 15                    | 41.5                          | 1.94             | 0.27           | 9       |
| 15                    | 69.3                          | 2.28             | 0.65           |         |
| 15                    | 60                            | 1.76             | 0.73           | 7       |
| 15                    | 97                            | 1.68             | 0.87           |         |
| 15.1                  | 140.0                         | 1.94             | 1.31           | 4       |
| 15.1                  | 190.0                         | 1.8              | 1.12           | 5       |
| 15.3                  | 70.1                          | 1.97             | 0.71           | Rnase   |
| 15.3                  | 55.8                          | 2.2              | 1.08           |         |
| 15.3                  | 51                            | 3.34             | 0.66           | Rnase   |
| 15.4                  | 97.0                          | 1.62             | 0.9            | 10      |
| 15.4                  | 82.0                          | 1.7              | 0.75           |         |
| 15.4                  | 82                            | 1.4              | 0.66           |         |
| 15.6                  | 53                            | 1.39             | 0.82           | Rnase   |
| 15.6                  | 65                            | 1.19             | 0.41           | Rnase   |
| 15.6                  | 53                            | 1.82             | 0.66           | Rnase   |
| 15.6                  | 63.7                          | 1.66             | 0.73           |         |
| 15.7                  | 61                            | 1.53             | 1              |         |
| 15.7                  | 87                            | 1.78             | 1.19           | Rnase   |
| 15.7                  | 72.8                          | 2.18             | 1.22           |         |
| 16                    | 169.0                         | 1.77             | 1.09           |         |
| 16                    | 79.6                          | 1.98             | 0.46           | Rnase   |
| 16                    | 94.9                          | 1.5              | 1.09           | Rnase   |
| 16                    | 50.6                          | 1.6              | 0.82           |         |
| 16                    | 67.7                          | 1.66             | 0.86           |         |
| 16                    | 70                            | 1.77             | 0.97           | Rnase   |
| 17                    | 395.0                         | 1.86             | 1.65           | 13      |
| 17                    | 223.0                         | 1.69             | 1.5            |         |
| 17.4                  | 113.0                         | 2.03             | 1.16           |         |
| 17.7                  | 262.0                         | 1.85             | 1.8            | 14      |
| 17.7                  | 172.0                         | 1.76             | 1.01           |         |
| 18.4                  | 213.0                         | 1.92             | 1.33           |         |
| 18.6                  | 108.0                         | 1.56             | 1.18           |         |
The difference between DNA quantities in our study and those of Bi et al. [8] was assessed using the same model (which in this case corresponds to an analysis of variance). A multiple linear regression model, with a robust estimator variance was used to take into account gestational age and type of study simultaneously (Bi vs. ours), as predictors of DNA quantities. Whatever the model considered, logarithmic transforms were used. The adequacy of these models was tested using residuals analysis and by looking at the coefficient of determination.

Table 3. Cont.

| Weeks gestational age | DNA yield per 1 ml of AF (ng) | A260/A280 ratios | A260/230 ratios | Samples |
|-----------------------|------------------------------|------------------|-----------------|---------|
| 18.8                  | 259,0                        | 1,92             | 1,48            |         |
| 19                    | 268,0                        | 1,92             | 1,65            |         |
| 19.7                  | 259,0                        | 1,94             | 1,51            |         |
| 20                    | 330,0                        | 1,78             | 1,6             | 12      |
| 20                    | 263,8                        | 1,81             | 1,55            |         |
| 20.1                  | 219,5                        | 1,82             | 1,4             |         |
| 20.1                  | 89,0                         | 1,31             | 0,86            |         |
| 20.3                  | 134,8                        | 1,9              | 1,51            |         |
| 20.3                  | 381,6                        | 1,83             | 1,74            |         |
| 20.3                  | 208,6                        | 1,84             | 1,55            |         |
| 20.3                  | 276,6                        | 1,8              | 1,83            |         |
| 20.6                  | 840,0                        | 1,82             | 2,01            | 18      |
| 20.6                  | 401,1                        | 1,83             | 1,77            |         |
| 20.6                  | 232,9                        | 1,77             | 1,81            |         |
| 20.7                  | 325,1                        | 1,84             | 1,77            |         |
| 20.7                  | 223,8                        | 1,76             | 1,52            |         |
| 21                    | 230,0                        | 1,87             | 1,47            | 11      |
| 21                    | 652,0                        | 1,84             | 1,97            |         |
| 21                    | 427,5                        | 1,84             | 1,97            |         |
| 21.1                  | 400,4                        | 1,84             | 1,77            |         |
| 21.1                  | 250,4                        | 1,96             | 1,89            |         |
| 21.6                  | 434,6                        | 1,8              | 1,97            |         |
| 21.7                  | 234,5                        | 1,98             | 1,18            |         |
| 21.8                  | 278,5                        | 1,73             | 1,37            |         |
| 22.1                  | 420,4                        | 1,89             | 1,94            |         |
| 22.7                  | 336,2                        | 1,75             | 1,69            |         |
| 22.8                  | 430,0                        | 1,79             | 1,89            | 16      |
| 23                    | 366,0                        | 1,86             | 2               |         |
| 24                    | 644,0                        | 1,81             | 0,37            | 19      |
| 25                    | 505,0                        | 1,86             | 1,8             | 17      |
| 26                    | 235,0                        | 1,87             | 1,42            | 20      |
| 26.7                  | 570,0                        | 1,87             | 1,93            | 21      |
| 28                    | 470,4                        | 1,86             | 1,95            |         |
| 29.7                  | 216,0                        | 1,94             | 1,59            |         |
| 30                    | 181,0                        | 2,29             | 1,24            | 22      |
| 30.8                  | 180,0                        | 1,86             | 1,98            |         |
| 32                    | 349,0                        | 1,82             | 1,45            |         |
| 32                    | 277,0                        | 1,82             | 1,66            |         |
| 33                    | 553,0                        | 1,86             | 1,83            | 15      |
| 33.1                  | 385,0                        | 1,84             | 1,89            |         |
| 33.1                  | 415,0                        | 1,91             | 1,71            |         |
| 34                    | 452,1                        | 1,88             | 1,23            |         |

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Results

DNA Extraction

We extracted between 28.3 and 840 ng of DNA per 1 ml of AF from each of the ninety uncultured samples (Table 3). There was a correlation between the duration of gestation and the amount of DNA (which is already known) and statistically significant differences were observed between the Bi et al. study [8] and our study (109.4 ng vs. 218.6 ng, respectively; p = 0.0147 See Table 4). Moreover, the multiple linear regression analysis showed the same results (Table 5).

For the subgroup of the 41 uncultured samples of amniocytes of less than 16 weeks of gestation with or without chromosomal abnormalities, between 28.3 and 238.7 ng of DNA per 1 ml of AF were extracted (Table 3). The quantities of DNA extracted from the 41 samples of uncultured amniotic fluid were greater than those using the protocol described by Bi et al. [8] (98.1 ng vs. 36.4 ng, respectively; p = 0.0003 See Table 4). The extraction protocol was reproducible and allowed us to obtain sufficient

Table 4. Comparison of DNA yield and gestational age with Bi et al. [2008] study.

| Gestation (wks) | Bi et al., 2008 | Our study - Mean (95% confidence limits) | Bi et al., 2008 | Our study |
|----------------|----------------|-----------------------------------------|----------------|----------|
| ≤16            | 36.4           | 87.3 (73.5–101.1)                       | 2              | 41       |
| >16–20         | 120.7          | 269.5 (204–335)                        | 10             | 23       |
| 21–22          | 191.8          | 372.2 (288.4–456.1)                    | 3              | 11       |
| 23–34          | no data        | 386.5 (304.9–468.3)                    | no data        | 15       |
| Total          | 109.4          | 218.6                                   | 15             | 90       |

Table 5. Results of multiple linear regression analysis performed to assess the independent relationship between DNA level, the study (ours vs. Bi et al. 2008) and gestational age.

| Variables                  | DNA level | β   | Robust SE | 95% CI β | P-value |
|---------------------------|------------|-----|-----------|----------|---------|
| Study (ours vs Bi et al. 2008) | -103       | 14.4 | -131; -74 | 0.0001   |
| Gestational age           | 20.2       | 2.75 | 14.7; 25.6| 0.0001   |
| Intercept                 | -276       | 43.3 | -362; -190| 0.0001   |

The multiple linear regression analysis model adjusted for gestational age ($R^2 = 0.42$).

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Figure 1. Genome views of five number and structural abnormalities characterized by array-CGH, extracted from 1 ml of uncultured amniotic fluid from patients at less than 16 weeks of gestation. (cases 1 to 5).
amounts of DNA with an average A260/A280 ratio of 1.92 for the 41 samples. The average A260/A230 ratio was dependent on the gestational age (0.83, 16 wg, 1.53 17–20 wg, 1.73 21–22 wg and 1.66, 23 wg). The average A260/A280 ratio for the ninety samples was 1.82, indicating that the DNA was of good quality. High-quality genomic DNA samples should have an A260/A280 ratio of 1.8 to 2.0, indicating the absence of contaminating proteins. The use of Rnase in 9 cases did not show any difference for the DO260/280 with or without Rnase (p = 0.699) (Table S1 and Table S2).

**Array-CGH**

**Ten samples <16 weeks of gestation.** Sensitivity: Chromosome imbalances in the five samples of uncultured amniotic fluid of less than 16 weeks of gestation analyzed by array-CGH 4*44 K Agilent were detected and characterized (Table 1). Three number abnormalities and two structural abnormalities (Figure 1

![Figure 2](image-url)
### Table 7. Prospective study of amniotic fluid (AF) by array-CGH.

| Study                        | Number of AF | Indication | Abnormalities detected by array-CGH % | Abnormalities not detected on karyotype % | Array types | DNA preparation                                      | Volume of AF |
|------------------------------|--------------|------------|---------------------------------------|-------------------------------------------|-------------|-----------------------------------------------------|--------------|
| Sahoo et al. [2006]          | 56           | AMA, AU    | 5                                     | 0                                         | BAC 1476 clones | Uncultured AF with amplification (n = 26) Cultured AF (n = 30) | 5 ml         |
| Miura et al. [2006]          | 13           | AU         | 46                                    | 0                                         | BAC 50 clones  | AF cffDNA                                            | 10 ml        |
| Choe et al. [2007]           | 118          | AMA, AU    | 5                                     | 0                                         | BAC 1440 clones | Uncultured AF with amplification                     | 4 ml         |
| Van den Veyver et al. [2008] | 254          | AMA, AU    | 5                                     | 2                                         | BAC 1476 clones | Uncultured AF with (n = 50) or without (n = 38) amplification | 5–7 ml       |
|                              |              |            |                                       |                                            |              |                                                     |              |
| Shaffer et al. [2008]        | 15           | AMA, AU    | 1.3                                   | 1.3                                       | BAC 4670 clones | Cultured AF (n = 151)                                  | no data      |
| Bi et al. [2008]             | 15           | AMA, AU    | 6.6                                   | 0                                         | Oligo 44 000   | Uncultured AF with (n = 2) or without (n = 13) amplification | 5–10 ml      |
| Kleeman et al. [2009]        | 50           | AU         | 2                                     | 2                                         | BAC 1887 clones | Uncultured AF (n = 6)                                  | 5–48 ml      |
|                              |              |            |                                       |                                            |              |                                                     |              |
| Coppinger et al. [2009]      | 188          | AMA, AU    | 3.8                                   | 2.7                                       | BAC 4685 clones | Cultured AF (n = 44)                                  | no data      |
|                              |              |            |                                       |                                            |              |                                                     |              |
| Maya et al. [2010]           | 204          | AMA, AU    | 2.6                                   | 1.1                                       | BAC 1476 clones | Uncultured AF (n = 123)                                | no data      |
|                              |              |            |                                       |                                            |              |                                                     |              |
| Park et al. [2011]           | 4033         | AMA, AU    | 1.8                                   | 0.27                                      | BAC 1440 clones | Uncultured AF with amplification                      | 4 ml         |
| Fiorentino et al. [2011]     | 938          | AMA, AU    | 3.3                                   | 0.9                                       | BAC 3161      | Uncultured AF (n = 924)                                | 5–15 ml      |
|                              |              |            |                                       |                                            |              |                                                     |              |
| Lee et al. [2012]            | 2996         | AMA, AU    | 1.2                                   | 1.1                                       | BAC 1093 clones | Uncultured AF (n = 19)                                | 15 ml        |
|                              |              |            |                                       |                                            |              |                                                     |              |

AMA, advanced maternal age; AU, abnormal ultrasound.

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and Table 1) were characterized in DNA from the uncultured amniotic fluid. The quality control from Agilent with DLRSp (Derivative Log Ratio Spread) showed from excellent to good quality for the five samples (DLRSp between 0.26 and 0.60). Detection of CNV showed an average of three per patient ranging from 400 bp to 600 kb; all were found in the Database of Genomic Variants (DGV).

**Twelve samples >16 weeks of gestation.** For the 12 samples of uncultured amniotic fluid with aneuploidy or structural abnormalities at more than 16 weeks’ gestation, all of the chromosomal imbalances were detected and characterized by array-CGH (Table 2 and Figure 2).

### Discussion

In recent years, there has been considerable progress in the use of array-CGH in the postnatal diagnosis of mental retardation [21]. Studies of fetal tissue with multiple malformations or spontaneous miscarriages revealed 10% of unbalanced rearrangements [22–26]. Larrabee et al. [1] were the first to test the array-CGH technique to analyze fetal DNA extracted from AF cffDNA and to compare the results obtained by array-CGH with those obtained with conventional karyotyping. In the literature, in a prenatal context, array-CGH analysis of cultured AF has been applied to marker or apparently balanced translocations, and has allowed genetic counseling [27,29].

Five retrospective [1,2,5,11,29] and twelve prospective studies [3,4,6,7,8,9,11,12,13,14,15,16] of prenatal array-CGH with different resolutions and involving uncultured amniotic fluid have been published (Table 6 and 7). Only one of them matched the aims of the present study, i.e. to describe a new method for DNA extraction from small quantities of AF, but the authors only tested two samples <16 WG [8]. Our results suggested that our improved protocol gave significantly higher quantities of DNA than did the Bi et al. [8] protocol (Table 4 and 5). Our new protocol for DNA extraction from uncultured AF generated DNA of superior quality in greater quantities for array-CGH from less AF. This protocol allowed the rapid analysis of the fetal genome without the need for cell culture, and therefore earlier diagnosis, or retrospective studies when only a small quantity of frozen AF is available. Even if the DLRSp are not-optimal according to the recommendation protocols, all the known chromosomal rearrangements were confirmed. In one case, the array-CGH also showed a duplication (5p15, case 4) that was not detected by prenatal karyotyping because the size of the abnormality was too small to be detected by karyotype. Therefore, this method should be restricted to certain cases when biologists only have a small quantity of sample available. For array-CGH analysis on an Agilent platform, a yield of 50 ng is sufficient to obtain results using the protocol described here, without background noise. Many post-natal reports have demonstrated the sensitivity, specificity and accuracy of array-CGH in detecting large and small imbalances. In our prenatal study, array-CGH showed reproducible results with high sensitivity and specificity in the analyses of 10 samples of uncultured amniotic fluid samples of less than 16 weeks of gestation. In our retrospective study, we were able to detect and characterize all of the rearrangements using 1 ml of uncultured AF before or after 16 weeks of gestation. We tested the DNA extraction protocol described by Rickman et al. [2] with 1 ml of uncultured AF without success (the amount of DNA extracted was between 33 and 130 ng from ten samples of uncultured amniotic fluid, gestational age ranging between 19 and 24 weeks). However, in prenatal studies, array-CGH is limited by the fact that this technique is unable to detect polyploidy and balanced chromosomal rearrangements. Furthermore, array-CGH has the potential to uncover unwanted information in the prenatal setting (genes that predispose for cancer, heterozygosity for autosomal recessive disease, carrier of X linked disease), which creates ethical problems in a prenatal context. In prenatal diagnosis, the detection of new CNV not reported in the Database of Genomic Variants (DGV), or variants with incomplete penetrance without ultrasound abnormalities could lead to major difficulties in genetic counseling.

In conclusion, this study describes a reproducible method for DNA extraction from only 1 ml of uncultured amniotic fluid with significantly greater quantities recovered in our study than in the Bi et al. study [8]. This improved protocol for DNA extraction from 1 ml of uncultured AF permits array-CGH analysis in prenatal diagnosis from patients at less than 16 weeks’ gestation. This technique can be applied to retrospective and prospective studies and benefits from a number of advantages: short turn-around time, absence of culture and high resolution. Until array-CGH becomes a standard technique for prenatal diagnosis, it should be considered a complement to the standard prenatal karyotyping.

### Supporting Information

**Table S1 Summary for variables (do260280) by categories of Rnase.**

**Table S2 Linear regression for variables (do260280) by categories of Rnase.**

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### Author Contributions

Conceived and designed the experiments: PC. Performed the experiments: CR MP NM ALM-B JT SEC. Analyzed the data: SA CT PC. Contributed reagents/materials/analysis tools: CT-R TR PS FH FM. Wrote the paper: LF PC.

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