DOI: 10.1002/pd.4793

ORIGINAL ARTICLE

Embryo-fetal erythroid cell selection from celomic fluid allows earlier prenatal diagnosis of hemoglobinopathies

Antonino Giambona[1]*, Gianfranca Damiani[2], Filippo Leto[1], Cristina Jakil[2], Disma Renda[1], Valentina Cigna[2], Giovanna Schillaci[2], Francesco Picciotto[2], Kypros H. Nicolaides[3], Cristina Passarello[1], George Makrydimas[4] and Aurelio Maggio[1]

[1]Unit of Hematology for Rare Diseases of Blood and Blood-forming Organs, Regional Reference Laboratory for Screening and Prenatal Diagnosis of Hemoglobinopathies, Palermo, Italy
[2]Unit of Prenatal Diagnosis, Hospital Villa Sofia Cervello, Palermo, Italy
[3]Harris Birthright Research Center for Fetal Medicine, King's College, London, UK
[4]Obstetrics and Gynecology, Ioannina University Hospital, Ioannina, Greece
*Correspondence to: Antonino Giambona. E-mail: a.giambona@villasofia.it

ABSTRACT

Objective Celocentesis, which involves aspiration of celomic fluid at 7–9 weeks' gestation, can potentially provide early prenatal diagnosis of single-gene disorders. The main barrier to wide acceptability of this technique is contamination of the sample by maternal cells. This problem can be overcome through selection of embryo-fetal erythroid precursors, which are found in celomatic fluid.

Method Embryo-fetal erythroid precursors were selected by an anti-CD71 MicroBeads method or by direct micromanipulator pickup of the cells selected on the basis of their morphology.

Results In our series of 302 singleton pregnancies at high risk for hemoglobinopathies, Celocentesis provided a sample of celomic fluid in all cases. In 100 (33.1%) samples, maternal contamination was absent or very low (<5%), and unambiguous results were obtained without the need for any preliminary procedures. In 160 (53%) cases, the contamination was between 5% and 60%, and selection of embryo-fetal erythroid precursors was successfully achieved by anti-CD71 MicroBeads. In 42 (13.9%) cases, the contamination was >60%, and selection of embryo-fetal cells was achieved by micromanipulation. In all 302 cases, there was concordance between DNA obtained from celomic fluid samples and fetal or newborn DNA.

Conclusions Celocentesis can be a reliable procedure for earlier prenatal diagnosis of fetal monogenic diseases.

Funding sources: Project Sistema di purificazione di cellule fetali per indagini prenatali precoci, Linea di intervento 4.1.1.1 del PO F.E.S.R. Sicilia 2007–2013. A grant supported by Foundation Franco and Piera Cutino ONLUS (Palermo, Italy).
Conflicts of interest: None declared

INTRODUCTION

In countries with a high prevalence of hemoglobinopathy carriers, the only realistic approach to control the birth of new patients with thalassemia major or sickle cell disease is population screening in combination with invasive prenatal diagnosis.[1–3] Prenatal diagnosis for hemoglobin disorders was initiated in 1970s by the use of globin chain synthesis analysis of fetal blood obtained by fetoscopy or placental aspiration at 18 weeks' gestation.[4] In the early 1990s, molecular definition of the thalassemia defects, development of procedures for their detection by DNA analysis and introduction of amniotic fluid sampling (amniocentesis) or chorionic villus sampling (CVS) led to early prenatal diagnosis at 16 and 11 weeks.[5,6]

Celocentesis, in which celomic fluid (CF) contains cells of fetal origin, can be carried out at 7–9 weeks' gestation.[7,8] Animal studies in baboons have shown that celocentesis may be a safe procedure.[9] However, studies in pregnant women reported contradictory results. One small study in 20 women undergoing celocentesis prior to pregnancy termination reported a 25% rate of fetal loss within a few days after the procedure.[10] In contrast, in a study of 108 women undergoing celocentesis 1–3 weeks before elective termination and 339 controls, the rate of fetal death during this interval was only 4.7% in the celocentesis group and 2.7% in the controls.[11]

Previous studies utilizing celocentesis for prenatal determination of fetal sex and diagnosis of single-gene defects reported variable success, ranging from 58% to 95%, because of low total DNA content and presence of maternal cell contamination (MCC).[12–16] The problem of low amount of DNA can be overcome with the use of sensitive PCR techniques as for pre-implantation diagnosis. However, maternal contamination remains the main

difficulty for prenatal diagnosis on CF. The objective of this study is first to describe the use of a micromanipulator to identify embryo-fetal cells by optical phase contrast microscopy and aspirate each one cell through a glass micropipette in cases of high maternal contamination and second to describe a work flow to obtain samples of CF suitable for prenatal diagnosis of monogenic genetic disorders.

MATERIALS AND METHODS

Subjects

During a 9-year period (2006–2015), 302 couples at risk for β-thalassemia or sickle cell disease (Table 1) asked for prenatal diagnosis by celocentesis in our hospital in Palermo, Italy. The couples were counseled that this technique is offered within the context of research because of limited data on risks and diagnostic accuracy and were advised that it may be preferable for them to undergo conventional prenatal diagnosis (CVS or amniocentesis). The institutional review board approved the study, and the participating couples provided written informed consent (hospital ethical committee authorization on date 26 January 2005, no. 80). The advice from the ethical committee was that if the results from celocentesis suggested that the fetus was not affected, the diagnosis should be confirmed by CVS at 12 weeks' gestation or amniocentesis at 16 weeks.

Sampling

Celocentesis was carried out at between 6^{+6} and 9^{+2} weeks' gestation by one of four operators (G. M., G. D., C. J. and F. P.). Sampling involved transvaginal sonography, insertion of a 20-G needle through the fornix into the celomic cavity and aspiration of a total of 1 mL of fluid into three different syringes (0.2, 0.2 and 0.6 mL). Contamination was higher in the first two samples, and although all samples were analyzed, the third was considered as the diagnostic sample.

Work-flow laboratory procedure

The CF samples were first evaluated for contamination. Second, embryo-fetal cells were selected, and finally, β-globin gene analysis was carried out. Our work-flow laboratory procedure is shown in Figure 1.

Table 1 **Phenotype of the couples requesting early prenatal diagnosis by celocentesis**

Phenotype of couples	Number of couples
β^{Thal}–β^{Thal}	239
β^{Thal}–β^{SCD}	44
Sicilian $\delta\beta^{Thal}$–β^{Thal}	8
β^{Thal}–$\beta^{Lepore\text{-}Boston\text{-}Washington}$	5
β^{SCD}–β^{SCD}	4
$\beta^{SCD\ affected}$–β^{SCD}	1
Sicilian $\delta\beta^{Thal}$–Sicilian $\delta\beta^{Thal}$	1

SCD, sickle cell disease.

Preliminary contamination evaluation

Genomic DNA of both components of at-risk couple was extracted using standard protocols.[17] One-tenth volume of each sample of CF was aspired and placed in an Eppendorf tube (0.5 mL). Cell pellet was collected by centrifugation at 12 000 rpm for 10 min and dissolved in 30 μL of 5% IstaGene matrix (Bio-Rad Laboratories, Hercules, CA, United States). The sample was incubated at 56 °C for 30 min and at 95 °C for 10 min, and supernatant (25 μL) containing DNA was removed and transferred into a clean Eppendorf tube. All samples underwent multiplex quantitative fluorescent PCR (QF-PCR) using small tandem repeats (STR) markers.[18,19] One multiplex PCR reaction was set up using 14 STR markers for chromosomes X, Y, 13, 18 and 21 selected on the basis of their highly polymorphic nature (AMXY; HPRT; SRY; DXS1187; DXS8377; D13S325; D13S634; D13S631; D18S499; D18S535; D18S386; D21S1435; D21S1411; D21S1442). The reaction was set in a final volume of 20 μL containing 5 μL CF DNA, 10 μL of 2X Platinum Multiplex PCR Master Mix (Life Technologies, Carlsbad, CA, United States), 3–25 pmol of each primer and H_2O to final volume. After denaturation at 95 °C for 2 min (hot-start procedure), the sample was subjected to 35 repeating cycles of amplification (95 °C 30 s, 60 °C 90 s and 72 °C 60 s) and a final extension of 30 min at 72 °C in a thermal cycling using the Gene Ampl 9700 (Applied Biosystems, Foster City, CA, United States). The PCR products (2 μL) were mixed with 15 μL of formamide and 0.4 μL of Genescan-500 Liz containing the reference molecular size standard. Capillary electrophoresis and analysis of the fluorescent PCR products and size standards were performed with ABI PRISM 3130 automated DNA sequencer and GENESCAN 4.0 software analyzer (Applied Biosystems, Foster City, CA, United States). All data assessed by the QF-PCR tests on CF were compared with the results obtained from parents. As reported by Allen *et al.*,[20] analysis of a paternal sample should be used for maternal contamination exclusion as this may raise nonpaternity issues. However, in cases of prenatal diagnosis of hemoglobinopathies, the identification of paternal mutations in the fetus is essential for the diagnosis. The aim is accurate prenatal diagnosis, and in cases of unknown father or when there is a paternity issue, all β-globin genes are analyzed by sequencing to exclude the presence of any other mutations. During the preliminary genetic counseling, couples were exhaustively informed that molecular analysis on the father DNA gives information on paternity.

Evaluation maternal contamination

In case of maternal cell contamination, QF-PCR shows characteristic alleles profiles with extra allele peaks of maternal origin for all chromosomes. The proportion of contamination can be assessed if maternal profile is exclusively heterozygous. It is possible to establish the percentage of maternal contamination in CF by comparing the area of extra maternal contaminant peak with the area of maternal. The ratio of these numerical data allows calculation of the percentage of the extra peak and then maternal contamination (area maternal peak – area contaminated peak = *N*; MCC% = area contaminated peak X100/N). Analysis of paternal pattern is further helpful

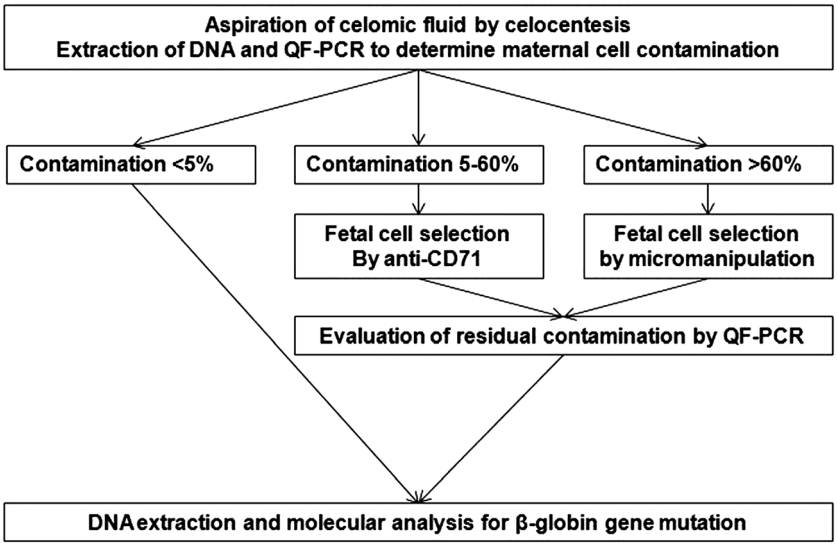

Figure 1 Work flow for celomic fluid analysis

for a more accurate assessment of the degree of maternal contamination (Figure 2).

Fetal cells in the celomic fluid

We have shown previously that the most common cells in CF are embryonic nucleated red blood cells.[21] Detailed analysis revealed a prevalent megaloblast morphology (Figure 3), CD71+/CD45− membrane antigens and mRNA expression of ε and γ globin, consistent with embryonic erythroid precursors. We used anti-CD71 antibody to isolate ENRBC. Although these antibodies are specific for any erythroid precursor of fetal and maternal origin, we have not identified maternal erythroblasts in CF as demonstrated by STR segregation analysis using QF-PCR.

Selection of fetal cells

Celomic fluid samples with no or very low (<5%) maternal contamination were successfully analyzed without preliminary

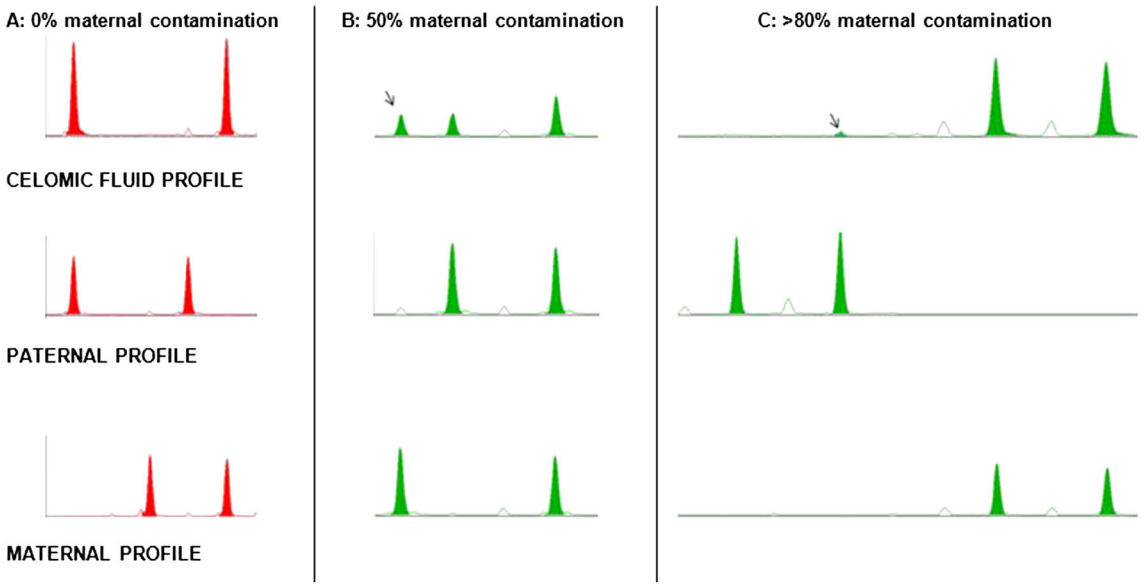

Figure 2 Evaluation of celomic fluid for maternal cell contamination by quantitative fluorescent PCR. (A) Electropherograms of amplification product from celomic fluid without maternal contamination using small tandem repeats (STR) markers specific for chromosome 13 (D13S631). In the electropherogram of celomatic fluid, there is one peak of paternal and one peak of maternal origin. (B) Electropherograms of amplification product from celomic fluid with about 50% of maternal contamination using STR markers specific for chromosome 13 (D13S325). In the electropherogram of celomatic fluid, there is one peak of paternal and two peaks of maternal origin. The arrow indicates the maternal extra contaminant peak, which is of the same area as that of the paternal peak. (C) Electropherograms of amplification product from celomic fluid with high maternal contamination (>80%). Use of STR markers specific for chromosome 21 (D21S1411) demonstrates the presence of a low paternal peak in the celomic fluid, which indicates a high maternal contamination. The arrow indicates the low paternal peak present in the celomic fluid

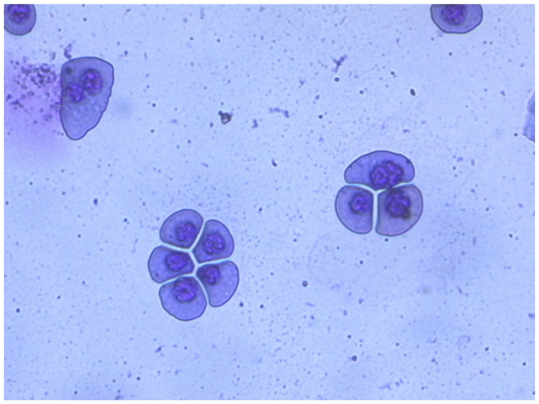

Figure 3 Cytospin and May Grunwald–Giemsa stained fetal erythroblast cells obtained from celomic fluid at 8 weeks' gestation. The cells are slightly larger than an erythrocyte, the nucleus is condensed, and the cytoplasm is of similar color to that of the erythrocyte This figure is available in colour online at wileyonlinelibrary.com/journal/pd

treatment. In samples with >5% maternal contamination, two different procedures were used to isolate embryo-fetal cells. The first technique involved positive selection of embryo-fetal erythroid precursors by anti-CD71 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The sample was placed in an Eppendorf tube and centrifuged at 1000 rpm for 7 min. The pellet was then dissolved in 50 μL of phosphate-buffered saline (PBS) and 2.5 μL of anti-CD71 (MicroBeads) was added and incubated at 4 °C for 15 min. Subsequently, PBS (500 μL) was added to the tube, centrifuged at 1000 rpm for 7 min, and the pellet redissolved in PBS (550 μL). The sample was then applied to a MiniMACS separation column (Miltenyi Biotec), and all the liquid containing no-fetal erythroid cells (negative selection) was eluted into an Eppendorf tube. After two washes of the column with 1 mL of PBS, additional 0.5 mL of PBS was added, and a plunger was used to elute selected fetal cells. DNA was extracted by 5% IstaGene matrix as previously reported and successively amplified.

The second procedure to isolate embryo-fetal cells was through the use of a micromanipulator. Embryo-fetal nucleated red cells in the CF were identified by optical phase contrast microscopy. These are roundish cells with a size about 12–16 μm in diameter and a high cytoplasmic nuclear ratio and nucleus polarized to one side of the cell near the wall (Figure 4A). Cells were aspirated one by one using a 45-μm glass micropipette (BioCare Europe) and placed into a drop of 0.9% NaCl in the same petri disk (Figure 4B). Each drop containing a group of embryo-fetal erythroid precursor cells was placed into a 0.2-mL Eppendorf tube. The sample was centrifuged at 10 000 rpm for 7 min, and supernatants were discarded. All samples obtained were subjected to DNA extraction by alkaline method[22] and DNA amplified using a nested PCR because the quantity of fetal DNA was too low to obtain PCR product using one step of amplification.

β-Globin gene analysis (MIM 141900)

After cell selections, maternal cell contamination test by QF-PCR was performed once again to be sure that all selected cells were of fetal origin. Samples were analyzed for β-globin gene (HBB MIM#141900). PCR was performed in 50-μL reactions using 20 ng of DNA, 200 μmol/L of each dNTP (Roche Diagnostics GmmH, Mannheim, Germany), 1X PCR buffer 1.5 mmol/L $MgCl_2$, 10 pmol/L of each primer and 1.5 units of Taq polymerase (Invitrogen, Valley Blvd, San Diego, CA, United States). After one cycle of denaturation at 96 °C for 5 min, the reaction was performed for 35 cycles of 94 °C 30 s, 55 °C 30 s and 72 °C for 45 s followed by 10 min at 72 °C. Amplified products were analyzed by the direct sequencing method using BigDye Terminator 3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, United States). Runs were performed on ABI PRISM 3130 DNA analyzer (Applied Biosystems), and results of CF single nucleotide polimorphisms (SNPs) in the β-globin gene were compared with those obtained from parent DNAs. Primer for amplification and sequencing are reported in Table 2. SNPs in β-globin gene were also used to check maternal contamination and for linkage analysis.

Laboratory procedures to avoid cross-contamination

Because PCR-based analysis of a single cell requires many cycles of amplification, it is vulnerable to the accidental introduction of contaminating DNA. During the PCR steps, sources of contamination include operator contamination

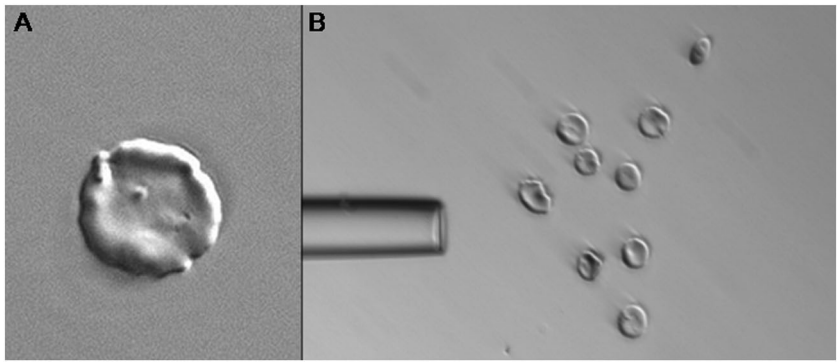

Figure 4 Fetal erythroid cell (12–16 μm in diameter) observed by optical phase contrast microscopy at 40× magnification (left). Fetal erythroid cells showing the same morphology by 40× optical phase contrast microscopy are aspirated one by one using a micromanipulator with 45-μm glass micropipette (right)

Table 2 Primer sequences for β-globin gene

Primer	Sequence (5′-3′)	Position (NG_000007.3)
β-Globin gene analysis: first PCR		
A (forward)	AGAGATATATCTTAGAGGGAGGG	70341–70363
B (reverse)	ACAATGTTAAGGCATTAAGTATAATAG	71270–71296
C (forward)	GTACGGCTGTCATCACTTAGACCTCA	70416–70441
D (reverse)	GAAAACAATTGTTATGAACAGCA	71200–71222
β-Globin gene analysis: nested PCR		
E (forward)	TGCATATTCATAATCTCCCTACTTT	71400–71424
F (reverse)	GAATAAGGCATATGCATCAGGG	72325–72346
G (forward)	GTACACATATTGACCAAATCAGGG	71498–71521
H (reverse)	TGTGCATTAGCTGTTTGCAGCCT	72291–72313
β-Globin gene analysis: sequencing		
Seq1 (forward)	GGCCAATCTACTCCCAGGAG	70467–70486
Seq2 (reverse)	TAAAAGAAACTAAAACGATCCTG	71175–71197
Seq3 (forward)	TAATACTTTCCCTAATCTCTTTCTTT	71590–71615
Seq4 (reverse)	CTGACCTCCCACATTCCCTT	72189–72208

The β-globin gene is sequenced from −204 bp downstream of the transcriptional start site to nt 257 in the Intron II of β-globin gene and from nt 361 in the Intron II nt 361 to 320 bp downstream of stop codon of β-globin gene (NG_000007.3).

and carry-over contamination, both of which were minimized using stringent conditions as for a pre-implantation genetic diagnosis laboratory. Physically separated working places were used for fetal cell selection, template preparation before PCR, setting up PCR reactions and post-PCR analysis. DNase-free and RNase-free thin-walled PCR tubes, special aerosol-resistant pipette tips and a dedicated (only for PCR) set of pipettes were used. PCR reactions were set up under a fume hood equipped with ultraviolet light. Microcentrifuge and disposable gloves were used only for PCR under the fume hood, the importance of changing gloves frequently was stressed, and contamination in the system was monitored by the inclusion of negative controls and blanks at all stages.

RESULTS

Celocentesis was successfully performed in all cases, and an adequate sample was obtained at first attempt in 298 cases and after the second attempt in four cases. The volume of CF used for diagnosis was between 450 and 870 μL. In 100 (33%) samples, there was no or very low (<5%) maternal cell contamination, and unambiguous results were obtained without the need for any preliminary procedures. In 202 (67%) samples, the maternal cell contamination was >5%, and selection of embryo-fetal cells was necessary. In 160/302 (53%), maternal cell contamination was between 5% and 60%, and selection of embryo-fetal erythroid precursors was successfully achieved by anti-CD71 MicroBeads. In the remaining 42 (14%) cases with maternal cell contamination >60%, residual maternal DNA was observed after selection with MicroBeads, and selection of embryo-fetal cells by micromanipulation was carried out. In two cases (0.6%), no embryo-fetal erythroid cells were observed. After selection by MicroBeads, a variable yield from 30 to 100 cells was obtained, while when we used the micromanipulator, we selected only a few units of cells (2–20 units).

β-Globin gene was amplified from all samples, and the corresponding paternal and maternal DNAs were studied as markers.

In two (0.7%) of the 302 cases, diagnosis was not possible from CF because of the absence of fetal cells; in these cases, prenatal diagnoses were made by CVS. In 68 (22.6%) of the 300 cases, the fetus was affected by β-thalassemia major or sickle cell/β-thalassemia, and 66 (97.1%) of these women chose to terminate the pregnancy. Two families decided to continue the pregnancy for ethical reasons despite the documented presence of an affected fetus, and diagnoses were confirmed after birth. In all 66 pregnancies that were terminated, the prenatal diagnosis was confirmed by molecular analysis of placental tissue. In 232 (77.4%) of the 300 cases, the fetus was diagnosed as being normal or a carrier for β-thalassemia or sickle cell disease (Table 3). The results obtained after celocentesis were confirmed by CVS or amniocentesis or after birth in 212. There are 12 more cases that have not delivered yet in which confirmatory diagnosis has not been carried out because women opted against further invasive testing. The total fetal loss following celocentesis was 3.4% (8 of 236 pregnancies; manuscript in preparation) (Table 3).

DISCUSSION

The findings of this study in a large number of pregnancies investigated by celocentesis demonstrate that embryo-fetal cell selection from CF allows reliable and early prenatal diagnosis of hemoglobinopathies. There were no false positive or negative diagnoses. This technique is attractive to parents because it provides prenatal diagnosis of genetic disease at least 4 weeks earlier than what can be achieved by the traditional procedures. This reduces anxiety of parents and

Table 3 **Overall results of prenatal diagnosis of hemoglobinopathies by celocentesis**

Pregnancies having celocentesis	302
Results of prenatal diagnosis	302
Affected	68
Heterozygote	161
Unaffected	71
No result	2
Method of confirmatory diagnosis[a]	288
Analysis of placental tissue after termination	66
Analysis of placental tissue after fetal loss	8
Analysis of samples from CVS or amniocentesis	153
Analysis of blood from the neonate	61
Diagnostic errors from celocentesis	0
Fetal loss following celocentesis	8

CVS, chorionic villus sampling.
[a]There are 12 continuing pregnancies where confirmatory diagnosis has not yet been carried out.

provides the option for medical termination of affected cases at 8–10 weeks' gestation, which is less traumatic and safer than second-trimester surgical termination. If this technique is proven to be safe for the pregnancy, it could be used for early prenatal diagnosis in populations with restrictive laws on abortion such as Orthodox Jewish that only allow termination before 40 days of post-conception. However, celocentesis remains an experimental technique and should be used only in specific research protocols. Although we performed prenatal diagnosis using celocentesis in more than 300 cases and had no false positive or false negative results, it would still be necessary to confirm such results in larger prospective studies.

In a previous study, we reported that human CF contains functional embryonic erythroid precursors that are megaloblasts and demonstrated the feasibility of DNA diagnosis of hemoglobinopathies in 25 of 26 singleton pregnancies.[23] The findings of this extended series demonstrate that selection of embryo-fetal cells by different methods, depending on the grade of maternal contamination, allows reliable prenatal diagnosis by celocentesis. Consequently, one of the main obstacles to widespread use of celocentesis has now been overcome.

In cases of maternal contamination of CF of between 5% and 60%, reliable embryo-fetal cell selection is possible by CD71 cell labeling and magnetic activated cell sorting (MACS). This methodology allows isolation of a large number of erythroblasts, but it is not sufficiently sensitive for samples with a higher degree of contamination. In the latter case, it is possible to use the micromanipulator to pick up even few morphologically recognizable fetal cells. Direct extraction of DNA from selected embryo-fetal cells and application of nested PCR permitted accurate and reliable molecular analysis. Investigation by QF-PCR was crucial to test the grade of maternal contamination when both MACS and micromanipulator technology were used.

Recent advances have allowed the widespread introduction of noninvasive prenatal testing using cell-free DNA in maternal blood. This technique is used as a screening test for trisomies especially trisomy 21 with a detection rate of 99%, at false positive rate of 0.1%.[24] However, regarding the prenatal diagnosis of β-thalassemia, only the paternal mutations can be diagnosed, and therefore, this technique could only reduce the rate of invasive testing by 50% when the parents have different mutations.[25] Furthermore, certain ethnic groups share only few mutations,[3] and it is therefore very common for both parents to have the same mutation precluding noninvasive prenatal testing.

CONCLUSION

The proposed clinical diagnostic flow-work protocol for contaminated CF samples allows selection of pure fetal cells, making celocentesis a reliable procedure for early prenatal determination of the mutation status of fetuses conceived by partners carrying already known Mendelian disorders.

ACKNOWLEDGEMENTS

This work was possible, thanks to at-risk couples who participated to this study. We also thank the following doctors for carrying out the preliminary assessment of at-risk couples: Margherita Vinciguerra, Monica Cannata, Filippo Cassarà and Anna Maria Crivello. We also thank BioDiagene Srl as partner of this project for selection of fetal cells.

WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Celocentesis may represent the most attractive procedure for the earlier prenatal diagnosis of monogenic disease, but the main barrier to wide acceptability of this technique is the contamination of the sample by maternal cells.

WHAT DOES THIS STUDY ADD?

- A clinical diagnostic flow-work protocol for contaminated celomic fluid samples makes celocentesis a reliable procedure for early prenatal diagnosis of fetal monogenic diseases.

REFERENCES

1. Cao A, Pirastu M, Rosatelli C. Prenatal diagnosis of inherited hemoglobinopathies. Haematologica 1990;75:169–77.
2. Loukopoulos D, Kaltsoya-Tassiopoulou A, Fessas P. Thalassemia control in Greece. Birth Defects Orig Artic Ser 1988;23:405–16.
3. Giambona A, Damiani G, Vinciguerra M, *et al.* Incidence of haemoglobinopathies in Sicily: the impact of screening and prenatal diagnosis. Int J Clin Pract 2015;69:1129–38.
4. Kan YW, Golbus MS, Trecartin R. Prenatal diagnosis of homozygous beta-thalassaemia. Lancet 1975;25:790–1.
5. Kan YW, Dozy AM. Antenatal diagnosis of sickle-cell anaemia by DNA. Analysis of amniotic-fluid cells. Lancet 1978;28:910–2.
6. Pirastu M, Kan YW, Cao A, *et al.* Prenatal diagnosis of beta-thalassemia. Detection of a single nucleotide mutation in DNA. N Engl J Med 1983;4:284–7.

7. Jurkovic D, Jauniaux E, Campbell S, *et al.* Celocentesis: a new technique for early prenatal diagnosis. Lancet 1993;26:1623–4.
8. Findlay I, Atkinson G, Chambers M, *et al.* Rapid genetic diagnosis at 7–9 weeks gestation: diagnosis of sex, single gene defects and DNA fingerprint from celomic samples. Hum Reprod 1996;11:2548–53.
9. Santolaya-Forgas J, Vengalil S, Kushwaha A, *et al.* Assessment of the risk of fetal loss after the celocentesis procedure using a baboon model. Fetal Diagn Ther 1998;13:257–60.
10. Ross JA, Jurkovic D, Nicolaides K. Celocentesis: a study of short-term safety. Prenat Diagn 1997;17:913–7.
11. Makrydimas G, Kaponis A, Skentou C, Lolis D. Short-term safety of celocentesis for the mother and the fetus. Ultrasound Obstet Gynecol 2002;19:243–5.
12. Jurkovic D, Jauniaux E, Campbell S, *et al.* Detection of sickle gene by celocentesis in early pregnancy: a new approach to prenatal diagnosis of single gene disorders. Hum Reprod 1995;10:1287–9.
13. Jauniaux E, Cirigliano V, Adinolfi M. Very early prenatal diagnosis on celomic cells using quantitative fluorescent polymerase chain reaction. Reprod Biomed Online 2003;6:4948.
14. Jouannic JM, Costa JM, Ernault P, Bénifla JL. Very early prenatal diagnosis of genetic diseases based on celomic fluid analysis: a feasibility study. Hum Reprod 2006;21:2185–8.
15. Jouannic JM, Tachdjian G, Costa JM, Bénifla JL. Celomic fluid analysis: the absolute necessity to prove its fetal origin. Reprod Biomed Online 2008;16:148–51.
16. Pietropolli A, Vicario R, Peconi C, *et al.* Transabdominal celocentesis as early source of fetal DNA for chromosomal and molecular diagnosis. J Matern Fetal Neonatal Med 2014;27:1656–60.
17. Miller SA, Dikes DD, Polesky HF. A simple salting-out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 1988;3:1215.
18. Adinolfi M, Pertl B, Sherlock J. Rapid detection of aneuploidies by microsatellites and quantitative fluorescent polymerase chain reaction. Prenat Diagn 1997;17:1299–311.
19. Cirigliano V, Ejarque M, Fuster C, *et al.* X chromosome dosage by quantitative fluorescent PCR and rapid prenatal diagnosis of sex chromosome aneuploidies. Mol Hum Reprod 2002;8:1042–5.
20. Allen S, Mountford R, Butler A, *et al.* Practice guidelines for the testing for maternal cell contamination (MCC) in prenatal samples for molecular studies. Guidelines ratified by the UK Clinical Molecular Genetics Society, 2008
21. Renda MC, Giambona A, Fecarotta E, *et al.* Embryo-foetal erythroid megaloblasts in the human celomic cavity. J Cell Physiol 2010;225:385–9.
22. Rudbeck L, Dissing J. Rapid, simple alkaline extraction of human genomic DNA from whole blood, buccal epithelial cells, semen and forensic stains for PCR. Biotechniques 1998;25:588–90.
23. Giambona A, Makrydimas G, Leto F, *et al.* Feasibility of DNA diagnosis of haemoglobinopathies on celocentesis. Br J Haematol 2011;153:268–72.
24. Gil MM, Quezada MS, Revello R, *et al.* Analysis of cell-free DNA in maternal blood in screening for fetal aneuploidies: updated meta-analysis. Ultrasound Obstet Gynecol 2015;45:249–66.
25. Xiong L, Barrett AN, Hua R, *et al.* Non-invasive prenatal diagnostic testing for β-thalassaemia using cell-free fetal DNA and next generation sequencing. Prenat Diagn 2015;35:258–65.