



Noninvasive Antenatal Diagnosis of Fetal RhD Status

Emilija Velkova^{1*} and Dijana Plaseska-Karanfilska²

¹*Institute for Transfusion Medicine of the Republic of Macedonia, Republic of Macedonia.*

²*Macedonian Academy of Sciences and Art, Skopje, Republic of Macedonia.*

Authors' contributions

This work was carried out in collaboration between both authors. Author EV designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Author DPK managed the analyses of the study. Both authors read and approved the final manuscript.

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ABSTRACT

Introduction: Fetal cell-free nucleic acids within the blood stream of a pregnant woman come from fetal genetic material which can be acquired by simple venipuncture that reduces any risk to a minimum. Fetal cell-free DNA can be detected in the mother's blood stream in the 5th gestation week at the earliest. That enables fetal genotyping at the earliest possible stage of pregnancy which is best done in the 12th gestation week.

Aim: To determine fetal RhD status at RhD negative pregnant women where the father is a heterozygote, Dd.

Materials and Methods: The research includes 1540 RhD negative pregnant women, out of which at 30 of them the RhD fetal status had been detected by a PCR technique from the mother's plasma. The RhD fetal status was confirmed after delivery by serologic analysis at 27 newborn babies.

All research patients were submitted to serologic immunohematology testing: blood group typing of red blood cell antigens, screening of irregular anti-red blood cell antibodies. Fetal RhD status was determined by the plasma of RhD negative pregnant women using the real-time PCR technology in

*Corresponding author: E-mail: emavelkova@yahoo.com;

the period from the 12th gestation week until the 31 gestation week. The biological fathers of all 30 fetuses were phenotyped as heterozygote to the RhD antigen. The results showed that 30% of the fetuses are RhD negative, and 70% are RhD positive.

Conclusion: The noninvasive fetal RhD genotyping is not only one precious tool in the management of RhD alloimmunised pregnancies, but it also allows antenatal anti-D immunoglobulin prophylaxis exclusiveness for only non-immunized RhD pregnant women carrying RhD positive fetus. Taking into consideration that 30% of the RhD negative pregnant women that carry a RhD negative fetus receive antenatal RhIG prophylaxis with no absolute need for it. At RhD alloimmunised pregnant women the noninvasive genotyping of the fetal blood group enables an easy and safe method in determination of a fetal risk from a hemolytic disease, and at the same time evading a vast laboratory and clinical monitoring of RhD antigen-negative fetal cases.

Keywords: Hemolytic Disease of the Newborn (HDFN); antibody; red blood cell antigen; alloimmunisation; sensibilisation; anti-D Ig prophylaxis (RhIG); Rh blood group system; fetomaternal haemorrhage (FMH); fetal DNK.

1. INTRODUCTION

Cell-free fetal nucleic acids running through the blood stream of a pregnant woman demonstrate a source of fetal genetic material which can be acquired by simple venipuncture. Contrary to invasive procedures, where fetal genetic material is acquired from fetal cells directly from the uterus through chorionic villous sampling between the 11th and 14th gestation week, or through amniocentesis after the 15th week, this acquisition of the mother's blood sample does not involve a procedure that can cause fetal loss [1,2].

It is well known that fetal cell-free DNA is released by trophoblastic cells that are subjected to apoptosis [3]. As a result of this apoptotic penetration fetal cell-free DNA in the mother's blood stream is usually comprised of fragments smaller than 150 (bp) by size, demonstrating its presence as mononucleosomal DNA [4]. Firstly, fetal cell-free DNA can be detected in the mother's blood stream in the 5th gestation week at the earliest [5,6], or 18 days after the embryo transfer[7].

Fetal cell-free DNA present within the frames of the mother's blood stream can be found in the rich background of the mother's cell-free DNA. The mother's cell-free DNA is mainly of hematopoietic origin, but transplanted models displayed also other tissues as a source of cell-free DNA [8].

The middle fraction concentration of fetal cell-free DNA in maternal plasma is approximately 9,7%, 9,0% and 20,4% during the first, second and third trimester, measured by digital PCR [8].

The concentration of fetal cell-free DNA in the mother's serum is lower than the one in the mother's plasma because of lysis of the mother's nucleic red blood cells during the process of coagulation that leads to increasing of the percentage of mother's DNA in the blood sample [9].

After delivery, the fetal cell-free DNA is purified by the mother's circulation within a few hours, with a semi-life of 16 minutes. This fast breakthrough shows that during pregnancy fetal cell-free DNA is constantly released in large amounts within the mother's circulation. Calculations suggest that there is a release rate of $2,24 \times 10^4$ copies in a minute. Unlike fetal cells that can survive within the mother's circulation for many years [9] fetal cell-free DNA has not been discovered in the blood stream of non-pregnant women [10], which makes it specific compared to the state occurring in current pregnancies.

The non-invasive foetal RhD genotyping is a precious tool in the management of RhD allosensitised pregnancies, especially for mothers who carry a RhD negative fetus, avoiding numerous unnecessary analyses during pregnancy.

On the other hand, antenatal anti-D immunoglobulin prophylaxis is exclusively intended for non-immunized RhD pregnant women carrying RhD positive fetus.

2. MATERIALS AND METHODS

The research includes 1540 RhD negative pregnant women, out of which at 30 of them the RhD fetal status had been detected by a PCR

technique from the mother's plasma. The RhD fetal status was confirmed after delivery by serologic immunohematology analysis at 27 newborn babies. There was also phenotyping of Rh D,C,c,E,e and K antigens including 200 samples from the fetal biological father. The phenotypisation was performed with two serologic mikroagglutination techniques in immunohematology, simultaneously: mikrogel (Biorad) and magnetic pearls (OrthoDiagnostic). The tests were done including the serum, plasma and red blood cells of vein blood, without anticoagulants and/or EDTA, not older than 24 hours.

The tests for newborns were done including vein and umbilical cord blood, without anticoagulants and/or EDTA, not older than 24 hours.

All research patients: pregnant women, biological fathers of the fetuses and newborns, were submitted to serologic immunohematology testing:

- Blood group typing of red blood cell antigens,
- Screening of irregular anti-red blood cell antibodies (indirect antihuman globulin test, enzyme test and direct antihuman globulin test)

The serology tests were done by mikroagglutination technique - DiaMed ID card, using two monoclonal anti-D reagents out of which one of them recognises DVI phenotype that is immunogenic for RhD negative mother.

Acquired results were analysed and elaborated by different statistical methods: all statistical series according to variables of interest are tabulated and graphically displayed; the analysis of the structure of the attributable statistical series is made with the coefficients of relations, proportions and rates.

Real-time PCR technique monitors the amplification of a targeted DNA molecule during the PCR in real time. It measured the amount of nucleic acids through the detection of a fluorescent signal which is proportional to the concentration of a double chain DNA in the PCR tube. The fluorescent signals are produced by fluorophores that are added to the reaction mixture. SYBR Green real-time PCR, SYBR Green introduces a colour that forms a complex with the double chain DNA which efficiently fluorescents. TaqMan analysis probes are

specific fluorescently marked oligonucleitic tests that also have a quencher residues and they hybridise with the targeted DNA.

The probe subsequently hydrolyses from the elongated Taq polymerase which results in the division of the fluorophore from its quencher. In both cases, the produced fluorescent signal can be used as a measure for the initial amount of the targeted sequence in reaction. The real-time PCR fluorescent signal is noticed in "the real time" at the end of each PCR cycle. The amount of the targeted sequence is then calculated automatically.

In quantification, by using the method of a standard curve, you can measure an unknown quantity on the basis of measures for a known quantity. First, you generate a standard curve and then the values of an unknown quantity are compared to the standard curve out of which you can extrapolate values.

It represents a noninvasive method for determination of the fetal RhD status by analysing the mother's plasma.

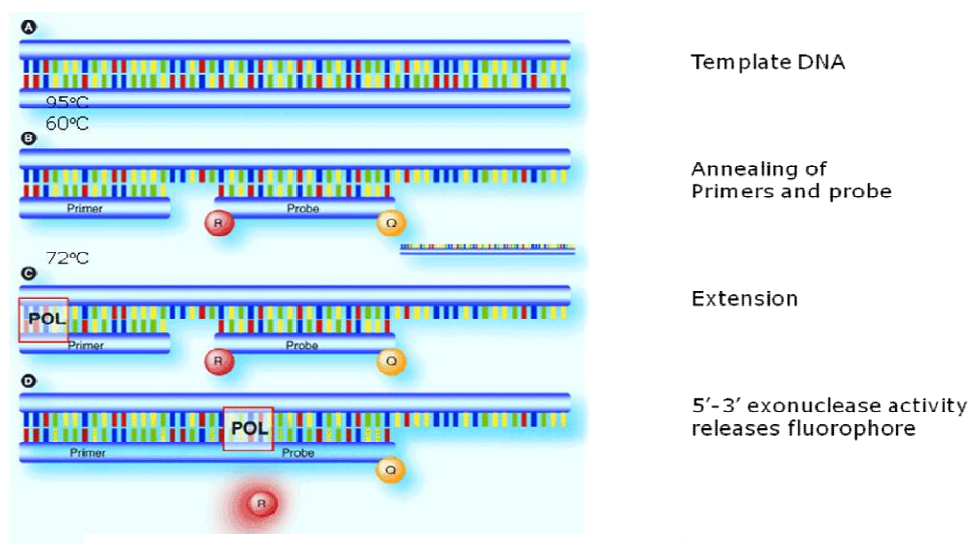
The fetal RhD status of the fetus was determined by RHD TaqMan system used as described by Chiu R and Coo.in 2001, in order to detect fetal RhD status. Specific primers were used to amplify egzon 5 (RHD5) and egzon 7 (RHD7) of the RHD gen, as well as the control fragment CCR5.

The primer sequences and the fluorescent probes were used from the Finning publication [11]. Cff DNA (Cell Free Fetal DNA) was extracted due to the QIAamp Circulating Nucleic Acid kit according to the producers protocol.

RHD5 primers and the probe are amplified only by the RHD gene, while RHD7 primers and the probe are amplified not only by the RHD gene, but by the RHD pseudogene as well.

2.1 Reaction Mixture

Each plate for real-time PCR amplification included RHD5, RHD7 and CCR5 amplification on patients, RHD negative control and controlling genome DNA (Promega) for the generation of standard curves. The reaction evolved in a volume of 25 μ L, out of which 20 μ L reaction mixture and 5 μ L cff DNA/negative control DNA/DNA as a standard.



Picture 1. Schematic display of a real-time PCR reaction

Table 1. Used primers for amplification

Target	Primer	5'-3' sequence	Final conc. (nM)	Size of amplicon (bp)
RHD5	Forward	CGCCCTCTTCTTGTGGATG	300	82
	Reverse	GAACACGGCATTCTTCTTTC	300	
RHD7	Forward	CAGCTCCATCATGGGCTACAA	300	75
	Reverse	AGCACCAGCAGCACAATGTAGA	300	
CCR5	Forward	TACCTGCTCAACCTGGCCAT	300	91
	Reverse	TTCCAAAGTCCCCTGGGC	300	

Table 2. Used fluorogenic oligonucleotic probes

Target	5'-3' sequence	5' Label	3' Label	Final conc. (nM)
RHD5	TCTGGCCAAGTTTCAACTCTGCTCTGCT	FAM	BHQ1	166
RHD7	AGCTTGCTGGGTCTGCTTGGAGAGATC	HEX	BHQ1	166
CCR5	TTTCTTCTTACTGTCCCCTTCTGGGCTC	FAM	BHQ1	166

2.2 Real-time Protocol and Amplification Conditions

The apparatus software is adjusted to the adequate template and type of samples according to the schedule that involved their plate application. The appropriate colour should be chosen on the reporter (FAM for RHD5 and CCR5 and HEX for RHD7, while BHQ1 (non-fluorescent quencher) as a bleacher.

3. RESULTS

All samples are analysed, and standard curves are generated for each set of primers. The basic line is adjusted between the 3rd and the 15th cycle, and the threshold is adjusted to 1.0.

A negative result is indicated by a Ct value ≥ 42 (no amplification), while a positive result is when the Ct value < 42 (there is amplification).

The angle of standard curves should be between -3.2 and -4.5.

Table number 3 is used for interpretation of results.

There was a determination of fetal RhD genotyping by the mother's plasma for 30 RhD negative pregnant women related to various gestation week of pregnancy, i.e. from 12 gestation week up till 31 gestation week, by using the method of Polymerase Chain Reaction (PCR).

There was also RhD phenotyping from the biological father in reference to 200 samples, at which the mother was previously typed as RhD negative. All 30 pregnant women included in the research are related to a biological father phenotyped as RhD heterozygote.

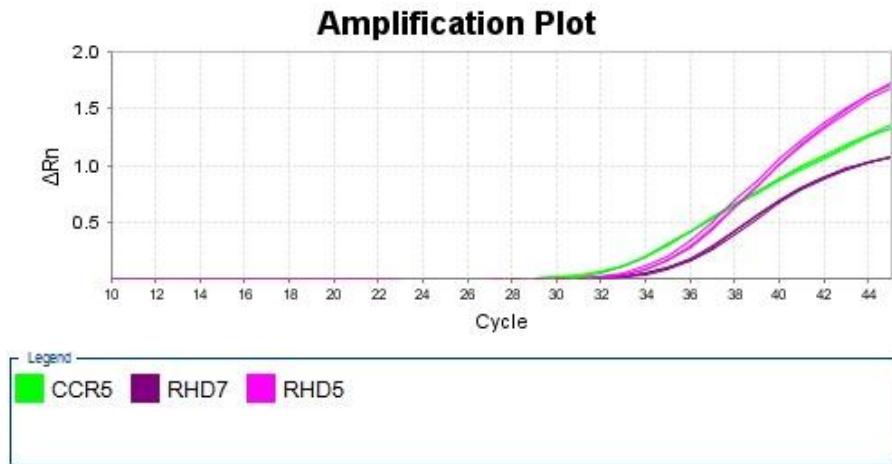
Acquired results demonstrated that 9 fetuses are RhD negative and 21 RhD positive. The test was repeated at the negative ones in the period of early pregnancy one more time after the 20th gestation week.

Table 3. Interpretation of results

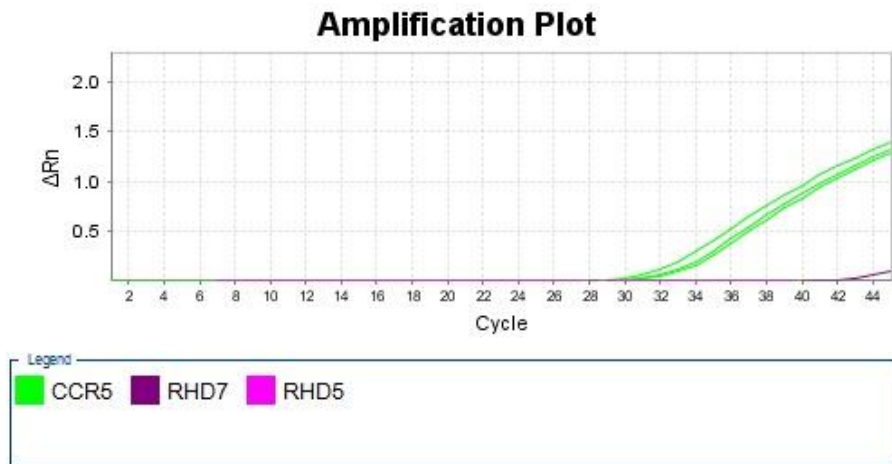
Number of replicates RHD5 <Ct42	Number of replicates RHD7 <Ct42	RHD genotype
0	0	RHD negative
0	1	RHD negative
1	0	RHD negative
1	1	RHD negative if Cts>38;
2-4	2-4	Inconclusive
0,1	5,6	RHD pseudogene
5,6	5,6	RHD positive if 5-6 Cts <42 (standard deviation should be no more than 1.5)

Table 4. RHD genotyping fetal DNA by the mother's plasma

No.	Sample	Gestation week	Fetal gender	Biological Father Rh genotype	Rh genotype	Notes
1	101	28	F	DcE/dce	-	
2	102	24	M	DCe/dCe	+	
3	103	15	/	DcE/dce	+	
4	104	18	F	DCe/dce	+	
5	105	26	M	DcE/dce	+	
6	106	22	M	DCe/dce	+	
7	107	30	F	DCe/dce	+	
8	108	33	M	DCe/dce	+	
9	109	23	M	/	+	
10	110	29	F	DcE/dce	+	
11	111	31	M	DCe/dCe	+	
12	112	26	F	DCe/dce	+	
13	113	27	M	DCe/dce	+	
14	114	31	M	DcE/dcE	+	
15	115	21	F	Dce/dce	-	RHD5 1+ 2- RHD7 1+ 2-
16	116	24	M	DCe/dce	+	
17	117	12	M	DCe/dce	+	RHD5 1- 2+ RHD7 1- 2+
18	118	22	F	DcE/dce	+	RHD5 1+ 2- RHD7 1- 2+
19	119	20	/	DCe/dce	+	
20	120	24	M	Dce/dce	+	
21	121	27	M	DCe/dce	+	
22	122	28	F	Dce/dce	+	
23	123	26	M	DcE/dcE	+	
24	124	17	/	DCe/dce	+	
25	125	29	F	DCe/dce	+	
26	126	22	F	DCe/dce	-	
27	127	27	M	DcE/dce	+	
28	128	19	M	Dce/dce	+	
29	129	26	M	DCe/dce	+	
30	130	27	F	DCe/dce	+	



Picture 2. Amplification plot of a real-time PCR related to RHD gene from cff DNA of a positive RHD fetus



Picture 3. Amplification plot of a real-time PCR related to RHD gene from cff DNA of a negative RHD fetus

4. DISCUSSION

Developed countries have introduced routine antenatal anti-D prophylaxis since 1960, combined with RhIG administration in high risk situations during pregnancy and delivery, such as miscarriage, terminate pregnancy, invasive antenatal diagnosis procedures, external version, Caesarean section, etc [12,13,14]. It was noted that there is a significant decrease in the rate of anti -D immunization and anti -D perinatal mortality [15].

Further decrease had been noticed in several studies that researched routine antenatal application of RhIG in order to prevent immunisation of undiscovered FMH during the

last trimester of pregnancy [14,15]. But even if postnatal and antenatal prophylaxis is combined, 0.1-0.3% of the women are at risk, and they still create RhD antibodies [16,17]. If the prevention risk factors that contribute to the rest of the immunisation can be identified, it will be possible to get even a greater decrease of HDFN.

A pregnant woman that is RhD negative is at risk of Allosensitization only if she carries an RhD positive baby, and that can happen only if the father is RhD positive. An RhD negative woman that has conceived a baby with an RhD negative man cannot carry an RhD positive baby, and cannot be allosensitized. Around one sixth (1/6) RhD negative women might get pregnant by a

man who is also RhD negative. There is no need of anti-D prophylaxis for them.

Undeniably, we should avoid application of antenatal prophylaxis for those women. There are opinions that it might require additional analysis that is not necessary, but it is a fact that they should be saved from possible side effects during application of anti-D, as well as avoid the trauma of unnecessary application and risks.

Nevertheless, it is necessary for the RhD negative woman to identify the biological father, and he should determine his Rh phenotype and possible genotype.

It has been discovered that 56% of Rh positive white race individuals are heterozygote to the RhD antigen. Thus, when the biological father is heterozygote RhD positive, there is 50% chance for the fetus to be Rh negative.

If the father is antigen negative, there is no chance for the fetus to be antigen positive, and therefore there is no risk of HBFN that can be caused by the mother's alloantibodies.

Nowadays it is possible to determine the antigen fetal status by a DNA test through the mother's plasma if the father is heterozygote antigen positive, in case of allosensitization of the mother by D,C,c,E and K antibodies.

Traditionally, genotyping of the fetal blood group is done by amniocentesis.

This invasive procedure involves a risk of miscarriage, can potentially cause maternal sensitization and increases the risk of FMH that can produce a larger amount of antibodies [18,19].

The discovery of cell-free fetal DNA in the plasma of pregnant women (since the end of the 20th century) demonstrated one noninvasive and safe method for determination of fetal blood group genotype with almost 100% accuracy [20,21].

At the moment, the noninvasive fetal RhD genotyping at D-allosensitized pregnant women is a routine clinical practice in several European states.

Moreover, following the grand research of fetal RhD genotyping since 2010, Denmark and Holland introduced a national program for fetal

RhD screening related to the determination of the fetal D antigen in order to apply immunoprophylaxis at not immunised D-negative pregnant women [22]. There was also a development of noninvasive genotyping analysis in determination of fetal blood group for the rest of clinically relevant red blood cell antigens.

In our research, we have used vein maternal blood taken in EDTA. Genotyping was done in the maternal plasma because fraction concentration of cell-free fetal DNA within the maternal serum is lower than the one in the maternal plasma (because of lyses of maternal nucleic red blood cells during coagulation) which produces percentage increase of maternal DNA in the blood sample [23,24,25].

The research included 1540 RhD negative pregnant women with serologic immunohematology testing. In order to determine fetal RhD status it is necessary to primarily determine the RhD zygote type of the biological father. Unfortunately, the biological father is sometimes difficult to determine if based on the women's report/declaration, besides only ½ of them can be a heterozygote. Only 30 pregnant women volunteered to participate in the research out of the total number of fetuses with RhD heterozygote biological father from the results acquired by testing samples of 200 fathers.

The total amount of 30 RhD negative pregnant women was tested by Real time PCR between the 12 and 31 gestation week. All pregnant women including the ones at which we have detected Allosensitization to the D antigen involved phenotyping of the Rh blood group system of the biological father.

The research related to the determination of the fetal RhD status included 30 case at which the biological father was a heterozygote, while the extraction of fetal DNA in the maternal serum was confirmed.

Result analysis demonstrated that 9 (30%) are RhD negative fetuses, and 21 (70%) are RhD positive. Science books percentage of RhD negative fetuses moves around 35% and 40%, depending on the author. In cases of a negative result, acquired by a sample in an early gestation maturity, the test was repeated after 2-3 weeks. The negative result might be a consequence of a lack of fetal DNA in the first trimester, thus it is desirable to get one more confirmation of the fetal RhD negative status by another test.

Regarding the fetal RhD screening, samples of the postnatal umbilical cord were sent to our lab in order to determine RhD serology of the newborn. Serology of the umbilical cord was done at 90% of the cases. Fetal RhD typing was positive at 20 out of 20 samples by plasma from women that had delivered a D-positive baby, accordingly the result is 100% sensitivity. Out of the total amount of 7 samples of plasma by women that had delivered a D-negative baby, fetal RhD typing was negative at 6 samples.

In reference to science literature, false negative results appear because of lack of enough DNA in the system of detection, because of failure in extraction, low fetal DNA concentration in the plasma sample, or unsuccessful PCR or mixed sample. False negative results represent the main concern in fetal RhD screening analysis since anti-D immunoprophylaxis will be unjustly excluded from the treatment of D-negative pregnant women that can result in risk of immunisation and potential morbidity and mortality by HDFN in subsequent pregnancies. According to statistics, this percentage is minimal and it amounts to 0.06% [26].

Numerous studies succeeded in fetal RhD genotyping at RhD negative mothers with almost 100% accuracy. The standard methodology is available and there are established international exterior schemes of quality.

In the last several years, a couple of research studies for RhD typing that use automated DNA extraction and accurate robotic handling were successfully presented, achieving a sensitivity of 99.7% to 99.9% [8,25,26].

Recently in many developed countries, routine antenatal anti-D prophylaxis where anti-D immunoglobulin is applied between 28 – 34 gestation week, became a standard care for D-negative pregnant women. This practice combined to postnatal prophylaxis brought about further reduction of maternal immunisation for more than 50% [27,28,29] Never the less, we have to pay attention to the unnecessary use of antenatal prophylaxis in women who carry RhD negative baby.

5. CONCLUSION

Collected data demonstrate that the noninvasive fetal RhD genotyping is not only a precious tool in the management of RhD alloimmunized pregnancies, but also allows antenatal RhIG

prophylaxis exclusively for those not immunised RhD negative pregnant women that carry RhD positive fetus.

The analysis of results that we have acquired in our study reveal the fact that 30% of RhD negative pregnant women receive antenatal RhIG prophylaxis, and have no need of it. Besides, these pregnant women can be saved from further unnecessary testing during pregnancy since there is no risk of HBFN.

Pregnant women that were alloimmunized in their previous pregnancies and/or transfusions, and have a confirmation of fetal RhD negative status, there is no risk of HBFN, and no further testing is necessary.

However, if the fetus inherits the implicated antigen, it implies the need of timely and careful follow up of fetal anaemia through a series of titers from maternal antibodies and other activities, fetal measures by Doppler ultrasound at peak systolic velocity in the middle cerebral artery, and finally intrauterine sample of fetal blood with possibility of intrauterine transfusion on time.

CONSENT AND ETHICAL APPROVAL

As per university standard guideline participant consent and ethical approval has been collected and preserved by the authors.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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