

## Fetal *RHD* and *RHCE* Genotyping in Plasma of Rh Negative Pregnant Women

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**Aim:** To assess the accuracy of *RHD* and *RHCE* genotyping by real-time PCR, through the analysis of fetal DNA in plasma samples of Rh negative pregnant women.

**Methods:** The study consisted in the collection of two tubes of blood (each with 3 ml) from 19 RhD negative pregnant women that attended the obstetric consultation at the District Hospital of Figueira da Foz, E.P.E. One of the samples was used to perform blood typing and the other was used for fetal DNA extraction. The Rh genotype was determined by real-time PCR with specific primers and Taqman<sup>®</sup> probes for *RHD*, *RHC*, *RHE*, *SRY* and *GLO* genes.

**Results:** In 88.2% of cases (15/17) the *RHD* genotype was concordant with the phenotyping data, with 1 false-positive result (5.9%) and a precision of 94% ( $p_{value}=0.001$ ;  $K=76.7\%$ ). For the *RHC* gene, there was concordance in 60% of cases (6/10), with 4 false-negative results (40%) and a precision of 60% ( $p_{value}=0.175$ ;  $K=31\%$ ). Relatively to the *RHE* gene, the concordance achieved was 100% (10/10), with a precision of also 100% ( $p_{value}=0.002$ ;  $K=100\%$ ).

**Conclusion:** The present study confirms the precision of fetal *RHD* and *RHE* genotyping in maternal plasma. Performing a similar study with a superior number of samples could allow the implementation of this non-invasive prenatal diagnostic test in laboratorial and clinical routine, in order to follow pregnancies at risk for developing Hemolytic Disease of the Fetus and Newborn.

**Key words:** Fetal DNA, maternal plasma, Hemolytic Disease of the Fetus and Newborn, Anti-D prophylaxis, non-invasive prenatal diagnosis

### Introduction

The Rh blood group system is the most polymorphic of all human blood groups and, after ABO system, the most clinically significant in transfusion practice.<sup>(1-3)</sup> The antigens (Ag) of this system (D, C, c, E, e) are encoded by the homologous genes *RHD* and *RHCE*, both located on chromosome 1.<sup>(4-6)</sup> The RhD negative phenotype results primarily from a deletion of the entire *RHD* gene, an alteration which is prevalent in Caucasians

(15-17%) and less common in Africans (5%), which in approximately 66% of the cases have an inactive gene (*RHD* $\psi$ ).<sup>(3,7,8)</sup> In Europeans, the C and c Ag have a prevalence of 70% and 80% respectively, and, in most populations, the E and e Ag have a frequency of about 30% and 98%.<sup>(6)</sup>

The formation of Rh antibodies (Ab) results from the exposure to cells with those Ag, generally through transfusions or pregnancy.<sup>(9-11)</sup> These Ab are involved in Hemolytic Transfusion Reactions, Auto-Immune Hemolytic Anemia and Hemolytic Disease of the Fetus and Newborn (HDFN).<sup>(2,12)</sup> HDFN is a significant cause of

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perinatal mortality and morbidity<sup>(9,13)</sup> and, in 50% of the cases, results from the transplacental passage of maternal RhD Ab, followed by their binding to fetal red blood cell Ag and subsequent destruction.<sup>(1,5,14)</sup> Besides the D Ag, the most common cause of HDFN are the c and Kell Ag.<sup>(3)</sup>

In the early 60s, Stern *et al* demonstrated experimentally that the administration of anti-D immunoglobulin (Ig) could prevent sensitization to the RhD Ag.<sup>(10)</sup> This Ig is produced from pooled human plasma<sup>(10,15)</sup> and injected in strict controlled doses, but its origin generates controversy about the viral security<sup>(4,16)</sup> and more recently about the transmission of prion type diseases.<sup>(4,15,16)</sup> Although in the future this Ig could be manufactured through recombinant or monoclonal products (which would eliminate the associated risks), there is still no perspective about its introduction in a foreseeable future.<sup>(4,10)</sup> Thus, the anti-D immunoprophylaxis is recommended to all RhD negative pregnant women undergoing invasive procedures or at the 28<sup>th</sup> week of gestation and until 72 hours *postpartum*.<sup>(7,10,11)</sup> However, in a predominantly Caucasian population, 38-40% of these women also carry an RhD negative fetus, and will receive the treatment unnecessarily.<sup>(7-10)</sup>

In the late 90s, Lo *et al* confirmed the existence of cell-free fetal deoxyribonucleic acid (cffDNA) in maternal circulation using real-time Polymerase Chain Reaction (PCR), a study that constituted the first demonstration of the cffDNA presence in maternal plasma.<sup>(12,17)</sup> This discovery has opened up new possibilities for non-invasive prenatal diagnosis, which represent a great advantage over the conventional methods (amniocentesis and chorionic villus sampling), associated with a potential risk of miscarriage of 0.5 to 1%<sup>(14,18)</sup> or isoimmunization by fetomaternal hemorrhage (FMH)<sup>(19-23)</sup> which in amniocentesis is of about 20%.<sup>(3)</sup>

It is thought that cffDNA is originated by the apoptosis of trophoblasts derived from the embryo<sup>(14,24)</sup> and that it consists the most in short DNA fragments, inferior to 313 basepairs.<sup>(14,25,26)</sup> It is also known that it is detectable in maternal circulation since the fifth week of gestation, that it has a half-life time of 16 minutes and that it is undetectable 2 hours *postpartum*.<sup>(14,27)</sup>

Besides the Y chromosome, the first genetic *locus* that captured the investigators' attention was the *RHD* gene.<sup>(28,29)</sup> However, the scarce concentration of cffDNA in maternal blood (3-6%, in early and late pregnancy)<sup>(28,30)</sup> and the difficulty to distinguish it from the maternal DNA background, were unavoidable obstacles to its clinical application.<sup>(22,29,31)</sup>

This analysis allows the evaluation of gene alleles

paternally inherited and that are not present in the mother's genome, such as the fetal RhD in RhD negative pregnant women.<sup>(19,21,32)</sup> Clinical applications of this biological phenomenon also include fetal aneuploidy detection, prenatal diagnosis of several genetic diseases<sup>(29,32,33)</sup> and identification of pregnancy complications.<sup>(31,34)</sup>

The prenatal determination of the fetal Rh genotype could lead to a substantial reduction of the use of anti-D Ig and, in the other hand; it would prevent the unnecessary exposure of pregnant women carrying an RhD negative fetus to this pool of human plasma that, despite strict control, is still associated with an elevated number of risks.

The aim of this study was to assess the concordance between *RHD* and *RHCE* genotyping by real-time PCR (through the analysis of fetal DNA extracted from plasma samples of RhD negative pregnant women) with the newborn Rh phenotype.

## Materials and Methods

### Study population and data collection

The study population consisted of 19 RhD negative pregnant women that attended the obstetric consultation at the District Hospital of Figueira da Foz, E.P.E. (HDFE, E.P.E.) from January to May of 2011 who were only included in the analysis after giving informed consent and filling an inquiry, with the aim to collect some relevant data for the work. The sex and Rh phenotype of the children were obtained after delivery.

The present study follows the principles established by the Helsinki Declaration. There aren't any ethical issues or conflict of interest and it was guaranteed maximum protection and confidentiality of the obtained data.

### Sample collection and processing

From each pregnant woman, two samples of 3 ml peripheral blood were collected into tubes containing tripotassium ethylenediamine tetraacetic acid (EDTA K<sub>3</sub>) (VenoSafe™, Madrid, Spain). Immediately after the collection, one of the samples was processed according to the methodology used by other researchers.<sup>(1,12,35)</sup> The plasma was first separated from whole blood by centrifugation at 3000g for 10 minutes and re-centrifuged

with the same speed and duration. The supernatant was stored in a new microtube at -80°C. The second sample was kept at 4°C until its processing at the Laboratory of Biomedical Sciences from the College of Health Technology of Coimbra, where it was determined the ABO blood group (cell and serum testing), Rh phenotype, screening and identification of irregular Alloantibodies (saline, enzymatic and Anti-Human globulin (AGH) media), Autoantibodies and Direct Antiglobulin Test (DAT), using ID-card technology (Diamed GmbH, Cressier, Switzerland) and specific reagents, such as Low Ionic Strength Solution (LISS), bromelin and commercial phenotyped cells (ID-DiaCell ABO/I-II-III e ID-DiaPanel) (Diamed GmbH, Cressier, Switzerland).

## DNA Extraction/Purification

DNA was extracted/purified from 200 µl of plasma samples, with a Quick-gDNA™ MiniPrep Kit (Zymo Research, Irvine, U.S.A.), according to the manufacturer's recommended protocol. The DNA was eluted in a final volume of 50 µl and stored at -20°C until further processing.

## Real-time PCR

The fetal sex and Rh genotype were determined by Real-Time PCR, performed with a MiniOpticon™ System (Bio-Rad Laboratories, Inc., Hercules, U.S.A.), using primers and TaqMan® probes synthesized by Thermo Fisher Scientific GmbH (Ulm, Germany) specific for the *RHD*, *RHCE*, *SRY* and *GLO* genes (Table I), as described by Hromadnikova *et al*<sup>(1)</sup> and other authors.

<sup>(26,32,34)</sup> The primers and probes were used with a final concentration of 300 and 200 nM, respectively. To increase the specificity of the assay, two regions of the *RHD* gene (exons 7 and 10) were investigated, and to confirm the presence of DNA in the sample, the *GLO* and *SRY* genes were amplified.

Each amplification reaction was set up in a final volume of 25 µl, containing, in addition to the primers and respective probe, 5 µl of the purified plasma sample, 5 µl of 10X PCR buffer, 200 µmol of each dNTP, 6 mM MgCl<sub>2</sub> and 1.5 U of i-Taq™ Polymerase, reagents from the i-Taq™ plus DNA Polymerase Kit (iNtRON Biotechnology, Inc., Sangdaewon-Dong, South Korea).

The PCR conditions were programmed with an initial step of denaturation at 95°C for 10 minutes and 50 cycles at 95°C for 15 seconds and 60°C for 1 minute. <sup>(1,12,34)</sup>

The amplification data was collected and analyzed by the Bio-Rad CFX Manager software version 1.6 (Bio-Rad Laboratories, Inc., Hercules, U.S.A.). In order to increase the sensitivity of the method, the genotyping was performed in duplicate for all samples and in each assay was tested a negative control (female individual with ccddee phenotype) and a positive control (two male individuals with CcDee and ccDEe phenotype).

The amplification results are represented by the Cycle threshold (C<sub>t</sub>). The samples were considered positive when one or more repetitions were positive and when the C<sub>t</sub> value was less than 42.

## Statistical analysis of the results

The results were analyzed with Statistical Package for the Social Sciences (SPSS) version 17.0.0 (SPSS,

**Table I Used primers and probes sequence.**

Gene	Exon	Primers (forward and reverse)	Probes
<i>RHD</i>	10	5' – CCT CTC ACT GTT GCC TGC ATT – 3' 5' – AGT GCC TGC GCG AAC ATT – 3'	5'-(FAM) TAC GTG AGA AAC GCT CAT GAC AGC AAA GTC T (TAMRA)-3'
<i>RHD</i>	7	5' – CTC CAT CAT GGG CTA CAA – 3' 5' – CCG GCT CCG ACG GTA TC – 3'	5'-(FAM) AGC AGC ACA ATG TAG ATG ATC TCT CCA (TAMRA)-3'
<i>RHC</i>	2	5' – CAT TGC TAT AGC TTA AGG ACT CA – 3' 5' – ATG ATT GTA CCA CTG GGA AG – 3'	5'-(FAM) CAA CAC CAA ACC AGG GCC ACC (TAMRA)-3'
<i>RHE</i>	5	5' – TGG CCA AGT GTC AAC TCT C – 3' 5' – TCA CCA TGC TGA TCT TCC T – 3'	5'-(FAM) AAG AAT GCC ATG TTC AAC ACC TAC TA TG (TAMRA)-3'
<i>SRY</i>	-	5' – TGG CGA TTA AGT CAA ATT CGC – 3' 5' – CCC CCT AGT ACC CTG ACA ATG TAT T – 3'	5'-(FAM) AGC AGT AGA GCA GTC AGG GAG GCA GA (TAMRA)-3'
<i>GLO</i>	-	5' – GTG CAC CTG ACT CCT GAG GAG – 3' 5' – CCT TGA TAC CAA CCT GCC CAG – 3'	5'-(FAM) AAG GTG AAC GTG GAT GAA GTT GGT GG (TAMRA)-3'

Inc., Chicago, U.S.A.), using the Chi-square of independence test and Cohen's Kappa, which relate the concordance between obtained and expected results. Diagnostic measures were also used, such as sensitivity, specificity and precision/accuracy.

## Results

The 19 RhD negative pregnant women included in the study were in a gestational period between 22 and 37 weeks and were aged 19 to 40 years (mean  $29.8 \pm 5.4$ ). Of these, 17 (89,5%) were tested during the 2<sup>nd</sup> trimester of pregnancy and 2 (10.5%) during the 3<sup>rd</sup> trimester.

When answering the questionnaire, all women (n=19; 100%) reported not having received blood transfusions in the last 3 months and never having done a transplant. These two questions are extremely relevant because if a pregnant woman had received a blood product or even an organ, it could possibly change the phenotyping results, since we could be testing the DNA from the donor instead. Regarding the Rh group of the male parent of the child, 16 women (84.2%) referred it to be Rh<sup>+</sup>, 1 (5.3%) Rh<sup>-</sup> and 2 (10.5%) didn't know or didn't remember.

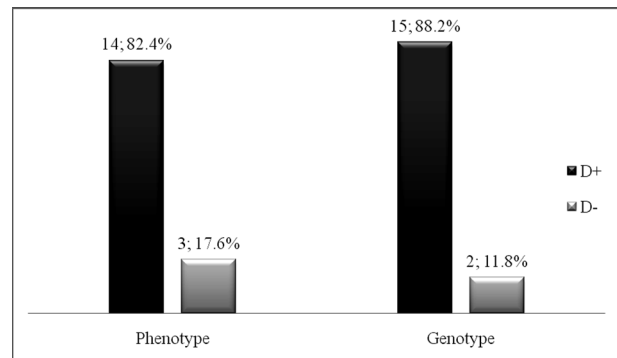
Relatively to the blood group typing, it was found that 16 pregnant women (84.2%) had the ccdee phenotype, 2 (10.5%) the cddEe phenotype and only 1 (5.3%) the Ccdee phenotype. Regarding the alloantibody screening, it was identified an anti-D Ab in samples 2, 5, 9 and 11 (n=4; 21%), anti-Fy<sup>a</sup> in sample 4 (n=1; 5.3%) and anti-Fy<sup>a</sup> and anti-Jk<sup>a</sup> in sample 8 (n=1; 5.3%).

From the initial study population, only 17 pregnant women were considered for the final analysis, since there were 2 drop-outs, due to the incapacity of establishing contact with them, presumably because both returned to their country of origin, since they had non-Portuguese nationality.

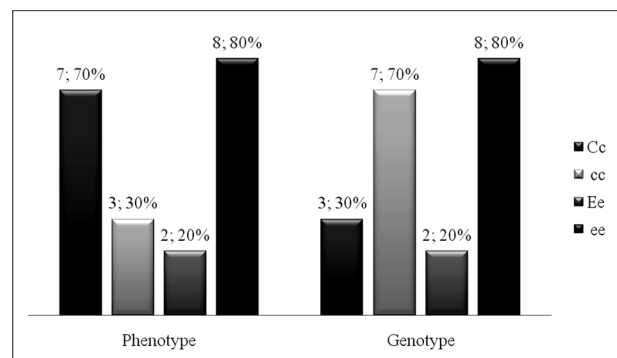
Given that HDFF, E.P.E. doesn't have a maternity, the births occurred in different hospitals; due to this fact, the collection of the *postpartum* data was dependent of the contact with the progenitors, making it impossible (in 7 cases), to obtain the child's complete information. Therefore, the number of samples considered for the concordance analysis between genotyping and phenotyping data was different (D Ag and fetal sex: n=17; C and E Ag's: n=10).

From the 17 children considered for the study, 10 (58.8%) were males and 7 (41.2%) were females. Regarding the D Ag, 14 samples (82.4%) were from RhD positive children and 3 (17.6%) from RhD negative

children (Figure 1). For the C and E Ag were included 10 samples; 7 (70%) had Cc phenotype, 3 (30%) cc phenotype, 2 (20%) Ee phenotype and 8 (80%) ee phenotype (Figure 2).



**Fig.1** Frequency of D positive and D negative newborns according to the genotype and phenotype.



**Fig.2** Frequency of C and E in newborns according to the genotype and phenotype.

Concerning the fetal sex genotyping by real-time PCR (Table II), for which 17 samples were considered, 9 (52.9%) revealed the presence of the *SRY* gene. In the same way, from the 17 samples considered for the *RHD* gene, the research was positive for 15 (88.2%) (Figure 1). For the *RHC* and *RHE* genes, of the 10 samples considered, 3 (30%) revealed to be positive for the *RHC* gene and 2 (30%) for the *RHE* gene (Figure 2). The *GLO* gene was detected in all samples.

Statistical analysis (Table III) was performed after comparison of the genotyping with the fetal sex and Rh phenotype of the children. Relatively to the *SRY* gene, the genotyping correctly predicted fetal sex in 82.4% of cases (14 of 17 samples), having occurred 1 false-positive (5.9%) and 2 false-negatives (11.8%), which led to obtaining a sensitivity of 80% and specificity of 86%, corresponding to a precision of 82% ( $p_{value}=0.008$ ;  $K=64.3\%$ ). In 88.2% of cases (15 of 17 samples), the predicted *RHD* genotype was in concor-

**Table II Fetal RHD, RHC, RHE and SRY genotyping results by Real-Time PCR and Rh phenotype and sex.**

Sample No.	Gestation weeks	Maternal phenotype	Fetal genotyping in maternal plasma				Rh phenotype	Sex
			RHD	RHC	RHE	SRY		
1	29	ccddee	Pos	Neg	Pos	Pos	ccDEe	♂
2	37	ccddEe	Pos	Pos	Pos	Neg	CcDEe	♀
4	32	ccddee	Pos			Pos	D	♂
5	36	ccddee	Pos	Neg	Neg	Pos	CcDee	♀
6	30	ccddee	Neg	Neg	Neg	Neg	ccddee	♂
8	27	ccddee	Pos	Pos	Neg	Pos	CcDee	♂
9	31	ccddee	Pos	Neg	Neg	Neg	CcDee	♀
10	28	ccddee	Pos	Pos	Neg	Pos	CcDee	♂
11	30	ccddEe	Neg	Neg	Neg	Pos	ccddee	♂
12	28	ccddee	Pos			Pos	D	♂
13	28	ccddee	Pos	Neg	Neg	Neg	CcDee	♀
14	28	ccddee	Pos	Neg	Neg	Neg	CcDee	♀
15	27	ccddee	Pos			Neg	D	♀
16	29	Ccddee	Pos			Pos	dd	♂
17	22	ccddee	Pos			Neg	D	♀
18	26	ccddee	Pos			Pos	D	♂
19	26	ccddee	Pos			Neg	D	♂

Legend – Neg: Negative; Pos: Positive;

**Table III Statistical Analysis and diagnostic measures between genotyping and phenotyping.**

	RHD	RHC	RHE	SRY
Concordance	88.2% (15/17)	60%	100%	82.4%(14/17)
Sensitivity	100%	43%	100%	80%
False-negatives	-	40% (4/10)	-	11.8% (2/17)
Specificity	67%	100%	100%	86%
False-positives	5.9% (1/17)	-	-	5.9% (1/17)
Precision	94%	60%	100%	82%
<i>p</i> value	0.001	0.175	0.002	0.008
<i>Cohen's Kappa</i>	76.7%	31%	100%	64.3%

Legend – *p*value: statistical significance if  $\leq 0.05$

Note: 17 samples were considered for RHD and SRY and 10 samples for RHC and RHE.

dance with the serologically determined phenotypes, with only 1 false-positive result (5.9%), for which the RHD was detect but the determined phenotype was RhD negative. The genotyping for this gene obtained a sensitivity of 100% and specificity of 67%, with a precision of 94% ( $p_{value}=0.001$ ;  $K=76.7\%$ ). Regarding the RHC gene, there was concordance in 60% of cases (6 of 10 samples), with 4 false-negative results (40%), a sensitivity of 43%, specificity of 100% and precision of 60% ( $p_{value}=0.175$ ;  $K=31\%$ ). For the RHE gene the concordance was 100% (10 of 10 samples), with a precision, sensitivity and specificity of also 100% ( $p_{value}=0.002$ ;  $K=100\%$ ).

It was not possible to establish any significant relation between the gestational age and the cfDNA con-

centration (which increases as the pregnancy progresses), once the population only included 2 samples collected in the 3<sup>rd</sup> trimester of pregnancy, while the remaining 15 were from the 2<sup>nd</sup> trimester.

## Discussion

The alloantibody screening revealed the presence of irregular Ab in 6 samples. The 4 samples (no. 2, 5, 9 and 11) that showed a positive result for anti-D Ab did belong to women with more than 28 weeks of gestational age; therefore, they had already received the anti-D Ig, which explains the presence of those Ab. Besides anti-D, the principal alloantibodies that lead to HDFN are those

directed against other Rh Ag (anti-c and anti-E) and anti-Kell, but also anti-Kidd (J<sup>k</sup>), anti-Duffy (F<sup>y</sup>) and Ab of the MNS blood group system, including anti-U. Thus, the alloantibodies detected in samples 4 (anti-Fy<sup>a</sup>) and 8 (anti-Fy<sup>a</sup> and anti-Jk<sup>a</sup>) are relevant in HDFN and a reason why these pregnancies should be monitored and followed closely.<sup>(36)</sup>

When comparing the obtained results in the present work with other studies it becomes necessary to notice the differences between the dimension of the study populations (which were highly superior in most of the papers) and the available laboratorial and methodological conditions. In order to avoid false-negative results, the genotyping involved 2 specific products of the *RHD* gene (exons 7 and 10), due to the genetic complexity of the variant forms, as recommended by other authors.<sup>(9,20)</sup>

The statistical results (Table II) revealed that fetuses born from RhD negative pregnant women can be genotyped for the *RHD*, *RHE* and *SRY* genes with an acceptable level of accuracy (94%, 100% and 82% respectively), but not for the *RHC* gene (60%). In similar works, the concordance level achieved by other investigators was mostly superior, as it can be seen on Table IV. The percentage of false-positive results (5.9% for *RHD* and *SRY*) could be justified, in part, by the available conditions to perform the PCR. Some of the standard basics to carry out this procedure couldn't be followed, such as the use of separated rooms to execute the cfDNA extractions and set up the amplification reactions, which possibly have resulted in the contamination of some samples. However, the use of real-time PCR offers an extra level of protection, since it consists of an optic detection system that obviates the need for any post-amplification manipulation or sample analysis (ex.: electrophoresis), decreasing the possibility of carryover contamination.

Still, the small population studied has many disad-

vantages and this study should be repeated with a much larger sample number, which would allow the conclusions to be generalized. It should also be designed a protocol to enhance the communication with the progenitors, in order to avoid the occurrence of possible drop-outs.

If the decision of undergoing immunoprophylaxis was dependent of the study's results, the application of this non-invasive prenatal diagnostic test before the 28<sup>th</sup> week of gestation would only cause 88.2% (n=15) of the considered pregnant women to receive the Anti-D Ig (from which 93.3% effectively carried an RhD positive fetus). From the included mothers, 82.4% had a RhD positive child and thus only 5.9% would have unnecessarily received the Ig, when compared to 17.7% without genotyping. Using this technique the treatment could have been avoided for 11.8% of the pregnant women, as well as the associated risk with the exposure to this human pooled blood product.

The false-positive incidence for *RHD* and *SRY* genes (5.9% in both cases) has a relatively limited importance, when compared to false-negatives (40% for *RHC* and 11.8% for *SRY*). Although there hasn't been verified any case in the present study, the occurrence of a false-negative result for the *RHD* gene could lead to the suspension of treatment with the Anti-D Ig, which would be associated with a potential alloimmunization and HDFN in subsequent pregnancies.

False-negative results may occur due to low concentration of cfDNA. Thus, the identification of an RhD negative fetus should be confirmed, to check if it is possible to detect another fetal marker in the sample. Taking sample 11 as an example, if the search is negative for *RHC* and *RHE* genes, but positive for *SRY*, the sample will correspond to a male fetus that paternally inherited the cde haplotype, confirming the presence of cfDNA in plasma and the specificity of the negative results for the

**Table IV Similar studies performed by other authors.**

Author and reference	Year	Studied gene	Concordance
Lo <i>et al</i> <sup>(12)</sup>	1998	<i>RHD</i>	100%
Rouillac-Le Sciellour <i>et al</i> <sup>(20)</sup>	2004	<i>RHD</i>	99.5%
González-González <i>et al</i> <sup>(32)</sup>	2005	<i>RHD</i> ; <i>DYS390</i>	90% ;92%
Hromadnikova <i>et al</i> <sup>(1)</sup>	2005	<i>RHD</i> ; <i>RHC</i> ; <i>RHE</i> ; <i>SRY</i>	100%
Minon <i>et al</i> <sup>(13)</sup>	2008	<i>RHD</i> ; <i>SRY</i>	100% ; 100%
Sesarini <i>et al</i> <sup>(19)</sup>	2009	<i>RHD</i> ; <i>SRY</i>	86.7% ; 94.6%
Geifman-Holtzman <i>et al</i> <sup>(6)</sup> (revision paper)	2009	<i>RHC</i> ; <i>RHE</i>	96.3% ; 98.2%
Cardo <i>et al</i> <sup>(11)</sup>	2010	<i>RHD</i>	97%
Akolekar <i>et al</i> <sup>(23)</sup>	2011	<i>RHD</i>	98.8%
Bombard <i>et al</i> <sup>(45)</sup>	2011	<i>RHD</i>	99.5%

other genes.

The relatively low precision observed for *SRY* can be rectified with the amplification of another Y chromosome marker, as it was done for the *RHD* gene. However, the positive internal control based on the detection of the *SRY* gene it's only applicable to pregnancies of male fetuses and, likewise, the *GLO* gene amplification only allows to conclude that there is DNA in the sample, but not its origin. Therefore, it is not possible to ascertain if a negative amplification for all genes is attributable to a female fetus or a false-negative result due to low quantity of cfDNA, as it has happened in sample 6.

Since the major issue of this approach is lacking an internal control that detects the presence of cfDNA when the PCR results are negative<sup>(20)</sup>, it was recently proposed the use of universal positive controls for fetal DNA, based on the detection of specific methylation markers.<sup>(14,21,22,38)</sup> One of those markers is the *SERPINB5* gene, hipomethylated in the placenta and hipermethyated in maternal blood cells.<sup>(22,38)</sup> However, this technique is dependent of bisulfite conversion, a method that can destroy a great portion of DNA.<sup>(21,22,33)</sup> More recent is the detection of the *RASSF41A* DNA sequence, that exhibits an opposite methylation pattern to that from *SERPINB5* gene (hipermethylated in the placenta and hipomethylated in maternal blood cells).<sup>(5,13,22,39)</sup>

Another problem of this approach is the coexistent background of maternal DNA in plasma. In order to overcome this obstacle, Dhallan *et al*<sup>(40)</sup> have tried to develop an enrichment method for cfDNA or suppression of the referred background; however, the results weren't reproducible by other authors.<sup>(41-43)</sup>

As a result of the high efficiency and convenience of blood collection instead of performing invasive prenatal diagnostic procedures, the fetal *RHD* genotyping is used when combined with other tests in various European countries, like United Kingdom, Czech Republic, Netherlands, among others.<sup>(44)</sup> This test is already a reality in Belgium's laboratorial routine<sup>(13)</sup> and offered (by request) in centers located in Bristol, Göttingen, Paris and Marseille<sup>(44)</sup>. In some countries, the implementation of this strategy shows to be cost-effective, as reported in Netherlands<sup>(13)</sup> and United Kingdom.<sup>(10)</sup> Still, a study performed by Szczepura *et al*<sup>(7)</sup> in 2011, doesn't support the introduction of this technique in England and Wales, referring that the annual savings will be relatively small. Studies made in several other countries as France<sup>(5)</sup> and United Kingdom<sup>(4)</sup>, have concluded that the implementation of this methodology would be beneficial.

Similarly, when combined with serologic methods,

the Rh genotyping is already an important tool to clarify discrepancies and ambiguous grouping results in transfusion medicine, a fact that should encourage the testing of *RHD* markers for non-invasive prenatal diagnosis of the RhD genotype.<sup>(44)</sup>

Since this analysis only requires a small blood sample, it could raise numerous ethical, social and legal implications, due to the ease with which the test can be performed, such as potential non-medical applications, as the choice of fetal sex.

## Conclusion

The present study confirms the accuracy of fetal *RHD* and *RHE* genotyping (94%;100%) in maternal plasma without the existence of false-negative results.

The feasibility of the fetal *RHD* and *RHE* genotyping in maternal plasma is highly desirable for economic reasons. First, it avoids unnecessary administration of anti-D Ig, a blood product, to approximately 40% of the RhD negative women, also pregnant with an RhD negative fetus<sup>(5,11-13)</sup> (17.7% in this study); second, there would be no need to perform invasive procedures with risks to both mother and fetus, which also allows to avoid sensitization, a possible result of FMH.

Thus, fetal genotyping in maternal plasma should be offered to all RhD negative pregnant women, in order to limit the use of antenatal immunoprophylaxis only to those carrying RhD positive fetuses and also to avoid false-negative results, which could result in subsequent immunization.

Performing a study with a larger number of samples could allow the implementation of this prenatal diagnostic test in clinical laboratory routine, but first, the Rh and fetal sex genotyping must be economically evaluated in Portugal, with the purpose of defining the impact on health care costs. In long term, depending on the development of the appropriate technology and of a robust evaluation, fetal DNA analysis may play an important role on the national programs of prenatal screening for all RhD negative pregnant women.

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