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ORIGINAL ARTICLE

Clinical application of midtrimester non-invasive fetal *RHD* genotyping and identification of *RHD* variants in a mixed-ethnic population

M. Grande[1], E. Ordoñez[2], V. Cirigliano[2], J. Cid[3], E. Grau[3], A. Pericot[1], I. Teixido[1], J. L. Marin[4] and A. Borrell[1]*

[1]Department of Maternal-Fetal Medicine, Institute Gynecology, Obstetrics and Neonatology, Hospital Clínic Barcelona, Catalonia, Spain
[2]Labco-General Lab, Barcelona, Catalonia, Spain
[3]Institut Català de la Salut, Barcelona, Catalonia, Spain
[4]Centre de Diagnostic Biomedic, Barcelona, Catalonia, Spain
*Correspondence to: Antoni Borrell. E-mail: aborrell@clinic.cat

ABSTRACT

Objective This study aims to assess the suitability of non-invasive prenatal *RHD* genotyping in non-immunized midtrimester pregnant women from a mixed ethnic population, to prevent unnecessary anti-D immunoglobulin prophylaxis and to identify *RHD* variants

Methods Rhesus D-negative pregnant women were offered fetal *RHD* genotyping at 24 gestational weeks. A total of 284 samples were tested for *RHD* status using multiplex rt-PCR amplification of exons 5 and 7 of the *RHD* gene and exons 6 and 10 in selected cases. Women carrying *RHD*-negative fetuses were counseled about their option to avoid routine antenatal anti-D immunoglobulin administration. Diagnostic accuracy of *RHD* genotyping was compared with postnatal Rhesus D serotyping.

Results A total of 184 positives (65%), 91 negatives (32%) and 7 cases (2.5%) compatibles with *RHD* variants were detected by *RHD* genotyping. No false negative results were found, and a single false positive was observed in a twin pregnancy. Genotyping was accepted when offered by 94% of women (284/302), and anti-D immunoglobulin was avoided in 95% (90/95) of *RHD*-negative fetuses.

Conclusions Non-invasive routine antenatal *RHD* genotyping at 24 weeks of pregnancy is a highly accurate method, resulting in the avoidance of 95% of unnecessary administrations of anti-D immunoglobulin, with no false negative results. © 2012 John Wiley & Sons, Ltd.

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INTRODUCTION

Non-invasive fetal *RHD* genotyping in maternal blood was initially applied in alloimmunized pregnant women to confirm the risk of fetal hemolytic disease, avoiding the need of genotyping in amniotic fluid.[1] More recently, it has been applied to Rhesus D (RhD)-negative pregnant women from the general population in order to avoid routine antenatal anti-D prophylaxis, when the fetus was found to be *RHD* negative.[2]

The *RHD* gene is located in chromosome 1, and it encompasses ten exons contiguous to the *RHCE* gene to form the *RH* locus.[3] In Caucasians, the vast majority of RhD negative individuals are homozygous for a complete deletion of the *RHD* gene.[4] However, in other ethnicities such as black Africans, several *RHD* variants have been described with an RhD-negative phenotype, such an inactive *RHD* gene called *RHDΨ* pseudogene harboring a 37-bp duplication in exon 4 and a nonsense mutation in exon 6[5] or the *RHD-CE-D* hybrid gene containing exons from both *RHD* and *RHCE*.[6,7]

Prenatal *RHD* genotyping can be performed in maternal plasma, using circulating cell-free fetal DNA (cffDNA) to detect fetal *RHD*-specific sequences, which are absent in RhD-negative women.[8–11] Several studies on *RHD* genotyping in individuals of Caucasian origin have reported an accuracy near to 100%, using sequences from two or three exons.[12–14] However, first trimester *RHD* genotyping may result in a high false negative rate, and limited data are available on *RHD* genotyping in mixed ethnic populations, including postnatal confirmation of plasma analysis and of the *RHD* variants.

The aim of our study was to screen our mixed-ethnic population in the late second trimester to increase the sensitivity of *RHD* detection by exploiting the increased amount of cffDNA in maternal plasma. We also aimed to assess

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the accuracy of *RHD* genotyping using *RHD* exons 5, 7 and 10 in our mixed-ethnic population, including the identification and postnatal confirmation of *RHD* variants and the evaluation of its implementation as a routine test within the Catalan public health service in Spain.

MATERIALS AND METHODS

Population and study design

From February 2010 to October 2011, 302 RhD-negative pregnant women from the general population, attending one of the six health centers of Barcelona-West health district, were offered fetal *RHD* genotyping in maternal blood, at 24–26 gestational weeks, at the time of second trimester blood testing. The study was approved by the Hospital Ethics Committee, and all samples were collected with informed consent. Pregnant women were counseled about their option to avoid routine antenatal anti-D immunoglobulin in the third trimester in the presence of *RHD*-negative fetus. Twin pregnancies were also included in the study.

Sample collection

Blood samples were collected in two different 3 mL EDTA-K vacutainer tubes and plasma separated by low speed centrifugation (10 min at 3000 rpm) within 24 h from extraction. A second high speed centrifugation of 10 min at 12 500 rpm and 4 °C was performed in all samples. Collected supernatants were coded and stored at −20 °C until further processing.

DNA extraction

DNA was extracted from 850 μL of plasma using the COBAS AmpliPrep® DNA/RNA automated extractor (Roche Diagnostics), lowering the final elution volume to 75 μL; 9 μL of this volume was used as PCR template.

Real-time PCR

All samples were tested using a single multiplex rt-PCR including the primers and probes for *RHD* exons 5 and 7 and the *DYS14* multicopy sequence on the Y chromosome. Primers and probes for the *RHD* exon 5 were adapted from Finning *et al.*[11] to also target the mismatches that allow discriminating the *RHDΨ* pseudogene (Table 1 and Figure 2).

The inclusion of one marker on the Y chromosome allows confirming the presence of fetal DNA in *RHD*-negative male fetuses. A second multiplex including *RHD* exon 10 and SRY was used to confirm *RHD*-negative fetuses on a second DNA extraction. Negative female results were reported following two independent assays performed in triplicate (six PCRs in total on two different DNA extractions), with positive amplification of the *β-actin* gene to confirm the presence of DNA and exclude the possibility of assay failure. PCR amplification of the *RHD* exon 6 was only used in selected cases to confirm the presence of an *RHD* variant.

All rt-PCR amplifications were performed in a final volume of 25 μL using the Gene Expression Master Mix (Applied Biosystems) for 50 repeating cycles using the 7300 Real-Time PCR System (Applied Biosystems). All samples were tested in triplicates; positive and DNA extraction negative controls were included in all PCR batches.

Table 1 **Primers and probes used for multiplex real-time PCR assays**

Target (amplicon size)	Name*	Sequence (5′-3′)
DYS14 (84bp)	DYS14F	GGGCCAATGTTGTATCCTTCTC
	DYS14R	GCCCATCGGTCACTTACACTTC
	DYS14P	(NED) TCTAGTGGAGAGGTGCTC (MGB)
RHD exon 5 (82 bp)	r5F	CGCCCTCTTCTTGTGGATG
	r5R	GAACA**C**GGCATTCTTCCTTT**C**
	r5P	(6-Fam) CTGGCCAAGTTTC (MGB)
RHD exon 7 (57 bp)	r7F	TGCTGCTGGTGCTTGATACC
	r7R	TAAGCCCAGTGACCCACATG
	r7P	(VIC) CGGAGCCGGCAAT (MGB)
RHD exon 10 (74 bp)	r10F	CCTCTCACTGTTGCCTGCATT
	r10R	AGTGCCTGCGCGAACATT
	r10P	(VIC) TACGTGAGAAACGCTCAT GACAGCAAAGTCT (TAMRA)
SRY (78 bp)	SRYF	TCCTCAAAAGAAACCGTGCAT
	SRYR	AGATTAATGGTTGCTAAGGACTGGAT
	SRYP	(6-Fam) CACCAGCAGTAACTCCCCA CAACCTCTTT (TAMRA)
RHD exon 6 (153 bp)	r6F	ACACGCTATTTCTTTGCAGACTTCT
	r6R	AGGTACTTGGCTCCCCCAA**C**
	r6P	(VIC) AGATAGCCCAGCCACAA GACCCAG (MGB)
β-Actin (137 bp)	β-actF	GCGCCGTTCCGAAAGTT
	β-actR	CGGCGGATCGGCAAA
	β-actP	(NED) ACCGCCGAGACCGCGTC (MGB)

Primers and probes in exons 5 and 6 are designed on the mismatches that distinguish *RHD* from *RHCE* (bold) and *RHD* from *RHDΨ* (double-underlined). Probes labeling for multiplex PCR amplification is also shown.
*F = forward primer, R = reverse primer, P = probe.

Sample analysis

Samples were scored as *RHD*-positive if both *RHD* exons 5 and 7 were detected in at least two replicates and *RHD*-negative in the absence of amplification for all replicates. Positive amplification for *DYS14* in at least two replicates was considered evidence of male fetuses. Positive results with a threshold cycle value (Ct) ≥42 for any target or samples with detectable amplification in only one of the replicates were deemed inconclusive and confirmed on a second DNA extraction.

Data analysis

Maternal and demographic data and details on antenatal *RHD* genotyping and postnatal RhD serotyping were registered in an SPSS database (Statistical Package for the Social Sciences). Sensitivity, specificity, positive predictive value and negative predictive value of antenatal *RHD* genotyping were compared

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with conventional RhD serotyping, carried on routinely in umbilical cord blood after birth. Variants were confirmed in newborn blood spots using a single multiplex rt-PCR including primers and probes for *RHD* exons 5 and 7 and 6 and 10 in selected cases. Data were stratified by ethnic origin, because of known *RHD* variability in non-Caucasian ethnicities.

RESULTS

Genotyping was accepted by 94% (284/302) of RhD-negative pregnant women, and a total of 284 plasma samples were collected from 268 singleton and 16 twin pregnancies. Our study population was of mixed ethnic origin including 84% Caucasians (*n* = 238), 12% Latin Americans (*n* = 35), 1.4% Magreb Africans (*n* = 4), 1.1% Pakistanis (*n* = 3), 0.4% Oriental (*n* = 1), 0.4% Sub-Saharan Africans (*n* = 1) and 0.7% (*n* = 2) from other ethnicities. Blood group distribution was as follows: 43% O negative (*n* = 123), 39% A negative (*n* = 112), 13% B negative (*n* = 36) and 3.9% AB negative (*n* = 11) (Table 2).

Two samples were not informative, as *RHD* exons were detected in amount compatible with a maternal origin; the maternal *RHD* positive result was also confirmed on DNA extracted from the correspondent buffy coat. These two samples were excluded from further analysis. Antenatal *RHD* genotyping resulted in 65% positive (184/282) and 32% negative (91/282) results (Figure 1). Negative female fetuses accounted for 44% (40/91) of negative results (Table 2).

RHD variants were suspected in 2.5% of samples (7/282) (Table 2), 1.3% (3/238) in those from Caucasian and 11.4% (4/35) from Latin American women. Three samples showed positive amplification for *RHD* exons 7 and 10 in the absence of exon 5, a pattern compatible with *RHDΨ* or *RHDVI* variant (Table 3, Figure 2). Two samples were from Latin American, and the third was from a Caucasian woman. These results were reported as likely *RHD* variants, with a positive phenotype, which was confirmed postnatally by RhD typing. Postnatal follow-up *RHD* genotyping, with further detection of the *RHD* exon 6, confirmed the presence of a possible *RHDVI* type 1 or 4 variant in two Latin American women, and the remaining variant was not confirmed, as all *RHD* exons, including exon 5, were postnatally detected.

The *RHD* exon 10 was only used to confirm *RHD* negative results, using a second PCR on a second DNA extraction. This was the only detectable *RHD* sequence in four samples, compatible with a hybrid *RHD-CE-D* variant (Table 3). Two of these samples were from Latin American and two from Caucasian women, the four cases being reported as *RHD* negatives. Postnatal RhD serotyping and genotyping confirmed the RhD status established at antenatal genotyping.

The single false positive result was observed in one sample from a twin pregnancy, showing *RHD* exons 5 and 7 amplification, while postnatal RhD typing revealed two RhD-negative newborns. Postnatal *RHD* genotyping confirmed the absence of exons 5, 6, 7 and 10 in one of the newborns, in agreement with the RhD-negative phenotype. However, amplification of *RHD* exons 5, 6, 7 and 10 was observed in the sibling, compatible with the antenatal *RHD* genotyping but in disagreement with the RhD typing. Paternal zygosity analysis revealed a heterozygous RhD positive status, which may explain the discordant *RHD* genotypes of siblings. However, a *de novo* mutation or rearrangement inactivating the *RHD* gene in one of the siblings is suspected as the only possible explanation of this discrepant result.

Rhesus D typing of the newborns was concordant with prenatal *RHD* genotyping in 99.6% (281/282) of the pregnancies, resulting in 100% (186/186) sensitivity, 99% (95/96) specificity, 99% (186/187) positive predictive value and 100% (95/95) negative predictive value.

Only 5% (5/95) of women carrying an *RHD*-negative fetus requested antenatal anti-D immunoglobulin. Thus, unnecessary prophylactic treatment could be avoided in 95% (90/95) of women carrying RhD-negative fetuses.

Table 2 Pregnancy characteristics and sampling results

Ethnic group	
Caucasians	238 (84%)
Latin Americans	35 (12%)
Magreb Africans	4 (1.4%)
Pakistanis	3 (1.1%)
Oriental	1 (0.4%)
Sub-Saharan Africans	1 (0.4%)
Other	2 (0.7%)
Maternal blood group serology	
O negative	123 (43%)
A negative	112 (39%)
B negative	36 (13%)
AB negative	11 (3.9%)
Fetal *RHD* genotyping/sex determination	
RHD positive/male	101 (36%)
RHD positive/female	83 (29%)
RHD negative/male	51 (18%)
RHD negative/female	40 (14%)
Suspected variant male	5 (1.8%)
Suspected variant female	2 (0.7%)

DISCUSSION

Our study demonstrates that midtrimester non-invasive fetal *RHD* genotyping by targeting exons 5, 7 and 10 (with the occasional inclusion of exon 6) is a highly accurate method to avoid unnecessary anti-D immunoglobulin administration in women carrying RhD-negative fetuses. Interestingly, *RHD* variants were found more frequently than expected in our mixed ethnic pregnant population.

Non-invasive fetal *RHD* genotyping has extensively been evaluated in immunized and non-immunized pregnancies since free fetal DNA in maternal plasma was identified.[9–11,15] The 99.6% of accuracy observed is higher than the overall 94.8% reported in a meta-analysis including 44 protocols for non-invasive fetal *RHD* testing[16] and similar to more recent reports (99.3–99.8%)[12–14] or to conventional postnatal blood serotyping. False negative results, which are really clinically relevant, were not observed, confirming that fetal genotyping

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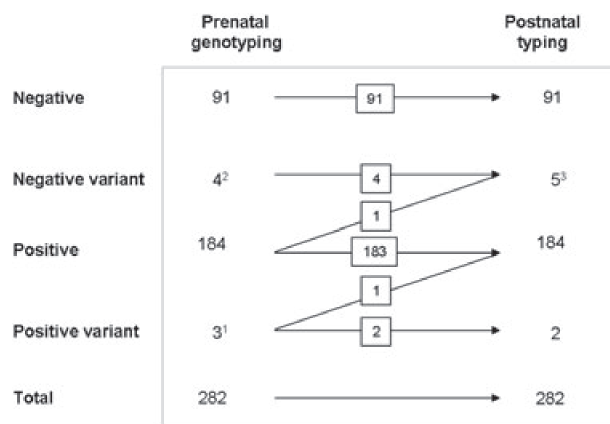

(1) Exons 6, 7 and 10 positive , exon 5 negative, compatible with DVI type 1 or 4. One case confirmed as positive for all exons at birth

(2) Exons 5, 6, and 7 negative, exon 10 positive compatible RHD-CE-D

(3) 1 RhD negative newborn with positive exons 5, 6, 7 and 10 compatible with *de novo* mutation

Figure 1 Results of fetal *RHD* genotyping in 282 pregnancies and the corresponding newborn RhD typing

Table 3 Results of testing 282 consecutive clinical samples for prenatal assessment of *RHD* genotypes and fetal sex

Prenatal results	*RHD* exon 5	*RHD* exon 7	*DYS14*	*RHD* exon 10	*SRY*	*RHD* exon 6	Anti-D immunoglobulin administration
Male positive (n = 101)	101	101	101	Not tested	Not tested	Not tested	yes
Male negative (n = 51)	—	—	51	—	51	Not tested	No
Female positive (n = 83)	83	83	—	Not tested	Not tested	Not tested	Yes
Female negative (n = 40)	—	—	—	—	—	Not tested	No
Male *RHDVI* type 1 or 4 (n = 1)	—	1	1	1	1	1	Yes
Female *RHDVI* type1 or 4 (n = 2)	—	2	—	2	—	2	Yes
Male *RHD-CE-D* (n = 4)	—	—	4	4	4	Not tested	No

can achieve a 100% negative predictive value if carried out late in the second trimester. False negative results have mainly been related to specific DNA extraction methods,[12] prolonged stored time before sample processing[17] and, particularly, early gestational age at maternal blood draw, because of the low amount of fetal DNA present in maternal plasma. Although recent first trimester studies have reported false negative rates ranging from 1.1% to 3.5%,[18–20] we assumed that false negative rates above 1% would have not been easily accepted by clinicians. Thus, we opted to perform cffDNA analysis at the time of routine midtrimester maternal blood sampling (about 24 weeks) when the amount of fetal DNA in maternal plasma is much higher.[21] Even in this case, the gestational age at reporting time, 1–2 weeks after sampling, is still well ahead the recommended gestational age for routine antenatal anti-D prophylaxis in the third trimester. Furthermore, the multicopy *DYS14* and the single-copy *SRY*-based internal positive control used together with *RHD* exons 5 and 7 or 10, respectively, at least confirmed the presence of cffDNA in male *RHD*-negative fetuses. Alternatively, the use of three Y-chromosome sequences with the *TGIF* marker has recently been reported to maximize the accuracy of the test,[20,22] whereas the hypermethylated DNA sequence of *RASSF1A* described as the first universal marker for cffDNA[23] is rarely used in daily routine because of the labor-intensive procedure involved in its analysis. Confirming the presence of fetal specific DNA markers in maternal plasma is of course of paramount importance during first trimester, to reduce false negative results. Different approaches have been used such as multiplex SNPs genotyping to detect paternally inherited polymorphisms.[20,22,24] Our study design targeting the *RHD* genotyping at 24 weeks using a screening multiplex rt-PCR

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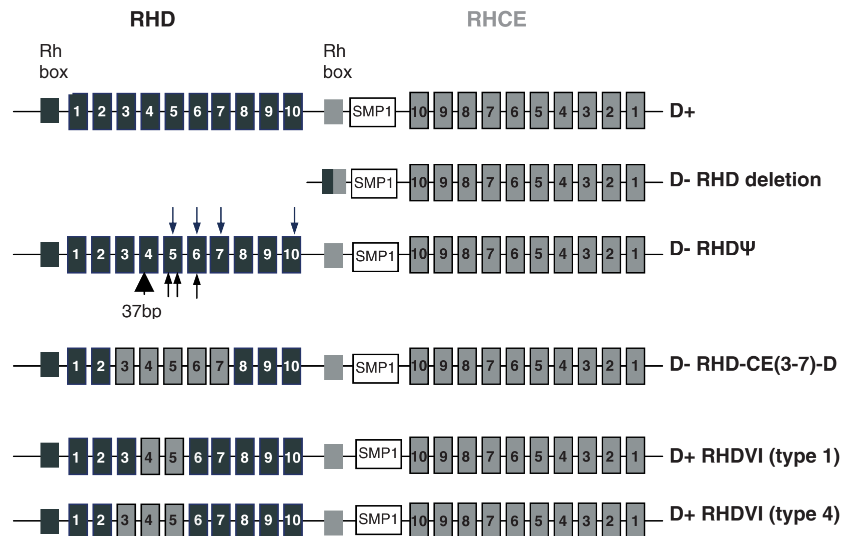

Figure 2 Diagram of Rh genes. RhD negative status (D⁻) is mostly due to the complete lack of the RHD gene. Primers and probes selected for the *RHD* exons 5 and 6 (top arrows) are designed on mismatches (bottom arrows) allowing discriminating true RhD-negative fetuses from cases carrying common *RHD* gene variants. The *RHD* pseudogene can be detected as lack of *RHD* exons 5 and 6 amplification in the presence of exons 7 and 10. Other Rh gene variants, such as *RHD-CE-D* (D⁻) hybrids (*RHD-CE(3-7)-D* shown here) or the *DVI* type 1 and type 4 variants, D positive (D⁺), can also be detected

including appropriate selection of *RHD* exons resulted in all RhD-negative fetuses being correctly identified.

There is no consensus about the *RHD* exons to be tested in fetal genotyping, with exons 4, 5, 7 and 10 the most widely used by different groups. *RHD* sequences selection is crucial to discriminate common *RHD* variants in multi-ethnic populations and to avoid false positive and negative results.[25] In our series, we screened all samples using exons 5 and 7, confirming negative results with exon 10 and extending the study to exon 6 only in selected cases. The inclusion of exon 5 has been proven useful to detect the *RHDΨ* pseudogene, which might give false positive results when testing only exons 7 and 10.[11] However, in our series, we observed three cases without amplification of this sequence, which could be identified as *RHDVI* variants (either type 1 or 4 and both RhD positive) by detecting amplification for the exon 6. The inclusion of this sequence was of diagnostic value to determine the fetal positive RhD status, allowing for the discrimination of this variant from the *RHDΨ*, even in one case in which newborn analysis confirmed the exon 5 amplification failure in maternal plasma. Although *RHDVI* type 1 or 4 has been described to account up to 88% *RHDVI* alleles in Spain,[26] the *RHDVI* variants were identified in women of Latin-American ethnicity.

The *RHD-CE-D* hybrid was the most frequent variant in our population and could only be detected when confirming the negative results for both exons 5 and 7 in a second DNA extraction, positive for the exon 10. In our experience, this second confirmation might have not been required as the negative *RHD* result for the first two exons would have agreed with the RhD negative result of the newborn. Our results confirm that screening protocols including only two *RHD* exons are not suitable for pregnant women of different ethnicities. Amplification of exons 7 and 10 or exons 5 and 7, for instance, might provide false positive or inconclusive results, respectively, in *RHDΨ* variants.[18,27] More recently, the use of a single *RHD* exon (4) has been reported to correctly assign the RhD status in *RHDΨ* and *RHD-CE-D*,[19] but this approach would fail in our population because it would provide a false negative result in *RHDVI* variants.

Newborn blood spots retesting was available and allowed to confirm all suspected variants. Neonatal follow-up raised the suspicion of a new rare variant causing the only false positive result observed in the course of this study. This was a twin pregnancy, in which all *RHD* exons 5, 6, 7 and 10 were detected in maternal plasma, and at birth in one of the two siblings, with discordant RhD-negative serology. Several RHD variants, such as *RHD-CE(8-9)-D* variant, have been described in Europeans as *RHD*-positive haplotype and negative serology with a prevalence of 1 : 15 512.[28] Interestingly, in this case, the rearrangement or mutation even must have arose *de novo* as one of the siblings inherited an *RHD*-negative allele while the other inherited an inactive *RHD* from the RhD⁺ father.

This study is strengthened by it being performed as an interventional study in a real clinical setting, the Catalan public health service, with antenatal visits being carried out in antenatal care centers for low risk women and in the hospital for high risk patients. All six antenatal care centers of our health district were involved in the study, resulting in a high uptake of pregnant women, which agreed in their vast majority both to participate (94%) and to avoid unnecessary prophylaxis (95%). One limitation of *RHD* genotyping in the second trimester might be the exclusion of women with early vaginal bleeding and threatened abortion or undergoing invasive procedures, commonly performed at 12–18 weeks.[29] However, these cases account for less than 10% of the pregnant women; thus, the approach of late genotyping would avoid immunoprophylaxis in less than 4% of women with *RHD*-negative fetuses. This 4% overtreatment would have a much smaller impact than the 1.1% to 3.5% undertreatment produced by first trimester false

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negative results.[18,19] On the other hand, the overall proportion of Caucasians in our population (84%) might be overrepresented considering that only 50% deliveries in our hospital are from women of this ethnical group. Difficulties to explain the aims of the study might have precluded a higher representation of other ethnicities (i.e. Latin Americans, Africans or Asians), which more frequently carry *RHD* variants.

Our results indicate that fetal genotyping with exons 5, 6 (in selected cases), 7 and 10 in our mixed population before 28 weeks of gestation is highly accurate to avoid unnecessary treatment in one third of the pregnant women screened, and it should be considered for further clinical application.

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WHAT'S ALREADY KNOWN ABOUT THIS TOPIC?

- Routine antenatal *RHD* genotyping performed in maternal plasma by real-time PCR is an accurate method to avoid the use of anti-D immunoglobulin when both the pregnant woman and the fetus are RhD negative.

WHAT DOES THIS STUDY ADD?

- Fetal genotyping at 24 weeks of gestation is highly accurate using probes for *RHD* exons 5 and 7 (10 and 6 in selected cases), minimizing the false negative results and allowing prenatal detection of *RHD* variants in our mixed ethnic population.

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