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Terminating Routine Cord Blood RhD Typing of the Newborns to Guide Postnatal Anti-D Immunoglobulin Prophylaxis Based on the Results of Fetal RHD Genotyping

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Mini-Summary

What does this study add to current knowledge?

- Our study adds more information and data needed to terminate routine cord blood RhD typing of the newborns to give postnatal anti-D immunoglobulin (Ig) to RhD-negative women, based on the result of fetal *RHD* genotyping, when genotyping predicts an RhD-positive fetus. This approach will streamline the maternity care.

What are the main clinical implications?

- NIPT using cell-free fetal DNA in maternal plasma to determine fetal RhD blood type aims identifying RhD-negative pregnant women with alloimmunization risk as they carry RhD-positive fetuses. We have shown high sensitivity of the fetal *RHD* genotyping assay that is essential not to risk false-negative results that will expose women to higher alloimmunization risk following discontinuation of cord blood RhD typing of the newborns. These women will then neither receive antenatal nor postnatal prophylaxis with anti-D Ig.

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Keywords
Fetal *RHD* genotyping · Alloimmunization · Noninvasive prenatal diagnosis · Cord blood RhD typing · Rhesus alloimmunization · Red cell alloimmunization

Abstract
Introduction: Targeted routine antenatal prophylaxis with anti-D immunoglobulin (Ig) only to RhD-negative pregnant women who carry RhD-positive fetuses (determined by fetal *RHD* genotyping) has reduced D-alloimmunization significantly when administered in addition to postnatal prophylaxis. Achieving high analysis sensitivity and few false-negative fetal *RHD* results will make RhD typing of the newborn redundant. Postnatal prophylaxis can then be given based on the result of fetal *RHD* genotyping. Terminating routine RhD typing of the newborns in cord blood will streamline maternity care. Accordingly, we compared the results of fetal *RHD* genotyping with RhD typing of the newborns. ***Methods:*** Fetal *RHD* genotyping was performed, and antenatal anti-D Ig was administered at gestational week 24 and 28, respectively. Data for 2017–2020 are reported. ***Results:*** Ten laboratories reported 18,536 fetal *RHD* genotypings, and 16,378 RhD typing results of newborns. We found 46 false-positive (0.28%) and seven false-negative (0.04%) results. Sensitivity of the assays was 99.93%, while specificity was 99.24%. ***Conclusion:*** Few false-negative results support the good analysis quality of fetal *RHD* genotyping. Routine cord blood RhD typing will therefore be discontinued nationwide and postnatal anti-D Ig will now be given based on the result of fetal *RHD* genotyping.

Introduction

Targeted routine antenatal anti-D prophylaxis (RAADP) with anti-D immunoglobulin (Ig) to RhD-negative pregnant women was implemented nationwide in Norway in 2016 [1]. Fetal *RHD* genotyping is performed at gestational week (GW) 24, and RAADP is given at GW28 only to those women with an RhD-positive fetus. RAADP in addition to routine postnatal prophylaxis reduces the risk of anti-D alloimmunization in women with RhD-positive newborns [2]. Postnatal anti-D prophylaxis is given only if the newborn is found to be RhD positive in routine serologic RhD typing of all newborns of RhD-negative women. The National Working Group that was established in 2015 to achieve consensus in management of the RAADP program has continued monitoring the challenges aiming to improve the routine [1]. Using local quality registries with comprehensive data, we have especially kept an eye on discrepancies between the results of fetal *RHD* genotyping versus newborns' serologic RhD typing [1]. Ensuring high analysis quality of fetal *RHD* genotyping is essential because without RhD typing of the newborns, false-negative results of fetal *RHD* genotyping may lead to higher D-immunization risk as the RhD-negative women will then neither receive antenatal nor postnatal anti-D Ig. Reliable test quality of fetal *RHD* genotyping will make RhD typing of the newborn redundant. Postnatal prophylaxis can then be administered based on the result of fetal *RHD* genotyping. Thus, aiming to reveal particularly false-negative results, we investigated discrepancies to the extent we could receive blood samples from the mother and blood or buccal swab from the newborns.

Materials and Methods

Methods that in total four laboratories, one in each health region, used for fetal *RHD* genotyping were previously described [1, 3]. In short, all performed fetal *RHD* genotyping at GW24 with assays targeting exons 7 and 10 in three of the laboratories, while the fourth used an assay targeting exons 5, 7, and 10.

Since these four regional laboratories only have the results of cord blood RhD typing when the women give birth at the same hospital, we invited 18 Norwegian laboratories including the four regional laboratories to join the study. These 18 laboratories receive the results of fetal *RHD* genotyping and perform immunohematologic pregnancy follow-up including antibody screens. They also perform routine serologic RhD typing of the newborns. They were asked to report the total number of positive, negative, and inconclusive results of fetal *RHD* genotyping between 2017 and 2020 together with the serologic RhD type of the newborns. We also asked them to report discrepancies between fetal *RHD* genotyping and serologic RhD type of the newborns.

Of the 18 we invited, the following 10 blood bank laboratories contributed with data: Nordland Hospital, Bodø and University Hospital of North Norway, Tromsø (Health Region North); St. Olavs Hospital, Trondheim (Health Region of Central Norway); Haukeland University Hospital, Bergen and Stavanger University Hospital, Stavanger (Health Region West); Vestfold Hospital, Tønsberg Vestre Viken Hospital, Drammen, Innlandet Hospital, Lillehammer, Akershus University Hospital, Lørenskog, and Oslo University Hospital, Oslo (Health Region South-East).

Sensitivity of the assay was calculated using the following formula: the number of true-positive *RHD* genotyping results/(the number of true-positive *RHD* genotyping results + the number of false-negative *RHD* genotyping results). Specificity calculation was as follows: the number of true-negative *RHD* genotyping results/(the number of true-negative *RHD* genotyping results + the number of false-positive *RHD* genotyping results).

Table 1. Overview of the annual birth numbers in Norway, estimated number of RhD-negative pregnant women in the population and the proportion of fetal *RHD* genotypings in relation to the estimated number of RhD-negative pregnant women in Norway, i.e., how many fetal *RHD* genotypings were performed in the total estimated number of RhD-negative pregnant women

Year	Annual number of births*	Estimated number of RhD-negative pregnant women in Norway**	The number of fetal *RHD* genotypings in the study	The proportion of fetal *RHD* genotypings in relation to the estimated number of RhD-negative pregnant women, %
2017	55,868	8,380	3,970	3,970/8380 = 47
2018	54,432	8,165	4,436	4,436/8165 = 54
2019	53,788	8,068	4,908	4,908/8068 = 61
2020	52,979	7,947	5,222	5,222/7947 = 66

*Data from Statistics Norway https://www.ssb.no/en. **Based on the assumption that 15% of the population in Norway is RhD negative.

Results

We present data from ten laboratories. Based on the assumption that 15% of the population in Norway is RhD negative, fetal *RHD* genotypings (n = 18,536) from 2017 throughout 2020 corresponded to almost 57% of the estimated number of RhD-negative pregnant women (Table 1). Newborns' serologic RhD typing results (n = 16,378), on the other hand, covered almost 50% of the estimated number of newborns of RhD-negative women in the whole country during the same period (Table 2). Sensitivity of the assays was 99.93%, while specificity was 99.24%.

As shown in Table 2, fetal *RHD* genotyping predicted an RhD-positive fetus in 61.5% of the samples, while 37.3% was RhD negative and 1.3% was inconclusive. Comparing fetal *RHD* genotyping with the newborn's RhD typing revealed discrepancies in 0.32% of the cases (n = 53). We found 46 false-positive (0.28%) and seven false-negative (0.04%) results, meaning that 46 women received anti-D prophylaxis unnecessarily. False-negative results are displayed in Table 3**.** In six of the seven false-negative cases, the women did not receive antenatal anti-D prophylaxis, but postnatal prophylaxis was given to all when the newborn was typed to be RhD positive.

Discussion

The clinical repercussion of terminating routine RhD typing will be streamlining the maternity care since it will not be necessary to take a cord sample and wait until the result is available to give the postpartum anti-D Ig. Consequently, postnatal anti-D prophylaxis can be given immediately after or in connection to delivery. Terminating routine RhD typing of newborns will also be timesaving regarding sampling and analysis, at the same time will mean an annual saving of the costs of serologic RhD typing. In 2022, 51,500 children were born in Norway. Assuming approximately 15% of the mothers being RhD negative, they gave birth to 7,725 babies who were routinely RhD typed. A serologic RhD typing costs about 11.34 EUR, implicating that almost 87,600 EUR will be saved annually. On the other hand, the major clinical drawback may be due to false-negative fetal *RHD* genotyping results that can lead to increased alloimmunization risk since neither antenatal nor postpartum prophylaxis will then be given. However, we will continue to monitor the number of D-immunized women to ensure a continuing high quality of the fetal *RHD* genotyping with high assay sensitivity with least possible false-negative results also after termination of serologic RhD typing of newborns by the following measures. First, The Norwegian National Advisory Unit on Immunohematology at Department of Immunology and Transfusion Medicine, Oslo University Hospital, Ullevaal that in 2019 initiated a national quality assessment program for fetal *RHD* genotyping with biannual workshops will continue the program. All the four Norwegian laboratories performing fetal *RHD* genotyping have participated in these national workshops, in addition to the external quality assessment program organized by Danish Institute for External Quality Assurance for Laboratories in the health sector (DEKS). Furthermore, data on occurrence of pregnancy-related anti-D both from National Transfusion Statistics and the Medical Birth Registry of Norway will give us the opportunity to unveil a possible increase in the number of de novo alloanti-Ds in case this altered strategy fails.

Interpretation of serologic RhD typing of the newborns is not always straightforward, for example, due to direct antiglobulin test positivity caused by ABO incompatibility or antenatal anti-D prophylaxis given during

Table 2. The results of fetal *RHD* genotyping and serologic RhD typing of the newborns together with discrepancies between these two results

	Fetal *RHD* positive, *n*	Fetal *RHD* negative, *n*	Fetal *RHD* inconclusive, *n*	Serologic RhD type of the newborns		Discrepancies	
				RhD positive, *n*	RhD negative, *n*	False positive, *n*	False negative, *n*
2017	2,431	1,483	56	2,292	1,332	13	2
2018	2,710	1,669	57	2,444	1,449	7	1
2019	3,054	1,787	67	2,753	1,544	13	3
2020	3,203	1,966	53	2,874	1,690	13	1
Total	11,398	6,905	233	10,363	6,015	46	7

False positive: fetal *RHD* positive, newborn RhD negative. False negative: fetal *RHD* negative, newborn RhD positive. The false-negative results were categorized as *RHD* negative in the Table and statistics and reported in discrepancies. One of the laboratories reported only results from 2019 to 2020. Another laboratory reported only 2018–2020.

Table 3. Overview of the false-negative results

Case number	Fetal *RHD* negative, serologic RhD type of the newborn positive	Antenatal anti-D prophylaxis	Postnatal anti-D prophylaxis
1	Strong indication of erroneous sample collection from a wrong newborn	No	Yes
2	Serologic RhD typing with weak (0.5+) reaction strength. Not re-typed. No information about DAT and ABO type of the newborn	No	Yes
3	Twin pregnancy with one newborn RhD negative, the other RhD positive	No	Yes
4–6	No more information could be obtained	No	Yes
7	One laboratory misinterpreted the amplification curves of fetal genotyping, another laboratory tested the same sample as *RHD* positive	Yes	Yes

DAT, direct antiglobulin test.

the third trimester and may necessitate a new sample to make a conclusion. However, in the spirit of patient blood management, unnecessary sampling should be avoided.

In Norway, some of the laboratories have fewer analysis numbers of fetal *RHD* genotyping. Therefore, we continued surveillance of the program over a longer period to reveal possible weaknesses and discrepancies and report here data from four full years beginning 4 months after rollout of the program.

All the Norwegian blood bank laboratories that perform immunohematologic pregnancy follow-up including antibody screens are organized as part of the hospitals with birth clinics [1]. In approximately 11.7% of the cases (2 161/18,536), we did not have the serologic RhD typing results of the newborns to verify the results of the fetal *RHD* genotyping. We assume this difference was mainly due to women giving birth at another hospital than the one routine antenatal immunohematologic follow-up took place at and the result of fetal *RHD* genotyping was registered. Furthermore, emergency events like profuse hemorrhage, urgent cesarean section may have led to omission of sample taking and/or administration of postnatal prophylaxis. False-positive results, i.e., fetal *RHD* genotyping predicting an RhD-positive fetus while newborn is typed to be RhD negative occur mostly due to fetal RhD variants [3, 4]. In these cases, the newborn has an *RHD* gene variant that is either weakly expressed or not expressed on the red blood cells and will therefore be typed as RhD negative serologically. Such false-positive results cannot be avoided and do not carry any alloimmunization risk for the mother.

False-negative results, on the other hand, with fetal *RHD* genotyping predicting an RhD-negative fetus while the newborn is typed to be RhD positive should be avoided because these women are at risk of alloimmunization, and they will not receive antenatal prophylaxis with anti-D Ig. Alloimmunization risk will be higher when routine serologic RhD typing of the newborns is terminated because these women consequently would not receive postnatal prophylaxis either. False-negative results can be due to errors in labeling the samples and/or patient identification as well as too low concentration of cell free fetal DNA or technical reasons like pipetting errors leading to suboptimal polymerase chain reaction. Howbeit, low amount of cellfree fetal DNA is not a concern for our RAADP program as the samples are collected at GW24 [1].

In addition, automated DNA extraction from 1 to 2 mL of EDTA plasma guards against false-negative results due to pipetting errors. In short, our study revealed seven false-negative results during the 4 years of data collection. In one of these cases, there was a strong indication of erroneous sample collection from the wrong newborn. In another case, the reaction strength of serologic RhD typing of the newborn was 0.5+. The newborn was not retyped in a new sample. We do not know whether direct antiglobulin test was positive and/or there was ABO-incompatibility between the mother and the newborn. In a third case, fetal *RHD* genotyping was negative in a twin pregnancy, but one of the newborns was RhD negative while the other one was RhD positive. Three more discrepancies were reported as false negative without any elaboration. We did not receive samples from these women or the newborns to further investigation. One of these three women was pregnant again 3 years thereafter and antibody screen was then negative. The last discrepancy case was a true false negative where the amplification curves were misinterpreted by one laboratory, but the sample was also tested to be positive in another laboratory, and the pregnant woman received antenatal prophylaxis.

In 2012, Clausen et al. [4] advocated continuation of serologic cord blood RhD typing of the newborns primarily when fetal *RHD* genotyping predicted an RhD-negative fetus not to risk false-negative cases. They were also concerned that a lack of antenatal *RHD* genotyping could lead to lack of postnatal anti-D prophylaxis in case cord blood RhD typing was terminated. Denmark, followed by the Netherlands subsequently terminated routine RhD typing in cord blood sample of the newborns 2 years after implementation of their RAADP programs as they documented high assay sensitivity and specificity [5, 6]. The Stockholm area in Sweden, where fetal *RHD* genotyping is based on a single exon (exon 4) and performed from GW10, discontinued serologic RhD typing of the newborns in 2020 after 10 years [7]. Sensitivity of our assays (99.93%) in all health regions was also very high and similar to reports by other groups [4, 6–15], while specificity was 99.24%.

Initially in Norway, antenatal anti-D Ig was given at the maternity outpatient clinics at the hospitals except for Health Region of Northern Norway but since September 2021, only general practitioners or midwives of the primary healthcare have received the results of fetal *RHD* genotyping and administered RAADP. We must therefore ensure that the results of fetal *RHD* genotyping will also be easily available for the birth clinics responsible for the postnatal prophylaxis administration. The procedures should not be too complicated to follow for the birth clinics. Therefore, the National Working Group for RAADP has decided that postnatal prophylaxis will be given without further serologic RhD typing of the newborns when fetal *RHD* genotyping was inconclusive. Serologic RhD typing of the newborns may yet be necessary, in a lesser extent, for cases fetal *RHD* genotyping has not been performed either because the pregnant women do not wish to take the test or may have moved to the country just before delivery.

Conclusion

Our data, with a low number of false-negative fetal *RHD* genotyping results, in addition to high sensitivity and specificity support that termination of serologic RhD typing of the newborns will be safe and justifiable. The National Working Group for RAADP agrees upon the conclusion. Postnatal anti-D prophylaxis will now be given based on the result of fetal *RHD* genotyping.

Acknowledgments

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Statement of Ethics

The study was conducted in accordance with the Declaration of Helsinki and the data protection officer for research at Oslo University Hospital, Oslo, Norway stated that additional approval was unnecessary since the study was already approved as an internal quality assurance study (2013/3268 and 21/10417) and all data reported from the other laboratories were numbers and no sensitive information was reported. Written informed consent from participants was not required in accordance with the local guidelines.

Conflict of Interest Statement

The authors declare no financial or other conflicts of interest in this work.

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Author Contributions

Çiğdem Akalın Akkök contributed with the concept and design of this study, interpreted the data, wrote the draft, and submitted the manuscript. Monica Stensrud, Mette Silihagen Bævre, and Inger Margit Alm conducted the statistical analysis. Monica Stensrud, Mette Silihagen Bævre, Inger Margit Alm, Ho Yi Wong, Ida Herud, Barbora Jacobsen, Dijanne Dicky Jannie Anne de Vos, Helena Eriksson Stjern, Janne Brit Barane, Tonje Espeland Bagås, Mona Rasmussen, Norunn Ulvahaug, Vendula Wamstad, Ingvild Hausberg Sørvoll, and Geir Tomter contributed equally with data and interpretation of data, in addition to drafting and editing of the manuscript.

Data Availability Statement

Data used in this manuscript are not publicly available but at a protected digital area dedicated to this study and created with permission from data protection officer for research at Oslo University Hospital, Oslo, Norway. Further inquiries can be directed to the corresponding author.

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