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# Perinatal prophylaxis with immunoglobulin anti-D and the impact on RhD sensitizations among pregnant women in Slovenia

## Perinatalna zaščita z imunoglobulinom anti-D in vpliv senzibilizacije na antigen RhD med nosečnicami v Sloveniji

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## Abstract

**Background:** Sensitizations to red blood cell antigens may be a relevant cause of foetal and neonatal- perinatal morbidity. Of all red blood cell antigens, only alloantibodies to antigen RhD (D) can be prevented during pregnancy using perinatal preventive inoculation with anti-D immunoglobulin (Ig anti-D). Nevertheless, new sensitizations to antigen D among pregnant women are detected. The purpose of this article is to determine the incidence of sensitizations to antigen D among pregnant women in Slovenia in the period from 1 January 2010 to 31 December 2020 and to identify the most likely causes for sensitizations.

**Methods:** We retrospectively reviewed the medical records and the laboratory data in the transfusion information system for pregnant women, in whom we detected antibodies anti-D for the first time, from 1 January 2010 to 31 December 2020. We identified the most likely causes of sensitisation to anti-D from the data. The research was conducted at the Blood Transfusion Centre of Slovenia in Ljubljana (ZTM) and the Centre for Transfusion Medicine at the University Medical Centre Maribor (CTM).

**Results:** We detected 69 new sensitizations to D antigen in the reviewed period, which means that 0.16% of D-negative (D-neg) pregnant women or 0.26% D-neg pregnant women at risk were sensitized. 45% of sensitizations occurred during pregnancy, 29% after childbirth of a D-positive (D-poz) child, 9% after previous abortion. Of the 45% sensitizations during pregnancy, 8 cases (26%) could be prevented, 1 case (3%) could not be prevented, other cases (71%) have most likely occurred as a result of silent foetomaternal haemorrhage (FMK). Of the 38% of sensitizations that occurred after a previous birth or abortion, 4 cases (15%) could be prevented; 2 cases after abortion, 1 case after childbirth abroad, 1 case of refused


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**Ključne besede:** RhD; imunizirajoči dogodki; preventivno vbrizganje; imunoglobulin anti-D

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protection. We could prevent 12 cases (17.5%) of all sensitizations that most likely occurred due to incomplete compliance with the guidelines for preventive inoculation with Ig anti-D (in 2 cases (3%) pregnant women refused protection), 1 case (1.5%) could not be prevented (the pregnant woman did not come to the gynaecologist despite the bleeding).

**Conclusion:** More than 80% of sensitizations occurred despite following guidelines for their prevention; 12 cases (17.5%) could be prevented (7 cases of incomplete perinatal inoculation with Ig anti-D, 1 case of missed postpartum protection and 2 after abortion, 2 cases of refused protection), one case (1.5%) could not be prevented (one pregnant woman did not visit a gynaecologist despite the bleeding). A new measure to prevent sensitization could be routine protection with Ig anti-D in the second trimester of pregnancy and additional educational programs before planning a pregnancy. These programmes should pay particular attention to identifying and responding to potential sensitizing events in pregnancy, blood type D, and the consequences of refusing inoculation with Ig anti-D. Targeted protection represents one of the measures in this area.

## Izvleček

**Izhodišča:** Senzibilizacije na eritrocitne antigene so lahko pomemben vzrok perinatalne obolevnosti plodov in novorojenčkov. Od vseh eritrocitnih antigenov lahko med nosečnostjo preprečujemo nastanek aloprotiteles le proti antigenu RhD (D), in sicer s perinatalnim preventivnim vbrizganjem imunoglobulina anti-D (Ig anti-D). Kljub temu odkrivamo med nosečnicami nove senzibilizacije na antigen D. Namen članka je določiti incidenco senzibilizacij na antigen D med nosečnicami v Sloveniji v obdobju od 1. 1. 2010 do 31. 12. 2020 ter prepoznati najverjetnejše vzroke za njihov nastanek.

**Metode:** Retrospektivno smo pregledali medicinsko dokumentacijo z anamnestičnimi podatki in izvide laboratorijskih preiskav v transfuzijskem informacijskem sistemu za nosečnice, pri katerih smo v izbranem obdobju prvič odkrili protitelesa anti-D. Iz dokumentacije smo razbrali najverjetnejše vzroke za nastanek anti-D. Raziskava je potekala na Zavodu RS za transfuzijsko medicino v Ljubljani (ZTM) ter na Centru za transfuzijsko medicino v Univerzitetnem kliničnem centru Maribor (CTM).

**Rezultati:** V obravnavanem obdobju smo odkrili 69 novih senzibilizacij na antigen D, kar pomeni, da se je senzibiliziralo 0,16 % D-negativnih (D-neg) nosečnic oz. 0,26 % D-neg nosečnic s tveganjem. Med nosečnostjo je nastalo 45 % senzibilizacij, 29 % po rojstvu D-pozitivnega (D-poz) otroka, 9 % po predhodni prekinitvi nosečnosti. Od 45 % senzibilizacij med nosečnostjo bi lahko preprečili 8 primerov (26 %), 1 primera (3 %) nismo mogli preprečiti, ostali primeri (71 %) pa so najverjetneje posledica tihih fetomaternalnih krvavitev (FMK). Od 38 % senzibilizacij, ki so nastale po predhodnem porodu ali prekinitvi nosečnosti, bi lahko preprečili 4 primere (15 %): 2 primera po prekinitvi nosečnosti, 1 primer po rojstvu D-poz otroka v tujini, 1 primer zavrnitve zaščite. Skupno bi lahko preprečili 12 senzibilizacij (17,5 %), ki so najverjetneje posledica nepopolnega upoštevanja smernic za preventivno vbrizganje Ig anti-D (med njimi sta 2 nosečnici (3 %) zaščito zavrnili), enega primera (1,5 %) nismo mogli preprečiti (nosečnica kljub krvavitvi ni prišla h ginekologu).

**Zaključek:** Več kot 80 % senzibilizacij je nastalo kljub upoštevanju smernic za njihovo preprečevanje, 12 primerov (17,5 %) bi lahko preprečili (7 primerov nepopolne perinatalne zaščite z Ig anti-D, 1 primer neizvedene zaščite po rojstvu D-poz otroka in 2 po prekinitvi nosečnosti, 2 primera zavrnjene zaščite), enega primera (1,5 %) pa kljub upoštevanju smernic nismo mogli preprečiti, ker nosečnica kljub krvavitvi ni obiskala ginekologa. Novi ukrep za preprečevanje senzibilizacij bi lahko bila rutinska zaščita z Ig anti-D še v drugem trimesečju nosečnosti in dodatni izobraževalni programi pred načrtovanjem nosečnosti. Posebno pozornost bi bilo potrebno nameniti prepoznavanju in ukrepanju ob možnih dogodkih, ko lahko pride do senzibiliziranja v nosečnosti, krvni skupini D ter ob posledicah zavrnitve zaščite z Ig anti-D. Ciljana zaščita že predstavlja enega od ukrepov na tem področju.

## 1 Introduction

Alloimmunization is defined as an immune response with the formation of antibodies against foreign antigens. In the case of haemolytic disease of the foetus and new-born (HDFN), it is the formation of maternal alloantibodies directed against antigens on the surface of the foetal red blood cells, which the foetus inherited from the father. HDFN can be caused by antibodies against more than 50 different red blood cell antigens, the most important of which are anti-D antibodies. The RhD (D) antigen is highly immunogenic and approximately 15–19% of the white population, 7% of the black population, and 1% of the Asian population are negative for it (1,2). In the event that D-positive foetal red blood cells are transferred into the bloodstream of the D-negative mother, the mother may begin to produce anti-D antibodies. Antigen D can be detected on foetal red blood

cells as early as 38 days after conception (3,4). Anti-D IgG antibodies are actively transported across the placenta into the foetal bloodstream, where they destroy foetal red blood cells, causing the development of severe anaemia, which in the worst cases leads to oedema and foetal death; this is called hydrops fetalis. If the mother and foetus are ABO compatible, the risk of immunization is 16%, if they are ABO incompatible it is 2%. The overall risk is therefore 13.2% (5). Sensitization can also be triggered by other causes, such as receiving a transfusion, organ transplantation, or, rarely, where naturally occurring antibodies are involved.

In the Laboratory for prenatal diagnostics at the Blood Transfusion Centre of Slovenia (ZTM) we keep a register of sensitized pregnant women. Despite a national screening programme for preventing sensitization among D-negative pregnant women, the emergence of new sensitizations to D antigen is observed. Therefore, the purpose of our study was to determine the incidence of sensitization to D antigen and to identify the most likely causes of anti-D formation among pregnant women in Slovenia in an 11-year period, from 1 January 2010, to 31 December 2020. The research was conducted at ZTM and at the Centre for Transfusion Medicine at the University Medical Centre Maribor (CTM).

## 2 History of prevention of sensitization to D antigen

During pregnancy, only sensitization to D antigen can be prevented by perinatal prophylaxis with anti-D immunoglobulin (Ig). The mechanism of action of anti-D Ig is still not completely understood today. It can act through the rapid clearance of D-positive foetal cells, interfere with the presentation of D antigen to dendritic cells and macrophages, and inhibit the activity of reactive B lymphocytes (5,6). The beginnings of preventing sensitization of D-negative pregnant women with anti-D Ig date back to the 1960s. At that time, the first experiments were performed in which D-negative male volunteers were injected with D-positive red blood cells, and the resulting antibodies were used to prevent sensitization. Anti-D Ig has been available in Europe and North America since 1968 (5). In 1969–1970, routine prophylaxis of D-negative pregnant women giving birth to a D-positive baby was introduced. Guidelines for the injection of anti-D Ig at termination of pregnancy, invasive diagnostic procedures, and other possible sensitizing events (hereinafter: event) during pregnancy have also been developed (6). Thus, the risk of immunization of D-negative pregnant women carrying a D-positive foetus was reduced from 16% to 1.6% (7). In the mid-1990s, the introduction of prenatal anti-D Ig injections followed, preventing sensitization due to undetected fetomaternal haemorrhage (FMH) in the last trimester of pregnancy. With this measure, the number of sensitizations was further reduced to 0.1–0.3% (6). In 1970, prophylaxis with postpartum anti-D Ig injection was first introduced in Slovenia, while prenatal prophylaxis has been mandatory in this country since 1994. Unnecessary injection of anti-D Ig to pregnant women carrying a D-negative child and the discovery of cell-free foetal DNA (cffDNA) in the mother's blood in 1997 led to the development of targeted protection. In targeted protection, the foetal *RHD (D)* genotype is determined from a peripheral blood sample of pregnant women and prenatal anti-D Ig injection is recommended only if the pregnant woman is carrying a D-positive foetus. Targeted protection was introduced in 2010 in Denmark, in 2011 in the Netherlands, in 2014 in Finland, followed by France, England, Sweden, and Belgium (7-9) and since January 2018 it has been implemented in Slovenia based on the Regulations (Official Gazette of the Republic of Slovenia, No. 32/18) (10,11). According to research, tests to determine foetal D from the peripheral blood of a pregnant woman have a sensitivity of 99.8% and a specificity of 94.2% (8).

When implementing a routine prophylaxis for D-negative pregnant women, it is also important to estimate FMH. We know acute or chronic FMH, which can occur spontaneously or as a result of various events in pregnancy. The incidence of FMH increases with gestational age: in the first trimester it occurs in 3–5%, in the second in 13–15%, in the third in up to 30%, and at birth in up to 50% of cases. Bleeding is greater than 1 ml in 1% of pregnant women and greater than 15 ml in 0.3% (1,12-13). Immunization can be caused by as little as 0.01–0.03 ml of FMH (14). Events in pregnancy that may cause FMH, and thus maternal sensitization, include: spontaneous or artificial termination of pregnancy, ectopic pregnancy, cluster mole, intrauterine foetal death, stillbirth, bleeding during pregnancy, invasive procedures during pregnancy (e.g. amniocentesis, chorionic villus biopsy), intrauterine therapeutic procedures (intrauterine transfusion, laser surgery), abdominal injuries, procedures in the third trimester (external cephalic version), long delivery, or completion of labour with surgery (15). The purpose of estimating FMH is that in cases where the amount of FMH exceeds the volume of foetal red blood cells, which is covered by a standard dose of anti-D Ig, injection of additional doses is recommended. The test used is the Kleihauer-Betke test, and in the case of greater FMH the flow cytometer

(FC) is used. The Kleihauer-Betke test is based on the principle that foetal haemoglobin is resistant to acid elution, while adult haemoglobin is eluted from red blood cells under acid action. During the examination, a smear of the pregnant woman's peripheral blood is taken, the adult haemoglobin is eluted with the elution solution, and the preparation is stained and dried and observed under a light microscope. Foetal red blood cells are coloured red, and adult red blood cells are visible as white-grey ("ghost") cells (15). In flow cytometry, specific anti-D antibodies labelled with fluorochromes are used to estimate FMH in a D-negative neonatal mother with a D-positive baby, and in other cases anti-HbF (foetal haemoglobin)/polyclonal CA (carbonic anhydrase) antibodies can be used. HbF-negative and CA-positive cells indicate adult red blood cells, and foetal red blood cells are HbF-positive and CA-negative (15-16).

## 3 Guidelines for preventing immunization to D antigen

The European Medicines Agency (EMA) has highlighted in the core Summary of Products Characteristics for human anti-D immunoglobulin for intramuscular (IM) use that the guidelines and recommendations for injection indications and injected anti-D Ig levels vary between countries (17). Guidelines of the British Committee for Standards in Haematology (BCSH), guidelines of the Royal College of Obstetricians and Gynaecologists (RCOG) (18), guidelines of the American Congress of Obstetricians and Gynaecologists (ACOG), guidelines of the Society of Obstetricians and Gynaecologists of Canada (SOGC) (19), and guidelines of the Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) (3,20-22) recommend that pregnant women receive Anti-D Ig protection as soon as possible, within 72 hours of the event. Some protection is also provided by receiving a dose within 10 days (RCOG guidelines) or within 28 days of the event (ACOG and SOGC guidelines). For events in pregnancies of less than the 12th week of gestation, the ACOG and RANZCOG injection guidelines recommend 250 IU, the SOGC guidelines recommend 250 IU, and in cases of termination of pregnancy, threatened abortion, or ectopic pregnancy, 600 IU of anti-D Ig. For events after the 12th week of gestation, ACOG and SOGC recommend injecting 1,500 IU and RANZCOG recommends 625 IU of anti-D Ig. The RCOG recommends injecting 250 IU for events before 20 weeks of gestation and 500 IU of anti-D Ig for events after 20 weeks of gestation as well as estimation of FMH. In addition, protection before the 12th week is recommended only in cases of ectopic pregnancy, molar pregnancy, therapeutic termination of pregnancy, and in cases of recurrent bleeding accompanied by abdominal pain and not ending before gestational week 12. The RCOG and BCSH guidelines also address the measures in cases of chronic bleeding in pregnancy; before 20 weeks of gestation, a dose of 250 IU every six weeks and after 20 weeks of gestation 500 IU every six weeks and estimation of FMH every two weeks is recommended. If the FMH is greater than 4 ml, it is necessary to inject additional 125 IU of anti-D Ig per 1 ml of foetal red blood cells IM or 100 IU of anti-D Ig per 1 ml of foetal red blood cells intravenously (IV), and to re-estimate the FMH 72 hours after IM or 48 hours after IV injection of anti-D Ig. For routine prenatal care, the SOGC and RCOG guidelines recommend a single dose of 1,500 IU or two doses of 600 IU of anti-D Ig, ACOG recommends a single dose of 1,500 IU, and RANZCOG two doses of 625 IU at 28 and 34 weeks of gestation. The amount of anti-D Ig administered after childbirth also varies. RCOG recommends injection of 500 IU and additional doses if the FMH is greater than 4 ml, ACOG recommends 1,500 IU, SOGC recommends 1,500 IU or 600 IU and additional doses if the FMH is greater than 6 ml, and RANZCOG recommends 625 IU or to decide depending on FMH (3,20-24). In European countries, different doses are injected after childbirth, ranging from 500 IU to 1,500 IU; for example, in the Netherlands 1,000 IU of anti-D Ig (6) and in Italy 1,250–1,300 IU of anti-D Ig (13).

There are two medications available in Slovenia with a standard dose of anti-D Ig, namely Rhophylac 1,500 IU/2ml (given into a vein or muscle, covering 30 ml of foetal blood) and Rhesonativ 625 IU/ml or 1,250 IU/2ml (injected into a muscle, covering 12.5 ml or 25 ml of foetal blood). According to the described guidelines (14, 20-24), for events before gestational week 12 or 20 a dose of 625 IU/ml is sufficient. After 20 weeks of gestation, the same dose can be injected, and the estimation of FMH determines whether additional doses of anti-D Ig are needed, or a higher dose, 1,500 IU, should be used (13). Post-partum, injecting at least 1,250 IU is recommended, which is achieved with both drugs (Rhesonativ 1,250 IU/2 ml, Rhophylac 1,500 IU/2 ml).

## 4 Data collection, method of work, definitions

We have retrospectively analyzed the results of laboratory tests in the blood transfusion information system and anamnestic data collected in medical documentation,

which, in accordance with the Regulations (Official Gazette of the RS, No. 32/18 of 11 May 2018), are kept in the Laboratory for prenatal diagnostics for each sensitized pregnant woman. The study included all pregnant women in whom, in the period from 1 January 2010 to 31 December 2020, anti-D antibodies were newly detected at the ZTM (Blood Transfusion Centre of Slovenia) and CTM (Centre for Transfusion Medicine Maribor). The results of the following laboratory tests were taken into consideration: Indirect Coombs test (ICT), antibody specification, estimation of FMH, determination of D antigen in partner and new-born, or determination of *D* in the foetus. Among the anamnestic data, we looked for data on events in pregnancy and data on prophylactic anti-D Ig injection at specific events within the routine pre- and postpartum prophylaxis. Detected antibodies were divided according to the time of their detection in pregnancy and according to the probable sensitizing event. Data on the number of births were obtained from the Statistical Office of the Republic of Slovenia (SORS). The data was analyzed using Excel.

The study design was approved by the Republic of Slovenia National Medical Ethics Committee (0120-432/2021/3, 19.10.2021).

## 5 Results

Within the analyzed period, there were between 19,300 and 22,300 births per year in Slovenia, a total of 229,508 births (38). Anti-D antibodies were newly discovered in 69 pregnant women. Given that 19% of our population is D-negative, 0.16% of D-negative pregnant women developed anti-D antibodies. However, considering the fact that 60% of D-negative pregnant women carry a D-positive foetus, anti-D was developed by 0.26% of D-negative pregnant women with high-risk pregnancies. Most likely, 68 of 69 cases (98.6%) were pregnancy-related, one case (1.4%) was related to receiving a D-incompatible transfusion in the past. No anamnestic data were available for 11 pregnant women (missing data or management at another centre). Four women were pregnant for the first time, in others anti-D was detected in one of the ensuing pregnancies. The time of antibody detection and the most likely sensitizing events are shown in Table 1.

Anti-D was developed by 20 pregnant women (29% of sensitizations) after the birth of a D-positive baby. In 19 of them, a FMH test was done after delivery. Prophylaxis was recommended according to the recommendations, and for more extensive bleeding, an additional dose of anti-D Ig determined by FC. Two pregnant women had previously given birth abroad, one of whom did not receive anti-D Ig prophylaxis after giving birth to a D-positive baby. One of the pregnant women refused anti-D Ig prophylaxis postpartum. This group also included six pregnant women (9%) who had had a previous abortion, and then anti-D was detected during pregnancy, two of whom did not receive adequate anti-D Ig prophylaxis in the past after the abortion. Therefore, previous pregnancies were the cause of sensitization in a total of 38%.

31 cases of sensitization (45%) most likely occurred due to FMH during pregnancy. Among them, anti-D was detected in four pregnant women who were pregnant for the first time, and the pregnancies proceeded

**Table 1:** Time of anti-D detection and probable sensitizing event.

| Probable sensitizing event: number (guidelines followed (number) + guidelines partially followed (number)) | | | | | |
|---|---|---|---|---|---|
| **Time of anti-D detection** | **birth of a D-positive baby** | **fetomaternal bleeding in pregnancy** | **other (termination of pregnancy, receiving a D-positive transfusion)** | **unknown** | **total: number (%)** |
| First examination in the first pregnancy (before gestational week 12) | 0 | 4 | 1 | 11 | 16 (23%) |
| First examination in the second or subsequent pregnancies | 20 (18+2) | 2 (1+1) | 6 (4+2) | 0 | 28 (41%) |
| Routine check-up at gestational week 28 | 0 | 19 (14+5) | 0 | 0 | 19 (27%) |
| Postpartum sample | 0 | 6 (2+3) | 0 | 0 | 6 (9%) |
| Total | 20 (29%) | 31 (45%) | 7 (10%) | 11 (16%) | |

unremarkably until the first examination, which means it occurred without a recognized cause. In one pregnant woman, anti-D was detected at the first examination during her second pregnancy; in the first pregnancy she gave birth to a D-negative child, so immunization most likely occurred in the early period of the second pregnancy. In another pregnant woman, anti-D was detected during her second pregnancy, at the first delivery, extensive chronic FMH was identified, she received the appropriate amount of anti-D Ig (FMH size determined by FC), but received additional doses later than 72 hours after delivery. In 19 pregnant women, anti-D was detected during pregnancy, and in six it was detected after delivery. Of these, eight pregnant women did not receive anti-D Ig prophylaxis during pregnancy; one bled in early pregnancy and did not visit a gynaecologist, one did not receive prophylaxis after bleeding, one did not receive prophylaxis after amniocentesis, two received partial prophylaxis (after amniocentesis, but not in the gestational week 28), in two pregnant women anti-D Ig prophylaxis was omitted in the gestational week 28, and one pregnant woman refused prophylaxis in the 28th week. Of the nine sensitizations with a known cause during pregnancy, eight were most likely due to incomplete compliance with guidelines: one of these pregnant women refused prophylaxis, and in one case the guidelines could not be followed (the woman did not visit a gynaecologist despite bleeding).

Recommendations for anti-D Ig injection were fully followed in 45 pregnant women (65.5%), and in 12 pregnant women (17.5%) they were not followed or were only partially followed: one pregnant woman did not receive protection after giving birth to a D-positive baby in the past abroad, two after termination of pregnancy in the past, six during pregnancy, one received prophylaxis postpartum after more than 72 hours, two refused prophylaxis (one during pregnancy, one after delivery); one case (1.5%) could not be prevented (bleeding without a visit to the gynaecologist), and one case (1.5%) was associated with receiving an inappropriate blood transfusion in the past. There was no data available for 11 cases (16%).

## 6 Discussion

Despite advances in diagnostic methods and obstetric protocols to prevent sensitization of D-negative pregnant women, 1–3 out of 1,000 D-negative pregnant women still develop anti-D antibodies. In our study, we found anti-D antibodies in 69 pregnant women over a period of 11 years, which means that 0.16% of pregnant women developed anti-D or 0.26% D-negative pregnant women at risk of developing anti-D (those carrying a D-positive foetus). The results are comparable to the results of studies after the introduction of prenatal prophylactic anti-D Ig injection (6,23-27). A study by McCauley et al. showed that the incidence of anti-D alloimmunization was 0.31% (27), and a study by Turner et al. (24) and McBain et al. (25) showed it was 0.3%.

The majority of immunizations in our study occurred during pregnancy (45%), after the birth of a D-positive baby (29%), or after termination of pregnancy (9%). In the first cases, immunizations were most likely due to partial compliance with guidelines in eight of 31 cases, which is 26% of sensitizations detected during pregnancy, one case (3%) could not be prevented (pregnant woman did not visit a gynaecologist despite bleeding), in other cases (71%) they were most likely due to silent FMH in pregnancy. In the second group, sensitization could be prevented in only two of 20 pregnant women (10%) who became sensitized after the birth of a D-positive baby (one case of omitted prophylaxis, one case of rejected prophylaxis), or in two of six pregnant women (33%) who did not receive prophylaxis after pregnancy termination. We can conclude that by strictly following the guidelines, we could additionally prevent 26% of sensitizations during pregnancy, 10% of sensitizations after the birth of a D-positive baby and 33% of sensitizations after pregnancy termination, which means a total of 17.5% of all sensitizations (12 cases) or 14.5% (10 cases), excluding two pregnant women who refused prophylaxis despite the explanation. Silent FMHs are an important cause of sensitization during pregnancy. The causes of anti-D development despite the recommended prophylaxis and the estimation of FMH could not be discovered in all cases after the birth of a D-positive baby, as we did not have data on whether the pregnant women actually received the recommended prophylaxis.

Numerous studies in the past have identified the importance of postpartum (28) and prenatal anti-D Ig injection and the need for strict compliance with anti-D Ig guidelines. A study in New Zealand showed that half of the new sensitizations to D antigen were associated with partial compliance or non-compliance with local anti-D Ig injection guidelines; 41% of pregnant women with a recognized event during pregnancy did not receive anti-D Ig prophylaxis, and 12.8% received incomplete prophylaxis (29). A 2010–2015 study in Northern Ireland found that as many as 51% of newly detected anti-D occurred after the birth of a D-positive baby, and in all but one case, the FMH was estimated and appropriate follow-up according to the guidelines were determined

after delivery. 21% of sensitizations occurred during pregnancy. One of three with a recognized event in pregnancy did not receive adequate prophylaxis, and in other cases the cause of sensitization was most likely silent FMH. Anti-D Ig was rejected by one pregnant woman. They found that only 4% of newly discovered anti-D sensitizations could be prevented, and in 96% of cases, the guidelines for injecting anti-D Ig were correctly followed (27). Comparing these results with our research shows that a higher proportion of sensitizations occurred after the birth of a D-positive baby (51% vs. 29% or 38%) while the proportion was lower during pregnancy (21% vs. 45%). The difference in the proportion of sensitizations after the birth of a D-positive baby can be partly explained by the difference in the recommended amount of anti-D Ig injection after birth; their guidelines recommend 500 IU of anti-D Ig postpartum (3,18), and in our country at least 1250 IU. A higher dose in our country probably means greater safety, as it covers a greater volume of FMH, although in both countries FMH is determined postpartum. The higher share of sensitizations during pregnancy in Slovenia can be explained by the higher share of incomplete compliance with the guidelines, 4% according to their study and 14.5% or 17.5% according to ours, although both studies showed that most sensitizations during pregnancy could not be prevented. They were most likely caused by silent FMH before gestational week 28. Both in our country and in theirs, cases of rejected anti-D Ig prophylaxis were considered. Despite the fact that in our country, after giving birth to a D-positive baby, pregnant women receive a high dose of anti-D Ig compared to the guidelines of other countries (3,6,13,18-22), the share of sensitizations after the birth of a D-positive baby is high, 29%. As we did not have access to data on whether pregnant women actually received the recommended postpartum prophylaxis, the result cannot be fully explained. However, according to research, the effectiveness of anti-D Ig prophylaxis could also be affected by the concentration of anti-D Ig in the blood, which is affected by the absorption of anti-D Ig from muscle or subcutaneous tissue, which may be related to body mass index (39).

As new antigen D sensitizations occur despite following the perinatal anti-D Ig injection guidelines, various studies have examined possible additional causes of sensitizations and possible new approaches to preventing them (6). The general risk factors for sensitization of pregnant women described in the literature are related to their general history (surgical procedures, blood transfusion, haematologic diseases), pregnancy and parity. Risk factors associated with pregnancy may include bleeding, abdominal injury, invasive procedures during pregnancy (chorionic villus biopsy, amniocentesis), Caesarean section, surgical removal of the placenta, increased postpartum haemorrhage, and an unfavourable history of pregnancy (abortion, stillbirth) (30-32). Various causes of D antigen sensitization are also described; for example, silent FMH before 28 weeks gestation (27), omitted perinatal anti-D Ig prophylaxis (27), insufficient absorption of anti-D Ig after being injected into a muscle (26), omitted FMH estimation and thus an undetected extensive FMH postpartum, or the pregnant woman refusing anti-D Ig prophylaxis for personal reasons (27). A study by Koelewijn et al., investigating the causes of sensitization to D antigen despite adequate perinatal prophylaxis with anti-D Ig, showed that additional risk factors for sensitization may include non-spontaneous delivery (Caesarean section, assisted vaginal delivery), overdue labour, receiving a blood transfusion during childbirth, and age (6). Non-spontaneous termination of labour poses a risk of increased FMH that is not covered by the standard dose. FMH is determined by the Kleihauer-Betke test or FC. The former involves manual and subjective counting of foetal red blood cells under a microscope, while the performance of the FC method can be influenced by the technical properties and validation of the method. In addition, blood loss into the peritoneal cavity may occur at the end of labour with surgical procedure, which cannot be detected with the Kleihauer-Betke test. The time of taking the sample is also important, because due to the time that the baby's red blood cells are absorbed into the mother's bloodstream, a sample should be taken 30–45 minutes after birth (15) or up to two hours after delivery (6,13,18,22). Overdue labour means prolonged exposure to foetal red blood cells, so in this case the preventive dose received in gestational week 28–30 is not enough. The half-life of anti-D Ig is 17–22 days, and routine prenatal prophylaxis theoretically provides a sufficient amount of anti-D to protect against 1 ml of foetal red blood cells or 2 ml of foetal blood for 12 weeks after injection (20). Several studies have shown that passive anti-D Ig was no longer detectable in the blood more than 12 weeks after injection, nor was it detected for several weeks before delivery (6,33-37). Receiving a blood transfusion is known to activate the immune system, which can trigger an immune response to foetal antigens, while also indicating a non-spontaneously completed labour and the risk of extensive FMH.

According to the results of our and previously mentioned research (6,27-29), the development of anti-D antibodies could be further prevented in several ways,

namely by preventing sensitization due to silent FMH before gestational week 28, with prevention in cases when passive anti-D Ig is no longer detectable in the blood due to the prolonged duration of pregnancy, and in cases when pregnant women do not visit a gynaecologist despite the events or they refuse prophylaxis with anti-D Ig. The first cases could be prevented with additional doses of anti-D Ig in the second trimester of pregnancy, e.g. at gestational week 16, and others with additional doses in case of overdue pregnancy. Other cases could be prevented by educational pre-pregnancy programmes that pay special attention to identifying and responding to pregnancy events, blood group D interpretation, and the consequences of omitting prophylaxis with anti-D Ig. Pregnant women who refuse prophylaxis can be offered additional support in deciding on getting anti-D Ig injection (targeted protection) by determining the foetal *D* genotype from peripheral blood.

## 7 Conclusion

Screening programmes for preventing sensitization to D antigen have shown great success in preventing the development of anti-D alloantibodies, thereby greatly reducing morbidity and mortality associated with HDFN. Nevertheless, 0.1–0.3% of D-negative pregnant women still become sensitized. Our research found that the share of newly discovered anti-D sensitizations in Slovenia was 0.16–0.26%, which is comparable to the results of other developed countries after the introduction of prenatal prophylaxis with anti-D Ig. By carefully following the guidelines for perinatal prophylaxis with anti-D Ig injection, only 14.5% or 17.5% sensitizations could be further prevented. Other sensitizations were most likely due to silent FMH in pregnancy. The latter could only be prevented by new approaches, such as routine preventive doses of anti-D Ig in the second trimester of pregnancy, additional doses of anti-D Ig in case of overdue pregnancy, by recognizing and understanding the importance of possible additional factors for antibody production, and by educating women of reproductive age. Cases when pregnant women refuse prophylaxis with anti-D Ig can today be successfully solved in our country with additional explanation and the introduction of targeted prophylaxis.

### Conflict of interest

None declared.

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