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C4D AS A FOOTPRINT OF MATERNAL ANTI-FETAL IMMUNITY IN RECURRENT MISCARRIAGE

A novel pathogenic mechanism

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Abstract

BACKGROUND The conceptus represents a foreign body to the maternal immune system. This 'natural' allograft is usually not rejected. In analogy with solid organ transplantation, we hypothesized that antibody-mediated rejection, characterized by activation of the classical complement system, could play a role in women with unexplained recurrent miscarriage. We therefore investigated the presence of placental C4d deposition, a marker for classical complement activation, as well as the presence of anti-HLA class I and II antibodies in women with recurrent miscarriage compared to control subjects.

METHODOLOGY AND PRINCIPLE FINDINGS We studied placental C4d deposition in 38 women with unexplained recurrent miscarriage (cases), a first control group of 22 women who experienced one spontaneous miscarriage but subsequently had live births and a second control group of 67 women who underwent elective termination of pregnancy. C4d depositions were found at the maternal side of the syncytiotrophoblast and a diffuse staining pattern was strongly associated with recurrent miscarriage (p=0,004) when compared tot control subjects. Presence of anti-HLA class I and II antibodies was determined in 28 out of 38 women in the recurrent miscarriage-group, of whom 11 (39%) had a positive titer for anti-HLA class I or II antibodies. Of those, 9 of 11(82%) had focal or diffuse placental C4d depositions, whereas in the 17 patients without anti-HLA antibodies diffuse placental C4d was present in only 4 patients (23%) (p=0,003).

CONCLUSIONS Placental C4d is present significantly more often in patients with unexplained recurrent miscarriage compared to control subjects. C4d deposition is found at the fetal-maternal interface, and may be interpreted as a footprint of antibody mediated trophoblast injury. We identified 9 out of 39 patients with unexplained recurrent miscarriage who had both placental C4d deposits and positive titers

for anti-HLA antibodies. This combination strongly suggests an antibody-mediated immune response, and might embody a new pathophysiologic mechanism responsible for recurrent miscarriages.

Introduction

About 1-3% of all couples will be confronted with recurrent miscarriage, which is defined as ▸3 consecutive miscarriages within 20 weeks of gestation.[1] In any recognized pregnancy there is a chance of miscarriage of about 10-15%. The large majority of these sporadic miscarriages are caused by fetal chromosomal aneuploidies.[2] In recurrent miscarriage, maternally derived underlying causes can be identified in a substantial proportion of women. Examples of such causes are uterine anomalies, endocrine disorders, autoimmune disorders such as SLE or antiphospholipid syndrome, thrombophilia or balanced translocations in the maternal (and/or paternal) DNA. However, in more than 50% of woman suffering from recurrent miscarriage, no causal factor can be identified.[1] This burden of continuous uncertainty has major impact on the lives of women and their partners. For clinicians, the lack of both etiological insight and evidence based therapeutic interventions makes the management of these patients complex and sometimes frustrating.

In analogy with solid organ transplantation it has been hypothesized that recurrent miscarriage of unknown etiology is a form of maternal anti-fetal allograft rejection. In the transplant world, allo-antibody mediated rejection (humoral rejection) has gained much attention since the discovery of the biomarker C4d in the early nineties.[3,4] C4d is a tissue-biomarker for classical complement activation, a powerful component of human innate immunity that plays an essential role in inducing tissue injury in many allo- and autoimmune settings. When antibodies (allo- or autoantibodies) bind or deposit, the classical complement pathway is activated via a cascade of enzymatic reactions. The formation of potent anaphylactoxins C5a and C3a and the formation of the membrane attack complex are the main results of this process.

C4d is a non-functional split product of classical complement activation that covalently attaches to cells and tissues. While antibodies dissociate over time, C4d stays anchored to the tissue, thereby acting as a footprint of recent antibody mediated tissue injury. Nowadays C4d is routinely used by transplantation pathologists all over the world.[5]

We have recently demonstrated that C4d is abundantly present in placentas of women with autoimmune mediated pregnancy losses caused by SLE and antiphospholipid syndrome.[6] Placental C4d was found at the fetal-maternal interface, and was strongly associated with intrauterine fetal death and severe forms of preeclampsia.[7-10] The concept of excessive complement activation as an important mediator of maternal anti-fetal immunity has shown to be relevant in settings other than autoimmune disease too. Lee *et al* recently published that C4d in fetal cord endothelium was associated with circulating maternal anti-HLA I antibodies in a setting of spontaneous preterm birth.[11] Furthermore, a recent cohort study of patients with severe preeclampsia demonstrated that 19% of women had mutations in complement regulatory genes. It was shown that such mutations are responsible for inadequate inhibition of complement activation at the fetal-maternal interface, serving as a basis for impaired trophoblast functioning and placental development.

In recurrent miscarriage of unknown etiology both auto- and alloantibodies could theoretically be involved. Auto-antibodies could for instance be antiphospholipid-like antibodies that are not picked up by current assays but have a similar effect on trophoblast cells. Allo-antibodies could be anti-HLA antibodies directed against fetal inherited paternal HLA antigens. We hypothesized that if an ongoing antibody-mediated process is responsible for miscarriage, C4d should be present at the fetal-maternal interface. We therefore aimed to investigate the presence of C4d deposition on trophoblast tissue of patients with unexplained recurrent miscarriage compared to control subjects. To further unravel the disease mechanism, we related placental C4d with the presence of circulating anti-HLAantibodies in

a subgroup of women, with the hypothesis that antibody-mediated rejection of the fetal allograft may indeed be responsible for a proportion of women with unexplained recurrent miscarriage.

Material and methods

ETHICS STATEMENT All tissue and serum samples were handled in a coded and anonymized fashion, according to the Dutch National Ethical guidelines (Code for Proper Secondary Use of Human Tissue, Dutch Federation of Medical Scientific Societies). This national guideline or code makes it possible to perform research with human material that came available within the framework of patient care. Subsequently, this human material can be used for research purposes when properly coded and anonymized.

PATIENTS We studied products of conception of 127 women, who were divided into three groups: A case-group of 38 patients with recurrent miscarriage of unknown etiology, a first control group of 22 healthy women with one spontaneous miscarriage who subsequently had normal pregnancies and live births, and a second control group of 67 women who underwent an elective termination of pregnancy due to medical reasons (i.e. fetal chromosomal anomalies) or social reasons.

The case-group consisted of 38 women diagnosed with recurrent miscarriage of unknown etiology, who were selected from a population of women enrolled in a clinical trial performed at the Leiden University Medical Center (Habenox trial, trial register number NVT0095962).[12] The Habenox trial investigated the effect of anticoagulant treatment on pregnancy outcome in women with unexplained recurrent miscarriage or thrombophilia. Recurrent miscarriage was defined as three or more consecutive first trimester miscarriages (<13 weeks), two or more second trimester miscarriages (13-24 weeks) or one third trimester miscarriage combined with at least one first trimester miscarriage. Patients with thrombophilia, defined as factor V Leiden mutation, prothrombin gene mutation, protein C or S deficiency, high factor VIII or presence of antiphospholipid antibodies were excluded for the current study. Other exclusion criteria were history of thromboembolism or bleeding disorders, allergy to aspirin or enoxaparin, uterine anomalies, cervical insufficiency, untreated thyroid disease, poorly treated diabetes mellitus, parental chromosomal abnormalities and pregnancies achieved by assisted reproductive techniques. We included all women of whom tissue samples of miscarriages were available in the archives of the pathology department of the Leiden University Medical Center, Leiden, the Netherlands.

For a first control group (sporadic miscarriage group) we included 22 tissue samples of miscarriages from women who experienced one spontaneous miscarriage, but subsequently had live births and uneventful pregnancies. None of the patients had a history of preeclampsia or Hemolysis Elevated Liver enzymes and Low Platelets (HELLP) syndrome, or any of the other exclusion criteria used in the case group.

For a second control group (elective termination of pregnancy group) we included 67 tissue samples of elective terminations of pregnancy, of which 21 terminations were performed for medical reasons (i.e. fetal chromosomal anomalies) and 46 for social reasons. Of 13 cases in this group we received tissue samples from an abortion clinic, therefore, of these patients we have no clinical information other than the gestational age of the pregnancy.

An overview of the clinical characteristics of all patients, derived from the case-record files, is given in table 1.

TISSUE SAMPLES OF MISCARRIAGES AND ELECTIVE TERMINATIONS OF PREGNANCY Products of conception were fixed in 4% buffered formalin and embedded in paraffin. Paraffin sections were routinely stained with HE. To study classical complement activation, immunohistochemical staining was performed for C4d (BI-RC4d, Biomedica Gruppe, Austria). Optimal antibody dilutions and incubation times for the different antibodies were pre-determined by means of titration on positive control sections. Endogenous peroxidase activity was blocked. Antigen retrieval was performed with 10 mM

citrate buffer (pH 6.0). A polyclonal rabbit anti-human C4d antibody (Biomedica Gruppe, Austria), was applied at a dilution of 1:80 in 1% BSA/PBS, and slides were incubated for one hour at room temperature. The slides were then incubated with a secondary antibody (anti-rabbit EnVision, K5007, Dako Cytomation, Denmark) for 30 minutes. Staining was visualized with diaminobenzidine (Dako Cytomation, Denmark) and counterstained with Haematoxylin.

QUANTIFICATION OF MORPHOLOGY AND IMMUNOHISTOPATHOLOGY Sections were evaluated by two experienced observers (IMB and DC) who scored the slides blinded to the patients' clinical data. Differences in scorings were resolved by re-reviewing the sections and coming to consensus. Positivity for C4d was scored semi-quantitatively. Staining intensity around syncytiotrophoblast was scored as 0, 1, or 2, with 0 representing the total absence of staining, 1 representing focal positive staining, and 2 representing diffuse staining of all syncytiotrophoblast cell- and basement membranes. Typical examples of staining patterns are given in figure 1.

SEROLOGY: ANTI-HLA ANTIBODIES AND ANTIBODY SPECIFICITY Serum samples from 28 patients from the recurrent miscarriage group were available for analysis. The samples were procured from 80 C storage and a Lambda Antigen Tray class I & II ELISA (One Lambda, Canoga Park, CA) was carried out to detect the presence of HLA class I and class II IgG antibodies. The ELISA was conducted according to protocol with OD readouts at 630 nm.

STATISTICAL ANALYSIS Categorical variables were compared using the Chi-square test and its trend version (linear-by-linear analysis). Differences in quantitative parameters between groups were assessed using one-way ANOVA (for data normally distributed) or the non-parametric Kruskall Wallis-test (for non-normally distributed data). All analyses were performed using SPSS statistical software package (version 16.0; Chicago, IL). A p-value less than 0,05 was considered statistically significant.

Results

CLINICAL CHARACTERISTICS OF CASES AND CONTROL SUBJECTS Table 1 shows the clincial characteristics of the study population. Differences between women with unexplained recurrent miscarriages and control subjects were observed in maternal age and gravidity (both p‹0.05). Women from the recurrent miscarriage group were treated with anticoagulant therapy during pregnancy in 25 out of 38 cases (67%). The medication used was a prophylactic dose of low molecular weight heparin (LMWH) in 15 cases (40%), aspirin in 10 cases (27%) or a combination of both in 6 cases (16%). None of the women with sporadic miscarriage used any medication during pregnancy. In the elective termination of pregnancy-group this information was not available.

IMMUNOHISTOCHEMICAL C4D STAINING IN CASES VERSUS CONTROL SUBJECTS Immunohistochemistry was performed on trophoblast tissue from miscarriage material from the three study-groups. When C4d was present on trophoblast tissue, positivity was detected at the fetal-maternal interface, on the maternal side of the syncytiotrophoblast, either in a focal or a diffuse staining pattern. Typical examples of C4d staining patterns are shown in figure 1A-C.

The presence of placental C4d and its distribution in either a focal or diffuse pattern differed significantly among the three groups in a chi-square linear by linear association analysis (p‹0.004). A diffuse C4d staining pattern in the placenta was present in 10 of 38 of women with unexplained recurrent miscarriage (26%), compared to 3 out of 22 in the sporadic miscarriage group (13%) and 7 out of 67 in elective abortions (10,4%)(p‹0,004). Detailed information on C4d staining in cases versus control subjects is given in table 2.

ANTI-HLA ANTIBODIES AND THEIR RELATION WITH C4D Table 3 shows the relationship between anti-HLA seropositivity and presence of C4d in placental tissue. In total, serum samples of 28 women with unexplained recurrent miscarriage were analysed.

Seropositivity for anti-HLA class I or II IgG antibodies was detected in 11 cases. Of those, 9 women (82%) also had placental C4d deposits in a focal or diffuse pattern. In 17 women without detectable anti-HLA antibodies, only 4 (23%) had focal or diffuse placental C4d staining (P=0,004).

Conclusion and discussion

Recurrent miscarriage is a devastating complication of pregnancy, affecting a large population of women worldwide. Many disease mechanisms for this multifactorial disorder have been identified, but in 50% of couples no underlying cause can be found.[1] For many years it has been questioned whether the fetus can indeed be interpreted as an 'allograft' and thus, miscarriage as 'rejection'.[1;13] In this study we demonstrate that antibody-mediated rejection of the fetal allograft may indeed be present in a subgroup of women with so far unexplained recurrent miscarriages.

C4d, a biomarker of classical complement activation and a footprint of antibody-mediated injury, was present in areas of active fetal-maternal exchange at the maternal side of the syncytiotrophoblast. Placental C4d in a diffuse staining pattern was present significantly more often in women with unexplained recurrent miscarriages compared to two control groups. Moreover, presence of C4d was associated to the presence of anti-HLA class I or II antibodies in the recurrent miscarriage group. Taken together, our data support the concept of an antibody-mediated immune response at the fetal-maternal interface, leading to miscarriage in a certain subgroup of patients with so far unexplained recurrent miscarriages.

Antibody deposition is present in the placenta under physiological conditions but because the placenta is strongly protected from spontaneous complement activation by regulatory mechanism such as Decay Accelerating Factor (DAF), Membrane Cofactor Protein (MCP) and CD59, this usually does not lead to extensive tissue damage.[14-16] Therefore, excessive complement deposition as we observed in certain women with recurrent miscarriage can be interpreted as a sign of local dysregulation of the placental complement system. In other words, there must be either 'excessive complement activation', or 'inadequate complement regulation'.

Too much complement activation may be caused by excessive antibody deposition.[6;17] Antiphospholipid antibodies are likely candidates, as they are associated with placental complement activation and impaired pregnancy outcome.[6] However, all women in our recurrent miscarriage population were tested for anticardiolipin IgG, IgM and lupus anticoagulant and were excluded from the study if any of these antibodies were positive.[12] Allo-antibodies could also be involved. Recently, Nielsen *et al* described that anti-HLA antibodies are related to a reduced live birth rate in women with recurrent miscarriage.[18] Interestingly, we found anti-HLA class I and II antibodies in serum samples of women with unexplained recurrent miscarriage, and demonstrated their potential trace at the fetal-maternal interface via C4d deposition. These antibodies are most likely directed against inherited paternal antigens expressed on trophoblast cells. Up to 30% of women have circulating anti-HLA antibodies during pregnancy, which usually do not predispose to higher risk of adverse pregnancy outcome, miscarriage or pre-eclampsia.[19;20] However, from transplantation settings we know that only some allo-antibodies cause rejection, depending on their antigenicity, their ability to activate complement and their avidity for the antigenic target. Furthermore, presence and detection of anti-HLA antibodies could also be a marker for a broader antibody response. This was shown previously in HLA identical family transplantations, where the presence of anti-HLA antibodies was a risk factor for worse outcome, although clearly anti-HLA antibodies themselves could not have caused any harm.[21] The specific allo-antibodies involved in recurrent miscarriage should be subject for further studies.

Apart from excessive activation, inadequate complement regulation at the fetal-maternal interface may also play a role. It was recently shown in a group of women with SLE and antiphospholipid syndrome, that up to 19% of patients who develop severe preeclampsia

have genetic mutations in genes encoding for complement regulatory proteins that are necessary to prevent damage of host tissue due to uncontrolled activation of complement.[22] These mutations were first described in populations with atypical Hemolytic Uremic Syndrome (aHUS) where they lead to widespread microthrombotic injury. In the study by Salmon *et al*, patients with these mutations developed severe forms of preeclampsia, intrauterine growth restriction and even third trimester intrauterine fetal death. They did not have signs of microthrombotic injuries in organs other than the placenta and did not present as HUS cases. It is possible that genetic defects in complement regulation may cause recurrent miscarriages. The excessive deposition in placental tissue of some of our patients is in line with this concept.

At present there is no evidence based treatment for women with unexplained recurrent miscarriage. None of the published randomized controlled trials investigating the effect of LMWH and aspirin in this population could detect a beneficial effect of these interventions on live birth rate.[12;23;24] In our population a substantial proportion of women was using LMWH, aspirin, or a combination of both at time of miscarriage. In our study, we could not find a relation between use of anticoagulation and presence of C4d, or presence of anti-HLA antibodies (data not shown). Clearly the current group is too small to draw definite conclusions.

Recurrent miscarriage, as is demonstrated by this study, is not a condition with a single cause. Progress in understanding the many different mechanisms that may lead to recurrent miscarriage is urgently needed. This is not a condition with a single cause, and the trials described above illustrate that searching for a single treatment for all patients is likely futile. Unraveling possible pathofysiological mechanisms for recurrent miscarriage in order to define patient tailored treatment strategies is essential. Our findings possibly identify a subgroup of patients in which complement activation plays an important role. This is especially interesting in the light of animal studies by Girardi *et al*, showing that heparin is beneficial in patients with antiphospholipid antibodies because it inhibits complement activation, and not because of its effects on the coagulation cascade.[25] Patients with recurrent miscarriage and evidence for excessive complement activation at the fetal-maternal interface could be the 'positive responders' to treatment with heparin or LMWH. Whether a positive C4d stain in placental tissue of a patient with multiple miscarriages may guide treatment is an interesting subject for further investigations.

TABLE 1 PATIENT CHARACTERISTICS

	RECURRENT MISCARRIAGE (CASES) (N=38)	SPORADIC MISCARRIAGE (CONTROLS) (N=22)	ELECTIVE ABORTION (CONTROLS) (N=67)
Mean maternal age in years (SD)	33,5 (5,4)	31,9 (6,7)	28,6 (7,7)
Mean gravidity (SD)	4,2 (2,1)	2,1 (1,0)	NA
Mean parity (SD)	0,8 (0,8)	1 (1,1)	NA
Previous miscarriage or fetal loss (%)	100	0	NA
Gestational age at miscarriage or abortion (wks) (SD in wks)	10,7 (3,8)	10,5 (2,0)	9,149 (3,1)
▸ 4 miscarriages n(%)	26 (68)	0	NA
Heparin therapy during pregnancy n(%)	15 (40)	0	NA
Aspirin during pregnancy n(%)	10 (27)	0	NA
Aspirin and heparin during pregnancy n(%)	6 (16)	0	NA

NA = No information available

TABLE 2 RECURRENT MISCARRIAGE AND C4D STAINING

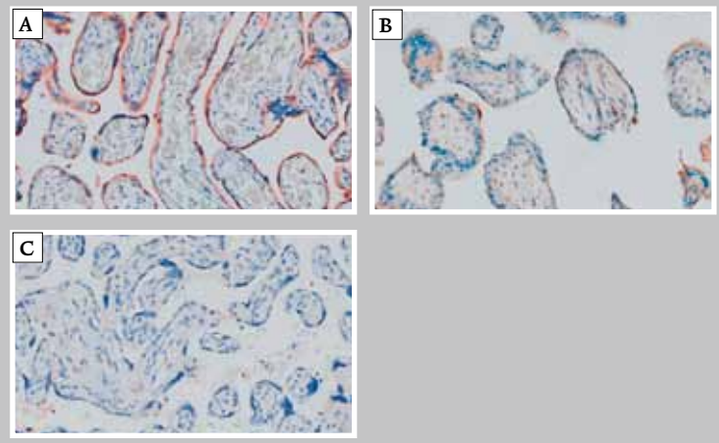
	NO C4D DEPOSITION	FOCAL C4D DEPOSITION	DIFFUSE C4D DEPOSITION	TOTAL
Recurrent miscarriage n(%)	16 (42,1)	12 (31,6)	10 (26,3)	38
Spontaneous miscarriage n(%)	7 (31,8)	12 (54,6)	3 (13,6)	22
Abortion on request n(%)	45 (67,2)	15 (22,4)	7 (10,4)	67
P ◂ 0,004 (Chi squared linear by linear analysis)				

TABLE 3 C4D AND ANTI-HLA ANTIBODIES IN 28 PATIENTS WITH UNEXPLAINED RECURRENT MISCARRIAGE

	NEGATIVE ANTI-HLA CLASS I OR II	POSITIVE ANTI-HLA CLASS I OR II
No or focal C4d deposition	13	2
Diffuse C4d deposition	4	9
	17	11

P = 0,004

FIG 1 EXAMPLES OF IMMUNOHISTOCHEMICAL STAINING PATTERNS OF PLACENTAL C4D



A. Diffuse C4d staining on trophoblast tissue derived from miscarriage material. C4d stains red and is positive at the fetal-maternal interface. Every fetal villus is fully covered with C4d deposits. No staining is visible within fetal villi, suggesting a maternal origin of complement activation.
B. Focal C4d staining. Between 10% and 50% of fetal villi show signs of C4d deposition. Some parts of the trophoblast layer stain positive, but other parts remain negative.
C. Negative C4d staining.

REFERENCES

1 Branch DW, Gibson M, Silver RM. Clinical practice. Recurrent miscarriage. *N Engl J Med* 2010; 363(18):1740-1747.

2 Sullivan AE, Silver RM, LaCoursiere DY, Porter TF, Branch DW. Recurrent fetal aneuploidy and recurrent miscarriage. *Obstet Gynecol* 2004; 104(4):784-788.

3 Collins AB, Schneeberger EE, Pascual MA, Saidman SL, Williams WW, Tolkoff-Rubin N *et al.* Complement activation in acute humoral renal allograft rejection: diagnostic significance of C4d deposits in peritubular capillaries. *J Am Soc Nephrol* 1999; 10(10):2208-2214.

4 Colvin RB. Dimensions of antibody-mediated rejection. *Am J Transplant* 2010; 10(7):1509-1510.

5 Sis B, Mengel M, Haas M, Colvin RB, Halloran PF, Racusen LC *et al.* Banff '09 meeting report: antibody mediated graft deterioration and implementation of Banff working groups. *Am J Transplant* 2010; 10(3):464-471.

6 Cohen D, Buurma A, Goemaere NN, Girardi G, le CS, Scherjon S *et al.* Classical complement activation as a footprint for murine and human antiphospholipid antibody-induced fetal loss. *J Pathol* 2011.

7 Salmon JE, Girardi G, Holers VM. Complement activation as a mediator of antiphospholipid antibody induced pregnancy loss and thrombosis. *Ann Rheum Dis* 2002; 61 Suppl 2:ii46-ii50.

8 Pierangeli SS, Vega-Ostertag M, Liu X, Girardi G. Complement activation: a novel pathogenic mechanism in the antiphospholipid syndrome. *Ann N Y Acad Sci* 2005; 1051:413-420.

9 Girardi G, Salmon JB. The role of complement in pregnancy and fetal loss. *Autoimmunity* 2003; 36(1):19-26.

10 Cohen D, Berger SP, Steup-Beekman GM, Bloemenkamp KW, Bajema IM. Diagnosis and management of the antiphospholipid syndrome. *BMJ* 2010; 340:c2541.

11 Lee J, Romero R, Xu Y, Kim JS, Topping V, Yoo W *et al.* A signature of maternal anti-fetal rejection in spontaneous preterm birth: chronic chorioamnionitis, anti-human leukocyte antigen antibodies, and c4d. PLoS One 2011; 6(2):e16806.

12 Visser J, Ulander VM, Helmerhorst FM, Lampinen K, Morin-Papunen L, Bloemenkamp KW *et al.* Thromboprophylaxis for recurrent miscarriage in women with or without thrombophilia. HABENOX: a randomised multicentre trial. *Thromb Haemost* 2011; 105(2):295-301.

13 Guleria I, Sayegh MH. Maternal acceptance of the fetus: true human tolerance. *J Immunol* 2007; 178(6):3345-3351.

14 Grennan DM, McCormick JN, Wojtacha D, Carty M, Behan W. Immunological studies of the placenta in systemic lupus erythematosus. *Ann Rheum Dis* 1978; 37(2):129-134.

15 Johnson PM, Natvig JB, Ystehede UA, Faulk WP. Immunological studies of human placentae: the distribution and character of immunoglobulins in chorionic villi. *Clin Exp Immunol* 1977; 30(1):145-153.

16 Bulla R, Bossi F, Agostinis C, Radillo O, Colombo F, De SF *et al.* Complement production by trophoblast cells at the feto-maternal interface. *J Reprod Immunol* 2009; 82(2):119-125.

17 Shamonki JM, Salmon JE, Hyjek E, Baergen RN. Excessive complement activation is associated with placental injury in patients with antiphospholipid antibodies. *Am J Obstet Gynecol* 2007; 196(2):167-5.

18 Nielsen HS, Witvliet MD, Steffensen R, Haasnoot GW, Goulmy E, Christiansen OB *et al.* The presence of HLA-antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth. *J Reprod Immunol* 2010; 87(1-2):67-73.

19 Harris RE, Lordon RE. The association of maternal lymphocytotoxic antibodies with obstetric complications. *Obstet Gynecol* 1976; 48(3):302-304.

20 Balasch J, Ercilla G, Vanrell JA, Vives J, Gonzalez-Merlo J. Effects of HLA antibodies on pregnancy. *Obstet Gynecol* 1981; 57(4):444-446.

21 Opelz G. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. *Lancet* 2005; 365(9470):1570-1576.

22 Salmon JE, Heuser C, Triebwasser M, Liszewski MK, Kavanagh D, Roumenina L *et al.* Mutations in complement regulatory proteins predispose to preeclampsia: a genetic analysis of the PROMISSE cohort. PLoS Med 2011; 8(3):e1001013.

23 Clark P, Walker ID, Langhorne P, Crichton L, Thomson A, Greaves M *et al.* SPIN (Scottish Pregnancy Intervention) study: a multicenter, randomized controlled trial of low-molecular-weight heparin and low-dose aspirin in women with recurrent miscarriage. *Blood* 2010; 115(21):4162-4167.

24 Kaandorp SP, Goddijn M, van der Post JA, Hutten BA, Verhoeve HR, Hamulyak K *et al.* Aspirin plus heparin or aspirin alone in women with recurrent miscarriage. *N Engl J Med* 2010; 362(17):1586-1596.

25 Girardi G, Redecha P, Salmon JE. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat Med* 2004; 10(11):1222-1226.