



HLA-C antibodies in women with recurrent miscarriage suggests that antibody mediated rejection is one of the mechanisms leading to recurrent miscarriage



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ABSTRACT

HLA-C is the only polymorphic classical HLA I antigen expressed on trophoblast cells. It is known that higher incidence of C4d deposition on trophoblast cells is present in women with recurrent miscarriage. C4d is a footprint of antibody-mediated classical complement activation. Therefore, this study hypothesize that antibodies against HLA-C may play a role in the occurrence of unexplained consecutive recurrent miscarriage.

Present case control study compared the incidence of HLA-C specific antibodies in 95 women with at least three consecutive miscarriages and 105 women with uneventful pregnancy. In the first trimester of the next pregnancy, presence and specificity of HLA antibodies were determined and their complement fixing ability. The incidence of HLA antibodies was compared with uni- and multivariate logistic regression models adjusting for possible confounders.

Although in general a higher incidence of HLA antibodies was found in women with recurrent miscarriage 31.6% vs. in control subjects 9.5% (adjusted OR 4.3, 95% CI 2.0–9.5), the contribution of antibodies against HLA-C was significantly higher in women with recurrent miscarriage (9.5%) compared to women with uneventful pregnancy (1%) (adjusted OR 11.0, 95% CI 1.3–89.0). In contrast to the control group, HLA-C antibodies in the recurrent miscarriage group were more often able to bind complement.

The higher incidence of antibodies specific for HLA-C in women with recurrent miscarriage suggests that HLA-C antibodies may be involved in the aetiology of unexplained consecutive recurrent miscarriage.

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1. Introduction

About 1–3% of all couples are confronted with recurrent miscarriage, which is internationally defined as ≥ 3 consecutive miscarriages before 20 weeks of gestation (Coulam, 1991). Recurrent miscarriage is a heterogeneous disorder. Possible etiologic factors include uterine anomalies, endocrine disorders, acquired thrombophilia (anti-phospholipid syndrome), hereditary thrombophilia, or balanced translocations in the maternal or paternal

DNA (Branch et al., 2010; Larsen et al., 2013). However, in many couples no causal factor can be identified (Branch et al., 2010; Rai and Regan, 2006).

During pregnancy the maternal immune system has to tolerate the persistence of the semi-allogeneic fetal cells. The extravillous trophoblasts, which is in direct contact with maternal cells, expresses only HLA-C and the non-classical HLA-E, -F and -G. Chimeric fetal cells in the peripheral circulation express all classical HLA class I and II antigens. Approximately 30% of healthy women develop HLA antibodies during pregnancy, the presence of these antibodies increases after 28 weeks of pregnancy and antibodies can still be present at time of a new conception (Regan et al., 1991; van Kampen et al., 2001). Therefore, the incidence of HLA antibodies in the first trimester is higher in multiparous women than in nulliparous women (van Kampen et al., 2001).

Abbreviations: LMWH, low molecular weight heparin; LUMC, Leiden University Medical Centre.

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Binding of antibodies to paternal HLA antigens of the fetus might lead to complement fixation and antibody mediated rejection of the fetus. In women with recurrent miscarriage an increased deposition of C4d, a marker of classical complement activation, was found at the maternal-fetal interface (Meuleman et al., 2015). In spontaneous preterm birth C4d in fetal umbilical cord endothelium was associated with circulating maternal anti-HLA class I antibodies (Lee et al., 2011). A recent meta-analysis showed no significant association between HLA antibodies and the occurrence of recurrent miscarriage (Lashley et al., 2013), however the included studies showed a high level of clinical and statistical heterogeneity. Interestingly, the role of HLA-C specific antibodies has not been studied yet, while from transplantation settings we know that a proportion of alloantibodies cause rejection, amongst others depending on their ability to activate complement (Loupy et al., 2013).

We hypothesize that antibody mediated reactivity plays a role in unexplained recurrent miscarriage. Therefore, the incidence of HLA antibodies, especially those directed against the only polymorphic classical HLA I antigen expressed on trophoblast (HLA-C), is compared between women with recurrent miscarriage and women with uneventful pregnancy.

2. Material and methods

2.1. Subjects

We performed a case control study. The case group was selected from the Habenox trial (NCT0095962) (Visser et al., 2011), a multicentre multinational trial which investigated thrombophylaxis (enoxaparin (low molecular weight heparin (LMWH)), aspirin, or combination of both) for women with recurrent miscarriage with and without thrombophilia. The clinical work-up included documentation of a standardised history from the couple, karyotyping of the couple, an ultrasound or hysteroscopy, and thrombophilia screening. Exclusion criteria for the Habenox trial were a history of thromboembolism or bleeding disorders, allergy to aspirin or enoxaparin, uterine anomalies, cervical insufficiency, untreated thyroid disease, poorly regulated diabetes mellitus, parental chromosomal abnormalities, and pregnancies achieved by assisted reproductive techniques. Hereditary thrombophilia was defined by the presence of a factor V Leiden mutation, increased factor II (prothrombin gene mutation), protein C or S deficiency, increased factor VIII, or antithrombin deficiency. Acquired thrombophilia (anti-phospholipid syndrome) was defined as the presence of IgG anticardiolipin antibodies or lupus anticoagulant or IgG β 2-glycoprotein I antibodies in repeated samples taken 3 months apart and at least 6 weeks after pregnancy (Giannakopoulos et al., 2009).

To obtain a more homogenous case group in this case control study, only women with three or more, consecutive miscarriages prior to the 20th week of gestation without uterine anomalies and parental chromosomal abnormalities were selected. Women with hereditary thrombophilia were not excluded, as the evidence that hereditary thrombophilia is associated with recurrent miscarriage is less clear (Larsen et al., 2013; McNamee et al., 2012).

The presence of HLA antibodies can be considered as a marker for a broader immune response (Opelz, 2005). Although the presence of anti-phospholipid antibodies is highly associated with recurrent miscarriage (Chan et al., 2011; Larsen et al., 2013), women with acquired thrombophilia were not excluded since anti-phospholipid antibodies are potential candidates for this broader antibody response. Sensitivity analyses were performed to evaluate the presence of HLA antibodies in women with and without acquired thrombophilia in the case group compared to control subjects.

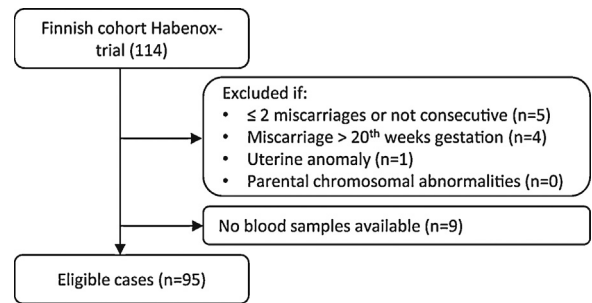


Fig. 1. Flow chart of the case group.

Both women with primary recurrent miscarriage (no history of live birth) and secondary recurrent miscarriage (history of (a) live birth(s)) were included. The Finnish women were selected, since blood samples were available from this group. Samples were taken at the first antenatal visit before enrolment in the Habenox trial, and every 4 weeks, till the pregnancy ended. This pregnancy was indicated as the index pregnancy. In the Habenox trial, 114 Finnish women were consecutive enrolled from January 2002 until December 2007 and 95 cases were eligible for this case control study (Fig. 1).

The control group consisted of women who had an uncomplicated index singleton pregnancy, ending in an uncomplicated live birth without congenital abnormalities, delivering at the department of Obstetrics at the Leiden University Medical Centre (LUMC) in the Netherlands between June 2003 and June 2012. In total 105 women were eligible and consisted of both nulliparous and multiparous women. All these women had no recurrent miscarriage or complicated pregnancy (preeclampsia, Hemolysis Elevated Liver enzymes and Low Platelets syndrome, preterm birth, preterm rupture of membranes, fetal growth restriction, perinatal death or maternal death defined as described below) in history and current pregnancy. Women were at the time of inclusion healthy individuals with an uneventful medical history. Samples were taken in the first trimester during pregnancy, which was indicated as the index pregnancy.

2.2. Variables and definitions

From the cases and control subjects data was collected from medical files. The following data was collected: personal characteristics, intoxications, use of medication, general medical history, information on previous blood transfusions and transplantations, outcome and complications of all previous pregnancies before the index pregnancy, gender of previous children, treatment for previous miscarriages, gestational age at blood sampling, outcome and complications of index pregnancy, medication use during index pregnancy, and neonatal characteristics.

Pregnancy induced hypertension was defined as systolic blood pressure above 140 mmHg and/or diastolic pressure above 90 mmHg, combined with proteinuria as preeclampsia, preterm birth as a delivery between 24 and 37 weeks gestation, fetal growth restriction as birth weight below the 2.3th percentile for gestational age and sex (Kloosterman, 1970), perinatal death as fetal loss after 22 weeks of gestation till 7 days after birth, and postpartum haemorrhage as blood loss above 1000 mL in the 24 h postpartum.

2.3. Antibody screening

The presence of HLA antibodies was determined in the first trimester (till 13 weeks gestation) of the index pregnancy, both in cases and control subjects.

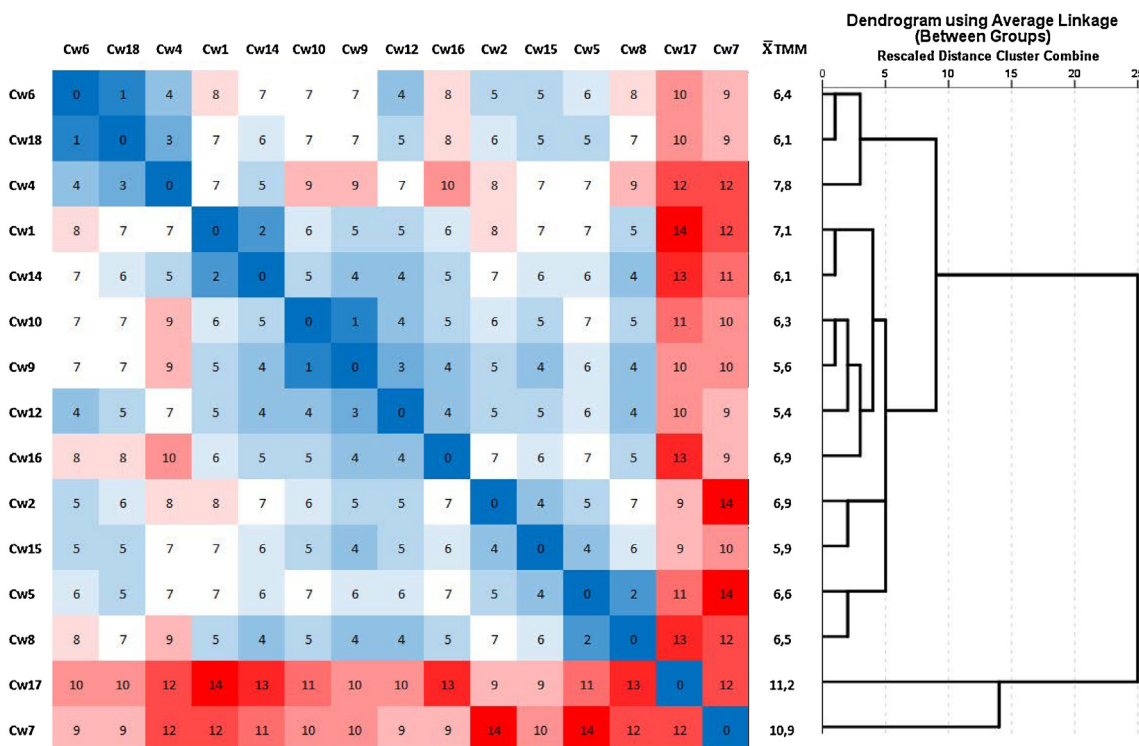


Fig. 2. Cross-analysis of single HLA-C antigen for triplet mismatches shown in a heatmap. The median number of triplet mismatches is colored white, increasing number of triplets are colored red and decreasing are colored blue. Mean triplet mismatches (\bar{X} TMM) of specific HLA-C alleles towards all the other HLA-C alleles and clustering between group using average linking are given. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The detection of HLA class I and II antibodies in maternal sera was performed by an enzyme-linked immunosorbent assay (LAT TM, One Lambda, CA) with readouts at 630 nm, detecting both complement fixing and non-complement fixing antibodies. Positive sera (ratio patient/control >2.0) was further tested for HLA antibody specificity by the standard NIH complement dependent cytotoxicity assay (Terasaki et al., 1978) against a panel of 54HLA-typed individuals and for their class I and II specificity with Luminex single antigen beads (Gen Probe, Stamford, CT) following the manufacturer's instructions. Purified anti-human C1q was used in the IgG SAB assay to detect complement fixing antibodies. A median fluorescence intensity >1000 was considered positive as reported elsewhere (Billen et al., 2008; Zoet et al., 2011).

2.4. Statistical analyses

For descriptive analysis of baseline characteristics between cases and control subjects the Mann Whitney *U* test was performed and for categorical variables the Chi-squared test, if expected counts were less than five Fisher's exact test was used.

HLA antibody presence and specificity (HLA-A, HLA-B, HLA-C, HLA class I, and II antibodies) were compared between cases and control subjects.

Bias can arise due to genetic differences between the Finnish case group and the Dutch control group. As we had special interest in HLA antibodies against the most polymorphic antigen on trophoblast the HLA-C antigen, we determined the relative immunogenicity of the different HLA-C antigens using the HLAMatchmaker program developed by Duquesnoy (2002) as previously described for HLA-A and -B (Dankers et al., 2004). The number of epitope (triplet) mismatches was calculated on basis of polymorphic amino acid configurations that represent defined areas of HLA epitopes on protein sequences of HLA-A, -B, and -C chains accessible to alloantibodies and depicted in a cross-analysis.

HLA-C*07 and C*17 showed the highest mean of triplet mismatches within the HLA-C allele (Fig. 2), suggesting that these are the most immunogenic antigens. The frequencies of the HLA-C alleles in the Finnish and Dutch population were compared using www.allelefreqencies.net and appeared to be comparable i.e. HLA-C*07 (0.30 vs. 0.33) and C*17 (0.022 vs. 0.004) between the Finnish and Dutch population.

Furthermore, potential confounding factors for the presence of HLA antibodies and the occurrence of recurrent miscarriage include an older age, acquired thrombophilia, thyroid autoimmunity, previous pregnancy complications, and having a boy prior to the index pregnancy.

More pregnancy complications are observed prior and subsequent to the miscarriages in women with recurrent miscarriage (Nielsen et al., 2010a; Yang et al., 2006). In a recent meta-analysis no beneficial or harmful effect of HLA alloantibodies was observed in pregnancy, however more studies are necessary to determine definite effect of HLA antibodies on pregnancy outcome (Lashley et al., 2013), and therefore pregnancy complications could still be a potential confounder for the presence of HLA antibodies.

Furthermore, previous studies showed that recurrent miscarriage is more often preceded by a firstborn boy than girl (Nielsen et al., 2008; Ooi et al., 2011). A boy prior to the index pregnancy is related with presence of HLA antibodies (Nielsen et al., 2010b), due to the higher incidence of pregnancy complications, if the child preceding the secondary recurrent miscarriage is a boy compared to a girl (Nielsen et al., 2010a).

Acquired thrombophilia was not measured in the control group, none of the cases and control subjects had thyroid disease, and control subjects were selected on basis of uncomplicated previous pregnancies. All these possible confounding factors, including having a boy prior to the index pregnancy, could not be included in the multivariate analysis. Therefore, the association between the presence of HLA antibodies and recurrent miscarriage was studied with

Table 1
Baseline characteristics of subjects.

	Recurrent miscarriage (N=95)	Uneventful pregnancy (N=105)
Maternal age at time of index pregnancy (years; median [IQR])	32 (29–35)	32 (27.5–35)
Body-mass index (kg/m ² ; median [IQR]) ^b	22.6 (20.7–25.3)	23.2 (20.7–26.0)
Maternal smoking ^c	10 (11.2%)	9 (8.6%)
Blood transfusions	0	0
Transplantation	0	0
Thrombophilia	24	n.t
V Leiden mutation	8	
Prothrombin gene mutation	2	
Protein C or S deficiency	1	
High factor VIII	0	
Anti-thrombin deficiency	0	
Anti-phospholipid syndrome	17	
Gravidity at time of index pregnancy (median [IQR])	5 (4–6) ^a	2 (1–3)
Parity at time of index pregnancy (median [IQR])	0 (0–1)	1 (0–1)
Nulliparous at time of index pregnancy	55 (57.9%)	51 (48.6%)
Previous miscarriages (range)	95 (100%) 3–8	23 (21.9%) 0–2
Previous termination of pregnancy (range)	6 (6.2%) 0–2	12 (11.5%) 0–2
Previous curettage	73 (76.8%) ^a	14 (13.3%)
Gestation blood sampling (days; median [IQR])	67.5 (44.5–70) ^a	77 (71–84.5)
Having a boy prior to the index pregnancy	26 (65.0%)	34 (63%)

Data are n (%) unless otherwise indicated, IQR interquartile range; n.t. not tested. All χ^2 tests or Mann-Whitney *U* test.

^a Compared with women with uneventful pregnancy $p < 0.001$.

^b 11% missing values (4 of 95 cases and 18 of 105 controls).

^c 4% missing values (6 of 95 cases and 2 of 105 controls).

uni- and multivariate logistic regression, adjusting for age as continuous variable in a multivariate logistic regression analysis using the enter method.

In the sensitivity analysis for parity the presence of HLA antibodies was compared between nulliparous, multiparous cases and control subjects.

Gravidity was not included in a sensitivity analysis because antibodies are rarely demonstrable before 28 weeks gestation (Regan et al., 1991). Of the 50 pregnancies ending in a spontaneous miscarriage, only two women developed antibodies during the index pregnancy (Regan et al., 1991). As previous curettages and the gestational age at the time of blood sampling can hypothetically affect the incidence of HLA antibodies, these parameters were included in sensitivity analyses.

Statistical analysis was performed using SPSS Statistics (version 20.0, IL, USA). Descriptive statistical analysis was performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, CA, USA, www.graphpad.com).

2.5. Ethic statements

Approval to use blood samples for research from the Finnish women in the case group was given (NCT0095962). The determination of HLA antibodies in serum of women with uneventful pregnancies was approved by the Ethic Committee of the LUMC (P13.048).

3. Results

3.1. Baseline characteristics of subjects

No differences between the cases and control subjects were observed with respect to maternal age, BMI, smoking habits, and

parity (Table 1). In both groups the proportion of nulliparous women was equal. None of the cases and control subjects had blood transfusions or transplantation in their medical history. In the case group the median consecutive miscarriage rate was 3, while 33 patients (34.7%) had 4 or more consecutive miscarriages. Of the 105 control subjects, 23 women (21.9%) had at least one miscarriage and 12 women (11.4%) had at least one termination of pregnancy before the index pregnancy. Overall no differences were observed in pregnancy complications between cases and control subjects (Table 2).

3.2. The incidence of HLA antibodies is higher in women with recurrent miscarriage

The incidence of HLA antibodies in the first trimester was significantly higher in women with recurrent miscarriage 31.6% vs. in control subjects 9.5% (OR 4.3, 95% CI 2.0–9.5) (Fig. 3a). After adjustment for age, in multivariate analysis, the estimator of interest did not change (adjusted OR 4.3, 95% CI 2.0–9.5). The contribution of HLA-C antibodies to the humoral sensitization was significantly higher in women with recurrent miscarriage i.e. 9 out of 95 cases compared to only one out of 105 control subjects (OR 10.8, 95% CI 1.3–87.6 and adjusted OR 11.0, 95% CI 1.3–89.0) (Fig. 3b). In 5 out of 9 women with recurrent miscarriage these HLA-C antibodies were C1q fixing antibodies, the HLA-C antibodies found in the women with an uneventful pregnancy were non-C1q fixing antibodies.

As anti-phospholipid antibodies had not been measured in the control subjects, we could not correct in multivariate logistic regression for the presence of anti-phospholipid antibodies, but even after exclusion of the 17 cases with anti-phospholipid syndrome, the incidence of HLA antibodies was still higher in the recurrent miscarriage group (OR 3.0, 95% CI 1.3–7.0).

Table 2
Characteristics of the ongoing index pregnancy in subjects.

	Recurrent miscarriage (N = 95)	Uneventful pregnancy (N = 105)
Live births	60 (63.2%)	105 (100%)
Gestational age (days; median [IQR])	281 (271–288)	281 (276–287)
Birthweight (gram; median [IQR])	3372.5 (3137.7–3957.5)	3460 (3147.5–3802.5)
Blood pressure (mmHg; median [IQR]) ^c		
Systolic	120 (115–130)	123 (120–130)
Diastolic	78 (73–90)	78 (71.2–80)
Pregnancy complications	19 (31.7%)	17 (16.2%)
Preterm birth	4 (6.8%) ^a	0 (0.0%)
Fetal growth restriction	0	0
Fluxus	8 (14.3%) ^b	1 (1.0%)
Pregnancy induced hypertension	10 (16.7%)	16 (15.2%)
Preeclampsia	1 (1.7%)	0
HELLP syndrome	0	0
Perinatal death	0	0
Maternal death	0	0

Data are n (%) unless otherwise indicated, IQR interquartile range, HELLP Hemolysis Elevated Liver enzymes and Low Platelets. All χ^2 tests or Mann-Whitney *U* test.

^a Compared with women with uneventful pregnancy $p < 0.05$.
^b Compared with women with uneventful pregnancy $p < 0.001$.
^c 6.0% missing values (9 of 60 cases and 1 of 105 controls).

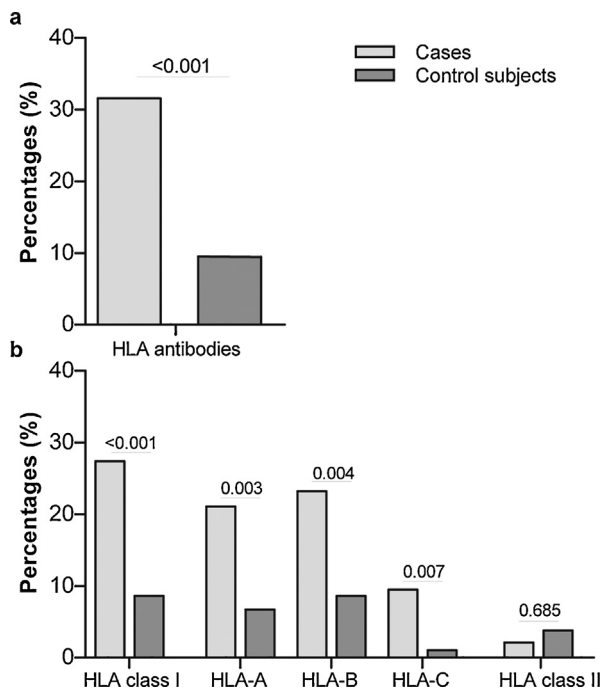


Fig. 3. Presence of HLA antibodies (a) and specific HLA antibodies (b) in cases and control subjects. HLA antibodies in the first trimester were significantly higher in women with recurrent miscarriage 31.6% vs. in control subjects 9.5% (a). For specific HLA antibodies, especially those directed against the only polymorphic classical HLA I antigen expressed on trophoblast (HLA-C) were increased. In 9 out of 95 cases HLA-C antibodies were detected compared to only one out of 105 control subjects (b).

Sensitivity analysis for parity showed that HLA antibodies were more often present in nulliparous cases than in nulliparous control subjects (29.1% vs. 0%, $p < 0.001$). No significant difference was observed between multiparous cases and control subjects in HLA antibodies (35% vs. 18.5%, $p = 0.070$). In nulliparous cases four women had HLA-C antibodies compared to none of the control subjects ($p = 0.050$). Furthermore, multiparous cases had significantly more often HLA-C antibodies than control subjects (12.5% vs. 1.9%, $p = 0.037$).

Previous curettage was not associated with an increase of HLA antibodies, neither in cases nor in control subjects (Fig. 4). In addition, no HLA antibodies were detected in nulliparous control

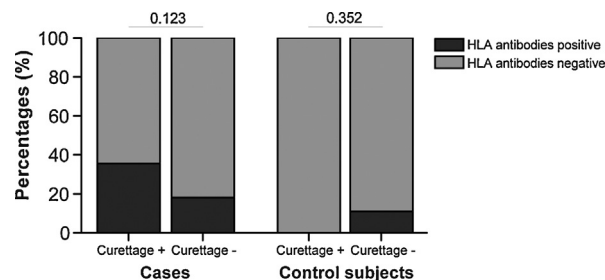


Fig. 4. Presence of HLA antibodies in cases and control subjects with and without previous curettages. No association between the presence of HLA antibodies in the cases or in the control subjects with and without previous curettage ($p = 0.123$ and $p = 0.352$ respectively).

subjects, although 7 control subjects had a termination of pregnancy before the index pregnancy and one control subject had two terminations of pregnancy. In the case group the mean gestational age at the time of blood sampling was comparable in women with and without HLA antibodies (61.4 vs. 58.0 days, $p = 0.391$). In the control group gestational age at time of blood sampling was significantly later in women with HLA antibodies (81.8 vs. 76.6 days, $p = 0.035$).

4. Discussion

The incidence of HLA-antibodies in the first trimester was found to be significantly increased in women with recurrent miscarriage. Particular relevant for the local situation in the placenta, is the observation that significantly more often HLA antibodies specific for HLA-C are found in women with recurrent miscarriage.

The strength of the study is that, in comparison to other studies (Lashley et al., 2013), a large homogenous case group of women with a history of recurrent miscarriage were selected. In addition sensitivity analysis for parity was performed. Finally, the detection of the HLA-C specificity of the HLA antibodies is important, considering that HLA-C is the only classical HLA antigen expressed on the trophoblast. Furthermore, by analysing the ability of the HLA-C antibodies to fix complement, a possible link can be made with the previously observed increased C4d deposition on the trophoblast in women with recurrent miscarriage (Meuleman et al., 2015).

HLA-antibodies in early pregnancy are associated with lower chance of a live birth in women with recurrent miscarriage (Nielsen et al., 2010b). However, in our study we could not evaluate pres-

ence of HLA antibodies related to pregnancy outcome because the use of medication. In our case group, all women used medication as part of the randomized control trial, i.e. enoxaparin, aspirin, or a combination of these two, during blood sampling for this study, which could influence pregnancy outcome. In vivo studies in mice showed that treatment with heparin prevents complement activation and protects mice from pregnancy complications induced by anti-phospholipid antibodies (Girardi et al., 2004). Recently, a similar effect of LMWH in women with at least two consecutive miscarriages or one late miscarriage has not been observed (Pasquier et al., 2015; Schleussner and Petroff, 2015), however the effect of LMWH in a homogenous group of women with recurrent miscarriage has not been studied yet.

Furthermore, the HLA typing of the fetus or products of conception from the index pregnancy was unknown, which made it impossible to determine whether the HLA antibodies were specific for the index pregnancy. Future research in a different group of women with recurrent miscarriage, without using medication, should focus on the presence of HLA antibodies in first trimester, whether these antibodies are child specific, and relate this to pregnancy outcome.

The fact that the gestational age at blood sampling was significantly higher in control subjects than in cases, could have affected the results. However, a later gestational age is supposed to be associated with a higher production of HLA antibodies (Regan et al., 1991), whereas less HLA antibody production was seen in the controls subjects, suggesting that the observed difference might even be an underestimation.

The presence of HLA antibodies can be considered as a marker for a broader immune response, as was previously shown in HLA identical family transplantations, where the presence of HLA antibodies was a risk factor for worse outcome, although HLA antibodies themselves could not have caused any harm (Opelz, 2005). Anti-phospholipid antibodies, which are highly associated with recurrent miscarriage (Larsen et al., 2013), are potential candidates for this broader antibody response. No correction could be made in multivariate logistic regression for the presence of anti-phospholipid antibodies since these antibodies were not measured in the control subjects. However, even after exclusion of cases with anti-phospholipid syndrome, a significant association between the presence of HLA antibodies and occurrence of recurrent miscarriage was present.

Some studies suggest that the presence of specific antibodies directed to the paternal HLA could be beneficial for a pregnancy by enhancing the development of maternal-fetal immunologic tolerance (Agrawal et al., 2002; Bartel et al., 2011; Coulam, 1992; Kishore et al., 1996; Pandey et al., 2005), others demonstrate that HLA antibodies against the paternal antigens are rarely demonstrable before 28 weeks gestation in uneventful pregnancy (Regan et al., 1991), which is in line with our finding that none of the nulliparous control subjects had HLA antibodies. In contrast, almost one third of the women with primary recurrent miscarriage produced HLA antibodies in the first trimester. The presence of HLA-C antibodies in women with primary recurrent miscarriage may partly explain the high incidence of C4d found in women with primary recurrent miscarriage shown previously (Meuleman et al., 2015). However, from transplantation settings it is known that not all alloantibodies cause rejection and that their ability to activate complement might play a determinative role (Loupou et al., 2013). The majority of the HLA-C antibodies in the case group were able to fix complement, whereas no complement fixing HLA-C antibodies were found in the control group.

In conclusion, in this study which included a homogenous well-defined group of women with recurrent miscarriage, a higher incidence of HLA antibodies was observed compared to women with an uneventful pregnancy. Especially, the presence of comple-

ment fixing HLA-C antibodies in relation to the selective expression of HLA-C on trophoblast tissue might explain the aetiology in a proportion of the women with recurrent miscarriage.

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References

- Agrawal, S., Pandey, M.K., Mandal, S., Mishra, L., Agarwal, S., 2002. Humoral immune response to an allogenic foetus in normal fertile women and recurrent aborters. *BMC Pregnancy Childbirth* 2, 6.
- Bartel, G., et al., 2011. Prevalence and qualitative properties of circulating anti-human leukocyte antigen alloantibodies after pregnancy: no association with unexplained recurrent miscarriage. *Hum. Immunol.* 72, 187–192.
- Billen, E.V., Voort, C.E., Christiaans, M.H., van den Berg-Loonen, E.M., 2008. Luminex donor-specific crossmatches. *Tissue Antigens* 71, 507–513.
- Branch, D.W., Gibson, M., Silver, R.M., 2010. Clinical practice. Recurrent miscarriage. *N. Engl. J. Med.* 363, 1740–1747.
- Chan, Y.Y., Jayaprakasan, K., Zamora, J., Thornton, J.G., Raine-Fenning, N., Coomarasamy, A., 2011. The prevalence of congenital uterine anomalies in unselected and high-risk populations: a systematic review. *Hum. Reprod. Update* 17, 761–771.
- Coulam, C.B., 1991. Epidemiology of recurrent spontaneous abortion. *Am. J. Reprod. Immunol.* 26, 23–27.
- Coulam, C.B., 1992. Immunologic tests in the evaluation of reproductive disorders: a critical review. *Am. J. Obstet. Gynecol.* 167, 1844–1851.
- Dankers, M.K., et al., 2004. The number of amino acid triplet differences between patient and donor is predictive for the antibody reactivity against mismatched human leukocyte antigens. *Transplantation* 77, 1236–1239.
- Duquesnoy, R.J., 2002. HLA Matchmaker: a molecularly based algorithm for histocompatibility determination. I. Description of the algorithm. *Hum. Immunol.* 63, 339–352.
- Giannakopoulos, B., Passam, F., Ioannou, Y., Krilis, S.A., 2009. How we diagnose the antiphospholipid syndrome. *Blood* 113, 985–994.
- Girardi, G., Redecha, P., Salmon, J.E., 2004. Heparin prevents antiphospholipid antibody-induced fetal loss by inhibiting complement activation. *Nat. Med.* 10, 1222–1226.
- Kishore, R., Agarwal, S., Halder, A., Das, V., Shukla, B.R., Agarwal, S.S., 1996. HLA sharing, anti-paternal cytotoxic antibodies and MLR blocking factors in women with recurrent spontaneous abortion. *J. Obstet. Gynaecol. Res.* 22, 177–183.
- Kloosterman, G.J., 1970. On intrauterine growth. The significance of prenatal care. *Int. J. Gynaecol. Obstet.* 8, 895–912.
- Larsen, E.C., Christiansen, O.B., Kolte, A.M., Macklon, N., 2013. New insights into mechanisms behind miscarriage. *BMC Med.* 11, 154.
- Lashley, E.E., Meuleman, T., Claas, F.H., 2013. Beneficial or harmful effect of antipaternal human leukocyte antibodies on pregnancy outcome? A systematic review and meta-analysis. *Am. J. Reprod. Immunol.* 70, 87–103.
- Lee, J., et al., 2011. A signature of maternal anti-fetal rejection in spontaneous preterm birth: chronic chorioamnionitis, anti-human leukocyte antigen antibodies, and c4d. *PLoS One* 6, e16806.
- Loupou, A., et al., 2013. Complement-binding anti-HLA antibodies and kidney-allograft survival. *N. Engl. J. Med.* 369, 1215–1226.
- McNamee, K., Dawood, F., Farquharson, R., 2012. Recurrent miscarriage and thrombophilia: an update. *Curr. Opin. Obstet. Gynecol.* 24, 229–234.
- Meuleman, T., Cohen, D., Swings, G.M., Veraar, K., Claas, F.H., Bloemenkamp, K.W., 2015. Increased complement C4d deposition at the maternal-fetal interface in unexplained recurrent miscarriage. *J. Reprod. Immunol.* 113, 54–60.
- Nielsen, H.S., Andersen, A.M., Kolte, A.M., Christiansen, O.B., 2008. A firstborn boy is suggestive of a strong prognostic factor in secondary recurrent miscarriage: a confirmatory study. *Fertil. Steril.* 89, 907–911.
- Nielsen, H.S., et al., 2010a. Frequency and impact of obstetric complications prior and subsequent to unexplained secondary recurrent miscarriage. *Hum. Reprod.* 25, 1543–1552.
- Nielsen, H.S., et al., 2010b. The presence of HLA-antibodies in recurrent miscarriage patients is associated with a reduced chance of a live birth. *J. Reprod. Immunol.* 87, 67–73.
- Ooi, P.V., Russell, N., O'Donoghue, K., 2011. Secondary recurrent miscarriage is associated with previous male birth. *J. Reprod. Immunol.* 88, 38–41.
- Opelz, G., 2005. Non-HLA transplantation immunity revealed by lymphocytotoxic antibodies. *Lancet* 365, 1570–1576.
- Pandey, M.K., Rani, R., Agrawal, S., 2005. An update in recurrent spontaneous abortion. *Arch. Gynecol. Obstet.* 272, 95–108.
- Pasquier, E., et al., 2015. Enoxaparin for prevention of unexplained recurrent miscarriage: a multicenter randomized double-blind placebo-controlled trial. *Blood* 125, 2200–2205.
- Rai, R., Regan, L., 2006. Recurrent miscarriage. *Lancet* 368, 601–611.

- Regan, L., Braude, P.R., Hill, D.P., 1991. A prospective study of the incidence: time of appearance and significance of anti-paternal lymphocytotoxic antibodies in human pregnancy. *Hum. Reprod.* 6, 294–298.
- Schleussner, E., Petroff, D., 2015. Low-molecular-weight heparin for women with unexplained recurrent pregnancy loss. *Ann. Intern. Med.* 163, 485.
- Terasaki, P.I., Bernoco, D., Park, M.S., Ozturk, G., Iwaki, Y., 1978. Microdroplet testing for HLA-A, -B, -C, and -D antigens. The Phillip Levine Award lecture. *Am. J. Clin. Pathol.* 69, 103–120.
- van Kampen, C.A., Versteeg-van der Voort Maarschalk, M.F., Langerak-Langerak, J., van, B.E., Roelen, D.L., Claas, F.H., 2001. Pregnancy can induce long-persisting primed CTLs specific for inherited paternal HLA antigens. *Hum. Immunol.* 62, 201–207.
- Visser, J., et al., 2011. Thromboprophylaxis for recurrent miscarriage in women with or without thrombophilia. HABENOX: a randomised multicentre trial. *Thromb. Haemost.* 105, 295–301.
- Yang, C.J., Stone, P., Stewart, A.W., 2006. The epidemiology of recurrent miscarriage: a descriptive study of 1214 prepregnant women with recurrent miscarriage. *Aust. N. Z. J. Obstet. Gynaecol.* 46, 316–322.
- Zoet, Y.M., Brand-Schaaf, S.H., Roelen, D.L., Mulder, A., Claas, F.H., Doxiadis, I.I., 2011. Challenging the golden standard in defining donor-specific antibodies: does the solid phase assay meet the expectations? *Tissue Antigens* 77, 225–228.