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ORIGINAL ARTICLE ***Early pregnancy***

Maternal KIR haplotype influences live birth rate after double embryo transfer in IVF cycles in patients with recurrent miscarriages and implantation failure

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STUDY QUESTION: In patients with recurrent miscarriages (RM) or recurrent implantation failure (RIF), does the maternal killer immunoglobulin-like receptor (KIR) haplotype have an impact on live birth rates per cycle after embryo transfer with the patient's own or donated oocytes?

SUMMARY ANSWER: After double embryo transfer (DET) in patients with the maternal KIR AA haplotype, a significantly increased early miscarriage rate was observed when the patient's own oocytes were used, and a significantly decreased live birth rate per cycle after embryo transfer was observed when donated oocytes were used.

WHAT IS ALREADY KNOWN: Interactions between fetal HLA-C and maternal KIR influence placentation during human pregnancy. There is an increased risk of RM, pre-eclampsia or fetal growth restriction in mothers with the KIR AA haplotype when the fetus has more HLA-C2 genes than the mother.

STUDY DESIGN, SIZE AND DURATION: Between 2010 and 2014, we performed a retrospective study that included 291 women, with RM or RIF, who had a total of 1304 assisted reproductive cycles.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Pregnancy, miscarriage and live birth rates per cycle after single or DET, categorized by the origin of the oocytes and the presence of maternal KIR haplotypes, were studied. KIR haplotype regions were defined by the presence of the following KIR genes: Cen-A/2DL3; Tel-A/3DL1 and 2DS4; Cen-B/2DL2 and 2DS2; as well as Tel-B/2DS1 and 3DS1.

MAIN RESULTS AND THE ROLE OF CHANCE: Higher rates of early miscarriage per cycle after DET with the patient's own oocytes in mothers with the KIR AA haplotype (22.8%) followed by those with the KIR AB haplotype (16.7%) compared with mothers with the KIR BB haplotype (11.1%) were observed ($P = 0.03$). Significantly decreased live birth rates per cycle were observed after DET of donated oocytes in mothers with the KIR AA haplotype (7.5%) compared with those with the KIR AB (26.4%) and KIR BB (21.5%) haplotypes ($P = 0.006$). No statistically significant differences were observed for pregnancy, miscarriage and live birth rates per cycle among those with maternal KIR AA, AB and BB haplotypes after single embryo transfer (SET) with the patient's own or donated oocytes. The large number of cases studied strengthens the results and provides sufficient power to the statistical analysis.

LIMITATIONS, REASONS FOR CAUTION: During the IVF procedure, DET induces the expression of more than one paternal HLA-C and the oocyte-derived maternal HLA-C in the oocyte-donation cycles probably behaves like paternal HLA-C. Because this was a retrospective study, we did not have data about the HLA-C of the parent, donor, chorionic villi, or infant, which is a limitation because we cannot show differences according to paternal or oocyte donor HLA-C1 and HLA-C2.

WIDER IMPLICATIONS OF THE FINDINGS: These new insights could have an impact on the selection of SET in patients with RM or RIF, and a KIR AA haplotype. Also, it may help in oocyte and/or sperm donor selection by HLA-C in patients with RM or RIF and a KIR AA haplotype.

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Key words: Maternal KIR / paternal HLA-C / embryo transfer / miscarriage rate / live birth rate

Introduction

Successful maternal adaptation to the semi-allogeneic fetus is a complicated process, which occurs in the uterus at the site of placentation (Arck and Hecher, 2013). The CD56bright CD16dim decidual natural killer (dNK) cells are the most abundant leukocyte population during the first trimester of human pregnancy (Moffett-King, 2002). The fetal extravillous trophoblast cells (EVTs) express class I HLA-C and non-classical HLA-G and HLA-E antigens, whereas the class I antigens HLA-A and HLA-B and class II antigens are absent (King *et al.*, 2000; Apps *et al.*, 2009).

Placentation is regulated by interactions between maternal killer immunoglobulin-like receptors (KIRs) expressed by the dNK. The KIR ligands, which are HLA-C molecules, are expressed by invading trophoblast cells (EVT) (Hiby *et al.*, 2004, 2008, 2010a,b). Hiby *et al* showed that invading EVTs are the principal site of HLA-C expression in the decidua basalis and that both maternal and paternal HLA-C allotypes are presented to KIRs on dNK cells (Hiby *et al.*, 2010a,b). Insufficient invasion of the uterine lining by trophoblasts and vascular conversion in the decidua (Burton *et al.*, 2009) are thought to be the primary defect in disorders such as recurrent miscarriage (RM), pre-eclampsia and fetal growth restriction (FGR; Arck and Heche, 2013).

In any pregnancy, the maternal KIR genotype could be AA (no activating KIRs), AB, or BB (1–10 activating KIRs) (Uhrberg *et al.*, 1997). KIR haplotype regions are defined by the presence of the following KIR genes: Cen-A/2DL3; Tel-A/3DL1 and 2DS4; Cen-B/2DL2 and 2DS2; as well as Tel-B/2DS1 and 3DS1 (Hsu *et al.*, 2002).

The HLA-C ligands for KIRs are divided into two groups: HLA-C1 and HLA-C2. The C1 group allotypes are ligands for the inhibitory receptors KIR2DL2 (B haplotype) and KIR2DL3 (A haplotype), and the C2 group allotypes are ligands for the inhibitory KIR2DL1 (A haplotype) and activating KIR2DS1 receptors (B haplotype). Of the two, C2 is a stronger ligand than C1 (Winter *et al.*, 1998). The *A* haplotypes contain mainly genes for inhibitory KIR, and *B* haplotypes have additional genes encoding activating KIR. Inhibitory KIR2DL1 (*A* haplotype) and activating KIR2DS1 (*B* haplotype) both bind HLA-C2. The presence of activating KIR2DS1 (B haplotype) confers protection from pregnancy disorders (Hiby *et al.*, 2010a,b), and its absence (A haplotype) increases the risk of pregnancy complications (Xiong *et al.*, 2013; Wang *et al.*, 2014).

Pregnancies are at increased risk of RM, pre-eclampsia, or FGR in mothers who are homozygous for KIR haplotype *A* (KIR AA) when the fetus has more HLA-C2 genes than the mother, and the additional fetal HLA-C2 alleles are of paternal origin (Hiby *et al.*, 2010a,b).

In vitro fertilization (IVF) cycles usually include one or two embryos per transfer, and donated oocytes, sperm or embryos are often used during assisted reproductive treatments (ART). Double embryo transfer (DET) induces the expression of more than one paternal HLA-C in the trophoblast cells. In the oocyte-donation cycles, the oocyte-maternal HLA-C probably behaves like paternal HLA-C (two 'paternal' HLA-C per trophoblast cell) given that it is possibly different from the mother receiving the embryo.

Based on the available previous knowledge, we hypothesized that the increased expression of paternal HLA-C after DET could be associated with more pregnancy disorders than after single embryo transfer (SET) in mothers with an inhibitory KIR haplotype (AA). We retrospectively studied 291 woman undergoing a total of 1304 cycles of recurrent implantation failures (RIF) or RM after ART to assess the influence of maternal KIR haplotype on the pregnancy, miscarriage rates and live birth rates after DET or SET when categorized by the origin of the oocytes (patient's own or donated oocytes).

Materials and Methods

Between September 2010 and February 2014, a total of 291 woman who experienced RM or RIF undergoing a total of 1304 cycles (51.8% with the patient's own oocytes and 29.7% with donated oocytes) recruited from the Instituto Valenciano de Infertilidad (IVI) Clinics (Madrid, Barcelona, Bilbao, Sevilla, Valencia, Spain) were studied. Patients with a poor reproductive outcome were referred to our Reproductive Immunology Department (10.5% of total of ART IVF or oocyte-donation cycles in Madrid, 2.5% in Barcelona, 2.0% in Bilbao, 1.5% in Sevilla and 0.4% in Valencia). From these, only patients who fulfilled the inclusion criteria were included in the present study.

Study population

Women with a history of RM (>3 unexplained miscarriages after ART) or RIF (>3 failed IVF cycles with good quality embryos or >2 failed donor oocyte cycles) undergoing a total of 1304 cycles (42.1% DET and 23.5% SET) were studied. A comprehensive fertility screening was performed for all women and their partners. This included a complete clinical history, physical examination, viral serology, hormonal analysis, male spermiogram and FISH analysis of the sperm and pelvic ultrasound. Genetic evaluations included the karyotype of both parents and tests for inherited thrombophilic disorders (factor V Leiden, prothrombin G20210A mutation, serum homocysteine and deficiencies of the anticoagulants protein C, protein S and anti-thrombin III). Hormonal analysis was performed for thyroid-stimulating hormone, thyroxin, prolactin, estrogen and progesterone. Immunological screening included measurement of anticardiolipin and anti-β-2-glycoprotein (IgG or IgM) antibodies (Orgentec Diagnostika Gmbh, Mainz, Germany) and lupus anticoagulant. The specific ART, i.e. IVF or donated oocytes with or without intracytoplasmic sperm injection, was selected in accordance with hormonal and sperm analysis for each couple. Only patients with normal blood pressure, viral serology, karyotype (both members of the couple), thrombophilic and immunological results in the screening process were included in this study. The KIR haplotypes were analyzed in all women. The KIR haplotypes were analyzed as part of our routine investigation in women with RIF or RM referred for immunology consultation.

Genomic DNA isolation and genotyping

Genomic DNA was obtained from maternal blood. All samples were obtained from Spanish individuals and all were of Caucasian origin. KIR typing was performed by PCR-SSO on Luminex devices (Lifecodes,

Immucor, USA). 2DL1, 2DL2, 2DL3, 2DL4, 2DL5, 2DS1, 2DS2, 2DS3, 2DS4, 2DS4N, 2DS5, 3DL1, 3DL2, 3DL3, 3DS1, 2DP1 and 3DP1 were analyzed using two amplification multiple mixes. KIR haplotype regions were defined by the presence of the following KIR genes: Cen-A/2DL3; Tel-A/3DL1 and 2DS4; Cen-B/2DL2 and 2DS2; as well as Tel-B/2DS1 and 3DS1.

Statistics

Categorical and continuous variables were expressed as proportions and means with 95% confidence intervals (CI), respectively. Odds ratios (OR) and 95% CI were also calculated for categorical variables. Categorical data were compared using the χ^2 analysis and Fisher's exact test where appropriate and also tests for trend for ordered haplotype categories AA-AB-BB. Continuous data were compared with the Student's *t*-test or analysis of variance tests followed by Bonferroni's *post hoc* analysis. A *P* value <0.05 was considered significant.

Statistical analysis was performed using the Statistical Package for Social Sciences, version 17 (SPSS, Inc., Chicago, IL, USA).

Ethical approval

Ethical approval for the study was obtained from IVI Ethical Committee. Patients provided written informed consent, and Institutional Review Board approval from our institution was obtained prior to initiation of the study (MAD-DA-07-2010-03). Data were gathered anonymously to avoid individual patient identification, consistent with data-protection rules at our institution.

Results

The mean age of our patients was 37.5 ± 10 (range 27–48) years and their body mass index ranged from 18.5 to 28 kg/m^2. The mean age of the patients' partners was 39 ± 10.5 (range 28–50) years, and the partner's sperm count ranged from 200 000 to 20 000 000 per milliliter. These couples had a mean of 6 ± 3 previous failed ETs, and had been trying to conceive for a mean of 7 ± 3 years. The mean age of the oocyte donors was 25.5 ± 7.5 (range 18–33) years. The starting dose for controlled ovarian stimulation was 150–300 UI recombinant follicle stimulating hormone (rFSH) for patients, and 125–150 UI rFSH for oocyte donors (Gonal F, Merck Serono, Madrid, Spain; Puregon, MSD, Madrid, Spain).

From a total of 1304 ART cycles, 1279 were included in the study; 25 cycles were not considered because the ETs were not performed at the data collection time (programmed ETs). In our cohort (1279 cycles), 51.8% of total IVF treatments with ETs were performed with the patient's own oocytes and 29.7% were with donated oocytes. The remaining ART cycles involved intrauterine insemination (3.5%), donor intrauterine insemination (0.2%), or oocyte cryopreservation (11.4%) and the remainder were canceled cycles (1.3%). Timed intercourse represented 2.1% of total cycles. Of the total cycles, 81.5% ($N = 1063$ cycles) were with ETs, including 673 cycles (51.7% from a total of 1304) with fresh embryos and 390 cycles (29.9% from total of 1304) with frozen embryos. From the total cycles performed with frozen embryo transfers, 16.8% were with the patient's own oocytes and 13.1% were with donated oocytes. The mean number of oocytes obtained in patients was 6 ± 3 and 14 ± 4 in donors. The mean number of frozen embryos per cycle was 2 ± 2 with the patient's own oocytes and 5 ± 2 with donated oocytes. The pregnancy rate per cycle was 27.4%. The miscarriage rate per cycle was 17.7%: 9% were early miscarriages and 8.8% were clinical miscarriages. The live birth rate per cycle was 9.6%. In 23.5% ($N = 306$) of the total cycles, SET was performed, whereas DET was performed in 42.1% ($N = 549$) of the total cycles. The frequency of KIR haplotypes in the study population was 29.1% KIR AA, 42.9% KIR AB and 28% KIR BB.

Pregnancy, miscarriage and live birth rates according to KIR haplotypes, oocyte origin and SET or DET

When we studied the pregnancy, miscarriage and live birth rates in our cohort per cycle with patients categorized by KIR haplotype, no statistically significant differences were observed among the AA, AB and BB haplotypes groups (Table I).When we analyzed all clinical variables (pregnancy, miscarriage and live birth rates per cycle) according to KIR haplotype and categorized by DET or SET, a higher miscarriage rate per cycle occurred after DET in mothers with a KIR AA genotype (34.2%) compared with those with the KIR AB (28.9%) or KIR BB haplotypes (23.6%) ($P = 0.03$). Also, statistically significant differences were observed when the rate of early miscarriage was analyzed among the KIR haplotypes groups when DET was performed: AA (19.9%), AB (14.7%) and BB (9.6%) ($P = 0.008$) (Table II). A significantly decreased live birth rate per cycle was also observed after DET in patients with the KIR AA haplotype (7.5%) compared with those with the KIR AB (17.3%) or KIR BB (15.7%) haplotypes ($P = 0.02$) (Table II).

Table I Pregnancy, miscarriage and live birth rates per cycle categorized by KIR haplotypes (*N* = number of cycles; *n* = variable/cycle).

	KIR haplotypes								
	AA (*N* = 369)			AB (*N* = 550)			BB (*N* = 360)		
	n	Rate, %	95% CI	*n*	Rate, %	95% CI	*n*	Rate, %	95% CI
Pregnancy	95	25.7	21.3–30.2	162	29.5	25.6–33.2	93	25.8	21.3–30.3
Miscarriage	69	18.7	14.7–22.7	101	18.4	15.1–21.6	57	15.8	12.0–19.6
Early miscarriage	39	10.6	7.4–13.7	50	9.1	6.7–11.5	26	7.2	4.5–9.9
Clinical miscarriage	30	14.4	5.3–10.9	51	14.2	6.8–11.7	31	14.0	5.7–11.5
Live birth	26	7	4.4–9.6	61	11.1	8.4–13.7	36	10	6.9–13.1

Data are expressed as proportions and 95% CI.

Table II Pregnancy, miscarriage and live birth rates per cycle categorized by KIR haplotype and DET or SET (*N* = number of cycles; *n* = variable/cycle).

KIR haplotypes	DET								
	AA (*N* = 146)			AB (*N* = 225)			BB (*N* = 178)		
	n	Rate, %	95% CI	*n*	Rate, %	95% CI	*n*	Rate, %	95% CI
Pregnancy	61	41.8	33.8–49–8	104	46.2	39.7–52.7	70	39.3	32.1–46.5
Miscarriage	**50**	**34.2**[a]	26.5–41.4	65	28.9	23.0–34.8	42	23.6	17.3–29.8
Early miscarriage	**29**	**19.9**[a]	13.4–26.3	33	14.7	10.0–19.3	17	9.6	5.2–13.9
Clinical miscarriage	21	14.4	8.7–20.0	32	14.2	9.7–18.8	25	14.0	8.9–19.1
Live birth baby	**11**	**7.5**[a]	3.2–11.8	39	17.3	12.4–22.2	28	15.7	10.4–21.0
	SET								
KIR haplotypes	**AA (*N* = 101)**			**AB (*N* = 140)**			**BB (*N* = 65)**		
	n	Rate, %	95% CI	*n*	Rate, %	95% CI	*n*	Rate, %	95% CI
Pregnancy	33	32.7	23.5–41.8	51	36.4	28.4–44.4	22	33.8	22.34–45.3
Miscarriage	18	17.8	10.3–25.3	30	21.4	14.6–28.2	15	23.1	12.8–33.3
Early miscarriage	9	8.9	3.3–14.5	14	10.0	5.03–15.0	9	13.8	5.4–22.2
Clinical miscarriage	9	8.9	3.3–14.4	16	11.4	6.1–16.7	6	9.2	2.2–16.3
Live birth	15	14.9	7.9–21.8	21	15.0	9.1–20.9	7	10.8	3.2–18.3

Data are expressed as proportions and 95% CI.
[a]Statistically significant differences between the KIR AA, AB and BB groups.

No statistically significant differences were observed in pregnancy, miscarriage (including early miscarriage) and live birth rates per cycle among maternal KIR AA, AB and BB haplotypes after SET (Table II).

We wanted to determine if there was any difference in any of the clinical variables studied in our cohort by KIR haplotype after DET or SET when categorized by the oocyte origin. Indeed, a higher rate of early miscarriage per cycle after DET when the patient's own oocytes were used occurred in those with the KIR AA (22.8%) haplotype, followed by those with a KIR AB haplotype (16.7%), when compared with mothers with a KIR BB haplotype (11.1%) ($P = 0.03$) (Table III). A significantly decreased live birth rate per cycle after DET with donated oocytes was observed in mothers with a KIR AA haplotype (7.5%) compared with those with a KIR AB (26.4%) and KIR BB (21.5%) haplotype ($P = 0.006$) (Table III).

No statistically significant differences were observed in the pregnancy, miscarriage or live birth rates per cycle between patients with the maternal KIR AA, KIR AB and KIR BB haplotypes after SET with the patient's own or donated oocytes ($P > 0.05$) (Table III).

From 97 newborns, 11 (11.3%) had a low birthweight. Three of these were born from triplet pregnancies after DET, five were from twin pregnancies after DET, and three were from singleton pregnancies. During a singleton pregnancy, one patient presented with pre-eclampsia and had a preterm delivery, and the infant had a low birthweight.

Discussion

We observed a higher miscarriage rate per cycle with DETs in mothers with a KIR AA haplotype compared with those with KIR AB and KIR BB haplotypes. Also, a significantly decreased live birth rate per cycle was observed after DET using donated oocytes in mothers who were homozygous for KIR AA compared with those with KIR AB and KIR BB haplotypes. This effect could be explained by the expression of more than one paternal HLA-C by EVT in a DET, and this increased expression of paternal HLA-C may affect placentation in mothers who are homozygous for KIR AA.

The decreased live birth rate after cycles with donated oocytes and DET in mothers who were KIR AA compared with those with the KIR AB and KIR BB groups may be due to increased expression of paternal HLA-C. In DET with donated oocytes there would be four non-self HLA-C expressed in the trophoblast cells (two HLA-C per EVT cells for each embryo transferred) compared with one (own oocyte cycle) or two paternal HLA-C (donated oocyte cycle) expressed by the EVT cells after SET. Expressing four paternal HLA-C in the EVT cells after DET with donor oocytes is more likely to result in at least one non-self HLA-C2 than with one's own oocyte after SET, and implantation or placentation failure probably occurs in mothers with the KIR AA haplotype.

Based on previous knowledge, the spiral arteries are remodeled by EVT cells and NK cells as a result of the interaction between KIRs expressed by dNK and their ligands, HLA-C molecules expressed by EVT. Pregnancies are at an increased risk of RM, pre-eclampsia, or FGR in mothers who are homozygous for the KIR AA haplotype than in women who are heterozygous (*AB*) or homozygous for group *B* haplotypes (*BB*) (Hiby *et al.*, 2004). This effect has been described in pregnant women the KIR AA haplotype when the fetus has more fetal HLA-C2 genes than the mother and the additional fetal HLA-C2 alleles are of paternal origin (Hiby *et al* 2010a,b). The absence of activating KIR (*A* haplotype, contains mainly genes for inhibitory KIR) favors pregnancy disorders, and this effect has been observed in single fetus pregnancies (Hiby *et al.*, 2008).

Table III **Pregnancy, miscarriage and live birth rates per cycle categorized by KIR haplotypes, DET, SET and oocyte origin (*N* = number of cycles; *n* = variable/cycle).**

	Own oocytes									Donor oocytes								
	DET																	
KIR haplotype	**AA (*N* = 79)**			**AB (*N* = 138)**			**BB (*N* = 99)**			**AA (*N* = 67)**			**AB (*N* = 87)**			**BB (*N* = 79)**		
	n	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**
Pregnancy	34	43.0	32.1–54.0	56	40.6	32.4–48.8	33	33.3	24.0–42.6	27	40.3	28.5–52.0	48	55.2	44.7–65.6	37	46.8	35.8–57.8
Miscarriage	28	35.4	24.9–46.0	40	29.0	21.4–36.5	22	22.2	14.0–30.4	22	32.8	21.6–44.1	25	28.7	19.2–38.2	20	25.3	15.7–34.9
Early miscarriage	**18**	**22.8**[a]	13.5–32.0	23	16.7	10.4–22.9	11	11.1	4.9–17.3	11	16.4	7.5–25.3	10	11.5	4.8–18.2	6	7.6	1.7–13.4
Clinical miscarriage	10	12.7	5.3–20.0	17	12.3	6.8–17.8	11	11.1	4.9–17.3	11	16.4	7.5–25.3	15	17.2	9.3–25.2	14	17.7	9.3–26.1
Live birth	6	7.6	1.7–13.4	16	11.6	6.2–16.9	11	11.1	4.9–17.3	**5**	**7.5**[a]	1.2–13.8	23	26.4	17.2–35.7	17	21.5	12.4–30.6
	SET																	
KIR haplotype	**AA (*N* = 59)**			**AB (*N* = 84)**			**BB (*N* = 48)**			**AA (*N* = 42)**			**AB (*N* = 55)**			**BB (*N* = 17)**		
	n	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**	***n***	**Rate%**	**95% CI**
Pregnancy	22	37.3	24.9–49.6	26	31	21.1–40.8	15	31.3	18.1–44.3	11	26.2	12.9–39.5	24	43.6	30.5–56.7	7	41.2	17.7–64.5
Miscarriage	12	20.3	10.1–30.6	18	21.4	12.6–30.2	10	20.8	9.3–32.3	6	14.3	3.7–24.8	11	20	9.4–30.6	5	29.4	7.7–51.0
Early miscarriage	6	10.2	2.4–17.9	8	9.5	3.2–15.8	6	12.5	3.1–21.8	3	7.1	−0.6–14.9	5	9.1	1.5–16.7	3	17.6	−0.4–35.7
Clinical miscarriage	6	10.2	2.4–17.9	10	11.9	5.0–18.8	4	8.3	0.5–16.1	3	7.1	−0.6–14.9	6	10.9	2.6–19.1	2	11.8	−3.5–27.0
Live birth baby	10	16.9	7.4–26.5	8	9.5	3.2–15.8	5	10.4	1.7–19.0	5	11.9	2.1–21.7	13	23.6	12.4–34.8	2	11.8	−3.5–27.0

Data are expressed as proportions and 95% CI.

[a]Statistically significant differences between the KIR AA, AB and BB groups.

IVF cycles usually include single or double embryos per transfer, and donated oocyte, sperm or embryos are often used during ART. After DET, the expression of more than one paternal HLA-C per trophoblast cell is induced. In oocyte-donation cycles, the oocyte-maternal HLA-C, which is genetically different from the mother's receptor, behaves as a paternal HLA-C and the expression of two non-self or 'paternal' HLA-C in the trophoblast cells and embryo is present in the decidua basalis.

In human populations, pregnancy disorders are predicted to reduce the frequency of group A KIR, HLA-C2, or both and this selection is thought to have originated during human evolution (Adams and Parham, 2001; Parham, 2004; Rajalingam *et al.*, 2004). An inverse correlation between the frequencies of the KIR AA haplotype and HLA-C2 has been observed. Populations with the highest frequency of KIR AA (Japanese and Koreans) have the lowest HLA-C2 frequencies, whereas populations with the lowest frequency of KIR AA (Aboriginal Australians and Asian Indians) have the highest HLA-C2 frequencies. Natural selection seems to have driven an allele-level group A KIR haplotype and HLA-C1 ligand to an unusually high frequency in the Japanese, such that the detrimental KIR AA-HLA-C2 combination does not significantly affect pregnancy outcomes in Japanese and Korean populations (Saito *et al.*, 2006).

This correlation provides evidence that selection for human reproductive success has adapted to the KIR and HLA-C genes and could be responsible for maintaining balanced polymorphisms between the HLA-C1 and HLA-C2 groups and the A and B KIR haplotypes (Adams and Parham, 2001; Parham, 2004; Rajalingam *et al.*, 2004).

However, this natural human evolution is not taken into consideration nowadays during ART, and our new findings show that the maternal KIR haplotype and fetal HLA-C have an impact on the live birth rate after IVF cycles, especially when DET is used.

Hiby (Hiby *et al.*, 2010a,b) and Faridi (Faridi and Agrawal, 2011) have reported differences in outcomes of medically unassisted pregnancies, and our study is the first one showing differences in ART patients. Assisted pregnancies differ from medically unassisted pregnancies, in that patients receive mostly DET with more non-self HLA antigens presented to the mother's KIR compared with 'normal' pregnancies. Furthermore, donor oocytes are often used in ART, and no other report has studied the impact of KIR-HLA-C on donor oocyte cycles. We believe that the decreased live birth rate after DET in mothers with the KIR AA haplotype when donor oocytes were used and the higher rate of miscarriages after DET when the patient's own oocytes were used is due to the expression of more non-self HLA-C per embryo transfer. We speculate that completing a normal pregnancy is possible only for mothers with the KIR AA haplotype who carry a baby with a least one HLA-C1 (non-self HLA-C1).

Because this was a retrospective study, data regarding the parents', donors', chorionic villi or babies' HLA-C was not available, and this is a limitation, as we could not show the differences according to paternal or oocyte donor HLA-C1 and HLA-C2.

We must assume that the retrospective nature of our study and the heterogeneity of the studied population, including several different ART approaches, ages, stimulation protocols and infertility etiologies, may bias our results to some extent. Moreover, when many variable are studied, some could result in being statistically significant by chance (by definition, one out of twenty). However, apart from the statistical significance, the association strength, namely the differences seen in different haplotype groups, in some cases is noticeably high, allowing greater confidence in the findings.

Hiby *et al.* (Hiby *et al.*, 2004, 2008, 2010a,b) performed larger cohort studies that analyzed both maternal and paternal genotypes, with a large control group, and demonstrated a clear difference between the KIR and HLA-C genotypes in patients with disorders such as RM, pre-eclampsia and FGR. Our results are concordant with those of Hiby *et al.* and could help in adapting ART to natural human reproductive evolution by aiming to prevent pregnancy disorders such as miscarriage, pre-eclampsia and low birthweight. However, larger studies are needed to show differences according to KIR and paternal/donor HLA-C in ART patients.

Our results and these new insights could have an impact on the selection of SET in patients with RM or RIF and a KIR AA haplotype. Also, our results may have clinical significance, helping with oocyte and/or sperm donor selection according to HLA-C in patients with RM or RIF and a KIR AA haplotype since HLA-C1/C1 donors are predicted to be safer and C2/C2 males or oocyte donors may be more 'dangerous' as identified by epidemiological studies (Skjaerven *et al.*, 2005; Hiby *et al.*, 2010a,b).

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Authors' roles

D.A. conceived the study, participated in its design, performed the immunologic evaluation of the patients, supervised the genetic analysis and drafted the manuscript. N.G. carried out the statistical analysis and critically revised the manuscript. J.L.V. carried out the genetic analysis of the samples. A.B. participated in the selection of patients, and collection of samples and data. P.A. participated in the collection of samples and data. A.R. supervised the data collection. J.A.G.V. participated in the design and coordination of the study and selection of patients, supervised the data analysis and critically revised the manuscript.

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Conflict of interest

None declared.

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