Placenta 75 (2019) 27–33

Contents lists available at ScienceDirect

Placenta

journal homepage: www.elsevier.com/locate/placenta

Fetal human leukocyte antigen-C and maternal killer-cell immunoglobulin-like receptors in cases of severe preeclampsia

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ARTICLE INFO

Keywords:
Pregnancy
Preeclampsia
HLA
KIR
NK cells

ABSTRACT

Introduction: The pathogenesis of preeclampsia may involve inadequate trophoblast invasion caused by excessive inhibition of uterine natural killer cells (uNK) by extravillous trophoblast cells (EVT). This may be the result of a combination of maternal killer-cell immunoglobin-like receptor (KIR) AA genotype and fetal human leukocyte antigen-C2 (HLA-C2) genotype. A few studies have reported a significantly increased frequency of the maternal KIR AA/fetal HLA-C2 combination in cases of preeclampsia compared to controls.

Methods: Study subjects were 259 cases of severe preeclampsia/eclampsia and 259 matched pregnant women without preeclampsia or eclampsia. All pregnancies were singleton pregnancies, and mothers were preferentially primigravidae. Blood samples from women and their newborns were obtained from the Danish National Birth Cohort (DNBC) and the Danish Neonatal Screening Biobank. Significant differences in the frequencies of KIR AA and HLA-C2 between cases and controls were investigated.

Results: No significant difference was observed between cases and controls in the frequency of maternal KIR AA (OR = 0.86, 95%CI = 0.60–1.23, $P = 0.41$), neither when the fetus carried an HLA-C2 allele (OR = 0.85, 95%CI = 0.52–1.38, $P = 0.51$), nor when the fetus carried an HLA-C2 allele more than its mother (OR = 0.75, 95%CI = 0.34–1.64, $P = 0.47$).

Conclusion: The Results show no influence of HLA-C/KIR genetic variation on the risk of severe preeclampsia, contrary to what some previous studies have observed. An explanation could be that severe preeclampsia represents a separate pathological entity compared to mild preeclampsia.

1. Introduction

Preeclampsia and other hypertensive disorders of pregnancy are some of the most important causes of maternal and perinatal morbidity and mortality worldwide. According to the World Health Organization (WHO), one in ten pregnant women is affected by hypertensive disorders, and preeclampsia alone accounts for one in seven maternal deaths [1,2]. Preeclampsia is defined as a hypertensive disorder that can complicate pregnancy after 20 weeks of gestation, and is classified as either early-onset if it debuts before 34 weeks of gestation, or late-onset if it debuts at or after 34 weeks of gestation [3]. If the condition is progressive and left untreated, it can be further complicated by seizures or coma, a condition known as eclampsia, and can ultimately end fatally. Preeclampsia can be classified as mild or severe depending on the degree of hypertension, proteinuria, maternal organ dysfunction and impact on fetal development [2,4].

Young women, primigravidae and multigravidae with different paternities have been shown to predominantly be the groups that are affected by preeclampsia, while multigravidae with unchanged paternity are affected to a lesser extent [5,6]. The hypothesis based on these observations is that repeated maternal exposure to antigens of the same partner protects the woman from preeclampsia in a subsequent pregnancy with the same partner. Alternatively, Skjærven et al. (2002) have suggested that the interbirth interval is more critical than change in paternity [7]. Both explanations would be in accordance with a role of the maternal immune system in the pathogenesis of preeclampsia; either because of some specific fetal/paternal-maternal immune interaction or because of the decline over time of pregnancy-induced immune memory cells of importance for a woman's future pregnancies [8].

The most abundant maternal immune cell present at the feto-

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https://doi.org/10.1016/j.placenta.2018.11.008
Received 4 July 2018; Received in revised form 27 October 2018; Accepted 21 November 2018

maternal interface is the uterine natural killer cell (uNK) [9,10]. The uNK cells are especially numerous in the decidua in the first trimester of pregnancy. The uNK cells are a distinct subset of NK cells with low cytotoxicity and are believed to play a key role in regulating fetal extravillous trophoblast cells (EVT) in order to establish a balanced placentation [11]. The function of the EVT cells is to invade the spiral arteries of the uterus and convert them into high-conductance vessels that are unaffected by maternal vasoconstriction [12]. If the uNK cells are highly inhibited by the EVT cells, the invasion of the trophoblast and remodeling of the spiral arteries may be insufficient resulting in too little blood reaching the fetus. This failure of adequate placentation is believed to be predisposing for preeclampsia, as well as for other disorders of pregnancy such as fetal growth restriction (FGR) [13–16]. On the other hand, Xiong et al. (2013) have proposed that if the invasion of the EVT cells is not sufficiently counteracted by the uNK cells there might be a risk of the placenta and the fetus growing uncontrollably into the uterus, acting almost like an invasive cancer, as seen in the disorder *placenta accreta* [17].

The EVT cells and the uNK cells interact through recognition of fetal HLA molecules by the killer-cell immunoglobin-like receptors (KIR) on the maternal uNK cells. The ligands for KIR are classical HLA class Ia molecules [18]. The EVT cells express four different HLA molecules: HLA-C, HLA-E, HLA-F and HLA-G. HLA-E, HLA-F and HLA-G are non-classical HLA class Ib molecules. They show a very low protein polymorphism in the population. HLA-C on the other hand, is a classical HLA class Ia molecule and is polymorphic. The HLA-C alleles expressed by the EVT cells will therefore vary depending on which alleles are contributed by the mother and which alleles are contributed by the father [18,19]. The polymorphic HLA-C alleles can be grouped according to the presence of either a C1 or C2 epitope. Both of these epitopes function as ligands for KIR [20].

KIR genotypes are diverse due to varying numbers and combinations of KIR genes, but also because of allelic polymorphism of the individual KIR genes. KIR genes are organized in two haplotypes, KIR A and KIR B. Therefore, an individual may carry one of the following three genotypes: KIR AA, KIR AB or KIR BB. Belonging to the KIR B haplotype are several genes coding for activating receptors, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5 and KIR3DS1 but only one inhibitory receptor, KIR2DL5. The KIR A haplotype has one activating receptor, KIR2DS4, though the most common KIR2DS4 allele includes a deletion making it inactive. The remainder of the genes belonging to the KIR A haplotype, KIR2DL1, KIR2DL3, KIR3DL1 and KIR2DP1 all code for inhibitory receptors [21]. The HLA-C2 epitope functions as ligand for the activating KIR2DS1 on the KIR B haplotype and the inhibitory KIR2DL1 on the KIR A haplotype. The HLA-C1 epitope is ligand for inhibitory KIR2DL2 and KIR2DL3 on KIR A and activating KIR2DS2 on KIR B [20,21]. Recent publications have proposed that the C2 inhibition of KIR2DL1 is stronger than the inhibition produced by C1 and KIR2DL3 [13].

Hiby et al. (2004) have suggested that a combination of maternal KIR AA and fetal HLA-C2 results in too much inhibition of uNK cells and presents a risk factor for preeclampsia [22]. Since the publication of these first findings, other studies investigating a possible role of the interaction between KIR and HLA-C in different disorders or syndromes such as preeclampsia, FGR, recurrent miscarriages and high-/low birth weight have to some degree reported similar results [15,17,23–27]. However, not all studies have been able to confirm this association, so this issue is still controversial [28], and only a few of the published studies have investigated the KIR/HLA-C genetic combinations in cases of preeclampsia. Furthermore, cases of mild versus severe preeclampsia might have different etiologies and pathogeneses [29,30], and none of the published studies have investigated the KIR/HLA-C genetic variation exclusively in cases of severe preeclampsia and eclampsia, nor did they focus on early-onset versus late-onset preeclampsia.

The aim of the present study was to examine the influence of maternal KIR genotypes and fetal HLA-C genotypes on the risk of developing severe preeclampsia or eclampsia. This was done by investigating, whether there were any of these genotypes, or combinations thereof, that were more prevalent or less prevalent among women with severe preeclampsia or eclampsia compared to pregnant women without preeclampsia.

2. Methods

2.1. Study design and study cohort

The current study was based upon blood samples from the Danish National Birth Cohort (DNBC) biobank and the Danish Neonatal Screening Biobank, part of the Danish National Biobank (DNB) at Statens Serum Institut, Copenhagen, Denmark. The design of the study was a nested case-control study, where cases were pregnant women with severe preeclampsia or eclampsia, and controls were pregnant women without preeclampsia and eclampsia. In total, 259 case mothers and 259 matched control mothers were included. In addition to the case and control women, samples from their newborns were included.

2.2. Inclusion and exclusion criteria

All pregnancies were singleton pregnancies, and mothers were preferentially primigravidae, though 77 controls and the same amount of cases were multigravidae. Cases had been registered in the Danish Hospital Discharge Registry with ICD-10 codes for severe preeclampsia or eclampsia, O14.1 or O15, respectively. Therefore, it can be assumed that they met the criteria for severe preeclampsia: confirmed systolic blood pressure $\geq$160 mmHg or diastolic blood pressure $\geq$110 mmHg, as well as signs of multiorgan dysfunction e.g. persistent headaches, pulmonary edema, liver or kidney impairment (e.g. proteinuria > 3 g/24 h), or fetal abnormalities (e.g. intrauterine growth restriction) [31]. Controls did not have pregnancy-related hypertensive disorders, according to the Danish Hospital Discharge Registry, and were matched on year and place of giving birth as well as number of previous pregnancies. Postpartum blood samples were collected from the mothers and the newborns for genomic DNA isolation and genotyping. Collected information about subjects was: sex of the newborn, birth weight of the newborn, placental weight, gestational age of the newborn at pregnancy termination, number of previous pregnancies and births.

2.3. DNA genotyping of HLA-C and KIR

After DNA isolation, typing of the KIR genotype into haplotypes A and B was carried out, as well as typing of HLA-C genotypes into HLA-C1 and HLA-C2 allele groups was performed in the mothers and newborns according to established and published protocols [32]. Briefly, HLA and KIR typing were carried out using the ScisGo-HLA v5.0, and ScisGo-KIR v3.0 (Scisco Genetics Inc., Seattle WA, USA) typing kits for HLA and KIR respectively, according to the manufacturers provided protocol. Briefly, genomic DNAs are added to the HLA and KIR amplicon mixes and PCR cycled through stage 1, followed by the addition of stage 2 buffer and a continuation through second stage PCR. Samples were pooled, quantified, and sequenced using a MiSeq reagent kit v2, 500 cycles (Illumina, San Diego, CA, USA). Data were analyzed on Sciscloud and validated using GeMS-UI v88 (Scisco Genetics Inc., Seattle WA, USA). HLA data were reported as 3-field and KIR data as gene copy number and haplotypes.

2.4. Statistical analysis

Two-by-two contingency tables and χ^2-tests were used to test for frequency differences in genotypes and combinations of maternal and fetal genotypes between the case and control groups. Tests with a *P*-value < 0.05 were considered significant. The magnitude of effect was estimated by odds ratios (OR) and associated 95% confidence intervals

Table 1
Characteristics of preeclampsia cases and controls.

	Controls		Preeclampsia		*P*-value[a]
	n		n		
Birth weight (mean ± SD[b]; g) and percentile[c]	254	3504.2 ± 550.4 (50th centile)	244	2535.4 ± 936.5 (25th centile)	< 0.0001 (0.001[d])
Percentage with SGA[e]	254	13.0% (33)	244	42.2% (102)	< 0.0001
Placental weight (mean ± SD; g)	249	643.9 ± 9	235	521.9 ± 12.5	< 0.0001
Gestational age (mean ± SD; days)	259	279.7 ± 13.3	259	251.6 ± 26.3	< 0.0001
Percentage of newborn males	259	49.8%	250	53.6%	0.39

[a] *P*-values were calculated using independent samples *t*-test or χ^2-test whenever appropriate.
[b] Standard deviation.
[c] Birth weight percentiles were obtained using the WHO birth weight percentiles calculator.
[d] *P*-value after adjustment for gestational age using Marsál et al.
[e] Small for gestational age: defined as birth weight under the 10th percentile. SGA individuals were identified using the WHO birth weight percentiles calculator.

(95%CI) (SPSS Statistics for Windows, Version 24.0, IBM Corp.).

3. Results

3.1. Basic clinical characteristics of the case and control groups

Table 1 shows the characteristics of the cases and controls. Compared to the newborns of the healthy pregnant control women, the average birth weight before and after adjustment [33] of newborns of preeclamptic women was significantly lower, and the frequency of small for gestational age (SGA) [34] newborns was significantly higher (OR = 4.76, 95%CI = 3.16–7.69, $P < 0.0001$). Other significant findings were lower average placental weight and average gestational age at pregnancy termination in the case group compared to the control group. Frequency of male newborns was similar in the two groups (OR = 1.16, 95%CI = 0.82–1.65, $P = 0.39$).

3.2. Frequencies of HLA-C genotype groups are similar in preeclampsia subjects and controls

Frequencies of HLA-C genotype groups in mothers and newborns were investigated in the case and control groups, and these are presented in Table 2. No significant differences were found between fetuses of preeclampsia cases and control women when using χ^2-test. Table 2 shows *P*-values for comparison of case and control fetuses.

3.3. Frequencies of KIR genotypes and frequencies of maternal KIR/fetal HLA-C genotype combinations are similar in preeclampsia cases and controls

Next, frequencies of KIR genotypes were investigated. Table 3 shows frequencies of maternal and fetal KIR AA, KIR AB and KIR BB in the case and control groups. The frequencies of all three genotypes were similar in mothers and newborns and in cases and controls. No significant differences were observed in frequencies of any of the KIR genotypes in the cases and controls. Table 4 provides an overview of the frequencies of the possible combinations of KIR and HLA-C within the two study groups. Table 4 classifies cases and controls into the respective maternal KIR genotypes and shows similar frequencies of fetal HLA-C1C1, -C1C2 and -C2C2 within these groups. Table 5 shows frequencies of maternal KIR/fetal HLA-C genotype combinations within cases and controls. In Table 5, mothers with activating maternal KIRs (KIR AB and KIR BB) are pooled into the same group, and the two groups with fetal HLA-C2 are joined together as well. No significant differences were observed in the frequency of any of the KIR/HLA-C combinations.

Table 2
Frequency of HLA-C genotype and allele groups in cases and controls.

HLA-C genotype	Controls		Preeclampsia		*P*-value[a]
	Mother (n = 258)	Fetus (n = 241)	Mother (n = 255)	Fetus (n = 229)	
C1C1	38.8% (100)	39.4% (95)	42.4% (108)	41.5% (95)	0.65
C1C2	49.2% (127)	46.9% (113)	45.1% (115)	45.0% (103)	0.68
C2C2	12.0% (31)	13.7% (33)	12.5% (32)	13.5% (31)	0.96
C1	63.4%	62.9%	64.9%	64.0%	
C2	36.6%	37.1%	35.1%	36.0%	

[a] Comparison of control and preeclampsia fetuses.

Table 3
Frequency of KIR genotype groups in cases and controls.

KIR genotype	Controls		Preeclampsia		*P*-value[a]
	Mother (n = 257)	Fetus (n = 242)	Mother (n = 257)	Fetus (n = 241)	
AA	37.4% (96)	33.9% (82)	33.9% (87)	35.1% (81)	0.41
AB	60.7% (156)	62% (150)	63.8% (164)	62.3% (144)	0.47
BB	1.9% (5)	4.1% (10)	2.3% (6)	3.4% (16)	0.76

[a] Comparison of control and preeclampsia mothers.

3.4. The KIR AA genotype is not increased in mothers with preeclampsia when the fetus carries an HLA-C2 allele

Table 6 assesses the data in a different way than Table 4, which explains why percentages in the two tables differ. Table 6 is comparable to Table 3 in the publication by Hiby et al. [15]. Frequencies of the inhibitory KIR AA genotype in the case and control groups within the HLA-C genotype groups are compared. The maternal KIR AA frequency in the group of preeclamptic women that had a fetus with at least one HLA-C2 allele was similar to the frequency in the group of preeclamptic women, who had a fetus with only alleles coding for HLA-C1. The same was true for the control group. Furthermore, when the fetus carried an HLA-C2 allele, the maternal KIR AA frequency was not higher in the case group than in the control group.

3.5. The KIR AA genotype is not increased in mothers with preeclampsia when the fetus carries an HLA-C2 allele more than its mother

When the fetus carries an HLA-C2 allele more than the mother, naturally, this HLA-C2 allele must have been inherited from the father. If the combination of fetal HLA-C2 and maternal KIR AA is in fact a risk factor for preeclampsia, this should be apparent when analyzing the KIR AA frequency in mothers of fetuses with one more HLA-C2 alleles than their mother. However, Table 6 shows that the frequency of maternal KIR AA in the severe preeclampsia cases was not significantly different from the frequency of KIR AA in the control women, no matter what the fetal HLA-C status was.

Table 4
Overview of the combinations of maternal KIR and fetal HLA-C in the control group and preeclampsia group. The table depicts fetal HLA-C genotype frequencies within the three maternal KIR genotype groups.

Maternal KIR:	Controls				Preeclampsia			
	AA (n = 91)[a]	AB (n = 143)	BB (n = 5)	Total (n = 239)	AA (n = 79)	AB (n = 145)	BB (n = 5)	Total (n = 229)
Fetal C1C1	38.5% (35)	39.9% (57)	60.0% (3)	39.7% (95)	40.5% (32)	42.1% (61)	40.0% (2)	41.5% (95)
Fetal C1C2	50.5% (46)	44.8% (64)	20.0% (1)	46.4% (111)	41.8% (33)	46.2% (67)	60.0% (3)	45.0% (103)
Fetal C2C2	11.0% (10)	15.4% (22)	20.0% (1)	13.8% (33)	17.7% (14)	11.7% (17)	0.0% (0)	13.5% (31)

[a] The number within the KIR genotype group with data on fetal HLA-C genotype.

Table 5
Frequencies of maternal KIR/fetal HLA-C combinations.

	Control (n = 239)	Preeclampsia (n = 229)	*P*-value
Maternal KIR/fetal HLA-C combination			
AA/C1C1	14.6% (35)	13.9% (32)	0.84
AA/C1C2 or C2C2	23.4% (56)	20.5% (47)	0.45
AB or BB/C1C1	25.1% (60)	27.5% (63)	0.55
AB or BB/C1C2 or C2C2	36.8% (88)	38.0% (87)	0.79

3.6. No maternal KIR/fetal HLA-C combination is increased in cases of preeclampsia with birth at gestational age < 34 weeks, preeclampsia with birth at or after 34 weeks of gestation, preterm birth, or small for gestational age

In Table 7, study subjects were assessed on other parameters: preeclampsia and birth at gestational age < 34 weeks, preeclampsia and birth at gestational age ≥34 weeks, SGA and preterm birth. Within these groups, the frequencies of maternal KIR/fetal HLA-C combinations were investigated. Sixty-eight mothers with preeclampsia gave birth before 34 weeks of gestation, and these mothers can with certainty be classified as early-onset preeclampsia cases. Fifty-four of these cases had information on maternal KIR genotype and fetal HLA-C genotype. A borderline significant association was found using the Pearson's chi-squared approximation test between having the maternal KIR AA/fetal HLA-C2 combination and being in the control group, while the confidence interval for the magnitude of effect included 1 (OR = 0.41, 95%CI = 0.17–1.00, $P = 0.045$). Mother-child couples with SGA children were compared to couples with children of median birth weight or above. No significant differences were observed in the frequencies of any KIR/HLA-C combination in cases of SGA, cases of preeclampsia with delivery at gestational age ≥34 weeks, or cases of preterm birth.

4. Discussion

In the current case-control study, it was investigated whether an association between developing preeclampsia and carrying certain KIR and HLA-C genetic variations could be observed. Case and control mother-child couples differed in certain baseline characteristics: gestational age of the child at pregnancy termination and birth weight of the child. Lower gestational age at birth would naturally result in a lower birth weight, but the difference in birth weight of case-children and control-children was statistically significant even after adjustment for gestational age. A study by Ødegård et al. (2000) found that birth weight reduction and preeclampsia severity might be connected: preeclampsia was associated with a 5% reduction in birth weight compared to the expected birth weight, while severe preeclampsia was associated with a 12% reduction [35], similar to what is observed in the severe preeclampsia cases in the current study. Though results of previous studies have been ambiguous, fetal sex and preeclampsia have been connected in several studies [36–39]. In the current study, no significant difference was observed in the sex of newborns in the case and control groups.

Hiby et al. (2004) found that mothers with the KIR AA genotype were at an increased risk of developing preeclampsia, especially when they were pregnant with a fetus possessing an HLA-C genotype with an HLA-C2 allele [22]. No association was found in mothers with preeclampsia who carried the KIR AA genotype when their fetus possessed only HLA-C1 alleles. The explanation for this was proposed to be

Table 6
Frequency of maternal KIR AA when the study population has been grouped according to fetal HLA-C status.

	Control	Preeclampsia	OR (95% CI)	*P*-value
Fetal HLA-C[a]				
All	37.4% (96[b])	33.9% (87[b])	0.86 (0.60–1.23)	0.41
Fetus has only C1	36.8%[c] (35)	33.7% (32)	0.87 (0.48–1.58)	0.65
Fetus has C1C1				
Fetus has C2	38.9% (56)	35.1% (47)	0.85 (0.52–1.38)	0.51
Fetus has C1C2 or C2C2				
Fetus has less C2 than mother	32.0% (16)	25.0% (12)	0.71 (0.29–1.71)	0.44
Fetus has C1C1 and mother has C1C2				
Fetus has C1C2 and mother has C2C2				
Fetus has same number of C2	41.1% (53)	40.8% (49)	0.99 (0.60–1.64)	0.97
Fetus has C1C1 and mother has C1C1				
Fetus has C1C2 and mother has C1C2				
Fetus has C2C2 and mother has C2C2				
Fetus has more C2 than mother	40.0% (22)	33.3% (18)	0.75 (0.34–1.64)	0.47
Fetus has C1C2 and mother has C1C1				
Fetus has C2C2 and mother has C1C2				

[a] No fetal HLA-C genotype was obtained in 30 preeclampsia cases and 20 controls.
[b] In mothers with KIR AA, no fetal HLA-C status was obtained in 5 controls and in 8 preeclampsia cases.
[c] Percentages differ slightly from the ones in Table 4. Table 4 focuses on fetal HLA-C genotype frequencies within the different maternal KIR genotype groups, while Table 6 focuses on maternal KIR genotype frequencies within the fetal HLA-C groups, and because of different numbers of missing variables, this results in different percentages.

Table 7
Frequency of maternal KIR/fetal HLA-C combinations in preeclampsia cases with delivery before 34 weeks of gestation, preeclampsia cases with delivery at or after 34 weeks of gestation, cases of small for gestational age and cases born preterm.

Maternal KIR/fetal HLA-C combination	Preeclampsia and delivery < 34 weeks of gestation[a] (n = 54)	Control (n = 239)	*P*-value
AA/C1C1	14.8% (8)	14.6% (35)	0.59
AA/C1C2 or C2C2	11.1% (6)	23.4% (56)	0.045[b] *OR = 0.41 (95% CI = 0.17-1.00)*
AB or BB/C1C1	35.2% (19)	25.1% (60)	0.13
AB or BB/C1C2 or C2C2	38.9% (21)	36.8% (88)	0.78
	Preeclampsia and delivery ≥34 weeks of gestation[a] (n = 175)	Control (n = 239)	
AA/C1C1	13.7% (24)	14.6% (35)	0.79
AA/C1C2 or C2C2	23.4% (41)	23.4% (56)	1.0
AB or BB/C1C1	25.1% (44)	25.1% (60)	1.0
AB or BB/C1C2 or C2C2	37.7% (66)	36.8% (88)	0.85
	Small for gestational age[c] (n = 121)	Median birth weight or above (n = 159)	
AA/C1C1	14.0% (17)	17% (27)	0.50
AA/C1C2 or C2C2	25.6% (31)	19.5% (31)	0.22
AB or BB/C1C1	24.8% (30)	22.6% (36)	0.67
AB or BB/C1C2 or C2C2	35.5% (43)	40.9% (65)	0.36
	Preterm[d] (n = 132)	Term (including postmature) (n = 336)	
AA/C1C1	15.2% (20)	14.0% (47)	0.75
AA/C1C2 or C2C2	19.7% (26)	22.9% (77)	0.45
AB or BB/C1C1	25.0% (33)	26.8% (90)	0.69
AB or BB/C1C2 or C2C2	40.2% (53)	36.3% (122)	0.44

[a] Preeclampsia subjects who gave birth before 34 weeks of gestation had early-onset preeclampsia with certainty. The group of preeclampsia cases who gave birth at or after 34 weeks of gestation likely contains cases of early-onset preeclampsia as well; however, these could not be identified in the current study set-up.
[b] Borderline significant *P*-value.
[c] Small for gestational age defined as birth weight under the 10th percentile.
[d] Birth before 37 weeks of gestation.

binding of HLA-C2 to KIR2DL1, one of the inhibitory receptors on the KIR A haplotype. This would result in too much inhibition of the uNK cells, since, as has been mentioned earlier, the HLA-C2 epitope has been shown to be a stronger ligand for inhibitory genes on the KIR A haplotype than HLA-C1. The current study found no difference in the frequency of maternal KIR AA in the control group and preeclampsia group. In a later publication by Hiby et al. [15], in which data and study subjects from two previous publications (Hiby et al., 2004 and Hiby et al., 2008) were supplemented with additional samples and analyses [18,22], an increased risk of preeclampsia was found once more, as well as an increased risk of FGR and RM, when mothers carried the KIR AA genotype and their fetus carried an HLA-C2 allele, especially when the fetus carried an HLA-C2 allele more than its mother (OR = 2.09, 95%CI = 1.24–3.51, $P = 0.007$) [15]. Once again, the current study could not confirm these Results. Furthermore, we did not observe a correlation between the maternal KIR AA/fetal HLA-C2 genetic combination and the risk of giving birth to a child with SGA, defined in our study as birth weight under the 10th percentile. Nor could a correlation to the risk of giving birth preterm, or a conclusive correlation to the risk of having preeclampsia and giving birth before 34 weeks of gestation be found. However, the current study was not designed to specifically study these pregnancy outcomes. When assessing new groups made across our data, or splitting our study groups into subgroups, we can no longer expect them to be matched on potential confounders. Also, the current study may not have the size to investigate these other outcomes. Only 68 cases of preeclampsia gave birth before 34 weeks of gestation. These cases are undoubtedly early-onset preeclampsia cases, but it is possible that the current study in fact included several more cases of early-onset preeclampsia. Delivery is the curative treatment for women with severe preeclampsia, but it may in some cases be postponed in order to undertake initial maternal stabilization and/or transfer the patient to facilities with adequate maternal and neonatal intensive care resources [40]. Interestingly, the combination of maternal KIR AA and fetal HLA-C2 was observed in 56 of the 239 control couples but only 6 of the 54 certain early-onset preeclampsia mother-child couples ($P = 0.045$, 95% CI = 0.17–1.00; Pearson's χ^2 approximation test). This is borderline significant, therefore, no conclusive association could be found between maternal KIR AA/fetal HLA-C2 and preeclampsia with delivery before 34 weeks of gestation in the current study. If certain maternal KIR/fetal HLA-C genotype combinations are a risk factor for developing preeclampsia, it would be tempting to hypothesize that these combinations would be most prevalent in cases of severe early-onset preeclampsia.

Hiby et al. (2010) found that carrying the telomeric region of the KIR B haplotype, the region containing the activating receptor KIR2DS1, was protective against development of preeclampsia. This was especially true when the fetus was HLA-C2-positive. Other maternal activating KIR genes were also associated with a decrease in the risk of preeclampsia compared to the KIR AA genotype [15]. In the current study, it was not possible to make inferences on the possible effects of having the maternal KIR BB genotype and maternal KIR BB/fetal HLA-C2 combination because of the low occurrence of these genotypes within our study population.

According to Guethlein et al. (2015), the evolution of HLA-C has been driven by KIR variation and expression, not the other way around [20]. The difference in the C1 and the C2 alleles of HLA-C is a

substitution in codon 80 changing asparagine in C1 to lysine in C2. The fact that the C2 epitope has been maintained in humans indicates that there must be some advantages to carrying an HLA-C2 allele, for example in the resistance against infectious diseases [20]. Hiby et al. (2004) stated that if the genetic associations they had observed were in fact causal for preeclampsia, having the KIR AA genotype and the HLA-C2 genotype within the same population posed an obvious problem. Hiby et al. suggested that the combination of KIR AA and HLA-C2 would be selected against and therefore vary within different geographical populations. Some populations would have a high prevalence of KIR AA and low prevalence of HLA-C2, if this combination provided protection against other diseases prevalent in this region, while other populations could have the opposite [22]. Saito et al. tested the hypothesis that couples consisting of Japanese women and Caucasian men should be at a higher risk of preeclampsia than couples consisting of Japanese women and Japanese men [28]. A number of studies was referenced to as having shown that the highest frequency of the KIR AA genotype was found among Japanese people, while Caucasians were found to have a 3.5 times higher frequency of HLA-C2 than the Japanese. However, an increased risk was not found in the 324 mixed couples that were included in the study by Saito et al. [28].

Ethnicity seems to be of importance in the development of preeclampsia. This may be due to different geographical populations' exposure to certain infectious diseases. Cytomegalovirus (CMV) has been the focus of several recent studies on the pathogenesis of preeclampsia, Results of which have been ambiguous [41–47]. Information about CMV serology would have been helpful in the current study. Genetics is another, associated, explanation for the role of ethnicity, and KIR/HLA-C interplay may be part of this explanation. It would also have been expedient to have included data on the ethnicity of the study subjects in the current study. Although the study subjects were registered with Danish civil registration numbers, and most of the population in Denmark is Caucasian, it is possible that certain ethnic minorities were more frequently represented than others among cases or controls.

The results of the current study do not support the hypothesis that maternal KIR AA in combination with fetal HLA-C2 is associated with an increased risk of preeclampsia, as has otherwise been presented by Hiby et al. [15,22], Nakimuli et al. [24] and Long et al. [25]. The method and sample size are comparable to those in the above-mentioned studies, however, selection criteria for preeclampsia cases differ between our study and previous studies. This is the first study investigating the role of KIR and HLA-C in the pathogenesis of preeclampsia that has been performed entirely on samples from cases with severe preeclampsia and/or eclampsia. It is possible that the mild and severe forms of preeclampsia, as well as early-onset and late-onset preeclampsia, respectively, represent separate pathological entities. This has been hypothesized before, e.g. by Sutherland et al. [29] and Stanek [30], and possibly by others as well.

The results of this study do not necessarily contradict previous findings tying KIR and HLA-C to preeclampsia. The conclusion made on basis of the presented results is, that no statistically significant effect of maternal KIR AA, fetal HLA-C2, or a combination of the two, could be found on the risk of developing severe preeclampsia in a large Danish study population. As for maternal KIR AA/fetal HLA-C2 and the risk of developing severe preeclampsia with delivery before 34 weeks of gestation, the results of this study were inconclusive and further investigations on the area are needed. Future studies on the influence of KIR and HLA-C on the risk of developing preeclampsia should restrict their criteria for case selection to either mild or severe preeclampsia, as well as early-onset and late-onset preeclampsia, or stratify preeclamptic cases according to preeclampsia severity and onset.

Causal inference is limited in a case-control study, including a nested case-control study. To understand the precise nature of the uNK cell response to HLA-C expressed on invading trophoblast cells, there is need for experimental studies verifying or disproving the associations found in observational studies, like the study by Hiby et al. [22]. Further investigations, whether they be observational or experimental, will help elucidate the mechanism of KIR and HLA-C interplay, and the possible role of variations and combinations of these molecules in the pathogenesis of preeclampsia.

Conflict of interest statement

The authors declared that they have no conflicts of interest to this work. We declare that we do not have any commercial or associative interest that represents a conflict of interest in connection with the work submitted.

Acknowledgements

Support for this work was generously provided by The Region Zealand Health Sciences Research Foundation and Zealand University Hospital. The Danish National Birth Cohort was established with support from the Danish National Research Foundation as well as the Danish Regional Committees, the Pharmacy Foundation, the Egmont Foundation, the March of Dimes Birth Defects Foundation, the Health Foundation and other minor grants. The Danish National Birth Cohort Biobank received support from the Novo Nordisk Foundation and the Lundbeck Foundation. Support for follow-up has been received from the Danish Medical Research Council, the Lundbeck Foundation, the Innovation Fund Denmark, the Nordea Foundation, Aarhus Ideas, the University of Copenhagen Strategic Grant and the Danish Council for Independent Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.placenta.2018.11.008.

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