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PERICONCEPTION, PREGNANCY AND CHILD OUTCOMES
COMMENTARY

Variation of maternal *KIR* and fetal *HLA-C* genes in reproductive failure: too early for clinical intervention

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Abstract A distinctive type of (uterine) natural killer (NK) cell is present in the uterine decidua during the period of placental formation. Uterine NK cells express members of the killer immunoglobulin-like receptor (*KIR*) family that bind to parental HLA-C molecules on the invading placental trophoblast cells. The maternal *KIR* genes and their fetal ligands are highly variable, so different *KIR*/*HLA-C* genetic combinations occur in each pregnancy. Some women only possess inhibitory *KIR* genes, whereas other women also express activating *KIR* genes. The overall signal that NK cells receive from paternal HLA-C on trophoblast depends on the ratio of activating and inhibitory KIR genes expressed by them. Therefore, NK cells provide a balance during placentation to ensure maternal survival and an adequately nourished fetus. Because inhibitory *KIR*s are found more frequently in women with defective placentation, e.g. pre-eclampsia, fetal growth restriction or recurrent spontaneous abortion, some fertility clinics suggest that women should be 'tissue typed' for their *KIR* genotypes. We explain why, presently, it is premature to introduce *KIR* and *HLA-C* typing to predict pregnancy outcome. In future, however, selecting for certain combinations of *KIR* and *HLA-C* variants in surrogacy, egg or sperm donation may prove useful to reduce disorders of pregnancy.

KEYWORDS: HLA-C, KIR, natural killer (NK) cells, placental disorders, tissue typing, trophoblast

Introduction

A critical period in human pregnancy occurs during the first trimester when the trophectoderm attaches to the uterine surface epithelium, with subsequent invasion of trophoblast cells through the decidua until they stop in the inner myometrium. Infiltration of placental cells deep into the uterus is essential, because trophoblast moves towards, and then transforms the spiral arteries to establish the fetal blood supply line. A range of evidence points to defects in placental–uterine interactions underpinning a spectrum of pregnancy disorders, such as pre-eclampsia, unexplained stillbirth, fetal growth restriction and recurrent spontaneous abortion (Brosens et al., 2011). Disordered interactions between trophoblast and uterus

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may also contribute to primary infertility and failed IVF, given the epidemiological overlap between all these conditions (Basso et al., 2003).

We hypothesize here that part of the regulation of placentation results from local immune recognition of trophoblast by decidual leukocytes, an idea that arose from two observations. First, cellular interactions in the decidua occur between cells from two genetically different individuals (allogeneic cells). Second, the crucial role that the decidua plays in the regulation of placentation is obvious from studying pregnancy disorders that arise when decidua is absent: in this scenario, known as placenta percreta/accreta, trophoblast invades relentlessly through the uterus, a situation that often occurs when the placenta forms over a previous caesarian section scar.

Key features of the immune system are specificity and memory. In human pregnancy, epidemiological and genetic studies of pre-eclampsia do point to 'partner specificity' (Lie et al., 1998). Furthermore, the protective effect of a first pregnancy against pre-eclampsia in subsequent pregnancies with the same father might be interpreted as 'memory' (Trogstad et al., 2001). A further important point is the high incidence (about 25%) of pre-eclampsia in oocyte donation pregnancies (Levron et al., 2014). Such embryos are derived from two allogeneic individuals and share no 'self' with the mother, a situation therefore analogous to a mother being exposed to two rather than one paternal genomes. No comparable studies have indicated that particular fathers might be 'risky' in recurrent spontaneous abortion or primary infertility.

Uterine NK cells and trophoblast HLA-C

Which immune cells and target antigens might be involved in pre-eclampsia? Our focus is on uterine natural killer cells (uNK), because they are the dominant population (about 70% of leucocytes) present during early placentation and have receptors that can bind to trophoblast ligands. Although T cells, the effector immune cells responsible for rejection of organ grafts, are also present in decidua (about 10–30% of leukocytes), there is no evidence to date that pregnancy failure in humans ever results from T-cell 'rejection' of the placenta (Moffett and Colucci, 2014). Indeed, no molecular recognition system whereby maternal T-cells might recognize and target trophoblast has yet been described convincingly. In contrast, uNK are distinctive immune cells that accumulate in large numbers around the infiltrating extravillous trophoblast (EVT) cells in the *decidua basalis*, and they have a range of receptors that can bind to ligands on EVT (Sharkey et al., 2008; Xiong et al., 2013). The uNK receptors of particular interest are called killer immunoglobulin-like receptors (*KIR*s), and they are a highly polymorphic family of genes with great variation between different individuals. Some members of the *KIR* gene family bind HLA-C molecules, the only polymorphic HLA class I molecule expressed by EVT (**Figure 1**). Therefore, in any pregnancy, individual women vary in the *KIR* genes that they have inherited and that are expressed by their uNK cells. The paternally derived *HLA-C* allotype expressed on the trophoblast will also differ in each pregnancy (even when from the same father) depending on which of the two paternal *HLA-C* alleles the fetus has inherited (**Figure 2**).

The possibility that the combinations of two variable gene systems, one in the mother and one in her fetus, might subtly determine the outcome of interactions between trophoblast and uNK cells led to initial studies on pre-eclampsia, a disorder with clear underlying defects in placentation. Pre-eclampsia is associated with certain combinations of maternal *KIR*/fetal *HLA-C* genetic variants in cohorts of both Africans and Europeans. Since then, studies of small cohorts of women affected by recurrent spontaneous abortion and fetal growth restriction have indicated a similar relationship. More recently, analysis of maternal *KIR* and fetal *HLA-C* variants in normal pregnancies resulting in a range of different birth weights has indicated that selective pressures on these two gene systems are operating to keep human birth weight between two extremes, both associated with high fetal and maternal mortality and morbidity (Hiby et al., 2014). Indeed, these *KIR* and *HLA* gene families are likely to be subjected to balancing or stabilizing selection via human birth weight, as the two extremes of the normal range are both deleterious (Karn and Penrose, 1951).

A synthesis of these results is shown in **Figure 3**. When expressed by a NK cell, individual KIRs can confer either an inhibitory or activating signal to the NK cell. The great complexity of KIR genetic polymorphism can be simplified by considering two main haplotypes, *KIR A* and *KIR B* that principally differ by the presence of additional activating *KIR* on the *B* haplotype. The *KIR A* haplotype has only seven *KIR* genes, three framework genes present in all individuals, and three inhibitory *KIR*s. The only activating *KIR* on the *A* haplotype, *KIR2DS4*, is generally non-functional. The *KIR B* haplotype has a varying number of additional *KIR*s, many of them activating. The particular *KIR*s present in the *KIR A* or *B* haplotypes whose products bind to HLA-C molecules are shown in **Figure 2**. All of the many (> 1000) *HLA-C* alleles present in populations can be divided into two groups, called here C1 and C2, based on which amino acid (asparagine or lysine) is present at position 80 of the HLA-C molecule. This region is where KIRs bind to the HLA-C molecules, the KIR-binding C1 or C2 epitope. Two KIRs bind to C2 epitopes, inhibitory *KIR2DL1* and activating *KIR2DS1*. Only one KIR binds to C1 epitopes and imparts a weakly inhibitory signal (*KIR2DL2/3*).

Our results show that when women who have inherited two *KIR A* haplotypes (known as a *KIR AA* genotype) encounter trophoblast expressing a C2 epitope, then trophoblast is more likely to fail to establish a good maternal blood supply to the placenta, with the outcome of poor fetal growth and an increased risk of pre-eclampsia. Furthermore, the risk seems greater if the fetal C2 is derived from a paternal rather than a maternal *HLA-C2* allele. The reasons for this are unknown, and it is also unclear why the effect of paternal C1 epitopes inherited by the fetus is neutral.

How do these genetic findings translate into functional differences in uNK cells? NK cell function is determined by the overall input of the activating and inhibitory signals the NK cells receive. Because activating *KIR*s are found only on *KIR B* haplotypes, an individual with two *KIR A* haplotypes (*KIR AA* genotype) only has *KIR* genes that encode receptors that will impart a strong inhibitory signal to uNK cells. The likely candidate *KIR* responsible for this deleterious effect on the *KIR A* haplotype is *KIR2DL1* because it binds strongly and specifically to C2 epitopes of HLA-C. In Europeans, when there is a pregnancy with a paternally derived C2 epitope, the mother

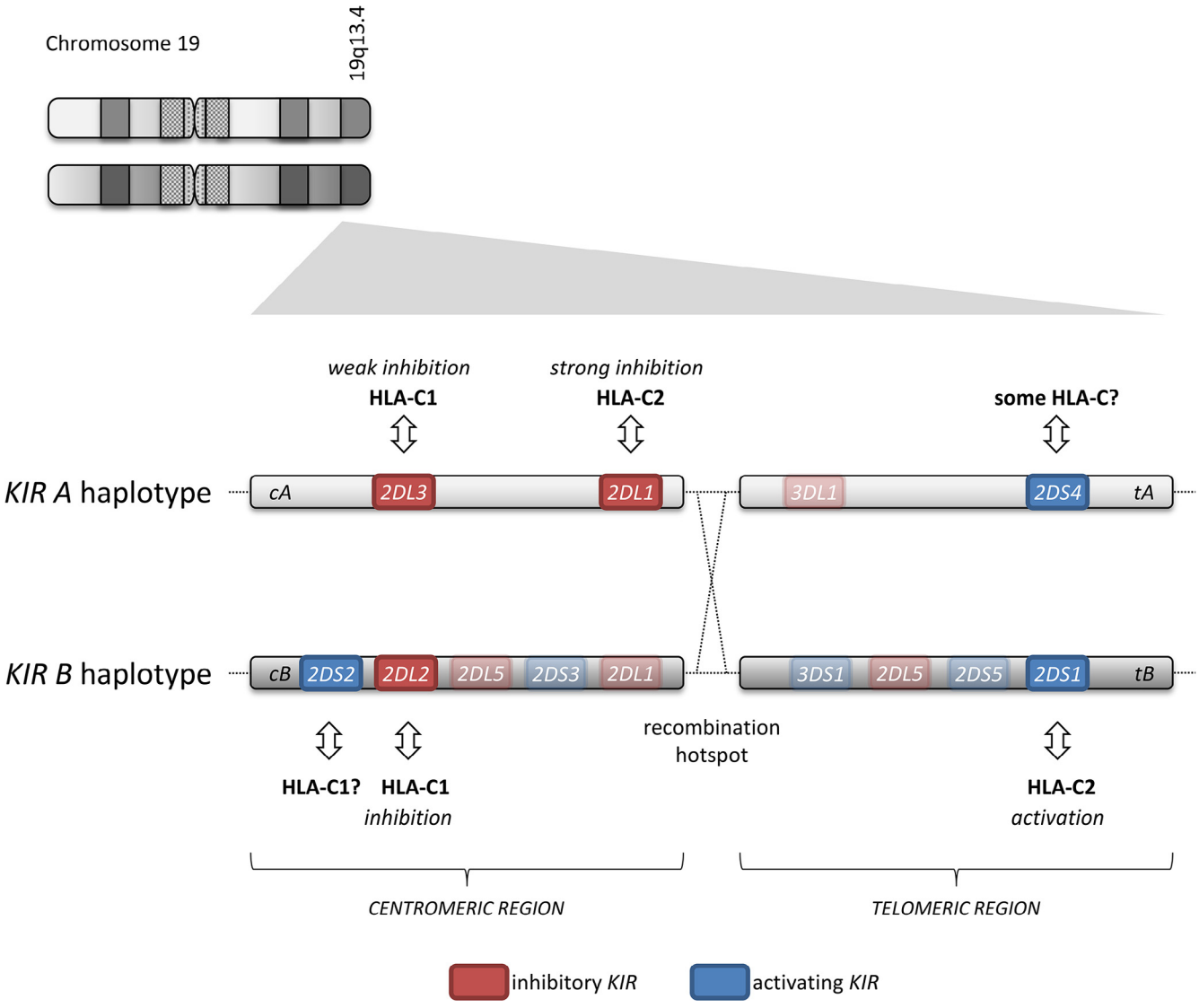

Figure 1 killer immunoglobulin-like receptor (*KIR*) variation. *KIR* haplotypes are a combination of centromeric and telomeric regions with extensive recombination in between. Only *KIR* genes encoding potential receptors for HLA-C molecules are shown. The combination of two *KIR A* haplotypes forms a *KIR AA* genotype, which will contain two copies of *KIR2DL1*, a potent inhibitory receptor for C2 epitopes. The *KIR2DL1* gene present on the *KIR B* haplotype has been reported as less functional in terms of expression, binding and signalling. HLA-C1 or HLA-C2 = *HLA-C* alleles bearing C1 or C2 epitope.

is protected when she has a *KIR B* haplotype that contains the activating KIR for C2 epitopes, *KIR2DS1*. Therefore, this combination has a protective effect, because, when a paternal C2 epitope is present, the strong inhibitory signal from *KIR2DL1* to uNK cells is counter-balanced by an activating signal.

Pre-eclampsia

The largest cohorts studied to date are from pregnant white British women, with controls who had not been previously pregnant, and had a normal full-term pregnancy selected from the same hospitals at the same time (Hiby et al., 2004, 2010). For women developing pre-eclampsia, an association of a maternal *KIR AA* genotype with a paternal *HLA-C* allele bearing a C2 epitope exists. These findings associated with risk of pre-eclampsia have now been confirmed in a smaller cohort of Ugandan women, but interestingly the protective *KIR* region is different in Africans compared with Europeans (Nakimuli et al., 2015). Further, large cohorts are needed in European, Asian and African populations to provide further validation. This is essential as the *KIR* and *HLA* regions have evolved rapidly with the frequencies of *KIR A* haplotypes and *HLA-C* alleles carrying the C2 epitope varying in different populations with a striking inverse correlation between the two. For example, Japanese have increased frequencies of *KIR A* haplotypes, but a reduced frequency of *HLA-C* alleles carrying the C2 epitope compared with Europeans (Hiby et al., 2004). This situation is likely to have evolved to avoid too many pregnancies with this risky combination given that *KIR* and *HLA-C* are located on separate chromosomes and segregate independently. This observation also gives rise to speculation that couples of mixed

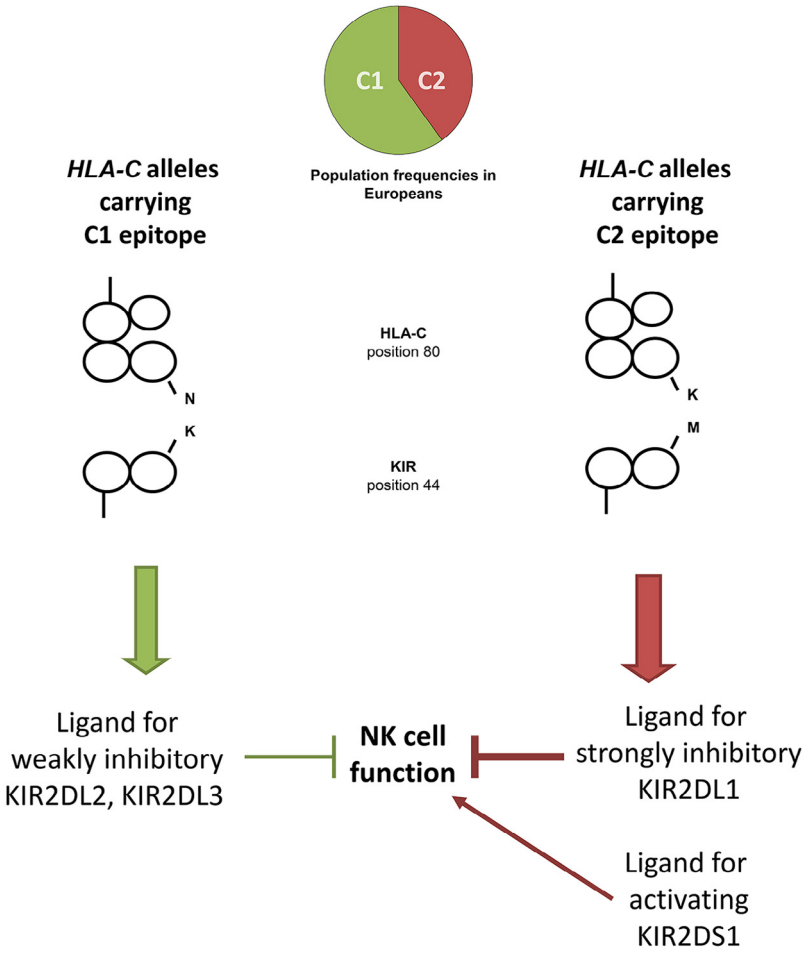

Figure 2 *HLA-C* variation. *HLA-C* alleles are divided into two groups according to a dimorphism at position 80 the α1 domain (C1: $Asn_{80,}$ C2: Lys_{80}). The pie chart shows the average distribution of *HLA-C* alleles with the C1 epitope (green) and the C2 epitope (red) in Europeans populations. Different KIRs bind to the C1 and the C2 epitope depending on a dimorphism at position 44. N, asparagine; NK, NK, natural killer; K, lysine.

ethnicity might be more or less at risk of pre-eclampsia. Large cohorts, however, would be needed to test this. Although one study found that the incidence of pre-eclampsia in 324 Japanese women with European partners was not higher than in Japanese women with Japanese partners (Saito et al., 2006), this study was only based on assumed frequencies of genotypes and no *KIR* and *HLA-C* genotyping of the couples was carried out (Moffett et al., 2006). The incidence of the KIR AA genotype is 60% in Japanese women. Frequencies of HLA-C alleles with the C2 epitope are 32% in Europeans but only 9% in Japanese. Around five out of 324 (1.5%) Japanese–European couples had pre-eclampsia. Given this sample size, the investigators have less than 50% power to detect any significant difference ($P < 0.05$) in the frequency of pre-eclampsia between Japanese–European couples compared with Japanese–Japanese couples for an odds ratio as large as 3. Therefore, the conclusions drawn by Saito et al. (2006) are untenable owing to the lack of power to recognize any effect, regardless of the direction. The study is thus statistically flawed.

If our own genetic results hold up in more powered studies, is there any role in the future for prediction and prevention of pre-eclampsia? First the accuracy of the genetic prediction needs to be improved. In addition to variation in numbers of *KIR* genes a woman inherits, there is also allelic variation at individual *KIR* loci. *KIR2DL1* (the inhibitory *KIR* for C2 epitopes) has four common alleles segregating in Europeans, whose products differ in expression level, binding avidity/affinity and signalling. The different HLA-C allotypes that all bear C2 epitopes may also bind differentially to different KIR allotypic variants. These two levels of allelic variation could explain the low accuracy, which might also be improved by combining the genetic information with models based on uterine artery Doppler ultrasound and maternal factors (Yu et al., 2005).

Of immediate concern is the risk to oocyte donation recipients and surrogate mothers who are reported to have such a high risk of pre-eclampsia, which could be linked to their higher risk of exposure to foreign C2 (64% versus 40% in a normal pregnancy). Ideally one might predict that mothers volunteering for surrogacy who have a *KIR AA* genotype are only given an embryo that lacks *HLA-C* alleles carrying a C2 epitope and only has those with C1 epitopes. Although this is a low risk, low-cost intervention, confirmation that the pre-eclampsia cases are indeed associated with fetal C2 is first needed. In such a study, and assuming a 25% risk of pre-eclampsia in oocyte donations versus 10% in other women

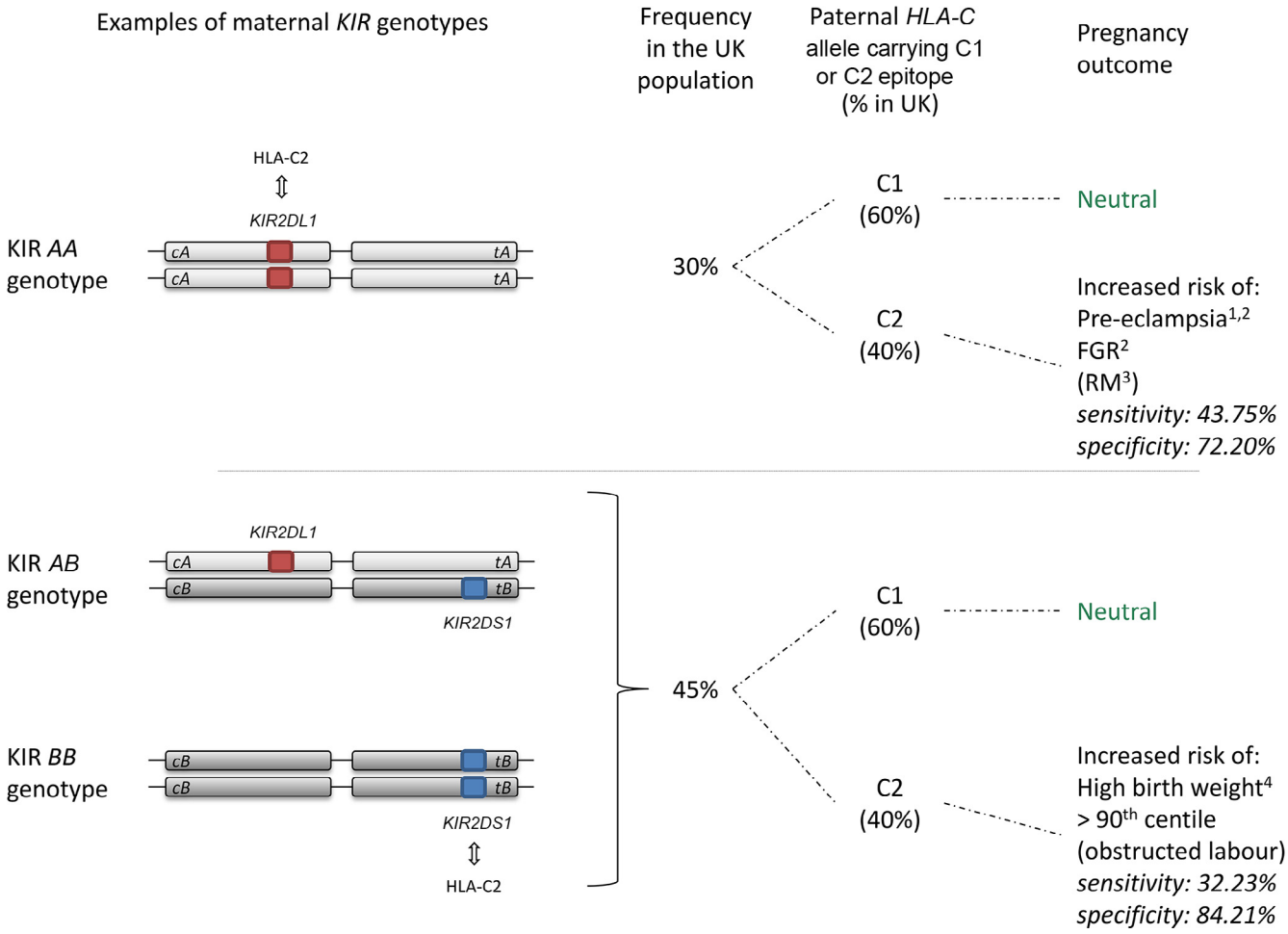

Figure 3 Genetic findings (colour codes as for **Figure 1**). [1]Hiby et al. 2004; [2]Hiby et al. 2010; [3]Hiby et al. 2008; [4]Hiby et al. 2014. HLA-C2 = *HLA-C* alleles carrying a C2 epitope.

undergoing assisted reproduction, 750 cases would be needed to detect with 90% power a significant (<0.05, two-sided) risk of pre-eclampsia associated with maternal *KIR AA* and a fetal C2 epitope.

Recurrent spontaneous abortion

Several studies of maternal *KIR* genes in recurrent spontaneous abortion have been published, although the findings are conflicting with no clear results (Moffett and Hiby, 2009). These studies have many problems, including very small patient numbers, questionable methodology for *KIR* typing, multiple testing, poorly matched controls and different clinical criteria for recruitment. For example, not all studies have made a simple division between *KIR A* and *KIR B* haplotypes but have analysed individual *KIR* genes. Clearly, at present, there is no rationale for introducing *KIR* typing into the clinic until these issues are resolved. Large well-characterized cohorts are needed with good controls to define clearly whether there are any particular *KIR* genotypes associated with failure of trophoblast to establish a blood supply in the first trimester.

Infertility and IVF

One study on *KIR* genotypes in women with infertility and failed IVF has been published, that points to a lower live birth rate in women with the *KIR AA* genotype (Alecsandru et al., 2014). Further studies would be of interest as it is not clear whether the failure could in some cases occur in the early days after implantation or from failure of attachment of the blastocyst to the surface epithelium. In the latter situation, uNK cells are unlikely to play a part.

Genotyping *KIR* and *HLA* in other clinical scenarios

The NK cells that are present in blood participate in responses to infectious agents and cancerous cells and modify the outcome of haematopoietic cell transplantation. Interestingly, genotyping for *KIR* and *HLA* is also being investigated in these areas of medicine to improve current treatments. For example, genotyping of *HLA-C* and *KIR* significantly improves the prediction of responses to treatment in patients with chronic hepatitis C and hepatitis

B virus infection (Suppiah et al, 2011; Stelma et al., 2016). Other studies in haplo-identical (half-*HLA* mismatched) haematopoietic cell transplantations for myeloid leukemia have shown that there is a better outcome if the donor has a *KIR B* haplotype (Cooley et al., 2014).

The future

Can these results be translated yet to any clinical situation in reproduction? In short, the answer must be 'not yet'. There are many reasons for this as outlined below:

- Most of the cohorts studied are small. Large numbers are required for sufficiently powered studies that involve two individuals, two complex gene system, and conditions with heterogeneous causes influenced by other genetic and environmental contributions. To give one example, a cohort of 3943 pregnancies would be needed to detect with 90% power a significant risk (<0.05, two-sided) of reduced birth weight (120 g less than the average 3350 g ± 450 g) linked to a combination of a given *KIR* allele and a given fetal *HLA-C* allele found in about 4% of the pregnancies. In the current state of knowledge, if maternal *KIR*/fetal *HLA-C* genetic combinations were used for screening, considering an incidence of pre-eclampsia of 4%, we would have the following: if the positive test result is for the women to be *KIR AA* (OR 1.54), the sensitivity (true positive rate) would be 36.92% and the specificity (true negative rate) 72.50%. If the positive test result is for the women to be *KIR AA* with a paternal HLA-C2 allele in the fetus (OR 2.02), the sensitivity (true positive rate) would be 43.75% and the specificity (true negative rate) 72.20%. Therefore, the performance of these screening tests, in those configurations, would not be useful to identify women at risk of pre-eclampsia.
- Cases and controls must be very carefully matched, particularly with ethnicity and genetic admixture because of the great diversity of *KIR* and *HLA* genes across different populations. Independent studies in different populations across the world are needed to replicate the original findings.
- The clinical characteristics should be identified carefully. For example, in women affected by recurrent spontaneous abortion or failed IVF, all known underlying causes for problems in pregnancy must be excluded. For pre-eclampsia, pre-existing hypertension, renal disease and placental malaria must be ruled out. In studying true fetal growth restriction, the gestational age is needed and cases with infectious, genetic, or other causes of low birth weight must be omitted.
- The population to select as ideal controls is not immediately obvious. Ideally, women with a history of normal pregnancies reaching a gestation of over 38 weeks, with birth weights in the middle of the normal range, no sponteous abortions or ectopic pregnancies would be used. This would correspond to a case-control design with extreme phenotypes, which would increase power. Cases and controls should still be matched in terms of unrelated diseases. Earlier studies have used normal first pregnancies.
- A family based design would also help avoid confounding factors to identify the genes responsible for defective placentation in pre-eclampsia.
- The *KIR* and *HLA* typing should be carried out in conjunction with laboratories devoted to tissue typing for transplantation in hospitals or to *KIR* and *HLA* diversity in a research setting. The UCLA International Cell Exchange offers validation for *KIR* typing and all research laboratories should be a part of this (http://www.hla.ucla.edu/cellDna.htm; Chazara and Moffett, 2015).
- Analysis of *KIR* variation has been a challenge given the huge diversity. There is a growing consensus that, because of the tight linkage disequilibrium of *KIR*, characteristic of the telomeric (*t*) and centromeric (*c*) regions of *KIR A* and *B* haplotypes, it is better to first use a broad distinction between *KIR AA* and all other genotypes and then between these centromeric and telomeric (*cA*, *tA*, *cB* and *tB*) regions. This strategy avoids implicating any *KIR* that may only be 'carried' in LD and not be a risk itself.
- If, from these studies, the associations of *KIR* and *HLA-C* variants with reproductive problems turn out to be robust, in the future it might be feasible to select sperm from donors who are C1C1 homozygous and carry no *HLA-C* alleles with a C2 epitope. In our cohorts we have always found a neutral effect of C1C1 babies whatever the mother's *KIR* genotype. The frequency in the UK of C1C1 men is about 36%.

Conclusion

The *KIR*/*HLA* system is likely to be associated with the primary stage in the pathogenesis of pre-eclampsia and defective placentation, and is unlikely to be related to the systemic symptoms. In keeping with this, similar *KIR*/*HLA-C* associations are found with recurrent spontaneous abortion and fetal growth restriction. This is one end of the spectrum (in terms of birth weight or low placental efficiency), with large babies at the other end. In this scenario birth weight is acting as a proxy for either a 'good' or 'bad' placenta. We have described how immune system genes are candidates for balancing the system and keeping the birth weight optimum for the population overall (Moffett and Colucci, 2015). Rarer combinations are found at the two extremes of birth weight. Obviously, multiple other genetic, nutritional and environmental influences operate to modify how uNK cells function in each pregnancy. By bringing together high-resolution genotyping with new approaches to understand the biology of uNK-trophoblast interactions, we will eventually make progress towards predicting and preventing complications of pregnancy.

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