A KIR B centromeric region present in Africans but not Europeans protects pregnant women from pre-eclampsia

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In Sub-Saharan Africans, maternal mortality is unacceptably high with >400 deaths/100,000 births compared to <10/100,000 in Europeans. One third of the deaths are caused by pre-eclampsia, a syndrome arising from defective placentation. Controlling placentation are maternal natural killer cells that use killer-cell immunoglobulin-like receptors (KIR) to recognize the fetal HLA-C molecules on invading trophoblast. We analysed genetic polymorphisms of maternal KIR and fetal HLA-C for 484 normal and 254 pre-eclamptic pregnancies at Mulago Hospital, Kampala, Uganda. The combination of maternal KIR AA and fetal HLA-C2 associates with pre-eclampsia (P=0.0318, OR 1.49). The KIR genes associated with protection are located in centromeric KIR B regions that are unique to Sub-Saharan African populations and contain the KIR2DS5 and KIR2DL1 genes (P=0.0095, OR 0.59). By contrast, telomeric KIR B genes protect Europeans against pre-eclampsia. Thus, different KIR B regions protect Sub-Saharan Africans and Europeans from pre-eclampsia, whereas in both populations the KIR AA genotype is a risk factor for the syndrome. These results emphasize the importance of undertaking genetic studies of pregnancy disorders in African populations with the potential to provide biological insights not available from studies restricted to European populations.

Uganda | Pre-Eclampsia | NK cells | Maternal mortality | KIR

Introduction

Although pre-eclampsia presents clinically with a diverse array of systemic symptoms, the underlying disease–causing mechanism starts with placentation when trophoblast cells invade the decidua. Here they transform the uterine spiral arteries into large vessels that form the feto-placental supply line (1, 2). In pre-eclampsia and other pregnancy disorders (fetal growth restriction (FGR), stillbirth, recurrent miscarriage), known collectively as the Great Obstetric Syndromes (GOS), trophoblast fails to invade optimally (3). Pre-eclampsia and other GOS occur in all populations but women of African ancestry are significantly more at risk and thus GOS are responsible for much of the high maternal and fetal mortality rates seen in Sub-Saharan Africa (SSA) (4). The genetic contribution to pre-eclampsia is supported by several studies and involves both maternal genes and the paternal genes inherited by the fetus (5, 6).

The wall of the uterus is the territorial boundary between two genetically different individuals: the mother and the fetus. The uterine mucosal immune system appears to define this maternal/placental boundary. The decidua must control placentation, because in its absence the trophoblast infiltrates to a dangerous extent, causing the condition of placenta percreta (7). The decidua contains an abundant population of specialized NK cells. These uterine NK cells (uNK) express Killer-cell Immunoglobulin-like Receptors (KIR) that recognize trophoblast HLA-C ligands (8, 9). Both *KIR* and *HLA-C* are genetically variable, resulting in many possible combinations of maternal *KIR* and fetal HLA-C ligands (10). The *KIR* region is defined by two groups of haplotype: *A* and *B*. The *KIR A* haplotype has seven *KIR* genes, all encoding inhibitory receptors apart from *KIR2DS4*. In contrast, the *KIR B* haplotype contains a variable number of additional *KIR* most of which encode activating receptors (11, 12). All HLA-C allotypes are KIR ligands and can be divided into two groups, carrying either C1 or C2 epitopes, that are distinguished by a dimorphism at position 80 and are recognized by different KIR (13). Within a human population the combination of *KIR* and *HLA* diversity distinguishes individuals. Worldwide human populations exhibit considerable differences and this is particularly true for Sub-Saharan African populations. They exhibit less linkage disequilibrium between the *KIR* genes

Significance

Pre-eclampsia is especially common in African women, and is a major cause of maternal death. The KIR genes we analyzed are carried by Natural Killer cells, immune cells that populate the uterus and are essential for successful pregnancy. KIR proteins bind HLA ligands on the implanting placental trophoblast cells. African and European women share similar risk associations for pre-eclampsia, but protection is associated with different KIR genes in the two populations. African women are protected by a combination of KIR B haplotype genes that is present almost exclusively in Africans. This study emphasizes the importance of studying diseases in Africans where the KIR/HLA genetic system is at its most diverse and maternal mortality rates are the highest in the world.

Reserved for Publication Footnotes

Table 1. Frequency of maternal *KIR* genotypes and *KIR* gene carriers

	Uganda Pre-eclampsia cases (n=251) n (%)	Uganda Controls (n=483) n (%)	P-value†	OR (CI)	UK Pre-eclampsia cases (n=729) n (%)*	UK Controls (n=592) n (%)*	P-value†	OR (CI)
***KIR* GENOTYPE**								
KIR AA	91 (36.3)	136 (28.2)	0.0256	1.45 (1.05-2.01)	266 (36.5)	163 (27.5)	0.0005	1.51 1.20-1.91
KIR AB	157 (62.5)	336 (69.6)	NS		456 (62.6)	424 (71.6)		
KIR BB	3 (1.20)	11 (2.28)	NS		7 (0.96)	5 (0.84)		
***KIR* GENES**								
2DP1	247 (98.4)	474 (98.1)	NS		NA	NA		
2DL1	247 (98.4)	476 (98.6)	NS		707 (97.0)	569 (96.1)	NS	
2DL2	132 (52.6)	293 (60.7)	0.0365	0.72 (0.53-0.98)	348 (47.7)	313 (52.9)	NS	
2DL3	222 (88.4)	414 (85.7)	NS		662 (90.8)	530 (89.5)	NS	
2DL5	138 (55.0)	316 (65.4)	0.0061	0.65 (0.47-0.88)	330 (45.3)	330 (55.7)	0.0002	0.66 (0.53-0.82)
3DL1	248 (98.8)	473 (97.9)	NS		600 (95.5)‡	517 (94.3)‡	NS	
3DS1	30 (12.0)	57 (11.8)	NS		211 (33.8)‡	242 (44.3)‡	0.0002	0.64 (0.51-0.81)
2DS1	52 (20.7)	114 (23.6)	NS		240 (32.9)	255 (43.1)	0.0002	0.65 (0.52-0.81)
2DS2	118 (47.0)	262 (54.2)	NS		349 (47.9)	317 (53.5)	NS	
2DS3	56 (22.3)	118 (24.4)	NS		185 (25.4)	175 (29.6)	NS	
2DS4	244 (97.2)	462 (95.7)	NS		703 (96.4)	560 (94.6)	NS	
2DS4 del	73 (29.1)	144 (29.9)	NS		632 (89.9)	474 (84.8)	NS	
2DS4 wt	171 (68.1)	318 (65.8)	NS		262 (37.3)	215 (38.5)	NS	
2DS5	94 (37.5)	243 (50.3)	0.0009§	0.59 (0.43-0.81)	205 (28.1)	214 (36.1)	0.0023	0.69 (0.55-0.87)

*Hiby et al. 2010
† Fisher's exact test with mid-p adjustment
‡ a number of individuals were not typed for this gene
§ P=0.0126 after Bonferroni correction
NA, not available; NS, not significant

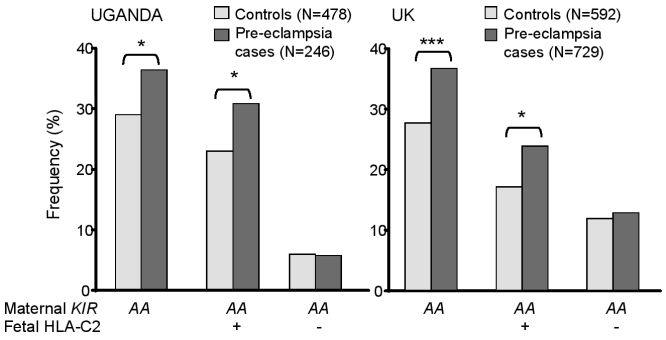

Fig. 1. Frequency of the KIR AA genotype alone and in combination with the fetal HLA-C carrier group in Uganda and in the UK.There was a significant difference in the *KIR AA* genotype frequencies between controls (grey bar) and pre-eclampsia cases (black bar) in both the Ugandan, *P=0.0256, OR 1.45, and the UK cohorts, ***P=0.0005, OR 1.51. The frequency of *KIR AA* genotypes is shown when combined either with a fetus carrying a C2 epitope or those lacking C2 and carrying only C1-bearing HLA-Callotypes. There is a significant risk of pre-eclampsia when a *KIR AA* women has a fetus carrying a C2 epitope for both cohorts; in Uganda *P=0.0318, OR 1.49, in the UK *P=0.0267, OR 1.46.

than other populations (14-16), and the *KIR* genes have greater allelic diversity (15, 16). A variety of diseases and clinical conditions have been associated with combinations of *HLA-C* and *KIR* genes. In previous case-control studies of pre-eclampsia in pregnant European women we showed that when the fetus carries a C2 epitope, maternal *KIR AA* genotypes are risk factors for pre-eclampsia, whereas the *KIR2DS1* gene of maternal *KIR B* haplotypes is protective (8, 17). In the case-control study reported here we test the hypothesis that these factors confer similar risk and protection to pregnant Sub-Saharan African women.

Results

Clinical characteristics of the cohort. This case-control study of pre-eclampsia involved 738 pregnant women at Mulago Hospital, Kampala in Uganda. More than 90% of cases and controls were Bantu, the largest ethnic group, with small numbers of Luo, Nilo-Hamites and other ethnic groups. The ethnicity of the male partners and the sex ratios of the singleton babies in all the groups were similar (Table S1). HIV+ women were not excluded from the analysis as there were similar numbers in both pre-eclamptic and control pregnancies (~5%) (Table S1) and similar results were found even when HIV+ women were omitted (Table S2). As expected, the gestational age at delivery and the birth weights were significantly lower in the pre-eclamptic cases compared to controls (P<0.001, Table S1, Figure S1).

Unlike European women, KIR B centromeric regions containing KIR2DS5 protect Ugandan women from pre-eclampsia.

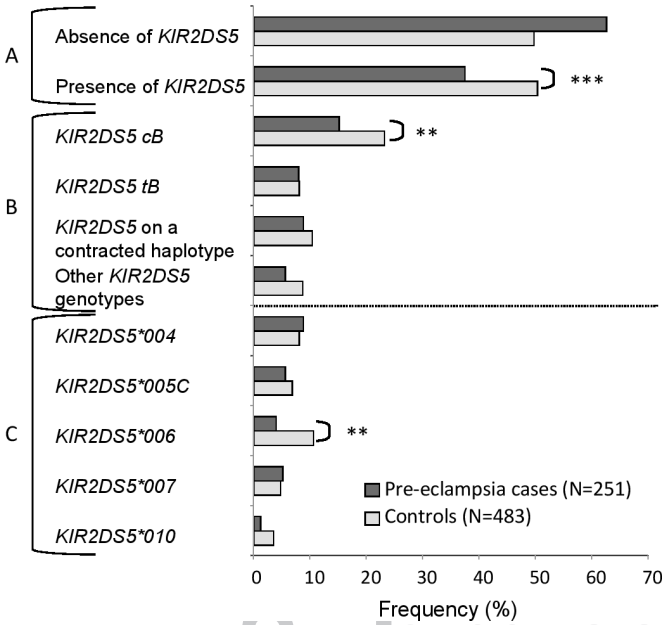

Fig. 2. Frequencies of the different genotypes carrying KIR2DS5 in controls and pre-eclampsia cases. *(A)* All controls (grey bars) and pre-eclamptic cases (black bars) were grouped according to whether they carried *KIR2DS5*. The presence of *KIR2DS5* protects women from pre-eclampsia. ***P=0.0009. OR 0.59. (P_c=0.0126 after Bonferroni correction)*; (B)* Women were grouped according to the location of *KIR2DS5* on the *KIR B* haplotype, centromeric = *cB*, telomeric = *tB*, contracted or other unusual genotypes. *KIR2DS5* on *cB* is significantly protective **P=0.0095, OR 0.59. *(C)* The carrier frequencies of those *KIR2DS5* alleles present on *cB* were compared between controls and pre-eclamptic cases. *KIR2DS5*005C* are those women where *KIR2DS5* is located on *cB*. Only *KIR2DS5*006* is significantly protective **P=0.0015, OR 0.3519

Table 2. Risk associated with the absence of *KIR2DS5* for the different maternal/fetal *HLA-C* combinations

Parameter	P-value*	OR (CI)
Effect of relative dose of maternal and fetal *HLA-C2* alleles		
Fetus had fewer *C2* alleles than the mother	0.7085	1.087 (0.69-1.69)
Fetus had the same number of *C2* alleles	0.1612	1.280 (0.91-1.80)
Fetus had more *C2* alleles than the mother	**0.0130**	1.724 (1.12-2.64)
Effect of origin of fetal *HLA-C2* allele		
Paternal origin	**0.0203**	1.795 (1.10-2.93)
Maternal origin	0.5222	1.162 (0.72-1.84)

*Fisher's exact test with mid-p adjustment

Maternal *KIR AA* genotype is increased in the pre-eclamptic pregnancies (P=0.0256, OR 1.45)(Table 1), particularly when combined with the presence of fetal *HLA-C* alleles encoding the C2 epitope, similar to our findings in Europeans (P=0.0318, OR 1.49)(Figure 1). We then analyzed which *KIR B* haplotype genes are protective. Three *KIR B* genes, *KIR2DL2*, *KIR2DL5* and *KIR2DS5,* are more frequent in controls than in women with pre-eclampsia. Of these three, only *KIR2DS5* is significantly protective for women with pre-eclampsia after Bonferroni correction (P=0.0009, P_c=0.0126, OR 0.59)(Table 1)(Figure 2A) (Table 3). In comparable studies on European women, protection was seen with *KIR2DS1* and not with *KIR2DS5* as shown here for African women (Table 1). Moreover, in the Ugandans, the telomeric *B* (*tB)* genes *KIR2DS1* and *KIR3DS1* are at similar low frequency in cases and controls (Table 1).

As *KIR* genes are in linkage disequilibrium, *KIR2DS5* could be itself protective or marking a nearby protective gene. *KIR2DS5* can be found in both the *KIR* centromeric *B (cB)* and telomeric *B* (*tB)* regions. To determine the location of *KIR2DS5* in our cohort, we grouped individual genotypes according to their combination of centromeric and telomeric *KIR* regions, based on previously described African *KIR* haplotypes (see Methods and Figure 3). Genotypes characteristic of expanded or contracted regions were also identified and shown to have similar frequencies in cases and controls.

Next, allele-level *KIR2DS5* typing was performed, which identified ten alleles that were assigned to *cB* or *tB* regions as described in Methods (Figure 4). *KIR2DS5*004, *006, *007,* and **010* are restricted to *cB*, whilst *KIR2S5*002, *003, *008, *009* and **011* are restricted to *tB. KIR2DS5*005* is the most frequent allele and the only one found in both *cB* and *tB* (Figure 4), pointing to it being the progenitor of all other *KIR2DS5* alleles. Our assignments of *KIR2DS5* alleles to *cB* or *tB* agree with those defined by complete *KIR* haplotype sequences and analysis of African and African-American families (15, 18, 19). With all this information, we were able to determine the centromeric or telomeric location of *KIR2DS5* for all *KIR2DS5*-carrying individuals.

Comparison of the frequency of the centromeric and telomeric *KIR2DS5* alleles in cases and controls shows that they differ in the protection they provide against pre-eclampsia. *KIR2DS5* is protective in Ugandan women when it is present in the *cB* region (*cB01* or *cB03*, P=0.0095, OR 0.59) (Figure 2B, Figure 3, Table S3). Furthermore, of all the *cB KIR2DS5* alleles, only *KIR2DS5*006* is significantly more frequent in controls than in pre-eclamptic pregnancies (P=0.0015, OR 0.35) (Figure 2C, Table S4). The dominant allele, *KIR2DS5*005,* has similar frequencies in both cases and controls even when we can unequivocally assign its location to *cB* and thus appears neutral. Consistent with the low frequency of *KIR2DS1* and *KIR3DS1* in Africans, *KIR2DS5* is less frequently present in *tB* than *cB*. When present in *tB* it has no effect, being at similar frequencies in controls and cases (Figure 2B, Table S3). Thus, the protective effect of *KIR B* is not just the absence of *KIR A* genes but also the presence of genes belonging to a particular subgroup of *cB* regions, *cB01* or *cB03* (Figure 3).

In Ugandan women, like European women, pre-eclampsia associates with maternal KIR AA genotype combined with fetal expression of paternal HLA-C2. We further examined the effect of different combinations of maternal *KIR* and fetal ligands, C1 and C2 epitopes of HLA-C allotypes. Considered alone, the C1 and C2 frequencies in mothers and babies do not significantly differ between cases and controls (Table S5). Using an extended Mantel-Haenszel test for linear trend, we find that *KIR AB* or *BB* genotype mothers carrying a C1C1 homozygous fetus have the least risk of pre-eclampsia, whereas a *KIR AA* mother carrying a C2 fetus has greatest risk (P=0.0122) (Figure S2, Table S6). Other genetic combinations have risks between these two extremes.

If the fetus has one more *HLA-C* allele encoding a C2 epitope than the mother, then the fetus must have inherited this C2 from the father. In this situation, the risk of pre-eclampsia in the absence of *KIR2DS5* is increased (P=0.0130, OR 1.72) (Table 2). To explore this further we defined the parental origin of the C2 for C1C2 heterozygous fetuses. When the single C2 is paternally inherited the risk of pre-eclampsia associated with the absence of *KIR2DS5* is greater (P=0.0203, OR 1.80) than when it is maternally inherited (NS, OR 1.16 CI 0.72–1.84) (Table 2). Taken together, these findings show that there is an increased risk of pre-eclampsia in women with a *KIR AA* genotype lacking

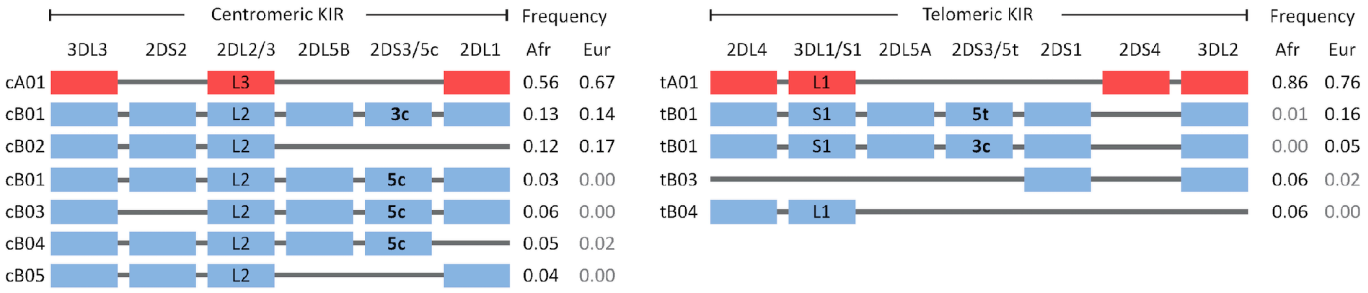

Fig. 3. Component genes of centromeric and telomeric KIR haplotype segments in African and European populations.The red segments together form the *KIR A* haplotype, all other combinations of centromeric and telomeric motifs form *KIR B* haplotypes. The gene content motifs are shown for the centromeric (left) and the telomeric regions (right). The frequencies of the different KIR regions in representative African and European populations is also shown (15, 39).

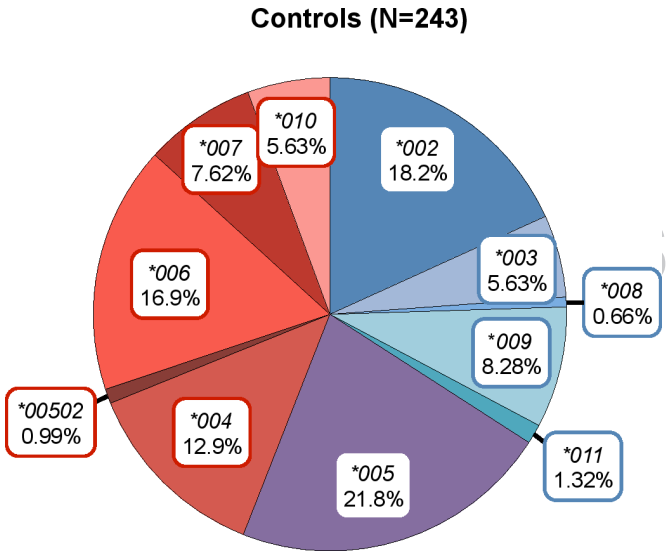

Fig. 4. Carrier frequencies of the different KIR2DS5 alleles found in the Ugandan population. *cB* alleles are in shades of red, *tB* alleles shades of blue, *KIR2DS5*005* (purple) is found in both *cB* and *tB*.

KIR2DS5 when the fetus has a *HLA-C* allele encoding a C2 epitope inherited from its father.

Recurrence of pre-eclampsia in Ugandan women is associated with maternal KIR AA genotype and fetal expression of paternal C2. The risk of recurrence of pre-eclampsia is known to be high (~20%) (20, 21). In our cohort were 24 pre-eclamptic women who had recurrence of a hypertensive disorder of pregnancy, a condition on the same spectrum as pre-eclampsia. The 45.8% frequency of the *KIR AA* genotype in these women with recurrent pre-eclampsia was even higher than the frequencies of 36.3% in the full cohort and 28.2% in controls. Ten of the eleven *KIR AA* pregnancies in this sub-cohort carried a C2 fetus.

Discussion

Our genetic study in an African population not only supports previous findings that certain combinations of maternal *KIR* and fetal *HLA-C* variants are associated with pre-eclampsia but also reveals the benefits of studying multiple populations including those most at risk of a disease. Pre-eclampsia occurs more commonly in African women and the symptoms are of severe, early onset disease associated with low birth weight and high mortality (4). Our findings have relevance to other disorders of pregnancy as unexplained stillbirth, fetal growth restriction and preterm labour are more common women with African ancestry and share the same underlying problem of defective placentation with reduced maternal blood flow to the placenta (4).

There is considerably more genetic diversity of KIR genes in Africans both at the level of KIR haplotypes and number of alleles at individual KIR loci (10, 15, 16). Despite this complexity, we find complete consistency with our studies of pre-eclampsia in Europeans: the risk is associated with a maternal *KIR AA* genotype combined with a paternally-derived HLA-C allotype carrying a C2 epitope in the fetus (8, 17). Recurrent pre-eclampsia frequently occurs in African women (24.6% in a recent Tanzanian study) and the high frequency of *KIR AA* genotypes in these women in our study is striking (45.8% compared to 28.2% in controls)(21). The KIR always present on the *KIR A* haplotype likely to confer this risk for is *KIR2DL1*, an inhibitory KIR with strict specificity for C2 epitopes (22). Thus, in women with a *KIR AA* genotype containing two copies of *KIR2DL1*, uNK will be strongly inhibited when confronted by HLA-C2+ trophoblast. There are at least 12 *KIR2DL1* alleles located in *cA* region in Africans compared to 1-5 in other populations (15). In the future analysis of larger cohorts, including more women with recurrent pre-eclampsia, should identify if there are particular *KIR2DL1* alleles responsible.

One clear difference that might partially explain the increased risk of pre-eclampsia in Africans is the higher frequency of C2-bearing HLA-C allotypes across SSA compared with elsewhere in the world (14). The probability of African women having a C2-positive partner or fetus is 80% compared to 64% for European women. Similarly the probability of African women having a fetus carrying a paternal C2 epitope is 55%, compared to 40% for European women (Table S4). Given the selective pressure that pre-eclampsia imposes on a population, there must be other scenarios where C2 epitopes are beneficial. *HLA-C* and *KIR* are immune system genes with roles in outcome from viral infections such as HCV and HIV (10, 23-25). In SSA C2 epitopes might be advantageous in responding to a range of pathogens, including malaria. Studies of how *HLA-C* and *KIR* variants affect responses to infection in SSA are still limited, especially in the crucial period from birth to adolescence.

We observed that *tB* regions containing *KIR2DS1* provide a protective effect for pre-eclampsia in Europeans (8). In contrast, we now show that in Ugandans KIR *cB* regions characterized by *KIR2DS5, KIR2DP1* and *KIR2DL1* (*cB01* and *cB03*) are protective. The low carrier frequency of *KIR2DS1* in SSA (1.4%-27.8%) compared to Europe (42.5%) also suggests that *KIR2DS1* does not play an important role in pregnancy success in Africans (14). One explanation for the different protective effect is that KIR2DS5, an activating KIR that likely evolved from a KIR specific for C2, does function like KIR2DS1 - although there is no evidence to date that the C2 epitope is a KIR2DS5 ligand (22). The single *KIR2DS5* allele in Europeans, *KIR2DS5*002*, is in tight LD with *KIR2DS1* and located in the *tB* region. Unlike Europeans though, *KIR2DS5* is polymorphic in Africans and African-Americans. We found 10 alleles in Ugandans, consistent with previous reports from African Americans

(IPD), located in both *cB* and *tB*, but most commonly found in those *cB* regions that also contain *KIR2DP1* and *KIR2DL1* (26, 27). The dominant allele, *KIR2DS5*005* is the only allele found in both *cB* and *tB* and is probably ancestral; when in either location it was similar in frequency between cases and controls. Of the *cB KIR2DS5* alleles, only *KIR2DS5*006* is significantly associated with protection from pre-eclampsia. KIR2DS5 can be expressed by European pbNK but we have been unable to demonstrate its expression on uNK using similar reagents (28-30). The functional effects of KIR2DS5 diversity await further investigation but certain KIR2DS5 allotypes do show different expression levels in transfected cells, similar to findings for other KIR variants (30). For example, allelic variation of KIR2DL1 affects expression levels at the cell surface, NK repertoire and affinity of binding (22, 31, 32). Furthermore, although no binding has been demonstrated of the European allele, *KIR2DS5*002*, to any HLA ligand, *KIR2DS5*006* might bind to C2 epitopes common in Africans (C*04, C*02, C*17, C*18)(15).

Another possibility is that *KIR2DS5* is in LD with other *KIR* on the protective *cB01* and *cB03* regions, notably *KIR2DL1*. The *cB KIR2DL1* allele present in Europeans, *KIR2DL1*004*, gives a weak inhibitory signal compared to the common *cA* allele, *KIR2DL1*003* (31). Thus, the protective effect of the *cB01* and *cB03* regions could either be due to KIR2DS5 activation or weaker KIR2DL1 inhibition, as both might counter-balance the strong inhibition conferred from *cA KIR2DL1* alleles. For both *KIR A* and *B* haplotypes, the particular *KIR2DS5* and *KIR2DL1* alleles involved are therefore important, but to investigate this will require much larger, clinically well-characterised cohorts. Our method to infer *KIR* regions allows a fairly simple analysis of *KIR* data from clinical cohorts in SSA compared to the complex sequencing needed to define the exact haplotypes (15). Hence, although this analysis does not unravel the complete complexity of *KIR* variants found, it can point to the regions conferring risk or protection. In this clinical context we have a clear pointer that the *cB01* and *cB03* regions, containing *KIR2DS5*, *KIR2DL1* and *KIR2DP1*, are providing protection from pre-eclampsia in Ugandan women.

In this African cohort, as in Europeans, a paternal rather than maternal origin of fetal C2 confers risk in women lacking *KIR2DS5* (8). Whether this effect is due to disparities between individual maternal and paternal HLA-C2 allotypes (allogeneic) and/or a dosage effect (more *HLA-C* alleles encoding C2 in the fetus than in the mother when C2 is paternally derived) is unresolved (8). This will require high-resolution genotyping of C1C2 mothers who have C1C2 babies (where the dosage is identical) in a large cohort (2000 cases and 4000 controls would be required).

The great diversity of *KIR* and *HLA-C* variants in SSA is maintained by balancing selection (10). The two contrasting functions of these immune system gene families in reproduction and immune responses to infection mean certain variants will be important at different stages of life in women, men, children and adults and in geographical regions with a range of different pathogens. We have previously argued that the selective pressures from reproductive success and immune response to pathogens are competing and have driven evolution of the *KIR A* and *B* haplotypes in humans compared to other hominids (10). Our combined studies of *KIR*/*HLA-C* variants in diverse European and African populations now suggest that the unusual reproductive strategies characteristic of modern humans compared to other hominids could also be a cause of balancing selection. The evolution of the large neonatal brain relative to a pelvis adapted for bipedalism means birth weight must be kept between two strictly defined limits. When babies are large (>95th centile) there is a risk of cephalo-pelvic disproportion and subsequent prolonged obstructed labour, birth asphyxia and post partum haemorrhage. Furthermore, these outcomes are also much more common in African women with associated features of pregnancy that favour smaller babies: earlier birth - the gestational age is reduced to 38 weeks, the head engages late into the pelvis and the baby matures earlier than in non-Africans (4). Thus, there is not only high mortality in mother and babies from pre-eclampsia (associated with low birth weight and still birth), but also at the other end of the normal birth weight spectrum. Both mothers and their babies benefit if the latter have intermediate birth weights and the two extremes of very low and high birth weight are selected against. The balance between these two extremes is partially determined at placentation when uNK allow trophoblast cells to access sufficient maternal oxygen and nutrients without starving the baby (defective trophoblast invasion) or risking uterine rupture (excessive trophoblast invasion) (3). In an African population, because of the greater risk of cephalo-pelvic disproportion (4), there is even greater selection for reduced fetal size with associated pre-eclampsia - this is consistent with the higher frequency of maternal *KIR AA*/paternal C2 combinations in SSA.

In Europeans, opposing *KIR*/*HLA-C* combinations are associated with the extremes of birth weight: a paternal C2 epitope is associated with both extremes, but in pre-eclampsia and low birth weight (<5th centile) the risk is with maternal *KIR AA* genotypes, whilst in high birth weight the association is with maternal *KIR2DS1* (33). Studies on how these genetic findings are translated in uNK functional differences are still limited but we found that when KIR2DS1+ uNK (isolated from UK patients) are activated by target cells expressing HLA-C2, there is increased production of soluble factors (eg GM-CSF) that enhance trophoblast invasion (34).

Thus, there is a balance between the *KIR A* and *KIR B* haplotypes in both populations but they differ in the regions of the *KIR B* haplotype that correlate with protection from pre-eclampsia. *tB* regions and *KIR2DS1* are infrequent in Africans compared to Europeans but the opposite is true for *cB* regions containing *KIR2DS5*. During the out-of Africa migrations it is possible that only individuals with *tB* with *KIR2DS1* moved away from SSA. Introgression of *KIR2DS1* from archaic humans is also a possibility (35). Our previous findings do indicate that *KIR2DS1* and *KIR3DS1* (both on *tB*) are selected against in SSA (14). Studying disorders of pregnancy in an African setting is important and informative; the high rates of pre-eclampsia as well as other major disorders of pregnancy including obstructed labour and stillbirth and the greater genetic diversity of *KIR* in SSA mean unravelling the role of the complex *KIR* and *HLA* systems will provide valuable genetic information to predict women who are at risk of a range of pregnancy disorders.

Materials and Methods

Ethics statement. Approval to conduct the study was given by the Higher Degrees Research and Ethics Committee of Makerere University College of Health Sciences and the Uganda National Council for Science and Technology (UNCST). The participants gave written informed consent to participate in the study. Withdrawal from the study never jeopardized health care and this was provided free to all women.

Study design. This study was conducted at Mulago National Referral and Teaching Hospital, located in Kampala, which functions as a tertiary referral center for Uganda. Mulago hospital is the busiest maternity hospital in Sub-Saharan Africa, with over 30,000 deliveries a year. Genomic DNA was obtained from maternal blood from unrelated healthy women (n=484) or women with pre-eclampsia or eclampsia (n=254) between July 2009 and June 2011. Umbilical cord samples were obtained from the babies for genomic DNA isolation. Pre-eclampsia was defined as hypertension of 140/90 mmHg or more, on more than one occasion at least 4 hours apart plus proteinuria of +2 or more by dipstick, both at 20 weeks or more of gestation. Eclampsia was diagnosed when a patient with pre-eclampsia had generalized tonic-clonic convulsions. Controls were women with a normal first pregnancy delivering at term (≥38 weeks) who were normotensive with no proteinuria. Excluded from controls were patients taking long term medication and patients with other diseases including chronic hypertension and renal disease but excluding HIV. Women who had received a blood transfusion within the last 3 months were also excluded. Cases and controls were consecutively recruited from the same catchment area during the study period. Data was collected

at the time of clinical examination of the participants using an interviewer-administered questionnaire and additional information was obtained from medical charts.

DNA isolation and genotyping. Maternal genomic DNA was isolated from 5 ml of blood using the QIAamp DNA Maxi Blood Kit (Qiagen). Fetal DNA was isolated from umbilical cord samples after overnight incubation with Proteinase K (Roche), purification with a protein precipitation solution (Qiagen) followed by ethanol precipitation. Twelve maternal *KIR* genes were typed for presence or absence by PCR-SSP using two pairs of primers per gene or allele as described previously (8, 14, 36). The *KIR* genes typed were *2DL1, 2DL2/3, 2DL5, 3DL1/S1, 2DP1, 2DS1, 2DS2, 2DS3, 2DS4* (including the deletion), and *2DS5*. All the samples were typed for *KIR2DL1* and *KIR2DP1* copy number and 28 selected samples were further investigated for additional *KIR* (*2DL4, 3DP1, 3DL2, 3DL3)* so that all 14 *KIR* genes were included (37). Individual genotypes were defined according to their combination of centromeric (*cA and cB*) and telomeric (*tA* and *tB*) *KIR* regions, based on previously described African *KIR* haplotypes (14, 15, 18). We first discriminated *KIR A* from *KIR B* regions on the basis of the presence/absence of *2DS2*, *2DL2/3*, *2DP1*, *2DL1*, *3DL1/S1*, *2DS1* and *2DS4*. There are common *cB* regions in Africans (Figure 3) that were identified in individuals with a *cB* region using information from the presence/absence of individual *KIR* genes and the copy number of *KIR2DL1* and *KIR2DP1* (18). Typically, *cB01* and *cB03* have *2DP1*, *2DL1*, *2DL5* and *2DS5* (or *2DS3*) whereas *cB02* lacks these genes. *KIR2DS5* alleles were genotyped by pyrosequencing, targeting exons 5, 6, and 7 (15). Then, by knowing which *KIR2DS5* alleles are present in individuals homozygous for either centromeric *A* (*cA*) or telomeric *A* (*tA*) regions, we could assign each of the ten *KIR2DS5* alleles to *cB* or *tB* (Figure 4). C1 and C2 were defined in maternal and fetal samples based on the primers and methods described previously (8, 36). *HLA-C* low resolution allelic typing was performed using a PCR-SSP method consisting of 21 reaction wells adapted from (38). Each well contained a final reaction volume of 10µl, consisting of 5x Green GoTaq Flexi Buffer (Promega), 0.2mM dNTPs (ThermoFisher), 1.25mM MgCl2 (Promega), 0.4U GoTaq DNA polymerase (Promega), 134nM 63/64 control primer (Eurogentec) and approximately 45ng DNA. PCR products were run on a 1% agarose gel and visualized using a UV and ethidium bromide.

Statistical analysis. Unless otherwise indicated, categorical data was analysed using the chi-square and Fisher's exact test with two-tailed mid-p adjustment and Student's t-test for continuous data. A P-value of ≤ 0.05 was considered to be statistically significant. The magnitude of the effect was estimated by odds ratios (OR) and their 95% confidence intervals (CI).

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Table S1 Clinical characteristics of the cohort

Characteristics	Pre-eclampsia cases n=254 n (%)	Controls n=484 n (%)	P-value*
Women's age (years)			
mean ± SD	24.8 ± 5.4	21.1 ± 2.8	< 0.001
range	(13.0-42.4)	(16.0-31.4)	
Parity			
Primigravidae	132 (52.0)	484 (100)	< 0.001
Multiparous	122 (48.0)	-	
Sex of baby			
Female	109 (46.0)	248 (51.2)	NS
Male	128 (54.0)	236 (48.8)	
Gestation age at delivery (weeks)			
mean ± SD	37.2 ± 3.7	39.8 ± 1.5	< 0.001
range	(24-44)	(38-45)	
Baby's birth weight (kg)			
mean ± SD	2.6 ± 0.8	3.1 ± 0.4	< 0.001
range	(0.7-4.5)	(2.0-4.5)	
Admission BP– systolic (mmHg)			
mean ± SD	163.8 ± 21.7	110.5 ± 7.1	< 0.001
range	(140-254)	(90-135)	
Admission BP– diastolic (mmHg)			
mean ± SD	110.2 ± 14.8	67.2 ± 6.2	<0.001
range	(90-160)	(60-85)	
HIV status			
Negative	241(94.9)	461 (95.2)	NS
Positive	13 (5.1)	23 (4.7)	

*Fisher's exact test with mid-p adjustment

Table S2 Summary of the results for HIV negative individuals

Parameter	P-value*	OR (CI)
Maternal *KIR AA*	0.0167	1.50 (1.08-2.10)
Maternal *KIR AA* and fetal HLA-C2+	0.0318	1.49 (1.04-2.15)
Presence of *KIR2DL2*	0.0179	0.68 (0.50-0.94)
Presence of *KIR2DL5*	0.0047	0.63 (0.46-0.87)
Presence of *KIR2DS5*	0.0009	0.58 (0.42-0.80)
Fetus had more *HLA-C2* alleles than the mother	0.0083	1.81 (1.17-2.79)
Paternal origin of fetal *HLA-C2* allele	0.0256	1.82 (1.08-3.07)
Presence of *KIR2DS5* centromeric	0.0089	0.58 (0.38-0.88)
Presence of *KIR2DS5*006*	0.0034	0.38 (0.18-0.74)
Trend of maternal KIR and fetal HLA-C combinations	0.0086†	NA

Pre-eclampsia cases (n=238), controls (n=460), all HIV negative
*Fisher's exact test with mid-p adjustment unless otherwise stated
†Extended Mantel-Haenszel chi square for linear trend
NA not available

Table S3 Frequency of the different *KIR2DS5* genotypes

	Pre-eclampsia cases (n=251) n (%)	**Controls (n=483) n (%)**	**P-value***	**OR (CI)**
Absence of *KIR2DS5*	157 (62.5)	240 (49.7)	0.0009	1.69 (1.24-2.31)
KIR2DS5 centromeric	38 (15.1)	112 (23.2)	0.0095	0.59 (0.39-0.88)
KIR2DS5 telomeric	20 (8)	39 (8.1)	NS	
KIR2DS5 on a contracted *KIR* haplotype	22 (8.8)	50 (10.4)	NS	
KIR2DS5 on other *KIR* genotypes	14 (5.6)	42 (8.7)	NS	

*Fisher's exact test with mid-p adjustment

Table S4 Frequency of the different *KIR2DS5* alleles

KIR2DS5 **allele location**	***KIR2DS5*** **allele**	**Pre-eclampsia cases (N=251) n (%)**	**Controls (N=483) n (%)**	**P-value***	**OR (CI)**
cB	**004*	22 (8.8)	39 (8.1)	NS	
cB	**00502*	2 (0.8)	3 (0.6)	NS	
cB	**006*	10 (4)	51 (10.6)	0.0015†	0.35 (0.17-0.69)
cB	**007*	13 (5.2)	23 (4.8)	NS	
cB	**010*	3 (1.2)	17 (3.5)	NS	
cB and *tB*‡	**005*	26 (10.4)	66 (13.7)	NS	
cB	**005C*	14 (5.6)	33 (6.8)	NS	
tB	**005T*	6 (2.4)	14 (2.9)	NS	
n.a.	**005* others	6 (2.4)	19 (3.9)	NS	
tB	**002*	28 (11.2)	55 (11.4)	NS	
tB	**003*	7 (2.8)	17 (3.5)	NS	
tB	**008*	2 (0.8)	2 (0.4)	NS	
tB	**009*	12 (4.8)	25 (5.2)	NS	
tB	**011*	1 (0.4)	4 (0.8)	NS	

*Fisher's exact test with mid-p adjustment

†P=0.0205 after Bonferroni correction

‡*KIR2DS5*005* can be found in both the *cB* and *tB* region. The more detailed analysis is given when the assignment to each region is possible.

Table S5 Frequency of maternal and fetal *HLA-C* genotypes

	Mothers		P-value	Fetuses		P-value
	Pre-eclampsia cases (n=251)	Controls (n=483)		Pre-eclampsia cases (n=247)	Controls (n=480)	
***HLA-C* genotype**						
HLA-C1C1	46 (18.3)	95 (19.7)	NS	45 (18.2)	106 (22.1)	NS
HLA-C1C2	132 (52.6)	245 (50.7)	NS	118 (47.8)	227 (47.3)	NS
HLA-C2C2	73 (29.1)	143 (29.6)	NS	84 (34)	147 (30.6)	NS
***HLA-C* group frequency**						
HLA-C1	224 (44.6)	435 (45)	NS	208 (42.1)	439 (45.7)	NS
HLA-C2	278 (55.4)	531 (55)	NS	286 (57.9)	521 (54.3)	NS

Table S6 Frequency of maternal *KIR* and fetal *HLA-C* combinations

Parameter	Pre-eclampsia cases (n=246) n (%)	Controls (n=478) n (%)	P-value*	Risk compared to baseline
Trend			0.0122	
***KIR AA* mother**				
Fetal *HLA-C2C2*	29 (11.8)	39 (8.2)		1.847
Fetal *HLA-C1C2*	46 (18.7)	67 (14)		1.705
Fetal *HLA-C1C1*	14 (5.7)	29 (6.1)		1.199
***KIR AB* or *BB* mother**				
Fetal *HLA-C2C2*	54 (22)	106 (22.2)		1.265
Fetal *HLA-C1C2*	72 (29.3)	160 (33.5)		1.118
Fetal *HLA-C1C1*	31 (12.6)	77 (16.1)		1

*Extended Mantel-Haenszel chi square for linear trend

Supplementary Figures.

Fig. S1. Birth weight (g) distributions of babies from control (n=484)(dotted line) and pre-eclamptic (solid line)(n=229) pregnancies. The birth weight (g) is shown on the x axis and the frequency (%) on the y axis."

Fig. S2. Linear trends in the frequencies of the maternal KIR and fetal HLA-C genotype combinations depicted in Table S5. Mothers were grouped as having either *KIR AA* or *KIR AB/BB* genotypes. Fetal *HLA-C* genotypes were defined as *HLA-C2C2*, *HLA-C1C2* or *HLA-C1C1*. In a comparison of pre-eclamptic and control pregnancies, there is a significant linear trend in frequencies. The most risk of pre-eclampsia is in pregnancies with a *KIR AA* mother and a *HLA-C2C2* or *HLA-C1C2* fetus. The least risk is with *KIR AB/BB* mothers with *HLA-C1C2* or *HLA-C1C1* fetuses. The data was analysed using an extended Mantel-Haenszel test for linear trend (p=0.0122).

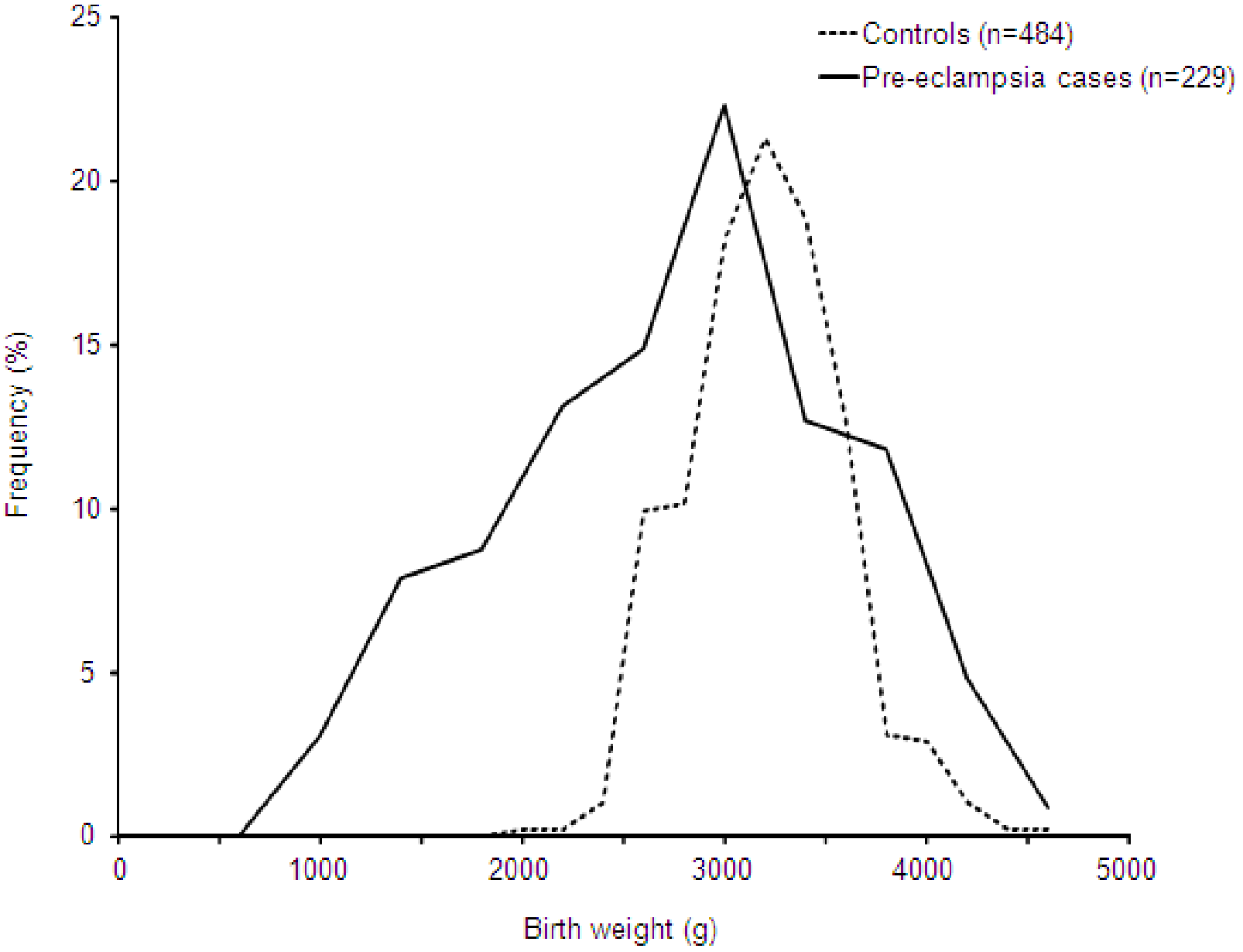
Controls (n=484)
Pre-eclampsia cases (n=229)
Frequency (%)
25
20
15
10
5
0
0
1000
2000
3000
4000
5000
Birth weight (g)

Figure S1

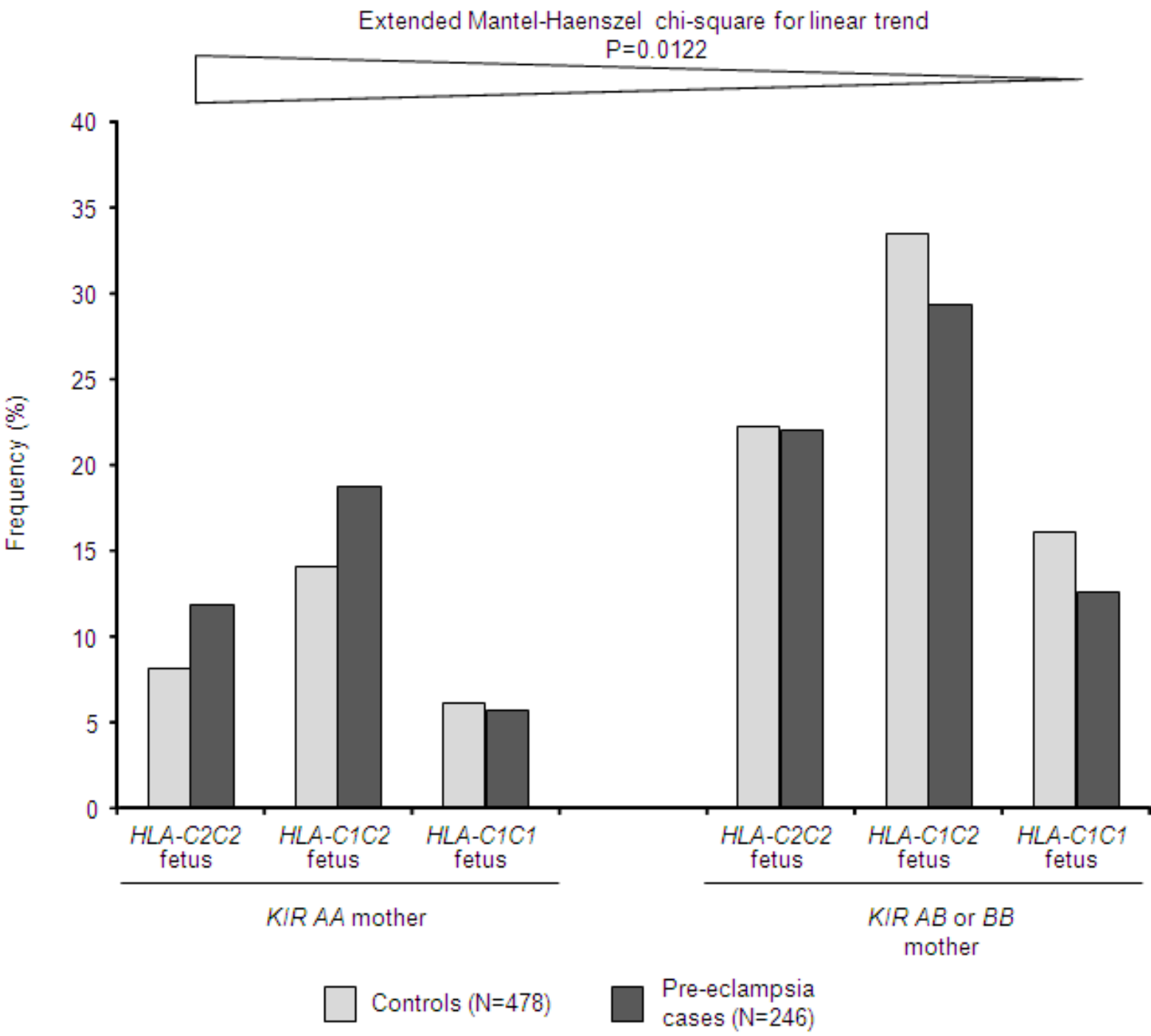
Extended Mantel-Haenszel chi-square for linear trend
P=0.0122
Frequency (%)
40
35
30
25
20
15
10
5
0
HLA-C2C2 fetus
HLA-C1C2 fetus
HLA-C1C1 fetus
HLA-C2C2 fetus
HLA-C1C2 fetus
HLA-C1C1 fetus
KIR AA mother
KIR AB or BB mother
Controls (N=478)
Pre-eclampsia cases (N=246)

Figure S2