Balancing Selection at *HLA-G* Modulates Fetal Survival, Preeclampsia and Human Birth Sex Ratio

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Abstract: The population sex ratio is thought to be maintained through balancing selection on rare phenotypes. However, empirical evidence for genetic influence has thus far proven elusive. We combined 1000 Genomes data and large cohorts to study human sex ratios. We found underrepresentation of male offspring in preeclampsia, a serious pregnancy disorder with uncertain pathogenesis. Genetic variation of fetal *human leukocyte antigen G* (*HLA-G*), regulating maternal anti-fetal immune responses, was found to be under balancing selection. Sex-linked downregulation of *HLA-G* and upregulation of *interferon alpha-1* (*IFNA1*) expression contribute to loss of fetal immunotolerance in preeclampsia and suggest hydroxychloroquine as a treatment option. Our findings indicate that an evolutionary trade-off between fetal immunotolerance and protection against infections promotes genetic diversity in *HLA-G*, thereby maintaining human sex ratios.

One Sentence Summary: Fetal *HLA-G* modulates human sex ratio.

Main Text: Whether human male-to-female sex ratios exhibit appreciable variation has been a subject of debate in the biological sciences. Recent large-scale data have confirmed an unbiased sex ratio at conception: equal numbers of X-bearing or Y-bearing sperm fertilize human eggs. However, the global birth sex ratio of 106 males for every 100 females, already seen in 20-week fetuses, indicates that more females are lost in early human pregnancies. In contrast, male fetuses might be at higher risk for late miscarriages and stillbirths *(1)*. Together, these findings suggest that undetermined fetal sex-specific mechanisms contribute to human pregnancy success and phenotypic variation in the sex ratio arises during pregnancy. Negative frequency-dependent selection has been proposed to keep sex ratios balanced *(2)*, however to our knowledge, there is a lack of empirical evidence across all species for a genetic link to sex ratio.

In humans, balancing selection at the human major histocompatibility (MHC) locus has been proposed to modulate a deficiency of human leukocyte antigen (HLA) homozygotes through maternal-fetal interaction *(3)*, without regard for sex-specificity. Increased HLA similarity in couples is associated with recurrent miscarriages, further supporting the need for the fetus to differ from maternal HLA *(4)*. Although the exact modifier locus remains unclear, only a limited pattern of HLA antigens (HLA-C, HLA-G, HLA-E, HLA-F) are expressed by fetal trophoblasts to prevent rejection by maternal immune cells *(5)*. Of these, HLA-G is the most studied due to its trophoblast-restricted expression and multiple isoforms inducing both local and systemic immunotolerance *(6)*.

Tens of studies, with still inconclusive results, have searched for the link between HLA-G, miscarriages, and the hypertensive pregnancy disorder preeclampsia *(7)*. While reduced HLA-G expression observed in the preeclamptic placenta is expected to facilitate maternal immune reactions to fetal alloantigens, its role in the pathogenesis is not yet clear *(8)*. Maternal immune maladaptation in preeclampsia, as one of the major theories, is supported by the shift from antibody-mediated T-helper 2 (Th2) and regulatory T cells (Treg) to cell-mediated T-helper 1 (Th1) responses, and by aberrant natural killer (NK) cell activity *(9, 10)*. This study

was motivated by the proposed but still disputed sex bias in preeclamptic births *(11)*, and particularly, the low male/female ratio in children of both women and men descending from a proband with preeclampsia *(12)*. We hypothesized that male fetal loss reflects failure in fetal HLA-G mediated immunotolerance in preeclampsia, stronger maternal immune responses towards male fetuses, and demonstrates the particular role of HLA-G in maternal-fetal interaction that affects fetal survival in human pregnancy.

We examined birth sex ratios (male/female) in a Finnish population cohort of 1.79 million live and stillbirths. This cohort included 38,752 preeclamptic births (2.2%). The sex ratio was 1.02 in preeclamptic and 1.05 in non-preeclamptic births (P=0.006), and the earlier the gestational age at birth, the lower the number of male offspring in preeclampsia (Spearman correlation coefficient, 0.80; P=0.007) (Fig. 1A). The sex bias was in striking agreement with Norwegian data on 1.82 million births and 44,000 preeclamptic pregnancies *(13)*, and with others *(14)*. Together with the data on unbiased human sex ratio at conception *(1)* and familial association of preeclampsia and miscarriages *(15)*, our results suggested male fetuses being lost before the onset of early preeclampsia, or linked to a higher risk of term disease, or both.

Figure 1.

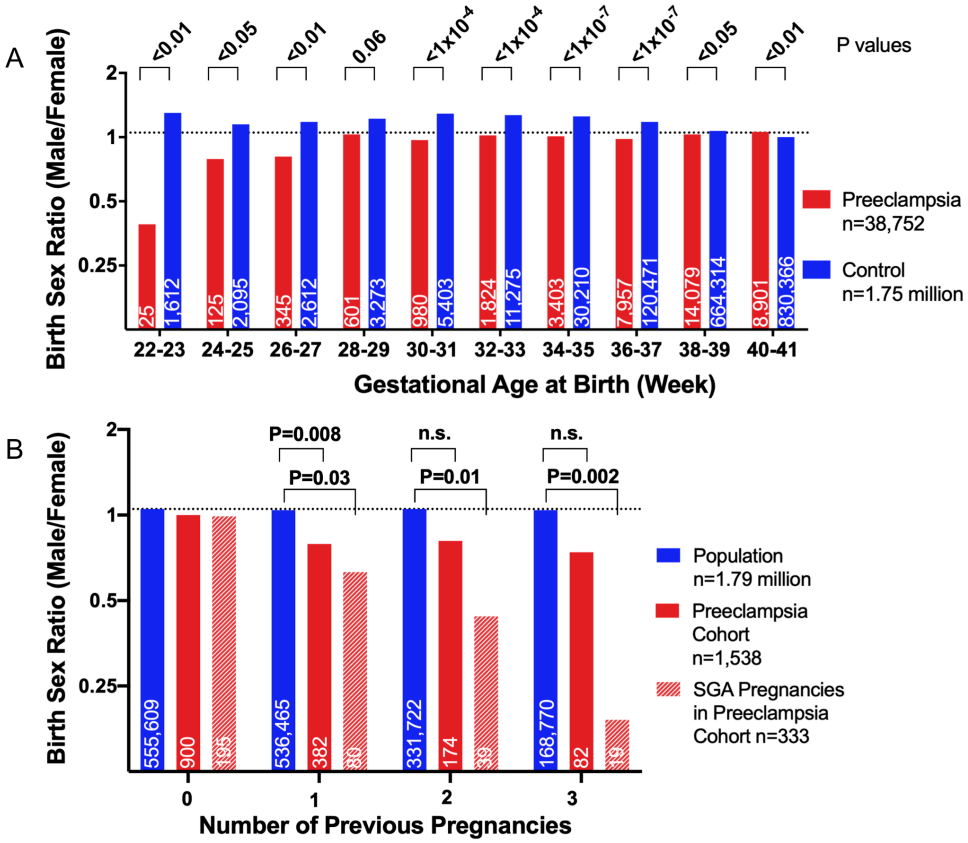

Fig. 1. Biased Birth-Sex Ratio in Preeclampsia. (A) The change from the female biased birth sex ratio in early preeclampsia to male biased in term disease, and the opposite pattern from male biased in early to balanced sex ratio in term births without preeclampsia. Preeclamptic births after 41 weeks were rare (n=512) and the sex ratio of 0.99 was comparable with that of the controls (data not shown). (B) The relationship between the number of previous pregnancies and the birth sex ratios in the Finnish population, in preeclamptic pregnancies of the FINNPEC series, and in a subgroup of preeclamptic pregnancies with small for gestational age (SGA) offspring. Numbers of individuals in each group are represented in the bars and P values (Chi-square test) for birth sex differences above the graphs. Dashed lines indicate the population birth sex ratio of 1.05 and n.s. denotes non-significant.

We studied the possible association between male fetal loss and preeclampsia. Using population-level data on women with one or more miscarriages, we demonstrated the reduced sex ratio of 0.98 in consecutive preeclamptic (n=6,469) compared with 1.04 in non-preeclamptic births (n=355,683; P=0.03). In the FINNPEC preeclampsia cohort (n=1,538), the higher number of previous pregnancies was associated with reduced number of male offspring in preeclamptic women, especially in pregnancies with small for gestational age (SGA) offspring (Fig. 1B). We also studied a Swedish stillbirth cohort (n=277), which showed increased intrauterine loss of male fetuses with the sex ratio of 1.18. Furthermore, pregnancy complications were overrepresented in male offspring in the Finnish preeclampsia family series *(16)*. Altogether, our results suggest that maternal immune reactions to fetal alloantigens, and stronger responses to the male-specific Y chromosomal histocompatibility (H-Y) antigen *(17)*, contribute to fetal survival and preeclampsia pathogenesis.

HLA-G downregulation is linked to its 3' untranslated regulatory region (3'UTR; Fig. S1) which is associated with distinct full-length haplotypes, and modulates mRNA stability and decay, microRNA targeting, and splicing *(6, 18)*. To uncover the link between *HLA-G* and the sex ratio, we studied *HLA-G* 3'UTR haplotype pairs, diplotypes, representing functional units. In the 1000 Genomes series (n=2504) *(19)*, sex ratios were modulated by the *HLA-G* 3'UTR diplotypes (Generalized linear model, GLM: $F_{21,24}$=2.27, P=0.03; Fig. S2). Rare diplotypes showed opposite patterns of sex ratios in preeclampsia offspring (Fig. 2A) and stillborn fetuses (Fig. 2B). The distribution of *HLA-G* 3'UTR diplotypes and the sex ratio differed between offspring from preeclamptic (Fig. 2A) and control pregnancies (Fig. 2C) in the FINNPEC series (GLM: $F_{13,21}$=9.15, P<0.001). We found a similar tendency in Africans versus Non-Africans in the 1000 Genomes data (GLM: $F_{1,24}$=8.47, P=0.008; Fig. S2), the Africans having the highest global prevalence of preeclampsia *(20)*. Altogether, rare diplotypes showed more variation in the sex ratio than the common ones, which were close to the balance of 1.0. These results support a pattern of negative frequency-dependent selection, a form of

balancing selection in which rare alleles are favored and the fitness of each allele decreases as its frequency increases *(21-23)*.

We further tested segregation of individuals homozygous for *HLA-G* 3'UTR (Fig. 2D). Decreased *HLA-G* homozygosity was observed in Africans (18.3% homozygous) versus Non-Africans (24.4%), and in the Swedish stillbirth cohort (18.4%) versus populations controls (25.7%), without sex-specific differences. Preeclamptic (26.8%) versus control offspring (26.3%) showed no differences in the overall homozygosity. However, homozygous males (31.7%; 35.2% in first pregnancies) versus females (22.3%; 21.4% in first pregnancies) were overrepresented in preeclamptic births in the FINNPEC cohort. The same association between homozygous male offspring and preeclampsia was observed in the preeclampsia family series *(16)*. Collectively, these results provide further support for balancing selection. While heterozygote advantage looks apparent for Africans, heterozygosity also shows an association with stillbirth in the Swedish cohort. In contrast, selection against *HLA-G* 3'UTR homozygous male offspring in preeclampsia supports the need for the fetus to differ from maternal HLA *(3)*, refines the locus for the findings of HLA similarity in couples with recurrent miscarriages *(4)*, and sheds light on the first-pregnancy predominance of preeclampsia *(24)*.

Figure 2.

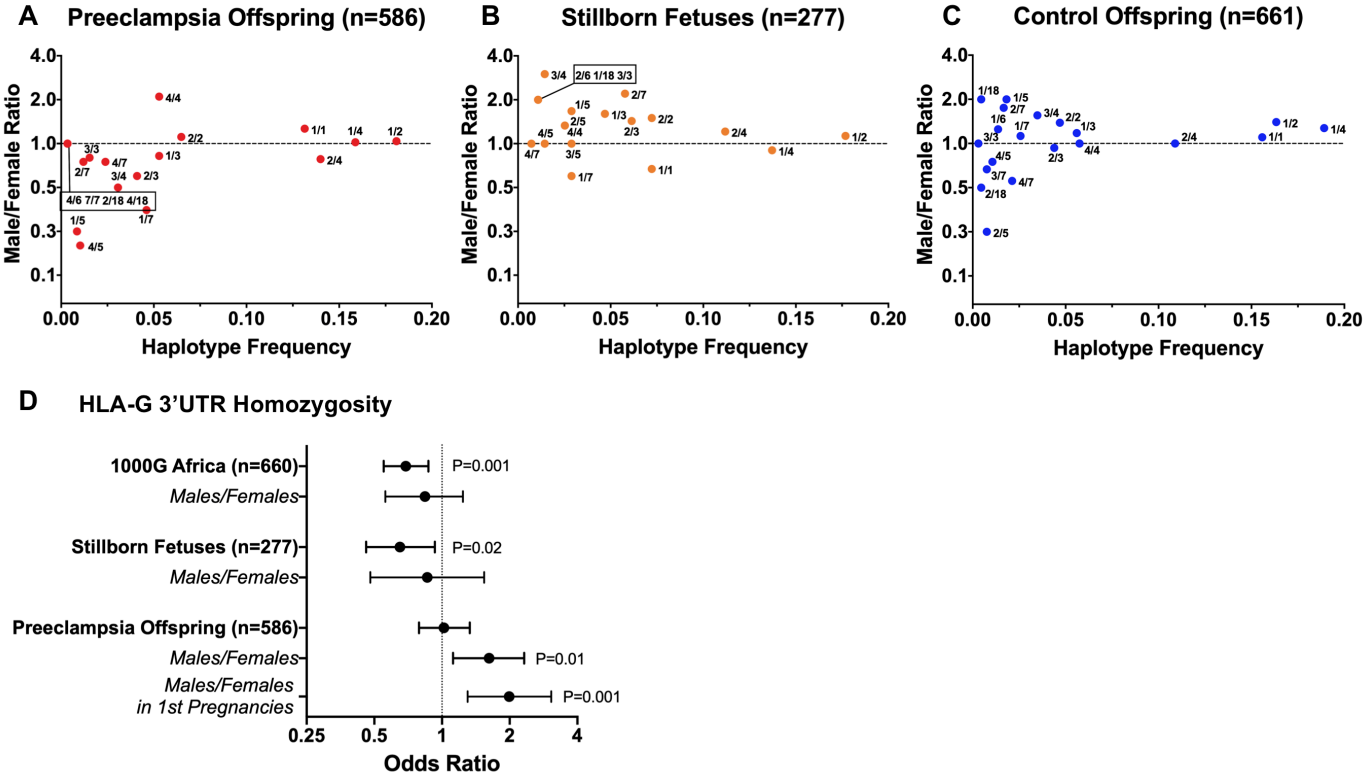

Fig. 2. *HLA-G* 3'UTR Haplotypes, Sex Ratio and Placental Expression. The distribution of *HLA-G* 3'UTR diplotypes (e.g. 1/2 defining the combination of UTR-1 and UTR-2) and the associated birth sex ratios in (A) preeclampsia, (B) stillborn fetuses, and (C) control offspring. The FINNPEC dataset (A and C) indicates that the *HLA-G* diplotype is the main determinant of the bias in the sex ratio (GLM; allele 1: $F_{5,21}$=0.44, P=0.82; allele 2: $F_{7,21}$=3.51, P=0.01; allele 1 by allele 2 interaction: $F_{13,21}$=9.15, P<0.001; haplotype frequency: $F_{1,21}$=0.67, P=0.42; maternal condition: $F_{1,21}$=3.58, P=0.07). Horizontal lines indicate the balanced sex ratio of 1.0. (D) The odds ratios (dots) and 95% confidence intervals (whiskers) for homozygous versus non-homozygous individuals for *HLA-G* 3'UTR. Only P values <0.05 are shown (Chi-Square test). 1000G= 1000 Genomes.

To support balancing selection on the HLA-G locus, we observed different frequencies of *HLA-G* 3'UTR diplotypes in offspring from preeclamptic and control pregnancies in the FINNPEC cohort: UTR-2 associated diplotypes dominated in preeclampsia (Fig. S3). The two major haplotypes, UTR-1 and UTR-2, differ for five polymorphic sites, mostly associate with the same HLA-G protein, and have global frequencies of 20-30% each *(18, 25)*. Of them, the evolutionarily most recent UTR-1 showed reduced frequency (P=0.01; OR 0.77, 95% CI: 0.63-0.94) and the ancestral haplotype UTR-2 increased frequency (P=0.01; OR 1.34, 95% CI: 1.07-1.69) in preeclamptic versus control offspring of first-time mothers. For UTR-2, the association remained when offspring from later pregnancies were included (P=0.04; OR 1.22, 95% CI: 1.01-1.47) (Table S1). The observed opposite effects of UTR-1 and UTR-2 on preeclampsia risk in first pregnancies support the advantage of divergent alleles and heterozygosity. Moreover, previous studies link UTR-2 to low expression, immune-mediated disorders, and pregnancy complications, and UTR-1 to high *HLA-G* expression *(6, 7)*. In line with this, the stillbirth cohort showed a similar but more significant protective effect of UTR-1 (P=0.0001; OR 0.65, 95% CI: 0.53-0.81). Conversely, UTR-5 was detected as a risk haplotype for stillbirth (P=0.03; OR 1.72, 95% CI: 1.06-2.79) (Table S2). UTR-5 is considered the oldest of all haplotypes and is closest to orthologous sequences in non-human primates *(25)*. We further confirmed that the 14 bp insertion polymorphism, previously implicated in pregnancy complications and present in haplotypes UTR-2, -5 and -7 *(7, 18)*, showed a modest association with both stillbirths (P=0.04; OR 1.24, 95% CI: 1.01-1.52) and preeclampsia (P=0.03; OR 1.21, 95% CI: 1.02 to 1.44).

To test the hypothesis of maternal immunoreactivity as the mechanism of fetal selection, we studied placental RNA expression of 136 genes (Fig. 3, Table S3, and Table S4), confirmed sex-linked *HLA-G* downregulation in preeclampsia (Fig. S4, Fig. S5, and S6), and found support for balancing selection (Fig. S7) *(16)*. No other immune-related genes showed compensatory effects. The marked upregulation of *interferon alpha-1* (*IFNA1*) in preeclampsia suggests that interferon signaling drives loss of fetal immunotolerance and might

be targeted by its inhibitors, such as hydroxychloroquine, in clinical trials of preeclampsia and miscarriages *(16)*.

Figure 3.

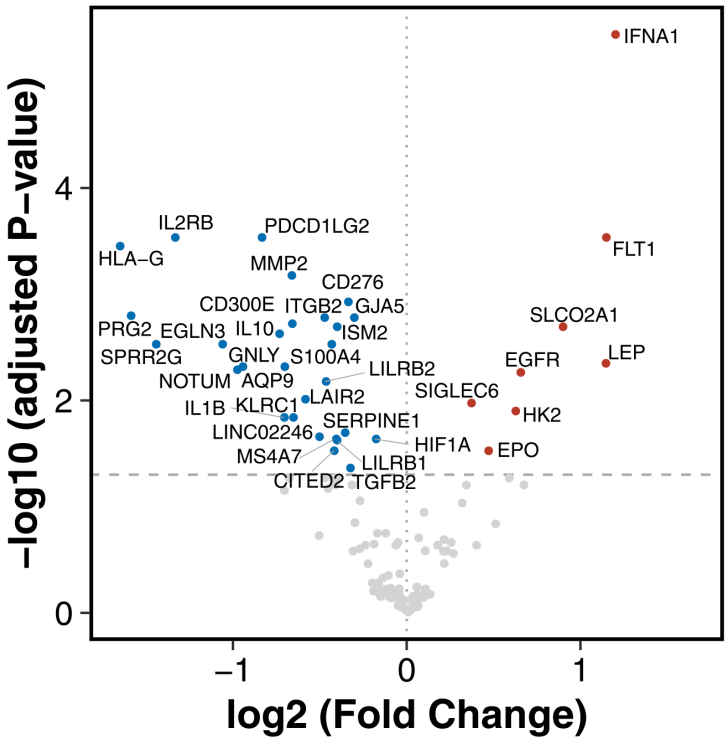

Fig. 3. Downregulation of *HLA-G* and Upregulation of *IFNA1* in Preeclampsia. Volcano plot of downregulated (blue dots) and upregulated (red dots) genes (P_{adj}<0.05) in placentas from women with severe preeclampsia (n=81) versus controls (n=63). Horizontal dashed line indicates P_{adj}=0.05 and vertical dotted line indicates no fold change.

We provide the first evidence of a gene modulating human sex ratio and support *HLA-G* as a sexually antagonistic locus determining maternal-fetal interaction and pregnancy success. Furthermore, we provide empirical evidence that balancing selection is acting on contemporary human populations and complicates human pregnancies. Balancing selection of *HLA-G* haplotypes clarifies the evolutionary paradox of preeclampsia and its highest prevalence in Sub-Saharan Africa *(26)*. How can a genetic trait potentially fatal for both the fetus and the mother show heritability estimates of 50% *(27)* and persist across human populations? We propose that low *HLA-G* expression, observed also in trophoblasts infected by *Plasmodium falciparum (28)* and some viruses (CMV, HSV) *(29)*, is advantageous in pathogen-rich environments to mount protective maternal immune responses, while concurrently detrimental, causing unfavorable maternal anti-fetal immune reactions, fetal loss, and preeclampsia *(16)*. In agreement with this scenario, ancestral fetal *HLA-G* haplotypes showed associations with preeclampsia and stillbirths in our series, thereby supporting their disadvantage in modern environments. Furthermore, high *HLA-G* expression is, however, associated with higher implantation rates *(30)* and uneventful pregnancies as presented here, but might confer an increased risk of offspring to malaria and other parasitic infections *(31, 32)*. These mechanisms might be in part mediated by the *fms-like tyrosine kinase 1* (*FLT1*) gene, as our results link balancing selection of *HLA-G* regulatory haplotypes also to asymmetry for placental *FLT1* expression. Notably, high fetal *FLT1* levels confer resistance against placental malaria and fetal loss *(33)*, and maternal hypertension and elevated sFLT1 levels are not specific to preeclampsia but arise in first-time mothers with placental malaria *(34, 35)*. To support their shared pathogenesis, placental malaria and preeclampsia show high rates and seasonal co-occurrence in Africa *(36)*, where both the *HLA-G* low-expressing "null allele" and high-expressing *FLT1* alleles are under positive selection *(33, 37)*, and the frequency and sex ratios associated with *HLA-G* 3'UTR haplotypes differ from other populations as shown here.

The majority of humans are HLA heterozygous for the need to provide more productive immune responses to pathogens *(3, 21)*. While *HLA-G* shows physiological expression almost uniquely in trophoblasts, its high expression that benefits the viability of fetuses during pregnancy may trade-off with *HLA-G* neoexpression, promoting immune evasion of viruses and malaria as well as tumors later in life *(6, 38)*. Therefore, the benefit of differential *HLA-G* expression may be dependent on the time and place, pathogens, and the tissue of expression, providing a role for balancing selection on immune-related traits beyond pregnancy in human evolution.

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Supplementary Materials:

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