HLA-G polymorphisms and soluble HLA-G protein levels in women with recurrent pregnancy loss from Basrah province in Iraq

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| Complete List of Authors: | Jassem, Raghed; University of Basrah, Basic Science, Dentistry College  
                          | Shani, Wafaa; University of Basrah, Biology, Science College  
                          | Loisel, Dagan; University of Chicago, Human Genetics  
                          | Sharief, Maysoon; University of Basrah, Gynecology and Obstetrics, Medicine College  
                          | Billstrand, Christine; University of Chicago, Human Genetics  
                          | Ober, Carole; University of Chicago, Obstetrics and Gynecology  |
| Keywords:           | HLA-G, recurrent pregnancy loss, soluble HLA-G, HLA-G genotype |
| Specialty:          | Reproductive Genetics |
**Title:** HLA-G polymorphisms and soluble HLA-G protein levels in women with recurrent pregnancy loss from Basrah province in Iraq

**Running Title:** HLA-G in Iraqi Women with RPL

**Authors:** Ragheed M. Jassem\(^1,^+\), Wafaa Sadoon Shani\(^2,^+\), Dagan A. Loisel\(^3,^+\), Maysoon Sharief\(^4\), Christine Billstrand\(^3\), and Carole Ober\(^3,5,*\)

\(^1\)Basic Science Department, Dentistry College, \(^2\)Biology Department, Science College, \(^4\)Gynecology & Obstetrics Department, Medicine College, University of Basrah, Basrah, Iraq
\(^3\)Department of Human Genetics, \(^5\)Department of Obstetrics & Gynecology, The University of Chicago, Chicago, IL, USA

\(^+\)These authors contributed equally.

\(*\)Address Correspondence to:

C. Ober

Departments of Human Genetics and Obstetrics & Gynecology

The University of Chicago

920 E. 58\(^{th}\) Street, Room 425

Chicago, IL 60637

TEL: 773-834-0753

FAX: 773-834-0505

EMAIL: c-ober@genetics.uchicago.edu
Abstract

Background: HLA-G is a nonclassical, class I major histocompatibility complex (MHC) gene that is constitutively expressed on placental cytotrophoblasts at the maternal-fetal interface, and likely plays a role in the maintenance of successful pregnancy. In this study, we investigated the role of HLA-G polymorphisms on risk for recurrent pregnancy loss (RPL) and on circulating levels of soluble (s)HLA-G in Iraqi women.

Methods: Blood samples were collected at 9 to 12 weeks gestation from 50 women with RPL and 50 healthy pregnant women seeking medical care in Basrah province, Iraq. DNA from these subjects was genotyped for six HLA-G polymorphisms that define eight of the most common alleles (or haplotypes). sHLA-G was measured in plasma collected in the first trimester of pregnancy.

Results: Median sHLA-G levels were significantly lower in the RPL cases compared to healthy controls (21.4 vs. 38.8 U/ml, respectively; \(P = 0.025\)), and decreased with increased maternal age \( (P = 0.0051)\). However, the frequencies of alleles at the six polymorphic sites or of the seven HLA-G haplotypes did not differ significantly between cases and controls \((P \geq 0.15\) and 0.15, respectively). In contrast, homozygosity for the C allele (CC) at a tri-allelic promoter polymorphism, \(-725C/G/T\), was associated with lower concentrations of sHLA-G compared to women with the CG or CT genotypes (median levels 21.1 vs. 40.1 vs. 42.6 U/ml, respectively; \(P = 0.0089\)). Genotype effects on sHLA-G levels were present in both the RPL cases and healthy controls, and were independent of maternal age.

Conclusions: HLA-G genotype and maternal age independently influence circulating concentrations of sHLA-G during the first trimester of pregnancy in Iraqi women. However,
neither HLA-G genotype nor sHLA-G levels in the first trimester of pregnancy were likely causes of pregnancy loss in these women.

Key Words: HLA-G, recurrent pregnancy loss, soluble HLA-G, HLA-G genotype
Maternal and fetal immune cells are in close contact during pregnancy, with apparent tolerance of maternal cells toward the fetus and vice versa. It has been suggested that HLA-G is an immunomodulatory molecule that contributes toward this tolerance, although the precise mechanisms are not completely known (Carosella et al., 2008; Hunt et al., 2005; Hviid, 2006).

HLA-G is considered a “non-classical” class I HLA due to its limited coding region polymorphism and restricted tissue distribution compared to classical class I HLA (HLA-A, HLA-B, HLA-C). Moreover, HLA-G transcripts have the unique property among HLA genes in that they undergo alternative splicing to generate at least seven transcripts and four protein isoforms (Fujii et al., 1994; Ishitani and Geraghty, 1992; Morales et al., 2003). Two soluble isoforms, referred to as G5 and G6, are present at relatively high levels in the maternal circulations during pregnancy (Hunt et al., 2000; Rizzo et al., 2009; Steinborn et al., 2007). Decreased levels of sHLA-G have been associated with poor implantation rates in in vitro fertilization (Rizzo et al., 2007), increased risk for pregnancy loss (Pfeiffer et al., 2000), and preeclampsia (Hackmon et al., 2007; Rizzo et al., 2009; Steinborn et al., 2007; Yie et al., 2005) in most, but not all (Steinborn et al., 2003), studies. In addition, specific polymorphisms in the HLA-G gene have been associated with sHLA-G levels (Chen et al., 2008; Hviid et al., 2004b) and with pregnancy outcomes (sporadic miscarriage, RPL, preeclampsia) (Aldrich et al., 2001; Hviid et al., 2004a; Larsen et al., 2010; Ober et al., 2003; Pfeiffer et al., 2001; Tan et al., 2008; Yan et al., 2006b; Yie et al., 2008) in some, but not all (Aldrich et al., 2000; Iversen et al., 2008; Lin et al., 2006; Vianna et al., 2007), studies.

To date, however, interrogation of multiple HLA-G polymorphisms, sHLA-G concentrations, and clinical outcomes has not been considered in the same study population. In
addition, nearly all studies of HLA-G and clinical outcomes have focused on women of
European or European American descent, even though the frequencies of HLA-G polymorphisms
differ considerably between racial and ethnic groups and minor allele frequencies for some
variants are too rare in European populations to assess their effects on sHLA-G levels or
pregnancy outcome. Therefore, comprehensive studies of HLA-G genotypes, sHLA-G levels, and
clinical outcomes in ethnically diverse populations are required to assess the functional or
clinical effects of all HLA-G variants.

To address these gaps and to comprehensively survey the effects of genetic variation in
HLA-G on both circulating levels of sHLA-G in the first trimester of pregnancy and RPL, we
initiated studies in women from the Basrah Province of Iraq, a population that has not previously
been studied for RPL, HLA-G polymorphisms, and sHLA-G concentrations.
Methods

Sample Composition

Women (N=50) classified as having recurrent pregnancy loss (RPL), defined by two or more consecutive miscarriages of less than 20 weeks gestation and a negative RLP evaluation, were recruited from the Obstetrics Unit at the Basrah Maternity and Childrens Hospital when they presented to Emergency with a miscarriage that became symptomatic between 9 and 12 weeks gestation. On evaluation, these women had normal levels of serum progesterone levels (>10 ng/mol) and normal thyroid function (T3 between 0.9-2.5 mIU/ml; T4 between 60-120 mIU/ml); antiphospholipid antibodies, anticardiolipin antibodies, antinuclear antibodies, and TORCH (toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus) studies were negative. These women were treated with either low dose aspirin or progesterone during the index pregnancy.

Control women (N=50) were healthy pregnant women, recruited between 9 and 12 weeks gestation from the same hospital as the women with RPL, and who delivered the index pregnancy at term. Controls had at least one child and no previous miscarriages, preterm deliveries, or stillbirths.

Three milliliters of EDTA-anticoagulated venous blood was collected from each woman with RPL prior to the infusion of intravenous fluids and from each control woman. Plasma was removed following centrifugation for 5 minutes at 3000 rpm and frozen in 100 μL aliquots. DNA was extracted from the remaining cells, using the phenol-chloriform extraction protocol of Sambrook et al. (Sambrook et al., 1989), and stored frozen. Frozen plasma and DNA were shipped on dry ice from Basrah, Iraq to Chicago for genotyping and sHLA-G studies.
All women signed informed consent. This study was approved by the Training and Development Division in the Basrah Health Office and the University of Basrah, and the Institutional Review Board at the University of Chicago.

**HLA Genotyping**

DNA samples were genotyped for six polymorphisms (Fig. 1) by SNaPshot (Applied Biosystems, Carlsbad, CA), using a modification of the protocol of Tan *et al.* (2008) that included the +3142G/C variant (Tan *et al.*, 2007). The six polymorphisms genotyped were (i) the -725C/G/T promoter variant (rs1233334) that has been associated with sporadic miscarriage (Ober *et al.*, 2003) and expression differences in reporter assays (Ober *et al.*, 2006), (ii) the +36G/A variant (rs1630185) in the untranslated exon 1 that tags two major *HLA-G* promoter haplotypes (Tan *et al.*, 2005) (iii) the 3’UTR 14bp insertion/deletion (indel) polymorphism (rs66554220) that has been associated with *HLA-G* transcript levels (Hviid *et al.*, 2003), circulated sHLA-G levels (Chen *et al.*, 2008), and preeclampsia (Larsen *et al.*, 2010; Moreau *et al.*, 2008), (iv) the 1597ΔC (1 bp deletion; rs41557518) in exon 3 that results in a null allele with respect to protein expression (Ober *et al.*, 1998) and has been associated with RPL (Aldrich *et al.*, 2001; Pfeiffer *et al.*, 2001), (v) the Thr258Met variant (rs12722482) that has been associated with preeclampsia (Moreau *et al.*, 2008; Tan *et al.*, 2008), and (vi) the +3142G/C variant (rs1063320) in the 3’UTR that disrupts a micro(mi)RNA target site and influences *HLA-G* expression levels in the presence of the miRNAs (Tan *et al.*, 2007).

These six variants define the following eight *HLA-G* alleles (referred to here as haplotypes) (Table I): G*010101 (not distinguishable here from the rare G*010108 and G*010104 haplotypes), G*010101b,c (distinguished from the common G*010101 by the

http://humrep.oupjournals.org
presence of the -725G and/or -1141T variants as described in (Ober et al., 2003; Tan et al., 2005),

$G^*010102, G^*010103, G^*0103 G^*0104, G^*0105N, G^*0106$. These $HLA-G$ haplotypes were

assigned to each woman manually based on the known allelic composition of the six variants on

each haplotype (Larsen et al., 2010; Ober et al., 1996; Tan et al., 2005). In three women (one

case, two controls), the genotypes at the six polymorphic sites were not consistent with two

known haplotypes, representing either genotyping error or identification of previously

unreported haplotypes. Because of limited DNA availability we could not differentiate between

the three possibilities, and these samples were excluded from analyses of haplotypes.

Measurements of sHLA-G Concentrations

sHLA-G ELISA kits were purchased from EXBIO (Vestec, Czech Republic)/BioVendor (Brno,

Czech Republic) for measurement of soluble G5 and shed transmembrane G1 in plasma samples,

according to the manufacturer’s instructions. All samples were run in duplicate; and mean

absorbances, measured at a wavelength of 450 nm, were determined for each subject. Calibration

curves based on the absorbance of calibrators of known concentration were used to determine the

concentration of sHLA-G in each sample.

Statistical Analyses

Frequencies of alleles at each of the six variants and for each of the eight haplotypes were

compared between RPL cases and healthy controls by the Pearson $\chi^2$ test, or a Fisher exact test if

cell counts were <5. Associations between individual polymorphisms or haplotypes and sHLA-G

levels were evaluated by nonparametric methods, using either the Wilcoxon rank sum test (to

compare 2 groups) or the Kruskal-Wallis test (to compare 3 or more groups). The effective

number of independent tests performed in the analysis of genetic associations with sHLA-G was
estimated given the correlation structure among polymorphisms using Li and Ji’s method (2005),
as implemented in the matrix spectral decomposition (matSpD) program (Nyholt, 2004). Li and
Ji’s method indicated that the six polymorphisms in HLA-G represented 5.19 independent
variables. Significant $P$-values from the tests of genetic associations were therefore corrected for
5.19 tests using the Bonferroni correction and are presented as $P_{\text{corrected}}$. Linear regression was
used to test for an association between log sHLA-G concentration and maternal age, and the
direction and strength of this association was estimated using the Pearson product-moment
correlation coefficient ($r$). Multivariate analysis of the combined effect of maternal age, genotype,
and RPL status on sHLA-G levels was performed on log-transformed sHLA-G measurements using a standard least squares regression. Analyses were performed using JMP software (SAS Institute Inc., Cary, NC), version 8.0.2.2. $P$-values < 0.05 were considered
significant.
Results

The RPL cases and healthy controls included in this study are described in Table II. Cases had significantly more pregnancies on average compared to the control women (mean 4.3 vs. 3.4, respectively), whereas control women had significantly more live born children on average compared to the RPL cases (mean 2.4 vs. 0.42, respectively). Circulating levels of sHLA-G in the first trimester were significantly lower in RPL cases compared to controls (median concentration = 21.3 U/ml and 38.8 U/ml, respectively; Wilcoxon rank sum test, \( P = 0.025 \)) (Fig. 2). Log sHLA-G concentration was also significantly negatively correlated with maternal age (F ratio = 8.2, \( r = -0.28 \), \( P = 0.0051 \)) (Supplementary data, Fig. S1), but not with number of prior pregnancies or live births (\( P > 0.50 \); data not shown).

We were able to successfully genotype 49 RPL cases and 48 controls. The genotype counts at each of the six variants were in Hardy-Weinberg equilibrium in cases only, in controls only, and in the combined sample (data not shown). The frequencies of alleles at each polymorphic site in the cases and controls are shown in Table III. None of the allele frequencies were significantly different between the two groups, although there was a trend toward a higher frequency of the insertion allele at the 14bp indel in the cases compared to controls (0.62 vs. 0.51; \( P = 0.12 \)).

The frequencies of each of the eight haplotypes in cases, controls and the combined sample of Iraqi women are shown in Table IV. Overall, the \( G^{*}010101 \) and \( G^{*}010102 \) haplotypes were the most common haplotypes, similar to other populations. However, all other haplotypes were relatively more frequent in the Iraqi women compared to many other populations (Hviid, 2006; Ober et al., 2003; Tan et al., 2005). For example, the frequency of the \( G^{*}0105N \) haplotype (carrying the null 1597\( \Delta C \) allele) was 0.083, among the highest frequency reported in populations of non-African ancestry (Hviid, 2006; Lin et al., 2009; Ober and Aldrich, 1997), and
the frequency of the G*0103 haplotype (carrying the -725T and the Thr31Ser alleles) was 0.072, also among the highest ever reported (Hviid, 2006). Nonetheless, haplotype frequencies did not significantly differ between cases and controls; although the relatively rare G*010103 haplotype occurred in six cases and only one control ($P = 0.12$) and the G*0104 haplotype was relatively more common in controls compared to cases ($P = 0.12$).

We next examined associations between HLA-G genotype and sHLA-G concentrations (Table V). Only one polymorphism, -725C/G/T, was significantly associated with sHLA-G levels in the combined sample (Kruskal-Wallis test; $P = 0.0089$); this association remained significant after Bonferroni correction ($P_{\text{corrected}} = 0.046$) (Fig. 3). The concentration of sHLA-G was lowest among women with the common CC genotype (median = 21.1 U/ml) compared to women with the CG (median = 40.1 U/ml) and CT (median 42.6 U/ml) genotypes (no women were homozygous for the G or T alleles). This pattern of association was similar in both the RPL cases and healthy controls.

Lastly, we assessed the combined effects of -725C/G/T genotype, maternal age, and case/control status on sHLA-G concentrations by linear regression. When tested individually in univariate regression analyses, the -725 genotype ($P = 0.0023$) and maternal age ($P = 0.0051$) were significantly associated with log sHLA-G concentration, while the effect of case/control status approached statistical significant ($P = 0.056$). The multivariate model that included all three of these predictor variables was highly significant (F ratio = 7.57, $P$-value = $1.37 \times 10^{-4}$), explaining 19.6% of the variance in log sHLA-G levels. Notably, both genotype and maternal age effects on log sHLA-G concentration remained significant in the multivariate model ($P = 0.012$ and $P = 0.0017$, respectively), whereas the effect of RPL status on log sHLA-G levels failed to achieve statistical significance ($P = 0.066$) when -725 genotype and maternal age are
included in the model. In the multivariate model, maternal age and -725 genotype account for 5.7% and 9.0%, respectively, of the total variance in log sHLA-G concentration. Overall, these results indicate that HLA-G genotype and maternal age independently influence circulating concentrations of sHLA-G during the first trimester of pregnancy in Iraqi women.
Discussion

We present here the first study of HLA-G in Iraqi women and examine the effects of genotypes on RPL and circulating levels of sHLA-G. We report a significant association between an HLA-G promoter polymorphism, -725C/G/T, and sHLA-G concentrations in the first trimester of pregnancy in women with RPL and in healthy controls. We show, in addition, that sHLA-G concentrations significantly decrease with increasing maternal age. Perhaps not surprisingly, sHLA-G concentrations were lower in women experiencing a miscarriage. However, because -725C/G/T genotype frequencies did not differ between RPL cases and healthy controls and the effect of pregnancy status (RPL case versus control) was reduced when genotype and maternal age were included in a multivariate model, we suggest that reduced concentrations of circulating sHLA-G in the RPL cases resulted from the miscarriage but were not causing the pregnancy loss.

There have been few studies of the HLA-G -725C/G/T polymorphism, but as part of a comprehensive study of variation in the promoter region of HLA-G, we previously reported an association between the -725G allele and sporadic miscarriage in fertile couples (the -725T allele was not surveyed at that time) (Ober et al., 2003). We subsequently demonstrated that the G allele was associated with increased HLA-G expression in a reporter assay (Ober et al., 2006), consistent with the findings in the current study (Fig. 3). In our earlier study, we reported that constructs carrying the T allele were not higher expressers in untreated JEG3 (placental) cells or in cells treated with IFN-β or with a CpG methylase (M. SssI). However, luciferase expression was 2-3 times higher for constructs carrying either the -725G or -725T alleles compared to those with the -725C allele in JEG3 cells treated with both IFN-β and M. SssI (see Figure 2D in Ober et al. 2006). That is, similar to the results in the current study, all promoters carrying the common -725C allele were associated with lower expression compared to promoters carrying
either the -725G or -725T alleles, under specific conditions. Although we do not know the
mechanism accounting for the genotype-specific effects on circulating sHLA-G concentrations in
the current study, these earlier experiments indicate that -725 alleles influence gene expression
differently in different cellular environments. The current study further shows that women who
are homozygous for the C allele are the lowest expressers of sHLA-G in the first trimester of
pregnancy.

Most previous studies of HLA-G genotypes and sHLA-G or HLA-G genotypes and
pregnancy outcomes were conducted in populations of European descent. Yet, most of the
known HLA-G alleles and haplotypes are relatively rare in European populations. As a result,
assessing the effects of the less common haplotypes or alleles on clinical phenotypes requires
studying non-European populations. This fact has been appreciated in studies of the null 1597ΔC
allele, which defines the G*0105N haplotype, because this allele occurs at relatively high
frequencies (0.05-0.12) in populations of African descent, but is quite rare in European and east
Asian populations (0-0.05) (Aldrich et al., 2001; Ishitani et al., 1999; Matte et al., 2000; Ober et
al., 1998; Tian et al., 2010; van der Ven et al., 1998). Surprisingly, the frequency of this allele is
also quite high in Iraqi women (0.083). This is consistent with recent reports of strikingly high
frequencies (0.18 and 0.14) of the 1597ΔC allele in Iranian and east Indian populations,
respectively (Abbas et al., 2004; Rahimi et al., 2010). It is possible, therefore, that this variant
occurs at highest frequency in the Middle East and South Asia, and not in Africa, as previously
thought (Aldrich et al., 2002; Ishitani et al., 1999).

Given the high frequency of the 1597ΔC allele in Iraqi women, it was unexpected that
this allele was not associated with sHLA-G concentrations because it is a proven null allele for
the G1 and G5 isoforms (Ober et al., 1998), which are the two isoforms measured by the ELISA
used in this study. This null allele has been associated with RPL in European and European American women (Aldrich et al., 2001; Pfeiffer et al., 2001), but not in Han Chinese women (Yan et al., 2006a), reflecting the heterogeneous nature of RPL and suggesting that other factors might compensate for low levels sHLA-G, as in our study. In contrast, there have been no previous studies of this polymorphism and circulating concentrations of sHLA-G using an HLA-G-specific ELISA. It is possible that maternal genotype for the null allele is not predictive of circulating levels or that there is compensation by the second, non-G*10105N allele in heterozygous individuals, both of which would reduce power to detect associations between the null allele and sHLA-G concentrations. Additional studies of both maternal and fetal genotypes, and in larger sample sizes will be necessary to evaluate these hypotheses.

Two other haplotypes occur at high frequencies in Iraqi women compared to European populations. The G*0106 haplotype, carrying the Met258Thr variant, occurs at frequencies <0.07 in most European populations (Hviid et al., 2001; Moreau et al., 2008; Ober et al., 2003; Sipak-Szmigiel et al., 2009), whereas the frequency of this haplotype in Iraqi women is 0.12. The -725T allele occurs at frequencies of 0.022 in European Americans and 0.102 in African Americans (Tan et al., 2005), and 0.12 in Iraqi women. In contrast, the -725G allele occurs at frequencies of 0.12 in European Americans and 0.068 in African Americans (Tan et al., 2005), and 0.11 in Iraqi women. As a result, the -725 CC genotype is less common in Iraqi women compared to either European American or African American subjects.

This study provides novel insights into the regulation of circulating levels of sHLA-G in early pregnancy and clinical outcome. First, the lack of an association between the -725 CC genotype and RPL, despite the observation of significantly lower sHLA-G concentrations in CC women, suggests that constitutively low levels of sHLA-G due to -725 genotype is not a cause of
recurrent pregnancy loss in these women. This would be consistent with the observation that CC is the most common genotype at this site, and indicates that the genotype-specific differences observed in this study are likely well within the range required for successful implantation and maintenance of pregnancy. Interestingly, even at 9-12 weeks of gestation in spontaneously terminating pregnancies, the genotype effects on sHLA-G concentrations are still apparent. Whether these genotype effects on sHLA-G concentrations remain throughout pregnancy, or whether they predict outcomes later in pregnancy remains to be determined. Second, we report an unexpected association between sHLA-G concentrations and maternal age that is independent of the HLA-G genotype effect. This observation needs to be replicated in additional studies, and examined in later trimesters of pregnancy. If confirmed, reduced concentrations of sHLA-G in the first trimester in older mothers may be a contributory mechanism, and a possibly marker, for the increased risk for adverse pregnancy outcomes in later pregnancy, such as preeclampsia and preterm birth, among these women (Cleary-Goldman et al., 2005; Cnattingius et al., 1992; Fretts et al., 1995), a hypothesis that can be examined in future prospective studies.

In summary, the relationship between HLA-G genotype, circulating sHLA-G concentrations in the first trimester, and adverse pregnancy outcomes remains complex. This study identifies two independent determinants of sHLA-G concentrations in the first trimester of pregnancy, including genotype at a promoter polymorphism, -725C/G/T, that was previously demonstrated to have functional effects on expression (Ober et al., 2006), and maternal age, a well established risk factor for adverse pregnancy outcomes throughout pregnancy. Further studies are required to elucidate the clinical effects of these observations throughout pregnancy and in ethnically diverse women.
Supplementary Data

Supplementary data are available at http:

Authors’ roles

R.M.J., W.S.S., D.A.L., M.S. and C.O. were involved overall study design, execution, and presentation. R.M.J., W.S.S. and M.S. performed the clinical activities, patient consenting, and acquisition of biological samples. R.M.J., D.A.L. and C.B. completed the genotyping and functional experiments. D.A.L., R.M.J. and C.O. performed the data processing and statistical analysis. R.M.J., W.S.S., C.O., M.S. and D.A.L. wrote and revised the manuscript. All authors contributed critical discussion, manuscript review, and gave final approval of the version to be published.

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Table I. Associations between six genotyped variants and eight imputed *HLA-G* haplotypes.

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<th>Haplotype</th>
<th>HLA-G Polymorphisms</th>
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<td>-725C/G/T</td>
</tr>
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<td>G<em>0101</em></td>
<td>C</td>
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<tr>
<td>G*010101b,c</td>
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<td>C</td>
</tr>
<tr>
<td>G*0106</td>
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*Includes the 010101, 010104, and 010108 haplotypes that cannot be differentiated by these six polymorphisms.*
Table II. Characteristics of the study sample. Means were compared by t-test; *P*-values are shown.

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<th>RPL Cases</th>
<th>Controls</th>
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<td>Births ± SD (Range)</td>
<td>0.42 ± 0.64</td>
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Table III. Frequencies of alleles for six HLA-G polymorphisms in 49 RPL cases and 48 healthy controls. Minor allele frequency differences between RPL cases and controls were assessed using the Pearson chi-square test in 2x2 contingency tables.

<table>
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<tr>
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<tr>
<td></td>
<td>Δ</td>
<td>0.071</td>
<td>0.094</td>
<td>0.57</td>
</tr>
<tr>
<td>Thr258Met</td>
<td>Thr</td>
<td>0.87</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Met</td>
<td>0.13</td>
<td>0.14</td>
<td>1.00</td>
</tr>
<tr>
<td>14bp indel</td>
<td>Ins</td>
<td>0.62</td>
<td>0.51</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Del</td>
<td>0.38</td>
<td>0.49</td>
<td>0.12</td>
</tr>
<tr>
<td>+3142G/C</td>
<td>G</td>
<td>0.69</td>
<td>0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>0.31</td>
<td>0.32</td>
<td>0.81</td>
</tr>
</tbody>
</table>
Table IV. Frequencies of eight HLA-G haplotypes in RPL cases (N=49), healthy controls (N=48), and the pooled sample (N=97). Frequency differences between cases and controls were assessed using the Pearson chi-square test in 2x2 contingency tables.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>RPL Cases</th>
<th>Controls</th>
<th>Pooled Sample</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>G*0101</td>
<td>0.22</td>
<td>0.20</td>
<td>0.21</td>
<td>0.65</td>
</tr>
<tr>
<td>G*010101b,c</td>
<td>0.092</td>
<td>0.13</td>
<td>0.11</td>
<td>0.46</td>
</tr>
<tr>
<td>G*010102</td>
<td>0.24</td>
<td>0.24</td>
<td>0.24</td>
<td>0.92</td>
</tr>
<tr>
<td>G*010103</td>
<td>0.061</td>
<td>0.010</td>
<td>0.036</td>
<td>0.12</td>
</tr>
<tr>
<td>G*0103</td>
<td>0.092</td>
<td>0.052</td>
<td>0.072</td>
<td>0.29</td>
</tr>
<tr>
<td>G*0104</td>
<td>0.092</td>
<td>0.17</td>
<td>0.13</td>
<td>0.12</td>
</tr>
<tr>
<td>G*0105N</td>
<td>0.071</td>
<td>0.094</td>
<td>0.082</td>
<td>0.57</td>
</tr>
<tr>
<td>G*0106</td>
<td>0.12</td>
<td>0.11</td>
<td>0.12</td>
<td>0.86</td>
</tr>
</tbody>
</table>
**Table V.** HLA-G genotype association with sHLA-G concentration in the pooled sample (N=97).

*P*-values were obtained for each polymorphism using either the Wilcoxon rank sum test or the Kruskal-Wallis test.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Genotype</th>
<th>Sample size</th>
<th>Median sHLA-G, U/ml (interquartile range)</th>
<th><em>P</em>-value for genotype association</th>
</tr>
</thead>
<tbody>
<tr>
<td>-725C/G/T</td>
<td>CC</td>
<td>66</td>
<td>21.1 (11.9-49.1)</td>
<td>0.0089</td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>18</td>
<td>40.1 (22.6-67.0)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>13</td>
<td>42.6 (22.7-76.3)</td>
<td></td>
</tr>
<tr>
<td>+36G/A</td>
<td>AA</td>
<td>37</td>
<td>23.6 (13.5-50.4)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AG</td>
<td>41</td>
<td>32.7 (13.6-61.6)</td>
<td>0.87</td>
</tr>
<tr>
<td></td>
<td>GG</td>
<td>19</td>
<td>28.1 (17.3-47.3)</td>
<td></td>
</tr>
<tr>
<td>1597ΔC</td>
<td>CC</td>
<td>81</td>
<td>28.1 (14.6-55.5)</td>
<td>0.73</td>
</tr>
<tr>
<td></td>
<td>CΔ</td>
<td>16</td>
<td>28.0 (11.8-47.5)</td>
<td></td>
</tr>
<tr>
<td>Thr258Met</td>
<td>Thr/Thr</td>
<td>74</td>
<td>26.2 (14.3-48.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Thr/Met</td>
<td>20</td>
<td>34.4 (15.1-69.7)</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Met/Met</td>
<td>3</td>
<td>21.3 (14.0-49.7)</td>
<td></td>
</tr>
<tr>
<td>14bp indel</td>
<td>Ins/Ins</td>
<td>36</td>
<td>25.2 (13.8-49.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ins/Del</td>
<td>38</td>
<td>28.0 (14.3-62.7)</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>Del/Del</td>
<td>23</td>
<td>28.6 (16.9-28.58)</td>
<td></td>
</tr>
<tr>
<td>+3142G/C</td>
<td>GG</td>
<td>49</td>
<td>26.8 (13.7-51.5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CG</td>
<td>35</td>
<td>32.4 (14.6-56.7)</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>13</td>
<td>24.2 (17.1-42.0)</td>
<td></td>
</tr>
</tbody>
</table>
1. HLA-G gene structure and location of six polymorphisms included in this study.

2. Soluble HLA-G plasma concentrations in RPL cases and controls. sHLA-G concentration differed significantly between RPL cases and controls (Wilcoxon rank sum test, $P = 0.025$). Boxes show interquartile range, horizontal lines show the median values and whiskers extend an additional 1.5 interquartile ranges from the boxes.

3. Soluble HLA-G plasma concentrations by -725C/G/T genotype. sHLA-G concentration was significantly associated with -725 genotype (Kruskal-Wallis test, $P = 0.0089$). Boxes show interquartile range, horizontal lines show the median values and whiskers extend an additional 1.5 interquartile ranges from the boxes. RPL cases are indicated by solid red diamonds and controls by open blue diamonds.
The graph shows the distribution of sHLA-G concentration (U/ml) in control women (n=50) and RPL women (n=50). The box plot indicates that the median concentration of sHLA-G is higher in control women compared to RPL women.
Supplementary Data

**Supplementary Figure S1.** Soluble HLA-G plasma concentration in the first trimester as a function of maternal age

![Graph showing soluble HLA-G plasma concentration as a function of maternal age.](image)

Log sHLA-G concentration was significantly negatively associated with maternal age (linear regression: $F$ ratio = 8.2, $r = -0.28$, $P = 0.0051$). RPL cases are indicated by solid red diamonds and controls by open blue diamonds.