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

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1 **Title:** *HLA-G* polymorphisms and soluble HLA-G protein levels in women with recurrent
2 pregnancy loss from Basrah province in Iraq
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4 **Running Title:** HLA-G in Iraqi Women with RPL
5
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24 **Abstract**

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

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

35 **Results:** Median sHLA-G levels were significantly lower in the RPL cases compared to healthy
36 controls (21.4 vs. 38.8 U/ml, respectively; $P = 0.025$), and decreased with increased maternal age
37 ($P = 0.0051$). However, the frequencies of alleles at the six polymorphic sites or of the seven
38 *HLA-G* haplotypes did not differ significantly between cases and controls ($P \geq 0.15$ and 0.15,
39 respectively). In contrast, homozygosity for the C allele (CC) at a tri-allelic promoter
40 polymorphism, -725C/G/T, was associated with lower concentrations of sHLA-G compared to
41 women with the CG or CT genotypes (median levels 21.1 vs. 40.1 vs. 42.6 U/ml, respectively; P
42 $= 0.0089$). Genotype effects on sHLA-G levels were present in both the RPL cases and healthy
43 controls, and were independent of maternal age.

44 **Conclusions:** *HLA-G* genotype and maternal age independently influence circulating
45 concentrations of sHLA-G during the first trimester of pregnancy in Iraqi women. However,

46 neither *HLA-G* genotype nor sHLA-G levels in the first trimester of pregnancy were likely

47 causes of pregnancy loss in these women.

48

49 **Key Words:** HLA-G, recurrent pregnancy loss, soluble HLA-G, HLA-G genotype

50 **Introduction**

51 Maternal and fetal immune cells are in close contact during pregnancy, with apparent tolerance
52 of maternal cells toward the fetus and vice versa. It has been suggested that HLA-G is an
53 immunomodulatory molecule that contributes toward this tolerance, although the precise
54 mechanisms are not completely known (Carosella *et al.*, 2008; Hunt *et al.*, 2005; Hviid, 2006).
55 HLA-G is considered a "non-classical" class I HLA due to its limited coding region
56 polymorphism and restricted tissue distribution compared to classical class I HLA (*HLA-A*, *HLA-*
57 *B*, *HLA-C*). Moreover, *HLA-G* transcripts have the unique property among HLA genes in that
58 they undergo alternative splicing to generate at least seven transcripts and four protein isoforms
59 (Fujii *et al.*, 1994; Ishitani and Geraghty, 1992; Morales *et al.*, 2003). Two soluble isoforms,
60 referred to as G5 and G6, are present at relatively high levels in the maternal circulations during
61 pregnancy (Hunt *et al.*, 2000; Rizzo *et al.*, 2009; Steinborn *et al.*, 2007). Decreased levels of
62 sHLA-G have been associated with poor implantation rates in *in vitro* fertilization (Rizzo *et al.*,
63 2007), increased risk for pregnancy loss (Pfeiffer *et al.*, 2000), and preeclampsia (Hackmon *et al.*,
64 2007; Rizzo *et al.*, 2009; Steinborn *et al.*, 2007; Yie *et al.*, 2005) in most, but not all (Steinborn
65 *et al.*, 2003), studies. In addition, specific polymorphisms in the *HLA-G* gene have been
66 associated with sHLA-G levels (Chen *et al.*, 2008; Hviid *et al.*, 2004b) and with pregnancy
67 outcomes (sporadic miscarriage, RPL, preeclampsia) (Aldrich *et al.*, 2001; Hviid *et al.*, 2004a;
68 Larsen *et al.*, 2010; Ober *et al.*, 2003; Pfeiffer *et al.*, 2001; Tan *et al.*, 2008; Yan *et al.*, 2006b;
69 Yie *et al.*, 2008) in some, but not all (Aldrich *et al.*, 2000; Iversen *et al.*, 2008; Lin *et al.*, 2006;
70 Vianna *et al.*, 2007), studies.

71 To date, however, interrogation of multiple *HLA-G* polymorphisms, sHLA-G
72 concentrations, and clinical outcomes has not been considered in the same study population. In

73 addition, nearly all studies of HLA-G and clinical outcomes have focused on women of
74 European or European American descent, even though the frequencies of *HLA-G* polymorphisms
75 differ considerably between racial and ethnic groups and minor allele frequencies for some
76 variants are too rare in European populations to assess their effects on sHLA-G levels or
77 pregnancy outcome. Therefore, comprehensive studies of *HLA-G* genotypes, sHLA-G levels, and
78 clinical outcomes in ethnically diverse populations are required to assess the functional or
79 clinical effects of all *HLA-G* variants.

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

84

85 **Methods**

86 Sample Composition

87 Women (N=50) classified as having recurrent pregnancy loss (RPL), defined by two or more
88 consecutive miscarriages of less than 20 weeks gestation and a negative RLP evaluation, were
89 recruited from the Obstetrics Unit at the Basrah Maternity and Childrens Hospital when they
90 presented to Emergency with a miscarriage that became symptomatic between 9 and 12 weeks
91 gestation. On evaluation, these women had normal levels of serum progesterone levels (>10
92 ng/mol) and normal thyroid function (T3 between 0.9-2.5 mIU/ml; T4 between 60-120 mIU/ml);
93 antiphospholipid antibodies, anticardiolipin antibodies, antinuclear antibodies, and TORCH
94 (toxoplasmosis, rubella, cytomegalovirus, herpes simplex virus) studies were negative. These
95 women were treated with either low dose aspirin or progesterone during the index pregnancy.
96 Control women (N=50) were healthy pregnant women, recruited between 9 and 12 weeks
97 gestation from the same hospital as the women with RPL, and who delivered the index
98 pregnancy at term. Controls had at least one child and no previous miscarriages, preterm
99 deliveries, or stillbirths.

100 Three milliliters of EDTA-anticoagulated venous blood was collected from each woman
101 with RPL prior to the infusion of intravenous fluids and from each control woman. Plasma was
102 removed following centrifugation for 5 minutes at 3000 rpm and frozen in 100 μL aliquots. DNA
103 was extracted from the remaining cells, using the phenol-chloriform extraction protocol of
104 Sambrook et al. (Sambrook *et al.*, 1989), and stored frozen. Frozen plasma and DNA were
105 shipped on dry ice from Basrah, Iraq to Chicago for genotyping and sHLA-G studies.

106 All women signed informed consent. This study was approved by the Training and
107 Development Division in the Basrah Health Office and the University of Basrah, and the
108 Institutional Review Board at the University of Chicago.

109 HLA Genotyping

110 DNA samples were genotyped for six polymorphisms (Fig. 1) by SNaPshot (Applied Biosystems,
111 Carlsbad, CA), using a modification of the protocol of Tan *et al*. (2008) that included the
112 +3142G/C variant (Tan *et al.*, 2007). The six polymorphisms genotyped were (i) the -725C/G/T
113 promoter variant (rs1233334) that has been associated with sporadic miscarriage (Ober *et al.*,
114 2003) and expression differences in reporter assays (Ober *et al.*, 2006), (ii) the +36G/A variant
115 (rs1630185) in the untranslated exon 1 that tags two major *HLA-G* promoter haplotypes (Tan *et*
116 *al.*, 2005) (iii) the 3'UTR 14bp insertion/deletion (indel) polymorphism (rs66554220) that has
117 been associated with *HLA-G* transcript levels (Hviid *et al.*, 2003), circulated sHLA-G levels
118 (Chen *et al.*, 2008), and preeclampsia (Larsen *et al.*, 2010; Moreau *et al.*, 2008), (iv) the 1597ΔC
119 (1 bp deletion; rs41557518) in exon 3 that results in a null allele with respect to protein
120 expression (Ober *et al.*, 1998) and has been associated with RPL (Aldrich *et al.*, 2001; Pfeiffer *et*
121 *al.*, 2001), (v) the Thr258Met variant (rs12722482) that has been associated with preeclampsia
122 (Moreau *et al.*, 2008; Tan *et al.*, 2008), and (vi) the +3142G/C variant (rs1063320) in the 3'UTR
123 that disrupts a micro(mi)RNA target site and influences HLA-G expression levels in the presence
124 of the miRNAs (Tan *et al.*, 2007).

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

128 presence of the -725G and/or -1141T variants as described in (Ober *et al.*, 2003; Tan *et al.*, 2005),
129 *G**010102, *G**010103, *G**0103 *G**0104, *G**0105N, *G**0106. These *HLA-G* haplotypes were
130 assigned to each woman manually based on the known allelic composition of the six variants on
131 each haplotype (Larsen *et al.*, 2010; Ober *et al.*, 1996; Tan *et al.*, 2005). In three women (one
132 case, two controls), the genotypes at the six polymorphic sites were not consistent with two
133 known haplotypes, representing either genotyping error or identification of previously
134 unreported haplotypes. Because of limited DNA availability we could not differentiate between
135 these two possibilities, and these samples were excluded from analyses of haplotypes.

136 <u>Measurements of sHLA-G Concentrations</u>

137 sHLA-G ELISA kits were purchased from EXBIO (Vestec, Czech Republic)/BioVendor (Brno,
138 Czech Republic) for measurement of soluble G5 and shed transmembrane G1 in plasma samples,
139 according to the manufacturer's instructions. All samples were run in duplicate; and mean
140 absorbances, measured at a wavelength of 450 nm, were determined for each subject. Calibration
141 curves based on the absorbance of calibrators of known concentration were used to determine the
142 concentration of sHLA-G in each sample.

143 <u>Statistical Analyses</u>

144 Frequencies of alleles at each of the six variants and for each of the eight haplotypes were
145 compared between RPL cases and healthy controls by the Pearson χ^2 test, or a Fisher exact test if
146 cell counts were <5. Associations between individual polymorphisms or haplotypes and sHLA-G
147 levels were evaluated by nonparametric methods, using either the Wilcoxon rank sum test (to
148 compare 2 groups) or the Kruskal-Wallis test (to compare 3 or more groups). The effective
149 number of independent tests performed in the analysis of genetic associations with sHLA-G was

150 estimated given the correlation structure among polymorphisms using Li and Ji's method (2005),
151 as implemented in the matrix spectral decomposition (matSpD) program (Nyholt, 2004). Li and
152 Ji's method indicated that the six polymorphisms in *HLA-G* represented 5.19 independent
153 variables. Significant *P*-values from the tests of genetic associations were therefore corrected for
154 5.19 tests using the Bonferroni correction and are presented as $P_{corrected}$. Linear regression was
155 used to test for an association between log sHLA-G concentration and maternal age, and the
156 direction and strength of this association was estimated using the Pearson product-moment
157 correlation coefficient (*r*). Multivariate analysis of the combined effect of maternal age, -725
158 genotype, and RPL status on sHLA-G levels was performed on log-transformed sHLA-G
159 measurements using a standard least squares regression. Analyses were performed using JMP
160 software (SAS Institute Inc., Cary, NC), version 8.0.2.2. *P*-values < 0.05 were considered
161 significant.

162 **Results**

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

172 We were able to successfully genotype 49 RPL cases and 48 controls. The genotype
173 counts at each of the six variants were in Hardy-Weinberg equilibrium in cases only, in controls
174 only, and in the combined sample (data not shown). The frequencies of alleles at each
175 polymorphic site in the cases and controls are shown in Table III. None of the allele frequencies
176 were significantly different between the two groups, although there was a trend toward a higher
177 frequency of the insertion allele at the 14bp indel in the cases compared to controls (0.62 vs.
178 0.51; $P = 0.12$).

179 The frequencies of each of the eight haplotypes in cases, controls and the combined
180 sample of Iraqi women are shown in Table IV. Overall, the *G**010101 and *G**010102 haplotypes
181 were the most common haplotypes, similar to other populations. However, all other haplotypes
182 were relatively more frequent in the Iraqi women compared to many other populations (Hviid,
183 2006; Ober *et al.*, 2003; Tan *et al.*, 2005). For example, the frequency of the *G**0105N haplotype
184 (carrying the null 1597ΔC allele) was 0.083, among the highest frequency reported in
185 populations of non-African ancestry (Hviid, 2006; Lin *et al.*, 2009; Ober and Aldrich, 1997), and

186 the frequency of the *G**0103 haplotype (carrying the -725T and the Thr31Ser alleles) was 0.072,
187 also among the highest ever reported (Hviid, 2006). Nonetheless, haplotype frequencies did not
188 significantly differ between cases and controls; although the relatively rare *G**010103 haplotype
189 occurred in six cases and only one control ($P = 0.12$) and the *G**0104 haplotype was relatively
190 more common in controls compared to cases ($P = 0.12$).

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

199 Lastly, we assessed the combined effects of -725C/G/T genotype, maternal age, and
200 case/control status on sHLA-G concentrations by linear regression. When tested individually in
201 univariate regression analyses, the -725 genotype ($P = 0.0023$) and maternal age ($P = 0.0051$)
202 were significantly associated with log sHLA-G concentration, while the effect of case/control
203 status approached statistical significant ($P = 0.056$). The multivariate model that included all
204 three of these predictor variables was highly significant (F ratio = 7.57, P-value = 1.37×10^{-4}),
205 explaining 19.6% of the variance in log sHLA-G levels. Notably, both genotype and maternal
206 age effects on log sHLA-G concentration remained significant in the multivariate model (P =
207 0.012 and $P = 0.0017$, respectively), whereas the effect of RPL status on log sHLA-G levels
208 failed to achieve statistical significance ($P = 0.066$) when -725 genotype and maternal age are

209 included in the model. In the multivariate model, maternal age and -725 genotype account for
210 5.7% and 9.0%, respectively, of the total variance in log sHLA-G concentration. Overall, these
211 results indicate that *HLA-G* genotype and maternal age independently influence circulating
212 concentrations of sHLA-G during the first trimester of pregnancy in Iraqi women.

213 **Discussion**

214 We present here the first study of HLA-G in Iraqi women and examine the effects of genotypes
215 on RPL and circulating levels of sHLA-G. We report a significant association between an *HLA-*
216 *G* promoter polymorphism, -725C/G/T, and sHLA-G concentrations in the first trimester of
217 pregnancy in women with RPL and in healthy controls. We show, in addition, that sHLA-G
218 concentrations significantly decrease with increasing maternal age. Perhaps not surprisingly,
219 sHLA-G concentrations were lower in women experiencing a miscarriage. However, because -
220 725C/G/T genotype frequencies did not differ between RPL cases and healthy controls and the
221 effect of pregnancy status (RPL case versus control) was reduced when genotype and maternal
222 age were included in a multivariate model, we suggest that reduced concentrations of circulating
223 sHLA-G in the RPL cases resulted from the miscarriage but were not causing the pregnancy loss.

224 There have been few studies of the *HLA-G* -725C/G/T polymorphism, but as part of a
225 comprehensive study of variation in the promoter region of *HLA-G*, we previously reported an
226 association between the -725G allele and sporadic miscarriage in fertile couples (the -725T allele
227 was not surveyed at that time) (Ober *et al.*, 2003). We subsequently demonstrated that the G
228 allele was associated with *increased* HLA-G expression in a reporter assay (Ober *et al.*, 2006),
229 consistent with the findings in the current study (Fig. 3). In our earlier study, we reported that
230 constructs carrying the T allele were not higher expressers in untreated JEG3 (placental) cells or
231 in cells treated with IFN-β or with a CpG methylase (M. *SssI*). However, luciferase expression
232 was 2-3 times higher for constructs carrying either the -725G or -725T alleles compared to those
233 with the -725C allele in JEG3 cells treated with both IFN-β and M. *SssI* (see Figure 2D in Ober
234 et al. 2006). That is, similar to the results in the current study, all promoters carrying the
235 common -725C allele were associated with lower expression compared to promoters carrying

236 either the -725G or -725T alleles, under specific conditions. Although we do not know the
237 mechanism accounting for the genotype-specific effects on circulating sHLA-G concentrations in
238 the current study, these earlier experiments indicate that -725 alleles influence gene expression
239 differently in different cellular environments. The current study further shows that women who
240 are homozygous for the C allele are the lowest expressers of sHLA-G in the first trimester of
241 pregnancy.

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

256 Given the high frequency of the 1597ΔC allele in Iraqi women, it was unexpected that
257 this allele was not associated with sHLA-G concentrations because it is a proven null allele for
258 the G1 and G5 isoforms (Ober *et al.*, 1998), which are the two isoforms measured by the ELISA

259 used in this study. This null allele has been associated with RPL in European and European
260 American women (Aldrich *et al.*, 2001; Pfeiffer *et al.*, 2001), but not in Han Chinese women
261 (Yan *et al.*, 2006a), reflecting the heterogeneous nature of RPL and suggesting that other factors
262 might compensate for low levels sHLA-G, as in our study. In contrast, there have been no
263 previous studies of this polymorphism and circulating concentrations of sHLA-G using an HLA-
264 G-specific ELISA. It is possible that maternal genotype for the null allele is not predictive of
265 circulating levels or that there is compensation by the second, non-*G**10105N allele in
266 heterozygous individuals, both of which would reduce power to detect associations between the
267 null allele and sHLA-G concentrations. Additional studies of both maternal and fetal genotypes,
268 and in larger sample sizes will be necessary to evaluate these hypotheses.

269 Two other haplotypes occur at high frequencies in Iraqi women compared to European
270 populations. The *G**0106 haplotype, carrying the Met258Thr variant, occurs at frequencies
271 <0.07 in most European populations (Hviid *et al.*, 2001; Moreau *et al.*, 2008; Ober *et al.*, 2003;
272 Sipak-Szmigiel *et al.*, 2009), whereas the frequency of this haplotype in Iraqi women is 0.12.
273 The -725T allele occurs at frequencies of 0.022 in European Americans and 0.102 in African
274 Americans (Tan *et al.*, 2005), and 0.12 in Iraqi women. In contrast, the -725G allele occurs at
275 frequencies of 0.12 in European Americans and 0.068 in African Americans (Tan *et al.*, 2005),
276 and 0.11 in Iraqi women. As a result, the -725 CC genotype is less common in Iraqi women
277 compared to either European American or African American subjects.

278 This study provides novel insights into the regulation of circulating levels of sHLA-G in
279 early pregnancy and clinical outcome. First, the lack of an association between the -725 CC
280 genotype and RPL, despite the observation of significantly lower sHLA-G concentrations in CC
281 women, suggests that constitutively low levels of sHLA-G due to -725 genotype is not a cause of

282 recurrent pregnancy loss in these women. This would be consistent with the observation that CC
283 is the most common genotype at this site, and indicates that the genotype-specific differences
284 observed in this study are likely well within the range required for successful implantation and
285 maintenance of pregnancy. Interestingly, even at 9-12 weeks of gestation in spontaneously
286 terminating pregnancies, the genotype effects on sHLA-G concentrations are still apparent.
287 Whether these genotype effects on sHLA-G concentrations remain throughout pregnancy, or
288 whether they predict outcomes later in pregnancy remains to be determined. Second, we report
289 an unexpected association between sHLA-G concentrations and maternal age that is independent
290 of the *HLA-G* genotype effect. This observation needs to be replicated in additional studies, and
291 examined in later trimesters of pregnancy. If confirmed, reduced concentrations of sHLA-G in
292 the first trimester in older mothers may be a contributory mechanism, and a possibly marker, for
293 the increased risk for adverse pregnancy outcomes in later pregnancy, such as preeclampsia and
294 preterm birth, among these women (Cleary-Goldman *et al.*, 2005; Cnattingius *et al.*, 1992; Fretts
295 *et al.*, 1995), a hypothesis that can be examined in future prospective studies.
296 In summary, the relationship between *HLA-G* genotype, circulating sHLA-G
297 concentrations in the first trimester, and adverse pregnancy outcomes remains complex. This
298 study identifies two independent determinants of sHLA-G concentrations in the first trimester of
299 pregnancy, including genotype at a promoter polymorphism, -725C/G/T, that was previously
300 demonstrated to have functional effects on expression (Ober *et al.*, 2006), and maternal age, a
301 well established risk factor for adverse pregnancy outcomes throughout pregnancy. Further
302 studies are required to elucidate the clinical effects of these observations throughout pregnancy
303 and in ethnically diverse women.

304 **Supplementary Data**

305 Supplementary data are available at http:

306

307 **Authors' roles**

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

315

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325

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467

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

Haplotype	*HLA-G* Polymorphisms					
	-725C/G/T	+36G/A	1597ΔC	Thr258Met	14bp indel	+3142G/C
*G**0101^	C	G	C	Thr	Del	C
*G**010101b,c	G	G	C	Thr	Del	C
*G**010102	C	A	C	Thr	Ins	G
*G**010103	C	A	C	Thr	Ins	G
*G**0103	T	G	C	Thr	Ins	G
*G**0104	C	A	C	Thr	Del	G
*G**0105N	C	A	Δ	Thr	Ins	G
*G**0106	C	A	C	Met	Ins	G

^Includes the 010101, 010104, and 010108 haplotypes that cannot be differentiated by these six polymorphisms.

469 **Table II.** Characteristics of the study sample. Means were compared by t-test; *P*-values are
470 shown.

	RPL Cases	Controls	*P*-value
Sample Size	50	50	
Mean Age ± SD (Range)	28.4 ± 6.2 yrs (20-40)	26.7 ± 6.0 yrs (17-39)	0.16
Mean Number of Pregnancies ± SD (Range)	4.3 ± 1.9 (2-14)	3.4 ± 1.4 (2-9)	0.0097
Mean Number of Live Births ± SD (Range)	0.42 ± 0.64 (0-2)	2.4 ± 1.4 (1-8)	7.8×10^{-14}

471

472 **Table III**. Frequencies of alleles for six *HLA-G* polymorphisms in 49 RPL cases and 48 healthy
473 controls. Minor allele frequency differences between RPL cases and controls were assessed
474 using the Pearson chi-square test in 2x2 contingency tables.

Polymorphism	Allele	RPL Cases	Controls	*P*-value
-725C/G/T	C	0.84	0.84	
	G	0.082	0.10	0.59
	T	0.082	0.052	0.41
+36G/A	A	0.57	0.61	
	G	0.43	0.39	0.54
1597ΔC	C	0.93	0.91	
	Δ	0.071	0.094	0.57
Thr258Met	Thr	0.87	0.86	
	Met	0.13	0.14	1.00
14bp indel	Ins	0.62	0.51	
	Del	0.38	0.49	0.12
+3142G/C	G	0.69	0.68	
	C	0.31	0.32	0.81

475

476 **Table IV**. Frequencies of eight *HLA-G* haplotypes in RPL cases (N=49), healthy controls
477 (N=48), and the pooled sample (N=97). Frequency differences between cases and controls were
478 assessed using the Pearson chi-square test in 2x2 contingency tables.

Haplotype	RPL Cases	Controls	Pooled Sample	*P*-value
*G**0101	0.22	0.20	0.21	0.65
*G**010101b,c	0.092	0.13	0.11	0.46
*G**010102	0.24	0.24	0.24	0.92
*G**010103	0.061	0.010	0.036	0.12
*G**0103	0.092	0.052	0.072	0.29
*G**0104	0.092	0.17	0.13	0.12
*G**0105N	0.071	0.094	0.082	0.57
*G**0106	0.12	0.11	0.12	0.86

479

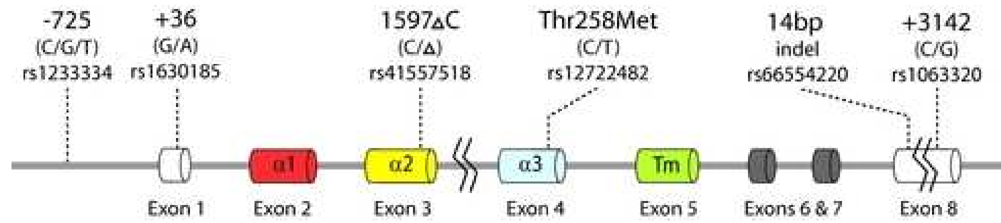
480 **Table V**. *HLA-G* genotype association with sHLA-G concentration in the pooled sample (N=97).
481 *P*-values were obtained for each polymorphism using either the Wilcoxon rank sum test or the
482 Kruskal-Wallis test.

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

483 **Figure Legends**

484

485 1. *HLA-G* gene structure and location of six polymorphisms included in this study.
486 2. Soluble HLA-G plasma concentrations in RPL cases and controls. sHLA-G concentration
487 differed significantly between RPL cases and controls (Wilcoxon rank sum test, *P* =
488 0.025). Boxes show interquartile range, horizontal lines show the median values and
489 whiskers extend an additional 1.5 interquartile ranges from the boxes.
490 3. Soluble HLA-G plasma concentrations by -725C/G/T genotype. sHLA-G concentration
491 was significantly associated with -725 genotype (Kruskal-Wallis test, *P* = 0.0089). Boxes
492 show interquartile range, horizontal lines show the median values and whiskers extend an
493 additional 1.5 interquartile ranges from the boxes. RPL cases are indicated by solid red
494 diamonds and controls by open blue diamonds.



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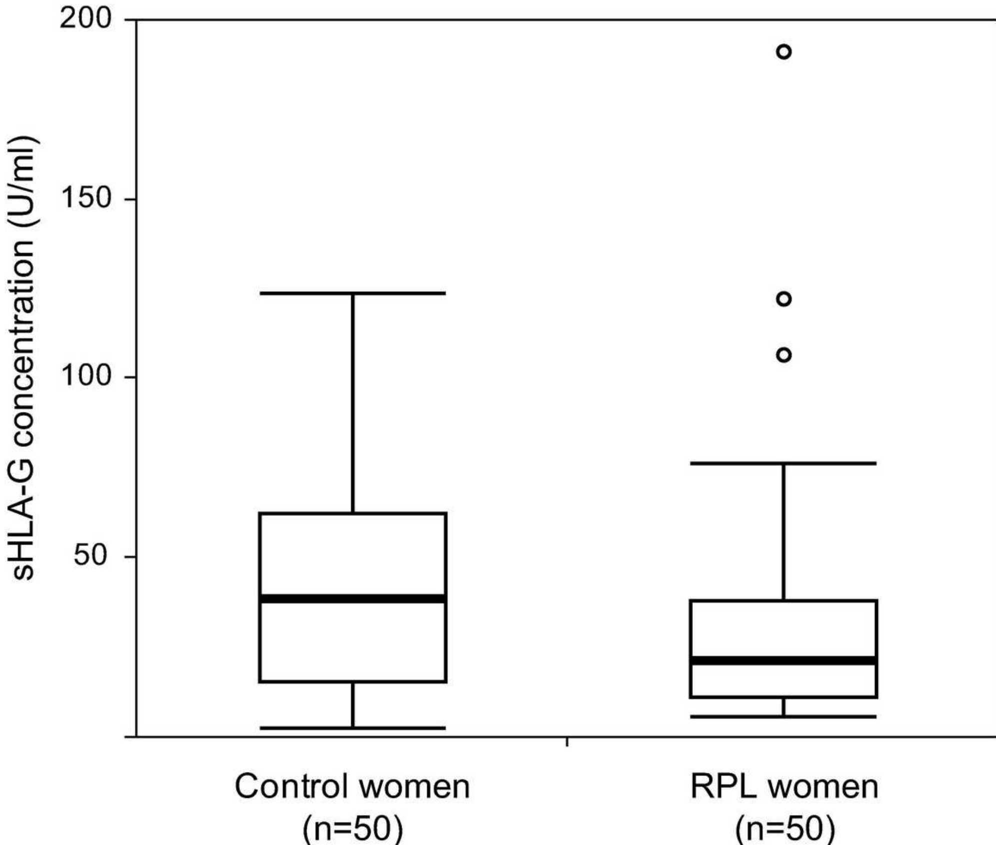

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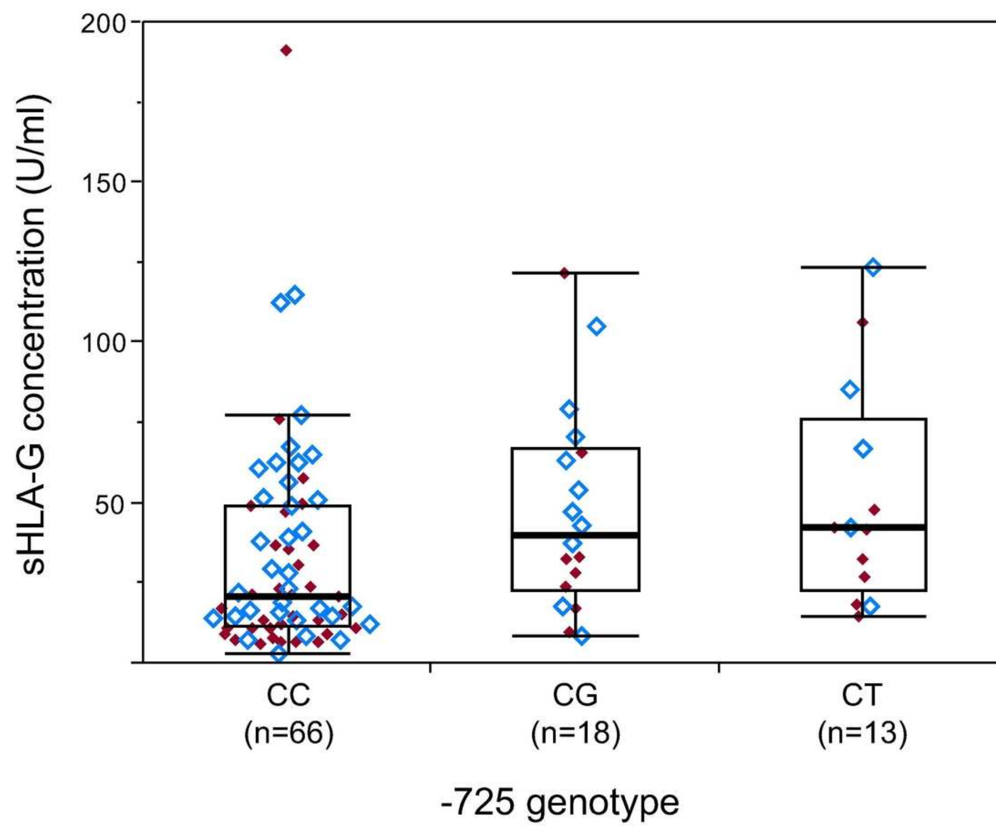

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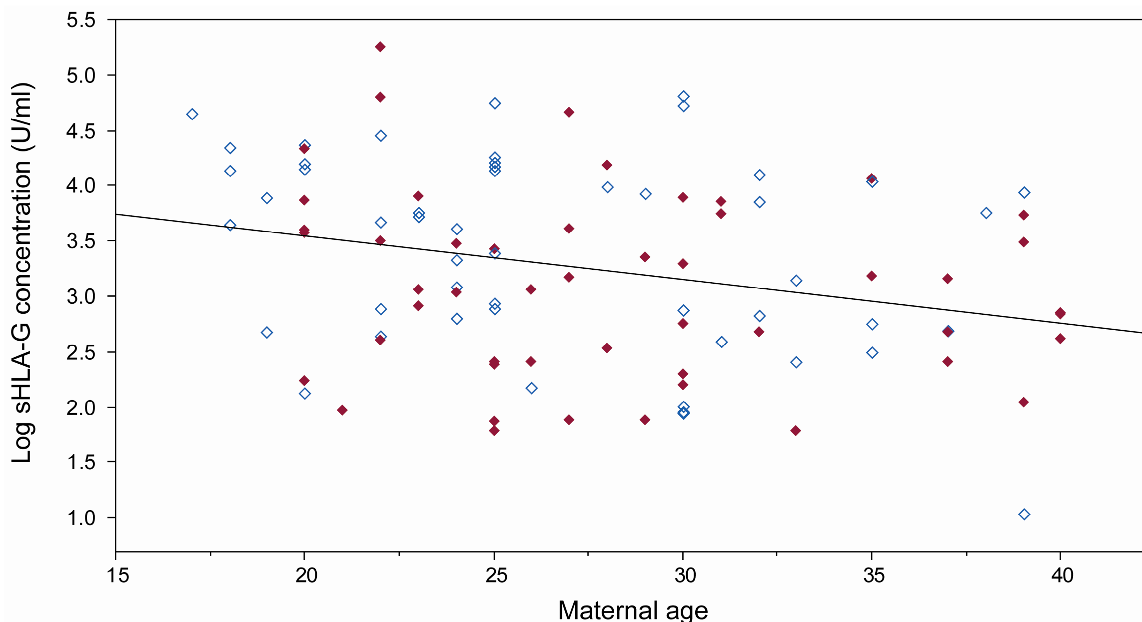
Supplementary Data

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



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.