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1 **sHLA-G1 and HLA-G5 levels are decreased in Tunisian women with**
2 **multiple abortion.**

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16 **Abbreviated Title: Decreased sHLA-G levels in abortion (37/45)**

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29

30 **ABSTRACT (words: 137/150)**

31 Pregnancy is associated with increased levels of soluble (s) Human leukocyte antigen (HLA)-G
32 molecules, while during abortion these molecules are decreased. To date, little is known about the
33 role of sHLA-G isoforms during abortion. In this study, we investigated the levels of total sHLA-G
34 and its isoforms: HLA-G1 (membrane shedded isoform) and alternative spliced HLA-G5 in plasma
35 samples obtained from 55 women who had experienced spontaneous abortion, 108 pregnant healthy
36 women and 56 non pregnant healthy women.

37 We found that pregnant women exhibited higher amounts of sHLA-G compared to either non
38 pregnant women or women with abortion. Among women who had experienced spontaneous
39 abortion, women with recurrent abortions (RSA) had lower sHLA-G than women with only one
40 abortion. In particular, RSA women were characterized by the absence of sHLA-G1 isoform,
41 suggesting a possible implication in abortion event.

42

43 **Keywords:** Pregnancy; abortion; sHLA-G; sHLA-G1; HLA-G5.

44 **Abbreviations:** sHLA-G=soluble HLA-G; sHLA-G1=shedding HLA-G1; HLA-G5 = HLA-G5;
45 RSA = recurrent spontaneous abortion.

46

47 1. INTRODUCTION

48 There is accumulating and compelling evidence that non-classical human leukocyte antigen (HLA)
49 class I molecule HLA-G is highly implicated in immune tolerance as an immune checkpoint [1]. Its
50 role substantiates its implication in immune cell regulation. Indeed, HLA-G can inhibit NK and
51 CD8+ T lymphocyte mediated cell lysis [2-3], dendritic cell antigen presentation [4] and T
52 lymphocyte CD4+ alloproliferation [5]. Additionally, HLA-G molecules up-regulate T lymphocyte
53 CD8+ apoptosis and enhance the production of regulator T cells [6]. These functions evidence the
54 importance of HLA-G molecules in fetus tolerance by the maternal immune system during
55 pregnancy. Indeed, HLA-G molecule expression was originally described at the feto-maternal
56 interface in trophoblast cells' surface [7-8].

57 The HLA-G gene expresses seven isoforms generated through alternate mRNA splicing: four
58 membrane-bound isoforms (HLA-G1, HLA-G2, HLA-G3 and HLA-G4) and three soluble isoforms
59 (sHLA-G: HLA-G5, HLA-G6 and HLA-G7). Additionally, it has also been demonstrated that
60 HLA-G1 could be cleaved by matrix metalloproteinases (MMP) generating HLA-G1 shedding
61 molecule (sHLA-G1) [9], in particular by MMP-2 [9]. However, the most studied isoforms of
62 sHLA-G consisted of sHLA-G1 and HLA-G5 [10].

63 Immune processes play a crucial role in abortion and especially in recurrent spontaneous abortion
64 (RSA) [11-12]. Several studies support the implication of HLA-G molecule in pregnancy
65 complications. Essentially, HLA-G isoforms, either membranous or soluble, are decreased in
66 pregnancy complications including preeclampsia and RSA [13]. Furthermore, a recent work
67 reported that uterine sHLA-G levels are altered in unexplained infertility [14].

68 Here we explored, for the first time, levels of sHLA-G in plasma samples from pregnant women in
69 comparison to women who had experienced spontaneous abortion (one or more abortion events)
70 and non-pregnant women. We also evaluated levels of membrane-shedded HLA-G1 (sHLA-G1)
71 and spliced HLA-G5 isoforms.

72

73 2. MATERIALS AND METHODS

74 2.1 Patients

75 Venous blood samples were collected from Tunisian participants, and plasmas were obtained by
76 centrifugation avoiding sHLA-G to be trapped and/or consumed by clots [15-16]. This study
77 implicated 55 women who have experienced spontaneous abortion recruited from the Basic Health
78 Group of Sousse ("Groupement de santé de base de Sousse"): 44 women with one abortion and 11
79 women with recurrent spontaneous abortions (RSA, with at least two spontaneous abortions [17-
80 19]). The mean age of these women equaled 34.83 ± 8.46 years (Mean \pm SD; Age range: 20-60
81 years).

82 Two age-matched control groups were recruited for this study. The first group consisted in 56
83 unrelated non-pregnant fertile volunteers women with a mean age 32.23 ± 11.72 (Age range: 18-63
84 years). Collected blood was off menstruating. The second group is composed by 108 healthy

85 women at different stages of uncomplicated pregnancy with a mean age 33.69 ± 8.43 (Age range: 18-
86 68 years). None of control groups had an abortion history or a complicated pregnancy.

87

88 **2.2 Soluble HLA-G dosage by Enzyme-Linked Immunosorbent Assay (ELISA)**

89 Sandwich ELISA was performed according to the Essen Workshop [20]. sHLA-G was measured by
90 enzyme-linked immunosorbent assay (ELISA) in plasma samples as previously reported [21-23],
91 using the MEM-G9 Monoclonal antibody (MAb; Exbio, Praha, Czech Republic) that recognizes
92 sHLA-G molecules associated to $\beta 2$ -microglobuline, or the 5A6G7 MAb (Exbio, Praha, Czech
93 Republic) that recognizes the HLA-G5, -G6 and -G7 isoforms. Standard supernatants of HLA-
94 G/721.221 were used for standard calibration curves generation. The intra-assay coefficient of
95 variation (CV) was 1.4 % and the inter-assay CV was 4.0 %. The limit of sensitivity was 1.0 ng/ml.

96

97 **2.3 Statistical Analysis**

98 Statistical analysis was performed with SPSS (16.0) and by Graphpad prism 5. Comparison
99 between ages and sHLA-G levels was evaluated respectively by unpaired t-test or Mann-Whitney
100 test. Fisher exact test was performed to compare HLA-G positive subjects' percentage. Two-tailed
101 p-value under 0.05 was considered as statistically significant.

102

103 **3. RESULTS**

104 **3.1 sHLA-G levels are decreased in women with abortion**

105 The three studied cohorts, women with abortion, pregnant and non pregnant women, presented
106 similar ages (Unpaired t-test: p (Abortion vs Non pregnancy) = 0.186; p (Abortion vs Pregnancy)
107 = 0.419).

108 The comparison of the three groups for sHLA-G plasmatic levels evidenced that women with
109 abortion had lower sHLA-G concentrations than pregnant women (Mean \pm SEM: 0.50 ± 0.30 ng/ml
110 and 2.8 ± 0.54 ng/ml, respectively $p < 0.0001$, Mann-Whitney test) (Table 1). Similarly, we observed
111 lower levels of both sHLA-G1 and HLA-G5 in women with abortion than in the pregnant ones ($p =$
112 0.0003 and $p = 0.0001$, respectively, Mann-Whitney test) (Table 1). On the contrary, no significant
113 difference in sHLA-G level was observed between non pregnant women and women with abortion
114 (Table 1).

115

116 **3.2 RSA women showed lower levels of sHLA-G**

117 Women with abortion were stratified according the number of abortions (one or more than one
118 abortion (RSA)). Both subgroups showed lower levels of sHLA-G compared to the pregnant
119 women (Pregnant versus one abortion: $p < 0.0001$; Pregnant versus RSA $p = 0.0046$, Mann-

120 Whitney). Interestingly, although without significance, the subgroup of RSA women showed lower
121 sHLA-G levels in comparison to the subgroup of women with only one abortion ($p = 0.53$;
122 percentage of positive samples = 9%) (Fig.1). No differences were observed between women with
123 abortion group and non pregnant women cohort.

124 The analysis of sHLA-G1 and HLA-G5 isoforms evidenced higher levels in pregnant women
125 compared to both abortion subgroups (Fig. 1).

126

127 **3.3 sHLA-G1 absence characterizes women with RSA.**

128 Considering the number of positive samples for sHLA-G and its isoforms in each group, we
129 reported a high positivity percentage in pregnant women (Fig. 1) (sHLA-G: 53.7%, sHLA-G1:
130 31.4%; HLA-G5: 37%) in comparison to both women with abortion subgroups ($p < 0.0001$, Fisher
131 exact test).

132 Interestingly, no positive samples for sHLA-G1 were found in the subgroup of RSA women
133 (women with RSA versus non pregnant women: $p < 0.0001$ and women with RSA versus women
134 with one abortion: $p = 0.01$; Fisher exact test) (Fig.1).

135

136 **4. DISCUSSION**

137 Pregnancy is an immunological paradox [24] in which the fetus, considered as “semi-allogenic”, is
138 maintained in the mother body through different immune-tolerance mechanisms particularly
139 brought by HLA-G molecules [21-25]. Indeed, a decrease in HLA-G expression is associated with
140 complications in pregnancy [23].

141 In accordance to previous studies showing that sHLA-G is increased in pregnant women plasma
142 [21,23], we have reported here the increase of this molecule in plasma samples from healthy
143 pregnant women. We also observed a decrease in total sHLA-G in women with abortion as
144 previously described [21,26]. Further investigations should shed light on the real mechanism
145 implicating sHLA-G and would explain whether low sHLA-G is the cause or only a by-product of the
146 spontaneous abortion. sHLA-G decrease could be attributed to the changes in pro-inflammatory and
147 anti-inflammatory cytokines balance associated to abortion [27]. In particular, previous studies
148 reported a decrease in IL (Interleukin)-10 expression, a positive regulator of HLA-G production in
149 normal pregnancy [28], in RSA women [29]. This defect in IL-10 secretion could in part explain the
150 observed decrease of sHLA-G expression in our cohort of women with abortion. Further studies
151 need to address this aberrant expression of cytokines to clearly substantiate their association to
152 HLA-G production.

153 To the best of our knowledge, this is the first time that a decrease in HLA-G5 and sHLA-G1
154 isoforms is reported in women with abortion. Similarly to preeclamptic women, where HLA-G5 is
155 the only isoform observed, even if with lower levels in comparison with healthy pregnant women
156 [24], we found only the HLA-G5 isoform in RSA women (Fig. 1). On the contrary, women with

157 only one abortion were characterized by the presence of sHLA-G1 isoform and also HLA-G5.
158 These data suggest a peculiar implication of the absence sHLA-G1 isoform in multiple abortion
159 sequelae.

160 As it is known that metalloproteinases, mainly MMP-2, are involved in HLA-G1 shedding [9] and
161 that the trophoblast cell invasion into the maternal endometrium is facilitated by the expression of
162 both HLA-G1 [30] with increased activity of MMP-2 [31], we think that RSA could be related to a
163 decreased cleavage of membrane-bound HLA-G1 molecules expressed by cytotrophoblast cells.
164 This hypothesis could be consolidated by previous studies showing that women with
165 spontaneous pregnancy termination presented increased levels of MMP-2 complexed with its
166 natural tissue inhibitor molecule TIMP-2 [31-32], suggesting a decreased activity of MMP-2
167 conducting to decreased HLA-G1 cleavage as well as low regular placentation and immune
168 reactivity towards fetal tissues. Further future studies should be addressed to clearly establish the
169 implication of MMP-2 activity deregulation in RSA.

170 To conclude, we reported, in this study, that abortion is associated to decreased plasmatic levels of
171 sHLA-G. Lower levels of sHLA-G1 isoform, in particular, seems to characterize RSA women,
172 suggesting a possible involvement in the abortion event.

173

174 5. AUTHOR CONTRIBUTIONS

175 Roberta Rizzo and Zidi Inès designed the experiments; Zidi Inès, Laaribi Ahmed Baligh, Nour Zidi
176 and Tlili Henda collected the samples; Bortolotti Daria, Zidi Inès and Rizzo Roberta wrote the
177 article. Bortolotti Daria, Zidi Nour and Zidi Inès performed the experiments. Rizzo Roberta, Zidi
178 Inès and Bortolotti Daria analyzed the data. Bouaziz Aicha, Di Luca Dario, Roberta Rizzo, and Zidi
179 Inès edited the manuscript.

180

181 6. REFERENCES

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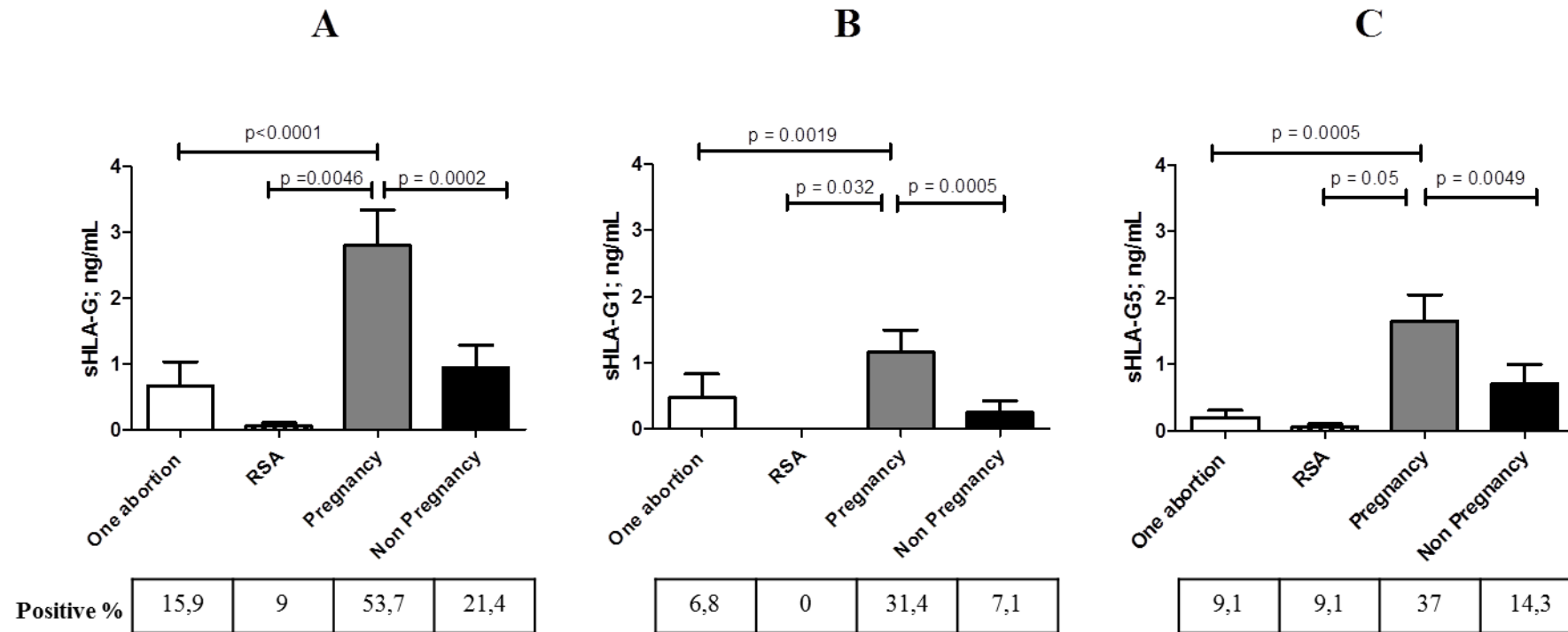
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- 263
- 264

Figure 1.



Box-plot of plasma total sHLA-G (A), sHLA-G1 (B), HLA-G5 (C) concentrations and positive expression percentages in women with abortion (=1 and >1 or RSA), pregnant, and non pregnant women. RSA= Recurrent spontaneous abortion, Mean± Standard Error of the Mean. P-values referred to concentrations were obtained by Mann-Whitney test.

Table 1. sHLA-G level characteristics in women with abortion and controls

Characteristics	Women with abortion (n=55)	Pregnant women (n=108)	Non pregnant women (n=56)	<i>P</i> -value ^a	<i>P</i> -value ^b	<i>P</i> -value ^c
sHLA-G*	0.50±0.30	2.80±0.54	0.95±0.33	< 0.0001	0.309	0.0002
sHLA-G1*	0.38±0.29	1.16±0.34	0.25±0.18	0.0003	0.761	0.0005
sHLA-G5*	0.17±0.09	1.64±0.40	0.70±0.29	0.0001	0.334	0.0049

The bold text indicates significant p-values.

*Mean±SEM (ng/ml).

^a women with abortion vs pregnant women (Mann-Whitney test)

^b woman with abortion vs non pregnant women (Mann-Whitney test)

^c pregnant women vs non pregnant women (Mann-Whitney test)