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ABSTRACT

Recurrent pregnancy loss (RPL) affects 1–2 % of couples who are trying to conceive. At some point, some couples do maintain a healthy pregnancy to term, but the underlying mechanism of RPL remains elusive. Human leukocyte antigen (HLA)-G is an immune modulatory molecule. Our group previously showed increased HLA-G levels in the decidua of term pregnancies after RPL, while other studies showed reduced soluble HLA-G (sHLA-G) blood levels in women with RPL. This led us to investigate sHLA-G levels in blood of women with RPL who had either a subsequent pregnancy loss (RPL-pregnancy loss) or a healthy term pregnancy (RPL-live birth), and compare these to healthy control pregnancies and non-pregnant controls. Soluble HLA-G concentrations were quantified by ELISA. Women with healthy term pregnancy had increased sHLA-G levels compared to non-pregnant controls. In contrast, RPL-live birth women at term did not have increased blood sHLA-G levels. Soluble HLA-G levels remained stable between first and third trimester. Interestingly, when comparing first trimester samples of RPL-live birth to RPL-pregnancy loss, sHLA-G levels also did not significantly differ. High sHLA-G levels in blood seem not to be crucial for an ongoing healthy pregnancy after RPL. However, since it was previously shown that women with RPL-live birth have increased HLA-G levels in term decidua compared to control pregnancies, the current data suggest that local and systemic immune regulation are not necessarily in concert. Further study of the contribution of fetus-derived HLA-G and HLA-G of maternal origin may provide more insight in the pathophysiology of RPL.

1. Introduction

Recurrent pregnancy loss (RPL) affects 1–2% of couples trying to conceive. In 50–70 % of these couples no underlying cause can be found, indicated as unexplained RPL (Diejomaoh, 2015; Youssef et al., 2020; Medicine, 2012). As of 2018, RPL is defined as two or more pregnancy losses before 24 weeks of gestation (RPL et al., 2018). No evidence-based therapeutic options are available and research on this pregnancy complication is confined due to the limited understanding of the pathophysiological mechanism. Furthermore, reliable predictive tools of pregnancy outcome are desirable (du Fossé et al., 2022). Previously, our group showed increased HLA-G levels in the decidua of term pregnancies after RPL (Craenmehr et al., 2019), while other studies showed reduced soluble HLA-G (sHLA-G) blood levels in women with RPL (Zidi et al., 2016; Kalotra et al., 2018). This led to the hypothesis that women with a history of RPL and subsequent healthy ongoing pregnancy also have increased blood sHLA-G levels compared to women without a history of RPL; thereby leading to the notion that this could be a potential biomarker for pregnancy outcome. This study is the first to compare sHLA-G levels for pregnancy outcome of couples with a history of RPL.

During pregnancy it is important to maintain immunological tolerance towards the semi-allogeneic fetus. One of the factors associated with immune tolerance in pregnancy is human leukocyte antigen (HLA)-G.HLA-G is an immune checkpoint molecule as it binds inhibitory receptors (e.g. ILT2 and ILT4) on several types of immune cells (T, NK, B, dendritic cells, and monocytes) resulting in inhibition of their effector functions (Carosella et al., 2015). The efficiency of HLA-G to reduce

Abbreviations: RPL, Recurrent Pregnancy Loss; HLA, human leukocyte antigen; sHLA-G, Soluble HLA-G; SBT, sequencing-based typing; NGS, next generation sequencing; 14 bp ins/del, 14 base pair insertion/deletion; UTR, untranslated region; GA, gestational age; NP, non-pregnant.

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$CD4^+$ T cell proliferation and cytokine production is similar to that of CTLA-4 (Saverino et al., 2002). It can also induce apoptosis of $CD8^+$ T cells (Fournel et al., 2000). Furthermore, HLA-G/ILT2 interaction on NK cells inhibits the polarization of lytic granules in the synapse and the production of IFN-y (Favier et al., 2010; Lesport et al., 2009). HLA-G levels have been shown to be low in blood of women with pregnancy loss (Pfeiffer et al., 2000; Zidi et al., 2016). Taken together, reduced levels of HLA-G could lead to a more inflammatory immune profile, as is observed in women with RPL (Emmer et al., 2000; Lee et al., 2013; Tsao et al., 2018).

HLA-G was first described in the placenta and is expressed by fetal extravillous trophoblast cells, which lack expression of classical HLA-class I antigens except HLA-C (Kovats et al., 1990). Maternal immune cells like monocytes and macrophages can produce and secrete HLA-G as well (Moreau et al., 1999; Morandi et al., 2007). Therefore, soluble HLA-G (sHLA-G) can be measured in plasma and serum of non-pregnant healthy individuals (Hunt et al., 2000; Rebmann et al., 1999). Blood sHLA-G levels are increased in pregnant women compared to non-pregnant women (Hunt et al., 2000). Throughout gestation some studies found stable sHLA-G levels (Alegre et al., 2007; Hunt et al., 2000) whereas others found a decrease in sHLA-G levels (Yie et al., 2005; Beneventi et al., 2017; Klitkou et al., 2015). However, when measuring levels of blood sHLA-G during pregnancy it is not known if the source is of fetal or maternal origin. Furthermore, the possible association with sHLA-G levels locally in the decidua, is not clear.

In contrast to studies showing reduced blood sHLA-G in RPL cases (Zidi et al., 2016; Kalotra et al., 2018), HLA-G levels in decidual tissue of women with RPL miscarriage are not reduced compared to elective abortion controls (Craenmehr et al., 2019). Furthermore, our research group showed that women with a history of RPL demonstrated increased decidual HLA-G levels in healthy term pregnancy compared to pregnant women without a history of RPL (Craenmehr et al., 2019). Taken together, these results lead to the question if women with a history of RPL and subsequent healthy ongoing pregnancy (RPL-live birth) also have increased blood sHLA-G levels compared to controls without a history of RPL. Secondly, we investigated if blood sHLA-G levels in RPL-live birth women differ from those in women with RPL and a subsequent pregnancy loss (RPL-pregnancy loss). Herein, we tested if sHLA-G in blood during first trimester could be a potential biomarker for pregnancy outcome.

In the current study we found that RPL-live birth women at term do not significantly increase sHLA-G levels compared to non-pregnant controls, while pregnant control women without a history of RPL have increased sHLA-G levels. Next to that, we found that sHLA-G levels at first trimester do not differ between RPL-live birth and RPL-pregnancy loss women.

2. Materials and methods

2.1. Study material

A total of 101 sodium heparin plasma samples from 71 individuals were retrospectively included in this study to form three case groups and two control groups (Fig. 1). For additional experiments we collected peripheral blood mononuclear cells for *HLA-G* typing. In this retrospective study, the case groups consisted of women with a history of at least three consecutive early pregnancy losses, following the international ESHRE guidelines from 2006 to define recurrent pregnancy loss (RPL)(Jauniaux et al., 2006). No underlying causes for RPL were found in the case groups after a full clinical workup. Exclusion criteria were age $\geq$ 40, BMI $\geq$ 35, smoking, and use of medication (e.g., progesterone). Of the 101 samples, 14 were from non-pregnant controls, 22 from pregnant controls, and 65 samples were from the cases. Of the cases, 9 samples were only used to study the influence of gestation age and reliability of sHLA-G measurement, since these 9 samples were from individuals of which another 1st trimester timepoint was included (e.g. 6 wks and 8 wks of gestation of the same pregnancy). The RPL-pregnancy loss group consists of 11 first-trimester samples from women with a history of RPL and another consecutive pregnancy loss at some point after sampling. This group consists of 9 individuals (samples come from different pregnancies). The RPL-live birth groups are women with a history of RPL and the samples are taken during an ongoing healthy pregnancy. The groups contain 23 first-trimester samples and 22 term samples from 26 individuals. From 19 individuals we had paired first-trimester and term samples (Fig. 1). All pregnant women visited the Department of Obstetrics and Gynaecology at the Leiden University Medical Center (LUMC) between 2009 and 2019. Control samples were obtained from non-pregnant healthy females (n = 14). All samples were obtained after informed consent and the study was carried out in accordance with the Declaration of Helsinki and with the guidelines

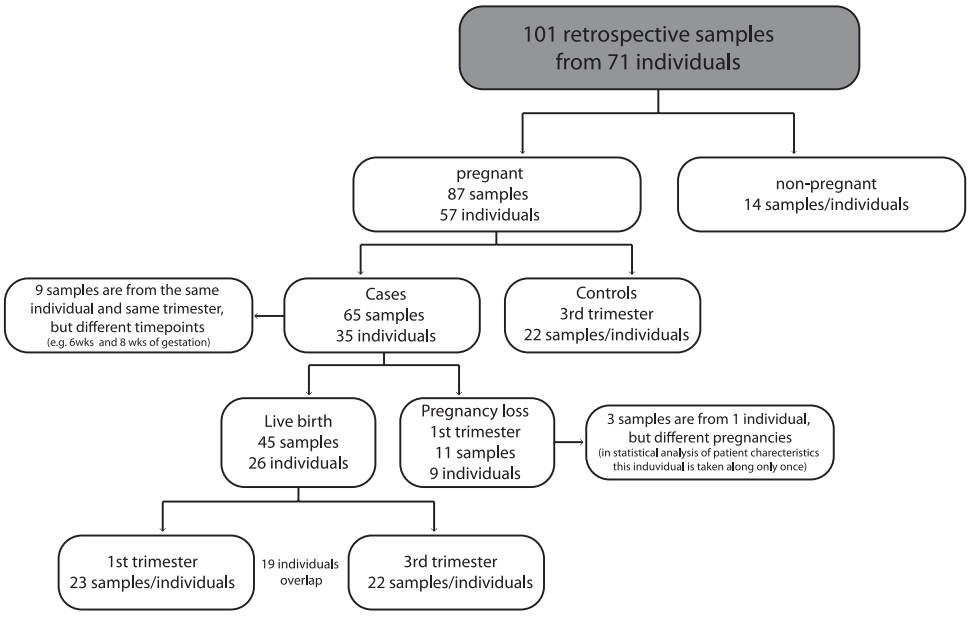

Fig. 1. Flowchart of individuals and samples studied. Five groups can be distinguished: non-pregnant controls, 3rd trimester pregnant controls, 1st trimester RPL-live birth, 3rd trimester RPL-live birth, 1st trimester RPL-pregnancy loss.

issued by the Medical Ethics committee of the LUMC (protocol P11.196).

2.2. sHLA-G ELISA

The concentration of sHLA-G protein (soluble HLA-G5 and shed transmembrane HLA-G1) in plasma samples were measured using a commercial sHLA-G ELISA kit (MyBioSource, San Diego, USA; MBS2516229) with a detection range of 0.313–20 ng/mL and a sensitivity of 0.188 ng/mL. ELISA assays were performed according to the manufacturer's instructions. All samples were run in duplicate using several dilutions (1:5–1:40). The mean absorbance of duplicates of each sample was measured at a wavelength of 450 nm and sHLA-G concentrations were determined using the standard curve per plate provided in the ELISA kit.

2.3. HLA-G polymorphisms

Peripheral blood was used to isolate DNA in order to determine the *HLA-G* genotype and 3′UTR haplotype of the women using an in-house sequencing-based typing (SBT) assay (Craenmehr et al., 2019) or next generation sequencing (NGS)-based assay (Drabbels et al., 2020). In short, in the SBT assay the 3′UTR of exon 8 was sequenced enabling the identification of polymorphisms including the 14-bp insertion/deletion (rs371194629), + 3142 C/G (rs1063320), + 3187 A/G (rs9380142), and + 3196 C/G (rs1610696). For the NGS-based assay the HLA-G gene was amplified with a PCR using HLA-G-specific primers, after which the PCR products were fragmented. The amplified and fragmented HLA-G gene was run on an Illumina Miniseq. The genotype of the samples was determined, and polymorphisms of interest were identified. The strength of the association between the 14-bp polymorphism and risk of RPL was calculated in three random effect models as previously done by Wang et al. (Wang et al., 2013). These are the co-dominant (14-bp ins vs 14-bp del), dominant (14-bp ins/ins and 14-bp ins/del vs 14-bp del/del) and recessive (14-bp ins/ins vs 14-bp ins/del and 14-bp del/del) models. Different combinations of 3′UTR polymorphisms from UTR haplotypes. UTR-1 and UTR-3 are associated with low sHLA-G expression, while URT-2, UTR-4, and UTR-7 are associated with high sHLA-G expression (Martelli-Palomino et al., 2013; Carlini et al., 2013; Di Cristofaro et al., 2013). Women with one 'low' and one 'high' haplotype were defined as 'medium' expressors.

2.4. Statistical analysis

Differences between groups were tested by Mann-Whitney U tests, chi-square tests or Wilcoxon test of paired samples. Correlation between BMI and concentration of sHLA-G was tested by a Spearman correlation. Values of $p < 0.05$ were considered to indicate statistical significance.

3. Results

3.1. Patient characteristics

Soluble HLA-G (sHLA-G) concentrations were measured in 101 samples (Fig. 1). To study the influence of gestational age (GA) at first trimester, from 9 individuals two timepoints during first trimester were measured. No significant correlation was found in the concentration of sHLA-G and GA in weeks (data not shown). In further analysis only one of the two first trimester timepoints was taken along, selected based on best GA match between the study groups.

Patient characteristics of the five study groups are shown in Table 1. Between the pregnancy groups there were no differences in parity, maternal age, and maternal BMI. The three RPL groups have a median of three pregnancy losses in their history whereas all the controls never experienced a pregnancy loss ($p < 0.001$). Furthermore, the gestational age differs between the 1st trimester RPL live birth group and the 3rd trimester pregnancy control group ($p < 0.001$).

3.2. Soluble HLA-G concentrations

We found that in the blood of RPL-live birth women the sHLA-G concentration was not significantly increased at 3rd trimester compared to non-pregnant controls ($p = 0.141$), while this was the case in 3rd trimester control pregnancies without a history of RPL ($p = 0.002$) (Fig. 2). Furthermore, there was a trend towards a decrease of sHLA-G concentration in the blood of RPL-live birth women at 3rd trimester compared to 3rd trimester control blood ($p = 0.108$) (Fig. 2).

We further focused on the women with a history of RPL but with an ongoing term pregnancy, as we were able to also measure blood sHLA-G levels in these women at the first trimester. No difference of sHLA-G concentrations between first and third trimester was observed, neither when comparing group medians of all samples ($p = 0.424$) (Fig. 3 A), nor when comparing samples in a pairwise fashion ($p = 0.165$) (Fig. 2B).

Next, we wondered whether blood sHLA-G levels were different during first trimester between women with RPL history who have ongoing pregnancy and those who suffer from pregnancy loss. We found no significant difference between the sHLA-G blood concentrations of RPL-live birth women compared to RPL-pregnancy loss women ($p = 0.513$) (Fig. 4).

3.3. Relationship of sHLA-G levels with BMI

We noticed a significant correlation between BMI and sHLA-G concentrations ($r = 0.651$, $p < 0.001$) (Fig. 5A). All 70 individuals were

Table 1
Patient characteristics of the individuals in the five study groups. Median (min-max), Mann-Whitney test.

	1st trim. RPL-live birth (n = 23)	3rd trim. RPL-live birth (n = 22)	*p*-Value[a]	1st trim. RPL-pregnancy loss (n = 9)	*p*-Value[b]	3rd trim. control pregnancy (n = 22)	*p*-Value[c]	NP control women (n = 14)
GA at time of blood collection (weeks+days)[d]	7 + 0 (4 +0 – 12 +3)	36 + 1 (35 +0 – 36 +4)	n.a.	5 + 0(4 +0 – 9 +3)	0.094	39 + 0 (37 +0 – 39 +0)	< 0.001 *	NK
Parity	0 (0–2)	0 (0–2)	0.996	1 (0–1)	0.406	1 (0–3)	0.067	NK
Previous miscarriages	3 (3–6)	3 (3–5)	0.293	3 (3–7)	0.818	0	< 0.001 *	NK
Maternal age[e]	35 (31–39)	34 (22–39)	0.667	36 (21–41)	0.670	33 (27–39)	0.251	NK
Maternal BMI	23.0 (19.2–34.1)	24.2 (19.2–34.1)	0.731	23.5 (21.8–25.2)	0.835	NK	n.a.	NK

RPL: recurrent pregnancy loss. *NP*: non-pregnant. *GA*: gestational age. *NK*: not known
[a] group 1st trim. RPL live birth and group 3rd trim. RPL live birth.
[b] group 1st trim. RPL live birth and 1st trim. RPL live pregnancy loss.
[c] 3rd trim. RPL live birth and 3rd trim. control pregnancy.
[d] No exact GA of time of blood collection is known for 23 samples (1st trim. RPL-live birth = 6, 3rd trim. RPL-live birth = 13, 1st trim. RPL-pregnancy loss = 4).
[e] Maternal age is unknown for two samples (1st trim. RPL live birth = 1, 3rd trim. RPL live birth = 1).

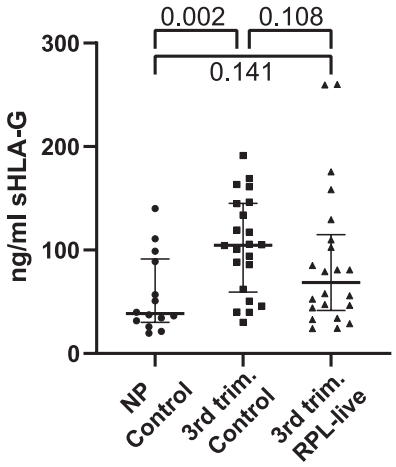

Fig. 2. Concentration of sHLA-G in blood of non-pregnant (NP) control women compared to third-trimester pregnant women without a history of RPL and to RPL-live birth women. In third-trimester samples, pregnant control women demonstrate increased sHLA-G levels compared to non-pregnant controls ($p = 0.002$). However, RPL-live birth women do not significantly increase sHLA-G levels compared to non-pregnant controls ($p = 0.141$). When comparing third-trimester control pregnancy to RPL- live birth there is a trend towards a decrease of sHLA-G levels in RPL-live birth women ($p = 0.108$). The graph shows medians with IQR. Differences between groups were tested with Mann-Whitney test.

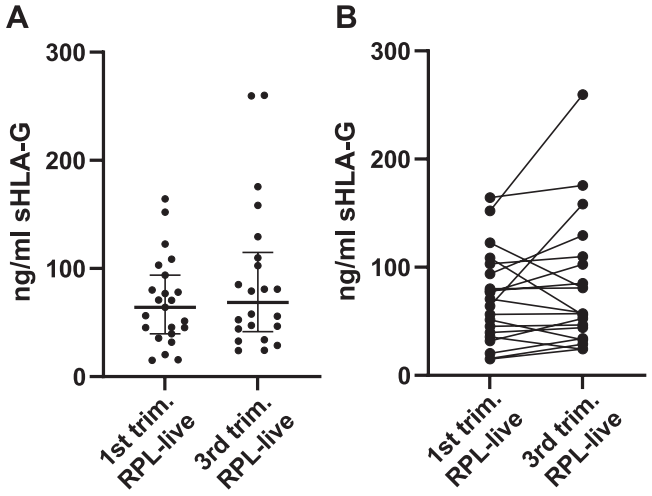

Fig. 3. Comparison of sHLA-G concentrations in blood during 1st trimester and 3rd trimester of live birth pregnancies in women with a history of RPL. (A) All samples of women with a history of RPL show there is no significant difference in sHLA-G between first and third trimester ($p = 0.424$). (B) When comparing the paired samples available in the dataset we also observed no significant difference in sHLA-G levels form first trimester to term ($p = 0.165$). Graphs show medians with IQR. Differences in the left figure were tested by Mann-Whitney test, and in the right figure with Wilcoxon test of paired samples.

included in this analysis. When an individual was in multiple study groups, the average concentration of sHLA-G was used. Therefore, we tested whether groups differed in sHLA-G levels after correcting for BMI. We found after correction no difference between in first trimester sHLA-G levels when comparing RPL-live birth to RPL-pregnancy loss ($p = 0.403$) (Fig. 5B). Additionally, we also found no difference between first- and third- trimester sHLA-G concentrations in the RPL-live birth women ($p = 0.451$) (Fig. 5 C). Furthermore, we did not observe any other correlation between patient characteristics (e.g. parity) and sHLA-G levels.

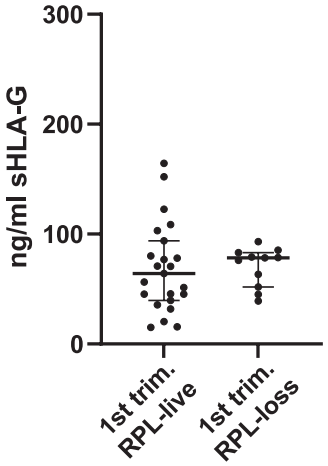

Fig. 4. Soluble HLA-G levels during first trimester in blood of women with a history of RPL. First-trimester samples of women with a history of RPL and subsequent ongoing pregnancy were not significantly different ($p = 0.513$) in sHLA-G compared to women with RPL and subsequent pregnancy loss. Graph shows medians with IQR. Difference was tested by Mann-Whitney test.

3.4. Equal distribution of 3′UTR HLA-G gene polymorphisms among groups

Since decreased sHLA-G levels may be associated with certain polymorphisms in the *HLA-G* gene, we examined the distribution of *HLA-G* polymorphisms within the RPL and pregnant control group. No difference was observed in the distribution of the 14-bp insertion/deletion polymorphism between women with a history of RPL and the pregnant control group (Supplementary Table 1). Similarly, no difference was observed for three other polymorphisms (+3142 C/G, +3187 A/G and +3196 C/G) between the RPL and control group (data not shown). Next to genotype analysis also 3′UTR haplotypes were determined and analyzed. Due to low sample size, we made three groups referring to UTR haplotypes associated with low (UTR-1 and UTR-3), or high (UTR-2, UTR-4 and UTR-7) expression of sHLA-G or a combination of a low and high haplotype (medium) (Martelli-Palomino et al., 2013; Carlini et al., 2013; Di Cristofaro et al., 2013). We found no significant differences between the RPL women and pregnant control women in the distribution of 3′UTRs described the be associated with low, medium or high HLA-G expression (data not shown). We also performed typing on the non-pregnant control group, but this group was too small to make comparisons with the RPL group or pregnant control group.

4. Discussion

There are no substantiated therapeutic treatment options for unexplained RPL as long as the understanding of the pathophysiology of RPL is limited. Furthermore, no suitable biomarkers are available for pregnancy outcome after unexplained RPL. Comparison of women with a history of RPL with subsequent ongoing pregnancy to those suffering from subsequent pregnancy loss may provide more insight into the underlying pathophysiology and may lead to the identification of predictive biomarkers for pregnancy outcome. Therefore, we compared sHLA-G levels between these two groups and found that sHLA-G is not a predictive marker for pregnancy outcome in the first trimester. In women with a history of RPL sHLA-G levels did not increase compared to non-pregnant controls and remained stable between the first and third trimester. At the same time, pregnant control women without a history of RPL did show increased levels of sHLA-G in the third trimester.

Previous literature shows an increase in sHLA-G levels in blood of pregnant women compared to non-pregnant individuals (Hunt et al., 2000; Rebmann et al., 1999; Alegre et al., 2007). This is in concordance

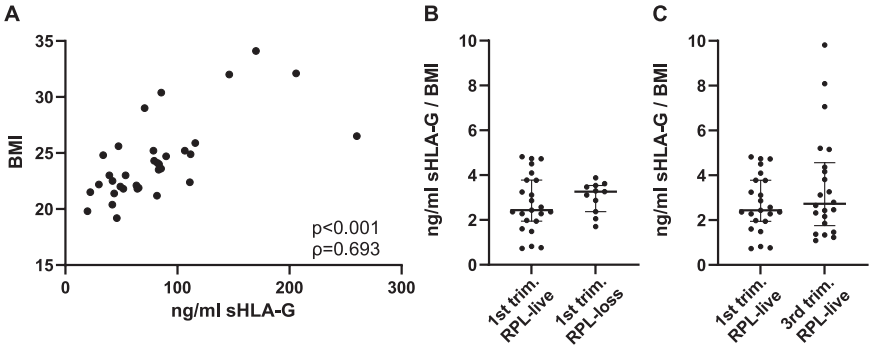

Fig. 5. Results for sHLA-G levels in women with RPL history after correction from BMI. (A) There is a positive correlation between BMI and sHLA-G concentrations (rho=0.693, $p < 0.001$). After correction for BMI (B) first-trimester samples of women with a history of RPL who have either a live birth pregnancy or a pregnancy loss did not significantly differ in sHLA-G levels ($p = 0.403$). (C) Similarly, when comparing first- and third-trimester samples of women with a history of RPL no significant differences were observed ($p = 0.451$). The first graph shows a Spearman correlation plot. While the other two graphs show medians with IQR and difference was tested with Mann-Whitney test.

with results from the current study, where women at term with no history of RPL demonstrated an increase in sHLA-G concentration. In contrast, RPL-live birth women did not significantly increase their sHLA-G levels compared to non-pregnant controls and compared to pregnant controls. Our research group previously showed that local HLA-G levels at the maternal-fetal interface (decidua) at term are increased in women with a history of RPL compared to term decidua from women without a history of RPL (Craenmehr et al., 2019). Since HLA-G can be secreted into the circulation, we hypothesized that women with a history of RPL may also have high sHLA-G levels in their blood at the time of 3rd trimester of an ongoing pregnancy. However, we found an opposite trend of lower sHLA-G levels compared to term controls. These data show that HLA-G levels in the decidua are not reflected in the peripheral blood sHLA-G levels. Mechanistically, this suggests that when there is not sufficient sHLA-G systemically, the placenta might compensate by locally providing more HLA-G. Findings from Djurisic et al. strengthen this point as they showed that sHLA-G levels in uterine blood (comprising of maternal and fetal blood, intervillous and amniotic fluids) isolated from first-trimester abortion material contains higher levels of sHLA-G than peripheral blood (Djurisic et al., 2015). Persson et al., however, did not observe a difference in placental blood and peripheral blood sHLA-G levels at term (Persson et al., 2021). In both studies HLA-G expression in the placenta was studied (membrane bound MFI and mRNA expression, respectively), but not directly compared to sHLA-G peripheral blood levels. To test this hypothesis further, future studies should focus on comparing sHLA-G in blood to membrane bound HLA-G in paired decidua samples.

Studies on possible differences between local and systemic HLA-G may also confirm the importance of the local microenvironment to maintain pregnancy. Many studies focus on peripheral blood to study pregnancy-related immunology. However, it has been shown before that the immune cell composition in peripheral blood is vastly different from that in the decidua (van der Zwan et al., 2020; Vento-Tormo et al., 2018). The same probably holds true for several other immunological factors including HLA-G. Somewhat contradictory is the fact that an ideal biomarker would be obtained from the peripheral blood. Indeed, when we examined sHLA-G as a possible biomarker for pregnancy outcome at first trimester we found that it cannot be used as a biomarker for pregnancy outcome. As described above, we initially hypothesized sHLA-G levels to be relatively high in RPL-live birth women both at first trimester and term whereas we expected sHLA-G to be relatively low in women with RPL and subsequent pregnancy loss. However, there was no difference in the sHLA-G levels between the two groups. Bates et al. also did not find a difference in cytokine profiles between RPL-live birth and RPL-pregnancy loss women, but did find differences between pregnant and non-pregnant controls (Bates et al., 2002). Together, these results raise the question of whether the maternal condition changes to attain a healthy ongoing pregnancy after RPL and, if so, whether there is a role of the fetus in contributing to a healthy ongoing pregnancy in women with RPL.

We found that in RPL-live birth there is no difference in blood sHLA-G levels between first trimester and third trimester. Some studies found a stable sHLA-G concentration (Alegre et al., 2007; Hunt et al., 2000) while others found a decrease (Yie et al., 2005; Beneventi et al., 2017; Klitkou et al., 2015) throughout gestation. Yie et al. observed a decrease in healthy pregnancy but not in their preeclamptic group, where sHLA-G levels were decreased compared to controls but stable over gestation (Yie et al., 2005). This is similar to our findings, where we observed in the RPL-live birth group a trend towards decrease compared to healthy pregnancy controls, but within the RPL-live birth group the sHLA-G levels were the same between first trimester and term.

Furthermore, we found a strong correlation between BMI and sHLA-G concentrations. In pregnancy it has been shown before that in obese women the sHLA-G levels are higher than in non-obese women (Beneventi et al., 2017). However, Persson et al. did not find a correlation between BMI and sHLA-G levels (Persson et al., 2021). Our cohort did include a few mildly obese women ($n = 8$, BMI ≥ 30; $n = 61$, BMI <30) but when these were excluded from the study, we still observed a significant correlation between BMI and sHLA-G concentrations ($p < 0.001$, data not shown). High amounts of adipose tissue cause increased low-grade inflammation, which is described to be balanced by increased amounts of Treg cells (Feuerer et al., 2009). Since HLA-G and Tregs are both immune regulatory, further studies could elucidate the role of HLA-G to balance inflammation mediated by adipose tissue. BMI was not documented for our control groups. Nevertheless, in the study groups with pregnant women BMI was no confounder, and after correcting for BMI in the other groups the main findings of the study were similar.

Lastly, we performed *HLA-G* typing for all women and focused on four polymorphisms in the 3′UTR of the *HLA-G* gene, which have been described to predispose to altered HLA-G expression, namely 14-bp insertion/deletion (rs371194629), + 3142 C/G (rs1063320), + 3187 A/G (rs9380142) and + 3196 C/G (rs1610696) (Rousseau et al., 2003; Chen et al., 2008; Yie et al., 2008; Tan et al., 2007; Dahl et al., 2015). It is believed that microRNAs bind differently to sites present in the 3′UTR of HLA-G mRNA depending on the polymorphisms in the 3′UTR region, thereby post-transcriptionally affecting the expression of HLA-G protein (Reches et al., 2020). There are contradictory results where some studies described that women with RPL have *HLA-G* gene constitutions that predispose to reduced HLA-G expression and secretion (Craenmehr et al., 2019; Xue et al., 2007; Christiansen et al., 2012; Monti et al., 2019), while other studies found, similarly to the current study, no difference between RPL and control groups (Vargas et al., 2011; Hviid et al., 2002; Kalotra et al., 2018). However, we cannot rule out that the difference in HLA-G levels between the women with RPL and pregnant control women is affected by genetic predisposition, as there seemed to be a slight trend in our study towards a higher frequency of the insertion phenotype in women with RPL, which is expected to be associated with lower sHLA-G production (Chen et al., 2008).

Our group size is too small to provide reliable results on the heterogeneity that is present in the HLA-G gene. Nevertheless, we have

included these data to provide more insight into the study population. In this retrospective study we ideally aimed to have similar number in the RPL-pregnancy loss and RPL-live birth groups. However, we obtained a lower amount of samples for the RPL-pregnancy loss group. This could have to do with the fact that previously women were referred to our clinic when they had a history of three or more pregnancy losses. After three or more pregnancy losses the ongoing pregnancy rate is 80.3 % (Youssef et al., 2020). Another limitation of the study is that sodium heparin was used as blood anticoagulant. Previous studies have indicated that blood plasma prepared with EDTA anticoagulant have significantly higher sHLA-G levels than heparin prepared blood (Poláková et al., 2011). However, in the current study we used sodium heparin in all study groups, we confirmed reproducibility (data not shown) and can therefore accurately compare the groups. However, these concentrations cannot be compared with other studies where EDTA was used as blood anticoagulant. Also, no distinction can be made between different sHLA-G isoforms using the commercial ELISA kit (MyBiosource).

In summary, no significantly increased blood sHLA-G levels in RPL-live birth women were found, in contrast to pregnant women without a history of RPL. Furthermore, in women with a history of RPL blood sHLA-G levels at first trimester did not differ between RPL-live birth and RPL-pregnancy loss. Therefore, systemic blood sHLA-G levels cannot be used as biomarker for pregnancy outcome in women with a history of RPL. High sHLA-G blood levels are obviously not a prerequisite to ensure an ongoing pregnancy. Given that local HLA-G levels in the decidua are not directly reflecting systemic sHLA-G levels in maternal blood, it is important to incorporate the local environment in the placenta to investigate underlying mechanisms of RPL and subsequent live birth pregnancy.

Author statement

All authors declare that they have seen and approved this final version of the manuscript. None of the authors have a conflict of interest.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jri.2022.103665.

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