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Human Leukocyte Antigen HLA-C, HLA-G, HLA-F and HLA-E placental profiles are altered in Early Severe Preeclampsia and Preterm Birth with Chorioamnionitis.

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1) **Condensation:** Placental Human Leukocyte Antigen expression is altered in the placenta and EVT of severe early onset PE pregnancies; HLA-G and HLA-C increased, HLA-F and E decreased. HLA_F and HLA-C are increased in the placenta of PTBs with chorioamnionitis.

2) **Short Title:** Dysregulation of placental HLA profiles in severe PE and PTB.

3) **AJOG at a Glance:**

A. **Why was this study conducted?** Increasing evidence suggests a dysfunctional maternal immune component to the etiology of severe preeclampsia (PE) and preterm birth (PTB). The extravillous trophoblast modulate maternal immunology by expressing non classical HLA proteins that interact with inhibitory receptors on the decidual immune cells. We hypothesised that dysregulation of HLA expression in the EVT of the placental bed would correlate with cases of PE.

B. **What are the key findings?** Expression levels of HLA-C, E, F, and G are significantly altered in the placenta and EVT in severe early onset PE pregnancies and PTB with chorioamnionitis from levels found in normal pregnancies. The potential to capitalize on these observations as early markers for prediction and to envision new therapeutic interventions are considered.

C. **What does this study add to what is already known?**

This novel study presents evidence of distinct changes in expression levels of HLA class I in the placental bed of pregnancies with severe early onset PE as compared to healthy pregnancies. Moreover, we show for the first time that placental levels of HLA-F and C are increased in the preterm placental with chorioamnionitis. The change in their collective expression pattern is a significant advance in our understanding of the immunological processes operating at the fetal maternal interface. As a practical application, the findings could be applied evaluate maternal serum
levels of HLA-C, E, F, G in early pregnancy as a predictor of severe preclampsia or maternal fetal rejection and preterm birth.
Abstract

**Background:** The extravillous trophoblast expresses each of the non-classical MHC class I antigens - HLA-E, F, and G and a single classical class I antigen HLA-C. We recently demonstrated dynamic expression patterns of HLA-C, G and F during early EVT invasion and placentation. **Objective:** In this study we investigate the hypothesis that the immune inflammatory mediated complications of pregnancy such as early preeclampsia and preterm labor, may show altered expression profiles of non-classical HLA. **Study Design:** Real time q-PCR, western blot and immunohistochemistry were performed on placental villous tissues and basal plate sections from term non-laboring deliveries, preterm deliveries and severe early onset preeclampsia both with and without small for gestational age neonates. **Results:** HLA-G is strongly and exclusively expressed by the EVT within the placental basal plate and its levels increase in pregnancies complicated by severe early onset PE with SGA neonates as compared to healthy term controls. HLA-C shows a similar profile in the EVT of PE pregnancies, but significantly decreases in the villous placenta. HLA-F protein levels are decreased in both EVT and villous placenta of severe early onset PE pregnancies both with and without SGA babies as compared to Term and PTB deliveries. HLA-E decreases in blood vessels in placentas from PE pregnancies as compared to Term and PTB deliveries. HLA-F and HLA-C are increased in the placenta of PTBs with chorioamnionitis as compared to idiopathic PTB. **Conclusion:** Dysregulation of placental HLA expression at the maternal fetal interface may contribute to the compromised maternal tolerance in PTB with chorioamnionitis and excessive maternal systemic inflammation associated with severe early onset PE.

**Key Words:** Human Leukocyte Antigen, placenta, extravillous trophoblast, preeclampsia, preterm birth
Introduction

Human pregnancy is one of the most interesting examples of immune tolerance seen in mammalian biology, where direct contact between the maternal immune system and the placental allograft results in adaptive maternal tolerance to paternally derived antigens. The human placenta expands during the first trimester of pregnancy giving rise to the invasive extravillous trophoblast (EVT) population. The EVT invade the decidua and myometrium to increase maternal blood flow to the developing placenta and fetus and transforming the uterine spiral arteries, uterine glands, and veins that supply maternal blood and nutrients to the placenta and fetus.\textsuperscript{1-4} A beneficial interplay between the EVT and the specialized resident maternal decidual leukocytes (uterine Natural Killer cells (uNK) and myeloid derived macrophage and T cells) within the decidua promotes immune-tolerance. It is mediated via cell-cell interactions between the human leukocyte antigen (HLA) class I ligands and killer immunoglobulin receptors (KIR), natural killer receptors (NKp), and ILT2/4 receptors (also known as LILRB1, LILRB2) receptors respectively.\textsuperscript{5-12} This process is disrupted early in pregnancy in cases of severe preeclampsia (PE), intrauterine growth restriction (IUGR) and preterm birth (PTB); as evidenced by the presence of untransformed uterine spiral arteries and a lack of endovascular EVT in the placental bed of such cases.\textsuperscript{13-17}

The EVT simultaneously express all three of the non-classical major histocompatibility complex (MHC) class I antigens, HLA-E, HLA-F and HLA-G.\textsuperscript{18-22} Levels of which change with gestational age, and tissue location (placenta vs membranes) and with fetal sex\textsuperscript{18,23} The low allelic polymorphism of HLA-G and its restricted expression to the EVT led to an early hypothesis that HLA-G functioned as a substitute for the local classical HLA-I antigens during pregnancy.\textsuperscript{24} Further, the reported low level of soluble HLA-G isoforms in maternal plasma at term and in preeclamptic (PE) pregnancies,\textsuperscript{25,26} together with the presence of activated macrophage expressing
the inhibitory HLA-G ligands, ILT2 and ILT4 in the placental bed of laboring and PE pregnancies suggested the hypothesis that HLA-G acts to promote maternal tolerance of the fetal allograft during pregnancy.

The placenta does not express the classical polymorphic MHC class I antigens with the exception of HLA-C, which is only expressed in the B2m-associated form. HLA-C like HLA-G is thought to play a role in the first 12 weeks of pregnancy during maximal EVT invasion into the decidua and myometrium, and the highest levels of uNK-specific KIRDL1/2 expression. HLA-C mismatched pregnancies have been shown to exhibit a decidual lymphocytic response to fetal EVT in decidual tissues, whereas HLA-C matched pregnancies do not. Placental HLA-C genotypes have also been suggested in the pathogenesis of PE, in that the fetal HLA-C C2 allele was reported to increase the risk of PE if associated with the maternal uNK KIR AA or KIRB haplotype phenotype. However, a study of severe PE pregnancies utilizing maternal and neonatal blood samples from the Danish National Birth Cohort and the Danish Neonatal Screening Biobank did not find any such association.

Our group has recently characterized the placental HLA profile across normal gestation demonstrating that first trimester EVT strongly express HLA-G, C and F but only express HLA-E in the very early 1st trimester placenta. In this study, we investigate placental HLA profiles in healthy pregnancies, preterm births, and severe early onset PE with appropriate and small for gestational age (AGA and SGA) babies with the hypothesis that the immune inflammatory mediated complications of pregnancy may show distinct and altered EVT specific expression profiles of non-classical HLA in comparison to healthy pregnancies.

Materials and Methods
Ethics

This study protocol was approved by the Mount Sinai Hospital Research Ethics Board, Sinai Health System (Placental Biobank REB# 02-0061A and Dr Lye’s laboratory REB# 12-0007E). All subjects donating placental tissues for research gave written informed consent in accordance with the Declaration of Helsinki. A material transfer agreement between OHSU and the Lunenfeld Tanenbaum Research Institute, Toronto allowed for transfer of tissue sections and de-identified clinical data between sites.

Tissue Collection

Frozen placental villous tissue and paraffin-fixed tissue sections were obtained from the Placental Biobank of Mount Sinai Hospital Toronto (all clinical information is detailed in Tables 1 for the IHC study and 2 for the villous placental study). Placental samples used in this study were from the following sources: (1) Term Normal (Healthy) Elective Caesarian Sections (ELCS) (38-41wk, n=22); (2) Preterm birth (PTB) (27-34wk, n=15); (3) Severe early onset PE with an appropriate weight for gestational age neonate (PE AGA, 28-34wk, n=20); and (4) Severe early onset PE with a small for gestational age (SGA) neonate (PE SGA, 28-34wk, n=21). Severe PE was diagnosed according to the most recent American College of Obstetricians and gynecologists (ACOG) criteria. The early severe PE group were delivered prematurely secondary to the diagnosis of severe PE and were not in labor. All PTB were from laboring deliveries 10 vaginal and 2 emergency Caesarian Sections, 7 of which were complicated by acute stage 2/3 chorioamnionitis identified by a placental pathologist at SHS. Frozen villous tissue was ground under liquid nitrogen in a mortar and pestle and RNA and protein extracted. Full thickness samples from the chorionic
plate to the placental bed were also collected for immunohistochemical analysis of the EVT in the placental bed.\textsuperscript{36}

**Real-time polymerase chain reaction**

RNA was isolated from all placentas using a Tissue Lyser II and RNeasy Universal Mini Kit (Qiagen, Toronto, Canada), and qPCR performed to assess levels of HLA-F and C as previously described.\textsuperscript{18}

**Western Blotting**

Equal amounts of total proteins from villous placenta samples (n of 12 per group) were extracted in High Salt Buffer and analyzed by Western blot analysis to detect levels of HLA-C and HLA-F as previously described (Table 2).\textsuperscript{18}

**Immunohistochemistry**

All immunohistochemistry (IHC) was performed at OHSU, Portland as previously described.\textsuperscript{37} Antigen retrieval specific to each anti-HLA antibody was performed as indicated Table 3. Samples (Table 1) were only included in the analysis if HLA-G positive EVT were determined to be present in the decidua i.e true placental basal plate. Term ELCS n=9, PTB (labor, vaginal delivery, no chorioamnionitis) n=8 and severe early onset PE AGA n=6; PE SGA n=5 (No labor, emergency Caesarian Section in all cases) were included in the final analysis. DAB intensity of 6 discrete regions was scored on digital images by two independent investigators blinded to patient group using Velocity Imaging software and a digitally calibrated scale of 0 - 5 (i.e no staining to very strong staining). The regions 1-6 were: 1) Proximal EVT (close to the decidual surface), 2) Distal
EVT (Deep within the decidua), 3) Decidual stromal cells, 4) Placental villous trophoblast, 5) Placental mesenchymal core and 6) Placental capillaries.

Statistical analyses

Statistical analysis of data was performed using Prism software on normally distributed data using one way ANOVA and Bonferroni post-testing. The non-parametric Kruskal Wallis test and Dunns post-test was used to analyze non-normally distributed ranked data such as the clinical parameters in Table 1 and 2 while the Mann Whitney U test was used to test for significant differences in IHC scoring intensities of HLA expression profile differences between patient groups. R software was used to perform multivariate analysis was used in the western blot analysis to assess for the impact of labor, infection, fetal sex and gravidity and gestational age. Graphical data is plotted as median ± S.D. P values of <0.05 as compared with respective controls were considered significant.

Results

EVT HLA-G and C expression is increased and HLA-F decreased in the preeclamptic placental bed

As HLA-G is exclusively expressed in the EVT, we utilized it as a marker for the EVT in the placental bed. Strong HLA-G staining was observed in the plasma membrane of all EVT (Fig 1). Interestingly, HLA-G expression in term- ELCS and idiopathic preterm birth- PTB groups decreased in the invaded distal EVT deeper within the decidual placental bed, when compared to proximal EVT found embedded in fibrin matrix at the decidual surface (p<0.05) (Fig 1 and 2A). A similar decrease in HLA-G expression in distal compared to proximal EVT was also noted in Early Severe PE without SGA but did not reach significance. However proximal, and distal HLA-G expression in the most severe PE group (PE with SGA) significantly increased above those
observed in healthy term controls (proximal p<0.01, distal p<0.0115) (Fig 2A). HLA-C demonstrated a general increase in the earlier gestational age samples i.e idiopathic PTB and PE, when comparing proximal to distal EVT, but due to the wide distribution of staining intensity within each group no statistical differences were observed between proximal and distal EVT (Fig 1 and 2B). However, while distal EVT of PE AGA group showed a trend of increased HLA-C expression (p=0.0568), the more severe PE SGA group revealed a significantly higher staining intensity of HLA-C in distal EVT in comparison to normal term not in labor (p<0.01) (Fig 1 and Fig 2B). In contrast, HLA-F increased in the distal EVT of term ELCS and idiopathic PTB pregnancies as compared to the respective proximal group (p<0.05) (Fig 2C). However, both proximal and distal EVT from severe early PE with or without SGA displayed a lower intensity of HLA-F staining as compared to the respective normal non-laboring term EVT (proximal p<0.05, distal p<0.01) (Fig 1 and Fig 2C).

**HLA-C levels are increased in the placentas of preterm births with chorioamnionitis and in laboring deliveries.**

Placental HLA-C mRNA expression levels do not demonstrate any differences between clinical groups (Fig 3A). However, HLA-C protein levels were significantly increased in the placenta samples from PTBs with chorioamnionitis (acute stage 2/3; confirmed by placental pathology), as compared to idiopathic PTB, term non-laboring ELCS, laboring vaginal deliveries and placentas from severe early onset PE with or without an SGA newborn (Fig 3B and C). HLA-C was also increased in the placentas from laboring vaginal deliveries as compared to non-laboring ELCS deliveries within each clinical group; except for the PE SGA samples which were all from ELCS
deliveries (p<0.05). There was no effect of fetal sex or gestational age or gravidity. IHC analysis of the pregnancies complicated by PE A lower staining intensity of HLA-C was noted in villous trophoblast, and the mesenchymal villous core in the PE AGA ELCS group as compared to idiopathic PTB placentas (Fig 4A and 4B). There was a decreased trend when comparing the PE SGA and PTB group. HLA-C expression was also significantly decreased in placental blood vessels in the PE AGA ELCS group when compared to Term placetas (Fig 4C).

**HLA-F mRNA and protein expression is decreased in placentas from pregnancies complicated by PE.**

HLA-F mRNA expression was found to be significantly increased in term ELCS samples compared to all other groups (p<0.05), but no differences were observed between PTB and early PE groups (Fig 5A). In the placental protein lysates the highest amount of HLA-F protein was detected in the term samples and samples from PTB with chorioamnionitis. Within the PTB group HLA-F protein levels were significantly increased in PTB with chorioamnionitis as compared to idiopathic PTB and all PE groups (p<0.01) (Fig 5B and 5C). There was no effect of labor, gravidity fetal sex in this analysis. HLA-F protein expression slightly decreased in placentas from both severe early PE with or w/out SGA as compared to term (p<0.05) (Fig 5B and 5C). HLA-F was expressed by villous syncytiotrophoblast and the villous stromal mesenchyme of term pregnancies as previously reported. Immunohistochemical analyses similarly revealed a small but non-significant decrease in HLA-F staining intensity in the trophoblast layer of early severe PE pregnancies (Fig 1).

**HLA-E expression is reduced in the feto-placental vasculature of PE placentas.**
HLA-E was very weakly detected in the EVT in the third trimester placental basal plate (Fig 6B) and due to a wide distribution observed in the staining intensity in the PTB and ELCS groups no significant differences were found in comparison to PE and PE SGA pregnancies (Fig 2D). In placentas from term ELCS and PE AGA pregnancies HLA-E showed a small but significant increase in mRNA levels as compared to idiopathic PTB (p<0.05) (Fig 6A). IHC showed that HLA-E was strongly expressed in the nuclei of the syncytio-cytotrophoblast bilayer of the placenta, and at lower levels in perivascular cells in the placental mesenchymal core and endothelial cells of placental vessels (Fig 6B and 4F). IHC revealed that HLA-E expression significantly decreased in placental vessels from PE AGA pregnancies compared to either idiopathic PTB or ELCS pregnancies (p<0.05) (Fig 4D).

Comment

Principal Findings

In this study we have shown that the EVT of the placental bed and placenta exhibit distinct and differential expression patterns of the non-classical class I HLA-G and HLA-F and the classical class I HLA-C in the pathological complications of pregnancy; PTBs with chorioamnionitis and severe early onset PE.

Results in the context of what is known

As expected, HLA-G was strongly expressed in the cell membranes of the EVT in the 3rd trimester placental bed. Prior work found strong cell surface HLA-G expression in early normal 1st trimester EVT transitioning to intracellular and weaker as gestation progressed into the 2nd trimester, a time
at which that active EVT invasion is reported to decrease.18,38 Similarly, we observed that HLA-G expression levels were low in the distal EVT deep within the placental bed at the myometrial junction, as compared to the proximal EVT at the maternal surface in both the PTB and term ELCS samples. HLA-G was also clearly restricted to the cell membrane of the EVT in the decidua. This may be related to the stationary status of the EVT in the 3rd trimester placental bed, as term EVT are known to undergo endoduplication of their nuclei and become fused large secretory cells.39 In line with previous reports,30 cytoplasmic HLA-C expression was also detected in EVT in the placental bed, and did not differ between the proximal and distal EVT in term ELCS group. HLA-C expression on the cell surface has been reported in both first trimester and term EVT.30 23

In contrast, in the PE placental bed, both HLA-G and HLA-C were highly expressed in the distal EVT as compared to their respective proximal EVT intensity. This was most profound in the severe PE group with an SGA newborn; a more severe form of PE related to placental dysfunction.40 We and others have recently shown that the Y153H mutation of the STOX1 transcription factor is enriched in the placentas in such cases41-43 and contributes to defective EVT invasion by preventing the acquisition of an invasive state.42,44 We suggest that the elevated HLA-G/C expression in these severe PE cases may therefore reflect this undifferentiated status of the EVT. The increase in HLA-G in the distal EVT of the placenta bed of PE pregnancies that we are reporting is in contrast with earlier reports of decreased HLA-G mRNA or protein levels in EVT of PE pregnancies.45,46 In our study we have utilized the pan HLA-G1 antibody 4H84 and separated proximal and distal EVT in our analysis while these earlier studies utilized antisense RNA probes or the 87G antibody (limited to frozen tissues) and considered the EVT as a whole unit.45 Additionally, our samples are all from severe early onset preterm PE pregnancies, while the earlier studies were a mix of preterm and term PE.26,34,47 Early onset PE occurring before 34 weeks of
gestation is attributed to defective EVT invasion and placentation\textsuperscript{42,48}, together with poor decidualization and disturbances in the decidual immune cell populations\textsuperscript{49,50} all of which contribute to failed uterovascular transformation, placental necrosis and higher levels of maternal morbidity.\textsuperscript{51} In contrast, late onset PE is not associated with defective EVT invasion or failed uterovascular transformation but appears to be a primarily a maternal cardiovascular inflammatory reaction leading to systemic endothelial activation.\textsuperscript{52,53} These studies suggest that the differences in PE subtype may account for the discrepancy in our findings with the previous studies.

HLA-F was expressed at higher levels in the distal invasive EVT as compared to the respective proximal groups in the idiopathic preterm and non-laboring term control samples. This is in accordance with our former results showing that HLA-F is increased on the cell surface of actively invading 1\textsuperscript{st} trimester trophoblast\textsuperscript{18} and provides validation to our hypothesis of an active role for HLA-F in EVT invasion. Increasing evidence also links high HLA-F expression with invasive cancer progression and metastasis\textsuperscript{54-56} further supporting a pro-invasive role for HLA-F. Importantly, the proximal and distal EVT of the PE samples displayed significantly lower intensity of HLA-F than that in the healthy controls. This too may reflect the reduced invasive capability of these cells in the PE condition.

HLA-E was low in all 3\textsuperscript{rd} trimester EVT. We have reported high HLA-E expression in EVT at 5 weeks of gestation but undetectable expression from 7 weeks onwards.\textsuperscript{18} Similar observations were made by Papuchova et al., who show that HLA-E is highly expressed in early EVT and decreases as gestation progresses to term.\textsuperscript{23} These studies are suggestive of a protective role for HLA-E in the earliest events of implantation but not in active EVT invasion. The role of HLA-E in the syncytiotrophoblast nuclei is currently unknown. However, we found a significant reduction in levels of HLA-E in the endothelial cells of the placenta vasculature of PE AGA pregnancies as
compared to controls. This endothelial expression of HLA-E is expected and it is thought to play a regulatory role in coagulation, inflammation and vascular homeostasis,\textsuperscript{57} all factors that are compromised by PE.\textsuperscript{58}

Interestingly, both HLA-C and HLA-F were significantly increased in the villous placenta in samples from preterm deliveries with acute stage 2/3 chorioamnionitis, as compared to idiopathic PTB. This data correlates well with the findings of Lee et al, who have previously reported that chronic chorioamnionitis and maternal anti-HLA class I seropositivity were associated with preterm labor and delivery, and fetal death.\textsuperscript{59,60} Our data therefore lends further support to the hypothesis that cellular and antibody-mediated anti-fetal rejection of the placenta and fetus is associated with immune infiltration of the placental tissues in preterm birth with chorioamnionitis.

**Clinical Implications**

The dysregulation of the non-classical HLA antigens in the EVT of the placental bed of PE pregnancies and the increase in HLA-F and C in infected PTB supports their important role in maternal fetal adaptive immunology. This data also lends further support for an immune component to the onset of early severe PE and PTB and a new avenue for potential preventive therapy.

**Research Implications**

Interestingly, in the IHC analysis both HLA-C and HLA-F protein levels were decreased in villous placenta from severe early onset PE cases, in comparison to the gestational matched idiopathic preterm samples. This may be attributable to the reported dysregulated differentiation and function of the villous mesenchyme in cases of PE.\textsuperscript{61,62} Studies have shown that HLA-C is transcriptionally cis-regulated at the promoter level by members of the NLR family of proteins including the
nucleotide-binding domain, leucine-rich repeat family, CARD domain-containing proteins

NLRC5 and NLRP2. NLRP2 has been shown to suppress cell surface expression of HLA-C without affecting HLA-E and HLA-G expression on EVT possibly implicating altered expression of NLRP2 and NLCR5 in the placenta of severe PE. The novel finding of reduced HLA-C and E expression in the feto-placental endothelial cells, that the maternal endothelial dysfunction and inflammation associated with PE extends to the feto-placental unit and supports the involvement of the NLRP inflammasomal pathway in PE. Further studies are warranted to characterize placental HLA expression in PE at term as well additional in-vitro studies to elucidate the interplay of HLA and their interaction with decidual NK cells. Lastly, if these findings translate into differences in the soluble forms of these antigens in maternal serum of early pregnancy they may prove to be functional early biomarkers for the onset of PE. Still, we do not know if soluble forms of HLA in the maternal plasma will correlate with placental levels across the course of gestation; this is the focus of our continuing studies.

**Strengths and Limitations**

The strength of our study derives from original and consistent findings that are a significant advance of our understanding of the immunology of pregnancy at the direct site of maternal fetal cellular interaction in the placental bed. The small sample number in the placental bed study and lack of non-laboring gestational matched controls for early severe PE are the primary limitation and so is the sex bias with greater numbers of males to female babies in our data set (Table 1), as well lack of PTB with chorioamnionitis in the IHC group.

**Conclusion**
Non-classical HLA expression is dysregulated in the EVT of the placental bed of severe early onset PE pregnancies; HLA-G and C increased, while F decreased. HLA-F and C increased in the placentas of PTB with chorioamnionitis. This altered MHC profile of the EVT and placenta may contribute to both failed EVT invasion and the altered maternal inflammatory immune response documented in the decidua of PE and PTB pregnancies.

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Author Contributions:

Conceptualization and project design: CED, and RH
Data acquisition, analysis and figures: CED, MD, HH.
Writing – original draft preparation: CED, MB and RH.
Writing – review & critical editing: CED, MB, JZ, HH, DEG, LM and RH
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Conflict of Interests

The authors declare no conflicts of interest.

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**Figure legends**

**Figure 1.** EVT HLA-G and F expression is increased while HLA-C decreases in the placental bed with preeclampsia. Representative images of HLA-G, C and F immunostaining of EVT resident in the placental basal plate are shown in serial sections utilizing HLA-G as an EVT marker for 3 independent patient samples from each of the following groups. 1) Term = term non-laboring deliveries, (n=9) 2) PTB = laboring idiopathic preterm birth before 36 weeks of gestation (n=8) and 3) PE = pregnancies complicated by severe early onset PE with (n=6) /without an SGA baby (n=6). A rabbit IgG negative control for the anti HLA-C and HLA-F antibodies is shown (upper right) - Rb IgG) EVT = extravillous trophoblast, Plac = placenta, Dec = decidua. Scale bar = 50 μm.

**Figure 2.** HLA-G, HLA-C and HLA-F are differentially expressed in the EVT of the placental bed of preeclamptic pregnancies. The intensity of HLA-G (A), HLA-C (B), HLA-F (C) and HLA-E (D) immunostaining in the EVT of the placental bed from Term (n=9), idiopathic PTB (n=8), PE (n=6) and PE SGA (n=6). Scoring was performed using Velocity Image analysis to generate a digital scale (0-5). Scoring was performed by two independent investigators CD and HH blinded to patient group. Mann Whitney U-test was used to assess differences in staining intensity between 1) Proximal and Distal EVT within groups (# p< 0.05) and 2) Proximal and Distal EVT across the groups (* p< 0.05, ** p<0.01).

**Figure 3.** HLA-C is decreased in the placentas of severe PE pregnancies and increased in PTB with chorioamnionitis. A: Fold change in HLA-C mRNA levels relative to the geometric mean of the housekeeping genes CYC1 and YWHAZ in the placenta of term Caesarian sections (Term n=20), preterm birth (PTB n=15), severe early onset preeclampsia with average for
gestational age fetal weight (PE AGA n=20) and small for gestational age fetal weight (PE SGA, n=21). No differences in HLA-C mRNA expression were observed across all groups. The PTB group is the reference control. B: Representative western blot for HLA-C showing a 40 kDa band corresponding to the positive control (HeLa cell lysate) C: Normalization with the housekeeping protein 1,4,3,3 zeta revealed that HLA-C protein expression was significantly increased in placentas from PTB with chorioamnionitis (n=7) as compared to idiopathic PTB (n=5) term and PE(n=12 in each group). There was an increase in levels of placental HLA-C in laboring samples as compared to placentas from elective non-laboring caesarian sections. ANOVA with Bonferroni post testing was used test for differences between groups. Multivariate analysis was used to assess the effect of fetal sex, gravidity, infection and labor. There was no effect of gestational age or gravidity. Different letters represent a significant difference of p < 0.01.

**Figure 4. HLA-C and E immunostaining is decreased in the villous placenta of severe PE pregnancies.** Graphs showing the scoring intensity of HLA-C and HLA-E immunostaining in the villous placental layers A: Villous syncytiotrophoblast (Syn), B: Villous core (Mes), and C and D: placental blood vessels of Term (BV) (n = 9), idiopathic PTB (n=10), PE AGA (n=6) and PE SGA (n=6). Staining intensity was scored using Velocity Image analysis to generate a digital scale (0-5). Scoring was performed by 2 independent investigators CD and HH blinded to patient group. Mann Whitney U-test was used to assess differences in staining intensity. * represents p < 0.05 E: Representative images of HLA-C immunostaining in the placentas of Term, PTB and PE pregnancies. F: Representative images of HLA-E immunostaining in the placentas of Term, PTB and PE pregnancies. Scale bar = 20 µM.
Figure 5. HLA-F protein levels increase in the placentas of PTB with chorioamnionitis pregnancies. A: Fold change in HLA-F mRNA levels relative to the geometric mean of the housekeeping genes CYC1 and YWHAZ in the placenta in preterm birth (PTB), term Caesarian sections (Term) and severe early onset preeclampsia with a normal weight baby (PE AGA) or a SGA baby (PE SGA). The 26-34wk PTB group is the reference control, n=20 in each group. HLA-F mRNA are significantly higher at term than in all other groups. B: Representative western blot using the anti HLA-F Abcam antibody is shown, a single 40 kDa protein band was detected corresponding to the positive control cell line PLH, the negative control cell line K562 showed no band. C: Quantification of the HLA-F band intensity compared to the house keeping protein 1,4,3,3 zeta showed a significant increase in HLA-F expression in PTB with chorioamnionitis as compared to idiopathic PTB (p<0.05) (n=7 vs n=5) or all cases of PE (n=12). ANOVA with Bonferroni post testing was used test for differences between groups. Multivariate analysis was used to control for the effect of fetal sex, gravidity, infection and labor. Labour, fetal sex and gravidity had no effect. Different letters represent a significant difference of p < 0.05.

Figure 6. HLA-E is weakly expressed in both EVT and placenta and decreases in the blood vessels of placenta from PEAGA deliveries. A: Fold change in HLA-E mRNA levels relative to the geometric mean of the housekeeping genes CYC1 and YWHAZ in the placenta in preterm birth (PTB, n=15), term Caesarian sections (Term, n=22) and severe early onset preeclampsia with a normal weight baby (PE AGA, n=20) or an SGA baby (PE SGA, n=21). The 26-34wk PTB group is the reference control. ANOVA with Bonferroni post testing was used test for differences between groups. Different letters represent a significant difference of p < 0.05. B: Representative images of HLA-E immunostaining of EVT resident in the placental basal plate are shown for 3
independent patient samples each from Term, PTB and PE patient groups. HLA-E is expressed in
the syncytiotrophoblast nuclei and the feto-placental blood vessels. Staining is decreased in the
endothelial cells in the PE placenta. Scale bar = 50 μm, EVT=Extravillous trophoblast,
Dec=Decidua, Plac=Placenta.
Table 1 Patient Demographics for EVT IHC Study

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Table 1 Patient Demographics for Villous Placental Study

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1 Table 2 - Antibodies, dilutions and antigen retrieval for placental immunohistochemistry

2 All antibodies were confirmed to not cross react with other HLA