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Johnsen, G.M.; Fjeldstad, H.E.S.; Drabbels, J.J.M.; Haasnoot, G.W.; Eikmans, M.; Storvold, G.L.; ... ; Staff, A.C.

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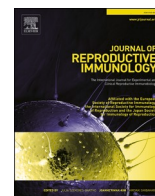
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## A possible role for HLA-G in development of uteroplacental acute atherosclerosis in preeclampsia

Guro M. Johnsen<sup>a,b,c,\*</sup>, Heidi E.S. Fjeldstad<sup>a,b</sup>, Jos J.M. Drabbels<sup>d</sup>, Geert W. Haasnoot<sup>d</sup>, Michael Eikmans<sup>d</sup>, Gro L. Størvold<sup>a,b,c</sup>, Patji Alnaes-Katjavivi<sup>b</sup>, Daniel P. Jacobsen<sup>b</sup>, Siccó A. Scherjon<sup>e</sup>, Christopher W.G. Redman<sup>f</sup>, Frans H.J. Claas<sup>d</sup>, Anne Cathrine Staff<sup>a,b</sup>

<sup>a</sup> Institute for Clinical Medicine, Faculty of Medicine, University of Oslo, Norway

<sup>b</sup> Division of Obstetrics and Gynecology, Oslo University Hospital, Norway

<sup>c</sup> Institute for Experimental Medical Research, Oslo University Hospital, Norway

<sup>d</sup> Department of Immunohematology and Blood Transfusion, Leiden University Medical Center, Netherlands

<sup>e</sup> Department of Obstetrics and Gynecology, University of Groningen, Netherlands

<sup>f</sup> University of Oxford, UK

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### ABSTRACT

HLA-G, a non-classical HLA molecule expressed by extravillous trophoblasts, plays a role in the maternal immune tolerance towards fetal cells. HLA-G expression is regulated by genetic polymorphisms in the 3' untranslated region (3'UTR). Low levels of HLA-G in the maternal circulation and placental tissue are linked to preeclampsia.

Our objective was to investigate whether variants of the 3'UTR of the HLA-G gene in mother and fetus are associated with acute atherosclerosis, a pregnancy specific arterial lesion of the decidua basalis that is prevalent in preeclampsia.

Paired maternal and fetal DNA samples from 83 normotensive and 83 preeclamptic pregnancies were analyzed. We sequenced the part of the HLA-G 3'UTR containing a 14-bp insertion/deletion region and seven single nucleotide polymorphisms (SNPs). Associations with acute atherosclerosis were tested by logistic regression.

The frequency of heterozygosity for the 14-bp polymorphism (Ins/Del) and the +3142 SNP (C/G) variant in the fetus are associated with acute atherosclerosis in preeclampsia (66.7 % vs. 39.6 %,  $p = 0.039$ , and 69.0 % vs. 43.4 %,  $p = 0.024$ ). Furthermore, the fetal UTR-3 haplotype, which encompasses the 14-bp deletion and the +3142G variant, is associated with acute atherosclerosis in preeclampsia (15 % vs. 3.8 %,  $p = 0.016$ ).

In conclusion, HLA-G polymorphisms in the fetus are associated with acute atherosclerosis. We hypothesize that these polymorphisms lead to altered HLA-G expression in the decidua basalis, affecting local fetomaternal immune tolerance and development of acute atherosclerosis.

### 1. Introduction

Preeclampsia, a hypertensive complication in pregnancy, affects at least 3% of pregnancies and confers a high risk of maternal and fetal mortality and morbidity (Ghulmiyyah and Sibai, 2012). Although its pathophysiology has not been completely unraveled, it is generally accepted that development of preeclampsia is secondary to placental dysfunction (Redman and Staff, 2015). There are likely multiple underlying maternal risk factors and pathophysiological pathways at play, involving the genetic background of both the mother and the fetus

(Hiby, 2004). These pathways may also be implicated in the development of acute atherosclerosis, a pregnancy-specific lesion of the uteroplacental spiral arteries that is predominantly observed in preeclampsia (Khong, 1991; Staff, 2020).

Typically, acute atherosclerosis comprises intramural lipid-filled foam cells, fibrinoid necrosis, and occasionally a perivascular mononuclear cell infiltrate (Robertson et al., 1976). Acute atherosclerosis is associated with placental pathology, severe preeclampsia, and adverse pregnancy outcomes (Khong, 1991; Stevens et al., 2013; Kim et al., 2015). We hypothesize that acute atherosclerosis is an inflammatory lesion, resulting from

\* Corresponding author at: Division of Obstetrics and Gynecology, Oslo University Hospital, Kirkeveien 166, 0450 Oslo, P.O.-Box 4956 Nydalen, 0424, Oslo, Norway.

E-mail address: [g.m.johnsen@medisin.uio.no](mailto:g.m.johnsen@medisin.uio.no) (G.M. Johnsen).

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multiple mechanisms leading to excessive decidual inflammation (Staff et al., 2014). One mechanism potentially responsible for this excessive inflammation may involve reduced feto-maternal immune tolerance in the decidua basalis, a physiological process where HLA-G is thought to play a role.

HLA-G is a non-classical MHC Class I antigen. In the placental bed, its expression is predominantly restricted to extravillous trophoblasts (EVTs) (Kovats, 1990; Hackmon, 2017). It can also be found in other organs (thymus, pancreas, and cornea), immune and epithelial cells, and mesenchymal stem cells (Persson, 2017). Several types of cancer, viral infections, and inflammatory diseases are associated with aberrant HLA-G expression (Persson, 2017).

HLA-G has immune suppressive effects, inhibiting both innate and adaptive immune functions by binding to immune cell receptors (Carosella, 2015). Specifically, HLA-G promotes feto-maternal tolerance by interacting with decidual T-cells, NK-cells, macrophages, and dendritic cells (Djurisic and Hviid, 2014; Rizzo, 2014; Carosella, 2015; Ferreira, 2017). It can be either membrane-bound or soluble (Persson, 2017). During the 1st trimester of pregnancy the concentration of soluble HLA-G in maternal circulation increases five-fold, then declines towards term (Hunt, 2000; Steinborn, 2007; Klitkou, 2015). Relatively low levels of circulating HLA-G are associated with preeclampsia, recurrent miscarriages, and infertility (Persson, 2017). We recently confirmed this finding in early- and late-onset preeclampsia (Jacobsen, 2020). Relatively low levels of HLA-G in the placenta are also associated with preeclampsia (Hara, 1996; Goldman-Wohl, 2000; Yie, 2004, Yie, 2005; Hackmon, 2007; Rizzo, 2009; Tang, 2015).

While classical HLA class I is highly polymorphic, the HLA-G coding region is less polymorphic (Castelli, 2014). Genetic variability in the HLA-G gene is mainly confined to the promoter region and the 3 prime untranslated region (3'UTR) (Castelli, 2014). The 3'UTR contains polymorphisms that post-transcriptionally affect the level of HLA-G expression (Hviid, 2006; Castelli, 2014). Most studied are the 14-bp insertion/deletion region (14-bp InsDel) and the single nucleotide polymorphism (SNP) +3142 C/G. The 14-bp InsDel region may affect mRNA stability, and some studies report an association between the insertion at this genomic region and decreased levels of soluble HLA-G in plasma (Hviid, 2004; Chen, 2008; Martelli-Palomino, 2013). Similarly, presence of a G nucleotide at the +3142 SNP position is associated with reduced HLA-G expression, an association thought to be secondary to enhanced affinity of microRNA molecules that negatively regulate gene expression (Tan, 2007; Castelli, 2009; Rizzo, 2014).

Several studies have shown an association between HLA-G polymorphisms and the occurrence of preeclampsia (O'Brien, 2001; Hylenius, 2004; Larsen, 2010; Quach, 2014; De Almeida, 2018) although the results are inconsistent (Iversen, 2008; Pabalan, 2015; Nilsson, 2016). In acute atherosclerosis, the role of HLA-G has not yet been explored. Since acute atherosclerosis occurs predominantly in preeclampsia, understanding the mechanisms behind acute atherosclerosis could provide insight into the underlying pathophysiology of preeclampsia. The goal of the current study was to determine whether the 14-bp InsDel region and seven additional polymorphisms in the 3'UTR of the HLA-G gene are associated with acute atherosclerosis.

## 2. Materials and methods

### 2.1. Patient inclusion, collection of biological material, and DNA isolation

Pregnant women were recruited prior to elective caesarian section after informed written consent, as previously described (Johnsen, 2018). We included paired mother and child samples from 166 pregnancies, diagnosed either with preeclampsia (PE, n = 83) or as normotensive (NT, n = 83). Pregnancies in each group were randomly selected. Preeclampsia was defined as new onset hypertension (blood pressure  $\geq 140/90$  mmHg) and new onset proteinuria ( $\geq 1+$  on dipstick, and/or protein/creatinine ratio  $\geq 30$  mg/mmol ( $\geq 0.3$  mg/mg)) at  $\geq 20$  weeks'

gestation (Roberts, 2003). The majority of preeclamptic pregnancies had early-onset preeclampsia, defined as delivery prior to 34 weeks' gestation (Tranquilli, 2013). Clinical characteristics of the pregnancy groups are shown in Supplemental Table 1. The study was approved by the Regional committee for Medical and Health Research Ethics in South-Eastern Norway, and performed according to the Helsinki Declaration. Maternal and fetal sources of DNA, the DNA extraction protocol, and the decidua basalis collection method are outlined in the Supplemental Methods.

### 2.2. Acute atherosclerosis evaluation

Acute atherosclerosis was identified based on histological staining of 3- $\mu$ m thick, formalin-fixed, paraffin-embedded decidua basalis tissue sections, as described in the Supplemental Methods. Acute atherosclerosis was evaluated prior to genetic sequencing (Alnaes-Katjavivi et al., 2016). The rate of acute atherosclerosis was 36 % (30/83) in preeclamptic pregnancies and 10 % (8/83) in normotensive pregnancies, comparable to the rates we previously found in our hospital population (Harsem et al., 2007; Alnaes-Katjavivi et al., 2016).

### 2.3. DNA sequencing of maternal and fetal HLA-G

We sequenced part of the HLA-G gene, specifically part of exon 8, which is found in the 3'UTR. The selected region contains the 14-bp insertion/deletion polymorphism (rs1704, +2961–2974 ATTTGTT-CATGCCT) and seven distinct SNPs (+3003C/T (rs1707), +3010C/G (rs1710), +3027A/C (rs17179101), +3035C/T (rs17179108), +3142C/G (rs1063320), +3187A/G (rs9380142), and +3196C/G (rs1610696)). The forward primer hybridizes at 71 bases before the intron 7/exon 8 border at position +2854 (when the 14-bp insertion is present), and is 699 bp or 713 bp long depending on whether the 14-bp insertion is present or absent. The region was amplified and sequenced by the Leiden Genome Technology Center as previously described (Drabbels, 2020). The results were interpreted using SBT Engine Software (GenDx, Netherlands). Eight distinct HLA-G polymorphism haplotypes were identified based on descriptions by (Castelli, 2010).

### 2.4. Statistical analyses

Statistical analyses were performed using SPSS version 25.0 (IBM). For clinical characteristics, non-parametric Mann-Whitney U tests (continuous variables) or Pearson chi-squared tests (categorical variables) were used. Allele frequency and genotype frequency of the 14-bp polymorphism and the SNPs were calculated for fetal and maternal samples. Haplotypes were composed based on the eight polymorphisms (Drabbels, 2020). For the seven distinct HLA-G haplotypes, the haplotype frequency was calculated. Associations with acute atherosclerosis were tested using logistic regression, presented as odds ratios (OR) with 95 % confidence intervals (CI). P-values < 0.05 were regarded as significant.

## 3. Results

### 3.1. HLA-G 3'UTR polymorphisms are not associated with preeclampsia

Preeclampsia, when compared to normotensive pregnancies, was not associated with any of the HLA-G 3'UTR polymorphisms in terms of allele frequency or genotype frequency, nor with any of the UTR haplotypes studied in the fetus (Supplemental Tables 2–4) or the mother (data not shown).

### 3.2. The genotype frequencies of the fetal 14-bp polymorphism and +3142 SNP are associated with acute atherosclerosis

The allele frequencies of the fetal (Table 1) and the maternal (data not shown) 14-bp polymorphism and the seven SNPs analyzed were not

**Table 1**  
Allele frequency of fetal HLA-G 3'UTR polymorphisms in acute atherosclerosis (AA).

		Total cohort				OR	P	Normotensive (NT) group				OR	P	Preeclampsia (PE) group				OR	P
		AA-		AA+				NTAA-		NTAA+				PEAA-		PEAA+			
		2n = 256		2n = 76				2n = 150		2n = 16				2n = 106		2n = 60			
		Count	%	Count	%			Count	%	Count	%			Count	%	Count	%		
<b>14-bp</b>	<b>Del</b>	165	64.5	49	64.5	1.00	0.997	100	66.7	11	68.8	1.1	0.866	65	61.3	38	63.3	1.09	0.797
	<b>Ins</b>	91	35.5	27	35.5			50	33.3	5	31.3			41	38.7	22	36.7		
<b>+3003</b>	<b>C</b>	41	16.0	8	10.5	0.62	0.24	26	17.3	0	0.0	n.a.		15	14.2	8	13.3	0.93	0.884
	<b>T</b>	215	84.0	68	89.5			124	82.7	16	100.0			91	85.8	52	86.7		
<b>+3010</b>	<b>C</b>	108	42.2	38	50.0	1.37	0.229	63	42.0	9	56.3	1.78	0.279	45	42.5	29	48.3	1.27	0.464
	<b>G</b>	148	57.8	38	50.0			87	58.0	7	43.8			61	57.5	31	51.7		
<b>+3027</b>	<b>A</b>	16	6.3	3	3.9	0.62	0.452	11	7.3	1	6.3	0.84	0.874	5	4.7	2	3.3	0.70	0.672
	<b>C</b>	240	93.8	73	96.1			139	92.7	15	93.8			101	95.3	58	96.7		
<b>+3035</b>	<b>C</b>	229	89.5	70	92.1	1.38	0.499	133	88.7	15	93.8	1.92	0.541	96	90.6	55	91.7	1.15	0.812
	<b>T</b>	27	10.5	6	7.9			17	11.3	1	6.3			10	9.4	5	8.3		
<b>+3142</b>	<b>C</b>	147	57.4	36	47.4	0.67	0.123	86	57.3	7	43.8	0.58	0.303	61	57.5	29	48.3	0.69	0.253
	<b>G</b>	109	42.6	40	52.6			64	42.7	9	56.3			45	42.5	31	51.7		
<b>+3187</b>	<b>G</b>	160	62.5	50	65.8	1.15	0.602	93	62.0	9	56.3	0.79	0.654	67	63.2	41	68.3	1.26	0.506
	<b>A</b>	96	37.5	26	34.2			57	38.0	7	43.8			39	36.8	19	31.7		
<b>+3196</b>	<b>C</b>	193	75.4	56	73.7	0.91	0.763	118	78.7	12	75.0	0.81	0.735	75	70.8	44	73.3	1.14	0.723
	<b>G</b>	63	24.6	20	26.3			32	21.3	4	25.0			31	29.2	16	26.7		

The table shows the total patient group, the normotensive group (NT) and the preeclampsia group (PE) separately. Associations of the allele frequencies of the different polymorphisms with acute atherosclerosis (AA) were tested using binary univariate logistic regression (\*significant p-value,  $p < 0.05$ ). OR, odds ratio; P, p-value; n.a., not applicable.

associated with acute atherosclerosis.

The genotype frequency of the Ins/Del variant of the 14-bp polymorphism in the fetus was significantly associated with acute atherosclerosis in preeclamptic pregnancies (Table 2). The fetal Ins/Del genotype was present in 66.7 % of PEAA + pregnancies compared to 39.6 % in PEAA- pregnancies (OR 9.52; 95 % CI 1.12–81.35; p-value 0.039).

The genotype frequency of the G/C variant of the fetal +3142 SNP was significantly associated with acute atherosclerosis both in the total cohort and in preeclamptic pregnancies (Table 2). In the total cohort, the fetal G/C variant was present in 67.6 % of AA + pregnancies compared to 50.8 % AA- pregnancies (OR 3.15; 95 % CI 1.12–8.89; p-value 0.030). In preeclamptic pregnancies, the fetal GC variant was present in 69.0 % of PEAA + pregnancies compared to 43.4 % PEAA- pregnancies (OR 4.13; 95 % CI 1.20–14.81; p-value 0.024). The genotype frequencies of the maternal 14-bp polymorphism and the SNPs were not associated with acute atherosclerosis (data not shown).

### 3.3. The fetal HLA-G UTR-3 haplotype is associated with acute atherosclerosis

Next, we assessed whether HLA-G haplotype was related to clinical outcome. The frequency of the UTR-3 haplotype in the fetus, which is the only UTR haplotype containing the 14-bp deletion variant combined with the +3142G variant, was significantly associated with acute atherosclerosis both in the total cohort and in preeclamptic pregnancies (Table 3). In the total cohort, the UTR-3 haplotype in the fetus was present in 17.1 % of AA + pregnancies compared to 7.0 % of AA- pregnancies (OR 2.73; 95 % CI 1.27–5.87; p-value 0.01). In preeclamptic pregnancies, the UTR-3 haplotype in the fetus was present in 15.0 % of PEAA + pregnancies compared to 3.8 % of PEAA- pregnancies (OR 4.5; 95 % CI 1.32–15.32; p-value 0.016).

Furthermore, we analyzed the diplotype frequency and found that the UTR-1/UTR-3 diplotype in the fetus was significantly associated with acute atherosclerosis both in the total cohort and in normotensive pregnancies (Supplemental Table 5).

UTR haplotype frequencies in the mother were not associated with acute atherosclerosis (data not shown).

### 3.4. Combined fetal and maternal HLA-G polymorphisms are not significantly associated with acute atherosclerosis

To investigate whether the UTR-3 haplotype was associated with acute atherosclerosis when present in the fetus and the mother concurrently, we created a univariate logistic regression model combining the presence versus absence of UTR-3 haplotype in the mother and the fetus in a categorical variable. The percentage of pregnancies where the fetus and the mother both had the UTR-3 haplotype was higher in pregnancies with acute atherosclerosis in the total cohort and in normotensive and preeclamptic pregnancies (Table 4); however this association was not statistically significant. In the total cohort both the fetus and the mother had the UTR-3 haplotype in 21.1 % of AA + compared to 10.9 % in AA- pregnancies (OR 2.33; 95 % CI 0.88–6.20; p-value 0.089). In preeclampsia both the fetus and the mother had the UTR-3 haplotype in 16.7 % of PEAA + compared to 3.8 % in PEAA- pregnancies (OR 5.25; 95 % CI 0.94–29.44; p-value 0.059).

## 4. Discussion

In the present study, we investigated a 14-bp InsDel polymorphism and seven SNPs located in the 3'UTR of the HLA-G gene. We found that certain HLA-G polymorphisms in the fetus were associated with decidual acute atherosclerosis. Furthermore, the 14-bp polymorphism and the seven SNPs are in strong linkage disequilibrium and are grouped in 43 different haplotypes based on these linkage associations (Castelli, 2010; Amodio and Gregori, 2020). In order to examine the significance of the combination of these polymorphisms relative to acute atherosclerosis, we studied the most common of these haplotypes (UTR-1 to UTR-8). The

remaining haplotypes are present in less than 1% of the population worldwide (Castelli, 2010). Overall, we demonstrated three main findings. First and secondly, acute atherosclerosis was associated with the fetal genotype frequencies of the heterozygous Ins/Del of the 14-bp polymorphism and the heterozygous G/C variant of the +3142 SNP. Our third and most interesting finding was that acute atherosclerosis was associated with the fetal UTR-3 haplotype, the only one of the eight haplotypes studied containing the 14-bp Del variant and the +3142G variant.

The 14-bp deletion is known to enhance mRNA stability, a feature that facilitates HLA-G protein expression (Castelli, 2014), while the insertion is linked to enhanced mRNA degradation and lower HLA-G expression (Castelli, 2009). This is in line with findings from prior studies that have linked the presence of the 14-bp insertion to decreased soluble HLA-G (Persson, 2017). The same goes for studies on membrane-bound HLA-G; the fetal Del/Del genotype is associated with higher HLA-G expression on trophoblasts compared to the Ins/Ins genotype (Djurisic, 2015). In summary, the evidence points towards the 14-bp insertion correlating with decreased HLA-G expression and the deletion with increased HLA-G expression, suggesting that the heterozygous genotype may be linked to intermediate expression of HLA-G. Our findings link the heterozygous 14-bp Ins/del polymorphism in the fetus with acute atherosclerosis in preeclampsia. Unlike us, some studies report an association between the 14-bp Ins/Ins genotype and severe preeclampsia in primiparas (Hylenius, 2004; Larsen, 2010) and early-onset preeclampsia (Zhang, 2012). Other studies, however, report no association with preeclampsia (Iversen, 2008; Vianna, 2007), which is consistent with our findings.

The +3142 SNP was the other polymorphism for which we demonstrate an association with acute atherosclerosis. *In silico* analyses predict that the +3142 G variant is related to decreased HLA-G expression (Tan, 2007). Consistent with this, a clinical study demonstrated that the +3142 CC genotype was associated with elevated sHLA-G levels in recipients of living-donor kidney transplants who had a higher susceptibility for cytomegalovirus infection (Guberina, 2017). Again, from this evidence in the literature one might infer that the heterozygous genotype of the +3142 SNP might be linked to an intermediate expression level of HLA-G. As for the clinical relevance of this polymorphism in pregnancy, we found that the heterozygous G/C variant of the +3142 SNP in the fetus is associated with acute atherosclerosis in preeclampsia, but did not find an association between this SNP and preeclampsia overall. Other studies of the +3142 C/G SNP have shown that the GG genotype is associated with severe preeclampsia (Larsen, 2010).

We consider our finding that the fetal UTR-3 haplotype is associated with acute atherosclerosis in preeclampsia to be the most interesting, as it speaks to the significance of the combination of polymorphisms in the context of pregnancy. We hypothesize that the association is linked to the impact this genetic combination has on HLA-G expression during pregnancy. UTR-3 is the only UTR haplotype of the eight most common haplotypes containing the 14-bp deletion combined with the +3142 G variant. In addition, the UTR-3 haplotype contains six other SNPs that are less studied, but that could still have an impact on HLA-G protein expression. In addition, we found that the frequency of the fetal UTR-1/UTR-3 diplotype was significantly associated with acute atherosclerosis. We consider that this result is driven by the effect of the UTR-3 haplotype as UTR-1 is the most common haplotype in the population worldwide.

HLA-G expression is partly regulated by the polymorphisms in the 3'UTR (Castelli, 2011). These polymorphisms are associated with mRNA stability and protein expression (Persson, 2017). At present it is not clear whether the UTR-3 haplotype is associated with high or low expression of HLA-G. An association between the UTR-3 and low levels of soluble HLA-G was reported outside of pregnancy (Di Cristofaro, 2015). Another study in healthy males reported that UTR-3 was associated with intermediate plasma levels of sHLA-G (Martelli-Palomino, 2013), and yet other studies point to UTR-3 being associated with high levels of HLA-G expression in seminal plasma (Craenmehr, 2019), and in dendritic DC-10 cells (Amodio and Gregori, 2020).

**Table 2**  
Genotype frequency of fetal HLA-G 3'UTR polymorphisms from pregnancies with acute atherosclerosis (AA).

		Total cohort						Normotensive (NT) group						Preeclampsia (PE) group					
		AA- n = 83		AA + n = 83		OR	P	NTAA- n = 75		NTAA + n = 8		OR	P	PEAA- n = 53		PEAA + n = 30		OR	P
		Count	%	Count	%			Count	%	Count	%			Count	%	Count	%		
<b>14-bp</b>	<b>DelDel</b>	53	41.4	13	34.2	1.96	0.406	31	41.3	4	50.0	0.77	0.832	22	41.5	9	30.0	4.09	0.209
	<b>InsDel</b>	59	46.1	23	60.5	3.12	0.150	38	50.7	3	37.5	0.47	0.545	21	39.6	20	66.7	9.52	0.039*
	<b>InsIns</b>	16	12.5	2	5.3	Ref.	–	6	8.0	1	12.5	Ref.	–	10	18.9	1	3.3	Ref.	–
<b>+3003</b>	<b>CC</b>	2	1.6	0	0.0	n.a.	–	2	2.7	0	0.0	n.a.	–	0	0.0	0	0.0	n.a.	–
	<b>CT</b>	37	28.9	8	21.1	0.64	0.317	22	29.3	0	0.0	n.a.	–	15	28.3	8	26.7	0.92	0.873
	<b>TT</b>	89	69.5	30	78.9	Ref.	–	51	68.0	8	100.0	n.a.	–	38	71.7	22	73.3	Ref.	–
<b>+3010</b>	<b>CC</b>	22	17.2	7	18.9	0.89	0.807	11	14.7	2	25.0	1.49	0.658	11	20.8	5	17.2	0.58	0.384
	<b>CG</b>	64	50.0	23	62.2	Ref.	–	41	54.7	5	62.5	Ref.	0.528	23	43.4	18	62.1	Ref.	–
	<b>GG</b>	42	32.8	7	18.9	0.46	0.106	23	30.7	1	12.5	0.36	0.360	19	35.8	6	20.7	0.40	0.108
<b>+3027</b>	<b>AA</b>	0	0.0	0	0.0	n.a.	–	0	0.0	0	0.0	n.a.	–	0	0.0	0	0.0	n.a.	–
	<b>AC</b>	16	12.5	3	7.9	0.6	0.438	11	14.7	1	12.5	0.83	0.869	5	9.4	2	6.7	0.69	0.664
	<b>CC</b>	112	87.5	35	92.1	Ref.	–	64	85.3	7	87.5	Ref.	–	48	90.6	28	93.3	Ref.	–
<b>+3035</b>	<b>CC</b>	103	80.5	32	86.5	Ref.	–	59	78.7	7	87.5	Ref.	0.873	44	83.0	25	86.2	Ref.	–
	<b>TC</b>	23	18.0	5	13.5	0.7	0.503	15	20.0	1	12.5	0.56	0.603	8	15.1	4	13.8	0.88	0.847
	<b>TT</b>	2	1.6	0	0.0	n.a.	–	1	1.3	0	0.0	n.a.	–	1	1.9	0	0.0	n.a.	–
<b>+3142</b>	<b>CC</b>	41	32.0	5	13.5	Ref.	–	22	29.3	1	12.5	Ref.	–	19	35.8	4	13.8	Ref.	–
	<b>GC</b>	65	50.8	25	67.6	3.15	0.030*	42	56.0	5	62.5	2.62	0.393	23	43.4	20	69.0	4.13	0.024*
	<b>GG</b>	22	17.2	7	18.9	2.61	0.135	11	14.7	2	25.0	4.00	0.278	11	20.8	5	17.2	2.16	0.318
<b>+3187</b>	<b>GG</b>	58	45.3	22	57.9	Ref.	–	35	46.7	5	62.5	Ref.	0.691	23	43.4	17	56.7	Ref.	–
	<b>AG</b>	19	14.8	2	5.3	3.6	0.102	11	14.7	1	12.5	1.57	0.694	8	15.1	1	3.3	5.91	0.109
	<b>AA</b>	51	39.8	14	36.8	2.61	0.232	29	38.7	2	25.0	0.76	0.828	22	41.5	12	40.0	4.36	0.188
<b>+3196</b>	<b>CC</b>	75	58.6	20	54.1	Ref.	–	46	61.3	5	62.5	Ref.	0.560	29	54.7	15	51.7	Ref.	–
	<b>CG</b>	43	33.6	15	40.5	1.31	0.49	26	34.7	2	25.0	0.71	0.692	17	32.1	13	44.8	1.48	0.422
	<b>GG</b>	10	7.8	2	5.4	0.75	0.72	3	4.0	1	12.5	3.07	0.369	7	13.2	1	3.4	0.28	0.249

The table shows the total patient group, the normotensive group (NT) and the preeclampsia group (PE) separately. Associations of the genotype frequencies of the different polymorphisms with acute atherosclerosis (AA) were tested using binary univariate logistic regression (\*significant p-value,  $p < 0.05$ ). The most common genetic variant was used as the reference for each variable, except for the 14-bp region and the +3142 SNP, where the reference value was set as the variant which in theory would give the highest level of HLA-G expression (i.e., DelDel for the 14-bp region and CC for the +3142 SNP) based on published studies. OR, odds ratio; P, p-value; n. a., not applicable.

**Table 3**  
Haplotype frequencies of HLA-G 3'UTR polymorphism from pregnancies with acute atherosclerosis (AA).

HLA-G 3'UTR Haplotypes	Total cohort																							
	AA- n = 256			AA+ n = 56			OR			P														
	Count	%		Count	%		Count	%		Count	%													
14-bp	+3003	+3010	+3027	+3035	+3142	+3187	+3196																	
UTR-1 Del	T	G	C	C	C	G	C	34.2	0.90	0.689	56	37.3	7	43.8	1.31	0.616	38	35.8	19	31.7	0.83	0.586		
UTR-2 Ins	T	C	C	C	G	A	G	23.8	1.07	0.894	32	21.3	4	25.0	1.23	0.735	29	27.4	15	25.0	0.89	0.741		
UTR-3 Del	T	C	C	C	G	A	C	7.0	17.1	2.73	0.01*	14	9.3	4	25.0	3.24	0.067	4	3.8	9	15.0	4.50	0.016*	
UTR-4 Del	C	G	C	C	C	A	C	15.6	10.5	0.64	0.27	26	17.3	0	0.0	n.a.	n.a.	14	13.2	8	13.3	1.01	0.982	
UTR-5 Ins	T	C	C	T	G	A	C	3.9	2.6	0.67	0.603	6	4.0	0	0.0	n.a.	n.a.	4	3.8	2	3.3	0.88	0.884	
UTR-6 Del	T	G	C	C	C	A	C	2.3	0.0	n.a.	n.a.	2	1.3	0	0.0	n.a.	n.a.	4	3.8	0	0.0	n.a.	n.a.	
UTR-7 Ins	T	C	A	T	G	A	C	6.3	3.9	0.62	0.452	11	7.3	1	6.3	0.84	0.874	5	4.7	2	3.3	0.70	0.672	
UTR-8 Ins	T	G	C	C	G	A	C	1	0.4	1	1.3	0.389	1	0.7	0	0.0	n.a.	n.a.	0	0.0	1	1.7	n.a.	n.a.
UTR-X								3.9	4	5.3	1.37	0.607	2	1.3	0	0.0	n.a.	n.a.	8	7.5	4	6.7	0.88	0.833

The 14-bp insertion/deletion and the SNP combinations comprising each haplotype are shown. The table shows the total patient group, the normotensive group (NT) and the preeclampsia group (PE) separately. Associations of the different UTR haplotypes with acute atherosclerosis (AA) were tested using binary univariate logistic regression (\*significant p-value, p < 0.05). OR, odds ratio; P, p-value; n.a., not applicable.

**Table 4**  
Multivariate logistic regression analysis of fetal and maternal UTR-3 haplotype combinations in acute atherosclerosis (AA).

	Total cohort																	
	AA- n = 128			AA+ n = 38			OR			P								
	Count	%		Count	%		Count	%		Count	%							
Neither have UTR-3	98	76.6	24	63.2	Ref.	0.095	56	74.7	4	50.0	Ref.	0.312	42	79.2	20	66.7	Ref.	0.143
Both have UTR-3	14	10.9	8	21.1	2.33	0.089	12	16.0	3	37.5	3.50	0.130	2	3.8	5	16.7	5.25	0.059
Only fetus has UTR-3	4	3.1	4	10.5	4.08	0.058	2	2.7	1	12.5	7.00	0.143	2	3.8	3	10.0	3.15	0.228
Only mother has UTR-3	12	9.4	2	5.3	0.68	0.629	5	6.7	0	0.0	n.a.	n.a.	7	13.2	2	6.7	0.60	0.546

Association of the UTR-3 haplotype with the occurrence of acute atherosclerosis were tested using multivariate logistic regression. The possible outcomes for mother and fetus were combined in one categorical variable. The reference category consisted of neither the mother nor fetus having the UTR-3 haplotype (\*significant p-value, p < 0.05). OR, odds ratio; P, p-value; n.a., not applicable.

We recently performed whole HLA-G gene amplification in the same patient population as in the present study and found a strong linkage disequilibrium between the UTR-3 haplotype and the HLA-G\*01:04 allele in the DNA coding sequence (Drabbels, 2020). The same association between UTR-3 and the 01:04 allele was related to low levels of serum soluble HLA-G and adverse outcome in lung transplant recipients (Di Cristofaro, 2015).

In the decidual tissue the main source of fetal HLA-G is the EVT that express membrane-bound and soluble forms of HLA-G during placentalization (Hackmon, 2017), both of which interact with receptors on maternal immune cells inhibiting their activation and proliferation, thereby inducing maternal-fetal tolerance (Ferreira, 2017). Reduced placental HLA-G expression is associated with impaired EVT invasion, and could potentially contribute to the impaired placentalization characteristic of early-onset PE (Goldman-Wohl, 2000). We hypothesize that altered HLA-G expression affects maternal-fetal tolerance, creating a local pro-inflammatory environment contributing to acute atherosclerosis development.

Another source of HLA-G expression in the decidua is maternal immune cells. However, we found no associations between maternal HLA-G polymorphisms and acute atherosclerosis. This is not surprising as HLA-G is primarily expressed by placental EVTs, and acute atherosclerosis is a focal decidual lesion. However, the percentage of pregnancies where both the mother and the fetus possessed the UTR-3 haplotype were higher in pregnancies with acute atherosclerosis (although this finding was not statistically significant). This is consistent with a prior report showing that the combined fetomaternal genotype (14-bp Ins/Del and +3142 C/G) was associated with maternal sHLA-G levels (Dahl, 2015).

Acute atherosclerosis is a subtype of preeclampsia. Yet, we found no association between the HLA-G 3'UTR polymorphisms analyzed and preeclampsia as a whole. This is in line with several other studies (Mando, 2016; Nilsson, 2016; Pabalan, 2015; Vianna, 2007). However, the literature is inconsistent (Persson, 2017), and none of these studies specifically looked at preeclamptic pregnancies with presence of decidual acute atherosclerosis. We hypothesize that some of these inconsistencies may be driven by the heterogeneity of preeclampsia; different gene polymorphisms may predispose to different subtypes of disease, all resulting in the same clinical syndrome.

The main intrinsic strengths of our study are the use of paired fetal and maternal samples, the decidual tissue collection method (Harsem et al., 2004), the well-defined histological characterization of acute atherosclerosis (Alnaes-Katjavivi et al., 2016), and the comprehensive clinical information.

The main extrinsic strength is that we divided preeclampsia into two phenotypes based on the presence or absence of acute atherosclerosis. No other studies have explored HLA-G polymorphisms relative to this uteroplacental artery lesion. Benton et al. proposed that distinct subtypes of preeclampsia could be identified by combining gene expression and histopathology analyses (Benton, 2018). They identified three preeclampsia subgroups based on placental gene expression patterns, which also showed distinct placental lesions (Benton, 2018). Similarly, our results suggest that studies of genetic polymorphisms in paired fetal-maternal samples combined with histological characterization of decidual lesions could help further delineate preeclampsia subtypes. The importance of combined fetal and maternal immunogenetic factors is further emphasized by our recent study demonstrating that acute atherosclerosis in preeclampsia is associated with the combination of maternal KIR-B and fetal HLA-C2 (Johnsen, 2018).

The main limitation of the present study is the small sample size for assessing genetic associations, especially in subgroups. The associations we found were not statistically significant after adjusting for multiple testing using Bonferroni correction, a conservative method for adjusting for false positive findings as a result of multiple testing. Hence, our findings should be confirmed in a larger cohort.

Our results must furthermore be interpreted with caution as HLA-G expression during pregnancy is modified by several transcriptional

and posttranscriptional mechanisms (Amodio and Gregori, 2020), as well as factors in the decidual milieu (Ferreira, 2017). Though further studies are needed to test causality, we speculate that HLA-G polymorphisms, individually or particularly in certain combinations as in the UTR-3 haplotype, alter local decidual immune tolerance and thereby promote a pro-inflammatory environment contributing to the formation of acute atherosclerosis and placental dysfunction. Insight into these mechanisms may help to identify novel biomarkers of preeclampsia subtypes and to develop targeted care for women with preeclampsia.

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### Declaration of Competing Interest

The authors report no declarations of interest.

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### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.jri.2021.103284>.

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