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ORIGINAL ARTICLE

Human pregnancy and generation of anti-angiotensin receptor and anti-perlecan antibodies

Gideon Hönger,[1] Heloise Cardinal,[2] Mélanie Dieudé,[2] Andreas Buser,[3] Irene Hösli,[4] Duska Dragun,[5] Marie-Josée Hébert[2] and Stefan Schaub[1]

1 Transplantation Immunology and Nephrology, University Hospital Basel, Basel, Switzerland
2 Research Centre, Centre hospitalier de l'Université de Montréal, Montreal, QC, Canada
3 Transfusion Medicine, University Hospital Basel, Basel, Switzerland
4 Department for Obstetrics and Fetomaternal Medicine, University Hospital Basel, Basel, Switzerland
5 Department of Nephrology and Intensive Care Medicine, Campus Virchow Klinikum, Charitè, Universitätsmedizin Berlin, Berlin, Germany

Keywords
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Correspondence
Stefan Schaub MD, MSc, Clinic for Transplantation Immunology and Nephrology, University Hospital Basel, Petersgraben 4, 4031 Basel, Switzerland.
Tel.: 0041 61 265 4533;
Fax: 0041 61 265 24 10;
e-mail: stefan.schaub@usb.ch

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Summary

Non-HLA antibodies against the angiotensin II type 1 receptor (AT_1R) and the C-terminal fragment of perlecan (i.e., LG3) are associated with the development of renal allograft rejection. It is currently unknown how humans develop anti-AT_1R or anti-LG3 antibodies. The aim of this study was to investigate whether pregnancy—as a model of sensitization to polymorphic proteins—induces anti-AT_1R and/or anti-LG3 antibodies. We included 104 samples from women obtained after physiologic full-term pregnancy and 80 samples from healthy non-sensitized controls (40 women and 40 men). Both anti-AT_1R and anti-LG3 antibody levels were lower in pregnancy samples than in controls (both $P < 0.05$). By multivariate analysis, male gender was an independent predictor for high anti-AT_1R antibody levels (OR 3.66, $P = 0.04$) and pregnancy was predictive for low anti-LG3 antibody levels (OR 6.53, $P = 0.0001$). There was no correlation of anti-AT_1R with anti-LG3 antibody levels, either in the pregnancy or in the control samples ($r^2 \leq 0.03$, $P \geq 0.26$). In conclusion, physiologic full-term pregnancy does not induce anti-AT_1R or anti-LG3 antibodies and may even lower their levels. Therefore, anti-AT_1R and anti-LG3 antibodies are likely not caused by allo-sensitization. The lack of correlation of anti-AT_1R with anti-LG3 antibodies suggests different mechanisms of generation, which remain to be elucidated.

Introduction

Acute antibody-mediated rejection (AMR) due to non-HLA antibodies is a rare, but well-described phenomenon in renal allograft recipients [1–5]. AMR is histologically characterized by inflammation in the vascular compartment (i.e., endothelialitis, glomerulitis, and peritubular capillaritis) with variable deposition of the complement fragment C4d in peritubular capillaries [6,7]. Recently, two distinct targets of such non-HLA antibodies have been identified. Dragun *et al.* [3] found that agonistic antibodies against the angiotensin II type 1 receptor (AT_1R) can induce AMR with the leading histological feature of endothelialitis. Similarly, Cardinal *et al.* [5] demonstrated that antibodies against a C-terminal fragment of the heparan sulfate proteoglycan perlecan (i.e., LG3) are associated with vascular rejection. Both anti-AT_1R and anti-LG3 antibodies may induce vascular compartment inflammation alone or in combination with donor-specific HLA antibodies [2–5,8].

Given the enormous polymorphism of HLA antigens, the production of HLA antibodies is mainly related to contact with foreign HLA antigens by transplants, blood transfusions, and pregnancies. Although the AT_1R gene has been extensively investigated and several polymorphisms were found, only one with a putative amino acid change in the extracellular domain implicated in antibody binding has been described yet [9,10]. Likewise, data regarding polymorphic amino acid residues on the LG3 fragment are not

available [11]. Thus, it is currently unknown how humans develop anti-AT_1R or anti-LG3 antibodies.

The aims of this study were to investigate (i) whether pregnancy—as a model of sensitization to polymorphic proteins—induces anti-AT_1R and/or anti-LG3 antibodies, (ii) whether sensitization to AT_1R and LG3 goes along with sensitization to HLA, and (iii) whether levels of anti-AT_1R and anti-LG3 antibodies correlate with each other, indicating a common mechanism of generation.

Materials and methods

Study population

As the distribution of anti-AT_1R and anti-LG3 antibodies in the general population or in pregnancy was unknown *a priori*, we used a convenience sample size to perform this study. The population for this IRB (local ethics committee)-approved study consisted of healthy controls (n = 80, forty women and forty men) and women with at least one full-term pregnancy (n = 104). Healthy controls were volunteer blood donors without any sensitizing event and no detectable HLA antibodies in the LabScreen® Mixed assay using a ratio cutoff of 1.5 (One Lambda, Canoga Park, CA, USA). In a previous study, sera of 301 women giving birth at the University Hospital Basel were obtained 1–4 days after delivery [12]. HLA antibodies were determined by single HLA antigen (SA) beads for class I and class II (iBeads Lot 1 and LabScreen® SA II Lot 9; One Lambda). A positive SA bead result was defined as a baseline normalized mean fluorescence intensity (MFI) >500. High-resolution HLA-A/B/Cw/DRB1 typing of the mother and the child was performed, which allowed defining the presence/absence of child-specific HLA antibodies for the A/B/Cw/DRB1 loci. To have a comparable sample size as the healthy controls and to include sufficient samples demonstrating clear sensitization against HLA molecules, we selected 40 women having the first and 40 women having the second live birth, stratified by the presence of child-specific HLA antibodies. In addition, we included all women having their ≥third live birth (n = 24). Thus, the (sub)groups for this study are as follows:

1. Healthy controls without any sensitizing event and no HLA antibodies (n = 80)
   - i. Forty women and forty men
2. Women with physiologic full-term pregnancies (n = 104)
   - i. Women with a first live birth (n = 40)
     1. Without any HLA antibody (n = 20)
     2. With child-specific HLA antibodies (n = 20)
   - ii. Women with a second live birth (n = 40)
     1. Without any HLA antibody (n = 20)
     2. With child-specific HLA antibodies (n = 20)
   - iii. Women with a ≥third live birth (n = 24)
     1. Without any HLA antibody (n = 4)
     2. With child-specific HLA antibodies (n = 11)
     3. With HLA antibodies, but not child specific (n = 9)

ELISA for anti-AT_1R and anti-LG3 antibodies

All samples were stored at −70 °C until they were analyzed in duplicate. Anti-AT_1R antibody ELISA was performed according to the instruction of the manufacturer (One Lambda). Delta optical density (delta OD; 450–620 nm) was measured using a Synergy™ H1 multimode microplate reader from BioTek®. Anti-AT_1R levels were determined using a four-parameter nonlinear regression curve fitting five standard sera included in the kit. For samples with delta OD values below that of the lowest standard sera (i.e., <2.5 U/ml), anti-AT_1R antibody levels were estimated using a linear regression of a double-reciprocal plot of the three lowest standards. The positive and negative control samples included on all plates were always within the predefined target levels. In our study, anti-AT_1R duplicates demonstrated good correlation (r^2 = 0.94); the intra-assay and interassay coefficient of variation (CV) were 5% and 13%, respectively.

Anti-LG3 antibody ELISA was performed on blinded samples at the Research Centre, Centre hospitalier de l'Université de Montréal, as reported previously [5]. Duplicates demonstrated good correlation (r^2 = 0.98). The observed intra-assay CV was 6%; the interassay CV was 11%. A positive and a negative control sample were included on every ELISA plate. The mean ± SD OD of the positive control sample was 0.23 ± 0.05, and the negative control revealed an OD of 0.05 ± 0.03. For the purpose of this study, anti-LG3 levels were calculated as the ratio of the sample OD divided by the positive control OD. An OD ratio >1 was used to define a positive anti-LG3 result.

Statistical analysis

We used JMP software version 10.0.2 (SAS Institute Inc., Cary, NC, USA) for statistical analysis. For categorical data, Fisher's exact test or Pearson's chi-square test was used. Parametric continuous data were analyzed by the Student's t-test. For nonparametric continuous data, the Wilcoxon rank-sum test was used for analysis. Nominal logistic regression was performed to determine independent predictors for a positive AT_1R (>17 U/ml, n = 24) or anti-LG3 (OD ratio >1, n = 76) antibody result. A P-value < 0.05 was considered to indicate statistical significance.

Results

Characteristics of the study groups

The characteristics of the study groups are summarized in Table 1. Male controls were significantly older than

the other groups (median 47 years vs. median 31–35 years, $P < 0.01$). In the whole pregnancy group, 90 of 104 women (88%) had a spontaneous delivery and 24 of 104 women (23%) had prior miscarriages. All women with child-specific HLA antibodies having the first or second live birth produced antibodies against class I molecules. In addition, 11 of 20 (55%) in the first live birth as well as 15 of 20 (75%) in the second live birth group also had child-specific antibodies against DRB1 molecules. In women having their ≥third live birth, 11 had child-specific HLA antibodies (5 class I only, 2 DRB1 only, and 4 class I and DRB1).

Anti-AT_1R and anti-LG3 levels in control and pregnancy samples

Anti-AT_1R antibody levels were significantly higher in control samples than in pregnancy samples (median 5.8 vs. 4.5 U/ml; $P = 0.03$). For a second way of analysis, we categorized anti-AT_1R antibody levels into "positive" (>17 U/ml), "at risk" (10–17 U/ml), and "negative" (<10 U/ml) as suggested by recent publications [4,13]. Although positive results were numerically higher in controls than in pregnancies (19% vs. 9%), the differences were statistically not significant ($P = 0.10$) (Fig. 1a). Anti-LG3 antibody levels were significantly higher in control samples than in pregnancy samples (median 1.10 vs. 0.77 OD ratio, $P = 0.0002$). Accordingly, the frequency of anti-LG3 positivity was higher in control than in pregnancy samples (58% vs. 29%, $P = 0.0001$) (Fig. 1b).

Factors affecting anti-AT_1R and anti-LG3 antibody levels

Anti-AT_1R antibody levels were different between male control, female control, and pregnancy samples assessed as a continuous and categorical variable (both $P = 0.04$). This was driven by higher levels in male controls. By contrast, anti-AT_1R antibody levels were not different between women having the first, second, and ≥third live birth ($P \geq 0.45$) (Fig. 2a).

Anti-LG3 antibody levels were different between male control, female control, and pregnancy samples assessed as a continuous and categorical variable ($P = 0.0006$ and $P = 0.0004$). Both control groups had higher anti-LG3 antibody levels than women after full-term pregnancy. By contrast, anti-LG3 antibody levels were not different between women having the first, second, and ≥third live birth ($P \geq 0.30$) (Fig. 2b).

Women with pregnancy-induced HLA antibodies had higher anti-AT_1R and anti-LG3 antibody levels than women without pregnancy-induced HLA antibodies (anti-AT_1R: median 6.3 vs. 3.5 U/ml, $P = 0.007$; anti-LG3: median 0.82 vs. 0.69 OD ratio, $P = 0.048$). Prior miscarriages ($n = 24$) did not lead to higher anti-AT_1R and anti-LG3 antibody levels compared with women without miscarriages ($n = 80$) ($P = 0.68$ and $P = 0.34$, respectively).

We found no correlation between age and anti-AT_1R or anti-LG3 antibody levels between male control, female control, and pregnancy samples ($r^2 \leq 0.03$; $P \geq 0.06$) (Fig. 3).

Next, we used a multivariate analysis to determine independent predictors for a positive anti-AT_1R (i.e., >17 U/ml) or anti-LG3 antibody (i.e., OD ratio >1) result [4]. For anti-AT_1R antibodies, male gender was the only independent predictor (OR 3.66; 95%-CI 1.06–15.06; $P = 0.04$), while pregnancy, miscarriages, age, and pregnancy-induced HLA antibodies were not (Table 2). Using a less stringent cutoff for a positive anti-AT_1R antibody test (i.e., >10 U/ml), the results were similar, but male gender reached only borderline significance (OR 2.75; 95%-CI 0.97–8.31; $P = 0.06$). For anti-LG3 antibodies, pregnancy was the only independent predictor (OR 0.15; 95%-CI 0.05–0.41; $P = 0.0001$),

Table 1. Characteristics of the study groups.

	Control males ($n = 40$)	Control females ($n = 40$)	Pregnancies ($n = 104$)		
			First ($n = 40$)	Second ($n = 40$)	≥Third ($n = 24$)
Age, median (range)	47 (21–75)*	32 (19–72)	31 (15–40)	32 (23–41)	35 (26–42)
Prior miscarriages, *n* (%)			-	16 (40%)	8 (33%)
Gestation week at delivery, median (range)			40 (36–41)	39 (36–41)	40 (37–41)
Spontaneous delivery, *n* (%)			37 (95%)	30 (76%)	23 (96%)
Male child, *n* (%)			14 (35%)	18 (45%)	16 (67%)
HLA antibodies, *n* (%)			20 (50%)	20 (50%)	20 (83%)
Class I CSA, *n*			20	20	9
DRB1 CSA, *n*			11	15	6

*$P < 0.01$ vs. other groups.
CSA, child-specific HLA antibodies.

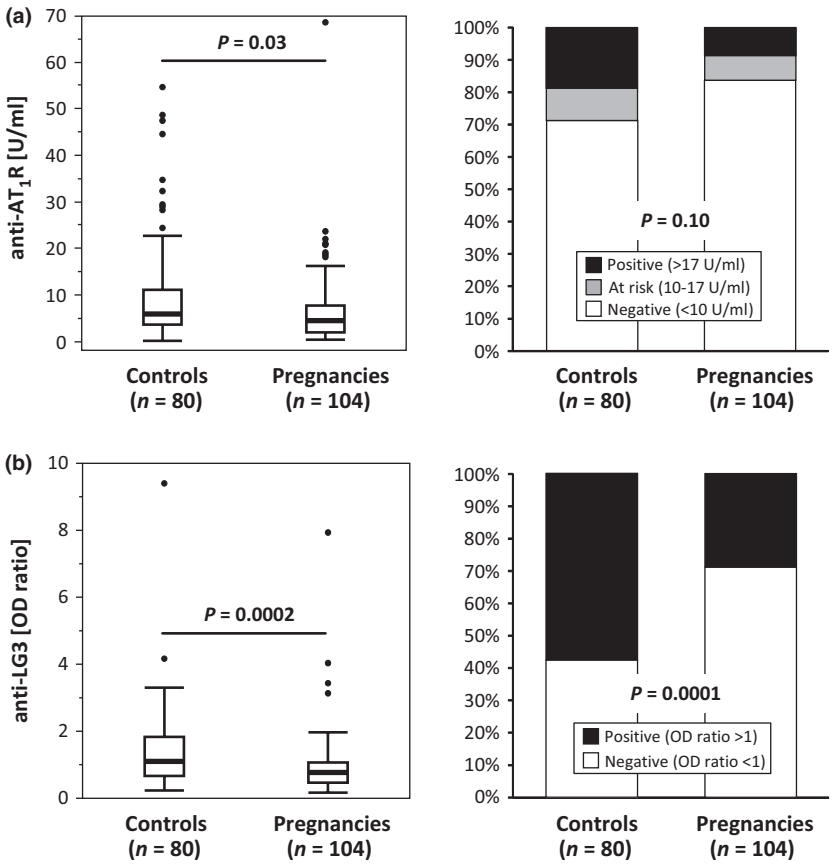

Figure 1 Anti-AT_1R and anti-LG3 antibody levels in healthy controls and women after full-term pregnancy. (a) anti-AT_1R antibody levels given as a continuous variable or as a categorical variable (i.e., positive vs. at risk vs. negative). (b) anti-LG3 antibody levels given as a continuous or categorical variable.

while male gender, miscarriages, age, and pregnancy-induced HLA antibodies were not (Table 2).

Correlation of anti-AT_1R with anti-LG3 antibody levels

Assessing all 184 samples, there was no correlation of anti-AT_1R with anti-LG3 antibody levels ($r^2 = 0.01$, $P = 0.13$). Furthermore, we found no correlation of anti-AT_1R with anti-LG3 antibody levels in the subgroups of female control, male control, and pregnancy samples ($r^2 \leq 0.03$, $P \geq 0.26$) (Fig. 4).

Discussion

From a mechanistic point of view, antibodies causing rejection of a renal allograft can be produced either following encounter with foreign antigens (i.e., alloimmune response) or as a reaction against self-antigens (i.e., autoimmune response). In this study, we investigated whether pregnancy—as a model for an alloimmune response against foreign polymorphic antigens—induces anti-AT_1R and/or anti-LG3 antibodies. Both anti-AT_1R and anti-LG3 antibody levels were not increased a few days after delivery of a full-term pregnancy compared with nonsensitized controls. This suggests that anti-AT_1R and anti-LG3 antibodies are not induced by an alloimmune response during physiologic pregnancy.

Interestingly, anti-LG3 antibody levels were lower in full-term pregnancy samples compared with nonsensitized female and male controls in the univariate as well as in the multivariate analysis. In addition, anti-AT_1R antibody levels were also slightly lower in pregnancy samples, although this was not significant in the multivariate model. These lower antibody levels might be related to the immune-modulating effect of the pregnancy, which can also influence the activity of some autoimmune diseases [14,15]. Therefore, this observation supports the hypothesis that anti-AT_1R and anti-LG3 antibodies could be generated by autoimmunity.

Male gender was the only statistically significant independent predictor for anti-AT_1R antibody positivity. We have no explanation for this finding, but it seems consistent

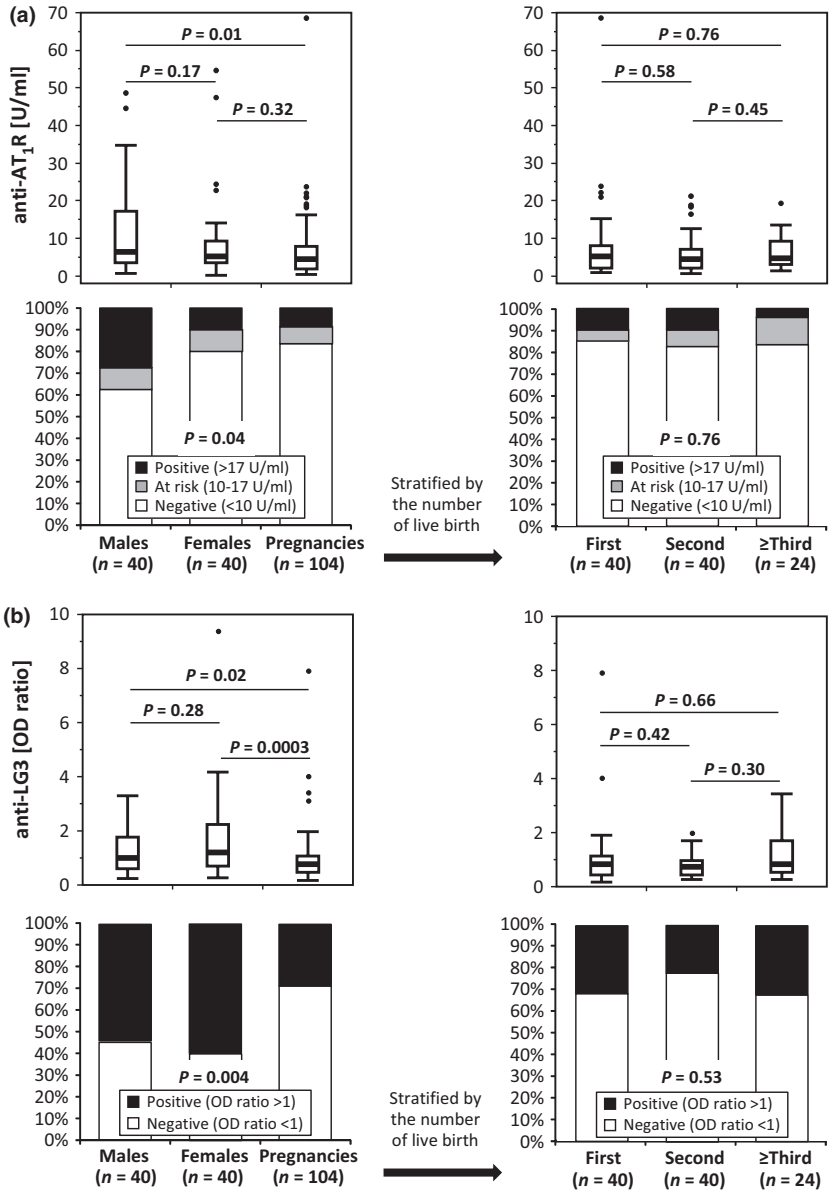

Figure 2 Anti-AT_1R and anti-LG3 antibody levels in control males, control females, and women after full-term pregnancy, which were further stratified by the number of live birth. (a) anti-AT_1R antibody levels given as a continuous variable or as a categorical variable (i.e., positive vs. at risk vs. negative). (b) anti-LG3 antibody levels.

with the first report regarding anti-AT_1R-associated AMR, where 63% were males compared with only 38% males in patients with HLA-antibody-related AMR [3].

Although both anti-AT_1R and anti-LG3 antibodies are likely induced by autoimmunity, the underlying mechanisms might be different, because there was no correlation between anti-AT_1R and anti-LG3 antibody levels. Several mechanisms can be proposed: (i) bystander production in the context of other autoimmune diseases or infections, (ii) molecular mimicry with infectious pathogens [16], and (iii) exposure to cryptic epitopes following endothelial cell stress/injury. The first possibility is very unlikely as none of the investigated females and males had a clinically apparent autoimmune disease. Exposure to cryptic epitopes might be a suggestive explanation for the generation of anti-LG3 antibodies, because LG3 is a fragment of the perlecan protein, which is produced following degradation by proteases that can occur during tissue injury [17]. Clearly, molecular, immunological, and epidemiological studies are needed to understand how and why anti-AT_1R and anti-LG3 antibodies are produced. Cardinal *et al.* found younger age as the only epidemiological factor associated with high pretrans-

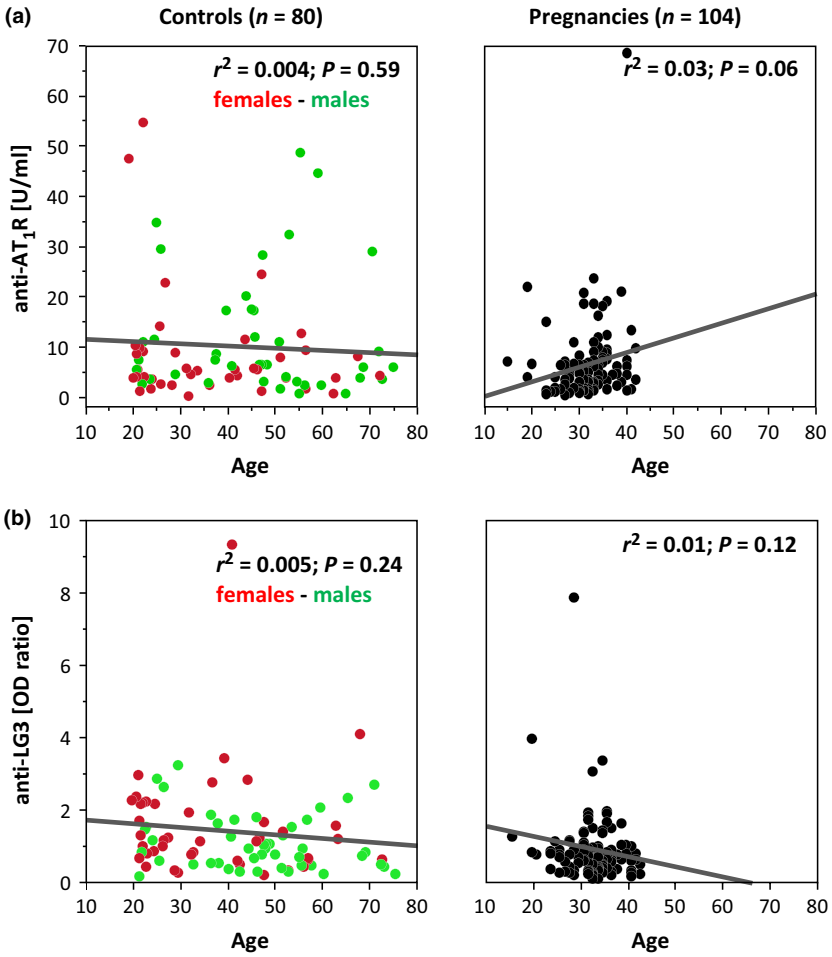

Figure 3 Correlation of anti-AT$_1$R and anti-LG3 antibody levels with age in healthy controls and women after full-term pregnancy. (a) anti-AT$_1$R antibody levels. (b) anti-LG3 antibody levels.

Table 2. Multivariate analyses to determine independent predictors for a positive anti-AT$_1$R or anti-LG3 antibody result. A positive anti-AT$_1$R antibody result was defined by a value >17 U/ml (n = 24), and a positive anti-LG3 antibody result was defined as an OD ratio >1 (n = 76).

	Anti-AT$_1$R antibody positivity			Anti-LG3 antibody positivity		
	OR	95% CI	*P*-level	OR	95% CI	*P*-level
Male sex	3.66	1.06–15.06	0.04	1.04	0.41–2.71	0.93
Pregnancy	0.48	0.06–2.69	0.41	0.15	0.05–0.41	0.0001
Miscarriages	0.39	0.02–2.36	0.35	0.84	0.27–2.31	0.74
Age, per year	0.99	0.96–1.03	0.71	0.97	0.95–1.00	0.07
HLA antibodies due to pregnancy	2.81	0.64–19.58	0.18	2.14	0.88–5.50	0.09

plant anti-LG3 antibody levels, but this was not noticed in the current study.

The missing generation of anti-AT$_1$R and anti-LG3 antibodies in physiologic and healthy pregnancy might also be related to the fact that the maternal immune system is not significantly exposed to these target antigens, which are mainly expressed on endothelial cells and the subjacent tissue. This is in sharp contrast to HLA molecules that are also expressed on hematopoietic cells crossing the placental barrier even in healthy pregnancy and thus enabling generation of HLA antibodies. However, in pathologic pregnancies, such as preeclampsia, extensive endothelial cell injury can occur and may provide the milieu for an immune response against AT$_1$R and LG3.

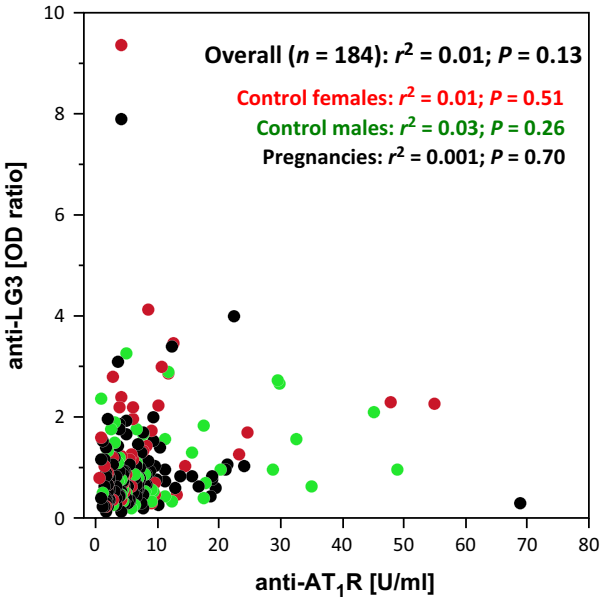

Figure 4 Correlation of anti-AT$_1$R with anti-LG3 antibody levels in the whole population (n = 184) and in subgroups [i.e., control females (n = 40), control males (n = 40), and women after full-term pregnancy (n = 104)].

As the mechanisms of generation of anti-AT$_1$R and anti-LG3 antibodies are unknown, it is currently difficult to define a "strict negative control group," which would be very important to establish a cutoff for a positive result. Indeed, 15 of 80 healthy controls (19%) had a positive anti-AT$_1$R antibody result (i.e., >17 U/ml) and 46 of 80 healthy controls (58%) had a positive anti-LG3 antibody result (i.e., OD ratio >1). Thus, in the absence of a "strict negative control group," the cutoff for a positive result has to be defined by the correlation of the antibody levels with clinically important outcomes (i.e., AMR, allograft survival). Recent studies were conducted using statistically derived anti-AT$_1$R antibody cutoff for particular clinical events. Giral *et al.* [13] investigated 599 renal allograft recipients and found that pretransplant anti-AT$_1$R antibody levels >10 U/ml were associated with a higher risk of acute clinical rejection episodes within the first 4 month post-transplant and a higher risk of allograft failure beyond 3 years post-transplant. Taniguchi *et al.* [18] defined a predictive anti-AT$_1$R antibody result by a value ≥15 U/ml and found that persisting pre- and post-transplant anti-AT$_1$R antibodies or *de novo* post-transplant anti-AT$_1$R antibodies are associated with a higher risk of allograft loss. Furthermore, in systemic sclerosis, a severe autoimmune disease with various organ manifestations, a statistically determined different anti-AT$_1$R antibody cutoff could define different organ pathologies [19]. Nevertheless, the most appropriate anti-AT$_1$R antibody cutoff for the prediction of AMR and/or allograft loss is still debated and will require further studies [20].

An intriguing observation in this study was the higher anti-AT$_1$R and anti-LG3 antibody levels in women with pregnancy-induced HLA antibodies compared with women without any pregnancy-induced HLA antibodies. This might point toward a broader humoral hypersensitivity in some women, which could predispose to both allo- and autoimmune responses. Although the associations between pregnancy-induced HLA antibodies and non-HLA antibody titers were not significant in multivariate analysis, the 95% CI comprised clinically interesting values. Larger experimental and clinical studies are needed to confirm and understand these relationships.

The advantage of this study is the reasonable population size with clearly defined sensitizing events and the reliable determination of pregnancy-induced allosensitization due to analysis of sera obtained 1–4 days after delivery. In addition, the robustness of our data is supported by the results of the multivariate analysis, which remained stable even after using different cutoffs for anti-AT$_1$R positivity.

Nevertheless, this study has also certain limitations. We did not perform genotyping of the AT$_1$R and perlecan genes to determine whether polymorphisms on the protein level can be excluded. However, our study indicates that anti-AT$_1$R and anti-LG3 antibodies are not induced by a classical route of allosensitization and thus provides circumstantial evidence that the AT$_1$R and the LG3 fragment are likely not polymorphic on the protein level. Notably, the "pregnancy-model" was instrumental in detecting and characterizing the HLA polymorphism [21,22]. In addition, despite extensive genetic investigations, only one putative amino acid change on the extracellular domain of the AT$_1$R has been described, which is likely rather rare [9,10].

Furthermore, it would have been ideal to have serum samples of the 104 pregnant women also before pregnancy to investigate the pregnancy-related evolution of anti-AT$_1$R and anti-LG3 antibodies. Unfortunately, we do not have prepregnancy sera from these 104 women. Indeed, it is rather difficult to obtain sera immediately before physiologic full-term pregnancy, because very few healthy women seek medical advice prior to pregnancy and would thus be available for such a study. As the female control group in our study (n = 40) had similar age than the pregnant women (median 32 years vs. 32 years; P = 0.57), it can be used as the "prepregnancy baseline" to investigate pregnancy-related changes in anti-AT$_1$R and anti-LG3 antibody levels.

In conclusion, healthy full-term pregnancy does not induce anti-AT$_1$R or anti-LG3 antibodies and may even lower their levels. Therefore, anti-AT$_1$R and anti-LG3 antibodies are likely not caused by allosensitization, but rather represent an autoimmune response due to a yet unknown

trigger. The lack of correlation of anti-AT_1R with anti-LG3 antibodies suggests different mechanisms of generation, which remain to be elucidated.

Authorship

GH, IH, and SS: participated in research design. GH, HC, AB, DD, MJH, and SS: participated in the writing of the manuscript. GH, HC, MD, and SS: conducted the research. GH, HC, DD, MJH, and SS: participated in data analysis. HC, MD, and MJH: contributed analytic tools.

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