The programmed cell death protein 1 (PD1) and the programmed cell death ligand 1 (PD-L1) are significantly downregulated on macrophages and Hofbauer cells in the placenta of preeclampsia patients

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Highlights

- Macrophages and Hofbauer cells are significantly downregulated in the placenta of preeclamptic patients.
- The majority of macrophages locally in the placenta are M2-polarized.
- PD1 is significantly downregulated on maternal macrophages of the decidua and on Hofbauer cells in the placenta of preeclamptic patients.
- PD-L1 is significantly downregulated on maternal macrophages of the decidua in the placenta of preeclamptic patients.

Abstract

Preeclampsia is a pregnancy-specific disease which is characterized by abnormal placentation, endothelial dysfunction, systemic inflammation and disruption of the immune system. The goal of this study was to characterize the PD-1/PD-L1 system, an important immune checkpoint system, on macrophages and Hofbauer cells (HBC) in the placenta of preeclamptic patients. The expression of the macrophage markers CD68 and CD163 as well as the proteins PD1 and PD-L1 in the placenta of preeclamptic patients was examined by immunohistochemistry and immunofluorescence in comparison to the placenta of healthy pregnancies. The numbers of CD68-positive and CD163-positive macrophages were significantly downregulated in the decidua ($p = 0.021$ and $p = 0.043$) and in the chorionic villi ($p < 0.001$ and $p < 0.001$) of preeclamptic patients. The majority of macrophages in the decidua and the chorionic villi were identified to be CD163-positive, indicating a predominantly M2-polarisation. The expression of PD1 on maternal macrophages of the decidua ($p < 0.001$) and on Hofbauer cells ($p < 0.001$) was shown to be significantly lower in preeclampsia. Looking at the protein PD-L1 the expression was proven to be downregulated on maternal macrophages in the decidua of preeclamptic patients ($p = 0.043$). This difference was only caused by a downregulation of PD-L1 expression in male offspring ($p = 0.004$) while there was no difference in female offspring ($p = 0.841$). The variation of the immune checkpoint molecules PD1 and PD-L1 in preeclampsia might play an important role in the development of inflammation seen in preeclamptic patients. It might thereby be an important target in the therapy of preeclampsia.

Keywords

preeclampsia, immune checkpoints, PD1, PD-L1, macrophages

1. Introduction

Preeclampsia (PE), defined as the new onset of hypertension in pregnancy after 20 weeks of gestation accompanied with proteinuria and/or another organ disfunction, is one of the most severe

complications of pregnancy. It affects 5% to 7% of pregnancies worldwide and is the main cause for high maternal and fetal morbidity and mortality. Literature distinguishes between two types of preeclampsia: Early-onset and late-onset preeclampsia. The former is defined as preeclampsia occurring before 34 weeks, the latter as preeclampsia after 34 weeks of gestation (Tranquilli et al., 2013; Valensise et al., 2008). The only effective treatment is the delivery of fetus and placenta, which remains a challenge in practice considering possible maternal and fetal complications of early delivery. Women affected by preeclampsia during pregnancy have an increased risk for developing cardiovascular diseases e.g. hypertension or ischemic heart disease in later life, which also highlights the importance and impact of this disease (Rana et al., 2019; Sukmanee and Liabsuetrakul, 2022; Vest and Cho, 2012; Xu et al., 2022). The exact etiology and pathogenesis of preeclampsia is still unknown and under current research. It is proven to be multifactorial and the placenta with its abnormal placentation by defective trophoblast invasion and elevated levels of antiangiogenic factors seem to play a central role (Rambaldi et al., 2019; Yeh et al., 2013).

Pregnancy leads to a big challenge for the maternal immune system because the semi-allogenic fetus has to be accepted and supported within the mother throughout the gestational period (Ander et al., 2019). The decidua, the maternal part of the placenta, normally upholds the immunological tolerance between mother and fetus. In preeclampsia it has been proven to become a site of inflammation and thereby plays an important role in the development of the disease (Vishnyakova et al., 2021). Not only in the decidua but also systemically the immune system in preeclampsia was proven to be upregulated into a pro-inflammatory state with for example a reduction of regulatory T-cells and anti-inflammatory cytokines and an incretion of CD4(+) T-cells and pro-inflammatory cytokines (Aggarwal et al., 2019; Harmon et al., 2016).

An important effector of local immunity in the placenta are macrophages, the second largest group of cells at the maternal-fetal-interface. These cells can either have pro-inflammatory and antimicrobial (M1-macrophage) or anti-inflammatory (M2-macrophage) functions depending on their polarization and activation status. During normal pregnancy the majority of macrophages are polarized as M2-macrophages, leading to maintenance of maternal-fetal-tolerance (Yao et al., 2019). During preeclampsia the existing data is conflicting with studies showing increased numbers of macrophages

(Huang et al., 2010; Li et al., 2016; Reister et al., 2001) and other studies showing decreased numbers in preeclampsia (Bürk et al., 2001; Williams et al., 2009). Regarding the polarization status of macrophages studies have proven a decrease in M2-macrophages and an increase in M1-macrophages in the placenta of preeclamptic women (Tang et al., 2013; Yang et al., 2017; Yao et al., 2019). The number of Hofbauer cells, the fetal macrophages within the chorionic villi which are mostly M2 phenotypes, has shown to be reduced in preeclampsia and might thereby contribute to inflammation due to the loss of feto-maternal tolerance (Reyes and Golos, 2018).

Macrophages are not only important for local immunity, but they also play a crucial role in tumor biology. Tumor associated macrophages (TAM) can either promote or restrict tumor growth and metastatic spread (Larionova et al., 2020). Studies showed that an upregulation of CD68, a pan-macrophage marker, and CD163, a M2-macrophage marker, inside the tumor can lead to tumor growth, invasion, progression and metastatic spread (Cencini et al., 2021; Larionova et al., 2020; Leisz et al., 2022; Yang et al., 2022). Because of the similarities of tumor biology and placenta biology, macrophages may also play an important role in placentation in normal pregnancies and the pathogenesis of pregnancy diseases. Therefore, this specific macrophage marker combination was used in this study.

Besides macrophages also immune checkpoint molecules like PD-1 and PD-L1 play an important role in maintaining immunological homeostasis and tolerance. Upon activation with its ligand, they negatively regulate effector immune cells like for example T-cells and thereby prevent dangerous immune attacks. With these mechanisms immune checkpoint molecules take part in immune regulation in infections, autoimmunity and tumor growth (Sharpe and Pauken, 2018). In addition, they play an important role in reproductive immunology and pregnancy (Mittelberger et al., 2022; Mohamed Khosroshahi et al., 2021). Investigations about the role of these immune checkpoint molecules in normal pregnancy and in the pathophysiology of preeclampsia are emerging suggesting multiple variations in preeclampsia compared to normal healthy pregnancy. Most of the research however focuses on peripheral blood immune cells. Little is known about the expression of the molecules in the placenta and thereby in the maternal-fetal-interspace (Mittelberger et al., 2022).

In order to investigate the variances in the polarization of macrophages as well as in the expression of the immune checkpoint molecules PD1 and PD-L1 this study was designed analyzing the placenta of 40 patients with preeclampsia and 40 healthy patients using immunohistochemistry and immunofluorescence staining.

2. Materials and Methods

2.1 Study subjects

This study was approved by the ethics committee of the Ludwig-Maximilian-University (LMU) Munich, Germany in July 2021. The placental tissue of 40 patients with preeclampsia (20 female offspring, 20 male offspring), who delivered by cesarean section in the University Hospital Augsburg in the years 2016-2020 were obtained and retrospectively included into the study after written informed consent. Patients with delivery after 30+0 pregnancy weeks that fulfilled the diagnostic criteria of preeclampsia were eligible for participation. Patients with HELLP-syndrome (hemolysis, elevated liver enzymes and low platelet count) or fetal growth restriction (FGR) were not excluded from the study. As a control group placental tissue of 40 healthy patients (20 female offspring, 20 male offspring) who delivered by cesarean section in the years 2018-2021 were obtained and also retrospectively included into the study after written informed consent. The control group was matched to the preeclampsia group in pregnancy week, fetal sex, and age of the mother +/- 5 years. In order to rule out confounders, healthy patients who fulfilled the following criteria were excluded from the study: fetal growth restriction, multiple pregnancy, gestational diabetes, overweight with a body mass index (BMI) > 30 kg/m², fertility treatment, signs for systemic inflammation in the blood, placentation disorders like placenta accreta/percreta/increta.

The placentas were preserved in buffered 4% formalin immediately after delivery. The samples were then dissected from the central part of the placenta in the institute for pathology in the University Hospital Augsburg, containing decidua, extravillious and villious trophoblasts. After fixation in buffered formalin the samples were then embedded in paraffin and cut with a sliding microtome to 2-3 µm slices.

The clinical details of the study population are shown in Table 1 (separated by time of onset) and Table 2 (separated by fetal sex).

Confounder analysis was done for all significant different clinical characteristics between the two groups by the linear regression model. The results can be seen in the supplementary table 1.

Table 1: Clinical details of the study population separated by time of onset

	Preeclampsia (n=40)		**Control (n=40)**	**p-value**
	Early onset (n = 18)	Late onset (n = 22)		
Maternal age at delivery, years	32.18 ± 4.96		32.57 ± 4.91	p = 0.718
	32.33 ± 4.19	32.05 ± 5.61	-	p = 0.858
BMI	29.34 ± 7.12		23.54 ± 3.31	**p < 0.001**
	30.42 ± 7.89	28.46 ± 6.47	-	p = 0.393
Gravidity	1.40 ± 0.73		2.03 ± 1.03	**p = 0.001**
	1.44 ± 0.78	1.36 ± 0.73	-	p = 0.798
Parity	1.30 ± 0.65		1.68 ± 0.80	**p = 0.007**
	1.33 ± 0.69	1.27 ± 0.63	-	p = 0.840
Gestational age at delivery	35.00 ± 2.06		35.00 ± 2.18	p = 0.957
	33.22 ± 1.48	36.45 ± 1.10	-	**p < 0.001**
Fetal birthweight, g	2089.73 ± 594.66		2362.50 ± 538.03	**p = 0.021**
	1677.44 ± 417.34	2427.05 ± 500.83	-	**p < 0.001**
Birth percentile	24.67 ± 23.22		45.68 ± 22.05	**p < 0.001**
	26.11 ± 20.93	23.43 ± 25.46	-	p = 0.426
APGAR 10 minutes	9.55 ± 0.60		9.53 ± 0.68	p = 0.973
	9.67 ± 0.49	9.45 ± 0.67	-	p = 0.427
Umbilical artery pH	7.26 ± 0.07		7.29 ± 0.09	**p < 0.001**
	7.26 ± 0.04	7.25 ± 0.09	-	p = 0.370
Placental weight	384.70 ± 125.10		460.13 ± 122.89	**p = 0.005**
	308.56 ± 79.11	447.00 ± 122.32	-	**p < 0.001**
HELLP-Syndrome	n = 9		n = 0	-
Fetal growth restriction	n = 5		n = 0	-

Table 2: Clinical details of the study population separated by fetal sex

	Preeclampsia (n = 40)	**Control (n = 40)**	**p-value**
	Preeclampsia female (n = 20)	Control female (n = 20)	
	Preeclampsia male (n = 20)	Control male (n = 20)	
Maternal age at delivery, years	32.18 ± 4.96	32.57 ± 4.91	p = 0.718
	31.65 ± 4.84	31.45 ± 4.15	p = 0.889
	32.70 ± 5.15	33.70 ± 5.44	p = 0.554

BMI	29.34 ± 7.12	23.54 ± 3.31	**p < 0.001**
	31.56 ± 8.69	24.00 ± 3.18	**p < 0.001**
	27.10 ± 4.24	23.08 ± 3.46	**p = 0.002**
Gravidity	1.40 ± 0.73	2.03 ± 1.03	**p = 0.001**
	1.40 ± 0.75	2.10 ± 1.17	**p = 0.035**
	1.40 ± 0.75	1.95 ± 0.89	**p = 0.043**
Parity	1.30 ± 0.65	1.68 ± 0.80	**p = 0.007**
	1.30 ± 0.66	1.80 ± 0.83	**p = 0.043**
	1.30 ± 0.66	1.55 ± 0.76	p = 0.221
Gestational age at delivery	35.00 ± 2.06	35.00 ± 2.18	p = 0.957
	34.95 ± 2.06	35.10 ± 2.20	p = 0.825
	35.05 ± 2.11	34.90 ± 2.22	p = 0.828
Birthweight, g	2089.73 ± 594.66	2362.50 ± 538.03	**p = 0.021**
	2002.80 ± 526.17	2371.05 ± 570.56	**p = 0.040**
	2176.65 ± 658.10	2353.95 ± 518.15	p = 0.350
Birth percentile	24.67 ± 23.22	45.68 ± 22.05	**p < 0.001**
	23.84 ± 22.93	48.70 ± 20.18	**p < 0.001**
	25.45 ± 24.06	42.65 ± 23.91	**p = 0.010**
APGAR 10 minutes	9.55 ± 0.60	9.53 ± 0.68	p = 0.973
	9.60 ± 0.50	9.60 ± 0.75	p = 0.640
	9.50 ± 0.69	9.45 ± 0.61	p = 0.718
Umbilical artery pH	7.26 ± 0.07	7.29 ± 0.09	**p < 0.001**
	7.26 ± 0.07	7.29 ± 0.10	**p = 0.006**
	7.25 ± 0.07	7.29 ± 0.07	**p = 0.038**
Placental weight	384.70 ± 125.10	460.13 ± 122.89	**p = 0.005**
	380.75 ± 115.95	462.75 ± 109.74	**p = 0.027**
	388.65 ± 136.55	457.50 ± 137.62	p = 0.121

2.2 Immunohistochemistry

In preparation for immunohistochemistry, the paraffin sections had to be deparaffinized with Roticlear® and afterwards bathed in 100% ethanol. To stop the endogenous peroxidase activity, the samples were then incubated in 3% H2O2 in methanol for 20 minutes and rehydrated in alcohol gradient to distilled water. In the next step, the slices were put in a high-pressure cooker for 6 min using boiling sodium citrate buffer with pH 6.0 (for CD68 and CD163), EDTA buffer with pH 8.0 (for PD-1) and EDTA buffer pH 9.0 (for PD-L1) for antigen retrieval.

After washing the slides in distilled aqua and PBS, they were treated for 5 min with a blocking solution (Reagent 1; ZytoChem Plus HRP Polymer System IgG kit (Mouse/Rabbit) by Zytomed) for

saturating electrostatic charges. Then tissue sections were incubated for 16 hours at 4°C with the respective primary antibody (against CD68, CD163, PD-1, PD-L1). After washing the slides with phosphate-buffered saline (PBS), the ZytoChem Plus HRP Polymer System IgG kit (Mouse/Rabbit) (Zytomed, Berlin, Germany) and liquid DAB (Diaminobenzidin) + Substrate Chromogen System (Agilent Technologies, Santa Clara, USA) was used for visualization of the bound primary antibodies. The slices were counterstained with Mayer 's acid hemalum for 2 min and stained blue for 5 min in tap water. In the following step the samples were dehydrated in an ascending series of alcohol, then treated with Roticlear® and cover slipped with RotiMount (Carl Roth, Germany).

All antibodies which were used in this study are listed in Table 2.

For the evaluation of the quantity of antigen-presenting macrophages and Hofbauer cells the number of cells was counted in three image sections at a magnification with a 40x lens. The total number of cells was then calculated by summing the three areas. For the evaluation of the intensity and distribution patterns of the antigen expression of PD-1 and PD-L1 in the extravillous trophoblast and the syncytiotrophoblast the semiquantitative immunoreactive score of Remmele (IRS) was used. The IRS is calculated by the multiplication of the grade of optical staining intensity (0 = none, 1 = weak, 2 = moderate and 3 = strong staining) and the percentage of positive staining cells (also divided into 4 categories: 0 = no staining, 1 = < 10% of the cells, 2 = 10–50% of the cells, 3 = 51–80% of the cells and 4 = more than 80% of the cells). The intensity, distribution pattern and counting of the immunochemical staining reaction were evaluated by two independent blinded observers. In 8 cases (n = 10%), the evaluation of the two observers differed. These cases were re-evaluated by both observers together. After the re-evaluation, both observers came to the same result. The concordance before the re-evaluation was 90%.

Table 2: List of the used primary antibodies

Antibody	**Isotype**	**Clone**	**Dilution**	**Source**
Anti-CD68	Rabbit IgG	Monoclonal; clone D4B9C	1:1000	Cell Signaling, USA
Anti-CD163	Mouse IgG1	Monoclonal; clone OTI2G12	1:2000	Abcam, UK
Anti-PD-1	Rabbit IgG	Polyclonal	1:100	Sigma Aldrich, USA

Anti-PD-L1	Rabbit IgG	Monoclonal; clone EPR19759	1:150	Abcam, UK

2.3 Immunofluorescence

The quadruple immunofluorescence staining allowed us to characterize specific antigens simultaneously. The same formalin-fixed and paraffin-embedded samples were placed in Roticlear® for 20 min for deparaffinization. Subsequently, the sections were panned in ethanol in order of descending concentrations (100%, 70%, 50%) and washed in distilled water. Unmasking of antigens was performed by a 5 min heat pretreatment in a pressure cooker with EDTA buffer, pH 9.0. After washing in distilled water and PBS for 4 min, incubation with immunofluorescence blocking buffer (Cell Signaling; Nr. 12411S) was performed to prevent unspecific staining. The solution was tipped off after 60 min and the primary antibodies were applied. CD68 was stained at a ratio of 1:3000 together with CD163 at a ratio of 1:4000 in dilution medium (Agilent; Nr. S302281-2) to differentiate cells in a double staining procedure. Incubation was performed for 16 h at 4 °C. After washing in PBS, the experimental room was darkened and the mixed secondary antibodies were applied: Cy-5-conjugated Goat-Anti-Mouse IgG antibody at a ratio of 1:100 (Dianova, Hamburg, Germany, 115-175-166) resulting in violet coloring and Cy-3- conjugated Goat-Anti-Rabbit IgG antibody at a ratio of 1:500 (Dianova, Hamburg, Germany, 111-165-144) resulting in red coloring. After 30 min of incubation, the excess secondary antibodies were washed off in PBS. Now the samples were blocked with Fab Fragment Donkey Anti-Goat IgG antibody (Dianova, Hamburg, Germany, 705-007-003) at a ratio of 1:50 in dilution medium (Agilent; Nr. S302281-2) for 30 minutes in the dark and afterwards washed in PBS for 4 minutes. In the next step the third primary antibody PD1 at a ratio of 1:100 in dilution medium (Agilent; Nr. S302281-2) or PD-L1 in a ratio of 1:100 in dilution medium (Agilent; Nr. S302281-2) was applied and incubated for 16 h at 4 °C. After washing in PBS, the secondary antibody Alexa Fluor 488-conjugated-Anti-Rabit IgG (Thermo Fisher, Massachusetts, USW, Nr. A-11008) in a ratio of 1:500 in dilution medium was applied and incubated for 30 minutes at room temperature in the dark, resulting in green coloring. The slides were once more washed in PBS for 4 minutes. In the dark, the preparations dried at room temperature and were cover slipped with

Mounting medium for fluorescence with DAPI (4′,6-diamidino-2-phenylindole), which stains the cell nuclei as a blue light impression.

The quadruple staining was evaluated and assessed using a fluorescence microscope (Keyence, Osaka, Japan).

2.4 Statistical Analysis

The statistical analysis was done using SPSS version 28. The data was first tested for normal distribution using the Kolmogorov-Smirnov test. For normal distributed data the t-test was used for comparing mean values. For data not following a normal distribution the Mann-Whitney U signed rank test was chosen to compare mean values. The comparison of mean values is illustrated by boxplots in which the boxes represent the range between the 25th and 75th percentile with a horizontal line at the median. The bars delineate the 5th and the 95th percentile. Dots represent outliers. Confounder analysis was performed by a Linear regression pathway for clinical data such as BMI, fetal weight and others. It was carried out to calculate the effect of clinical characteristics on macrophages/checkpoint molecules. Findings with p-values < 0.05 were considered significant.

3. Results

3.1 Downregulation of CD68 and CD163 in the decidua of preeclamptic placentas

The number of CD68 ($p = 0.021$) and CD163 ($p = 0,.43$) positive macrophages in the decidua were both shown to be significantly downregulated in the placenta of preeclamptic patients compared to healthy subjects (Figure 1). The lower expression of CD163-positive maternal macrophages was mainly due to a downregulation in female offspring ($p = 0.02$) while there was no significant difference in male offspring ($p = 0.364$). Compared to control placentas, the downregulation of CD68-positive and CD163-positive maternal macrophages was only seen in late-onset preeclampsia ($p = 0.037$ and $p < 0.001$) while there was no difference in early-onset preeclampsia ($p = 0.148$ and $p = 0.801$). This is illustrated in the boxplots in Figure 1G and 1H. The majority of maternal

macrophages in the decidua were identified as CD163 positive, both, in preeclamptic and healthy placentas. This is seen in Figure 3, parts A and B.

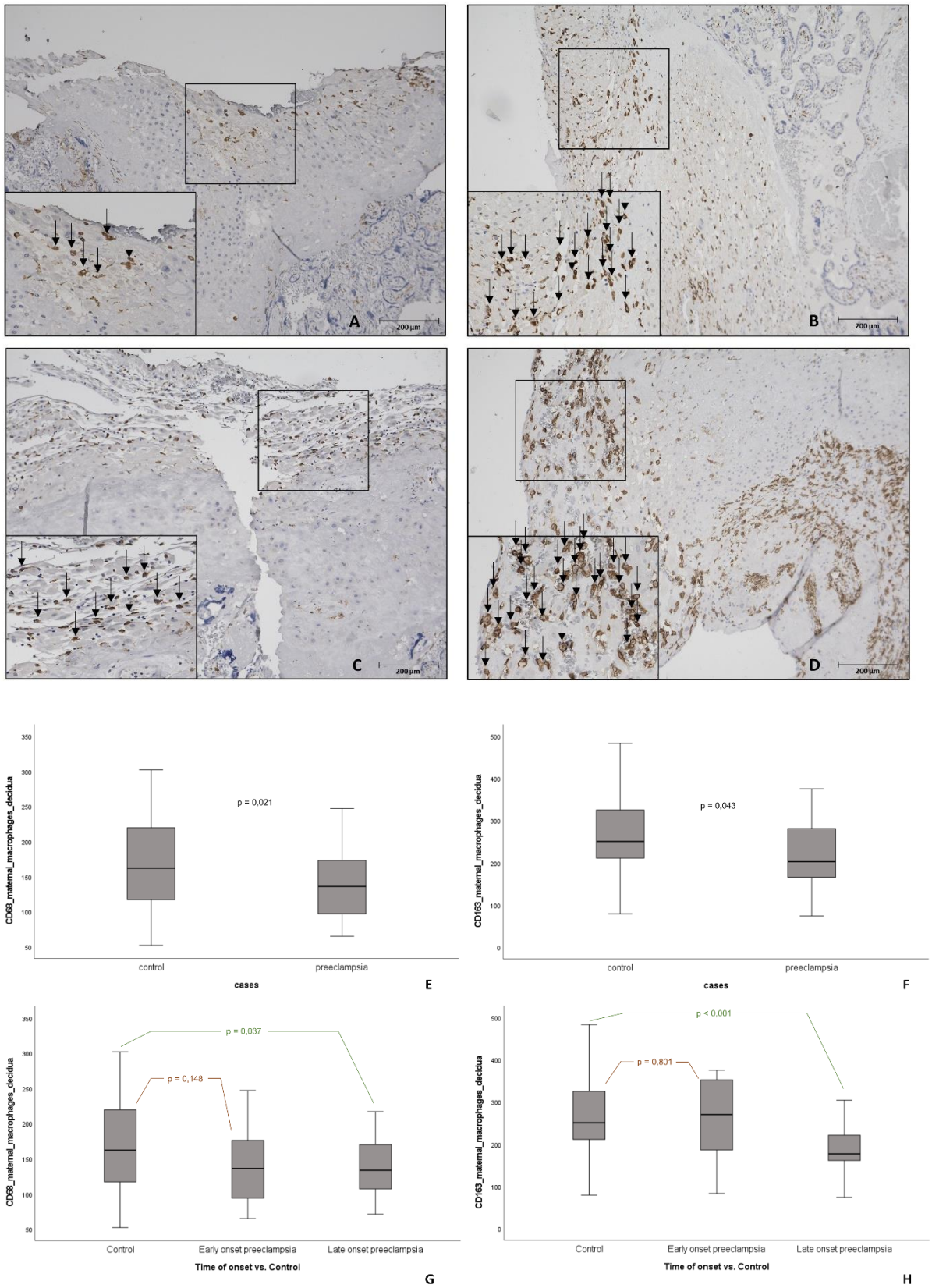
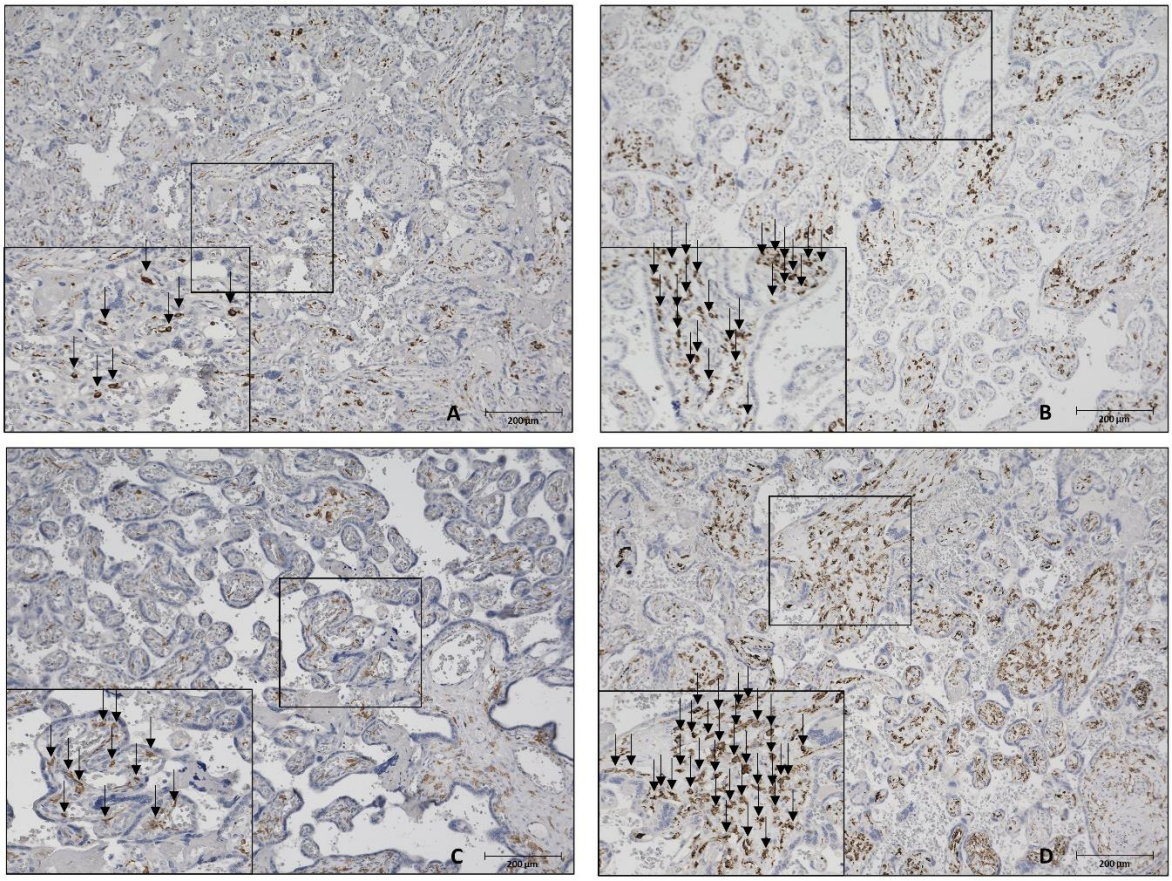


Figure 1: Representative slides of immunohistochemical staining for CD68 expression in the decidua of a preeclamptic patient (A) and healthy control (B) and for CD163 expression in the decidua of a preeclamptic patient (C) and healthy control (D). Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens. Scale bars are 200 µm. The black frame shows where the magnified inserts are taken from. The arrows mark the CD68- and CD163-positive macrophages. The significant downregulation of both macrophage markers in preeclampsia is illustrated in boxplots E and F. The downregulation of CD68-positive and CD163-positive macrophages only in late onset preeclampsia is illustrated in part G and H. .

3.2 Downregulation of CD68 and CD163 in the chorionic villi of preeclamptic placentas

The same difference in the expression of CD68 and CD163 was shown in Hofbauer cells within the chorionic villi. CD68 ($p < 0.001$) and CD163 ($p < 0.001$) were proven to be significantly downregulated in the chorionic villi of preeclamptic patients compared to healthy subjects without a sex-specific difference (Figure 2). No difference in the expression was seen between early onset and late onset preeclampsia. Exactly like in the decidua, the HBCs in both groups expressed more CD163 compared to CD68 (seen in Figure 3, parts C and D).



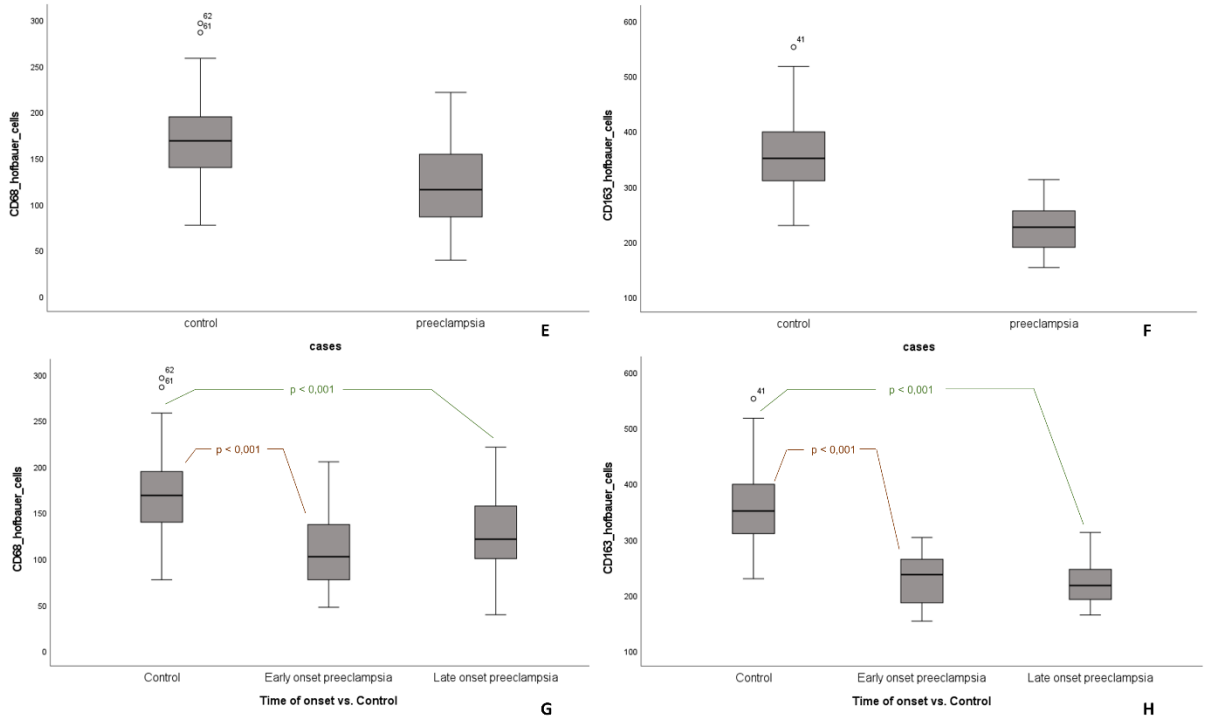


Figure 2: Representative slides of immunohistochemical staining for CD68 expression in the chorionic villi with its HBCs in a preeclamptic patient (A) and healthy control (B) and for CD163 expression in the chorionic villi in a preeclamptic patient (C) and healthy control (D). Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens. The black frame shows where the magnified inserts are taken from. Scale bars are 200 µm. The arrows mark the CD68- and CD163-positive HBCs. The significant downregulation of both macrophage markers in preeclampsia is illustrated in boxplots E and F. The comparison between early onset/late onset preeclampsia and the control group is shown in the boxplots G and H.

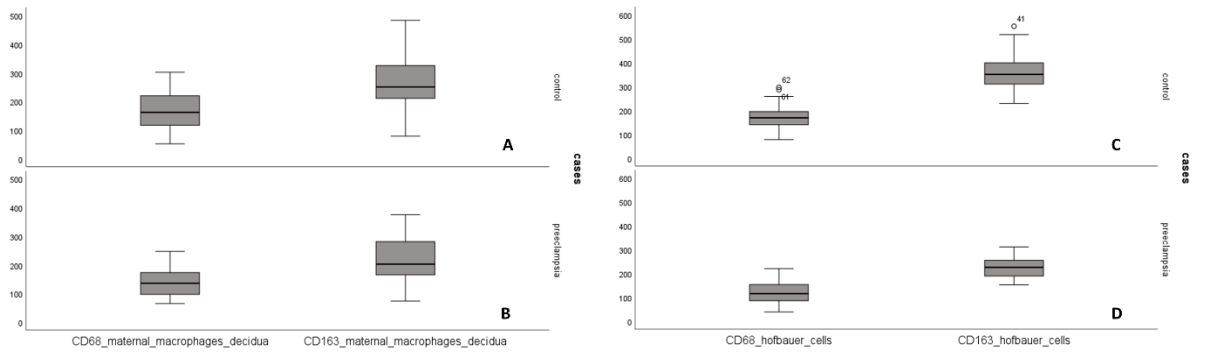
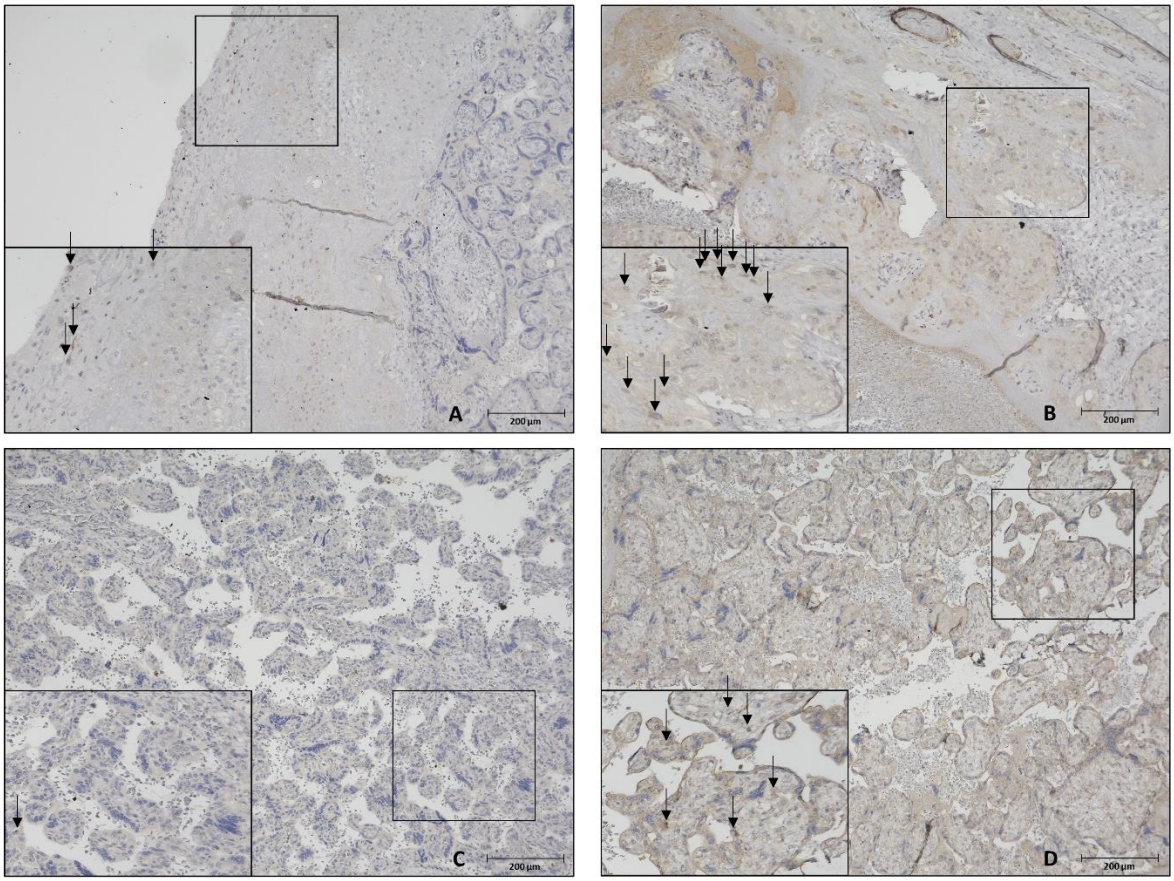


Figure 3: The boxplots show the predominant expression of CD163 in maternal macrophages of the decidua in control placentas (A) and preeclampsia placentas (B) and the predominance of CD163 in HBCs of the chorionic villi in control placentas (C) and preeclampsia placentas (D). This indicates a M2-polarisation of most macrophages.

3.3 Downregulation of PD-1 in preeclamptic placentas

The number of PD-1 positive macrophages were proven to be significantly downregulated both in the decidua ($p < 0.001$) and in the chorionic villi ($p < 0.001$) of preeclamptic patients. This is illustrated in Figure 4. The immuno-reactive score of PD-1 expression in the extravillious trophoblast ($p < 0.001$) and the syncytiotrophoblast ($p < 0.001$) were shown to be significantly lower in preeclampsia placentas compared to healthy placentas. Within the syncytiotrophoblast the downregulation was stronger in male offspring ($p = 0.002$) compared to female offspring ($p = 0.192$). This can be seen in the supplementary Table 2. Except for that, no other sex-specific differences or a difference between early and late onset preeclampsia was seen.



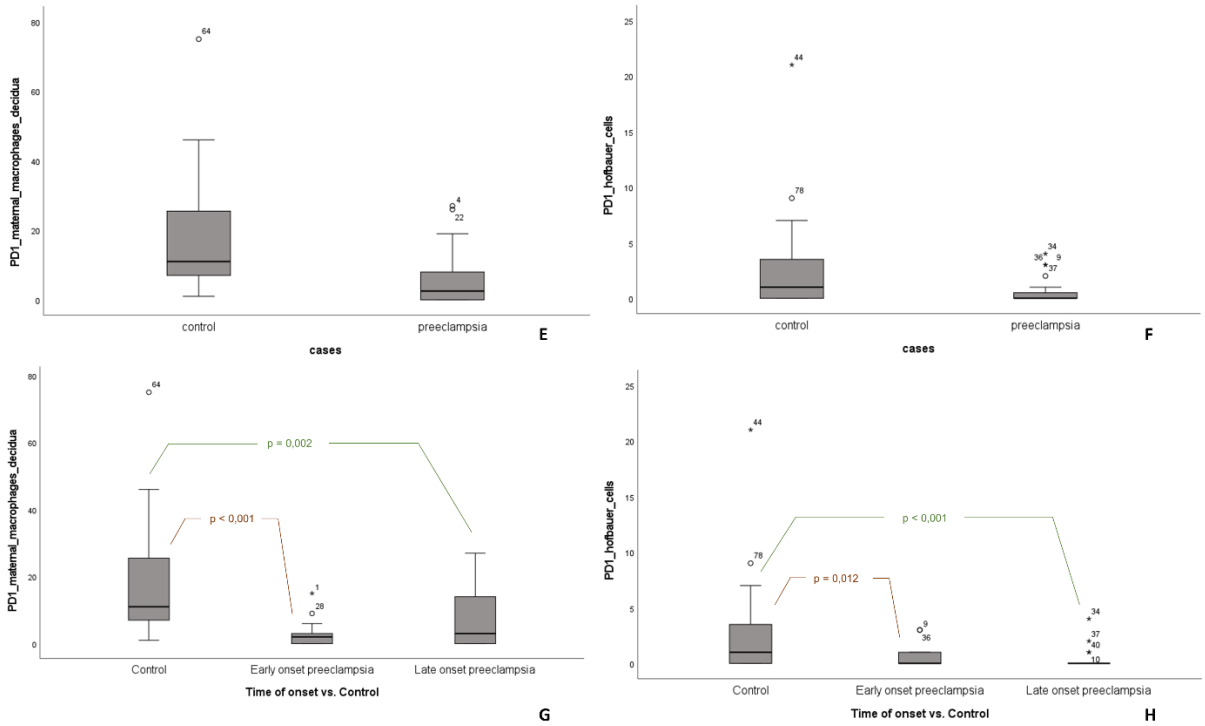
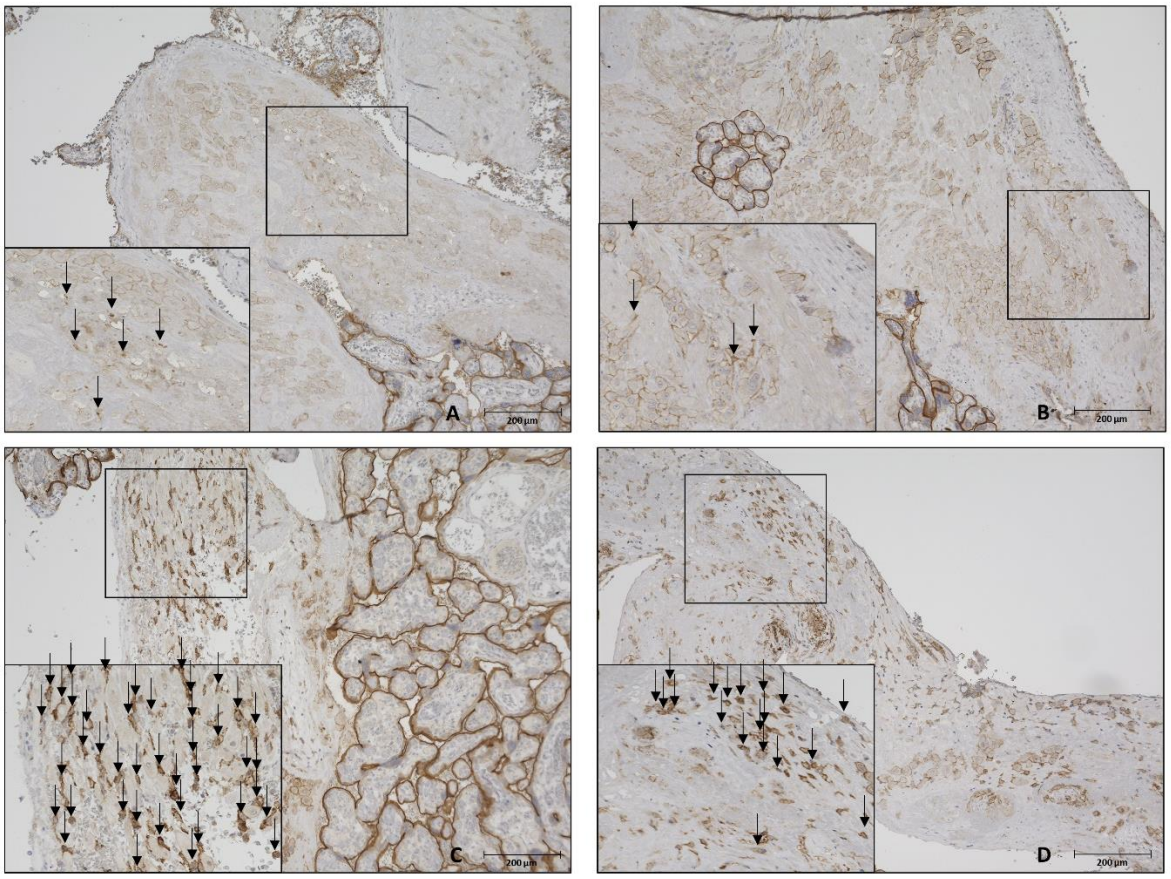


Figure 4: Representative slides of immunohistochemical staining for PD1 expression in the decidua of a preeclamptic patient (A) and healthy control (B) as well as the chorionic villi of a preeclamptic patient (C) and a healthy control (D). Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens. The black frame shows where the magnified inserts are taken from.The arrows point to the PD1-positive macrophages and HBCs. Scale bars are 200 µm. The significant downregulation of PD1 in preeclampsia is illustrated in boxplots E and F. The comparison between early onset/late onset preeclampsia and the control group is shown in the boxplots G and H.

3.4 Downregulation of PD-L1 on maternal macrophages in the decidua of preeclamptic placentas

The immunohistochemical analysis of PD-L1 expression showed a significant downregulation of PD-L1 positive maternal macrophages in the decidua of preeclamptic patients compared to healthy controls (p = 0.043, see Figure 5E). Looking at the different sex of the offspring this was only caused by a significant downregulation of PD-L1 expression in male preeclamptic placentas compared to male control placentas (p = 0.004). In contrast there was no difference in the PD-L1 expression in decidual macrophages in female offspring (p = 0.841). The comparison of PD-L1 positive macrophages within the preeclamptic group showed a significant lower expression in the male compared to the female preeclamptic placentas (p = 0.009). This is illustrated in Figure 5F. Comparing the PD-L1 expression on maternal macrophages between early onset/late onset preeclampsia and the control group showed a significant downregulation only in early onset preeclampsia (p = 0.005) while

there was no difference in late onset preeclampsia (p = 0.513). This can be seen in Figure 5G. No statistically significant difference between the two groups was found in the PD-L1 expression in Hofbauer cells, extravillious trophoblasts and syncytiotrophoblast.



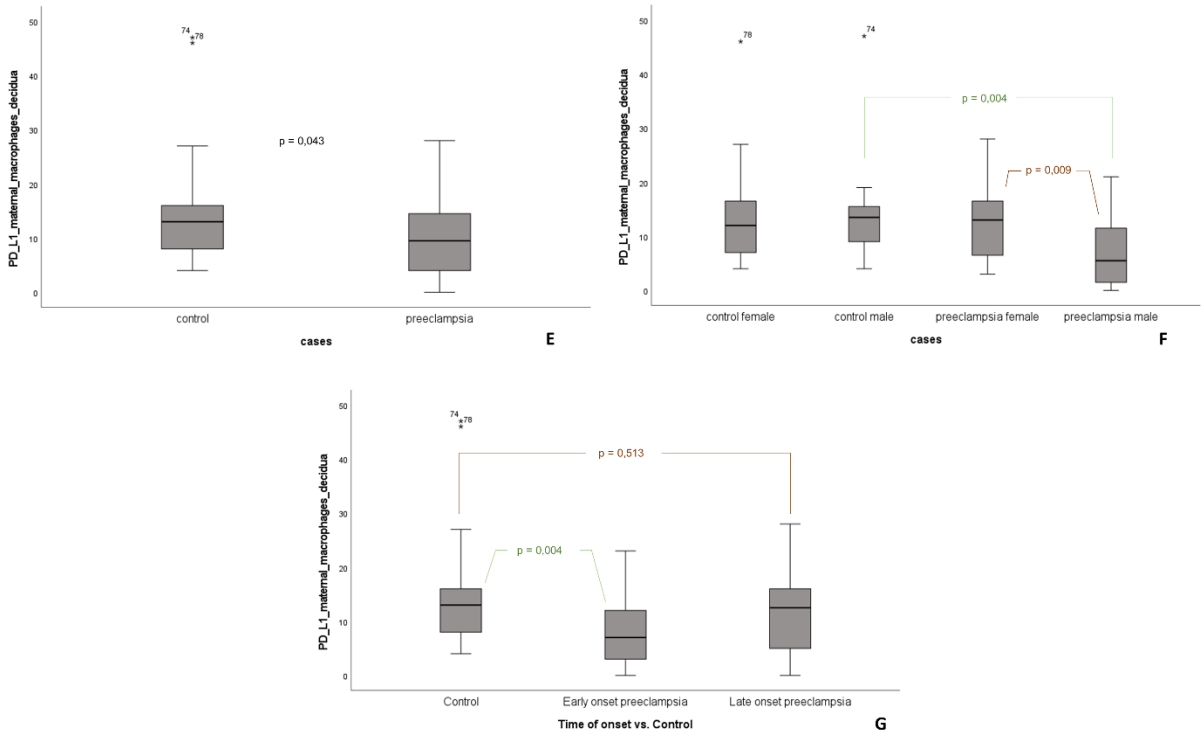


Figure 5: Representative slides of immunohistochemical staining for PD-L1 expression in the decidua of preeclamptic patients (A male, B female) and healthy controls (C male, D female). Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens. The black frame shows where the magnified inserts are taken from. Scale bars are 200 µm. The arrows mark the PD-L1-positive macrophages. The significant downregulation of PD-L1 on maternal macrophages in preeclampsia is illustrated in boxplot E, with a sex-specific difference shown in boxplot F. The comparison between early onset/late onset preeclampsia and the control group is shown in the boxplots G.

3.5 Quadruple Immunofluorescence Staining of PD1 and PD-L1 with CD68 and CD163

Quadruple Immunofluorescence staining was used to simultaneously characterize the antigens PD1 and PD-L1 together with the macrophage markers CD68 and CD163. Thereby the expression of PD1 and PD-L1 on macrophages within the decidua and the chorionic villi was proven.

The following figure 6 exemplarily shows the PD1 expression in the chorionic villi of a preeclamptic (parts A-D) and a control placenta (parts E-H). The expression of all three antigens in the same cell results in a yellow-orange coloring (parts D and H). The downregulation of PD1 on HBCs in preeclampsia can be seen in the arrow-marked areas.

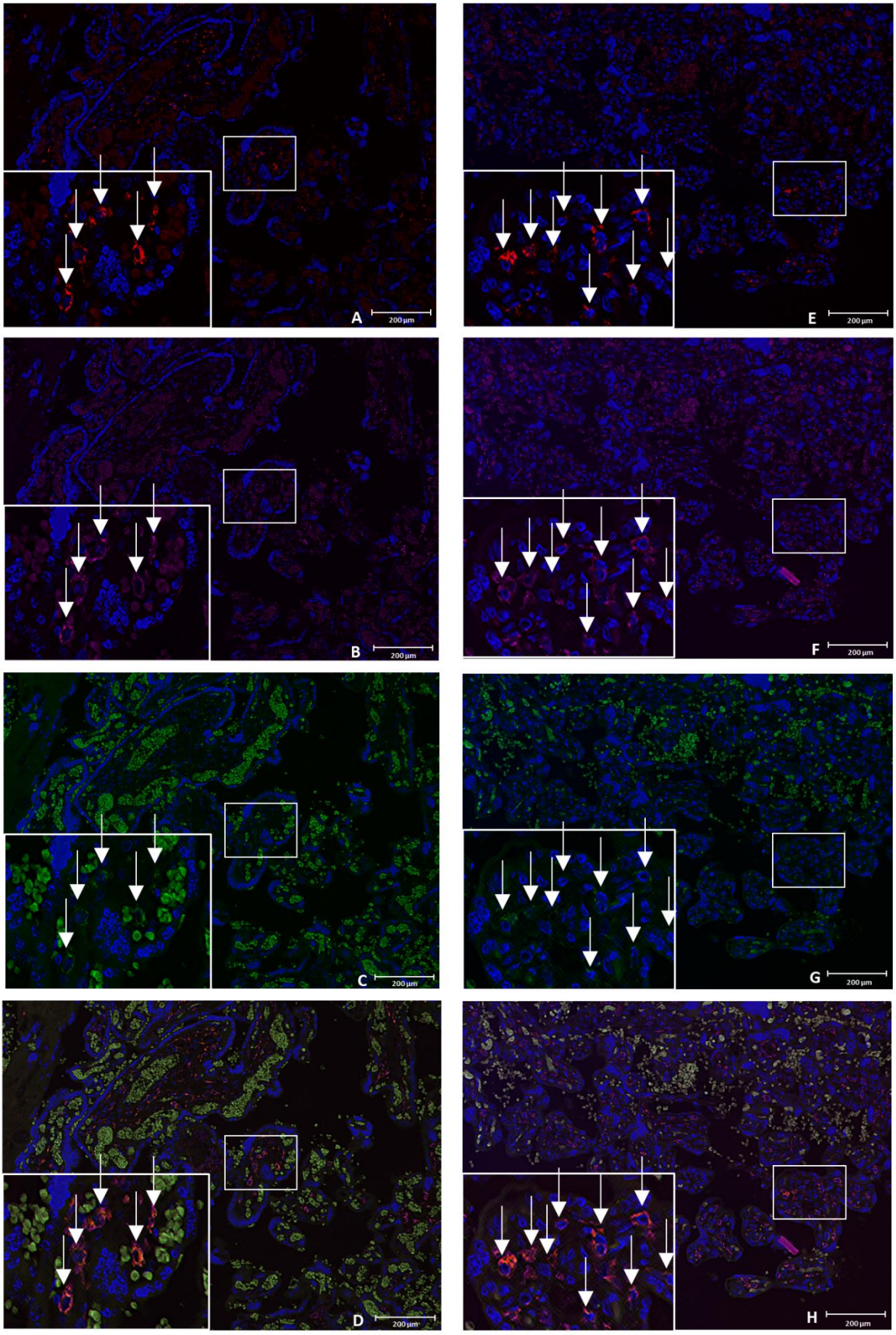


Figure 6: Representative slides of quadruple immunofluorescence staining in the chorionic villi of a preeclamptic placenta (A-D) and a control placenta (E-H). Parts A and E show the immunofluorescence of CD68 (red), parts B and F the

immunofluorescence of CD163 (pink) and parts C and G the immunofluorescence of PD1 (green). In the parts D and H all three markers are shown together in one picture. The arrows mark the HBCs which are PD1 positive. Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens with additional zoom. The white frame shows where the magnified pictures are taken from. Scale bars are 200 µm.

Figure 7 exemplarily shows the PD-L1 expression in the decidua of a preeclamptic (parts A-D) and a control placenta (parts E-H). The expression of all three antigens in the same cell results in a yellow-orange coloring (parts D and H). The downregulation of PD-L1 on the decidual macrophages in preeclampsia can be seen in the arrow-marked areas.

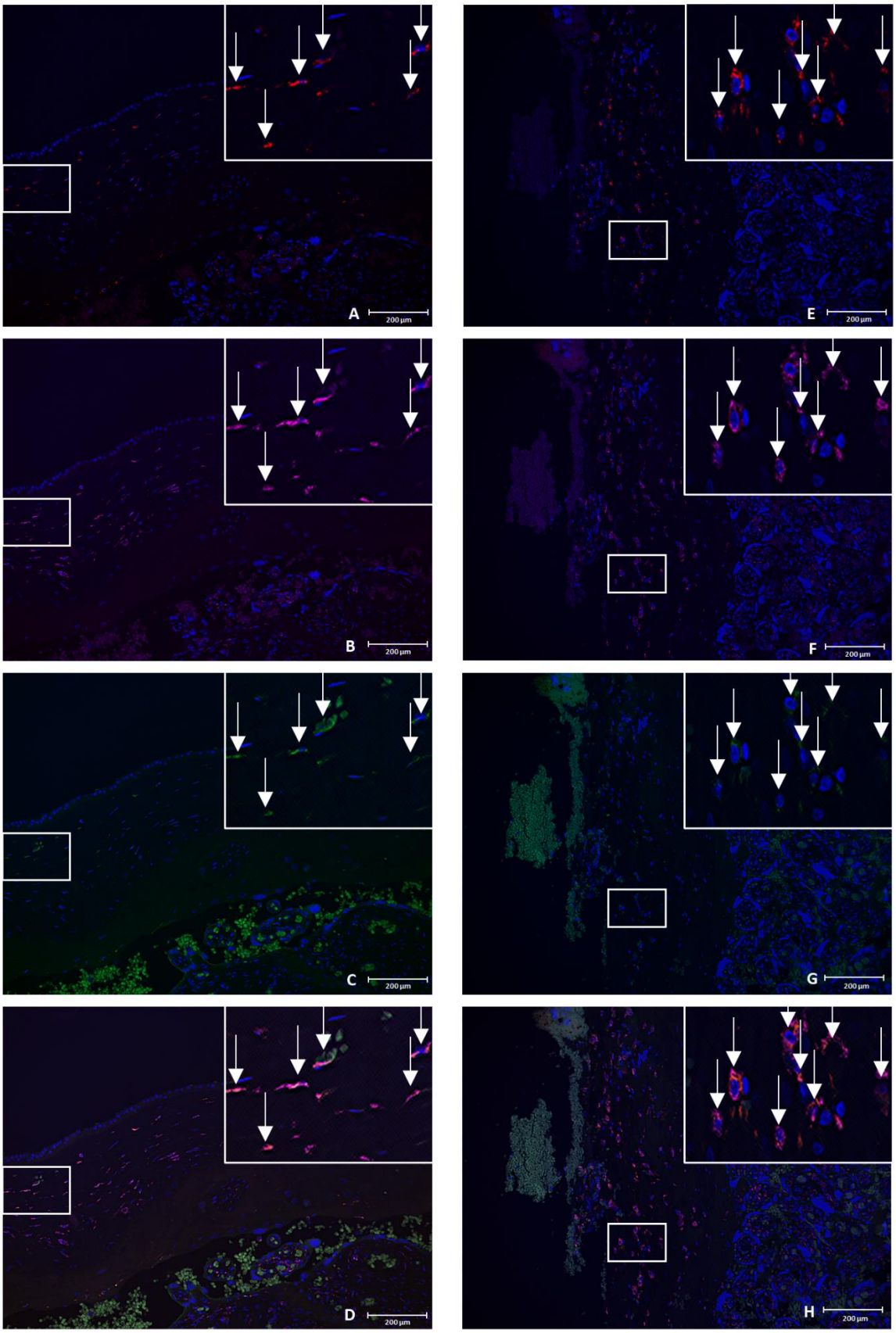


Figure7: Representative slides of quadruple immunofluorescence staining in the decidua of a preeclamptic placenta (A-D) and a control placenta (E-H). Parts A and E show the immunofluorescence of CD68 (red), parts B and F the

immunofluorescence of CD163 (pink) and parts C and G the immunofluorescence of PD-L1 (green). In the parts D and H all three markers are shown together in one picture. The arrows mark the HBCs which are PD-L1 positive. Pictures were taken with a 10x lens. The inserts represent a magnification with a 40x lens with additional zoom. The white frame shows where the magnified pictures are taken from. Scale bars are 200 µm.

4. Discussion

Preeclampsia is a complex and heterogenous disease of pregnancy triggered by impaired placental development and maternal vascular instability. Several studies have proven a proinflammatory state of the immune system being one of the key players in the development of preeclampsia (Aggarwal et al., 2019; Harmon et al., 2016; Rana et al., 2019; Vishnyakova et al., 2021; Yeh et al., 2013). The well matched and adjusted immune responses which are essential for formation and maintenance of a pregnancy and upholding the maternal-fetal-tolerance are thereby disrupted (Mor and Cardenas, 2010).

Within this study, we investigated the status of the PD1/PD-L1 system, an important immune checkpoint regulatory system, on macrophages in the placenta of preeclamptic and control patients by immunohistochemistry and immunofluorescence staining. The macrophages were studied by staining for CD68 (pan-macrophage marker) and CD163 (M2-polarization).

We observed a significant downregulation of both macrophage markers in the decidua and the chorionic villi in preeclampsia, revealing a decrease of macrophages. This matches part of the existing conflicting data of macrophages in preeclampsia with some studies showing an increased number (Huang et al., 2010; Li et al., 2016; Reister et al., 2001) and others showing a decreased number of macrophages (Bürk et al., 2001; Williams et al., 2009). In contrary to previous studies, demonstrating a reduction of M2-polarized macrophages and a predominance of M1-polrized macrophages in preeclampsia (Tang et al., 2013; Yang et al., 2017; Yao et al., 2019), our data showed an overall reduction of macrophages in preeclampsia and a predominance of M2-polarization in both groups, the preeclamptic and control patients. Our results of reduced number of Hofbauer cells are in concordance with the results of the study described by Tang et al. (Tang et al., 2013). They also used gestational

week matched placentas as controls as we did and discovered a downregulation of HBCs in severe preeclampsia. The reduction of M2-polarized HBCs could lead to a reduction of anti-inflammatory and pro-angiogenic responses in the placenta and thereby promote pro-inflammation and anti-angiogenesis seen in the PE-placenta (Rambaldi et al., 2019; Vishnyakova et al., 2021; Yeh et al., 2013). While high expression of CD68 and CD163 is associated with tumor growth and invasion in tumor biology (Larionova et al., 2020; Leisz et al., 2022; Yang et al., 2022), the significant downregulation of both markers shown in this study could explain at least in part the defective trophoblast invasion seen in preeclampsia. The downregulation might thereby play a crucial role in the pathogenesis of preeclampsia. Further studies, also with additional macrophage markers, are needed to investigate this hypothesis. The reduced number of macrophages observed in this study could also lead to reduced phagocytosis and reduced scavenging of cytotrophoblast and syncytiotrophoblast affected by apoptosis in preeclampsia (Crocker et al., 2003; DiFederico et al., 1999; Ishihara et al., 2002). This could result in higher placental damage and thereby also affect the pathogenesis of the disease.

The PD1 expression on decidual macrophages was shown to be significantly downregulated in preeclampsia, which is consistent with the study performed by Hu et al (Hu et al., 2022). In addition, our study also showed the downregulation of PD1 on Hofbauer cells within the chorionic villi. The activation of the PD1/PD-L1 system by PD-L1 binding to its receptor PD1 leads to an attenuation of the immune system amongst others through the inhibition of T-cell proliferation, the down-regulation of pro-inflammatory T-cell activity and a reduction of cytokine production (Chikuma, 2016; Francisco et al., 2010; Miko et al., 2019; Riley, 2009). A reduction of PD1 in preeclampsia could thereby lead to an amplification of the immune system leading to a pro-inflammatory state. Hofbauer cells are early local macrophages within the chorionic villi which migrate to other organs during fetal development. They are important for fetal immune defense and the feto-maternal tolerance. A pro-inflammation within HBCs could thereby lead to inflammation in other fetal organs causing end-organ dysfunction in the fetus. We also saw a significant downregulation of the PD1 receptor on extravillious trophoblast and syncytiotrophoblast within the placenta of preeclamptic patients compared to healthy controls. This is consistent to previous data shown by Zhang et al. and might contribute to the

Treg/Th17 imbalance seen in preeclampsia leading to proinflammation (Mittelberger et al., 2022; Zhang et al., 2018).

The protein PD-L1, the ligand of the immune checkpoint system, was shown to be significantly downregulated on maternal macrophages in the decidua of preeclamptic patients. This could again contribute to the pro-inflammatory state of the immune system seen in this disease, like illustrated above. Looking at the different sex of the offspring showed that this downregulation was only due to a decrease of the protein in male offspring while there was no variation seen in female offspring. Also, the comparison between the different sex within the preeclampsia group showed a significant lower PD-L1-expression on maternal macrophages in male offspring. Since many years it is well known that there is a male disadvantage in premature and low-birthweight neonates resulting in a higher mortality (Vu et al., 2018). The exact causes are still not fully understood. In preeclampsia many studies showed a higher rate of neonatal complications such as low birthweight, respiratory distress syndrome, hematological changes such as thrombocytopenia, necrotizing enterocolitis and bronchopulmonary dysplasia (Backes et al., 2011; Cetinkaya et al., 2012; Habli et al., 2007; Hansen et al., 2010; Kalagiri et al., 2015; Mouna et al., 2017; Soliman et al., 2020). Chun et al performed a population-based cohort study analyzing the long-term postnatal health of children delivered by preeclamptic mothers. The study showed a higher risk for endocrine, metabolic and nutritional diseases as well as diseases of the blood for children exposed to preeclampsia (Wu et al., 2009). All these studies unfortunately didn’t differ between the different sex of the offspring. The downregulation of PD-L1 on maternal macrophages in the placenta of preeclamptic patients with male offspring seen in our study could lead to more proinflammation and thereby to sex-specific complications in later life such as inflammatory diseases. Further studies are needed to investigate the sex-specific differences in morbidity and mortality in offspring of preeclamptic patients.

Confounder analysis by calculation of linear regression was performed for each significantly different variable between the two study groups. The results in Table 1 (supplementary) show, that particularly the expression of CD68 and CD163 within the Hofbauer cells are affected by the clinical characteristics such as BMI, fetal weight, birth percentile and umbilical artery pH. The confounder results indicate a systemic influence of the gestational disease preeclampsia especially on fetal

macrophages (Hofbauer cells). Not only placental weight, but also BMI, fetal birthweight, birth percentile, umbilical artery pH, gravidity and parity seam to strongly influence the number of fetal Hofbauer cells within placental villi, which is an astonishing result. This needs further research and cannot be explained within this study. Questionable are programming interactions between mother and fetus and thereby an influence on later life diseases. It could also influence the fact that severity of preeclampsia is reduced with an increasing number of pregnancies within the same woman. The placental weight influences nearly all evaluations within the placenta. This is not surprising because the size and weight of the placenta has an impact on the number of cells and molecules that can be observed.

Because 14 out of the 40 preeclampsia cases within this study are also affected by HELLP-syndrome or FGR it cannot be answered if these conditions during pregnancies have an impact on the given results. Real confounding is unlikely because they often go hand in hand with preeclampsia. Future studies are needed which investigate the macrophage markers and immune checkpoint molecules in HELLP-syndrome and FGR-cases.

An important strength of our study is the selection of macrophage markers, especially CD68 and CD163, because of their importance in tumor biology. This study might thereby show new insights in the pathogenesis of preeclampsia. Another strength is the differentiation of the shown data between female and male offspring, which might point out to a difference in prognosis depending on the fetal sex. This needs to be investigated further.

A limitation of the study is the significant difference in clinical data between the groups such as BMI. Residual confounding cannot be excluded. In future studies more markers regarding the polarization status of macrophages should be used in order to differ between differently polarized macrophages. In order to get more representative data, the numbers of counted image sections could be raised in future studies.

In conclusion our results indicate that the downregulation of PD1 and PD-L1 on macrophages might contribute to the pro-inflammatory environment seen in preeclamptic patients. The reduced numbers of CD68- and CD163-positive macrophages might play a crucial role in the defective trophoblast

invasion. The immune checkpoint system and the macrophage expression could play an important role in the pathogenesis of preeclampsia and point out a new target in the therapy of this pregnancy specific disease.

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