SUPPLEMENTARY MATERIAL

Lymphocyte characterization of decidua basalis spiral arteries with acute atherosis in preeclamptic and normotensive pregnancies.

Running title: Decidual lymphocytes and acute atherosis

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Keywords
acute atherosis, decidua, immunohistochemistry, perivascular infiltrate, placenta, preeclampsia

Supplementary material

Supplemental Methods

Patient recruitment

Singleton pregnant women were included prior to cesarean section to the Oslo Pregnancy Biobank study between 200-2013. All women provided informed written consent. The study was approved by the Regional Committee for Medical and Health Research Ethics of South-East Norway, and conducted according to the Declaration of Helsinki. None of the women had any pre-pregnant chronic disease and no pregnancies with fetal structural/chromosomal abnormalities were included. Preeclampsia (PE) was defined as new onset hypertension (blood pressure ≥140/90 mmHg) and new onset proteinuria (≥1+ on dipstick, or ≥30 protein/creatinine ratio) ≥20 weeks of pregnancy.

Acute atherosis evaluation

Decidua basalis acute atherosis (AA) was investigated by immunohistochemistry (IHC) on sections of formalin fixed paraffin embedded tissue as previously described (Alnaes-Katjavivi *et al.* 2016) and in the IHC protocol provided below. Briefly, decidua basalis tissue sections stained with H+E, Ck7+PAS, and desmin+PAS where used to identify spiral arteries (>140 μm diameter), and to confirm the presence of trophoblasts and the decidua basalis nature of the tissue, while CD68+PAS was used to identify CD68+ foam cells (Supplemental Figure 1). Traditionally PVI is a prerequisite for AA. However, we previously found that PVI (as assessed by H&E staining) was not unique to spiral arteries containing CD68+ foam cells (Alnaes-Katjavivi *et al.* 2016). Consequently, we did not conclude PVI as a mandatory prerequisite for AA diagnosis (Alnaes-Katjavivi *et al.* 2016). Instead, we defined AA as the presence of a minimum of two adjacent intramural vacuolated CD68+ (foam) cells in the wall of at least one spiral artery.

Immunohistochemistry protocol for AA evaluation and lymphocyte quantification

Immunohistochemistry was performed using an automated procedure on a Ventana Benchmark XT (Roche). Briefly, 3 µm sections of formalin-fixed paraffin embedded decidua basalis tissue samples were cut and dried at 60ºC for 1h, and 37ºC overnight. Antibodies were diluted to the desired concentration (see Table below) with EnVision Flex Antibody Diluent from Agilent Technologies/Dako (K8006) and filled in the antibody dispenser of the machine. The slides were placed in the stainer and the Ventana staining procedure was started, this procedure included deparaffinization and pretreatment for antigen retrieval with CC1 Standard Cell Conditioner 1 (pH 9) for 1h followed by incubation with the antibody at 37ºC for 32 min. Then signal amplification with Amplifier kit (Roche) was performed. The antibody staining was detected using ultraView Universal DAB detection KIT (Roche) or EnVision FLEX DAB+ Chromogen detection kit (Dako). The slides were removed from the automated stainer and rinsed in water before manual PAS staining was performed.

PAS staining was performed by firstly incubating the slides in 1% periodic acid with agitation for 10 min, followed by rinsing in several changes of tap water for 10 min. Secondly, the slides were incubated with Schiff's reagent for 10 min and rinsed in tap water for 10 min. The slides were counterstained with Foot's hematoxylin for 10 min and rinsed in distilled water, followed by a short dip in 4% acetic acid solution for differentiation, and a rinse in distilled water. Then the slides were incubated with bluing agent Scott's tap water for 1 min, and rinsed in distilled water. Finally the slides were dehydrated in 100% Ethanol for 1 min, and then in Xylene for 1 min. The slides were dried in RT for 5 min before they were mounted and sealed with a cover slip using Coverquick 4000 (Chemi-teknik).

Antibody information and order of serial sections stained:

Slide no.	Antibody	Manufacturer	Catalog no.	Clone	Dilution	Detection kit
1	Hematoxylin & Eosin					
2	Desmin	Roche	760-2513	DE R-11	Ready to use	
3	Cytokeratin 7	Dako	M7018	OV-TL 12/30	1/300	ultraView
4	CD68	Dako	M0814	KP1	1/1000	ultraView
5	Hematoxylin & Eosin					
6	FOXP3	Abcam	20034	236A/E7	1/200	EnVision Flex
7	CD3	Roche	790-4341	2GV6	Ready to use	ultraView
8	CD8	Dako	M 710301	C8/144B	1/150	EnVision Flex
9	CD56	Cell Marque	156R-95	MRO-42	1/500	ultraView

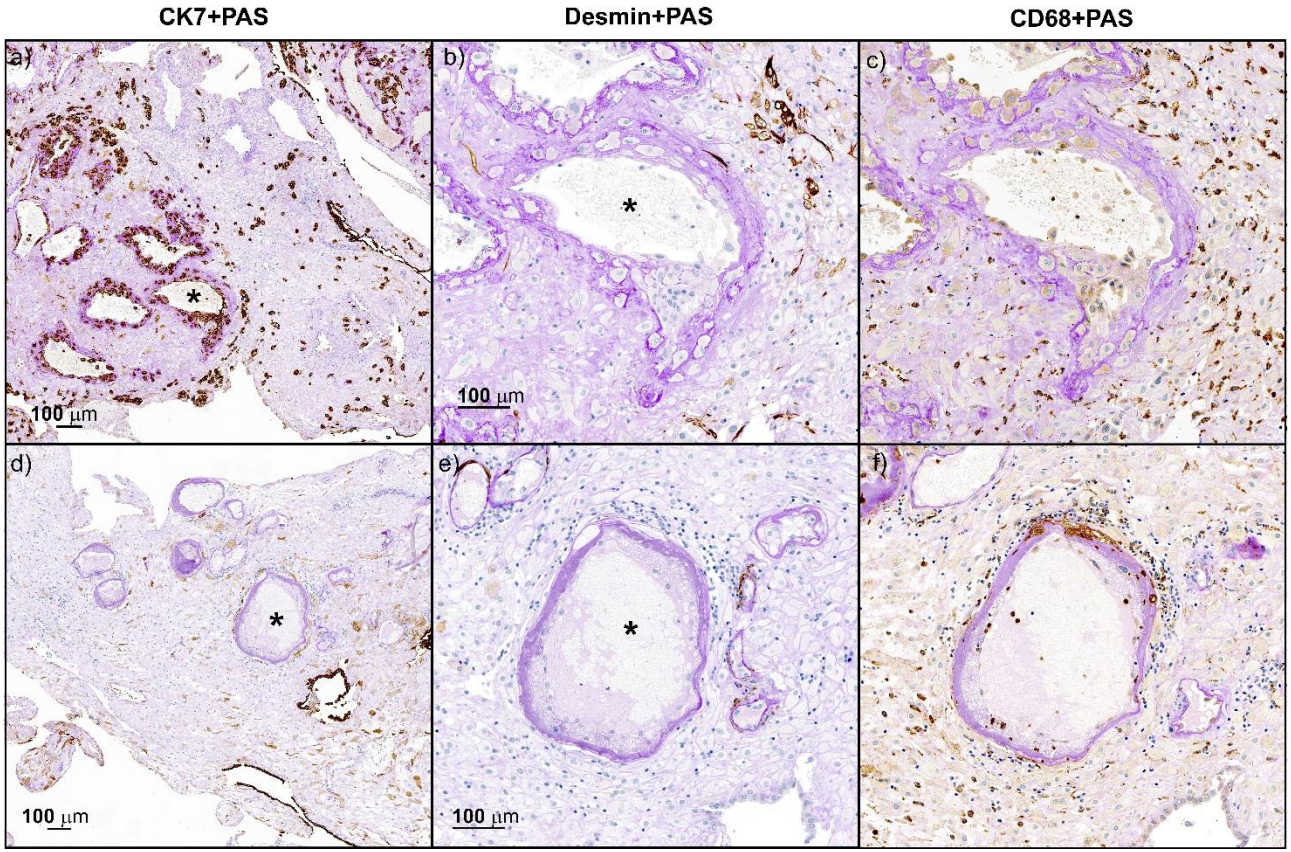
Supplemental Table 1. Clinical characteristics of women providing a decidual sample with spiral arteries identified (n=91), according to pregnancy groups. Gestational week (GW) at delivery was defined according to dating by routine ultrasound screening at GW 17-20. Blood pressure (BP) before gestational week 20 is the median value for the mean of all blood pressures registered before the 20th week of gestation, per woman. Newborn gender specific baby weight percentiles were calculated according to Norwegian fetal growth curves (20). Normotensive controls had uncomplicated pregnancies without clinical evidence of fetal growth restriction (all birth weights were above the 10th birth weight percentile). Data are presented as median values, percentages, or *rates*. Statistically significant differences (p-values <0.05) comparing preeclamptics with AA or without AA, and normotensives with or without AA, using the Mann-Whitney test, are indicated by an asterisk (*).

	Preeclampsia with AA n=27	**Preeclampsia without AA n=27**	**Normotensive with AA n=10**	**Normotensive without AA n=27**	**Total n=91**
Age at delivery (years)	32	33	34	33	33
BMI, pre-pregnancy (kg/m^2)	24.5	22.5	22.0	21.7	22.8
BMI, at delivery (kg/m^2)	29.3	29.8	27.5	27.5	28.6
Gestational age at delivery (weeks^{+days})	32+4	34+1*	39+0	39+0	36+1
Mean Systolic BP <20 GW (mmHg)	117	115	108	114	115
Mean Diastolic BP <20 GW (mmHg)	69	70	67	67	70
Systolic BP at delivery (mmHg)	160	160	119	125	150
Diastolic BP at delivery (mmHg)	104	99*	70	70	93
BW percentiles (%)	1.3	3.3	50.4	50.9	29.1

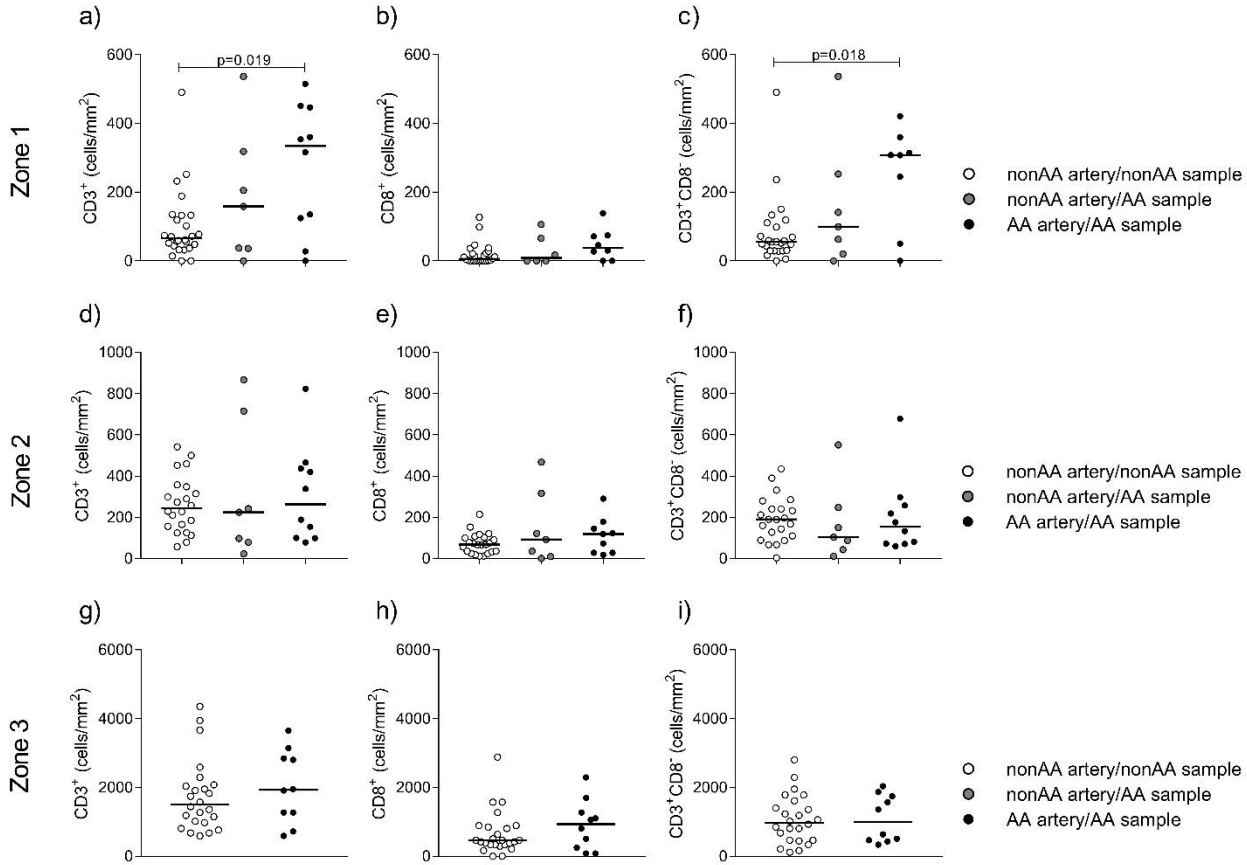
Supplemental Table 2. Median cell counts and *(min-max)* values for the lymphocytes investigated in preeclampsia and normotensive pregnancies in the pre-defined zones: 1) intramural, 2) perivascular, and 3) interstitial. The spiral arteries were divided in three categories: a) arteries without AA in samples without AA (nonAA artery/ nonAA sample), b) arteries without AA in samples with AA (nonAA artery/AA sample), and c) arteries with AA in samples with AA (AA artery/AA sample).

	Zone 1 (intramural)			**Zone 2 (perivascular)**			**Zone 3 (interstitial)**	
	nonAA artery/ nonAA sample	nonAA artery/ AA sample	AA artery/ AA sample	nonAA artery/ nonAA sample	nonAA artery/ AA sample	AA artery/ AA sample	nonAA sample	AA sample
Preeclampsia								
CD3+	3 *(0 – 59)*	3 *(0 – 11)*	8 *(0 – 50)*	23 *(6 – 69)*	38 *(5 – 124)*	45 *(5 – 165)*	9 *(3 - 32)*	17 *(4 – 39)*
CD8+	0 *(0 – 25)*	1 *(0 – 4)*	3 *(0 – 16)*	8 *(2 - 47)*	10 *(1 – 45)*	9 *(1 – 89)*	4 *(0 – 15)*	4 *(1 – 16)*
CD3+CD8-	2 *(0 – 34)*	2 *(0 – 6)*	7 *(0 – 35)*	12 *(0 – 40)*	21 *(2 – 123)*	38 *(2 – 124)*	6 *(2 – 26)*	11 *(2 – 37)*
FoxP3+	0 *(0 – 1)*	0 *(0 – 0)*	0 *(0 – 1)*	1 *(0 – 11)*	1 *(0 – 4)*	0 *(0 – 3)*	0 *(0 – 1)*	0 *(0 – 1)*
CD56+	0 *(0 - 21)*	0 *(0 – 4)*	0 *(0 - 5)*	12 *(1 – 46)*	13 *(1 – 40)*	17 *(0 – 120)*	3 *(0 - 11)*	3 *(0 – 16)*
Normotensive								
CD3+	6 *(0 – 47)*	11 *(0 – 35)*	17 *(0 – 74)*	44 *(9 – 116)*	63 *(3 – 146)*	51 *(5 – 136)*	12 *(5 – 34)*	17 *(5 – 29)*
CD8+	1 *(0 – 23)*	5 *(0 - 16)*	2 *(0 – 40)*	11 *(2 – 48)*	27 *(0 – 97)*	15 *(1 – 65)*	4 *(0 – 24)*	7 *(1 – 18)*
CD3+CD8-	4 *(0 - 37)*	8 *(0 – 30)*	15 *(0 – 51)*	34 *(0– 78)*	29 *(1 – 52)*	28 *(4 – 88)*	7 *(1 – 22)*	9 *(2 – 16)*
FoxP3+	0 *(0 – 2)*	0 *(0 – 0)*	0 *(0 – 0)*	1 *(0 – 5)*	0 *(0 – 6)*	0 *(0 – 6)*	0 *(0 – 1)*	1 *(0 – 5)*
CD56+	1 *(0 - 16)*	0 *(0 – 2)*	0 *(0 – 3)*	15 *(0 – 72)*	17 *(6 – 36)*	18 *(3 – 69)*	3 *(0 – 11)*	2 *(0 – 14)*

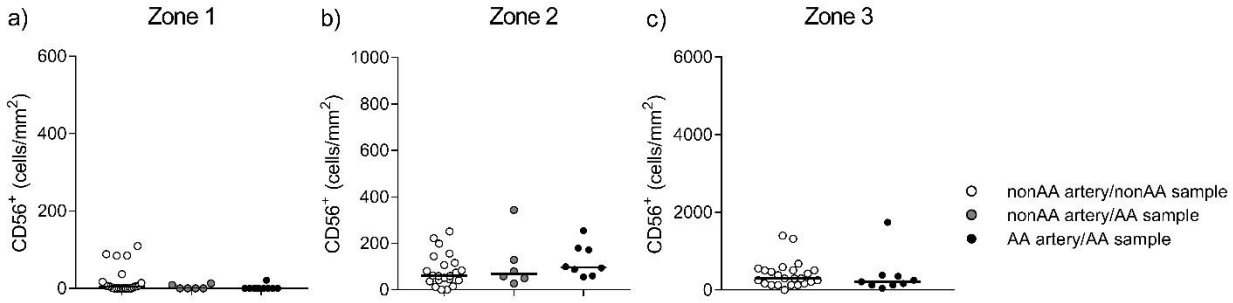
Supplemental Figure 1. Example of staining of serial sections of decidua basalis tissue to identify spiral arteries and AA. Slides were stained with (from left to right) desmin+PAS, CK7+PAS, CD68+PAS. The top row shows representative images of a spiral artery from a PE patient without AA (nonAA artery/ nonAA sample) characterized by: a) presence of CK7-positive trophoblasts and intramural fibrinoid (bright purple upon PAS staining) in the vessel wall, b) complete absence of intramural smooth muscle cells (no desmin stain), and c) absence of intramural CD68-positive foam cells. The bottom row shows representative images of a spiral artery from a PE patient with AA (AA artery/ AA sample) characterized by: c) absence of CK7-positive trophoblasts and normal intramural fibrinoid and presence of fibrinoid necrosis (visible as a grey-pink material) in the vessel wall, d) absence of intramural smooth muscle cells (no desmin stain), and e) presence of intramural CD68-positive foam cells.



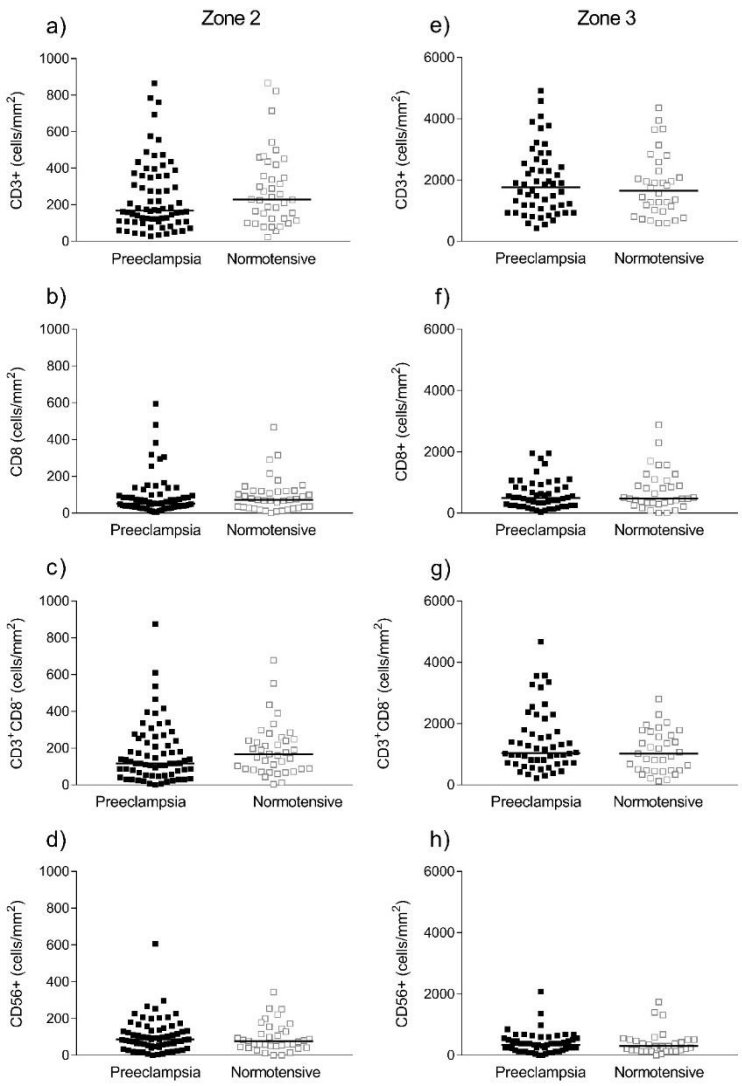
Supplemental Figure 2. T-cell concentration in the pre-defined Zones 1-3 in normotensive pregnancies.
Dot plots of median T-cell concentrations per patient in the pre-defined counting zones. Each plot shows the T-cell concentration in the three categories of spiral arteries: 1) arteries without AA in samples without AA (nonAA artery/nonAA sample, white dots), 2) arteries without AA in a sample with other arteries affected by AA (nonAA artery/AA sample, grey dots), and finally 3) arteries with AA lesions (AA artery/AA sample, black dots). The different plots show: a) CD3+ cells, b) CD8+ cells, and c) CD3+CD8- cells (assumed to be CD4+ cells) in Zone 1(intramural area). d) CD3+ cells, e) CD8+ cells, and f) CD3+CD8- cells (assumed to be CD4+ cells) in Zone 2 (perivascular area). g) CD3+ cells, h) CD8+ cells, and i) CD3+CD8- cells (assumed to be CD4+ cells) in Zone 3 (interstitial infiltrate area). The p-values were calculated using the Mann Whitney test, a p-value <.05 was considered statistically significant.



Supplemental Figure 3. NK-cell concentration in the pre-defined Zones 1-3 in normotensive pregnancies.
Dot plots of median NK-cell concentrations per patient in the pre-defined counting zones. Each plot shows the NK-cell concentration in three categories of spiral arteries: 1) arteries without AA in samples without AA (nonAA artery/nonAA sample, white dots), 2) arteries without AA in a sample with other arteries affected by AA (nonAA artery/AA sample, grey dots), and finally 3) arteries with AA lesions (AA artery/AA sample, black dots). The different plots show: a) CD56+ cells in Zone 1(intramural area), b) CD56+ cells in Zone 2 (perivascular area), and CD56+ cells in Zone 3 (interstitial infiltrate area). The p-values were calculated using the Mann Whitney test, a p-value <.05 was considered statistically significant.



Supplemental Figure 4. T-cell and NK-cell concentrations in preeclamptic as compared to normotensive pregnancies in Zone 2 and 3, regardless of the presence or absence of AA. Zone 2: a) CD3+, b) CD8+, c) CD3+CD8- d) CD56+ and Zone 3: e) CD3+, f) CD8+, g) CD3+CD8- h) CD56+. The p-values were calculated using Mann Whitney U-test, a p-value <.05 was considered statistically significant.



References

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