ONLINE SUPPLEMENT

Maternal angiotensin increases placental leptin in early gestation via an alternative RAS-pathway - suggesting a link to preeclampsia

Short title

Maternal AngIV and leptin in preeclampsia

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Methods

Sample Collection: First Trimester placental tissue

Placental tissue was collected from electively terminated gravidities with informed consent of healthy patients (with GA 5 – 11 weeks). Exclusion criteria were a maternal age under 18, maternal BMI >25, maternal pathologies (self-reported). Ethical approval was obtained from the Medical University of Graz Ethics Committee (31-019 ex 18/19; 26-132 ex 13/14).

Sample Collection: Term Control Tissue

Term samples were collected in the University Hospital of the RWTH Aachen, Germany. Sampling was approved by the local ethical committee (EK 148/07) and informed consent was obtained from each participating woman. Immediately after delivery, a random tissue sample (1x1x1cm) of the medial third of the placenta was cut from vital cotyledons that were macroscopically free of infarct areas or other obvious pathologies. Amnion and decidua were dissected and remaining tissue were rinsed twice in NaCl 0.9% 4°C to remove blood and then snap frozen in liquid nitrogen and stored at -80°C until processing.

Sample Collection: Serum collection from patients undergoing assisted reproductive technology (ART)

This prospective observational study was performed at a single academic tertiary hospital (Department of Obstetrics and Gynecology, Medical University of Graz, Austria) between September 2019 and January 2020. The study was approved by the institutional review board (Ethics committee at the Medical University of Graz, Austria; 30-514 ex 17/18). Patients were invited to participate if they were 18 years or older and presented with an indication for in vitro fertilization. Eligible patients were approached by members of the research team when they arrived at the outpatient clinic. Written informed consent was obtained from all participants.

Sample Collection: Preeclamptic placentae from Graz

Study samples were recruited retrospectively immediately after delivery at the inpatient clinic of the Department of Obstetrics and Gynecology, University Hospital Graz, Austria between 2018 and 2019. Preeclampsia was defined according to the ISSHP guidelines (Brown MA, Pregnancy Hypertension, 2018). 27 patients (41%) received prophylactic low dose aspirin, as a result of the first trimester preeclampsia screening test or because of risk factors such as history of preeclampsia in a previous pregnancy. Low dose aspirin was started before the 16th week gestational age, as recommended. The study was approved by the local Ethics committee at the Medical University of Graz (26-132 ex 13/14) and informed consent was obtained from each participating woman.

Sample Collection: Preeclamptic placentae from Oslo

Pregnant women were recruited prior to elective caesarian section after informed written consent, as previously described[1], from women with either preeclamptic (or normotensive pregnancies. Preeclampsia was defined as new onset hypertension (blood pressure ≥140/90 mmHg) and new onset proteinuria (≥1+ on dipstick, or ≥30 protein/creatinine ratio) at ≥20 weeks gestation. Early-onset preeclampsia was defined as delivery prior to gestational week 34. Placental villous tissue biopsies were cut from the center of central normal appearing cotyledons, and were snap frozen in liquid nitrogen and stored at −80°C until use. 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.

Sample Collection: Early gestation low and high-risk population

Determination of uterine artery resistance indices (RI) was performed in women attending a clinic for termination of pregnancy in the first trimester, as previously described[2] at St. George's Hospital (London, UK). Ethical committee approval and full written consent were obtained (reference, 01.96.8 and 01.78.5). Inclusion criteria were singleton pregnancy, GA of 9 to 14 weeks by crown-rump length (assigned by transvaginal measurement in accordance with local unit clinical policy), normal fetal anatomy, and nuchal translucency with no known

maternal medical condition or history of recurrent miscarriage. High-resistance cases were defined as a mean RI >95th percentile with bilateral diastolic notches. Normal-resistance cases had a mean RI of <95th percentile. Previous studies have determined that cases with UtAD RI >95th percentile in the first trimester had a 5-fold higher risk of developing PE compared to cases with UtAD RI <95th percentile[3]. Tissue obtained from first-trimester surgical terminations of pregnancy was collected and rinsed in ice-cold phosphate-buffered saline. Placental villous tissue was separated from the decidua by blunt dissection and was randomly sampled and divided. Approximately 100 mg of tissue was snap frozen in liquid nitrogen in two to three aliquots and stored at −70°C until use.

Cell Culture

For treatments, culture medium was supplemented with Angiotensin II at a working concentration of 0.1 µM. Candesartan was used at 0.1 µM, while DMSO at the same volume served as solvent control. Cells or placental explants were treated with 0.1µM AngII (Sigma-Aldrich, Merck, Vienna, Austria) for indicated timepoints. Recombinant human Leptin (Gibco® Life Technologies) was added to the medium with a concentration of 100 ng/ml. AngIV (Sigma-Aldrich, Merck, Vienna, Austria) was used at 4 nmol working concentrations for the timepoints indicated.

Viability Assay

To test for possible cytotoxicity of the compounds used for treatment, the lactate dehydrogenase (LDH) release was analyzed. Culture medium supernatants of the cells were centrifuged at 2000rpm for 5min and LDH release determined with an LDH cytotoxicity detection kit (TaKaRa Clontech) according to the manufacturer's protocol, measured at 490nm with SparkTM 10M (Tecan). Positive high controls were cells treated with Triton X-100 (molecular SIGMA biology, cell culture medium background and low untreated controls were used to test for cytotoxicity of each of the compounds' highest concentration and longest time point as used in cell culture protocols.

RNA isolation and RT-qPCR

Placental tissue was homogenised in RNA Lysis Buffer (AllPrep DNA/RNA/Protein Mini, Qiagen, Austin, Texas) using an UltraTurrax (IKA) and cells were lysed in RNA Lysis Buffer (peqlab, VWR International, Avantor, Darmstadt, Germany) using cellscraper. RNA was isolated according to the manufacturer's instructions (peqlab, VWR International, Avantor, Darmstadt, Germany or AllPrep DNA/RNA/Protein Mini, Qiagen, Austin, Texas). RNA quality was determined using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Quality check was followed by reverse transcription of 1µg total RNA per reaction using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's manual. qPCR was performed with Blue S'Green qPCR Kit (Biozym, CityVienna, Austria) using a Bio-Rad CFX96 cycler and specific primers for RAS components, and fetal sex determination, (see below in Table S1). Ct values and relative quantification of gene expression were automatically generated by the CFX Manager 3.1 Software (Bio-Rad Laboratories; Hercules, CA, USA) using the expression of first trimester placenta specific reference genes TBP, HPRT1, 18s as a reference chosen by their calculated M-Value.

In situ mRNA detection based on padlock probe technology

In situ padlock probe procedures were performed as described previously [4]. In short, the target-mRNA is reverse transcribed and linear oligonucleotides (ie padlock probes) bind with their 5´- and 3´parts to the complementary cDNA sequencing forming a circular DNA structure. After ligation, this circular DNA structure is amplified forming micron sized DNA structures which can be targeted by fluorescent probes[5]. For oligonucleotide sequences see Table S2.

Protein isolation and immunoblotting

Placental tissue was homogenized in Lysis Buffer (AllPrep DNA/RNA/Protein Mini, Qiagen, Austin, Texas) using an UltraTurrax (IKA) and protein was isolated according to the manufacturer's instructions (AllPrep DNA/RNA/Protein Mini, Qiagen, Austin, Texas).

Cells were washed with PBS and lysed in RIPA buffer (Sigma-Aldrich, Saint Louis, MO, USA) including protease inhibitor cocktail (Roche Diagnostics; Mannheim, Germany). Cell lysates were centrifuged at 8000×g and 4 °C for 10 min. The concentration of total tissue protein was determined in clear supernatants according to the Lowry method. Additionally, 30 µg total protein were applied to precast 10% Bis-Tris gels (NuPAGE, Novex, Life Technologies). Blotting on a 0.45 µm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK) was followed by analysis of blotting efficiency by Ponceau staining (Ponceau S solution, Sigma Aldrich). Membranes were cut in horizontal strips at molecular weight ranges for target proteins. Various primary antibodies for target proteins were used at tested concentrations (see Table S3) and as reference for normalization monoclonal anti-beta actin antibody (12.4 ng/mL, clone AC-15, abcam, Cambridge, UK) were applied to membrane strips overnight at 4 °C. HRP conjugated rabbit anti-goat (1:3000, Dako) and anti-mouse IgG (1:3000, Bio-Rad), respectively, were used as secondary antibodies and incubated on membranes for 2h at RT. Immunodetection was performed with a chemiluminescent immunodetection kit (Western Bright chemiluminescence Substrate Quantus, Biozym, Austria) according to the manufacturer´s instructions. Images were acquired with FluorChem Q System (Alpha Innotech, Cell Bioscienes, Santa Clara, CA, USA). Band densitometry was performed using Image Studio quantification software (Image Studio™ Lite Software version, Li-Cor Biosciences, Germany).

Immunofluorescence Staining

Formalin fixed and paraffin embedded (FFPE) first trimester and term placenta sections were deparaffinized and subsequently labelled with primary antibodies (see Table S3) and secondary goat anti-mouse or anti-rabbit Alexa Fluor 555/633/488 (1:200, Invitrogen, Carlsbad; CA, USA) for 30 min each. After three washing steps in PBS and a 10-min blocking step with irrelevant mouse IgG1 (10 µg/ml, DakoCytomation) the sections were labelled with FITC conjugated anti-keratin antibody (Dako, Denmark). Nuclei are stained with DAPI by mounting sections with Vectashield (Vector Laoratories, Burlingame, CA, USA).

FACS Analysis

BeWo cell experiments were analysed with the BD FACSCanto II flow cytometer and BD FACSDiva software (BD Bioscience). Data analysis was performed with FlowJo v.10 (FlowJo LLC).

Serum Angiotensin Measurement

Serum conditioning for equilibrium analysis was performed at 37 °C followed by stabilization through the addition of an enzyme inhibitor cocktail (Attoquant Diagnostics, Vienna, Austria) as described previously[6]. The samples then underwent C-18-based solid-phase-extraction and were subjected to LC-MS/MS analysis using a reversed-phase analytical column operating in line with a Xevo TQ-S triple quadruple mass spectrometer (Waters). Previous results have shown similar qualitative outcomes when comparing the quantification of circulating (stabilized immediately at blood drawing) and equilibrium angiotensin peptide levels[6]. Stabilized equilibrated serum samples were further spiked with stable isotope labelled internal standards for each angiotensin metabolite at a concentration of 200 pg/ml. Internal standards were used to correct for peptide and steroid recovery of the sample preparation procedure for each analyte in each individual sample. Analyte concentrations were reported in pM and are calculated considering the corresponding response factors determined in appropriate calibration curves, on condition that integrated signals exceeded a signal-to-noise ratio of 10.

Overrepresentation Analysis

Differentially expressed genes were examined with the functional enrichment analysis web tool WebGestalt (WEB-based Gene SeT AnaLysis Toolkit)[7,8].

References

1. Herse F, Dechend R, Harsem NK, Wallukat G, Janke J, Qadri F, Hering L, Muller DN, Luft FC, Staff AC. Dysregulation of the circulating and tissue-based renin-angiotensin system in preeclampsia. In: *Hypertension*. ; 2007. doi:10.1161/01.HYP.0000257797.49289.71

2. Prefumo F, Sebire NJ, Thilaganathan B. Decreased endovascular trophoblast invasion in first trimester pregnancies with high-resistance uterine artery Doppler indices. *Hum Reprod*. Published online 2004. doi:10.1093/humrep/deh037

3. Leslie K, Whitley GSJ, Herse F, Dechend R, Ashton S V., Laing K, Thilaganathan B, Cartwright JE. Increased Apoptosis, Altered Oxygen Signaling, and Antioxidant Defenses in First-Trimester Pregnancies with High-Resistance Uterine Artery Blood Flow. *Am J Pathol*. Published online 2015. doi:10.1016/j.ajpath.2015.06.020

4. El-Heliebi A, Kashofer K, Fuchs J, Jahn SW, Viertler C, Matak A, Sedlmayr P, Hoefler G. Visualization of tumor heterogeneity by in situ padlock probe technology in colorectal cancer. *Histochem Cell Biol*. Published online 2017. doi:10.1007/s00418-017-1557-5

5. Weibrecht I, Lundin E, Kiflemariam S, Mignardi M, Grundberg I, Larsson C, Koos B, Nilsson M, Söderberg O. In situ detection of individual mRNA molecules and protein complexes or post-translational modifications using padlock probes combined with the in situ proximity ligation assay. *Nat Protoc*. 2013;8(2):355-372. doi:10.1038/nprot.2013.006

6. Basu R, Poglitsch M, Yogasundaram H, Thomas J, Rowe BH, Oudit GY. Roles of Angiotensin Peptides and Recombinant Human ACE2 in Heart Failure. *J Am Coll Cardiol*. Published online 2017. doi:10.1016/j.jacc.2016.11.064

7. Zhang B, Kirov S, Snoddy J. WebGestalt: An integrated system for exploring gene sets in various biological contexts. *Nucleic Acids Res*. Published online 2005.

doi:10.1093/nar/gki475

8. Liao Y, Wang J, Jaehnig EJ, Shi Z, Zhang B. WebGestalt 2019: gene set analysis toolkit with revamped UIs and APIs. *Nucleic Acids Res*. Published online 2019. doi:10.1093/nar/gkz401

9. Vento-Tormo R, Efremova M, Botting RA, Turco MY, Vento-Tormo M, Meyer KB, Park JE, Stephenson E, Polański K, Goncalves A, et al. Single-cell reconstruction of the early maternal–fetal interface in humans. *Nature*. Published online 2018. doi:10.1038/s41586-018-0698-6

Supplemental Tables and Figures

Table S1. Primer Sequences

Gene	*Forward*	*Reverse*
TBP	TGACCCAGCATCACTGTTTC	CCAGCACACTCTTCTCAG CA
LEP	GTGCGGATTCTTGTGGCTTT	AGGAGACTGACTGCGTGT GT
LEPR	CGTTAAAGCTCTCGTGGCAT	AATCCTCTAAGGCACATCC CAG
LNPEP	AGGGATGAGCAATACACCGC	CCCACAATGAAAGCAACCAA G
MAS1	GTGGGGTTTGTTGAGAATGG G	GCGATAGACAGGTGGGTGAT G
ACE2	TGGTGGGAGATGAAGCGAGA	AACAGAGATGCGGGGTCAC A
ACE	GTGGAACGAGTATGCCGAGG	GTGGAACGAGTATGCCGAG G
AGT	CTATCTCCCCGGACCATCCA	ACTCTGTGGGCTCTCTCTCA
REN	GAGGCTGTTTGATTATGTCGT GA	TGGTGAGCGTGTATTCTTTG C
ENPEP	GTGACAACCCCTGATGAAATA AC	AGCCTACTTGCCTCTTCCAG
AGTR2	GATCTGGTGCTATTACGTCCC	GCCTAAACACACTCCTTCAA AA
AGTR1	CTATGGAATACCGCTGGCCC	TGCAGGTGACTTTGGCTACA
DDX3Y	AGTAGAGGCAACCGGCAGTA	TGCACTGGAGTAGGACGAGT A
XIST	GACACAAGGCCAACGACCTA	TCGCTTGGGTCCTCTATCCA
DPP3	AAG CTG GAA CGG GTG ATC CTA	CCA GAG AGA ACA TAA GCT CCC C
RNPEP	AGT ACA ACG GGG TGA TAG AAG A	AGA CAA GGG TTC TCC ATT CCT

Table S2. Primer sequences of Padlock Probe Detection Sequence

Reverse transcription primer

name	sequence 5' → 3'
RV_AGTR1_1	GTGCAGGCTTCTTGGTGGAT
RV_AGTR1_2	GCGTGCTCATTTTTGTTG
RV_AGTR1_3	GGGAATCCAGGAAAAGAAAA
RV_AGTR1_4	GCCTTCTTTAGGGCCTTCCA
RV_AGTR1_5	GATCAGAAAAGGAAACAGG
RV_AGTR1_6	AATGATGATGCAGGTGAC
RV_AGTR1_7	CAAGCATTGTGCGTCGAAGG
RV_AGTR1_8	GAAACTGACGCTGGCTGAAG
RV_AGTR1_9	GGGCCAGCGGTATTCCATAG
RV_AGTR1_10	GCCAGTGCTAAATTCAA
RV_CGB_1	CTCAGCAGCAGCAAC
RV_CGB_2	AGCGGCTCCTTGGAT
RV_CGB_3	TTGACGGTGATGCACA
RV_CGB_4	TCATGGTGGGGCAGTAG
RV_CGB_5	GACTCGAAGCGCACA
RV_CGB_6	AGGTCAAGGGGTGGT
RV_CGB_7	TTTGAGGAAGAGGAGT

Padlock probe

plp_AGTR1_1	/5Phos/GTAAGCTCATCCACCAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCGGCTATCACCGCCCCTCAGATAAT
plp_AGTR1_2	/5Phos/CCACTCAAACCTTTCAACAAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCGCCTATCCCCCCAAAAGCCAAATC
plp_AGTR1_3	/5Phos/CTGGGTTTCCTGTTTAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCGTCTATCGGGCCTGACCAAAAATATA
plp_AGTR1_4	/5Phos/CAATGAAGTCCCGCCAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCGACTATCTGGCTATTGTTCACC
plp_AGTR1_5	/5Phos/GCCAGTGTTTTTCTTAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCTGCTATCATGAAGCTGAAGACTGTG
plp_CGB_1	/5Phos/CTGCTGCTGTTGCTAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCCACTATCGAGATGTTCCAGGGG
plp_CGB_2	/5Phos/GGCTGTGGAGAAGGAAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCAGCTATCCCATCAATGCCACCCT
plp_CGB_3	/5Phos/GCAACTACCGCGATGTAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCACCTATCTGCCTCAGGTGGTGT

plp_CGB_4	/5Phos/GCAGCACCACTGACTAACCTCAATGCACATGTTTGGCTCCAATGCGTCTATTTAGTGGAGCCATCTATCATGTGCACTCTGCCGCC

Detection probes

Lin33	Cy3 - CCTCAATGCACATGTTTGGCTCC

/5Phos/ indicates phosphorylation on the 5’-end of the padlock probes; Hybridization arms which are complement to the target cDNA sequence are underlined; reporter sequences for the detection probe is highlighted in red and the 5’-end of the detection probe is Cy3 labeled

Table S3. List of Antibodies

Antibody		***Species***	***Distributor***
Cytokeratin-7	OV-TL (1352 P)	rabbit	Thermo Scientific
β-HCG	RB-059-A	rabbit	Neomarkers Thermo Scientific
CD31	Ab18364	rabbit	Abcam
CD34	M7165	mouse	Dako
Leptin	sc-48408	mouse	Santa Cruz
β-actin	AC-15	mouse	abcam
LNPEP (IRAP)	sc-365300	Mouse	Santa Cruz

Table S4. Overrepresentation Analysis of Array Data

Gene Set	Description	Size	Expect	Ratio	P-value
GO:0001659	temperature homeostasis	7	1.1235	3.5604	0.011182
GO:0007565	female pregnancy	7	1.1235	3.5604	0.011182
GO:0009653	anatomical structure morphogenesis	45	7.2222	1.5231	0.019988
GO:0032386	regulation of intracellular transport	8	1.2840	3.1154	0.020234
GO:0044706	multi-multicellular organism process	8	1.2840	3.1154	0.020234
GO:0106106	cold-induced thermogenesis	5	0.80247	3.7385	0.027376
GO:0120161	regulation of cold-induced thermogenesis	5	0.80247	3.7385	0.027376
GO:1990845	adaptive thermogenesis	5	0.80247	3.7385	0.027376
GO:0033157	regulation of intracellular protein transport	5	0.80247	3.7385	0.027376
GO:0046822	regulation of nucleocytoplasmic transport	5	0.80247	3.7385	0.027376

Table S5. Baseline characteristics of control population

Two independent study populations are shown. First trimester placental tissue was collected from electively terminated gravidities with informed consent of healthy patients (with GA 5 – 11 weeks). Exclusion criteria were a maternal age under 18, maternal BMI >25, maternal pathologies (self-reported). Term samples were collected in the University Hospital of the RWTH Aachen, Germany. Sampling was approved by the local ethical committee (EK 148/07) and informed consent was obtained from each participating woman. Values are the mean and SD in brackets.

				First Trimester Control	**Third Trimester Control**
n				198	52
Fetal		**sex (f)**	*%*	58.5	44.1
		sex (m)	*%*	41.5	55.9
		CRL	*cm*	1.90 (4.5)	
		weight	*g*		2513 (987)
		percentile	*%*		45.1 (21.48)
		gestational age	*d*	54 (11.8)	245 (33.6)
Maternal		**age**	*years*	26.45 (6.15)	31.79 (5.43)
	prepregnancy	**BMI**	kg/m^2	-	23.87 (4.87)
	gestational	**weight**	*kg*	59.17 (12.04)	77.62 (14.66)
		BMI	kg/m^2	21.83 (2.86)	28.14 (5.21)
	prepregnancy	**smoking**	*%*	-	75
		non-smoking	*%*	-	25
	In pregnancy	**smoking**	*%*	47.1	11.8
		non-smoking	*%*	52.5	88.2
	Placental	**volume**	cm^3	1.72 (1.47)	
	Birth Mode	**SVD**	*%*	-	20.6
		prim CS	*%*	-	41.2
		sec CS	*%*	-	36.8
		VE	%	-	1.5

Table S6. Baseline characteristics of ART patients

Gestational age	*d*	48.4 (3.5)
Height	*m*	163.3 (4.8)
Weight	*kg*	58.9 (15.6)
BMI	kg/m_2	22.0 (4.9)
Smoking	*n*	2
Non-Smoking	*n*	8

n=10, values shown as mean (SD)

Table S7. Baseline characteristics of PE study population Graz

Patients were treated with aspirin or not (low dose aspirin daily starting from before the 16th gestational week); in a linear regression model significant predictors of placental LNPEP mRNA expression were aspirin treatment and fetal sex, whereby aspirin intake in control groups predicted higher LNPEP levels compared to PE or control without low dose aspirin groups, and females had lower LNPEP mRNA expression ($p<.05$). PE-preeclamsia; eoPE: early-onset PE (delivery <34 gestational weeks); loPE: late-onset PE (delivery ≥34 gestational weeks). Values shown as mean (SD).

			ctrl	**PE**	***eoPE***	***loPE***
n			15	27	12	15
Low dose aspirin	**+**	*[n(%)]*	6 (40)	9 (55.6)	7 (58.3)	5 (53.3)
	-	*[n(%)]*	9 (60)	15 (33.3)	4 (33.3)	8 (33.3)
Birth Mode	**Prim. CS**	*[n(%)]*	8 (53.3)	23 (85.2)	11 (91.7)	12 (80)
	Sec. CS	*[n(%)]*	3 (20)	2 (7.4)	1 (8.3)	1 (6.7)
	SVD	*[n(%)]*	3 (20)	2 (7.4)	0 (0)	2 (13.3)
	VE	*[n(%)]*	1 (6.7)	0 (0)	0 (0)	0(0)
Fetal Sex	**male**	*[n(%)]*	11 (73.3)	13 (48.1)	8 (66.7)	5 (33.3)
	female	*[n(%)]*	4 (26.7)	14 (59.1)	4 (33.3)	10 (66.7)
Gestational Age		*[days (mean(SD)]*	255.6 (18.95)	237.6 (21.33)	217.4 (12.6)	253.8 (9.5)
Placental weight		*[g (mean(SD)]*	544.0 (169.9)	406.2 (171.2)	296.4 (57.0)	499.2 (181.8)
Fetal	**weight**	*[g (mean(SD)]*	2759.1 (644.0)	1879.2 (632.0)	1340.2 (345.6)	2341.3 (411.0)
	length	*[cm (mean(SD)]*	46.54 (4.2)	43.64 (3.7)	40.1 (2.5)	45.64 (14.6)
Maternal	**BMI prepregnancy**	*[kg/m_2 (mean(SD)]*	25.91 (4.7)	25.93 (5.8)	24.75 (4.5)	26.94 (6.8)
	weight prepregnancy	*[kg (mean(SD)]*	69.7 (14.2)	71.7 (18.7)	69.4 (16.5)	73.6 (20.7)
	weight at delivery	[kg (mean(SD)]	83.5 (16.5)	85.9 (21.2)	82.1 (15.8)	88.32 (24.3)

Table S8. Baseline characteristics of PE study population Oslo

PE-preeclamsia; eoPE: early-onset PE (delivery <34 gestational weeks); loPE: late-onset PE (delivery ≥34 gestational weeks). Values shown as mean (SD).

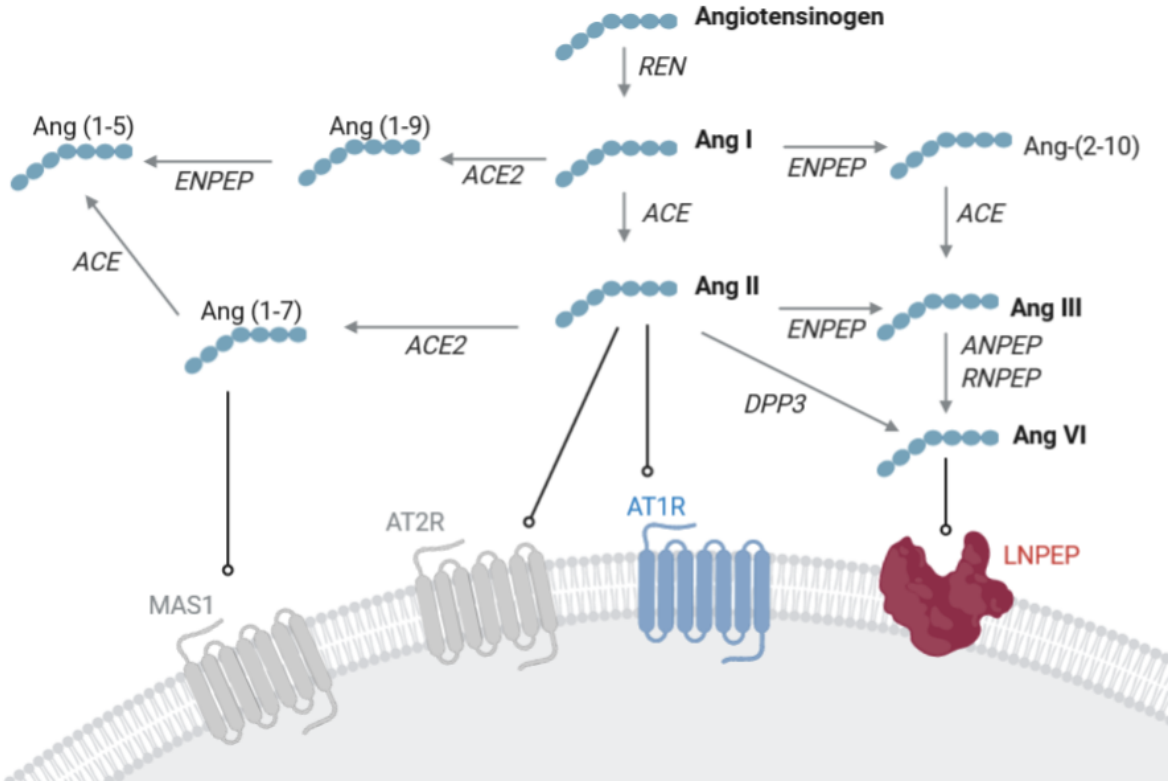
			ctrl	**PE**	***eoPE***	***loPE***
n			14	27	13	14
Fetal Sex	**male**	*[n(%)]*	8 (57.1)		6 (46.2)	8(57.1)
	female	*[n(%)]*	6 (42.9)		7 (53.8)	6(42.9)
Gestational Age		*[days (mean(SD)]*	276.4 (6.4)	234.2 (24.8)	213.2 (14.4)	253.6 (13.8)
Placental weight		*[g (mean(SD)]*	610.0 (124.9)†	400.0# (151.1)	314.7 (134.2) ¶	478.3 123.7)¶
Fetal	**weight**	*[g (mean(SD)]*	3613.1 (328.2)	2056.8 (902.6)	1311.0 (363.2)	2749.3 (659.6)
	length	*[cm (mean(SD)]*	48.1 (6.0) †	42.5 (6.4)§	39.3 (3.5)#	45.4 (7.2)#
Maternal	**BMI prepregnancy**	*[kg/m_2 (mean(SD)]*	22.4 (3.0)	25.0 (4.9)	25.4 (5.1)	24.6 (4.9)
	weight prepregnancy	*[kg (mean(SD)]*	63.5 (9.6)	69.4 (11.9)	69.9 (10.7)	68.9 (13.4)
	weight at delivery	[kg (mean(SD)]	78.9 (7.5)	85.5 (12.0)	84.6 (10.4)	86.3 (13.7)

Table S9. Baseline characteristics of study population with uterine artery resistance index (lower-risk vs. higher-risk)

		lower risk	**higher risk**
n		8	9
maternal age	*years*	25 (6.50)	29 (7.43)
mean RI		0.74 (0.11)	0.88 (0.03)
GA	*days*	73.3 (5.6)	72.9 (3.6)

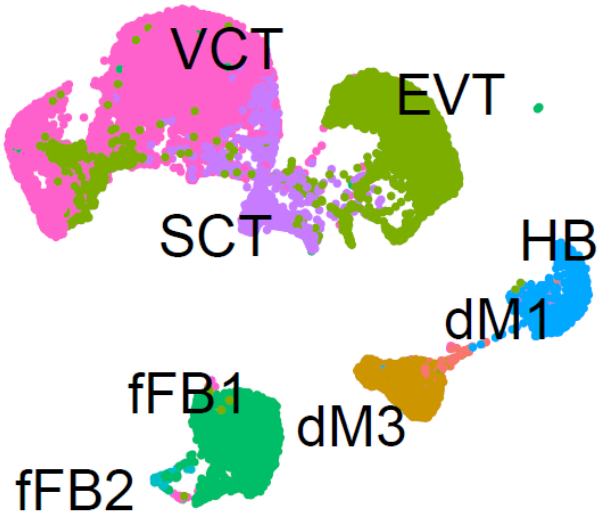
Values are shown in mean (SD).

Figure S1. Extended RAS



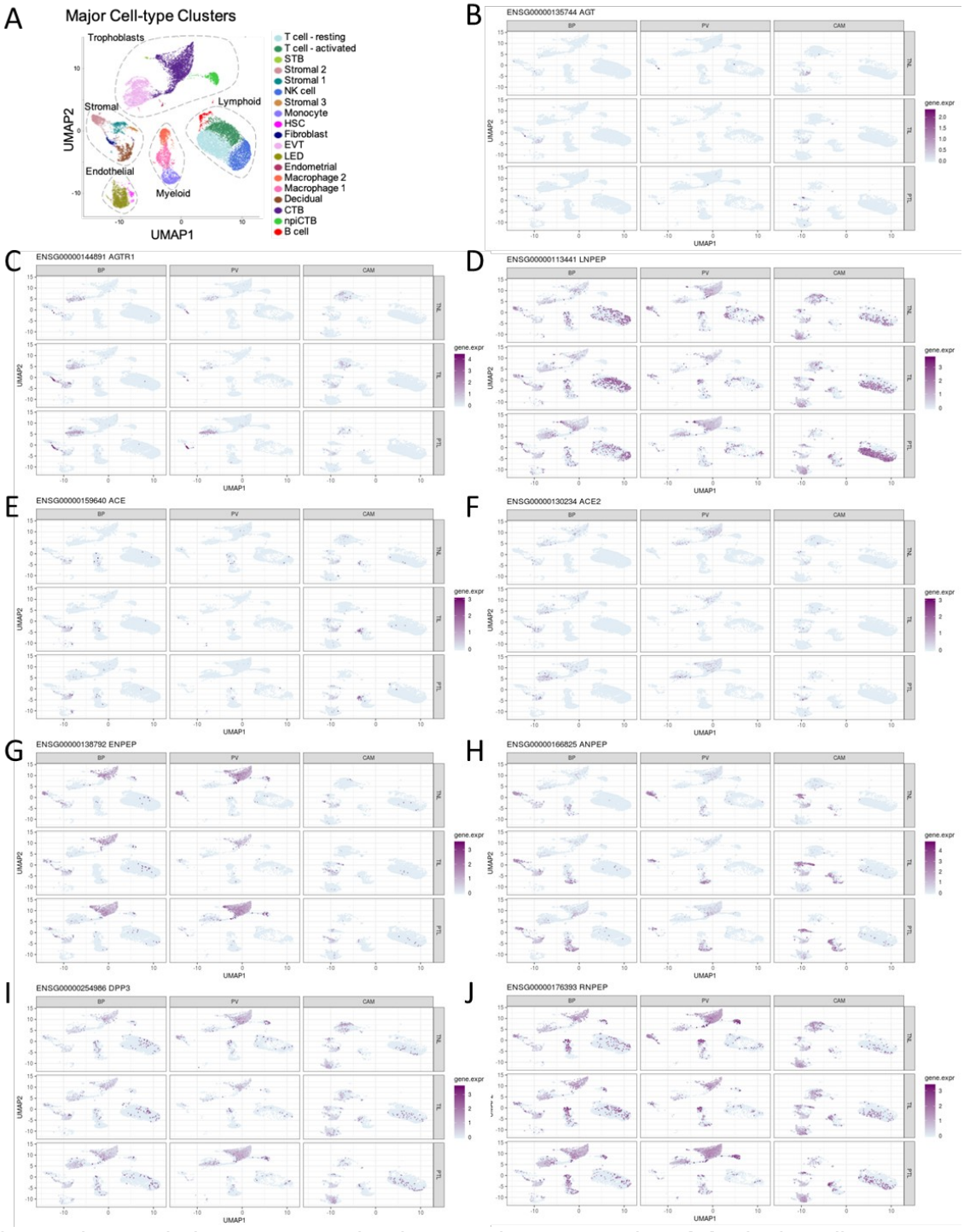
Extended RAS with multiple angiotensin conversion products (Ang = Angiotensin) and conversion enzymes (ACE and ACE2: angiotensin conversion enzyme; REN: renin; ANPEP: alanyl aminopeptidase; ENPEP: glutamyl aminopeptidase; RNPEP: arginyl aminopeptidase; DPP3: dipeptidyl peptidase 3; arrows show conversion) and receptors (AGTR1/2: angiotensin II receptor type 1 or 2; MAS1: MAS1 proto-oncogene G-protein coupled receptor; LNPEP: leucyl and cystinyl aminopeptidase = angiotensin IV receptor = placental leucine aminopeptidase; arrows with circular ends show ligand acting on receptor);
AngIII is a substrate for LNPEP and is degraded to AngIV (arrow not shown).

Figure S2. scRNA-seq data from first trimester placental tissues



UMAP and tSNE embeddings of scRNAseq data is shown with the cell type annotations used in expression heatmaps. Data for first trimester placenta was obtained from Vento-Tormo et.al, 2018 [9]; VCT: villous cytotrophoblast; SCT: syncytiotrophoblast; EVT: extravillous trophoblast; HB: Hofbauer cells; dM1 – dM3: decidual macrophages; fFB1-2: fetal fibroblast;

Figure S3. Extended RAS in scRNA-seq data from term and preterm samples



Placental extended RAS expression in control term samples. **(A)** Single cell RNA seq data from 25 scRNA-seq libraries published by Pique-Regi et al., 2019 who are kindly providing their data via http://placenta.grid.wayne.edu/ Pique-Regi et al., annotation used for samples obtained from chorioamniotic membrane (CAM), placental villi (PV), basal plate (BP). Extended RAS expression with **(B)** angiotensinogen AGT **(C)** angiotensin II receptor type 1 AGTR1 **(D)** leucyl and cystinyl aminopeptidase LNPEP and conversion enzymes (E) angiotensin conversion enzyme ACE **(F)** ACE2 **(G)** ENPEP: glutamyl aminopeptidase **(H)** ANPEP: alanyl aminopeptidase; **(I)** DPP3: dipeptidyl peptidase **(J)** RNPEP: arginyl aminopeptidase; not shown REN, MAS and AGTR2 (no placental expression or not found in data set)

Figure S4. RAS-associated components in scRNA-seq data

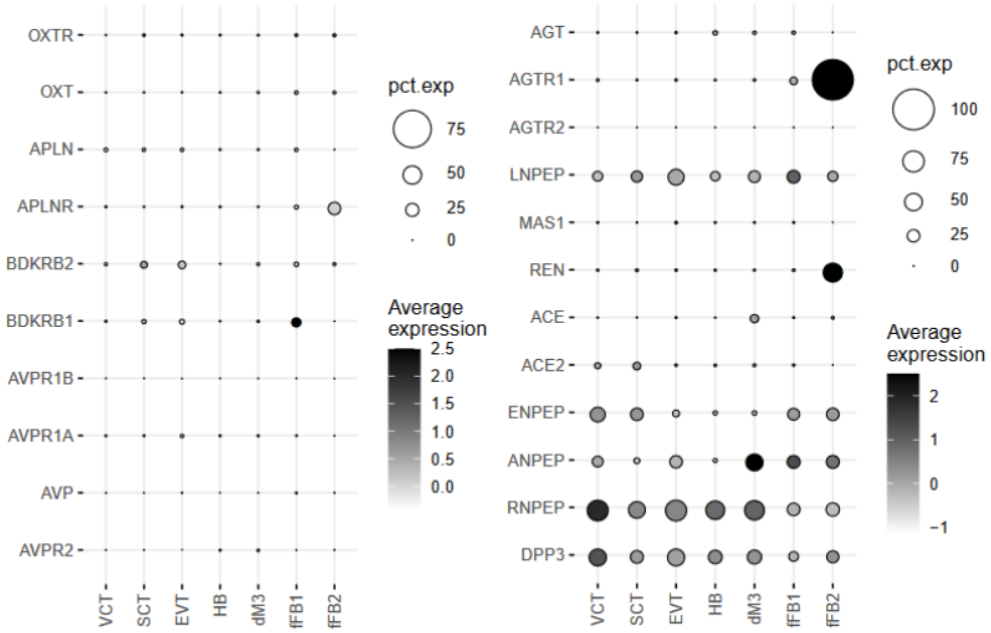
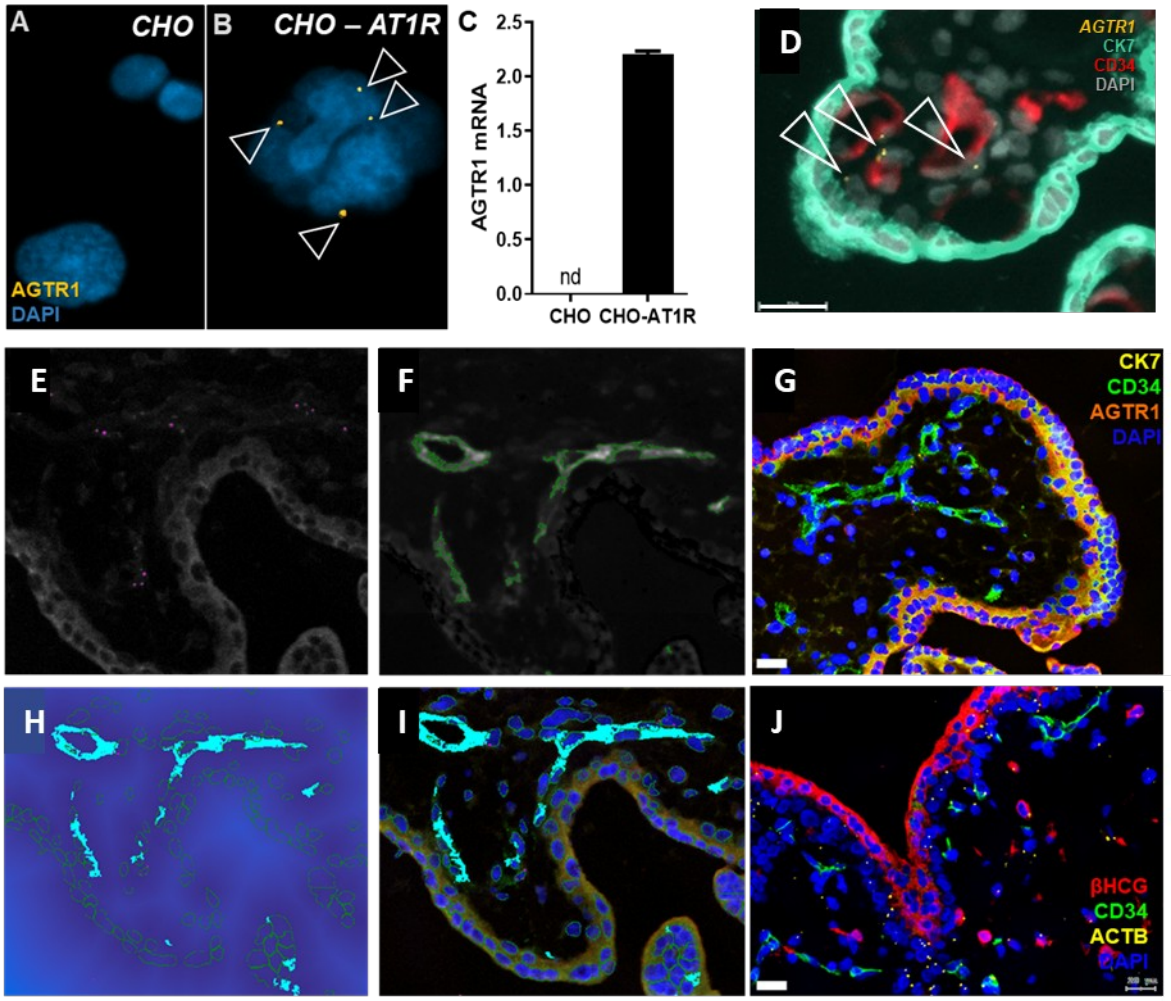


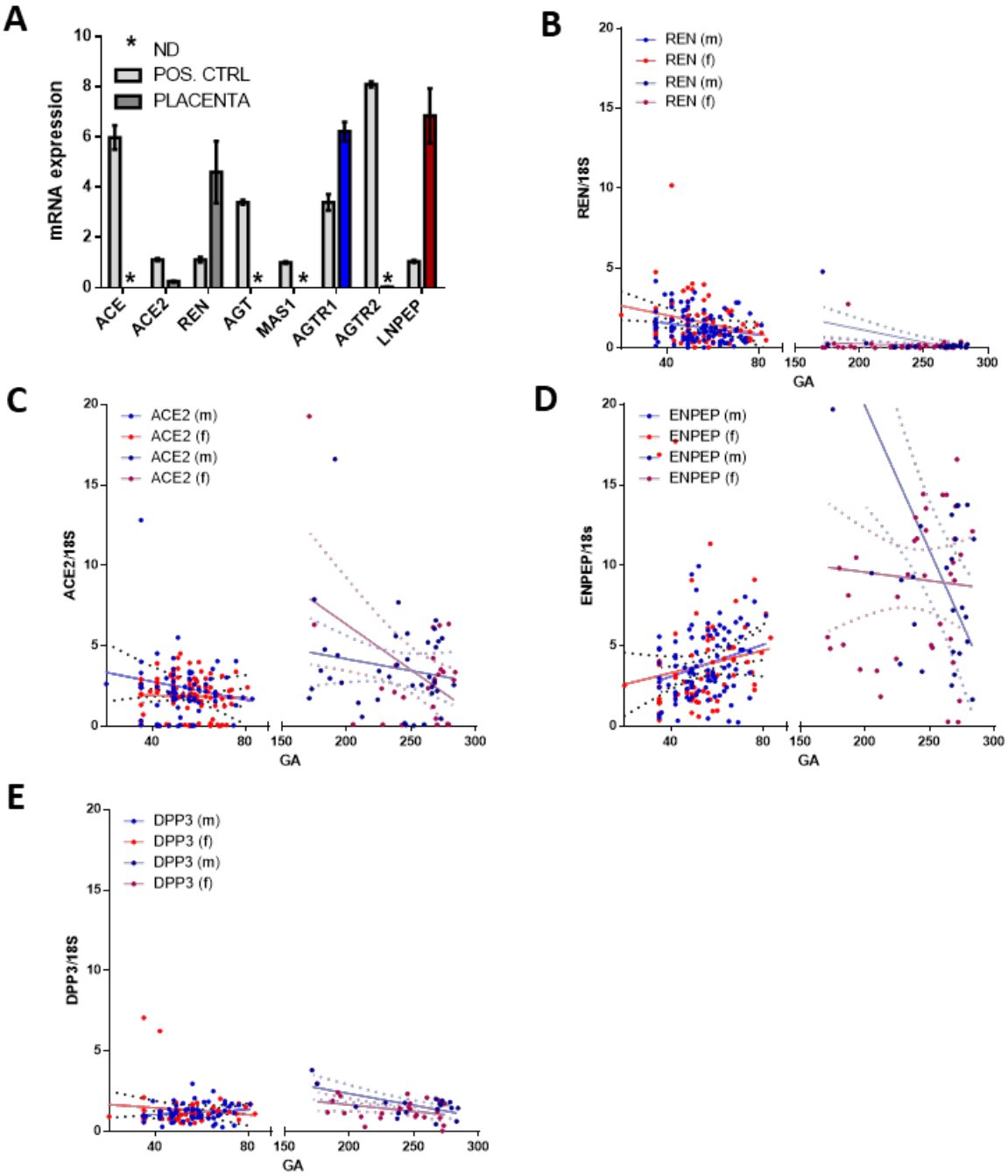
Figure from placental scRNA-seq data from Vento-Tormo et al, Nature 2018. Left-hand side: No RNA expression can be found on the maternal site for following genes and very low expression in foetal fibroblasts: OXTR = oxytocin receptor, OXT oxytocin, APLN apelin, APLNR apelin receptor, BDKRB1/2 bradykinin receptor B1 and B2, AVPR1A-1B-2 vasopressin receptor 1A – 1B – 2, AVP vasopressin. Cell types: VCT villous cytotrophoblast, SCT syncytiotrophoblast, EVT extravillous trophoblast, HB Hofbauer cells, dM3 Macrophages, fFB1/2 foetal fibroblasts. – On the right-hand side: see MAS1 as receptor to Ang1-7 lacking expression in placental villi.

Figure S5. In situ padlock probe mRNA detection and quantitative image analysis.



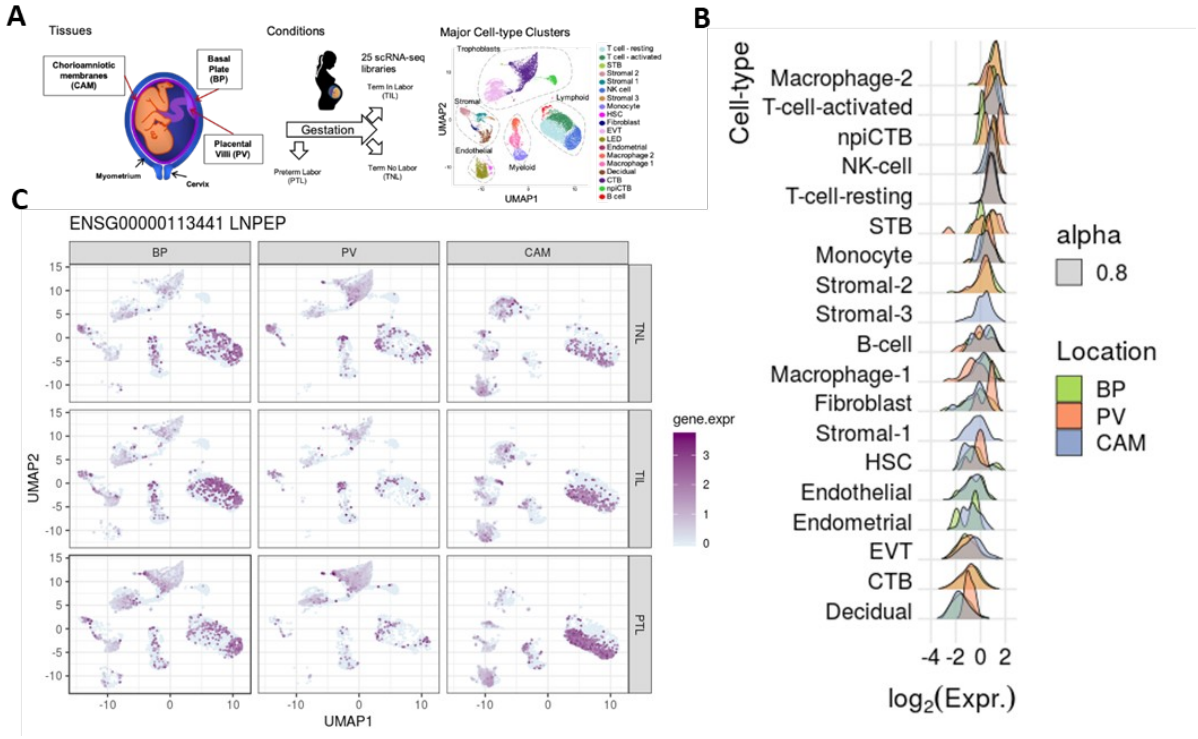
(A) CHO cells not expressing *AGTR1* as negative control for *AGTR1* padlock probes and **(B)** showing AGTR1 overexpressing CHO cells (CHO-AT1R) as positive control, with yellow dots representing rolling circle amplification products of *AGTR1* padlock probes with a Cy3 backbone **(C)** *AGTR1* expression in CHO and CHO-AT1R cells as validated by qPCR (norm. to *GAPDH*) **(D)** full four channel image showing *AGTR1* transcripts (arrows) **(E)** **S**ingle channel for autofluorescence correction, marking padlock probes signals in pink **(F)** Channel with CD34 staining, note the automatic vessel recognition based on fluorescence intensity thresholds **(G)** Full 4-channel immunofluorescence slidescan used to detect *AGTR1* transcripts **(H)** Vessels (light blue) against distance mappings; cell nuclei in green **(I)** Combined image of vessel areas, nuclei and immunofluorescence image **(J)** Part of slidescan used to calculate *ACTB* transcripts to vessel distance based on the automated analysis software settings.

Figure S6. Placental extended RAS expression



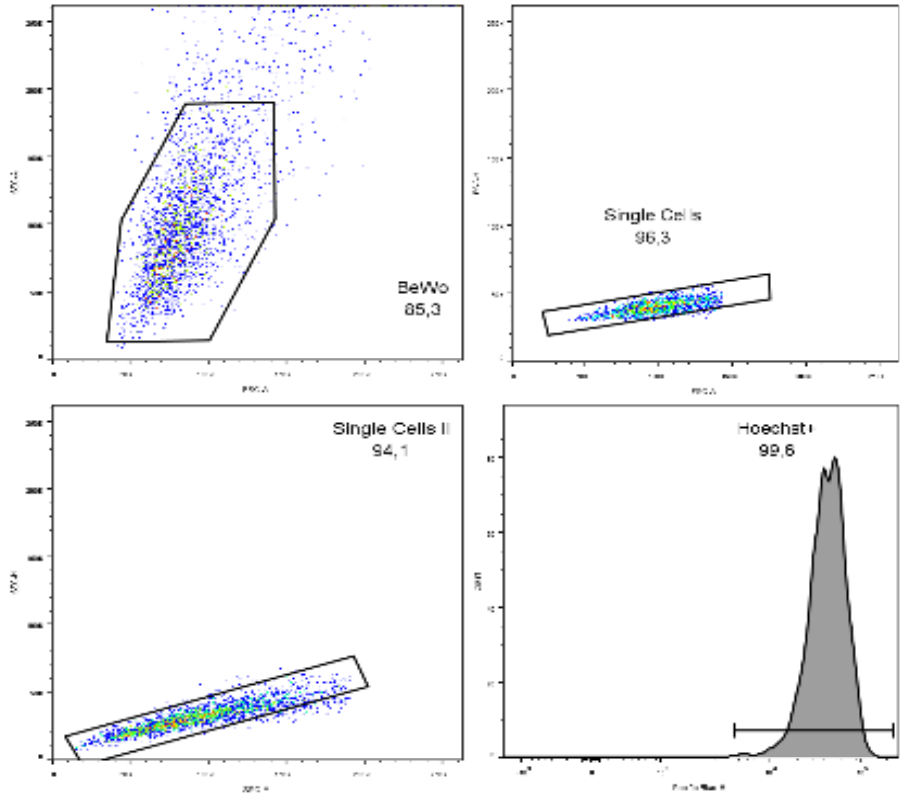
(A) First trimester placental RAS mRNA expression compared to positive controls: *MAS1*: Brain, *AGTR1*: Kidney, *AGTR2*: Lung; *LNPEP*: Brain **(B-E)** Expression of *REN, ACE2, ENPEP, DPP3* across gestation in healthy male (m) and female (f) control placentae (line represents linear regression and 95% confidence bands; n=252); Extended RAS with multiple angiotensin conversion enzymes (REN: renin; ACE2: angiotensin conversion enzyme 2; ENPEP: glutamyl aminopeptidase; DPP3: dipeptidyl peptidase.

Figure S7. Placental *LNPEP* is not altered in preterm labour compared to term deliveries.



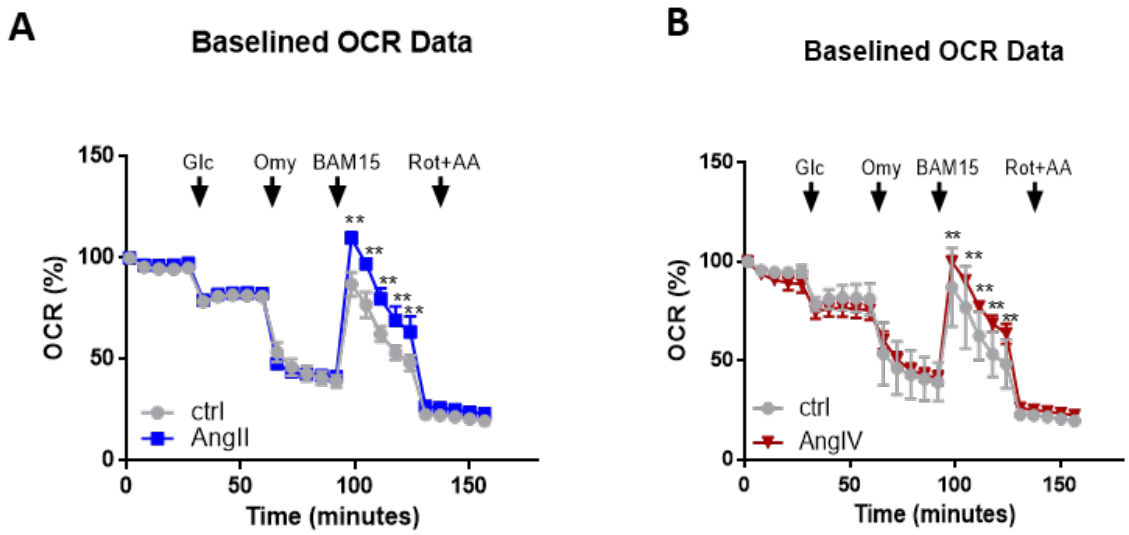
(A) Sampling and scRNA-sequencing chorioamniotic membranes (CAM), basal plate (BP) or placental villi (PV) from term no labor (TNL), term in labor (TIL) and preterm labor (PTL) was examined; Data is from 25 scRNA-seq libraries published by Pique-Regi et al., 2019 who are kindly providing their data via http://placenta.grid.wayne.edu/ **(B)** *LNPEP* expression in chorioamniotic membranes (CAM), basal plate (BP) or placental villi (PV) across all cell types was unchanged between term no labor (TNL), term in labor (TIL) and preterm labor (PTL) **(C)** Overlapping expression levels of *LNPEP* in major cell types (see A for cell type description, plots show UMAP projection of scRNA-seq data).

Figure S8. Gating strategy for normalizing Seahorse XF metabolic assays to cell number.



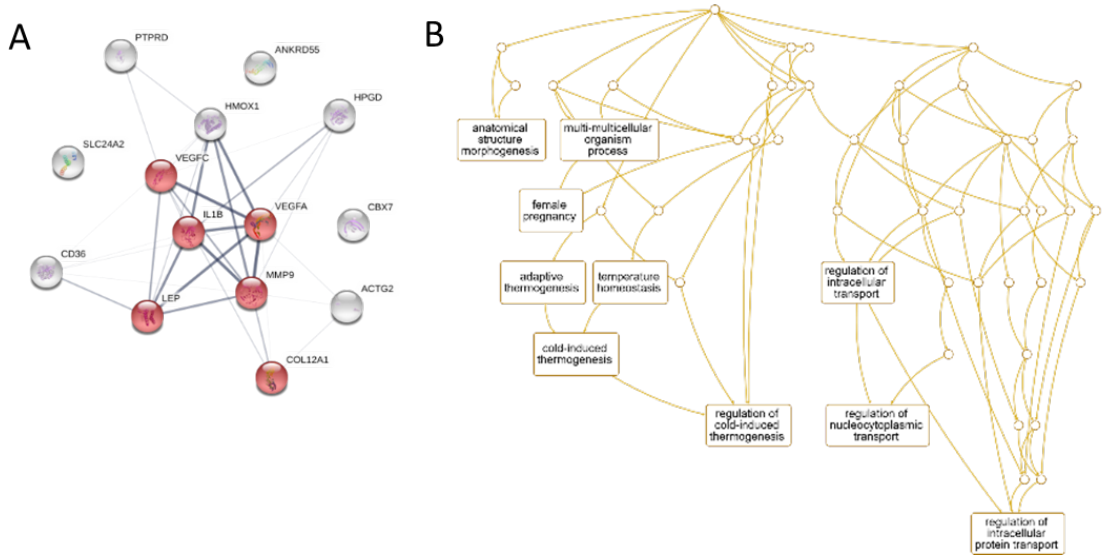
Cells were stained with Hoechst and only positive cells were sorted. BeWo cells from experiments were analyzed with the BD FACSCanto II flow cytometer and BD FACSDiva software (BD Bioscience). Data analysis was performed with FlowJo v.10 (FlowJo LLC).

Figure S9. Baselined data from Seahorse XF metabolic assays.



The data shows the significantly elevated max. OCR after BAM15 administration in **(A)** AngII and **(B)** AngIV pretreated samples to compensate the initially lower baseline OCR (n=3; sig<0.05).

Figure S10. Protein-protein interaction networks and overrepresentation analysis.



(A) Protein-protein interaction network; the nodes represent the significantly regulated genes from the array (p>0.05, regulation >1.5fold). Nodes colored in red are annotated to UniProt-Keyword „Secreted“ (KW-0964). Edge thickness shows increasing confidence levels (>0.15; PPI enrichment p=0.0023). **(B)** Over-Representation Analysis of significantly regulated genes, top GO terms include adaptive thermogenesis, female pregnancy and regulation of nucleocytoplasmic transport (enrichment categories Biological Process, p<0.059