Hypertension

PREECLAMPSIA

Maternal Angiotensin Increases Placental Leptin in Early Gestation via an Alternative Renin-Angiotensin System Pathway

Suggesting a Link to Preeclampsia

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ABSTRACT: Various studies found an association of different renin-angiotensin system (RAS) components with gestational duration and preterm birth, as well as with preeclampsia. Approximately 25% of first-time pregnant women develop a mild to severe hypertension in pregnancy or even preeclampsia. Based on recently published single-cell RNA-sequencing, we hypothesized an alternative RAS function in placenta and furthermore, an implication in hypertensive disorders in pregnancy. Placental RAS expression and localization was analyzed via quantitative polymerase chain reaction and in situ mRNA padlock probes. Tissue was collected from first-trimester elective termination (n=198), from healthy third-trimester controls (n=54), from early-onset preeclamptic (n=54) and age-matched controls (n=29), as well as first-trimester placentae from women with a high uterine artery resistance index (high-risk for preeclampsia, n=9) and controls (n=8). Serum levels of Ang (angiotensin) I to IV from women before and after conception were measured via mass spectrometry (n=10). Placental explants were cultured in 2.5% oxygen with Ang II, candesartan, and leptin. Seahorse XF96 MitoStress assays assessed trophoblast metabolism. Here, we show that maternal angiotensin acts on placental LNPEP (leucine aminopeptidase), that is, angiotensin IV-receptor and fetal angiotensin on placental AGTR1 (angiotensin II receptor type 1). Maternal circulating RAS shifts towards Ang IV in pregnancy. Ang IV decreases trophoblastic mitochondrial respiration and increases placental leptin via placental LNPEP. Lower placental LNPEP in preeclampsia and in first-trimester patients at high-risk for preeclampsia suggests a new alternative route in maternal RAS signaling and may contribute to hypertension and disease in pregnancy. The study shows how hypertensive disorders in pregnancy may be connected metabolic alterations that finally seem to contribute to the multifactorial disease in pregnancy, preeclampsia.
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Key Words: candesartan ■ leptin ■ oxygen ■ respiration ■ trophoblasts

Blood pressure regulation in pregnancy is crucial to pregnancy outcomes. Approximately 25% of first-time pregnant women develop a mild to severe hypertension in pregnancy or even preeclampsia[1] and 37% develop elevated blood pressure but not overt hypertension.[2] Women diagnosed with gestational hypertension experience increased morbidity, including augmented rates of caesarean deliveries, abruptio placentae, and acute renal dysfunction.[1] Depending on the country of origin, 10% to 50% of women initially diagnosed with gestational hypertension end up developing preeclampsia in as short a period as 1 to 5 weeks.[3,4] Conversely, chronic hypertension ranks second in risk factors for women developing preeclampsia (16%).[5]

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PREECLAMPSIA

Novelty and Significance

What Is New

- Renin-angiotensin system (RAS) receptor localization and missing angiotensinogen expression in placenta implicates a maternal and fetal circulating RAS.
- Maternal angiotensin acts on placental LNPEP (leucine aminopeptidase), fetal angiotensin (Ang) on placental AGTR1 (Ang II receptor type 1)
- Ang III and Ang IV increase in pregnancy but not Ang I and Ang II.
- Ang IV acting on LNPEP decreases trophoblastic mitochondrial respiration and subsequently upregulates placental leptin.
- Leptin downregulates placental LNPEP.
- Preeclamptic placentae and placentae from an early gestation high-risk population have decreased LNPEP compared with controls.

What Is Relevant

- RAS in pregnancy seems to have a shift towards an Ang IV/LNPEP/leptin pathway.
- The alternative RAS-pathway in pregnancy may explain some origins and effects of hypertensive disorders in pregnancy that are not yet elucidated.
- This Ang IV-accentuated RAS-pathway may play a role early in pregnancy when developing PE.

Summary

Maternal circulating RAS shifts towards Ang IV in pregnancy, decreases trophoblastic mitochondrial respiration, and increases placental leptin via placental LNPEP, that is, the Ang IV receptor. Lower placental *LNPEP* in PE and in early gestation higher-risk patients implicates a new alternative RAS mechanism contributing to hypertension in pregnancy.

Nonstandard Abbreviation and Acronyms

ACE	angiotensin-conversion enzyme
AGTR1	angiotensin II receptor type 1
Ang	angiotensin
ANPEP	alanyl aminopeptidase
ARB	angiotensin receptor blocker
ART	assisted reproductive techniques
AT1R	angiotensin II receptor type 1
GLUT4	glucose transporter type 4
hCG	human chorionic gonadotropin
IRAP	insulin-regulated aminopeptidase
LNPEP	leucine aminopeptidase
MAS1	MAS1 proto-oncogene G-protein coupled receptor
OCR	oxygen consumption rate
RAS	renin angiotensin system
REN	renin
scRNA-seq	single-cell RNA-sequencing
SMA	smooth muscle actin

However, not only pregnancy and immediate maternal and fetal morbidities are relevant to this topic, both mother and child suffer from an increased cardiovascular risk throughout their life following gestational hypertension and preeclampsia.[6,7]

Primary factors determining blood pressure are the sympathetic nervous system, plasma volume, vasopressin, and the renin-angiotensin (Ang) system. While the physiological blood regulation in a nonpregnant state is fairly well understood, the pathophysiology of gestational hypertension is not fully elucidated. Antihypertensive treatment of nonsevere gestational hypertension does not reduce the incidence of complications such as preeclampsia, placental abruption, preterm birth, or perinatal death.[8] ARB (Ang receptor blocker) and ACE (ACE inhibitor) exposure during pregnancy have been associated with fetal oliguria, renal dysfunction, and death.[9] Delivery is still the only effective treatment for preeclampsia.

Physiologically, Ang II regulates systemic blood pressure via the AT1R (Ang II receptor type 1) and has thus been focus of extensive research regarding hypertensive disorders in pregnancy, such as preeclampsia.[10–12] Various studies found an association of different renin-Ang system (RAS) components with gestational duration and preterm birth (*AGTR2*[13]), as well as with preeclampsia.[14–16] The crucial role of Ang II during pregnancy is suggested to be mainly mediated by AT1R (*AGTR1*), which is expressed in the placenta throughout gestation.[17,18] Besides the classical RAS-pathway, including the substrate angiotensinogen (*AGT*), the enzymes renin (*REN*), and Ang-converting enzyme (*ACE*), the resulting peptides Ang I and II and their receptors type 1 (*AGTR1*) and type 2 (*AGTR2*), there is an extended RAS[18,19] (see Figure S1 in the Data Supplement). Enzymes such as ACE2 convert Ang II to Ang (1–7) that acts on the Mas receptor. Aminopeptidase N (*ANPEP*), aminopeptidase A (*ENPEP*), dipeptidylpeptidase III (*DPP3*) convert products to the final Ang IV acting on AT4R (Ang IV receptor), also called IRAP (insulin-regulated aminopeptidase), oxytocinase, vasopressinase, or placental leucinyl aminopeptidase (*LNPEP*), which produces a soluble form only in human pregnancy.[20]

The novel technology single-cell RNA-sequencing (scRNA-seq) reveals a new insight into these complex

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systems at the feto-maternal interface. Recent scRNA-seq data published by Vento-Tormo et al[21] led us to revisit the localization of placental RAS components, which revealed a unique picture of the placental RAS, showing a segregated localization of components in different cell types, differently than previously suggested.[17,18] Based on this obtained novel picture of the placental RAS, we further on hypothesized an implication in hypertensive disorders in pregnancy.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. For a detailed description of patient cohorts and sampling methods, cell culture methods, viability assay, RNA isolation, RT quantitative polymerase chain reaction, gel electrophoresis, protein isolation, immunoblotting, immunofluorescence staining, FACS analysis, over-representation analysis,[22,23] serum Ang measurement in the Data Supplement.

Sample Collection

Samples from different patient biobanks were used to validate data: Placental tissue was collected from electively terminated gravidities of healthy patients in Graz, Austria (with gestational age 5–11 weeks; n=198). Healthy third-trimester samples were collected in the University Hospital of the RWTH Aachen, Germany (n=54). Serum samples were collected in a patient cohort undergoing assisted reproductive techniques (ART) at the Center for Gynecological Endocrinology and Reproductive Medicine, University Hospital Graz, Austria. Preeclamptic placentae and respective controls were sampled at the University Hospital of Graz, Austria (n=42) and within an independent cohort[15] at the Oslo University Hospital, Norway (n=33). Placental tissue from early gestation of patient populations at a lower and higher risk of developing preeclampsia (uterine artery resistance index >95 percentile: high risk for developing preeclampsia) and then undergoing elective terminations of pregnancy was sampled at St. George's Hospital, London, United Kingdom (n=16).[24,25] All participating women gave informed written consent, and all studies were approved by the local ethics committee. Villous samples were used and washed in PBS before processing and RNA or protein extraction. For a detailed description, please see the Data Supplement.

Serum Ang Measurement

The equilibrium levels of 4 different RAS Ang peptide metabolites (Ang I, Ang II, Ang III, Ang IV) in serum samples from ART patients were quantified by liquid chromatography-mass spectrometry/mass-spectroscopy performed at a service provider laboratory (Attoquant Diagnostics, Vienna, Austria) using previously validated and described methods.[26] For a detailed description, please see the Data Supplement.

Placental Explants

First-trimester placental tissue was collected from women undergoing an elective termination of pregnancy before week 12. Placental tissue was processed within 1 to 4 hours after the surgical intervention. Subsequently after washing in PBS (pH 7.0, 37 °C; Gibco, Life Technologies (TM), Thermo Fisher Scientific, Vienna, Austria), placental tissue was dissected under a stereoscopic microscope to obtain placental explants as described.[27] After culturing placental explants, tissue was homogenized via UltraTurrax (IKA) in RNA Lysis Buffer (peqlab, VWR International, Avantor, Darmstadt, Germany), and RNA was isolated according to the protocol provided (peqlab, VWR International, Avantor, Darmstadt, Germany).

Placental explants (n=12) were cultured in DMEM/F12 (1:1, Gibco) without supplements in a hypoxic workstation under physiological conditions with 2.5% oxygen for indicated time points at 37 °C for 3, 6, and 24 hours. Candesartan was used in its active form (Selleckchem, Munich, Germany) at a concentration of 0.1 µmol/L, Ang II was used at 0.1 µmol/L (Sigma-Aldrich, Merck, Vienna, Austria), Ang IV at a concentration of 4 nmol (Sigma), Leptin at a concentration of 100 ng/mL (Gibco).

In Situ mRNA Detection via Padlock Probes

In situ detection of mRNA with padlock probe technology was used to localize *AGTR1* and *CGB* mRNA transcripts as described previously.[28,29] In short, oligonucleotides and padlock probes were designed to capture all 5 mRNA transcript variants of *AGTR1* and the one mRNA transcript of *CGB*. Padlock probe technology allows detection of mRNA transcripts directly within tissue samples, preserving its original location. An important advantage of padlock probes are their high specificity in strong contrast to several AGTR1 (Ang II receptor type 1) antibodies showing only low specificity.[30] For further details and the oligonucleotide sequences, please visit the Data Supplement.

Quantitative Analysis of Padlocks In Situ

Slides were scanned with a TissueFAXS system in confocal mode and marker expression in virtual slides was quantified by StrataQuest Contextual Image Cytometry software (TissueGnostics GmbH, Austria). Briefly, the analysis pipeline was programmed to detect placental fetal vessels via fluorescence intensity in the according channel (CD34 immunofluorescence staining). Background subtraction was obtained by an additional autofluorescence imaging. Padlocks were identified with a pipeline based on punctual fluorescence intensity. Distances of padlocks to fetal vessels were calculated automatically.

Seahorse XF Experiments

BeWo cells (ATCC clone, 4×10^5 per well) were seeded onto a Seahorse XFe96 microplate and incubated for 24 hours in DMEM/F12 medium (1:1, supplemented with glucose, pyruvate, 10% v/v FBS, 1% v/v penicillin+streptomycin; Gibco Life Technologies) at 37 °C before conducting the Seahorse XF Cell Mito Stress Test. Compounds were either added to culture medium provided with the Seahorse XF Cell Mito Stress Test Kit (Seahorse XF, Agilent Technologies, Santa Clara, CA) when an incubation time of max. One hour was chosen or to the cell culture medium when a preincubation protocol was chosen. For treatments, the according culture medium was supplemented with Ang II at a working concentration of 0.1 or 1 µmol/L, Ang IV at 0.1, 1, 4, 10, and 40 nmol/L. Extracellular acidification rate

and oxygen consumption rate (OCR) were determined adding D-Glucose to a final concentration as found in the culture medium (17.5 mmol/L), oligomycin to a final concentration of 1 µmol/L, BAM15 to a final concentration of 1 µmol/L, and rotenone and antimycin to a final concentration of 1 µmol/L each (compounds all from Sigma Aldrich, Germany). For normalization, cells were detached after the Seahorse XFe Mito Stress test, and a nuclear staining with Hoechst was performed. Cell count was acquired with a BD FACSCanto II cell analyzer and analyzed with FlowJo v.10.

Single-Cell RNA-Sequencing Data

scRNA-sequencing data were aligned and quantified using the Cell Ranger Single-Cell Software Suite (version 3.0, 10x Genomics) 13 against the GRCh38 human reference genome. Cells with fewer than 300 detected genes and for which the total mitochondrial gene expression exceeded 20% were removed. Mitochondrial genes and genes that were expressed in fewer than 3 cells were also removed. Further bioinformatical analysis was done using R package Seurat[31] and RStudio; Uniform Manifold Approximation and Projection and t-distributed stochastic neighbor embedding projections were imported into Cell Loupe Viewer (version 3.0, 10x Genomics) for visualization and cell annotation purposes.

Sc-RNAseq data published by Pique-Regi et al[32] was used to evaluate effects of preterm labor, labor, and nonlabored term deliveries (Gene Expression Omnibus accession GSE114037, and ArrayExpress accession E-MTAB-6701), data from Vento-Tormo et al[21] was used to analyze RAS expression.

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 25 and R 3.5.0. Graphs were created with GraphPad Prism 7. Some cases were excluded following outlier testing. Obtained data was first analyzed for normal distribution with the Shapiro-Wilk test, and the statistically appropriate tests were then chosen according to the results. Alpha was set at 0.05 and when needed adjusted for multiple testing and alpha-error-accumulation with a Dunn or Holm-Sidak post hoc test. Homogeneity of variances was tested for by Levene test and, when appropriate, Welch correction was used. A linear regression model was applied in the cohort data for the analysis of possible predictors.

RESULTS

Maternal and Fetal Angs Have Different Target Placental RAS Receptors

We analyzed expression levels of RAS components in annotated scRNA-seq data from first trimester placentae (Figure 1A; annotation Figure S2). *AGT* (gene encoding for the Ang II precursor angiotensinogen) was not detectable in placental tissue and must, therefore, be supplied by either the maternal or the fetal circulation. We further investigated the expression of Ang-converting enzymes *REN*, *ACE*, *ACE2*, *ENPEP*, *ANPEP*, *RNPEP*, *DPP3* (Figure S1, modified from[19]), which were partially present in the placenta to convert exogenous Ang depending on cell type (Figure 1A). Out of 4 known Ang-receptors, only *LNPEP* and *AGTR1* were expressed in placental tissue in both third-trimester and first-trimester placenta (Figure 1A; Figures S3 and S4). The cell cluster annotated as syncytiotrophoblast expressed *LNPEP* as the only Ang receptor, whereas *AGTR1* was exclusively expressed in cells annotated as fibroblasts. Two other RAS receptors, *MAS1* and *AGTR2*, were lacking placental expression. The same expression patterns were found in third-trimester placentae (Figure S3).

Since both active receptors AGTR1 and LNPEP (leucine aminopeptidase) showed a different cell type expression, we hypothesized 2 independent RAS-pathways acting on the developing placenta, one of them fetal directed towards *AGTR1* and the other maternal directed towards *LNPEP* as the target receptor. We, therefore, validated their localization in first-trimester placental tissue sections. *CGB* transcripts were localized with padlock probe technology combined with an immunofluorescence staining for β-hCG (human chorionic gonadotropin β subunit), showing mRNA transcripts localized with the protein (Figure 1B, left). *CGB* encoding for β-hCG, a marker for syncytiotrophoblast (Figure 1B, red arrow), was expressed in the same cluster as *LNPEP* (Figure 1C, red arrow). An immunofluorescence staining against LNPEP shows its localization in the syncytiotrophoblast layer (Figure 1C, left).

scRNA-seq expression shows *AGTR1* in cells expressing *ACTA2*, encoding for SMA (smooth muscle actin), a vascular smooth muscle cell marker (Figure 1D and 1E, blue arrows). Immunofluorescence staining with endothelial marker CD31 reveals that cells around fetal vessels are stained for SMA (Figure 1D, right). *AGTR1* transcripts were localized in first-trimester placental tissue sections using specific padlock probes for mRNA in situ hybridization (Figure S5) and were found to be localized, in line with the scRNA-seq data, around fetal vessels (Figure 1E, right).

Both localizations were concordant to scRNA-seq data, although we suggest the fibroblast population, annotated as fibroblasts, represents the myofibroblast population surrounding developing fetal-placental vessels. To systematically evaluate the proximity of *AGTR1* transcripts to fetal vessels, *AGTR1* padlock signals (Figure 1F) were quantitatively analyzed for their distance from the vessels with image analysis software (TissueGnostics StrataQuest; n=5; Figure S5) and compared with the distribution of *ACTB* transcripts in serial tissue sections (n=3). Left-skewed distribution of *AGTR1* compared with a normal distribution of *ACTB* transcripts shows *AGTR1* closer to fetal vessels (3.6 versus 66.0 µm median distance from fetal vessels).

Serum Ang IV Increases in Pregnancy

We then validated results via quantitative polymerase chain reaction and found *LNPEP* and *AGTR1* to be

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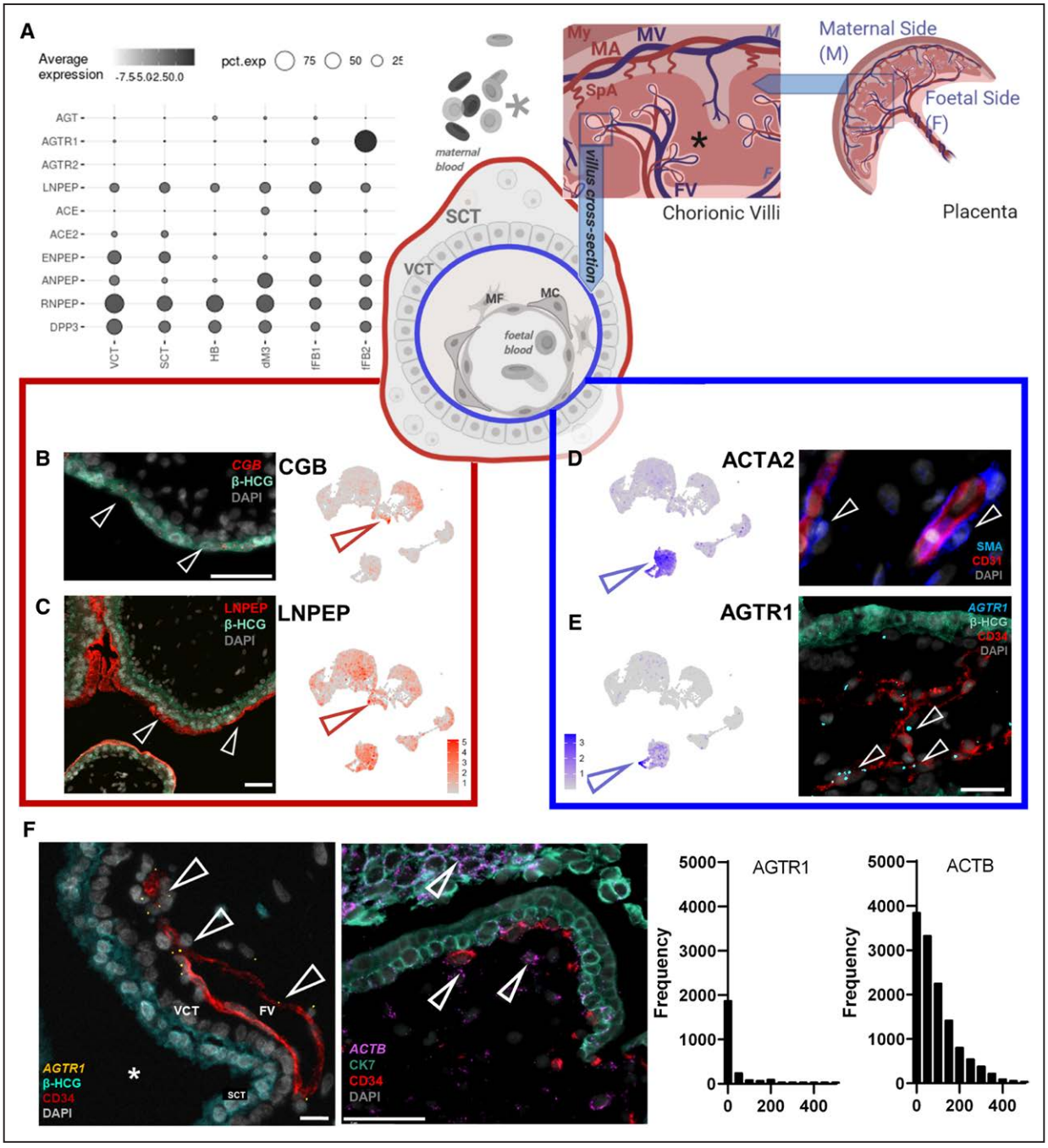

Figure 1. Maternal Ang (angiotensin) and fetal Ang II have different target receptors.
First-trimester villous tissue was used, see schematic representation of a first-trimester chorionic villus cross section (**center**). **A**, Expression and localization of renin Ang system (RAS) components in single-cell RNA-sequencing data from first trimester: *LNPEP* is expressed in the syncytiotrophoblast (SCT), *AGTR1* in fibroblasts (fFB2). *AGT* is not expressed in placenta. Dot circumference and intensity show expression level and percent expression. **B**, SCT marker β-hCG (human chorionic gonadotropin; *CGB*; **right**) expression in SCT cluster in scRNA-seq data (Uniform Manifold Approximation and Projection, red arrow) from first trimester placentae, localization of β-hCG (green) by immunofluorescence staining and *CGB* mRNA transcripts (red, arrow) by padlock probe technology (scale bar=50 µm; **left**). **C**, *LNPEP* expression in SCT cluster scRNA-seq data (**right**), localization of β-hCG (green) and LNPEP (red, arrow) by immunofluorescence staining (n=3, scale bar=50 µm; **left**). **D**, Vascular smooth muscle cell (VSMC) marker *ACTA2* (smooth muscle actin [SMA]) expression in scRNA-seq data (**left**) and localization of SMA (blue) by immunofluorescence staining (CD31=red; **right**). **E**, *AGTR1* expression in fFB2 cluster in scRNA-seq data (**left**). *AGTR1* transcripts (blue, arrow) via padlock probe technology around fetal vessels (FV, red) in first-trimester placenta (combined with immunofluorescence staining; FV: CD34, red; SCT: β-HCG, green; scale bar=50 µm; n=10, gestational age weeks 7 and 11; **right**). **F**, *AGTR1* transcripts (yellow) and actin beta transcripts (ACTB, pink) in first trimester placenta, combined with β-hCG (green) and CD34 (red) immunofluorescence staining (n=3, scale bar=50 µm). Histogram showing the transcript to vessel distance of *AGTR1* transcripts (median is 3.6 µm from fetal vessels) and ACTB transcripts as control (median is 66.0 µm from fetal vessels). MA indicates maternal artery; MC, myocyte; MF, myofibroblast; MV, maternal vein; My, myometrium; SpA, spiral artery; and VCT, villous cytotrophoblast. *intervillous space filled with maternal blood.

expressed in placental tissue, while *MAS1* and *AGTR2* were not detectable and *AGT* expression showed no placental expression, concordantly to the scRNA-seq data (n=8, Figure 2A; Figure S6). Expression levels across gestational age in healthy first trimester and third-trimester placentae (n=252; Table S5) were determined for placental RAS receptors *AGTR1* and *LNPEP*, showing an overall increase across gestation (Figure 2B and 2C). This was validated by protein expression data showing a significant increase in LNPEP from first to third trimester (Figure 2D). Additionally, *REN*, *DPP3*, *ENPEP*, and *ACE2* were also analyzed and displayed dynamic expression patterns across gestation (Figure S6). Furthermore, we analyzed fetal sex effects, maternal age, body mass index, and smoking habits with a linear regression model but did not see any effects on AGTR1 and LNPEP expression (Table S5). We next analyzed serum levels of maternal Ang I to IV (Figure 2E). Serum was collected from women undergoing ART at 2 time points, nonpregnant before embryo-transfer and after confirmed pregnancy around week 7 (Table S6) to compare intrapersonally the changes during pregnancy. The peptides Ang I (mean±SEM, 56.9±6.6 pmol/L) and Ang II (mean±SEM, 210.5±26.4 pmol/L) as measured by mass spectrometry showed no significant increase in first trimester. On the contrary, serum Ang III (mean±SEM, 2.6±0.6 pmol/L) and Ang IV (mean±SEM, 4.6±1.0 pmol/L), the 2 peptide hormones resulting from Ang I and Ang II, were significantly increased in pregnancy (Figure 2E). Interestingly, Ang III is converted to Ang IV as a substrate of LNPEP, and Ang IV acts as an inhibitor of LNPEP activity.[33,34]

Maternal Ang II Has a Metabolic Role in Trophoblasts via LNPEP

Next, we were interested in the effect that Ang may have on the syncytiotrophoblast layer, that is in direct contact with maternal blood. As shown by scRNA-seq data, the syncytiotrophoblasts express LNPEP but not AGTR1. LNPEP is known as AT4R, but also as IRAP (insulin-regulated aminopeptidase) residing in vesicles with GLUT4 (glucose transporter type 4)[35] and binding to acyl-coA-dehydrogenases at the cytosolic end.[36] We, therefore, investigated metabolic effects induced by Ang II and Ang IV in the trophoblastic cell line BeWo. We used Seahorse XF metabolic assays measuring the extracellular acidification rate and OCR after 1-hour Ang II treatment (Figures S7 and S8). Injection of glucose lead to an increase of extracellular acidification rate but, surprisingly, to a decrease in OCR. This pinpoints towards the highly glycolytic character of these cells. Basal OCR levels are decreased after Ang II treatment, but mitochondrial respiration after administration of the uncoupler BAM15 is unchanged (Figure 3A, Figure S8). BeWo cells showed a similar expression patterns of Ang receptors as found for syncytiotrophoblast by scRNA-seq (Figure 3B). Given that enzymes required to convert Ang II into Ang IV (*ENPEP*, *DPP3*, *ANPEP*, *RNPEP)* are expressed in BeWo cells, but no *AGTR1* expression was found (Figure 3B), we suggested a shift from an Ang II to an Ang IV pathway. We, therefore, repeated the metabolic assay with Ang IV treatment for 1 hour. Basal OCR remained decreased when treating cells with Ang IV (Figure 3C), again leaving maximal respiration unchanged compared with controls (Figure S8). Overall, both Ang II and Ang IV reduced mitochondrial metabolism but had no effect on their maximal potential. Glycolytic capacity was decreased (Figure 3D). These effects of decreased mitochondrial metabolism are consistent between Ang II and Ang IV treatment of trophoblasts (Figure 3E and 3F).

Ang II Leads to an Increase of Leptin in a Placental Secretory Response

We next aimed to analyze the effect of Ang II on placental villous explants, a model most close to the situation found in vivo. We incubated villous explants with 0.1 μmol/L Ang II for 6 hours under physiological oxygen concentrations of 2.5% and analyzed transcriptional changes of genes involved in placental pathologies and genes associated with Ang pathways (Figure 4A). Analysis of the differentially expressed genes showed a significant upregulation of *HMOX1, COL12A1, HPGD, ACTG2*, *LEP*—downregulated genes were *MMP9*, *IL1B*, *CD36*, *ANKRD55*, *CBX7*, *SLC24A2*, *VEGFC*, *VEGFA*, *PTPRD* (Figure 4B). Among these genes, *LEP* encoding leptin, had the highest fold change expression (3.98-fold). Analyzing the differentially regulated genes in an over-representation analysis, GO terms such as thermogenesis, female pregnancy, and nucleocytoplasmic transport were found to be significantly enriched (Table S4 and Figure S9). Further on, we validated array results in placental explants with the AT1R-blocker candesartan over different time points (3, 6, and 24 hours; n=10; Figure 4C) and found that a significant upregulation of leptin mRNA expression after 6-hour 0.1 μmol/L Ang II treatment could not be inhibited by candesartan (Figure 4C, middle), suggesting mediation of Ang II effects by LNPEP and not AGTR1. This led us to the conclusion that Ang II may mainly act on LNPEP in placental ex vivo tissue culture, resulting in an upregulation leptin. We, therefore, investigated whether leptin might regulate LNPEP expression as a feedback loop. We treated placental explants with a leptin concentration found physiologically in pregnancy (100 ng/mL) and detected a decreased LNPEP mRNA expression (Figure 4D) and LNPEP protein expression (Figure 4E).

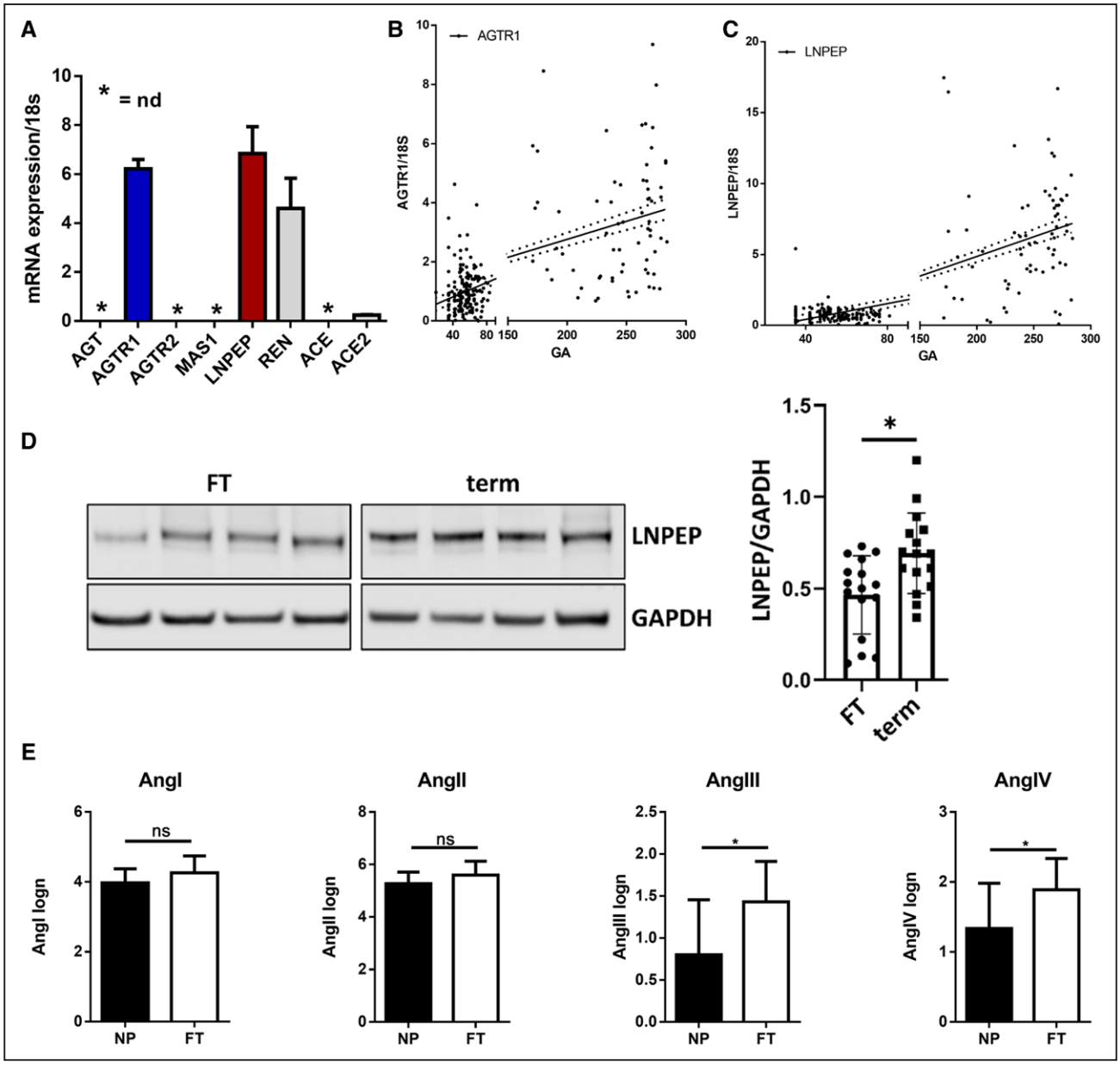

Figure 2. Maternal Ang (angiotensin) IV acting on placental renin (REN) Ang system (RAS) receptor LNPEP increases in pregnancy.

A, Quantitative polymerase chain reaction Validation of scRNA-seq data expression patterns of RAS components, 2 RAS receptors in placenta are AGTR1 (Ang II receptor type 1) and LNPEP, while no endogenous angiotensinogen (AGT) is expressed in human first-trimester placenta (n=8; mean and SEM; *not detected). **B**, AGTR1 and (**C**) LNPEP mRNA expression across gestation (n=252; nonlinear regression line; Table S5); (**D**) LNPEP protein expression (data are represented as mean with SEM; normality was tested by Shapiro-Wilk test followed by Mann-Whitney test; *$P \leq 0.05$). **E**, Log-transformed values of Ang I–IV serum levels before and after conception in ART patients; Ang III and Ang IV show a significant increase in pregnancy (Ang I and Ang II=ns, normality was tested by Shapiro-Wilk test; paired *t* test with graph showing mean and SD for Ang I, II, IV, and Wilcoxon-signed-rank test with median and IQR for Ang III, sig. level 0.05; n=10; patient characteristics in Table S6). ACE indicates Ang-converting enzyme; FT, first trimester; GA, gestational age; and NP, nonpregnant.

LNPEP-Dependent Ang II Effects May Lead to LNPEP Downregulation in Preeclampsia

While some of LNPEP functions such as degrading oxytocin or vasopressin have been elucidated, it is still unclear why low soluble LNPEP serum levels are associated to pregnancy pathologies like gestational diabetes, fetal death, or hypertensive disorders in pregnancy.[20,37]

To determine whether only shedding or transcription is disturbed in preeclampsia, placentae from healthy and preeclamptic patients were analyzed for LNPEP expression. A significant downregulation of *LNPEP* gene expression was found in early and late-onset preeclampsia (Figure 5A; Tables S7 and S8) compared with uncomplicated pregnancies. Furthermore, we investigated possible early gestational effects and analyzed

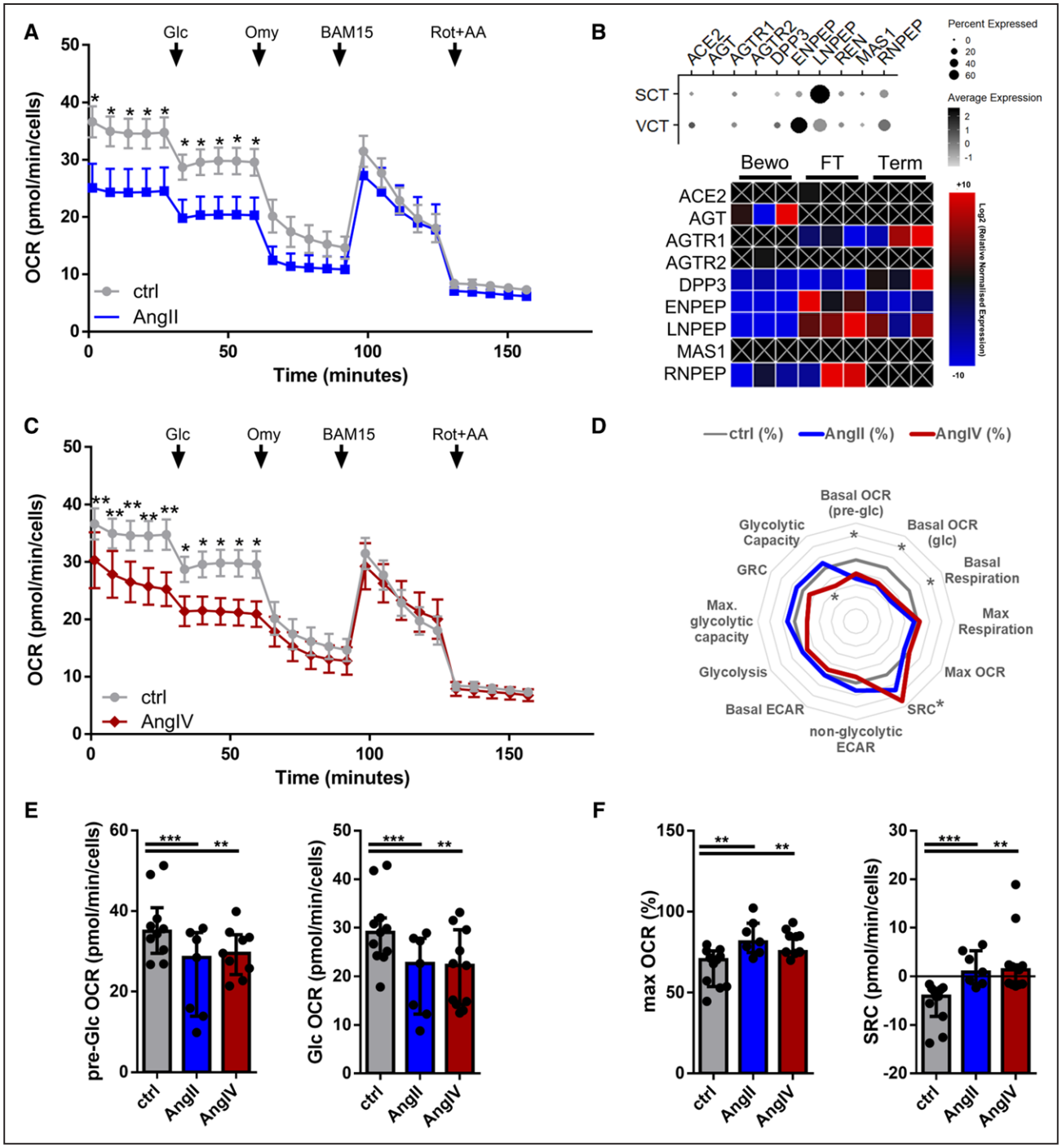

Figure 3. Maternal Ang (angiotensin) II and Ang IV have a metabolic role in trophoblasts.
A, Ang II (0.1 µmol/L) downregulates basal oxygen consumption rate (OCR) in BeWo trophoblast cells (mean and SEM, Seahorse XF data normalized to cell count; unpaired *t* test with Welch correction, $P<0.05$). **B**, scRNA-seq data of syncytiotrophoblast (SCT) and villous cytotrophoblasts (VCT). Expression analysis of renin Ang system (RAS) components by quantitative polymerase chain reaction shows LNPEP as only Ang receptor expressed in BeWo cells (term and first trimester placentae served as positive controls, n=3 for each; X=not detected; blue=low, red=high expression). **C**, Ang IV (4 nmol/L) downregulates basal OCR (mean and SEM; normalized, *t* test with Welch correction, $P<0.05$). **D**, Metabolic profile of Ang II and Ang IV treated cells relative to control (significant changes marked *$P<0.05$). **E**, Ang II and Ang IV alter metabolism similarly, significantly decreasing basal OCR levels (**F**) while maintaining a higher spare respiratory capacity (1 h pretreatment with 0.1 µmol/L Ang II or 4 nmol/L Ang IV; graph shows median and interquartile range, Mann-Whitney U test, Seahorse XF data normalized to cell count; $P<0.05$). AA indicates antimycin; ACE, Ang-converting enzyme; AGTR1, Ang II receptor type 1; DPP3, dipeptidyl peptidase 3; ECAR, extracellular acidification rate; ENPEP, glutamyl aminopeptidase; FT, first trimester; Glc, glucose; GRC, glycolytic reserve capacity; Omy, oligomycin; RNPEP, arginyl aminopeptidase; Rot, rotenone; and SRC, spare respiratory capacity.

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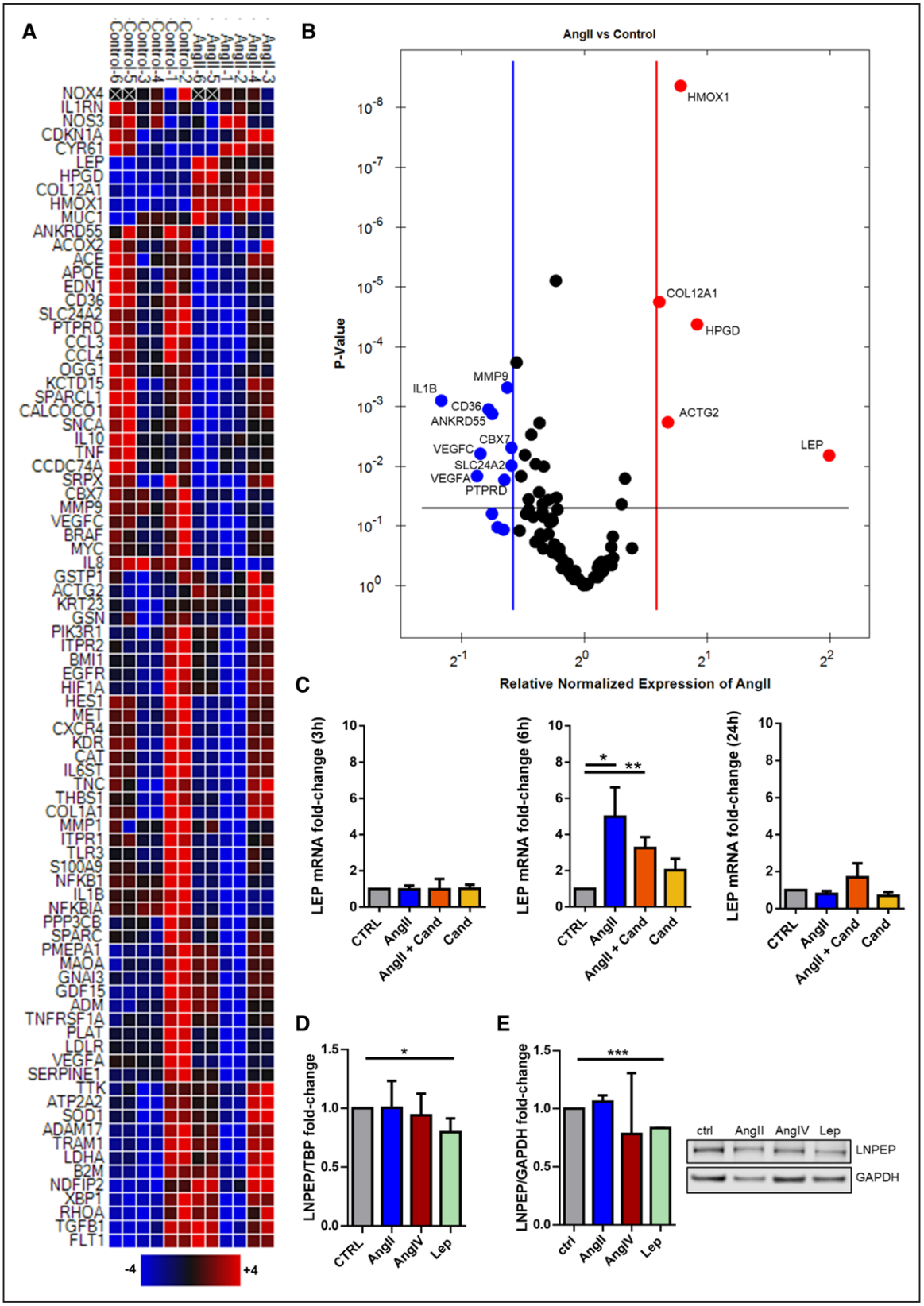

Figure 4. Exogenous Ang (angiotensin) II leads to an increase of leptin, while leptin downregulates LNPEP in first trimester placental explants.

A, Heat map showing the gene expression in first-trimester placental explants incubated with 0.1 µmol/L Ang II for 6 h (at O2 2.5% physiological concentration; n=3). **B**, Volcano plot showing significantly regulated genes (P>0.05, regulation >1.5 fold). Significantly upregulated (red) are *HMOX1, COL12A1, HPGD, ACTG2, LEP*–downregulated genes (blue) are (*Continued*)

first-trimester placenta samples from women classified by uterine artery Doppler ultrasound scanning as having a higher or lower risk of developing preeclampsia (uterine artery resistance index >95 percentile: high risk for developing preeclampsia) and then undergoing elective terminations of pregnancy.[24] *LNPEP* expression was significantly decreased (Figure 5B; Table S9) in the placentae derived from women with a high risk for preeclampsia.

Based on our data, we suggest that the downregulation of LNPEP in high-risk pregnancies or preeclampsia may be the result of a first trimester leptin-driven negative feedback loop initiated by maternal Ang IV (Figure 5C).

DISCUSSION

While there is broad consensus that the human placenta contains its own RAS from very early gestation onwards, our current study provides substantial experimental evidence to challenge the existing doctrine of cellular distribution of placental RAS receptors. This current study identified *LNPEP* as the sole RAS receptor at the maternal-placental interface, that is, the syncytiotrophoblast. We localized *AGTR1* exclusively at fetal-placental vessels, whereas neither *AGTR2*, nor *MAS1* were detected in human placenta. Previous studies localized AT1R in a broad range of placental cells, including the syncytiotrophoblast, villous cytotrophoblasts, villous stroma cells, Hofbauer cells,[17,38] AT2R staining was identified in both villous and extravillous trophoblast.[39] When investigating the expression of MAS1 (MAS1 proto-oncogene G-protein coupled receptor), apelin, bradykinin, oxytocin, or vasopressin, we saw in the scRNA-seq data from Vento-Tormo et al, that none of these were expressed in villous placental tissue in the first trimester (Figure S4). Based on our findings, we propose a novel concept that gives insight into a shift to an alternative RAS pathway in human pregnancy. Accordingly, maternal Ang II acts on the placenta by a shift towards its derivatives Ang III and Ang IV that are acting on the target receptor LNPEP, driving responses at the syncytiotrophoblast. The syncytiotrophoblast layer in direct contact with placental intervillous maternal blood, and thus maternal Ang-derivatives, responds with a decrease in basal mitochondrial respiration. Leptin increases and in a novel pathway, downregulates LNPEP—well in line with the results of lower expression of LNPEP we found in preeclamptic placentae as well as in first trimester higher-risk patients. Previous studies also identified high maternal leptin in serum and placenta as a hallmark of preeclampsia.[40,41]

Leptin has long been investigated in cardiovascular research for its involvement in endothelial dysfunction, aneurysm, atherosclerosis—also in association with Ang and RAS components.[42–44] Previous studies by Than et al[45] show that leptin positively correlates with blood pressure and that *AGT* plays a central role in functionally connecting differentially expressed features in preeclampsia. We are the first to show a direct relationship between maternal Ang and elevated placental leptin levels. This also allowed us to propose a pathophysiological mechanism around placental LNPEP. LNPEP is known as AT4R, oxytocinase, vassopressinase, placental LNPEP, but also as IRAP residing in vesicles with GLUT4 and binding to acyl-coA-dehydrogenases at the cytosolic end.[35,36] The concept may explain the appearance and interdependence of hypertension, metabolic disorders in the form of maternal obesity or gestational diabetes, activation of cell-mediated immunity, and preeclampsia.

Maternal susceptibility genes in preeclampsia identified LNPEP in decidua[46] together with *AGT*[47] and in maternal blood with *ANPEP*.[48] SNPs in LNPEP and associated sites were related to prematurity shown in maternal triads and to gestational length in general.[49] Maternal soluble form only in human pregnancy activity increases between the fourth and 38th weeks of gestation,[50] leading to accelerated metabolism of its substrates such as vasopressin or oxytocin. However, its activity decreases in preterm delivery and severe preeclampsia.[51] Its soluble fraction in maternal plasma is decreased with severe preeclampsia when compared with mild preeclamptic or normotensive pregnancies.[52] In line with this observations, levels of serum copeptin—a by-product of vasopressin hydrolysis from its precursor—were significantly elevated in women who subsequently developed preeclampsia, compared with controls across gestation.[53,54] Hence, our data on decreased LNPEP in first trimester placental tissue from women with a higher risk of developing preeclampsia fits well in this concept and indicates that a disturbed LNPEP activity may also mirror its vasopressinase activity.[51,52] In the overriding cohort[24] of early pregnancies with a high risk for developing preeclampsia, there was no difference in the body mass index from high-risk and control groups. This heightens the suggested importance of the renin-Ang system in preeclampsia. Altogether, LNPEP seems to have a fundamental role in sustaining a pregnancy.

Figure 4 Continued. *MMP9, IL1B, CD36, ANKRD55, CBX7, SLC24A2, VEGFC, VEGFA, PTPRD* (**C**) shows the relative LEP mRNA expression of placental explants incubated with Ang II (0.1 µmol/L) and AT1R (Ang II receptor type 1)-blocker candesartan (1 µmol/L) for 3 h (**left**), 6 h (**middle**), and 24 h (**right**). LEP expression showed a 4.97-fold increase in first trimester placental explants after 6 h treatment, with no change at 3 or 24 h. No Ang II type 1 receptor blocker effect can be detected at 6 h. $P<0.05$; 3 h: n=4; 6 h: n=10; 24 h: n=8, graph shows median and range. **D**, LNPEP mRNA expression after 6 h Ang II, Ang IV, leptin treatment of first trimester placental explants: LNPEP is significantly downregulated after 6 h leptin treatment (fold change; mean and SD, n=3). **E**, LNPEP protein expression in first trimester placental explants treated with Ang II (0.1 µmol/L), Ang IV (4 nmol/L), and leptin (10 ng/mL) for 6 h, normalized to GAPDH (AUC fold change shown, graph shows mean and SD, n=3). Test used: 1-way ANOVA and Holm-Sidak post hoc test.

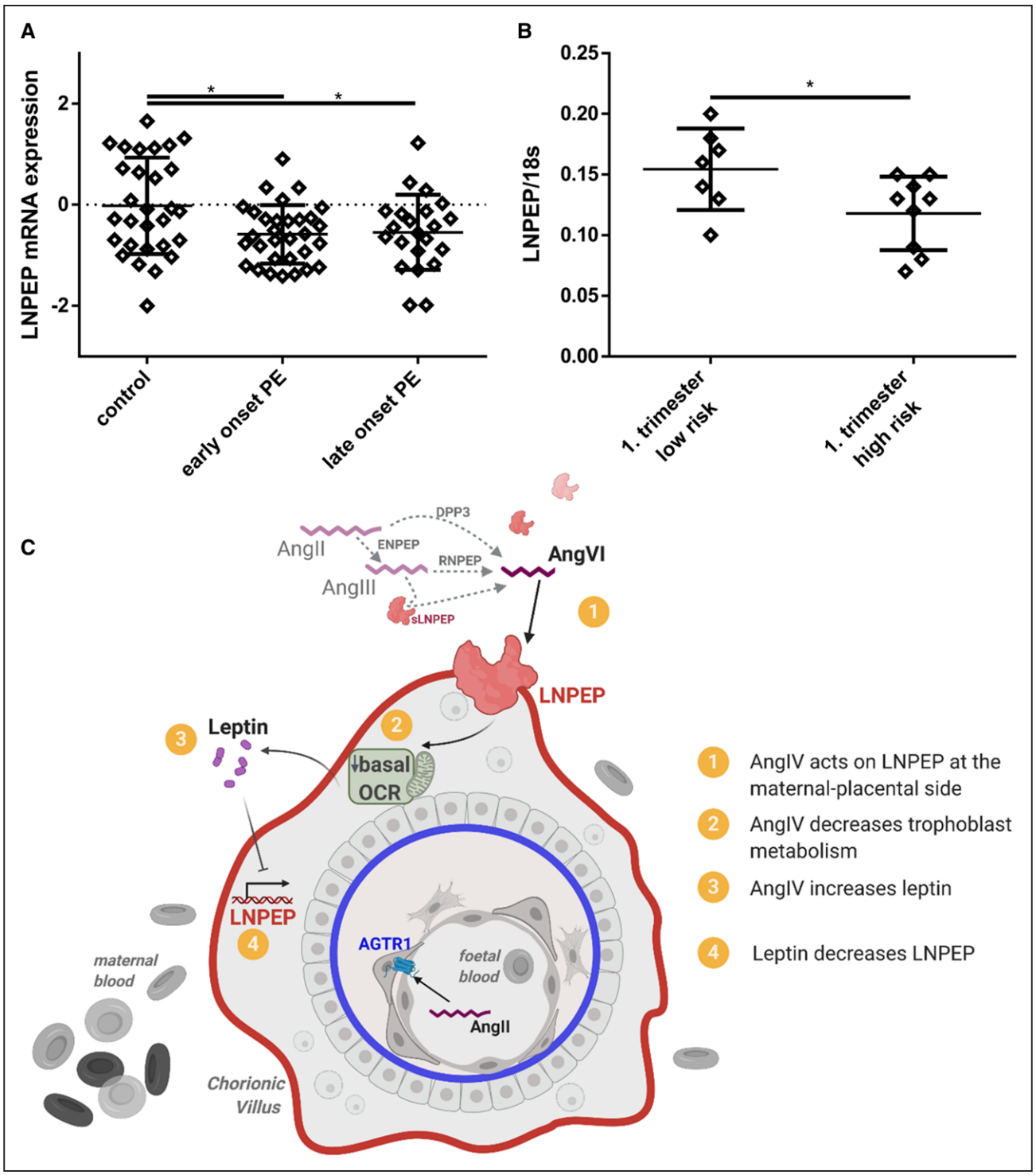

Figure 5. LNPEP-dependent Ang (angiotensin) II effects may lead to LNPEP downregulation in preeclampsia (PE).
A, *LNPEP* mRNA is differentially expressed in early and late-onset PE (eoPE, loPE) when compared with controls (data z-transformed; 1-way ANOVA with multiple comparisons; $P<0.05$; see Tables S8 and S9 Graz and Oslo cohort). **B**, *LNPEP* expression is lower in first trimester placentae from women with a high uterine artery resistance index, indicating high-risk for PE (n=16; Mann-Whitney *U* test, $P<0.05$). **C**, Graphical summary of the proposed mechanistic pathway based on the results. Maternal and fetal Ang II and its conversion product Ang IV have different target receptors. Maternal Ang IV, that increases in pregnancy, acts on trophoblastic LNPEP and decreases basal oxygen consumption rate (OCR) in mitochondrial respiration, which in turn may drive an increase in leptin expression. Increased leptin levels decrease LNPEP expression on an mRNA and protein level.

A strength of this study is that we confirmed reduced placental LNPEP expression in 2 independently recruited cohorts with preeclamptic patients. It is difficult to distinguish between preeclampsia effects and confounding factors related to preterm delivery and labor, unless gestational-age matched controls are available.

As expected, our uncomplicated control pregnancy group has a significantly higher mean gestational age than the preeclamptic group (Graz cohort: 253.8±4.1 days (n=27) controls and 236.9±3.5 days (n=37) preeclamptic pregnancies; Δ=16.9 days), meaning that a reduced placental LNPEP expression could result from lower gestational age, differences between birth mode, general risk factors for preterm birth. We addressed these possible confounders by investigating available scRNA-seq data from Pique-Regi et al[55] to evaluate effects of preterm labor, labor, and nonlabored term deliveries (Figure S10). We found no difference in LNPEP expression between these entities neither in villi, decidua nor chorioamniotic membranes. Hence, we conclude that LNPEP expression is likely not altered by labor stress, preterm birth, or risk factors that pertain to these pregnancy pathologies, but rather suggest it to be a preeclampsia-specific feature worth further investigation.

We could use smaller sample sizes for assessing a high-risk population and ART patients without the risk of overseeing confounders by using a large cross-sectional observational cohort of healthy first trimester placental samples to adjust for maternal and fetal characteristics. Using serum from women before undergoing ART and after a successful pregnancy in first trimester was the best method to intrapersonally adjust Ang levels in nonpregnant and pregnant women's serum by choosing a cohort that was documented to be nonpregnant and then pregnant and thus excluding variability between individuals as well as concentrating on changes in pregnancy.

In line with our findings, both LNPEP and ANPEP (Ang II induces placental AP-A) have previously been found to be expressed on the apical microvillous surface of the syncytiotrophoblast.[56] On top, ANPEP hydrolyzing Ang II to Ang III in pregnancy.[57] No longitudinal technically reliant data and composition of Angs in pregnancy was available up to date, and our data measured by mass spectrometry comparing prepregnancy and pregnancy levels of Angs in the same patients is unique. These new findings give an insight into entirely different, LNPEP-centered baseline conditions regarding RAS in pregnancy. RAS components degrading Ang II to Ang III[58] were significantly increased in preeclampsia compared with control pregnancies, such as placental ERAP1 and 2, increased in transcriptome profiling of preeclamptic tissues and higher AP-A (*ANPEP*) activity in preeclampsia.[47,59,60] This suggests an even higher shift towards Ang III and Ang IV, acting on placental LNPEP in pregnancies complicated by preeclampsia.

The importance of placental AGTR1 in early gestation depends on whether the fetal circulating RAS is established. We were not able to assess the availability of AGT in the fetal circulation in our study. While there are reports of Ang in term cord blood and thus the fetal circulation,[61] there are no earlier measurements of plasma AGT in the fetal circulation yet. scRNA-seq of fetal hepatocytes in 15 to 21 post-conception week synthesizing *AGT*[62] is the earliest available data, limiting comparison to our data from 7 post-conception week. However, this fact only adds to the importance of the maternal circulating RAS in this early pregnancy period before establishment of fetal RAS acting on placental *AGTR1* in second trimester.[62]

Our results suggest a general shift of the maternal circulating RAS towards increased Ang IV levels with onset of pregnancy, which are met by the appearance of placental LNPEP at the maternal interface. The Ang IV-induced decrease of basal mitochondrial respiration in trophoblasts may result in an attempt to rescue the placental metabolism by increasing leptin. Leptin in turn downregulates LNPEP expression, suggesting a negative feedback loop regulation of the Ang IV/leptin/LNPEP axis. We found that LNPEP, involved in vasopressin clearance and antigen cross-presentation, was downregulated in early and late-onset preeclampsia. More importantly, we could show that this effect was already observable in first trimester placentae from women that had a higher risk for developing preeclampsia based on a high uterine artery resistance index.

PERSPECTIVES

The study suggests for the first time the mechanisms by which hypertensive disorders in pregnancy may be connected to altered leptin and cellular metabolism. Furthermore, our results indicate a new pharmacological RAS target. Since LNPEP is shed and found in maternal plasma only in pregnancy, it could serve as a novel target. In contrast to ARBs and ACE inhibitor, it may potentially be nonteratogenic and serve as a more selective approach. Using LNPEP as pharmaceutical target may lower detrimental effects such as decreased mitochondrial respiration or increased maternal leptin levels that finally seem to contribute to the multifactorial disease in pregnancy, preeclampsia.

ARTICLE INFORMATION

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Disclosures

None.

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