Dissertation

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

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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the "Guidelines of the Medical University of Graz on Good Scientific Practice".

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Abbreviations and Definitions

(s)LNPEP	(soluble) leucyl and cystinyl aminopeptidase = angiotensin IV receptor = placental leucine aminopeptidase
AA	antimycin
ACE	angiotensin conversion enzyme
ACEI	ACE-inhibitor
AGTR1/2	angiotensin II receptor type 1 or 2 (gene)
AT1R/AT2R	angiotensin II receptor type 1 or 2 (protein)
Ang	Angiotensin
ANPEP	alanyl aminopeptidase
ARB	Angiotensin receptor blocker
ART	assisted reproductive techniques
AT1R	angiotensin II receptor type 1
BMI	body mass index
CHO	Chinese hamster ovarian cells
CI	Confidence interval
CK7	cytokeratin 7
CRL	crown-rump-length
CS	caesarean section
ctrl	control
DAPI	4′,6-diamidino-2-phenylindole
dM	decidual macrophages
DMSO	dimethyl sulfoxide
DPP3	dipeptidyl peptidase 3
ECAR	extracellular acidification rate
ECFC	endothelial colony forming cells
ENPEP	glutamyl aminopeptidase
eoPE	early onset preeclampsia
EVT	extravillous trophoblast
f	female
FACS	fluorescence-activated cell sorting
fFB1-2	foetal fibroblast
FFPE	Formalin fixed and paraffin embedded
GA	gestational age
GEM	gel beads in emulsion

Glc	glucose
GLUT4	glucose transporter type 4
HB	Hofbauer cells
hCG	human chorionic gonadotropin
IL (as in IL-6)	interleukin
IRAP	insulin regulated aminopeptidase
LDH	Lactatdehydrogenase
loPE	late onset preeclampsia
m	male
M1-family	The peptidases of family M1 are dependent on a single zinc ion for activity, many of them being aminopeptidases.
MAS1	MAS1 proto-oncogene G-protein coupled receptor
MMP11	matrix metalloproteinase 11
NaCl	sodium chloride
ND	not detected
NGS	next generation sequencing
OCR	oxygen consumption rate
Omy	Oligomycin
OR	Odds ratio
PBS	phosphate-buffered saline
pcw	Post conception week
PE	preeclampsia
P-LAP	placental leucine aminopeptidase
PTL	preterm labour
RAS	renin angiotensin system
REN	renin
RI	resistance index
RIPA Buffer	Radioimmunoprecipitation Assay Buffer
RNPEP	arginyl aminopeptidase
Rot	rotenone
RR	Relative risk
scRNA-seq	single-cell RNA-sequencing
SCT	syncytiotrophoblast
SD	standard deviation
SNP	single nucleotide polymorphism

SVD	spontaneous vaginal delivery
TIL	Term in labour
TNL	term no labour
TOP	termination of pregnancy
tSNE	t-distributed stochastic neighbour embedding
UMAP	*Uniform Manifold Approximation and Projection*
UMI	Unique molecular identifier
UtAD	Uterine artery Doppler
VCT	villous cytotrophoblast
VE	vacuum extraction
Wnt	Wingless Int-1

Abstract in German

Das plazentare Renin-Angiotensin-System (RAS) und dessen Komponenten spielen eine Rolle in der Präeklampsie. Durch Einzelzell-RNA-Sequenzierungsdaten (scRNA-seq), die im Gegensatz zu vorherigen Studien auf eine andere Verteilung der plazentaren RAS-Rezeptoren schließen ließen, entstand die Hypothese, dass ein alternatives RAS hypertensive Schwangerschaftspathologien erklären könnte.
Mittels scRNA-seq und *in situ* mRNA Hybridisierung durch padlock probes wurde die Lokalisation des plazentaren RAS untersucht. Plazentagewebe wurde von frühen elektiven Schwangerschaftsabbrüchen und gesunden Termin-Plazenten (n=252) gesammelt, Plazentagewebe von gesunden Kontrollen und Frauen mit Präeklampsie aus zwei unabhängig voneinander rekrutierten Kohorten wurde untersucht (n=49). Zusätzlich wurde Plazentagewebe von Frauen, die einen Schwangerschaftsabbruch hatten, und bei denen man im ersten Trimester auf dem Resistance Index der uterinen Arterien basierend ein erhöhtes Risiko für Präeklampsie feststellen konnte (Hochrisiko) und deren Kontrollen (n=16), untersucht. Die Serumspiegel von Angiotensinen wurden per LC-MS/MS im Serum von Patientinnen vor der Schwangerschaft und bei Schwangerschaft durch assistierte Reproduktionstechniken gemessen. Plazenta-Explants wurden bei 2,5% Sauerstoff mit AngII, dem AT1R-Blocker Candesartan und Leptin inkubiert. Der Trophoblast-Metabolismus wurde per Seahorse XF96 MitoStress Assays gemessen.

AngIII und AngIV Serumspiegel stiegen in der Schwangerschaft im Vergleich zu präkonzeptionellen Werten an, AngI und AngII jedoch nicht. AngII-Rezeptor AGTR1 konnte bei fötalen Gefäßen lokalisiert werden, AngIV-Rezeptor LNPEP, der als Plazentare Leucin-Aminopeptidase bekannt ist, im Syncytiotrophoblasten – beide Ergebnisse waren in scRNA-seq Daten ebenso replizierbar. Leptin-Expression war durch *in vitro* AngII-Behandlung von Plazenta-Explants erhöht, jedoch nicht durch Candesartan inhibiert. AngIV änderte den Trophoblast-Metabolismus in vitro. LNPEP-Expression war in Plazenta-Explants, die mit Leptin behandelt waren, signifikant niedriger, ebenso wie in präeklamptischen Plazenten und frühen Hochrisiko-Plazenten.

Maternales AngII wirkt als AngIV auf den plazentalen LNPEP-Rezeptor und scheint den Trophoblast-Metabolismus zu regulieren. Aufgrund der verminderten metabolischen Leistung kann Leptin steigen, was wiederum LNPEP hinunterreguliert. Der AngIV/LNPEP/Leptin Signalweg könnte die Entstehung eines präeklamptischen Phänotypen bereits in der frühen Schwangerschaft erklären.

Abstract in English

A role for placental Renin-Angiotensin-System (RAS) components was shown in preeclampsia. Investigating single cell RNA-sequencing (scRNA-seq) data showed differently expressed components than previously suggested. We hypothesised that an alternative maternal RAS pathway may explain pregnancy hypertensive disorders.

We investigated placental RAS localisation via scRNA-seq and in situ padlock-probes and the influence of maternal and foetal factors on expression. Placental tissue was collected from early electively terminated gravidities of healthy patients, from healthy term controls (n=252), from early-onset preeclamptic and early control pregnancies (n=49), as well as first trimester placentae from women with a high uterine artery resistance index (high-risk) and controls (n=16). Serum levels of angiotensins from patients undergoing assisted reproductive technology before and after conception were measured via LC-MS/MS. Placental explants were cultured in 2.5% oxygen with AngII, AGTR1-blocker Candesartan and leptin. Seahorse XF96 MitoStress assays assessed trophoblast metabolism.

AngIII and AngIV serum levels increased when pregnant, but not AngI and AngII. AGTR1 was located around foetal vessels, LNPEP was located in syncytiotrophoblast, both concordant with scRNA-seq data. In vitro studies with placental explants showed upregulation of leptin expression by AngII not inhibited by Candesartan. AngIV showed alteration of trophoblast mitochondrial metabolism in vitro. LNPEP expression decreased in explants treated with leptin, and was significantly downregulated in preeclamptic and early high-risk placentae.

Maternal AngII may act as AngIV on placental LNPEP, encoding for angiotensin IV receptor AT4R and known as placental leucine aminopeptidase, seemingly involved in regulating trophoblast metabolism. Leptin may be upregulated following decreased metabolism, in turn downregulating LNPEP expression. This AngIV/LNPEP/Leptin pathway may explain early gestational contributions toward a preeclamptic phenotype.

Introduction

Hypertension in Pregnancy

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 (Hauth *et al.*, 2000), and 37% develop elevated blood pressure but not overt hypertension (Porcelli *et al.*, 2020).

In pregnancy, a systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg defines pregnancy. A lower cut-off point of a systolic blood pressure 130 to 139 mmHg or diastolic blood pressure 80 to 89 mmHg has been endorsed by the American College of Cardiology and the American Heart Association for diagnosing hypertension in nonpregnant individuals.

Women diagnosed with gestational hypertension experience increased morbidity, including augmented rates of caesarean deliveries, abruptio placentae, and acute renal dysfunction (Hauth *et al.*, 2000). Depending on the country of origin, ten to fifty percent of women initially diagnosed with gestational hypertension end up developing preeclampsia in as short a period as one to five week (Saudan *et al.*, 1998; Barton *et al.*, 2001). Conversely, chronic hypertension ranks second in risk factors for women developing preeclampsia (16% (Bartsch *et al.*, 2016)). However, not only pregnancy and immediate maternal and foetal morbidities are relevant to this topic, both mother and child suffer from an increased cardiovascular risk throughout their life following gestational hypertension and preeclampsia (Magnussen *et al.*, 2009; Alsnes *et al.*, 2017; Birukov *et al.*, 2020).

Primary factors determining blood pressure are the sympathetic nervous system, plasma volume, vasopressin, and the renin-angiotensin system. While the physiological blood regulation in a non-pregnant state is fairly well understood, the pathophysiology of gestational hypertension is not fully elucidated. Antihypertensive treatment of non-severe gestational hypertension does not reduce the incidence of complications such as preeclampsia, placental abruption, preterm birth, or perinatal death (Webster *et al.*, 2017). Angiotensin receptor blocker (ARB) and ACE-inhibitor (ACEI) exposure during pregnancy have been associated with foetal oliguria, renal dysfunction, and death (Cox, Anderson & Cox, 2003). Delivery is still the only effective treatment for preeclampsia. (Nonn *et al.*, 2021)

Preeclampsia

Preeclampsia is defined as a hypertensive disorder of pregnancy that presents progressively over gestation with multisystem disorders. New onset of hypertension and proteinuria or a significant end-organ dysfunction in the last half of pregnancy or even post-partum are features of this disease. There are many hypotheses about its aetiology such as placental and maternal vascular dysfunction but no definite cause is yet found. Approximately 85 percent of late onset, i.e. after the 34th week of gestation, pre-eclamptic disease courses are of good foetal and maternal outcomes but present with an increased risk of serious morbidity or mortality (Desforges, Cunningham & Lindheimer, 1992). So called early onset preeclampsia which makes up the remaining 10 percent of cases carry an additional high risk for preterm birth and increased maternal and foetal morbidity and mortality. Several studies found that women with preeclampsia are at increased risk for cardiovascular, renal, and chronic hypertensive disease over their life time.

Preeclampsia can be divided into early onset and late onset preeclampsia, depending on whether the disease manifests before or after the 34th week of gestation. Clinical features and diagnostic criteria overlap, but early onset preeclampsia has more severe maternal and foetal as well as placental findings and poorer prognosis to both maternal and foetal outcomes (Lisonkova & Joseph, 2013; Harmon *et al.*, 2015) which led some researchers and clinicians to suggest two diseases with different pathophysiology (Redman, Sargent & Staff, 2014). However, biological variance remains an explanation for differences in early and late onset preeclampsia.

Prevalence and risk factors

For a systematic review of hypertensive disorders in pregnancy, a logistic model was developed to estimate the global and regional incidence of preeclampsia and eclampsia. Based on this, approximately 4.5 percent of worldwide deliveries were complicated by preeclampsia, depicting a wide variation across regions (Abalos *et al.*, 2013).

Even earlier onset of preeclampsia before the 34th week of gestation is less common with 0.3 percent than after the 34th week with 2.7 percent incidence (Lisonkova *et al.*, 2014).

Risk factors predisposing for preeclampsia include pregnancy-specific factors such as past history of preeclampsia, multi-foetal gestation, but also pre-existing pathologies such as hypertension, pregestational diabetes, chronic kidney disease, and some autoimmune

diseases (antiphospholipid syndrome, systemic lupus erythematosus). These factors are the ones contributing to the highest rise in relative risk to develop the disease.
Past history of preeclampsia for instance increases the risk in a subsequent pregnancy 8-fold (RR 8.4, 95% CI 7.1-9.9; (Bartsch *et al.*, 2016)), recurrence was reported to be as high as 25-65 percent (Sibai, El-Nazer & Gonzalez-Ruiz, 1986; Gaugler-Senden *et al.*, 2008).

Pre-existing diseases such as pregestational diabetes increase the relative risk by 4-fold (RR 3.7, 95% CI 3.1-4.3; (Bartsch *et al.*, 2016)). However, the exact pathogenetic pathway contributing to the development is not clear yet and is thought to be linked to a variety of factors such as underlying vascular disease, i.e., diabetic nephropathy, obesity, altered insulin resistance. Prevalence of preeclampsia in women with type 1 diabetes was 17%, five to six times more than in the background population (Vestgaard *et al.*, 2018).

Pre-existing hypertension as well as elevated blood pressure within the normotensive range showed an increased odds ratio to developing pre-eclampsia (OR 3.8-17.1; (Vestgaard *et al.*, 2018)). Pre-existing chronic hypertension is relatively rare in women of reproductive age and thus accounts for only 5-10 percent of preeclampsia cases (Roberts & Redman, 2017).

Interestingly, newer studies suggest a dose-response like association curve of hypertension and preeclampsia which becomes clinically evident when blood pressure rises are diagnosed from elevated (120-129/80 mmHg) to stage 1 hypertension (130-139/80-89 mmHg) and furthermore, the classical overt stage 2 hypertension >140/90 mmHg (Reddy *et al.*, 2020; Sutton *et al.*, 2020).

The risk of preeclampsia doubles with each 5 to 7 kg/m^2 increase in pre-pregnancy body mass index, persisting when adjusting for confounders such as chronic hypertension, diabetes mellitus, and such (O'Brien, Ray & Chan, 2003). Even though overweight and obesity are responsible for a relatively minor relative risk increase, the high prevalence in the end results in accounting for approximately 40 percent of preeclampsia cases (Roberts & Redman, 2017).

Clinical Features and Diagnosis

Late onset preeclampsia accounts for over 85 percent of cases, whereas 10 percent of preeclampsia cases develop before the 34^{th} week of gestation – the remainder is recognised post-partum usually 48 hours after delivery (Al-Safi *et al.*, 2011).

The clinical features such as maternal hypertension and proteinuria defining this disease spectrum, are highly variable in patients. Approximately 25 percent of women develop severe hypertension.

Diagnostic criteria are a new-onset hypertension after 20 weeks of gestation and/or one of the more nonspecific symptoms such as headache, visual abnormalities, epigastric pain, and new-onset dyspnoea. Whereas most pregnant women develop oedema, acute weight gain (>2.3 kg/week) and facial oedema allow to diagnose women who develop preeclampsia. The rapid development of oedema is due to a capillary leak derived from endothelial dysfunction and increased sodium retention. This is also part of the reason for the pathophysiognomic finding of proteinuria, defined as ≥0.3 g protein in a 24-hour urine specimen or protein ≥2+ (30 mg/dL) on a random midstream urine specimen test strip ("dipstick" urinalysis). However, this feature may be a late finding since capillary and glomerular leakage are so advanced as to become clinically apparent at that point (Barton *et al.*, 2001), leading to worse prognoses for foetal outcomes.

Further laboratory testing includes a complete blood count with platelets, serum creatinine levels, liver chemistries including aspartate aminotransferase (AST) and alanine aminotransferase (ALT).

As a prognostic marker the sFlt-1/PlGF ratio can be determined. These factors should reflect the balance of angiogenic (PlGF) and anti-angiogenic (sFlt-1) components. NICE guidelines state the use of PlGF testing used with standard clinical assessment and subsequent clinical follow-up to help rule-out pre-eclampsia in women presenting with suspected pre-eclampsia between 20 weeks and 34 weeks plus 6 days of gestation. However, when pre-eclampsia is not ruled-out using a PlGF-based test result, the result should not be used to diagnose (rule-in) pre-eclampsia and thus makes sFlt-1/PlGF ratio not an appropriate diagnostic tool for early risk detection (National Institute for Health and Care Excellence, 2019).

Renin-Angiotensin-System

Physiologically, angiotensin II (AngII) regulates systemic blood pressure via the angiotensin II receptor type 1 (AT1R) and has thus been focus of extensive research regarding hypertensive disorders in pregnancy, such as preeclampsia (Malha *et al.*, 2018; LaMarca *et al.*, 2008; Herse *et al.*, 2008; Quitterer *et al.*, 2019). Various studies found an association of different renin-angiotensin system (RAS) components with gestational duration and preterm birth (*AGTR2* (Zhang *et al.*, 2017)), as well as with preeclampsia (Herse *et al.*, 2008, 2007; Herse & Lamarca, 2013). The crucial role of AngII during pregnancy is suggested to be mainly mediated by AT1R (*AGTR1*), which is expressed in the placenta throughout gestation (Cooper *et al.*, 1999; Williams *et al.*, 2010; Lumbers & Pringle, 2014; Pringle *et al.*, 2011). Immunohistochemical stainings showed AT1R in cell columns and syncytiotrophoblast and some expression around foetal blood vessels (Tower *et al.*, 2010; Pringle *et al.*, 2011; Williams *et al.*, 2010), AT2R in cytotrophoblasts (Tower *et al.*, 2010; Cooper *et al.*, 1999), LNPEP, i.e. AT4R, in the syncytiotrophoblast (Williams *et al.*, 2010), see Figure 1.

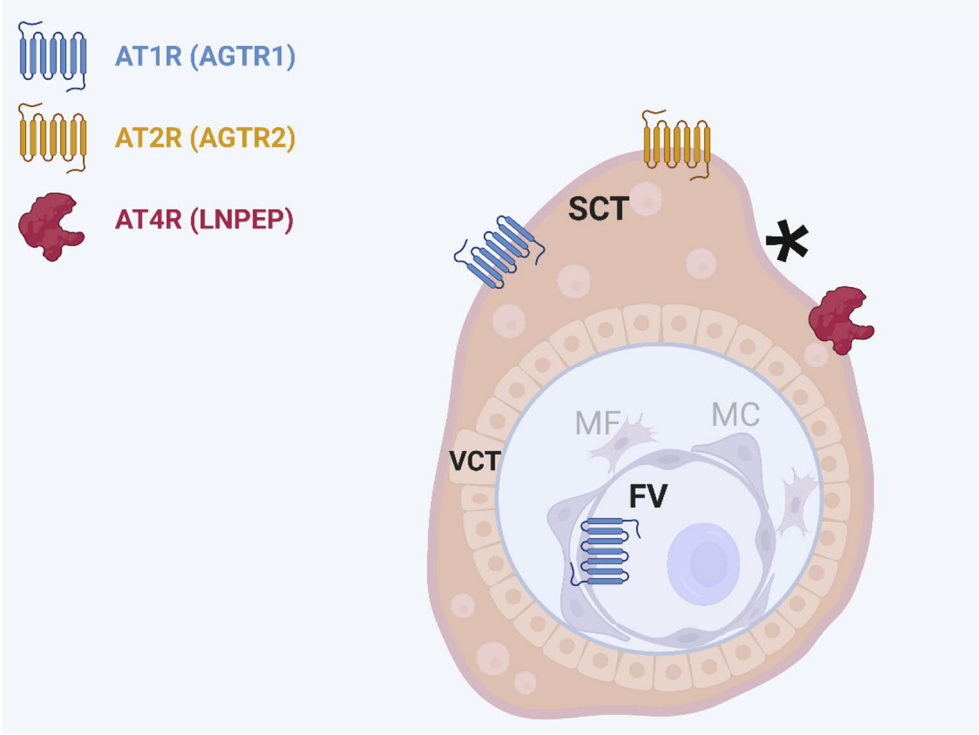

Figure 1. Immunohistochemical localisation of placental RAS in literature. *Previously published literature shows immunohistochemical stainings of RAS components in the foeto-placental unit* (Pringle *et al.*, 2011; Tower *et al.*, 2010; Cooper *et al.*, 1999; Williams *et al.*, 2010). *SCT=syncytiotrophoblast,*

VCT=villous cytotrophoblast, asterisk=intervillous space, FV=foetal vessel, MF=myofibroblast, MC=myocyte; AGTR1/2: angiotensin II receptor type 1 or 2; LNPEP: leucyl and cystinyl aminopeptidase = angiotensin IV receptor = placental leucine aminopeptidase

Besides the classical RAS-pathway, including the substrate angiotensinogen (*AGT*), the enzymes renin (*REN*) and angiotensin-converting enzyme (*ACE*), the resulting peptides angiotensin I and II and their receptors type 1 (*AGTR1*) and type 2 (*AGTR2*), there is an extended RAS ((Nehme *et al.*, 2015; Lumbers & Pringle, 2014); see Figure 2). Enzymes such as ACE2 convert AngII to Ang(1-7) that acts on the Mas receptor. Aminopeptidase N (*ANPEP*), Aminopeptidase A (*ENPEP*), Dipeptidyl peptidase III (*DPP3*) convert products to the final AngIV acting on angiotensin IV receptor (AT4R), also called insulin regulated aminopeptidase (IRAP), oxytocinase, vasopressinase, or placental leucinyl aminopeptidase (*LNPEP*), which produces a soluble form only in human pregnancy (sLNPEP) (Ito *et al.*, 2004a).

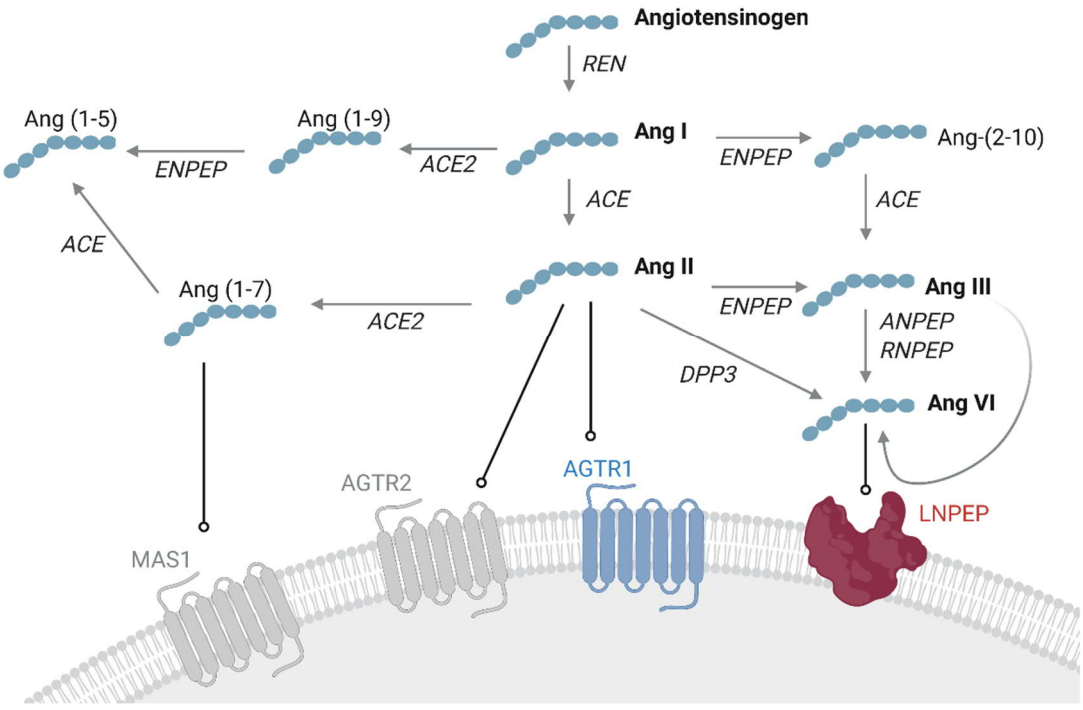

Figure 2. Extended RAS. *Extended RAS with multiple angiotensin conversion products (Ang = Angiotensin) and conversion enzymes (ACE and ACE2: angiotensin conversion enzyme; REN: renin; ANPEP: alanyl aminopeptidase; ENPEP: glutamyl aminopeptidase; RNPEP: arginyl aminopeptidase; DPP3: dipeptidyl peptidase 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. Figure reproduced under the CC-BY Licence from Nonn *et al.*, 2021.

LNPEP

The leucyl and cystinyl aminopeptidase is a metalloprotease and M1 aminopeptidase that has several functions, one of them is being a receptor for AngIV and is therefore known as angiotensin IV receptor (AT4R). Its cofactor is zinc, binding one zinc ion per subunit. Its aminopeptidase activity leads to an AngIII cleavage to AngIV, but also hydrolysis of oxytocin and vasopressin and may be involved in the inactivation of neuronal peptides in the brain, where the AT4R is suggested to be involved in cardiovascular homeostasis, cognition, and behavioural processes (Jackson et al., 2018). The blood brain barrier inhibits peripheral angiotensin peptides to pass through, thus a local RAS production can be found within the brain with a focus in the forebrain and connecting the hypothalamus and medulla. AT4R is a type II aminopeptidase located in cortical and hippocampal neurons as well as in basal ganglia which activation leads to improved cognition, cell signal transmission, synaptic remodelling and was shown to have both antioxidant and anti-inflammatory effects in the brain (Costa-Besada *et al.*, 2018; Chai *et al.*, 2000; Wright, Kawas & Harding, 2015).

Peptidases belonging to the M1 family are – across species - often spatiotemporally regulated with patterns allowing for strict regulation of activity since they are crucially involved in cell cycle and normal growth and development (Peer, 2011). LNPEP is membrane-spanning with a relatively long cytoplasmic domain that contains motifs for stimulus dependent translocation to the membrane and vesicular distribution (Tsujimoto *et al.*, 2008). Located at this cytoplasmic domain are motifs binding tankyrase, a scaffolding protein that regulates Wnt/β-catenin pathway in trophoblasts involved in progenitor cell regulation and GLUT4-associated vesicle residing LNPEP translocation to the membrane (Mariotti, Pollock & Guettler, 2017; Sadler *et al.*, 2019; Knöfler & Pollheimer, 2013). Tankyrases are known to intervene in many physiological profiles such as cell growth and survival by disturbing the Wnt pathway (Lakshmi *et al.*, 2016).

LNPEP shows a variety of functions involved in glucose and energy homeostasis when regarded as insulin-responsive aminopeptidase located in vesicles associated with the glucose

transporter 4 (GLUT4-storage vesicles, GSV). LNPEP deficiency in mice was shown to alter glucose metabolism.

Vasopressinase is also called placental leucine aminopeptidase (P-LAP) for its high abundance in pregnancy. The pregnancy serum form is released by being cleaved presumably by ADAM12 into the soluble leucyl-cystinyl aminopeptidase as found only during pregnancy (Ito *et al.*, 2004b). Oxytocinase activity of LNPEP is the only known to degrade the uterotonic peptide oxytocin and its activity increases over gestation (Nomura *et al.*, 2005). Interestingly, LNPEP was decreased in preterm delivery suggesting a higher level of serum oxytocin, known to induce labour (Kozaki *et al.*, 2001; Kim *et al.*, 2013).

Leptin

Leptin, a pleiotropic hormone, is involved in implantation and pregnancy sustenance (Maymó *et al.*, 2011). In women with spontaneous abortions leptin levels are abnormally low and leptin can have immunomodulating effects. It is known that it exerts antiapoptotic effects, both the early and late events of apoptosis, through the MAPK pathway in the placenta (Pérez-Pérez *et al.*, 2008), which may be one of the reasons for this important role. Leptin also induces protein synthesis via PI3K and MAPK pathways additionally, which is why the expression of leptin is highly regulated in placenta.

First trimester placental villi secrete 50-fold higher levels of leptin compared to term villi and this is upregulated by IL1A, 17b-estradiol and IL6 (Chardonnens *et al.*, 1999). Interestingly, human chorionic gonadotropin (hCG) induces leptin expression in trophoblast cells via MAPK and involving cAMP (Maymó *et al.*, 2009), a molecule that is very important in trophoblast function such as syncytialisation (Forstner *et al.*, 2020).

Histological structure of the human placenta

The human placenta is a temporary organ developed for the duration of pregnancy. As the interface between maternal and foetal sides it enables nutrition, oxygen exchange, and hormonal crosstalk. Pregnancies end with the delivery of the baby - and the placenta.

Early developing placenta

The extra-embryonic membranes, from which it derives, develops when the blastocyst is further differentiating into the embryoblast (inner cell mass) and the trophoblast (trophectoderm). The embryoblast will develop into the later embryo, whereas the trophoblast will make up the embryonic appendices. The blastocyst produces the “decidual reaction” i.e. transformation of the endometrium into decidua and in a crosstalk the trophectoderm differentiates (see Figure 3). Trophoblast cells are very heterogeneous, originating from stem cell populations that continuously differentiate into extravillous trophoblasts or residentiary cytotrophoblasts (Okae *et al.*, 2018). The extravillous trophoblast cells derive from cytotrophoblast cells and migrate and invade the decidua and with it maternal vessels and uterine glands (Weiss *et al.*, 2016), whereas syncytiotrophoblast is formed by fusing cytotrophoblasts. With time, structures reorganise and show floating villi surrounded by maternal blood. While trophoblast plugs are first present allowing only histiotrophic nutrition, maternal blood circulation around the villi is established from week 6 onwards (Roberts *et al.*, 2017). In its mesenchymal core foetal vessels, macrophages (called Hofbauer cells in the placenta) and fibroblast populations can be found. Some villi anchor the placenta to the decidua, which is restructured as well to serve the developmental purpose of the placenta (see Figure 4).

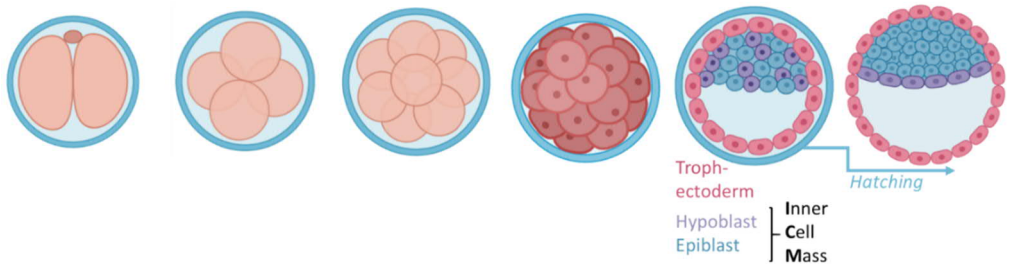

Figure 3. Preimplantation stages. *Embryonal preimplantation stages following conception (Carnegie stage 1-3). In the blastocyst (first and second from right) the trophectoderm later develops into the placenta.*

The placenta is meeting the metabolic needs of the growing foetus across gestation by constantly changing morphology of their villi. The human placenta is haemochorial, discoid, pseudo-cotyledonic, decidual and chorio-allantoic. All these features assure metabolic exchange processes across the placental barrier and that endocrine stimuli to both the maternal and foetal circulation are effective.

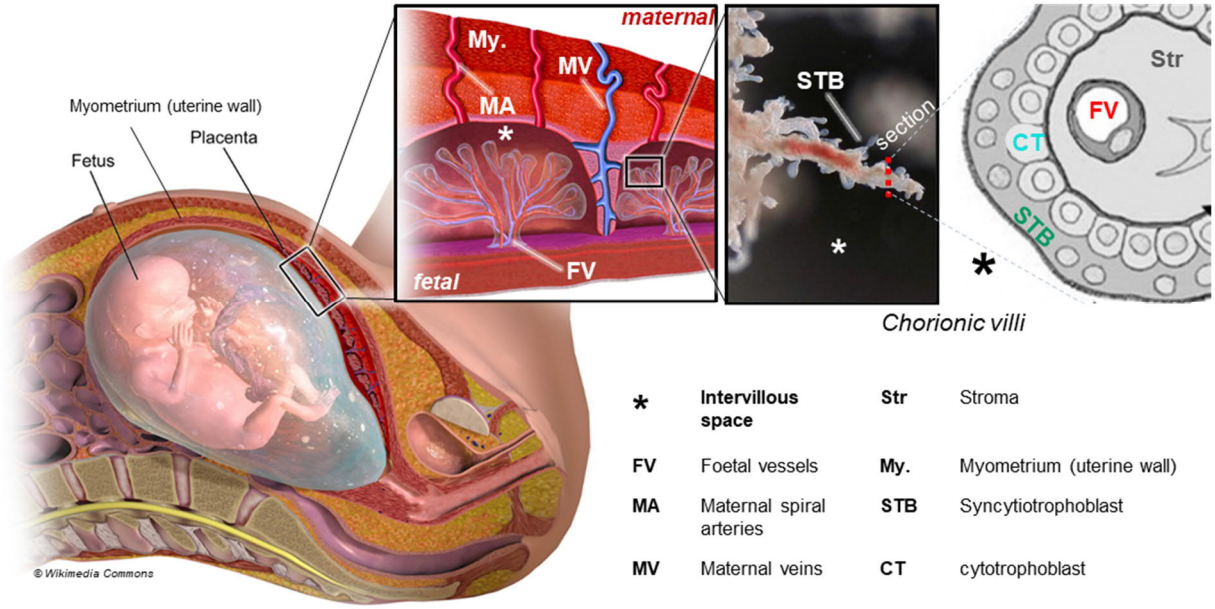

Figure 4. Human placenta. *Human placenta showing how maternal and foetal circulation is separated by a trophoblast layer (STB: syncytiotrophoblast and CT: cytotrophoblast). Maternal spiral arteries open into the intervillous space, which surrounds chorionic villi and is filled with maternal blood. Maternal blood leaves the intervillous space via maternal veins. From the foetal side, foetal vessels in the chorionic villi create a fine network. Exchange between the two systems sees a barrier of trophoblasts, which cover the chorionic villi.*

Extravillous Trophoblast

The mononucleated cytotrophoblasts form cell columns at the tips of villi that anchor and attach to the uterine wall. Within these cell columns, extravillous trophoblasts develop, then detach from the cell columns (Plessl *et al.*, 2015; Fock *et al.*, 2015). Interstitial trophoblast cells may even migrate into the inner third of the myometrium but certainly deep into the decidua,

anchoring the placenta to the mother (figure 5). Extravillous trophoblasts do not only invade interstitial decidual tissue but can be found in the walls of uterine spiral arteries, interacting with maternal endothelial cells and involved for vessel remodelling during placentation (Weiss *et al.*, 2016; Moser *et al.*, 2010; Ji *et al.*, 2013). Trophoblasts in uterine veins, so called endovenous trophoblasts, or those invading into uterine glands (endoglandular trophoblasts) and into uterine lymph vessels (endolymphatic trophoblasts) can be found regularly, suggesting an undirected invasion (Moser *et al.*, 2018). The expression of epithelial, mesenchymal and endothelial markers changes during these processes of invasion and migration.

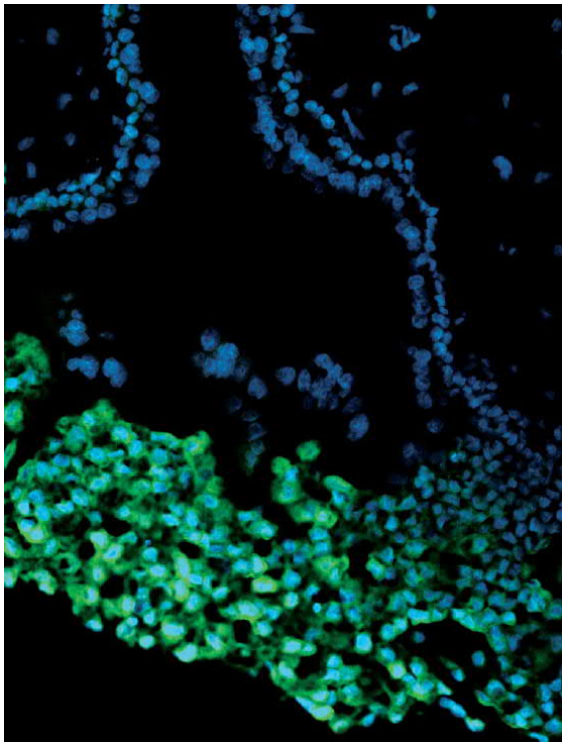

Figure 5. Extravillous trophoblasts in a cell column. *Immunofluorescence staining of a first trimester human placenta, showing an anchoring villus. Cell nuclei are stained with 4',6-diamidino-2-phenylindole = DAPI (blue) and a double layer of syncytiotrophoblast and cytrotrophoblasts can be seen. Extravillous trophoblasts are stained with the specific marker HLA-G (green).*

Syncytium

Fusion of highly proliferative and mononucleated cytotrophoblast at the outer layer then forms a multinucleated layer, the syncytium cells (Simpson, Mayhew & Barnes, 1992). Early on in the developing placenta, this multinucleated – syncytial - cell mass forms lacunae that are subsequently filled with maternal blood. Later, the chorionic villi are floating in the surrounding

by maternal blood covered by a layer formed of syncytiotrophoblast (Huppertz & Gauster, 2011; Knöfler & Pollheimer, 2013; Gauster & Huppertz, 2008; Gauster *et al.*, 2009).

The syncytiotrophoblast is primarily involved in the exchange of nutrients and waste (figure 6) as well as in the hormone production of among others human chorionic gonadotropin (hCG) and human placental lactogen (hPL).

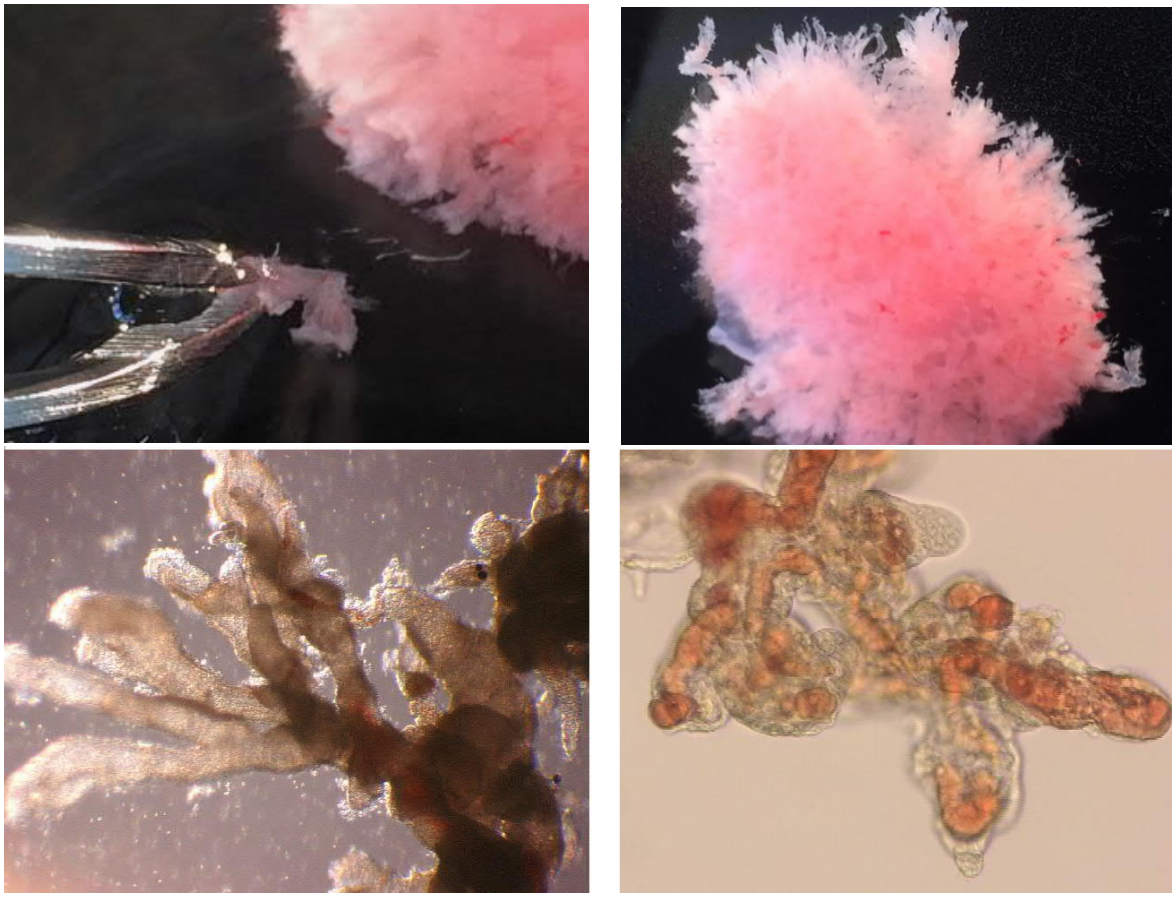

Figure 6. First trimester placental explant. *Placental Explant of a first trimester placenta (GA = 8 weeks). Placental villous tissue from human first trimester (n=7) was rinsed in buffered saline and dissected into small pieces of ~ 5 mg moist mass. Placental explants were cultured in DMEM/F12 (1:1, Gibco) supplemented with 10% FCS, penicillin/streptomycin, and L-glutamine in a hypoxic workstation (BioSpherix) under 2.5% oxygen at 37 °C. Lower Panel shows a picture of chorionic villi covered by a syncytiotrophoblast layer that constitutes the barrier to the surrounding maternal blood (left). Foetal erythrocytes can be seen running through foetal vessels within the chorionic villi (light microscopy image from Sabine Maninger; right hand side).*

Single-Cell RNA sequencing reveals novel cell types and subpopulations

The novel technology scRNA-seq reveals a new insight into these complex systems at the feto-maternal interface. Technologies on a single-cell resolution enable to understand complex processes driven by multiple factors such as recently published literature in high-ranking journals shows (Vento-Tormo *et al.*, 2018; Suryawanshi *et al.*, 2018). Information from single-cell approaches can give a more detailed view of exact processes and roles in affected tissues and help understand complex crosstalk such as receptor-ligand interactions regulating metabolic processes between different celltypes.

Placental single-cell sequencing cell atlas

Considering the methodological approach to obtain scRNA-seq data and process it, it is a new approach to gain insight into cell-specific functional processes as well as crosstalk between cells (Figure 7).

There are three major subsets of decidual natural killer cells that have distinctive immunomodulatory and chemokine profiles, and Vento-Tormo et al. used scRNA-seq to predict the cell-type specificity of cell–cell communication via these molecular interactions in order to identify many regulatory interactions that prevent harmful innate or adaptive immune responses in this reproductive environment. It reveals the cellular organization of the decidua and placenta of the maternal–foetal interface, and the interactions that are critical for placentation and reproductive success. (Vento-Tormo et al., 2018).

Vento-Tormo et al. showed the bioinformatically calculated differentiation trajectory to either VCT or EVT and additionally predicted the ligand-receptor interactions that they suggested to be likely to regulate processes involved in differentiation. They also suggested that possibly adverse adaptive and innate immune responses to placental development were reduced, where the largest subset of decidual immune cells, natural killer cells, were probably mediating trophoblast invasion and coordinating multiple immunomodulatory pathways. Similar to tumour cell immune evasion, maternal immune responses were found by Vento-Tormo et al. to be diminished by cell-surface expression of checkpoint inhibitors such as PD1, PDL1 or TIGIT, tethered ligand–receptor complexes, secreted proteins, and small molecules such as adenosine or steroid hormones (Vento-Tormo *et al.*, 2018).

Suryawanshi et al. on the other hand identified several subtypes from *ex vivo* placental tissue, such as both first- and third-trimester VCTs showing a subpopulation of proliferating cells expressing MKI67 and TOP2A, that they suggest are likely reflecting the continuous process of VCT to EVT differentiation throughout the pregnancy (Suryawanshi *et al.*, 2018).

They also found third-trimester EVTs to highly express the protease matrix metalloproteinase 11 (*MMP11*), which is specific for substrates such as IGFBP1 and COL6A3, both of which were highly expressed by decidual stromal cells in their data. MMP12 was also expressed by first-trimester EVTs, known for its role in dampening the inflammation process, rather than MMP11 degrading collagen possibly indicating a possible shift of EVTs from being anti-inflammatory in the early stage to highly invasive in the later stage of pregnancy (Suryawanshi *et al.*, 2018).

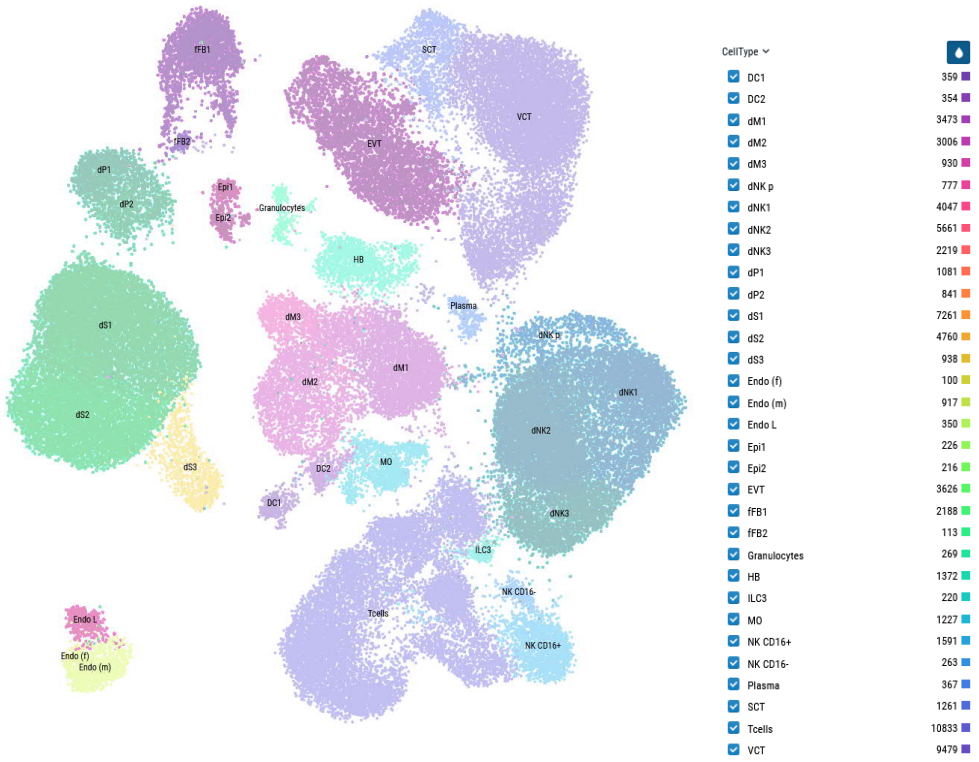

Figure 7. scRNA-seq data from first trimester placental tissues. *UMAP embeddings of scRNA-seq data is shown with the cell type annotations used in expression heatmaps. Data for first trimester placenta was obtained from Vento-Tormo et.al, 2018* (Vento-Tormo *et al.*, 2018) *and visualised with cellxgene v0.16.4* (Megill *et al.*, 2020) *and reproduced under the MIT license; "d" for decidual derived cells, "f" for foetal-derived cells: VCT: villous cytotrophoblast; SCT: syncytiotrophoblast; EVT: extravillous trophoblast; HB: Hofbauer cells; fFB1-2: foetal fibroblast; Endo: endothelial cells.*

1. Single cell transcriptomics

Generally speaking, the basic question defines the different needs in the pre-platform protocols (Table 1). To detect and analyse cell populations that are underrepresented in the tissue of origin, pre-enrichment is crucial. If the goal is to have an undirected approach to sequencing in order to detect yet unknown cell populations, enrichment might bias the representation of cell populations in the final data sets.

Table 1. Different approaches to sequence single cells in published articles using placental tissues

	Suryawanshi et al., Science Adv. 2018	**Vento-Tormo et al., Nature 2018**	**Liu et al., Cell Res 2018**	**Tsang et al., PNAS 2017**
Sample processing	Needle aspiration, enzymatic digestion, cell strainer	Scalpel, enzymatic digestion, cell sieve	Scalpel, enzymatic digestion, cell strainer, Percoll, enrichment (pos. Selection)	Enzymatic digestion, cell strainer,
Single cell solution	Trypan Blue, automated cell counting	FACS / DAPI		microscope
Sc-RNAseq	Droplet (Drop-Seq and Chromium 3′ 10X) – NextSeq 500	droplet (10x Chromium) – HiSeq 2000	Smart-seq2 + (low throughput) - HiSeq 2000	Droplet (Chromium 3′ 10X) – NextSeq

While there are multiple commercial platforms that developed different approaches to sequence single cells, the principle is similar (table 2). Since it is the cheapest and most widely available technique, droplet-based sequencing on a 10x Genomics platform is used here as the basic example to get scRNA-seq data, which is transcriptomic sequencing data gathered from single cells in a tissue compound.

Table 2. Various methods available for single cell sequencing

	Application	Sample per run	Costs per sample	Reliability	sensitivity	Cell recovery	Cell #
Chromium 	RNA (3‘, 5‘), DNA, CNV, Immune, ATAC	1-8	+++	++++	+	65%	800-80.000
inDrop 	RNA (3‘)	1	++	++	+	20%	100-40.000
ICELL8 	RNA (3‘, full-length), ATAC	1-8	+ - ++++++++	+++	+++	5-15%	<1800
C1 	RNA, DNA, diverse	1/2	++++++	++	+++	2.5-10%	<800
FACS-seq 	RNA, DNA, diverse	1	+++- ++++++	+	++	100%	<400
CellenOne 	RNA (3‘, full-length), DNA, CNV, Immune, ATAC	1	+ - ++++++++	?	+++	90%	5000

The most crucial part in scRNA-seq that enables the manifold possibilities of new approaches to data analysis is certainly the bioinformatical post-processing after receiving raw sequencing data output. While most of it is similar to bulk sequencing, meaning the classical sequencing of tissue samples without reattribution to single cells, some problems need to be solved in terms of the problem that data needs annotation not only to sample but also to single cells as the origin of transcripts. To achieve single cells being barcoded, in droplet based sequencing techniques a microfluidics partitioning approach is used. Cells are mixed with the reagents needed to lyse cells and for reverse transcription (RT), a gel bead is used for providing barcode sequences. Each droplet, also called GEM (gel beads in emulsion), thus ideally contains a gel bead with unique barcodes, a single cell, RT reagents, lysis buffer (see Figure 8).

The oligonucleotide primer that are introduced via the gel bead surface contain different sections. The sequencing adapter depends upon the next generation sequencing (NGS)

platform used, the barcode is the unique molecular address for each GEM, thus a single cell. The unique molecular identifier (UMI) on the other hand is used to later be able to accurately quantify each transcript. It only exists once in a given run.

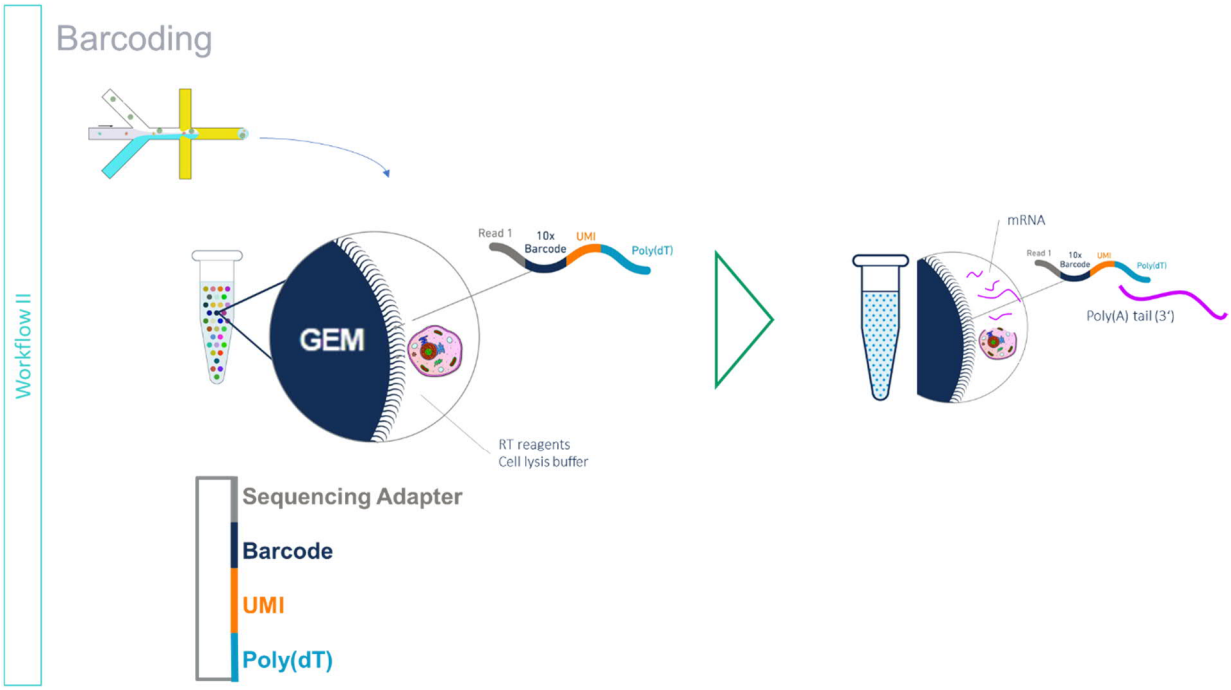

Figure 8. Principles of barcoding in drop-seq techniques. *Microfluidics are used to create water-in-oil based emulsions of so-called functional GEMs, containing a single Gel Bead with barcodes, a single cell and RT reagents. GEMs are a reaction vesicle in which a single cell is lysed the gel bead is dissolved to release identically barcoded RT oligonucleotides and then reverse transcription occurs. Gel beads that are added to droplets contain oligonucleotide sequences that add a unique code with different functional units to single cell mRNA. The cell is lysed within the droplet and its mRNA attaches with its poly(A)-tail to the poly(dT) tail of the gel bead sequence.*

Crucial to sequenced single cell RNA data is post-processing like creating gene-cell matrices to facilitate expression analyses but also quality control measures to exclude technically faulty droplets. A barcode analysis reattributes aligned reads to a cell. However, some limitations occur – since cell-to-barcode processing is mechanical, it might occur that a droplet with one set of barcodes either encloses no cell or two and more. Before expression analysis it is therefore necessary to perform quality control protocols to exclude non-relevant data.

The data analysis from Vento-Tormo et al. (Vento-Tormo *et al.*, 2018) used cells with fewer than 500 detected genes and for which the total mitochondrial gene expression exceeded 20% were removed. A small number of transcripts per cell barcode is often an indicator for cell encapsulation of cells death, premature cell rupture, or empty droplets which only captured ambient mRNAs. Cut-off levels are standardly chosen based on the tissue examined, for instance blood cells may have lower levels of detected genes since they are rather quiescent compared to actively dividing cancer cells for example. High expression levels of mitochondrial genes could be an indicator of poor sample quality, leading to a high fraction of apoptotic or lysing cells. The process of apoptosis is dependent on a cascade of signalling events that includes the increased expression of mitochondrial genes and the activation of caspases (Wang, 2001; Newmeyer & Ferguson-Miller, 2003). A cut-off above 10% is suggested for human samples (Osorio & Cai, 2020).

UMAP, uniform manifold approximation and projection, is a statistical approach for dimension reduction of high-dimensional data and project it as here two dimensionally. Different data sets can be summed up, i.e. “integrated”. However, analyses are extensive and ever-evolving and can be used for a plethora of scientific questions. Downstream analyses — such as shared nearest neighbour graph-based clustering as seen in UMAP and calculated computationally, differential expression analysis between samples, and visualisation (see Figure 7) —are mostly performed using the R package Seurat.

2. *Adding spatial context*

Recently scRNA-seq data has been put into spatial context. One of the limitations of scRNA-seq data is that even if cell populations are distinguished quite well, it is achieved purely by bioinformatical analyses. One of the examples where this limitation is well visualised is for instance with the problem of cell populations interspersed in the tissue of origin. In scRNA-seq these cells will be statistically similar based on similar transcriptomes and thus adjacent in purely computed embeddings such as tSNE or UMAP - but when using *in situ* methods to find those cells it would soon be clear that they are in fact not adjacent, such as was done here in this dissertation. The spatial context of scRNA-seq data is therefore not unimportant to answer certain hypotheses (Figure 9).

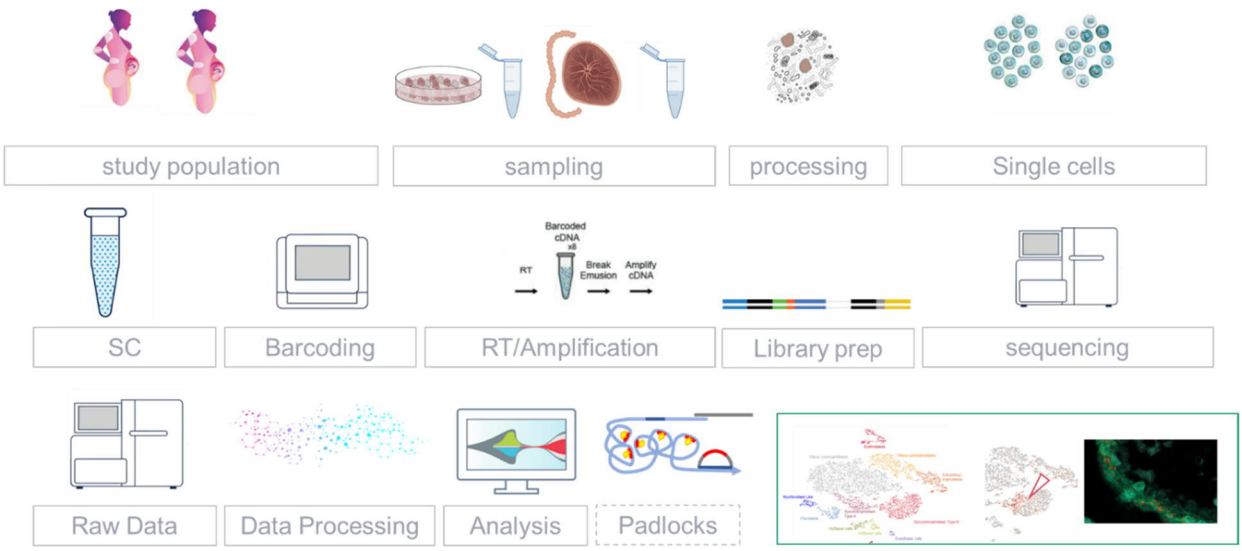

Figure 9. Summary of scRNA-seq workflows and spatial context as used in this dissertation. *To distinguish localisation of certain cell populations as found in scRNA-seq, padlock probes were used to find the mRNA transcripts in situ relating to the spatial distribution of the scRNA-seq signal (green box).*

These new insights into the complex array of subpopulations of cell types that are part of an interdependent interactome of receptor-ligand pairs and other processes like metabolic shifts shows how highly orchestrated and poorly understood placental and thus embryonic and foetal development is. The method of using scRNA-seq allows an entirely new model of how to study placental functions based on celltype-specific expression of system components and their validation *in situ.*

Justification of the research question, aim of the dissertation, hypotheses, novelty value and limitations of the research topic

RAS components have not yet been specifically investigated in the published works, but were sequenced as part of the overall placental transcriptome. The raw scRNA-seq data published by Vento-Tormo et al. (Vento-Tormo *et al.*, 2018) was an excellent basis to explore RAS expression and function and indeed demonstrated a unique picture of the placental RAS differently than previously suggested (Williams *et al.*, 2010; Lumbers & Pringle, 2014). Here, RAS components showed a segregated localisation in different cell types.

Based on this, maternal and foetal angiotensin derivates would be two separate systems contrary to published opinions. The novelty and relevance of the work is justified by using a novel single-cell based approach combined with *in situ* spatial information and the investigation of pathways crucial to early development.

This led us to the hypothesis, that maternal angiotensin impacts hypertensive disorders in pregnancy by an alternative RAS pathway.

Material and Methods

Methods were similarly described in Nonn *et al.*, 2021.

Tissue Collection

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). 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 of the placenta (storage at +4°C immediately by midwives), a tissue sample (1x1x1cm; randomly chosen by the person sampling) of the medial third of the placenta was cut from vital cotyledons that were macroscopically free of infarct areas or other obvious pathologies that are assumed to have happened during delivery. This should avoid sampling degraded RNA in infarcted areas and ensure a yield high enough for further analysis, well knowing that it might skew towards possibly inaccurate phenotypes on either side of disease and healthy samples. 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.

All different samples were as quickly as possible processed to isolate RNA and reversely transcribe it into the more stable cDNA. Storage did not exceed recruitment periods.

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). Placental tissue was immediately after surgical extraction put in culture medium (DMEM/F12 1:1, 1g/dL glucose, Gibco®, Life Technologies (TM), Thermo Fisher Scientific, Vienna, Austria) on +4°C and then processed within 1-4 hrs after the surgical intervention. Amnion and decidua were dissected and remaining tissue were rinsed twice in NaCl 0.9% 4°C to remove blood, then snap frozen in liquid nitrogen and stored at -80°C until processing.

Sample Collection: Third trimester Control Tissue

Third trimester 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 Gynaecology, 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 fertilisation. 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. Serum samples for the determination of serum angiotensin levels were collected in VACUETTE® with CAT Serum clot activator (Greiner Bio-One, Kremsmünster, Austria), stored at +4°C until processing. Tubes were centrifuged at +4°C at 2000 rpm for 10min and the supernatant then stored at -80°C until analysis.

Sample Collection: Pre-eclamptic placentae from Graz

Study samples were recruited retrospectively immediately after delivery at the inpatient clinic of the Department of Obstetrics and Gynaecology, 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. Early-onset preeclampsia was defined as delivery prior to gestational week 34. Sampling was performed from each of the four quadrants of the

placenta, maternal and foetal membranes were removed and villous tissue was washed in PBS. Samples were frozen in liquid nitrogen and stored at -80°C until use.

Table 3. ISSHP Guidelines

Hypertension	
Defined as systolic BP ≥140 and/or diastolic BP ≥90 mmHg.BP should be repeated to confirm true hypertension.	If BP is severe (systolic BP ≥160 and/or diastolic BP ≥110 mmHg), then the BP should be confirmed within 15 minutes
Gestational Hypertension	
Gestational hypertension is persistent de novo hypertension that develops at or after 20 weeks' gestation in the absence of features of preeclampsia.	
Preeclampsia	Preeclampsia is gestational hypertension accompanied by ≥1 of the following new-onset conditions at or after 20 weeks' gestation:
• Proteinuria • Other maternal organ dysfunction, including: • AKI (creatinine ≥90umol/L; 1 mg/dL) • Liver involvement (elevated transaminases, eg, alanine aminotransferase or aspartate aminotransferase >40 IU/L) with or without right upper quadrant or epigastric abdominal pain	• Neurological complications (examples include eclampsia, altered mental status, blindness, stroke, clonus, severe headaches, and persistent visual scotomata) • Hematological complications (thrombocytopenia–platelet count <150000/μL, disseminated intravascular coagulation, hemolysis) • Uteroplacental dysfunction (such as fetal growth restriction, abnormal umbilical artery [UA] Doppler wave form analysis, or stillbirth)

Sample Collection: Pre-eclamptic placentae from Oslo

Pregnant women were recruited prior to elective caesarean section after informed written consent, as previously described(Johnsen *et al.*, 2018), 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 centre 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 (Prefumo, Sebire & Thilaganathan, 2004) 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 foetal 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 (Leslie *et al.*, 2015). 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.

Placental explants

First trimester placental tissue was collected from women undergoing an elective termination of pregnancy (TOP) before week 12, with an ethical vote from the Medical University of Graz and after obtaining informed consent from the patients. Placental tissue was processed within 1-4 hrs after the surgical intervention, while in the mean time being stored in culture medium at +4°C (see above). Subsequently to a wash 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 (Siwetz *et al.*, 2016). Tissue was then homogenised 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 physiologic conditions with 2.5% oxygen for indicated time points at 37°C for 3h, 6h and 24h, while being submerged in wells with 4ml of medium.

Cell Culture

For treatments, culture medium was supplemented with freshly prepared treatment dilutions, stock solutions were stored at -20°C for the maximum time indicated by the distributor. Angiotensin II was used at a working concentration of 0.1 µM (stock solution 1mM diluted in H_2O). Candesartan, an AT1R blocker (ARB), was used at 0.1 µM (1 mM stock solution in DMSO, then diluted in H_2O). For timepoints as indicated cells or explants were treated with 0.1µM AngII (Sigma-Aldrich, Merck, Vienna, Austria). Recombinant human Leptin (Gibco® Life Technologies) was added to the medium with a working concentration of 100 ng/ml as measured in maternal serum (100 ng/ml = 6.25 nmol; stock concentration 1mg/ml in 0.1% BSA). AngIV (Sigma-Aldrich, Merck, Vienna, Austria) was used at 4 nmol working concentrations for the timepoints indicated in figures. DMSO at the same volume served as solvent control substance.

BeWo cells were purchased from the European Collection of Cell Cultures (ECACC) and were cultured in DMEM/F12 (1 : 1, Gibco, life technologies; Paisley, UK) supplemented with 10% FCS (Gibco), penicillin/streptomycin (Gibco), and L-glutamine (Gibco) in a humidified atmosphere of 5% CO_2 at 37 °C. Cells between passage 10 and 20 were used for in vitro experiments. DMSO served as the isovolumetric vehicle control for treatments and was applied at a final concentration of maximum 0.001% (v/v).

Isolation of primary cells

Isolated mRNA for both isolated primary cells (endothelial colony forming cells = ECFC, trophoblasts) were kindly provided by the Perinatal Research Laboratory, Department of Gynaecology and Obstetrics, University Hospital of the Medical University of Graz. For isolation and cultivation of ECFCs, mononuclear cells were isolated from cord blood by density gradient centrifugation and plated onto 6-well tissue culture plates. Cells were maintained in endothelial cell growth medium 1 [EGM-1; Lonza, Basel, Switzerland; supplemented with supplier-recommended concentrations of human recombinant epidermal growth factor,

fibroblast growth factor, VEGF, ascorbic acid (vitamin C), hydrocortisone, and recombinant insulin-like growth factor] with 10% FBS at 5 × 10^7 cells/well on collagen-coated sixwell plates (BD Bioscience, Heidelberg, Germany) and incubated at 37°C in an atmosphere of 5% CO_2. Passages 2–4 were used in experiments. To further characterize the isolated ECFCs and to confirm their phenotype, flow cytometric analyses using surface markers CD31, CD34, CD133, VEGFR-2, and CD45 as well as appropriate isotype controls as described (Duda *et al.*, 2007) with minor modifications were used. ECFC colonies with cobblestone morphology appeared after a few days. After reaching confluency, cells were transferred into cell culture flasks and further expanded. For the isolation of first trimester trophoblasts, placental villi were digested with Dispase/DNase and Trypsin (Gibco, Invitrogen). After Percoll (Gibco) gradient centrifugation, cells were incubated with magnetic beads conjugated with anti-CD90 and anti-CD45 (Dako) antibodies for 30 min and placed on a DynaMag-15 magnet (Thermo Scientific) to remove fibroblasts and common leukocyte antigen expressing cells. Functional activity and purity of trophoblast isolations were assessed by secretion of human chorionic gonadotropin (hCG) (Dade Behring) and immunocytochemical staining for cytokeratin 7 (Dako, 1:750) and human leukocyte antigen (HLA)-G (BD-Biosciences, 1:500), respectively. Only functionally active preparations with a purity ≥95% were used (Blaschitz *et al.*, 2000).

Viability Assay

To test for possible cytotoxicity of the compounds used for treatment, the lactate dehydrogenase (LDH) release was analysed. Culture medium supernatants of the cells were centrifuged at 2000rpm for 5min and LDH release determined with an LDH cytotoxicity detection kit (TaKaRa Clontech, Saint-Germain-en-Laye, France) according to the manufacturer's protocol, measured at 490nm with SparkTM 10M (Tecan, Männerdorf, Switzerland). Positive high controls were cells treated with Triton™ X-100 for maximum cytotoxicity (Sigma-Aldrich, Merck, Vienna, Austria), cell culture medium background and low untreated controls were used to test for cytotoxicity of each of the compounds' highest concentration (AngII, AngIV, Candesartan) 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 cell scraper. 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, foetal 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 via padlock probes

In situ detection of mRNA with padlock probe technology was used to localise AGTR1 and CGB mRNA transcripts. For application of the *in situ* detection padlock probe method samples will be dehydrated using an ethanol series of 70%, 85%, and 99.5% for 1 min each and stored at −80 °C until use. In situ mRNA detection using padlock probes is based on in situ reverse transcription of the target mRNA and linear oligonucleotide sequences, i.e. padlock probes, showing a cDNA target binding sequence at each end and a binding sequence for detection probes for visualisation (Figure 10; Table S2). After the ligation process a circular oligonucleotide is formed and amplified to increase possible fluorescent detection probe sequences (26–28).

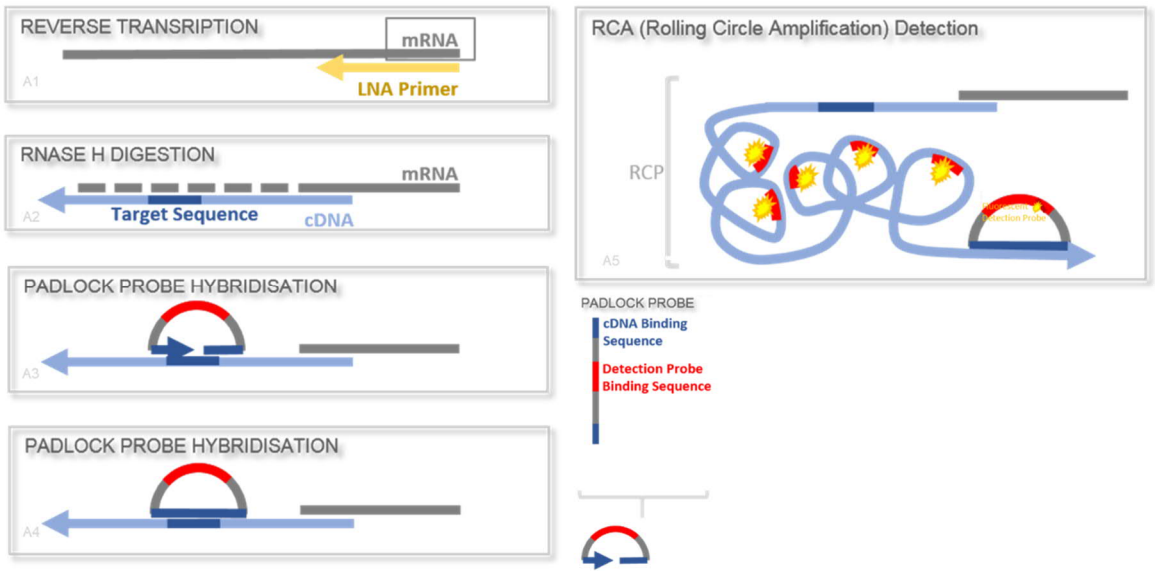

Figure 10. Principle behind in situ detection of mRNA with padlock probe technology. *(A) Schematic representation of the procedure, adapted from Larsson et al., 2010, Nature Methods and Weibrecht et al., 2013 Nature Methods. (A1) mRNA in situ is reversely transcribed by using so-called locked nucleic acid primers (LNA primers) (A2) the target sequence is now on the synthesised cDNA strand while the original mRNA is digested using a RNase (A3) Padlock probes specific to the target sequence are hybridised to it (A4) the padlock probe is ligated to form a circular oligonucleotide (A5) amplification occurs and produces a rolling circle amplification product (RCP), detection probe binding sequences (red) are thus multiplied and the final fluorescent detection probe that binds to the sequences creates a dot-like transcript detectable by fluorescent light microscopy (B) The linear oligonucleotide sequence, i.e. padlock probe, showing a cDNA target binding sequence at each end and a binding sequence for detection probes for visualisation. After the ligation process a circular oligonucleotide is formed.*

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 foetal 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 foetal vessels were calculated automatically.

Gel Electrophoresis

Loading dye (6x, Thermo Fisher) was added to unpurified PCR products and loaded with onto a 1% agarose gel (agarose: Biozym; supplemented with 0.01% GelRed, Biotium). Images were acquired with FluorChem Q System (Alpha Innotech, Cell Bioscienes, Santa Clara, CA, USA), the bands of DNA fragments were compared to a DNA ladder (100 bp, Thermo Fisher). Running time was 60min.

Immunoblotting

Placental tissue was homogenised 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).

After incubation, the cells were washed with PBS and lysed in RIPA buffer (Sigma-Aldrich, Saint Louis, MO, USA) including protease inhibitor cocktail (1:25, 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; running time 60min). Blotting on a 0.45 µm nitrocellulose membrane (Hybond, Amersham Biosciences, GE Healthcare Life Sciences, Little Chalfont, UK; transfer time 1:30h) 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 normalisation monoclonal anti-beta actin antibody (12.4 ng/mL, clone AC-15, abcam, Cambridge, UK) was used. Antibodies 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) were used as secondary antibodies and incubated on membranes for 2h at RT (as indicated in Table S3). 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).

Immunofluorescence Staining

Deparaffinised first trimester and term placenta FFPE sections were labelled with primary antibodies (see Table S3) and goat anti-mouse or anti-rabbit Alexa Fluor 555/633/488 (1:200,

Invitrogen, Carlsbad; CA, USA) for 30 min each. For deparaffinisation, FFPE slides were washed in the xylene-substitute TissueClear® (Sakura, Germany) and then in a descending alcohol series (100%, 96%, 70% v/v) followed by distilled water for rehydration. After three washing steps in PBS and a 10-min blocking step with irrelevant mouse IgG1 (10 µg/ml, DakoCytomation) the sections were be labelled with FITC conjugated anti-keratin antibody (Dako, Denmark). Nuclei are stained with DAPI by mounting sections with Vectashield (Vector Laboratories, Burlingame, CA, USA).

FACS Analysis

BeWo cells 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 Angiotensin Measurement was performed by Attoquant Diagnostics, Vienna, Austria. Briefly, 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. It serves to achieve a stable composition of the highly instable degradable RAS-components such as AngI, AngII, AngIII, and AngIV. 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 (Prefumo, Sebire & Thilaganathan, 2004; Basu *et al.*, 2017; Kovarik *et al.*, 2015). 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 that statistically significant were examined with the functional enrichment analysis web tool WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) (Zhang, Kirov & Snoddy, 2005; Liao *et al.*, 2019).

Seahorse XF Experiments

BeWo Cells were seeded onto a 96-well Seahorse XF cell culture microplate and incubated for 24h in DMEM/F12 medium (1:1, supplemented with Glucose, Pyruvate, 10% FBS, 1% Penicillin + Streptomycin; Gibco® Life Technologies) at 37°C before conducting the Seahorse XF Cell Mito Stress Test Kit. Compounds were added to culture medium provided either with the Seahorse XF Cell Mito Stress Test Kit (Seahorse XF, Agilent Technologies, Santa Clara, CA, United States) when an incubation time of max. 1 h was chosen or to the cell culture medium. For treatments, the according culture medium was supplemented with Angiotensin II at a working concentration of 0.1 µM or 1µM, Angiotensin IV at 0.1, 1, 4, 10 and 40 nmol and Leptin at 1, 10, 100 ng/ml. ECAR and OCR were determined adding D-Glucose to a final concentration as found in the culture medium (17.5µM), Oligomycin (Mitochondrial Complex V Inhibitor) to a final concentration of 10µM, BAM15 (Mitochondrial Uncoupler) to a final concentration of 10µM, and Rotenone (Mitochondrial Complex I Inhibitor) and Antimycine (Mitochondrial Complex III Inhibitor) to a final concentration of 10µM each. Cell number for normalisation was determined via flow cytometry with a Hoechst staining (1:500).

Single Cell RNA sequencing data

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 three cells were also removed. Further bioinformatical analysis was done using R package Seurat and RStudio Apps (Heatmaps); UMAP and tSNE projections were imported into Cell Loupe Viewer (version 3.0, 10x Genomics) for visualisation and cell annotation purposes.

Sc-RNAseq data published by Pique-Regi et al. was used to evaluate effects of preterm labour, labour and no labour term deliveries by accessing data at http://placenta.grid.wayne.edu/ (GSE114037).

Statistical Analysis

Statistical analysis was performed with IBM SPSS Statistics 25 and R 3.5.0. Graphs were created with GraphPad Prism. Excluded cases were due to outlier testing. Obtained data was first analysed 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's and Holm-Sidak post-hoc test. Homogeneity of variances was tested for by Levene's test and, when appropriate, Welch's correction was used. A linear regression model was applied in the cohort data for the analysis of possible predictors.

Results

Results were similarly described and figures were modified from Nonn *et al.*, 2021 under the CC-BY licence.

Maternal and foetal angiotensins have different target placental RAS receptors

We analysed expression levels of RAS components in annotated scRNA-seq data from first trimester placentae (Figure 11 left; annotation see Figure 11 right). *AGT* (gene encoding for the AngII precursor angiotensinogen) was not detectable in placental tissue and must therefore be supplied by either the maternal or the foetal circulation. We further investigated the expression of Ang-converting enzymes *REN, ACE, ACE2, ENPEP, ANPEP, RNPEP, DPP3* (Figure 2, modified from (Nehme *et al.*, 2015)), which were partially present in the placenta to convert exogenous Ang depending on cell type (Figure 11). Out of four known Ang-receptors, only *LNPEP* and *AGTR1* were expressed in placental tissue in both third trimester and first trimester placenta (Figure 11; Figure 12). The cell cluster annotated as syncytiotrophoblast (SCT) expressed *LNPEP* as the only Ang receptor, whereas *AGTR1* was exclusively expressed in cells annotated as fibroblasts (fFB2). Two other RAS receptors, *MAS1* and *AGTR2*, were lacking placental expression. The same expression patterns were found in third trimester placentae (Figure 12).

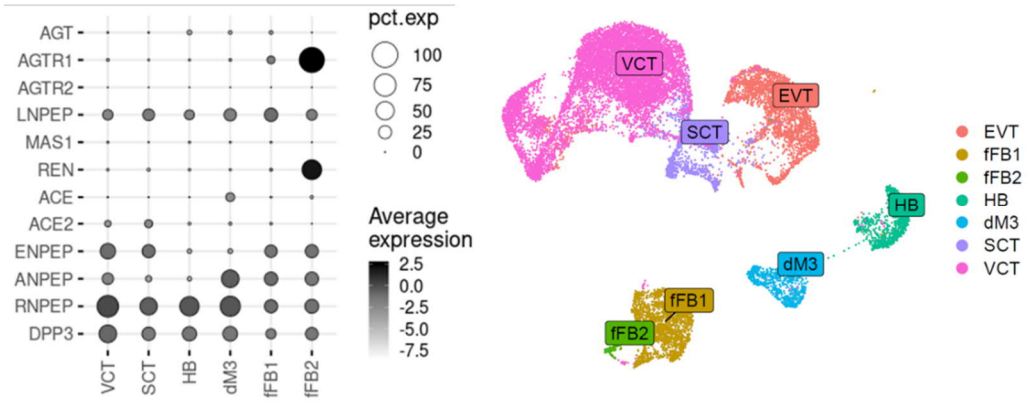

Figure 11. Placental RAS Expression in First Trimester. *Dot-plot of RAS component expression in first trimester placenta (sc-RNA-seq data from Vento-Tormo et al. 2018). UMAP and tSNE embeddings of scRNAseq data is shown with the cell type annotations used in the expression dot-plot on the left-hand side. Raw data and cell type annotation for first trimester placenta was obtained from Vento-Tormo*

et.al, 2018 (Vento-Tormo *et al.*, 2018) *and computed i.e. visualised with our own tool; Annotation by Vento-Tormo et al.: VCT: villous cytotrophoblast; SCT: syncytiotrophoblast; EVT: extravillous trophoblast; HB: Hofbauer cells; dM1 – dM3: decidual macrophages; fFB1-2: foetal fibroblast 1 and 2;*

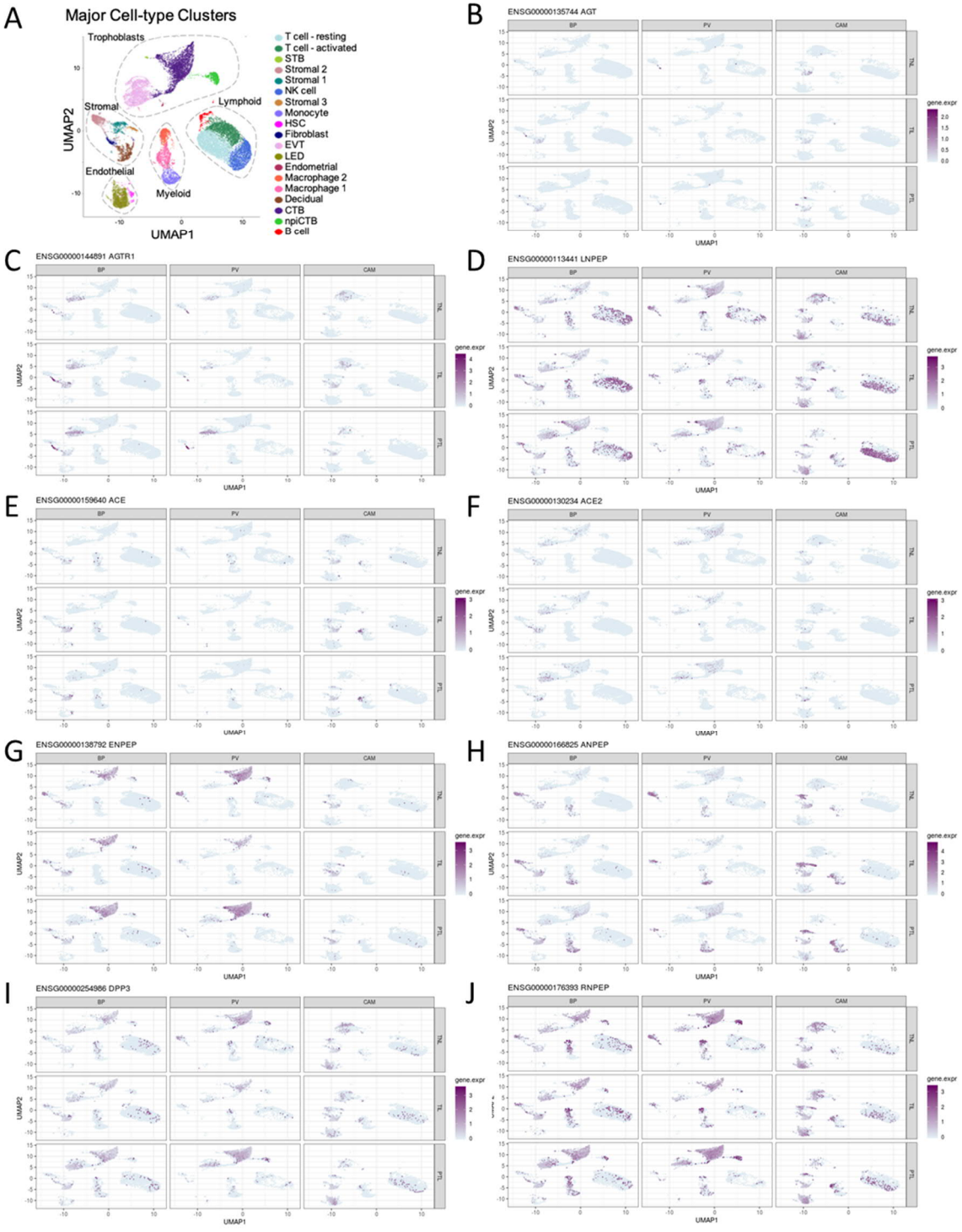

Figure 12. Extended RAS in scRNA-seq data from term and preterm samples. *Placental extended RAS expression in control term samples. (A) Single cell RNA seq data from 25 scRNA-seq libraries published by Pique-Regi et al., 2019 who are kindly providing their data via http://placenta.grid.wayne.edu/ Pique-Regi 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)

Since both active receptors AGTR1 and LNPEP showed a different cell type expression (as shown above in Figure 11A), we hypothesised two independent RAS pathways acting on the developing placenta, one of them foetal directed towards *AGTR1* and the other maternal directed towards *LNPEP* as the target receptor (Figure 13A). We therefore validated their localization in first trimester placental tissue sections (Figure 13B-E). *CGB* transcripts were localised with padlock probe technology combined with an immunofluorescence staining for β-hCG, showing mRNA transcripts localized with the protein (Figure 13B, left). *CGB* encoding for human chorionic gonadotropin beta subunit (β-hCG), a marker for syncytiotrophoblast (Figure 13B, red arrow), was expressed in the same cluster as *LNPEP* (Figure 13C, red arrow). An immunofluorescence staining against LNPEP shows its localization in the syncytiotrophoblast layer (Figure 13C, left).

scRNA-seq expression shows *AGTR1* in cells expressing *ACTA2*, encoding for smooth muscle actin (SMA), a vascular smooth muscle cell marker (Figure 13D-E, blue arrows). Immunofluorescence staining with endothelial marker CD31 reveals that cells around foetal vessels are stained for SMA (Figure 13D, right). *AGTR1* transcripts were localised in first trimester placental tissue sections using specific padlock probes for mRNA *in situ* hybridisation (Figure 13E, right) and were found to be localised, in line with the scRNA-seq data, around foetal vessels (Figure 13E, blue arrow).

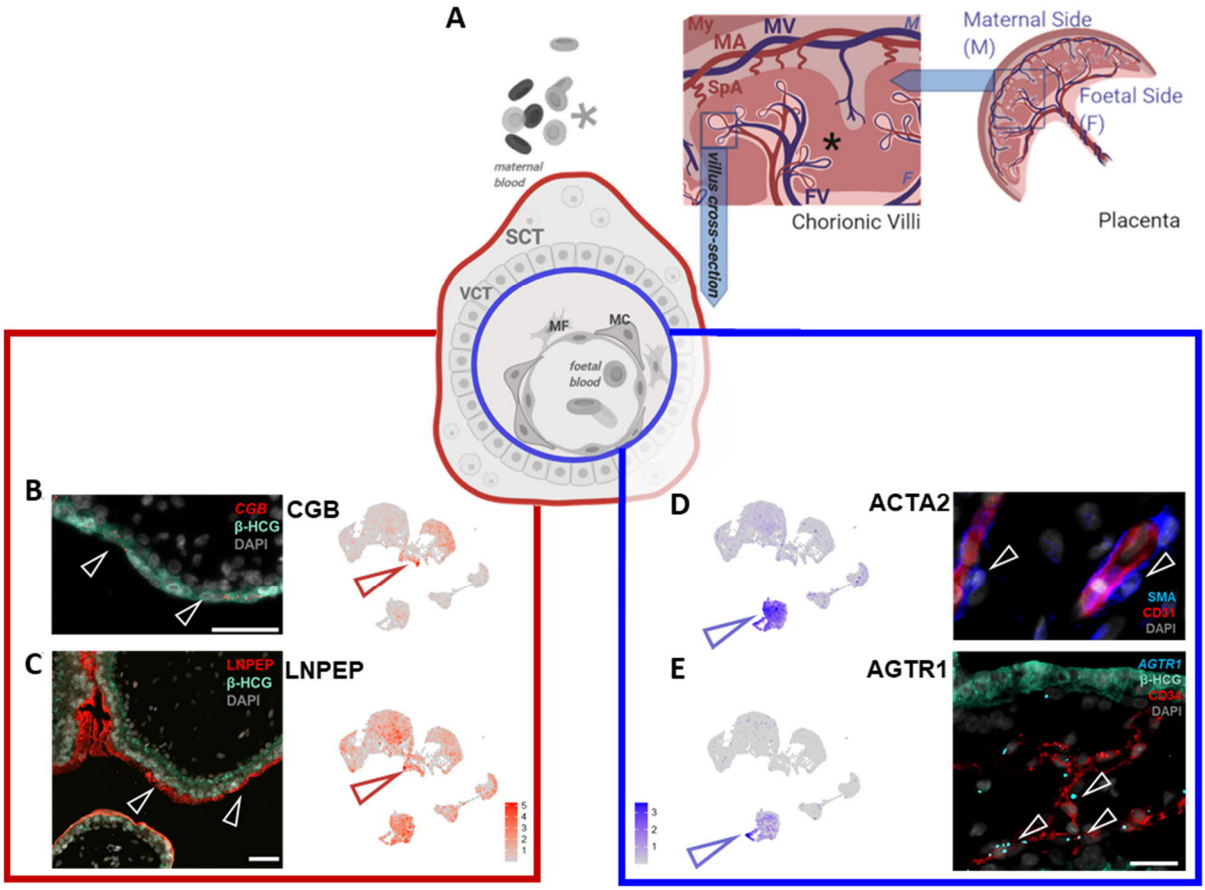

Figure 13. Different target receptors for maternal and foetal angiotensin. *First trimester villous tissue was used, see schematic representation of a first trimester chorionic villus cross section). Expression and localization of RAS components in single cell RNA-sequencing data from first trimester: 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. (A) Chorionic villus samples were used showing villous cross-sections. (B) Syncytiotrophoblast (SCT) marker β-hCG (CGB; right) expression in SCT cluster in scRNA-seq data (UMAP 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 = 50µm; 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 = 50µm; 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 (foetal vessel = FV, CD31 = red; right) (E) AGTR1 expression in fFB2 cluster in scRNA-seq data (left). AGTR1 transcripts (blue, arrow) via padlock probe technology around foetal vessels (FV, red) in first trimester placenta (combined with immunofluorescence staining; FV: CD34, red; SCT: β-HCG, green; scale bar = 50µm; n=10, GA weeks 7 and 11; right). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

Both localisations were concordant to scRNA-seq data, although we suggest the fFB2 population, cell-typed as “foetal fibroblast 2” by the original authors Vento-Tormo et al., rather represents the myofibroblast population surrounding developing foetal-placental vessels. As seen in Figure S1, CD34 as an endothelial marker is expressed in “fEndo” (foetal endothelial cells), while it is not expressed in “fFB2” (arrow), on the other hand ACTA2 (smooth muscle actin) is expressed in the cluster originally called “fFB2” by Vento-Tormo et al. and rather being foetal early smooth muscle cells i.e. myofibroblasts. To systematically evaluate the proximity of specific *AGTR1* transcripts (Figure 14A-C) to foetal vessels, *AGTR1* padlock signals (Figure 14D, G) were quantitatively analysed for their distance from the vessels with image analysis software (TissueGnostics StrataQuest; n=5; Figure 14D-I) and compared to the distribution of *ACTB* transcripts in serial tissue sections (n=3, Figure 14J). Left-skewed distribution of *AGTR1* compared to a normal distribution of *ACTB* transcripts shows *AGTR1* closer to foetal vessels (3.6µm vs 66.0µm median distance from foetal vessels, Figure 14K-N). To validate these findings, we used isolated primary cells with similar characteristics and analysed them via qPCR. CD34 positive endothelial colony forming cells (ECFC) expressed AGTR1 significantly higher than isolated first trimester trophoblasts (pT; Figure 15). Specific padlock probes for AGTR1 were used due to non-specificity of antibodies (Figure S2, (Benicky *et al.*, 2012; Herrera *et al.*, 2013)).

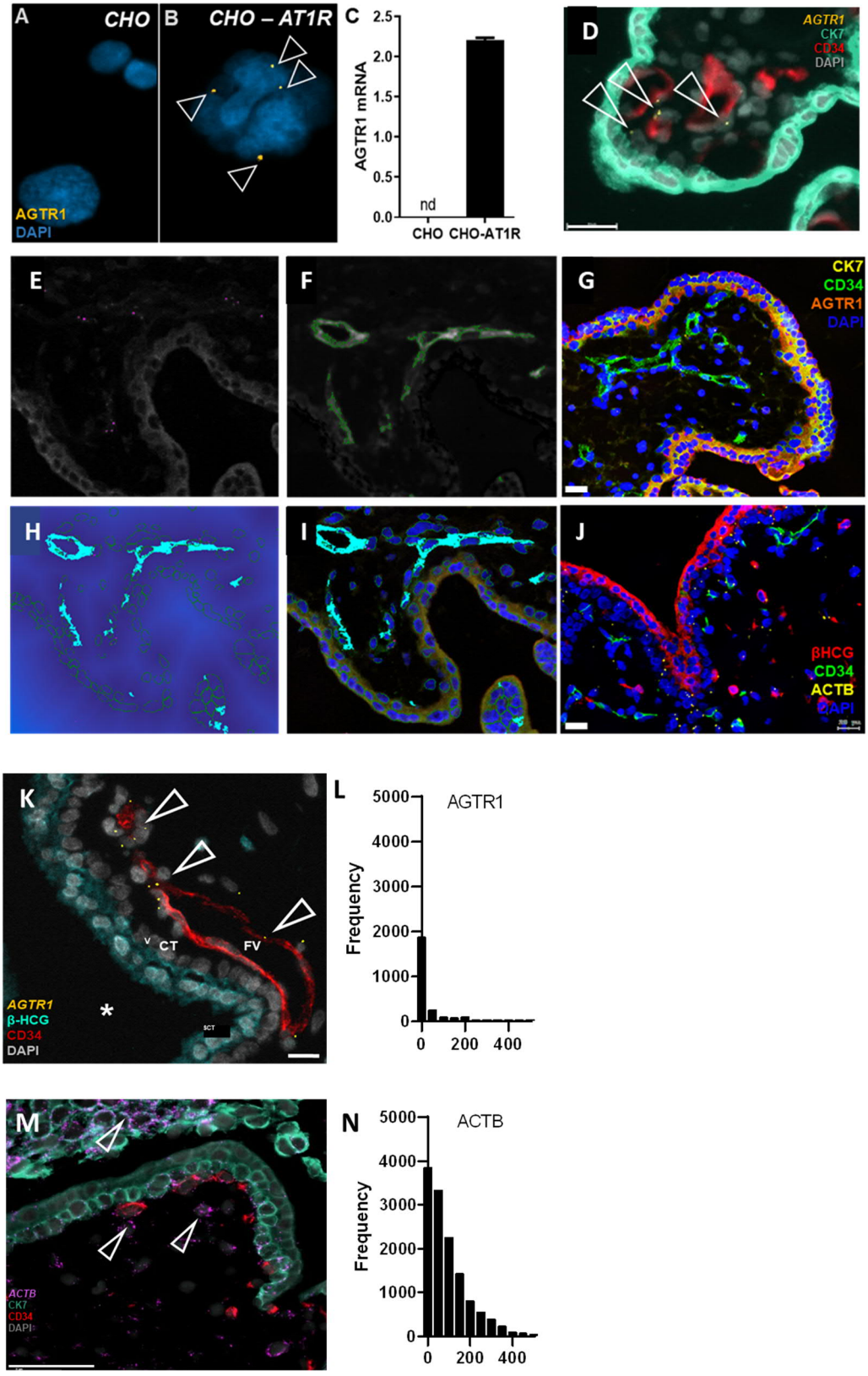

Figure 14. Padlock Probe specificity and quantification of AGTR1 transcripts.

(Figure 14) (*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) Single 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 slide-scan 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 slide-scan used to calculate ACTB transcripts to vessel distance based on the automated analysis software settings. (K) AGTR1 transcripts (yellow) and (M) actin beta transcripts (ACTB, pink) in first trimester placenta, combined with β-hCG (green) and CD34 (red) immunofluorescence staining (n=3, scale bar = 50µm). (L, N) Histogram showing the transcript to vessel distance of AGTR1 transcripts (median is 3.6 µm from foetal vessels) and ACTB transcripts as control (median is 66.0 µm from foetal vessels). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021*.*

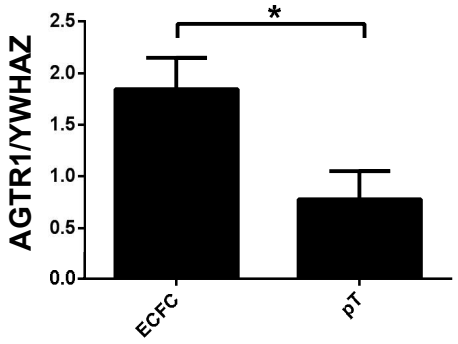

Figure 15. AGTR1 expression in isolated primary cells. *Preliminary data (ECFC n=4; primary first trimester trophoblasts n=2) shows that CD34-positive endothelial colony forming cells have a significantly higher expression of AGTR1 compared to first trimester trophoblasts (sig level <0.05; Mann Whitney U).*

Serum AngIV increases in pregnancy

We then validated results via qPCR and found *LNPEP* and *AGTR1* to be 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 16A). 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 16B-C). Additionally, *REN, DPP3, ENPEP* and *ACE2* were also analysed and displayed dynamic expression patterns across gestation (Figure S3, 3). Furthermore, we analysed foetal sex effects, maternal age, BMI, and smoking habits with a linear regression model, but did not see any effects on AGTR1 and LNPEP expression (Table S5). Protein expression of LNPEP was concordant to mRNA expression data across gestation (n=16, Figure 16D). We next analysed serum levels of maternal Ang I-IV (Figure 16E). Serum was collected from women undergoing assisted reproductive techniques (ART) at two time points, non-pregnant before embryo-transfer and after confirmed pregnancy around week 7 (Table S6). AngI and AngII showed no significant increase in first trimester (Figure 16E). On the contrary, serum AngIII and AngIV, the two peptide hormones resulting from AngI, AngII, and AngIII, were significantly increased in pregnancy (Figure 16E). Interestingly, AngIII is converted to AngIV as a substrate of LNPEP and AngIV acts as an inhibitor of LNPEP activity (Jackson *et al.*, 2018; Tsujimoto *et al.*, 1992).

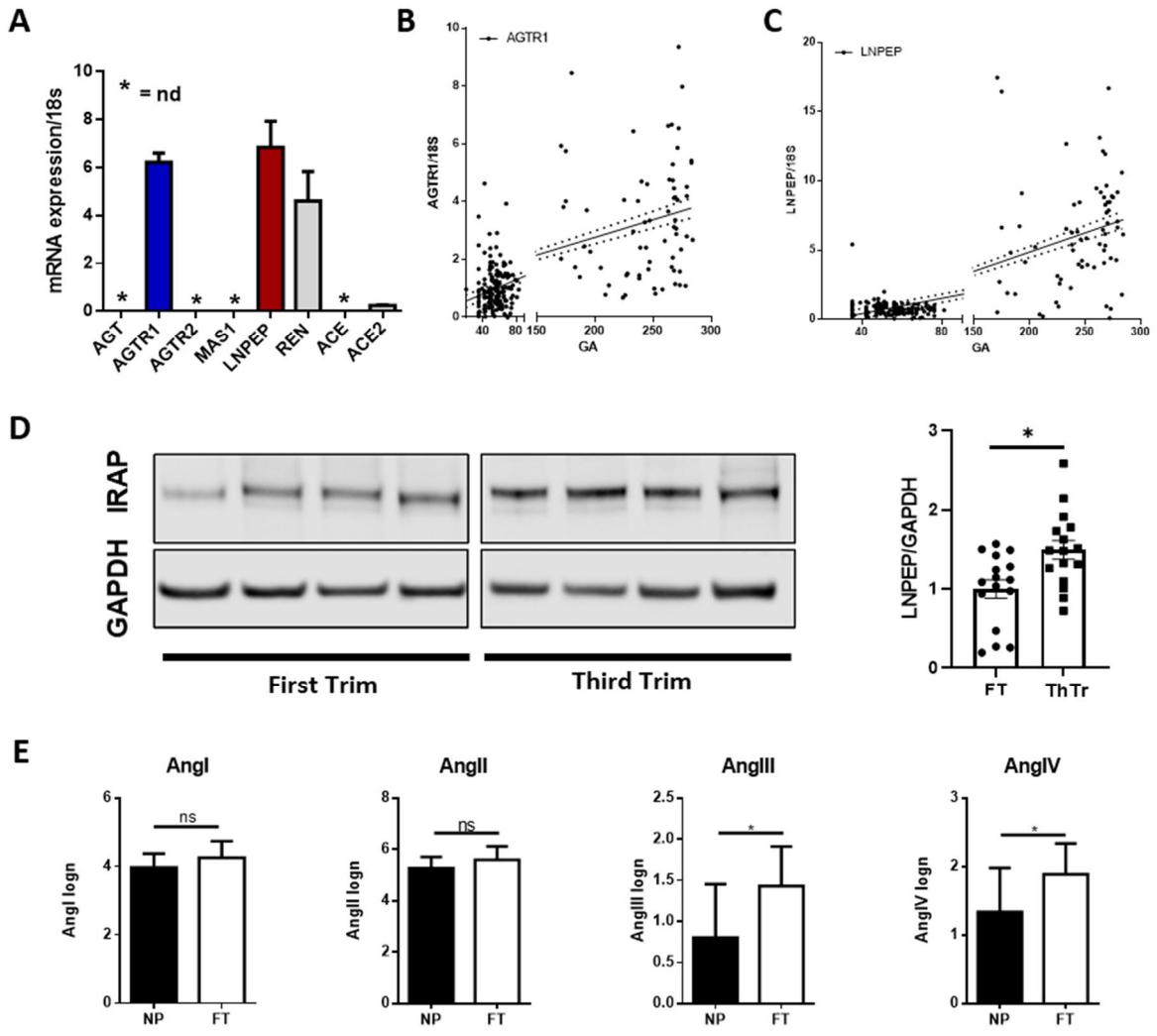

Figure 16. Maternal AngIV acting on placental RAS receptor LNPEP increases in pregnancy. *(A) qPCR Validation of scRNA-seq data expression patterns of RAS components, two RAS receptors in placenta are AGTR1 and LNPEP, while no endogenous angiotensinogen (AGT) is expressed in human first trimester placenta (n=8; mean and SEM; * = not detected) (B) AGTR1 and* (C) *LNPEP mRNA expression across gestation (n=252; non-linear regression line; Table S5); (D) LNPEP protein expression (data are represented as mean with SEM, n=16 each group; normality was tested by Shapiro-Wilk test followed by Mann-Whitney test; *p ≤ 0.05) (E) Log-transformed values of angiotensin I-IV serum levels before and after conception in ART patients; AngIII and AngIV show a significant increase in pregnancy (AngI and AngII = ns, normality was tested by Shapiro-Wilk test; paired t-test with graph showing mean and SD for AngI, II, IV and Wilcoxon-signed-rank test with median and IQR for AngIII, sig. level 0.05; n=10; NP = nonpregnant, FT = first trimester; patient characteristics in Table S6). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

Maternal AngII 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 insulin-regulated aminopeptidase (IRAP) residing in vesicles with glucose transporter type 4 (GLUT4) (Albiston et al., 2007) and binding to acyl-coA-dehydrogenases at the cytosolic end (Katagiri et al., 2002). We therefore investigated metabolic effects induced by AngII and AngIV in the trophoblastic cell line BeWo. We used Seahorse XF metabolic assays measuring the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) after 1h AngII treatment (Figure S5). Injection of glucose lead to an increase of ECAR but, surprisingly, to a decrease in OCR. This pinpoints towards the highly glycolytic character of these cells. Basal OCR levels are decreased after AngII treatment, but mitochondrial respiration after administration of the uncoupler BAM15 is unchanged (Figure 17A). BeWo cells showed a similar expression patterns of angiotensin receptors as found for syncytiotrophoblast by scRNA-seq (Figure 17B). Given that enzymes required to convert AngII into AngIV (ENPEP, DPP3, ANPEP, and RNPEP) are expressed in BeWo cells, but no AGTR1 expression was found (Figure 20B), we suggested a shift from an AngII to an AngIV pathway. We therefore repeated the metabolic assay with AngIV treatment for 1h. Basal OCR remained decreased when treating cells with AngIV (Figure 17C).

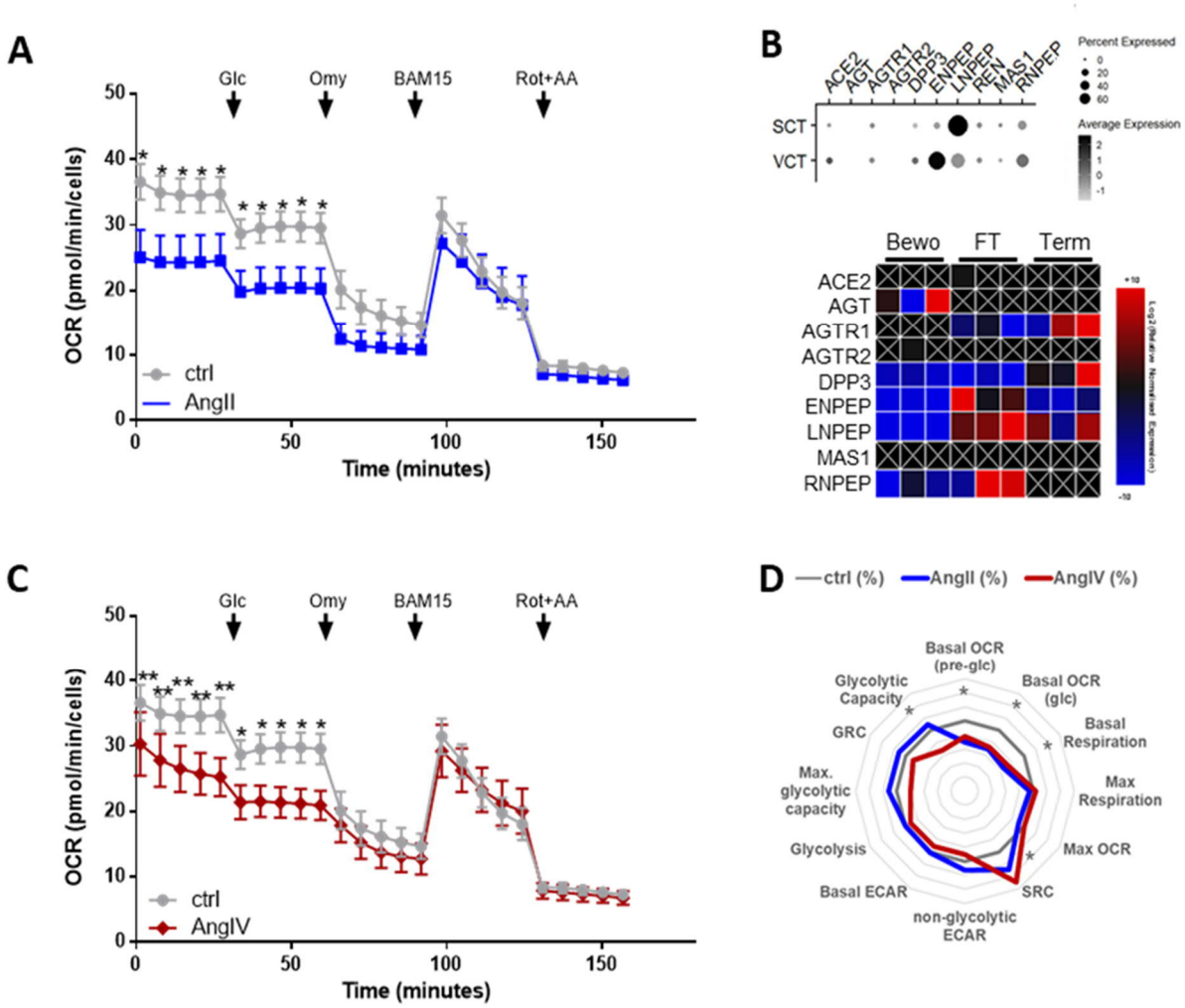

Figure 17. Metabolic Metabolic role of maternal AngII. *(A) 0.1μM AngII downregulates basal oxygen consumption rate (OCR) in BeWo trophoblast cells (graph shows mean and SEM, Seahorse XF data normalised to cell count; unpaired t-test with Welch's correction; Glc = Glucose, Omy = Oligomycin, Rot+AA = Rotenon + Antimycin) (B) Expression analysis of RAS components by qPCR shows LNPEP as only Ang receptor expressed in BeWo cells (term and first trimester placentae as positive controls, n=3 for each; X = not detected; blue = low, red = high expression); Expression data in syncytiotrophoblast (SCT) and villous cytotrophoblasts (VCT) in scRNA-seq data, showing the similarity of the RAS in vivo and the trophoblast cell line BeWo (C) 4 nmol AngIV downregulates basal OCR in BeWo cells (graph shows mean and SEM, normalised to cell count; unpaired t-test with Welch's correction) (D) Metabolic profile of AngIV treated cells relative to control (significant changes marked * = p<0.05; unpaired t-test with Welch's correction; ECAR = extracellular acidification rate; GRC*

= *glycolytic reserve capacity; SRC = spare respiratory capacity; glc = Glucose). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

Overall, both AngII and AngIV reduced mitochondrial metabolism but had no effect on their maximal potential. Glycolytic capacity was decreased (Figure 17D). These effects of decreased mitochondrial metabolism are consistent between AngII and AngIV treatment of trophoblasts (Figure 18). However, mitochondrial response potential did not change (Figure 19) and no cytotoxicity could play a role in the decreased metabolism (Figure S6).

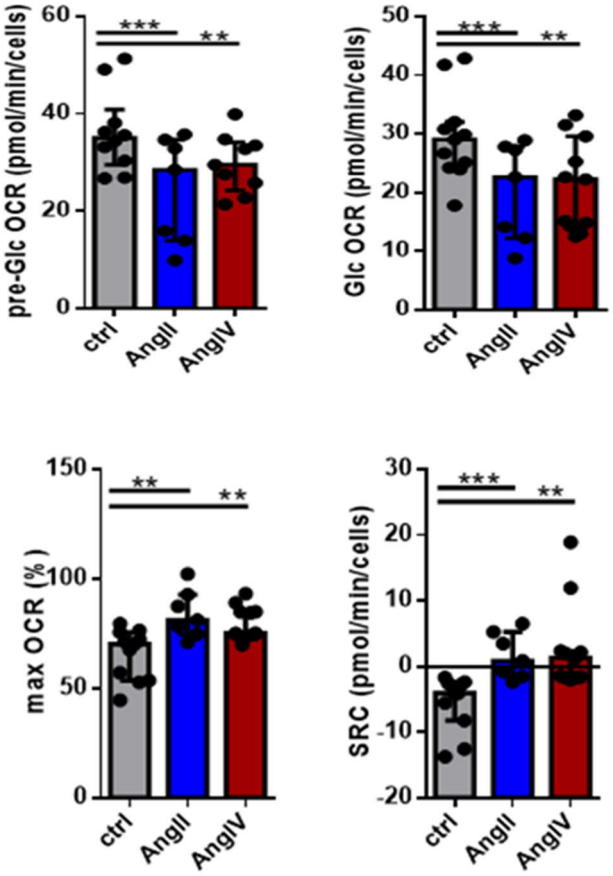

Figure 18. No alteration between AngII and AngIV effects on metabolism. *AngII and AngIV similarly alter metabolism, significantly decreasing basal OCR levels while maintaining a higher spare respiratory capacity (1h pre-treatment with 0.1µM AngII or 4 nmol AngIV; graph shows median and interquartile range; Seahorse XF data normalised to cell count; p<0.05; Mann-Whitney U test and t-test as appropriate based on distribution as calculated by Shapiro-Wilk) Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

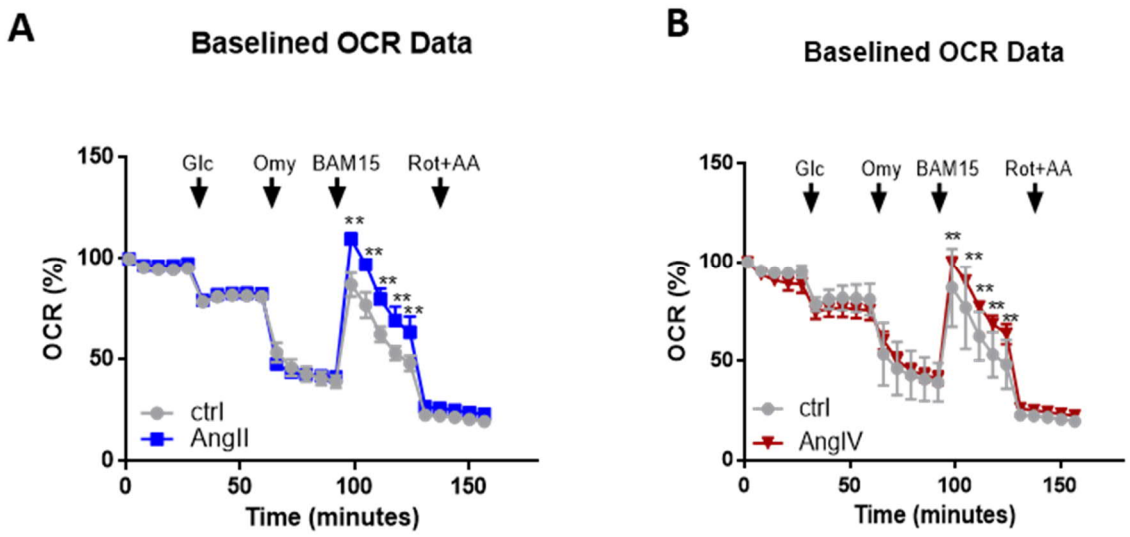

Figure 19. No change in mitochondrial response potential. *The data shows the significantly elevated max. OCR after BAM15 administration in (A) AngII and (B) AngIV pre-treated samples to compensate the initially lower baseline OCR (n=3; sig<0.05; Mann Whitney U test; normalised basal OCR before glucose injection is set at 100%, levels after that are relative to this, seeing a baselined data visualisation). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

AngII leads to an increase of leptin in a placental secretory response

We next aimed to analyse the effect of AngII on placental villous explants, a model most close to the situation found *in vivo*. We incubated villous explants with 0.1µM AngII for 6h under physiological oxygen concentrations of 2.5% and analysed transcriptional changes of genes involved in placental pathologies and genes associated with angiotensin pathways (Figure 20A). 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 20B). Among these genes, *LEP* encoding leptin, had the highest fold change expression (3.98). Analysing 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 4, Figure 21).

Further on, we validated array results in placental explants with the AT1R-blocker candesartan over different time points (3h, 6h, 24h; n=10; Figure 20C) and found that a significant upregulation of leptin mRNA expression after 6h 0.1µM AngII treatment could not be inhibited by candesartan (Figure 20C, centre), suggesting mediation of AngII effects by LNPEP and not AGTR1. This led us to the conclusion that AngII 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 20D) and protein expression (Figure 20E). AngIV showed no cytotoxicity in villous explants (Figure S6).

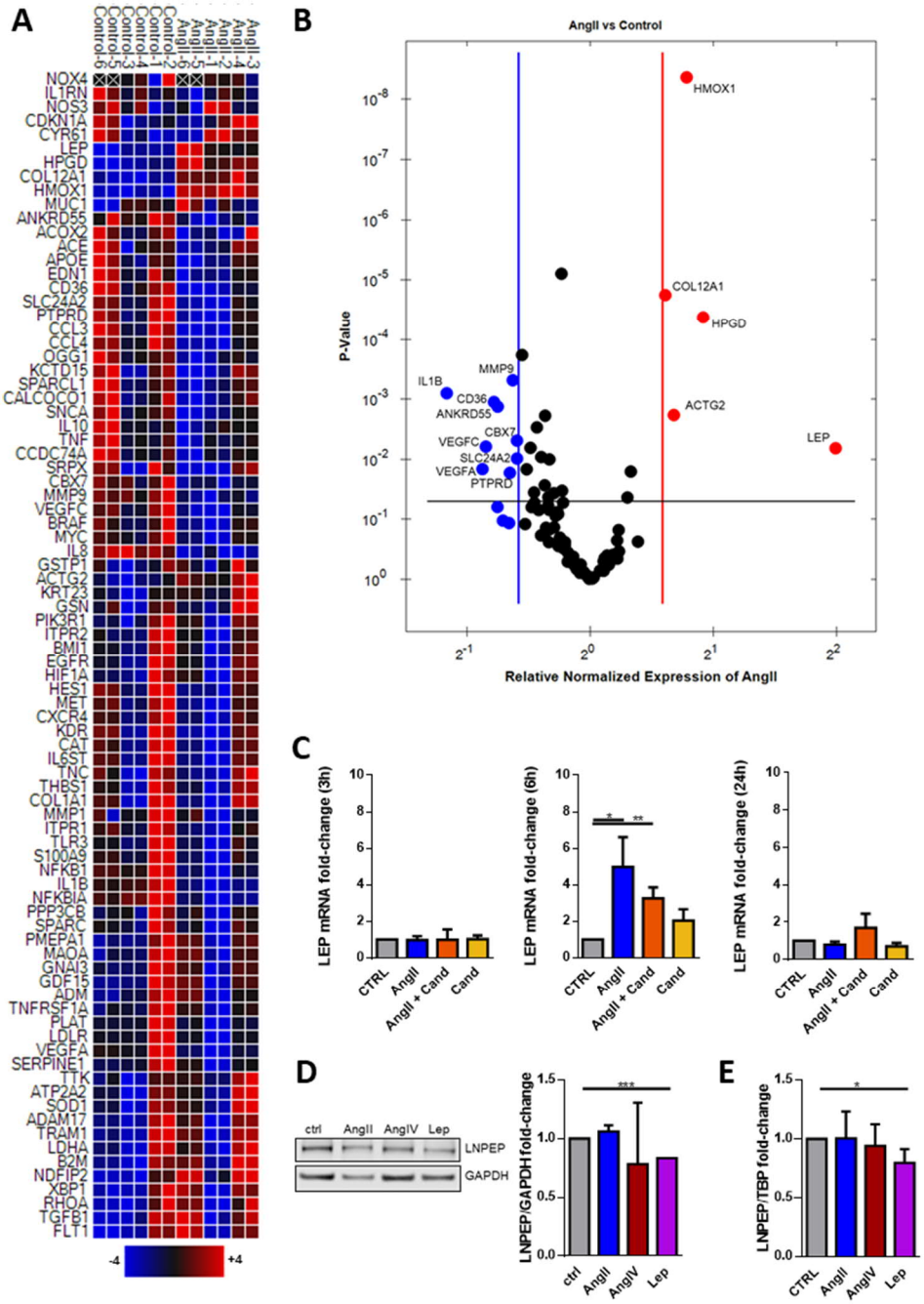

Figure 20. AngII leads to an increase in leptin in placental explants. *(A) Heat map showing the gene regulation in first trimester placental explants incubated with 0.1µM AngII for 6h (at O2 2.5% physiological concentration; n=3) (B) Volcano plot showing significantly regulated genes (p>0.05, regulation >1.5 fold). Significantly upregulated (red) are HMOX1, COL12A1, HPGD, ACTG2, LEP – downregulated genes (blue) are MMP9, IL1B, CD36, ANKRD55, CBX7, SLC24A2, VEGFC, VEGFA, PTPRD (C), (D) (E) show the relative LEP mRNA expression of*

placental explants incubated with AngII (0.1µM) and AT1R-Blocker Candesartan (1µM) for 3h (C), 6h (D), and 24h (E). LEP expression showed a 4.97-fold increase in first trimester placental explants after 6h treatment, with no change at 3h or 24h. No AngII type 1 receptor blocker effect can be detected at 6h. (DMSO was used as solvent control for candesartan at 1:10'000; sig<0.05; 3h: n=4; 6h: n=10; 24h: n=8, graph shows median and range). (F) LNPEP protein expression in first trimester placental explants treated with AngII (0.1µM), AngIV (4nmol), and leptin (10 ng/mL) for 6h, normalised to GAPDH (A.U.C. foldchange shown, graph shows median and IQR, n=3). (G) LNPEP mRNA expression after 6h AngII, AngIV, leptin treatment of first trimester placental explants: LNPEP is significantly downregulated after 6h leptin treatment (foldchange; median and IQR, n=3). Figure reproduced and adapted under the CC-BY Licence from Nonn *et al.*, 2021.

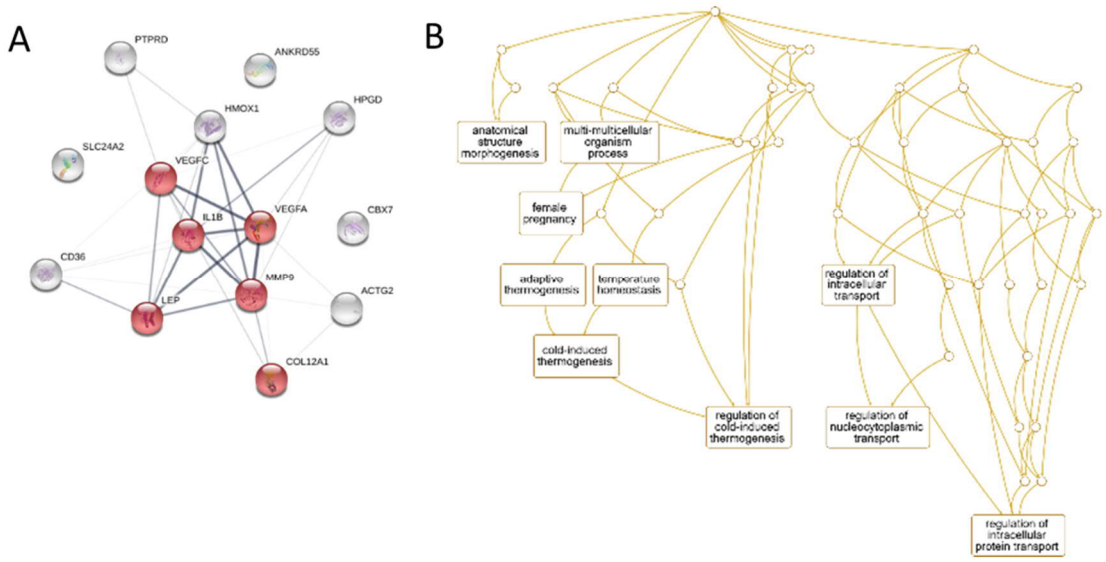

Figure 21. Protein-protein interaction networks and overrepresentation analysis. *(A) Protein-protein interaction network; the nodes represent the significantly regulated genes from the array (p>0.05, regulation >1.5fold). Nodes coloured in red are annotated to UniProt-Keyword „Secreted" (KW-0964). Edge 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). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

Table 4. Overrepresentation Analysis of Array Data

ORA was computed using webGestalt tool (Liao *et al.*, 2019)

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

LNPEP-dependent AngII 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, foetal death, or hypertensive disorders in pregnancy (Tian, Huang & Wen, 2016; Ito *et al.*, 2004a).

To determine whether only shedding or transcription is disturbed in preeclampsia, placentae from healthy and preeclamptic patients were analysed for LNPEP expression. A significant downregulation of *LNPEP* gene expression was found in early and late onset preeclampsia (Figure 22A; Table S7, S8) compared to uncomplicated pregnancies. Furthermore, we investigated possible early gestational effects and analysed first trimester placenta samples from women classified by uterine artery Doppler ultrasound scanning as having a higher or lower risk of developing preeclampsia (Leslie *et al.*, 2015). *LNPEP* expression was significantly decreased (Figure 22B; Table S9) in the placentae derived from women with a high risk for preeclampsia.

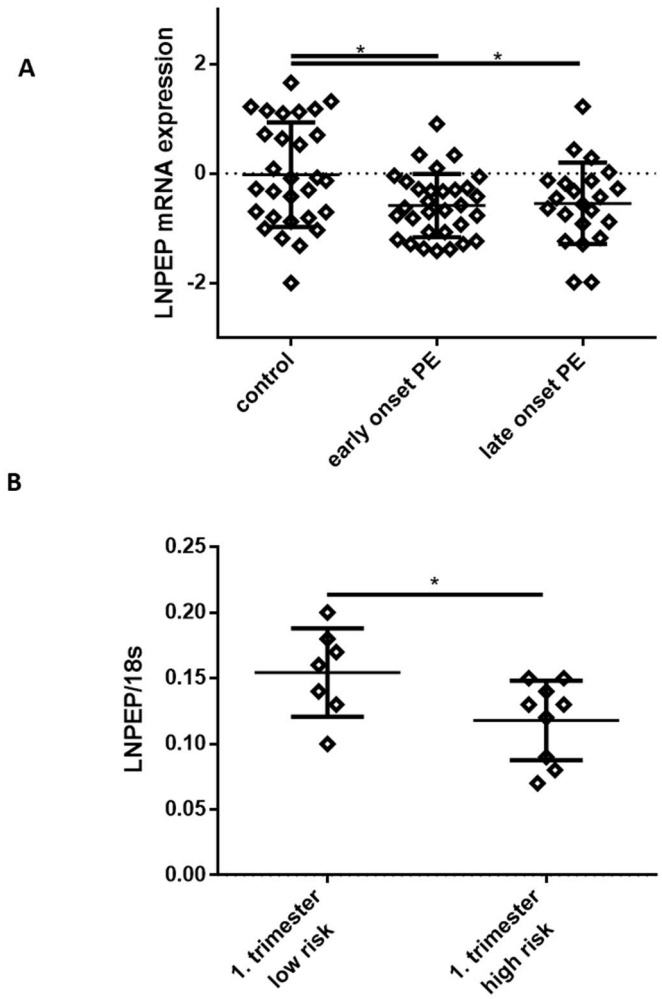

Figure 22. LNPEP is downregulated in preeclampsia and early high risk placenta. *(A) LNPEP mRNA is differentially expressed in early and late onset preeclampsia (eoPE, loPE) when compared to controls (data z-transformed; one-way ANOVA with multiple comparisons; sig.level = .05; for baseline characteristics see Table S7-S9) (B) LNPEP expression is lower in first trimester placentae from women with a high uterine artery resistance index, indicating high-risk for preeclampsia (n=16; p=0.0388; unpaired t-test). Figure reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

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 AngIV (Figure 23).

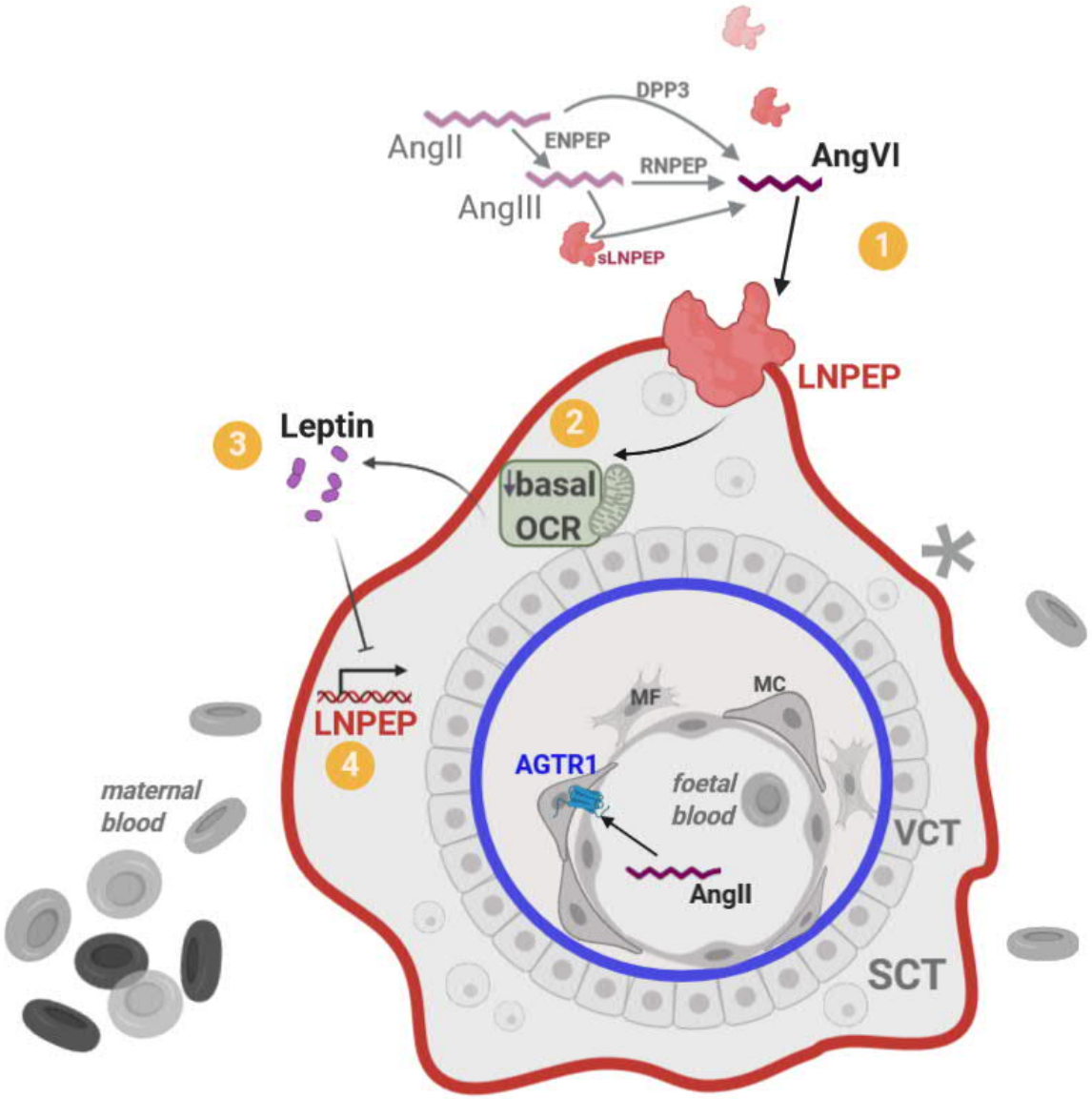

Figure 23. Graphical summary of suggested mechanistic pathway of the alternative RAS at the maternal-placental interface. *Graphical summary of results. Maternal and foetal angiotensin II and its conversion product AngIV have different target receptors. Maternal AngII-levels influence trophoblast metabolism by being converted to AngIV acting on placental LNPEP at the maternal-foetal interface. Foetal AngII acts on AGTR1 expressed in foetal myofibroblasts around vessels. AngIV, that increases when pregnant, acts on LNPEP (1) and decreases basal OCR (2), which in turn may drive an increase in leptin expression that is mediated by AngIV (3). Increased leptin levels decrease LNPEP expression on an RNA and protein level (4). Schematic drawing reproduced and adapted under the CC-BY Licence from* Nonn *et al.*, 2021.

Conclusion

Maternal circulating RAS shifts towards AngIV in pregnancy, decreases trophoblastic mitochondrial respiration and increases placental leptin via placental leucine aminopeptidase (LNPEP), i.e. the angiotensin IV receptor. Lower placental *LNPEP* in preeclampsia and in early gestation higher-risk patients implicates a new alternative RAS mechanism contributing to hypertension in pregnancy.

Discussion

In this study, we found RAS receptor distribution at the maternal-foetal interface different to previously published literature. Based on our findings we propose a novel pathophysiological concept to hypertension in pregnancy that gives insight into a shift to an alternative RAS pathway in human pregnancy. Maternal AngII acts by a shift towards its derivatives AngIII and AngIV and its target receptor LNPEP driven responses at the maternal-placental side. The trophoblast layer in contact to maternal blood, and thus maternal Ang-derivatives, responds with a decrease in basal mitochondrial respiration. In turn, leptin increases and downregulates LNPEP – well in line with the results of lower expression of LNPEP we found in pre-eclamptic placentae as well as early on in first trimester high risk patients and previous studies identifying high maternal leptin in serum and placenta as a hallmark of preeclampsia (Tsai *et al.*, 2011; Nishizawa *et al.*, 2011; Kang *et al.*, 2011; Sørensen *et al.*, 2010; Winn *et al.*, 2009; Sitras *et al.*, 2009; Enquobahrie *et al.*, 2008; Reimer, 2002).

Leptin has long been investigated in cardiovascular research for its involvement in endothelial dysfunction, aneurysm, atherosclerosis - also in association with angiotensin and RAS components (Faulkner, Bruder-Nascimento & Belin De Chantemèle, 2018; Xue *et al.*, 2016; Moreau, Messenger & Ciriello, 2015; Wang *et al.*, 2013; Kamber *et al.*, 2015; Huby *et al.*, 2015; Packer, 2018; Wallace *et al.*, 2001; Ben-Zvi *et al.*, 2016; Fukui *et al.*, 2013; Nonn *et al.*, 2021). Placental and serum leptin are also found to be consistently increased in hypertensive disorders in pregnancy (Tsai *et al.*, 2011; Nishizawa *et al.*, 2011; Kang *et al.*, 2011; Sørensen *et al.*, 2010; Winn *et al.*, 2009; Sitras *et al.*, 2009; Enquobahrie *et al.*, 2008; Reimer, 2002), and indeed preeclampsia shares common risk alleles with cardiovascular diseases (Valenzuela *et al.*, 2012; McGinnis *et al.*, 2017; Majander *et al.*, 2013; Inkeri Lokki *et al.*, 2017b, 2017a; Johnson *et al.*, 2012, 2013). Than et al. have shown that incubation of first trimester explants with preeclamptic mothers' serum results in a similar gene expression pattern as observed in preeclamptic placentae. They show that leptin is positively correlated to blood pressure and that AGT plays a central role in functionally connecting differentially expressed features in preeclampsia (Than *et al.*, 2018). We are the first to show a direct relationship between maternal Ang and elevated leptin levels in the placenta. This also allowed us to propose a pathophysiological mechanism around placental LNPEP. LNPEP is known as AT4R, oxytocinase, vassopressinase, placental leucine aminopeptidase, but also as insulin-regulated aminopeptidase (IRAP) residing in vesicles with GLUT4 and binding to acyl-coA-

dehydrogenases at the cytosolic end (Katagiri *et al.*, 2002; Albiston *et al.*, 2007). The concept of this alternative RAS-pathway acting in pregnancy at the maternal-foetal interface 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 with its biomarker or biological correlates in the placenta or maternal and foetal circulation (Nonn *et al.*, 2021). The blood pressure regulating hormone Angiotensin II is shifting towards Angiotensin IV and thus alters trophoblast metabolism and might alter glucose metabolism via LNPEP (Belman *et al.*, 2015) as well as antigen cross-presentation via LNPEP (Nunes-Hasler *et al.*, 2017). The subsequent upregulation of leptin adds to a metabolic disbalance – in the third trimester high maternal serum leptin of women with type 2 diabetes mellitus correlates with glycaemic levels when taking oral glucose tolerance test, HbA1c, gestational hypertension, and preeclampsia rates (Kapustin *et al.*, 2020).

The involvement of LNPEP in preeclampsia was shown by sequencing studies, where maternal susceptibility genes were identified in decidua (Yong *et al.*, 2014) together with AGT (Yong *et al.*, 2015) and in maternal blood with ANPEP (Khaliq *et al.*, 2020b). Single nucleotide polymorphisms (SNPs) in LNPEP and relevant sites such as its regulators were related to prematurity shown in maternal triads and to gestational length in general (Kim *et al.*, 2013; Nonn *et al.*, 2021). Its soluble form was downregulated in pregnancies that ended in foetal death, but also in other pregnancy pathologies (Tian, Huang & Wen, 2016). Altogether, LNPEP seems to have a fundamental role in pregnancy sustenance. sLNPEP activity increases between the 4th and 38th weeks of gestation (Ananthakrishnan, 2016), leading to accelerated metabolism of its substrates such as vasopressin or oxytocin. However, its activity decreases in preterm delivery and severe preeclampsia (Nomura *et al.*, 2005). Its soluble fraction in maternal plasma was shown to decrease with severe preeclampsia when compared to mild preeclamptic or normotensive pregnancies (Khaliq *et al.*, 2020a). Already at 16 weeks of gestation elevated levels of copeptin, a by-product of vasopressin hydrolysis from its precursor, are associated with increased preeclampsia risk (Yeung *et al.*, 2014; Sandgren *et al.*, 2015; Yeşil *et al.*, 2017), indicating that a disturbed LNPEP activity may also mirror its vasopressinase activity (Nomura *et al.*, 2005; Khaliq *et al.*, 2020a). In line with this observations, levels of serum copeptin were significantly elevated in women who subsequently developed PE, compared to gestational age matched controls at 10–14 weeks of gestation (Jadli *et al.*, 2017). In fact, maternal copeptin levels are increased in PE cases over a wide time span in gestation, since its concentration is significantly higher in pregnant women who developed preeclampsia in comparison with controls in the first, second, and third trimesters

(Santillan *et al.*, 2014). 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 (Nomura *et al.*, 2005; Khaliq *et al.*, 2020a). In the overriding cohort (Leslie *et al.*, 2015) of early pregnancies with a high risk for developing preeclampsia there was no difference in the BMI from high-risk and control groups. This heightens the suggested importance of the renin-angiotensin-system in preeclampsia. Altogether, LNPEP seems to have a fundamental role in sustaining a pregnancy. TH17-associated cytokine interleukin (IL)-17 was elevated in the maternal plasma and placenta following vasopressin infusion (Scroggins *et al.*, 2018). Interestingly, LNPEP polymorphisms in association with AGT (Liu *et al.*, 2018a) also seem to play a role in cardiovascular disease and septic shock mortality and the vasopressin response of these pathologies (Li, Habtemichael & Bogan, 2020; Ramírez-Expósito *et al.*, 2016). Platelets of pre-eclamptic women had a different intracellular calcium response after vasopressin administration than that of controls (Zemel *et al.*, 1990) and plasminogen activator inhibitor type (PAI)-1 expression levels were diminished in LNPEP knockouts (Niwa *et al.*, 2015), perhaps adding to a thrombogenic phenotype in preeclampsia that can be altered by low dose aspirin. Low dose aspirin intake was assessed in our study and did show a nonsignificant effect on lowering placental LNPEP expression in a regression model, but was limited to a low-powered pilot study and needs reaffirmation with a larger cohort. Low-dose aspirin, if started before second trimester, reduces the risk for pre-eclampsia (Hoffman *et al.*, 2020; Rolnik *et al.*, 2017). LNPEP acts as oxytocinase, thus degrading oxytocin, a uterotonic peptide. Interestingly, prostaglandins – synthesised by cyclooxygenases, which in turn is inhibited by aspirin – are uterotonic. An interplay and feedback loop between these two agents may be plausible. Cyclooxygenase type 2 (COX-2), which functions in a negative feedback loop in TGFβ and ERβ signalling pathways, positively impacts ERβ action through its effect on the expression of a number of steroidogenic enzymes, such as CYP19A1 highly expressed in syncytiotrophoblast, in the ERβ ligand metabolic pathway (Liu *et al.*, 2016). There is evidence that LNPEP is downregulated by oestrogen (Mustafa *et al.*, 2004), which makes an impact via aspirin plausible.

Part of the explanation of preeclampsia is that of “immune maladaptation”, and LNPEP exclusively identifies an endosomal compartment required for MHC class I cross-presentation (Saveanu *et al.*, 2009) and is required in the endosome-to-cytosol pathway of antigen cross-presentation (Embgenbroich & Burgdorf, 2018; Nunes-Hasler *et al.*, 2017). STIM1 functions as a calcium sensor in the endoplasmic reticulum and activates the "store-operated" ORAI1 calcium ion channels in the plasma membrane and its deficiency inhibited LNPEP recruitment

to this endosomal compartment (Nunes-Hasler *et al.*, 2017), suggesting that intracellular calcium concentrations are involved in antigen cross-presentation. Polymorphisms in LNPEP were associated with autoimmune disease such as juvenile idiopathic arthritis (Chiaroni-Clarke *et al.*, 2014; Hinks *et al.*, 2013) or psoriasis (Cheng *et al.*, 2014; Zhen *et al.*, 2019) but were also differentially expressed in high versus low antibody responders to small-pox vaccines (Haralambieva *et al.*, 2012). In addition, LNPEP deletion enhances T-cell receptor expression in basal conditions (Evnouchidou *et al.*, 2020), while it is known that in preeclampsia T-cell populations are imbalanced showing a higher circulating TH1/TH2 lymphocyte ratio (Saito *et al.*, 1999). Downregulation of LNPEP may contribute to impaired immunotolerance at the foeto-maternal interface.

A strength in this study was that we could reproduce findings of reduced placental LNPEP expression in two independently recruited cohorts with preeclamptic patients. Preeclampsia features earlier deliveries and thus makes it difficult, firstly, to find healthy controls with a similar gestational age and, secondly, to distinguish between preeclampsia effects or confounding factors related to preterm delivery and labour if no matched controls are available. Our controls have a significantly higher mean gestational age than the preeclamptic samples (Graz cohort: 253,8 ± 4,110 n=27 controls and 236,9 ± 3,543 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 this possible confounder by investigating available scRNA-seq data from Pique-Regi et al. to evaluate effects of preterm labour, labour and no labour term deliveries (Figure S7) found no difference in LNPEP expression between these entities neither in villi, decidua nor chorioamniotic membranes (Pique-Regi *et al.*, 2019). We conclude that LNPEP expression is not altered by labour stress, preterm birth or risk factors that pertain to these pregnancy pathologies but suggest it to be a preeclampsia specific feature worth further investigation. (Nonn *et al.*, 2021)

Receptor localisation of RAS components was investigated previously (Cooper *et al.*, 1999; Pringle *et al.*, 2011; Williams *et al.*, 2010), indicating AGTR1 expression at the syncytium. However, lack of specificity of used antibodies against AT1R led to misinterpretation (Herrera *et al.*, 2013; Benicky *et al.*, 2012), while by using specific novel methods such as padlock probe-based *in situ* hybridisation and scRNA-seq we were able to establish an entirely new concept of placental RAS receptor distribution. Both LNPEP and ANPEP were previously found to be expressed on the apical microvillous surface and less on the basal infoldings of the syncytiotrophoblast (Ito *et al.*, 2003), with LNPEP predominantly found in a microsomal

fraction (Ino *et al.*, 2003). On top, AngII induces placental AP-A (ANPEP) hydrolysing AngII to AngIII in pregnancy (Nakamura *et al.*, 2000). Previously it was only shown using EIA kits that AngII generally increased while angiotensin(1-7) concentration decreased across pregnancy (Khlestova *et al.*, 2018), but no longitudinal technically reliant data to compare pre-pregnancy and pregnancy levels and composition of angiotensins was available. Our data measured by mass spectrometry shows a significant increase of maternal AngIII and AngIV. Based on our results, AGTR1 does not seem to play a role in the maternal-placental communication, whereas angiotensin IV receptor LNPEP does.

The original cell annotation by Vento-Tormo et al., that was published along with the data in Nature 2018, was claiming that this cell population was “fFB2”, so one of the two subtypes of foetal fibroblasts in the villi. Given that our own data (ACTA2 staining coinciding with AGTR1 padlocks but not with CD34, except some artefact overlap given the z-stacks compressed in the immunofluorescence picture chosen) and the expression of endothelial and smooth vascular markers in their own scRNA-seq data, we concluded that the cell population in question is a myofibroblast population, based on nomenclature postulated by Benirschke, Kaufmann and Baergen (Benirschke, Kaufmann & Baergen, 2006). In this, they describe that until the end of second month stromal cells remain largely undifferentiated as mesenchymal cells expressing only vimentin (VIM). They then aquire desmin expression (DES) and may be called early myofibroblasts as soon as they express α-smooth muscle actin (ACTA2), and fully differentiated myofibroblasts once found expressing γ- smooth muscle actin (ACTG2). Given our samples from gestational week 7, we concluded to call this AGTR1-expressing population “myofibroblasts”.

This is substantiated by a shift of maternal serum angiotensin derivatives towards an AngIV accentuated RAS pathway as found in our longitudinal measurement of serum angiotensin I – IV levels in assisted reproductive therapy patients before and after successful implantation. Taken together with LNPEP as the only placental angiotensin target receptor at the maternal side, these new findings give an insight into entirely different baseline conditions regarding RAS in pregnancy.

The importance of placental AGTR1 in early gestation depends on whether the foetal circulating RAS is established. We were not able to assess the availability of AGT in the foetal circulation in our own study. While there are reports of angiotensin in term cord blood and thus the foetal circulation (Zhang *et al.*, 2013), there are no earlier measurements of plasma AGT in the foetal circulation yet. scRNA-seq of foetal tissues in 15-21 post-conception week (pcw)

shows that hepatocytes synthesise AGT and make this a highly expressed RAS component contributing to the top ten differentially expressed genes of foetal liver markers (Segal *et al.*, 2019). This being the earliest report of foetal AGT thus limits our ability to draw a conclusion as to when AGTR1 expressed in first trimester foetal placental vessels, as shown in our data from 7 pcw, is met by circulating foetal AGT and angiotensin derivatives. We can therefore assume the onset of functionality of foetal and foeto-placental systemic RAS in second trimester latest (15-21 pcw; see Figure 24).

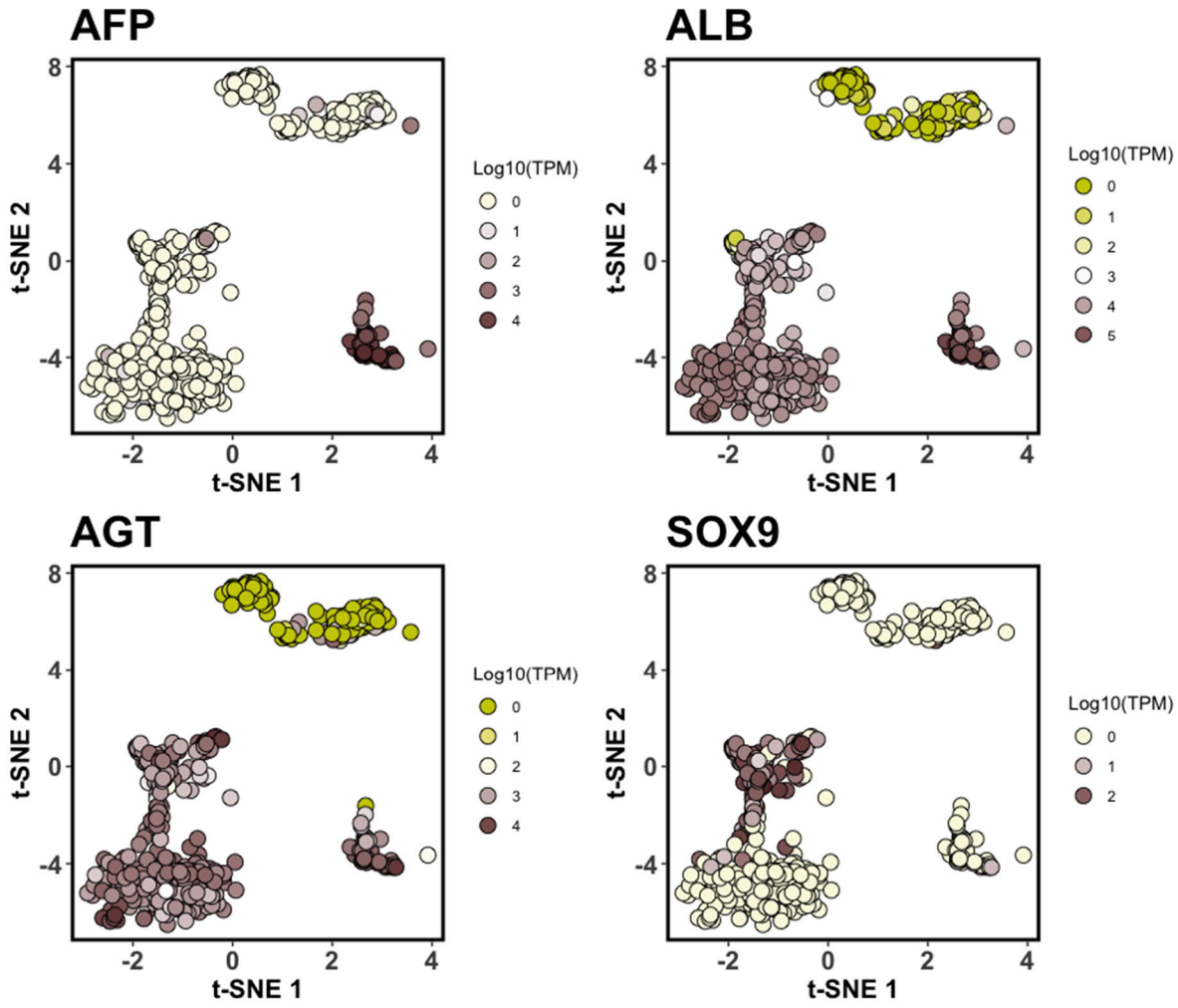

Figure 24. AGT expression in foetal liver in second trimester. *AGT appears to be highly expressed in foetal liver hepatocytes from 2nd trimester, as well as biliary progenitors from foetal liver, adult hepatocytes and adult BECS. Data available GEO: Series GSE130473. Graph kindly provided by Joe Segal to show AGT expression (Segal et al., 2019).*

However, this fact only adds to the importance of the maternal circulating RAS in this early pregnancy period. Studies have shown that ERAP, endoplasmic reticulum aminopeptidase, is

involved in part in the endosome-to-cytosol pathway of antigen cross-presentation and is otherwise a RAS component degrading AngII to AngIII (Hattori *et al.*, 2000). Both ERAP1 and 2 were revealed to be significantly increased in transcriptome profiling of preeclamptic placental tissues (Yong *et al.*, 2015; Xu, Wu & Wu, 2020). AP-A (*ANPEP*) hydrolyses AngII to AngIII and has a higher activity in pre-eclampsia compared to control pregnancies (Hariyama *et al.*, 2000). This suggests a more severe shift towards AngIII and AngIV, acting on placental LNPEP in pregnancies complicated by preeclampsia.

When investigating the expression of MAS1 (receptor to Ang1-7), 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. Oxytocin and vasopressin are substrates to LNPEP and its soluble form (abbreviated here as sLNPEP), the AngIV receptor which inactivates both hormones. However, they do not bind to the receptor like AngIV does. The receptors for oxytocin and vasopressin are not expressed in placental tissue (see Figure 25).

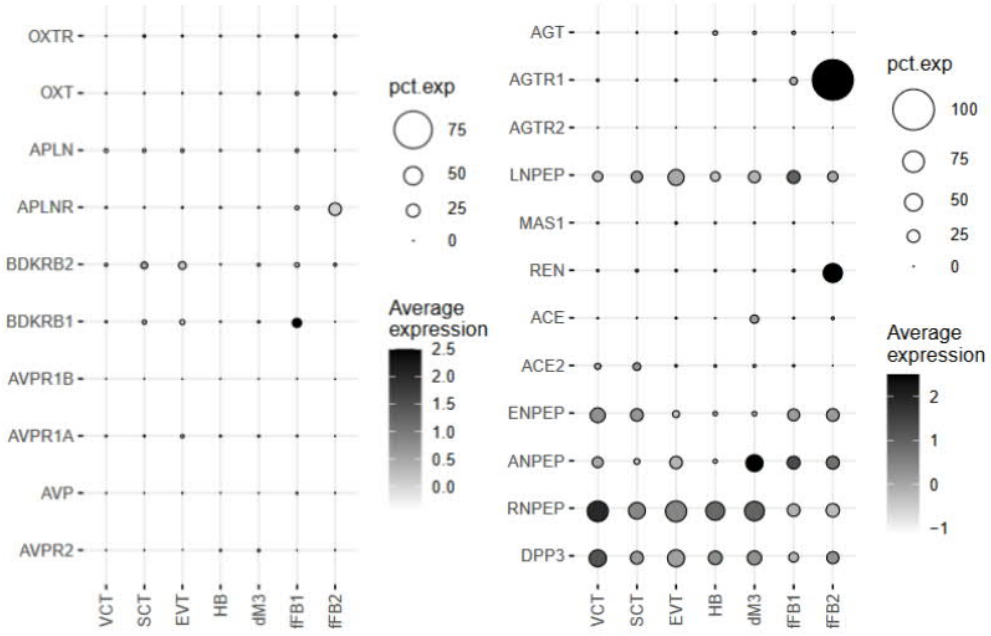

Figure 25. Extended RAS and associated components in placenta. *Heatmap from placental scRNA-seq data from Vento-Tormo et al, Nature 2018. No RNA expression can be found on the maternal site for following genes and very low expression in foetal fibroblasts: OXTR = oxytocin receptor, OXT oxytocin, APLN 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.

Blocking AGTR1 in BeWo cells was not additionally performed because the complete transcriptional lack of it was enough to conclude the effect through this LNPEP pathway. Indeed, the only RAS receptor available in BeWo cells is LNPEP. However, the lacking effect of blocking AGTR1 by candesartan in villous explants and thus the lack of an AngII-effect is shown in Figure 20, where leptin increases by adding AngII and despite candesartan being added to AngII. Concluding with this information additional to that in Figure 17, and with the data from Vento-Tormo et al. and Pique-Regi et al. (see Figure 11) where we found that all conversion enzymes are present and expressed at the maternal side, that AngII is converted to AngIV. Additionally, only LNPEP is present at the outer layer of the placenta (maternal side), therefore AGTR1 would not have any direct "anatomical" relevance for maternal AngII when expressed on the foetal side (see Figure 13 and graphical summary Figure 23).

Concerning the synthetic blocking of the peptidase LNPEP, thus imitating the effect of AngIV on LNPEP, underlined the AngIV-mediated effects (Figure 26). Some additional preliminary experiments on the metabolic effect of HFI-419, a synthetic LNPEP-agonist and thus inhibiting the peptidase action of LNPEP like AngIV, showed that both AngIV and HFI-419 inhibited LNPEP and decreased OCR levels. The preliminary experiment shows that the decrease in metabolism as measured by ECAR and OCR is LNPEP dependent.

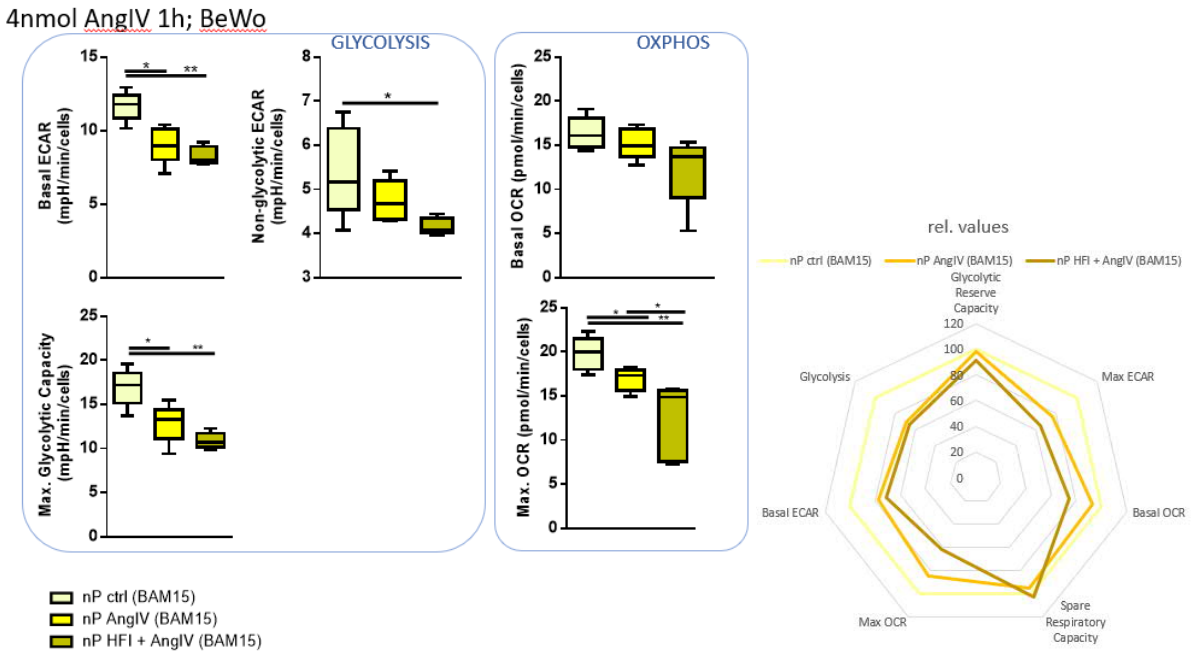

Figure 26. AngIV and synthetic LNPEP-inhibitor HFI-419 have similar effects on trophoblast metabolism. *We showed that the specific LNPEP-agonist HFI-419 acting on syncytiotrophoblast-like cells (BeWo cell line), which represent the layer of trophoblasts in contact to maternal blood, has an effect congruent to AngIV (n=3; Kruskal-Wallis test with Mann-Whitney-U test with adjusted significance levels). Basal ECAR (extracellular acidification rate) was measured before glucose injection.*

A novel pathway of AngIV decreasing mitochondrial respiration by acting on trophoblast LNPEP was shown in this work. The downstream action of LNPEP altering mitochondrial respiration is not yet elucidated, but it is known that AngIV causes a concentration-dependent rise in intracellular calcium concentration levels in Madin-Darby bovine kidney cells (Handa, Harding & Simasko, 1999), rat hippocampal neurons (Davis *et al.*, 2006), the carotid body (Fung *et al.*, 2007), and peripheral artery endothelial cells (Chen, Patel & Block, 2000). LNPEP is upregulated by hypoxia (Fung *et al.*, 2007) and its downstream pathways are regulated by both PI 3-kinase- and ryanodine-sensitive Ca(2+) stores (Chen, Patel & Block, 2000). Ang IV increases intracellular calcium to enhance NO synthase to modulate superoxide production (Bennion *et al.*, 2015) and reduced mitochondrial metabolism was shown to decrease cytotrophoblast differentiation (Dorothée Poidatz *et al.*, 2014). Ca^{2+} ions play a fundamental role in cell death mediated by oxidative glutamate toxicity or oxytosis and cause increases in cytosolic reactive oxygen species and mitochondrial dysfunction (Maher *et al.*, 2018), suggesting that LNPEP alters mitochondrial function via calcium influx.

Moreover, AngIV has been suggested to reduce the amino acid metabolism, which is the prominent form of energy source in first trimester. LNPEP produces a number of amino acid fragments needed to be then further metabolised (Zervoudi *et al.*, 2011). AngIV acts as an inhibitor to LNPEP enzymatic activity, thus perhaps reducing the availability of amino acid fragments. Glomerular arginine transport by CAT (Cationic Amino Acid Transporter)-2 protein decreased during pregnancy and increased in sFlt-1 transfected pregnant and virgin dames, which effect in turn was abolished by L-arginine treatment (Shashar *et al.*, 2019) and 4-hydroxyglutamate and C-glycosyltryptophan are early predictors of preeclampsia in women (Sovio *et al.*, 2020). Interestingly, it was shown that sLNPEP activity was associated with foetal growth restriction (Gopalaswamy, Balasubramaniam & Kanagasabapathy, 1983; Hensleigh, Cheatum & Spellacy, 1977) as was reduced amino acid transport (Hayward *et al.*, 2016) via mTOR signalling and the involvement of leptin (Winterhager & Gellhaus, 2017).

The use of a cross-sectional observational cohort where first trimester placental samples were gained by early termination of pregnancy before 12th week of gestation allows us to adjust for confounding by maternal age and BMI, foetal sex, gestational age and smoking status. It lies in the nature of early terminations that no pregnancy outcome is available. Placentae that would not reach a term delivery due to early pregnancy loss, that occur at a base risk of approximately 15% (Magnus *et al.*, 2019), are thus included in the cohort. We addressed this selection bias by recruiting a high number to have higher power when assessing first trimester placentae and investigating maternal and foetal predictors in statistical models. Furthermore, we could use smaller samples for assessing a high-risk population and ART patients without the risk of overseeing confounders. Using serum from women before undergoing ART and after a successful pregnancy in first trimester, was the best method to intra-personally adjust angiotensin levels in non-pregnant and pregnant women's serum by choosing a cohort that was documented to be non-pregnant and then pregnant and thus excluding variability between individuals as well as concentrating on changes in pregnancy. The sample size is very small because of the special recruitment circumstances and the novel method of measurement, and is therefore hard to interpret statistically when it is extremely skewed. We did match the samples inter-individually by maternal age, gestational age, BMI and comorbidities. However, we intended to further diminish possible confounding factors but because of the small sample size were not able to perform standard statistical stratification and adjusting for factors such as age, BMI, etc. We therefore intended to qualitatively assess an increase of the Ang peptides in pregnancy and used the fold change to show this. We now chose a statistically more accurate method and log-transformed the data to make highly skewed distributions less skewed for making patterns in the data more interpretable and for helping to meet the

assumptions of inferential statistics. We tested for normality using the Shapiro-Wilk test and then used Wilcoxon-signed-rank or paired t-test accordingly.

Our results suggest a general shift of the maternal circulating RAS towards increased AngIV levels in pregnancy, which are met by the appearance of placental LNPEP at the maternal side. According to our findings, placental AGTR1 only plays a role for the foetal circulating RAS once it is established in the second trimester latest. AngIV possibly acts as an inhibitor of LNPEP aminopeptidase activity and may increase intracellular calcium levels. The AngIV-induced decrease of basal mitochondrial respiration in trophoblasts may result in a rescue of metabolism by increasing placental leptin. Leptin in turn downregulates LNPEP expression, suggesting a negative feedback loop regulation of the AngIV/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 that had a high risk for preeclampsia based on a high uterine artery resistance index. (Nonn *et al.*, 2021)

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Appendix

Supplemental material is partly reproduced from Nonn *et al.*, 2021.

Table S1. Primer Sequences

Gene	*Forward*	*Reverse*
TBP	TGACCCAGCATCACTGTTTC	CCAGCACACTCTTCTCAGCA
LEP	GTGCGGATTCTTGTGGCTTT	AGGAGACTGACTGCGTGTGT
LEPR	CGTTAAAGCTCTCGTGGCAT	AATCCTCTAAGGCACATCCCAG
LNPEP	AGGGATGAGCAATACACCGC	CCCACAATGAAAGCAACCAAG
MAS1	GTGGGGTTTGTTGAGAATGGG	GCGATAGACAGGTGGGTGATG
ACE2	TGGTGGGAGATGAAGCGAGA	AACAGAGATGCGGGGTCACA
ACE	GTGGAACGAGTATGCCGAGG	GTGGAACGAGTATGCCGAGG
AGT	CTATCTCCCCGGACCATCCA	ACTCTGTGGGCTCTCTCTCA
REN	GAGGCTGTTTGATTATGTCGTGA	TGGTGAGCGTGTATTCTTTGC
ENPEP	GTGACAACCCCTGATGAAATAAC	AGCCTACTTGCCTCTTCCAG
AGTR2	GATCTGGTGCTATTACGTCCC	GCCTAAACACACTCCTTCAAAA
AGTR1	CTATGGAATACCGCTGGCCC	TGCAGGTGACTTTGGCTACA
DDX3Y	AGTAGAGGCAACCGGCAGTA	TGCACTGGAGTAGGACGAGTA
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

Table S2a: 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

Table S2b: Padlock probe

plp_AGTR1_1	/5Phos/GTAAGCTCATCCACCAA**CCTCAATGCACATGTTTGGCTCC**A ATGCGTCTATTTAGTGGAGCCGGCTATCACCGCCCCTCAGAT AAT
plp_AGTR1_2	/5Phos/CCACTCAAACCTTTCAACAAA**CCTCAATGCACATGTTTGGC TCC**AATGCGTCTATTTAGTGGAGCCGCCTATCCCCCCAAAAGCCAA ATC
plp_AGTR1_3	/5Phos/CTGGGTTTCCTGTTTAA**CCTCAATGCACATGTTTGGCTCC**A ATGCGTCTATTTAGTGGAGCCGTCTATCGGGCCTGACCAAAAATATA
plp_AGTR1_4	/5Phos/CAATGAAGTCCCGCCAA**CCTCAATGCACATGTTTGGCTCC** AATGCGTCTATTTAGTGGAGCCGACTATCTGGCTATTGTTCACC
plp_AGTR1_5	/5Phos/GCCAGTGTTTTTCTTAA**CCTCAATGCACATGTTTGGCTCC**A ATGCGTCTATTTAGTGGAGCCTGCTATCATGAAGCTGAAGACTGTG
plp_CGB_1	/5Phos/CTGCTGCTGTTGCTAA**CCTCAATGCACATGTTTGGCT CC**AATGCGTCTATTTAGTGGAGCCCACTATCGAGATGTTCCA GGGG
plp_CGB_2	/5Phos/GGCTGTGGAGAAGGAAA**CCTCAATGCACATGTTTGG CTCC**AATGCGTCTATTTAGTGGAGCCAGCTATCCCATCAATG CCACCCT
plp_CGB_3	/5Phos/GCAACTACCGCGATGTAA**CCTCAATGCACATGTTTGG CTCC**AATGCGTCTATTTAGTGGAGCCACCTATCTGCCTCAGG TGGTGT
plp_CGB_4	/5Phos/GCAGCACCACTGACTAA**CCTCAATGCACATGTTTGG CTCC**AATGCGTCTATTTAGTGGAGCCATCTATCATGTGCACT CTGCCGCC

Table S2c: 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 bold; 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 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.

Table S5				First Trimester Control	Third Trimester Control
n				198	52
Foetal		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 foetal 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: preeclampsia; eoPE: early-onset PE (delivery <34 gestational weeks); loPE: late-onset PE (delivery ≥34 gestational weeks). Values shown as mean (SD).

Table S7			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)
Foetal 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)
Foetal	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-preeclampsia; 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
Foetal 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)¶
Foetal	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)

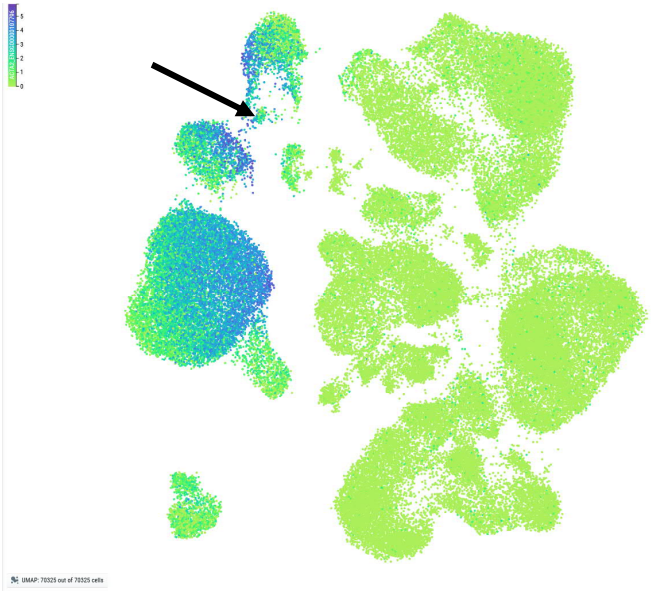
		lower risk	higher risk
n		8	9
maternal age	*years*	25 (6.50)	29 (7.43)
mean RI		0.74 (0.11)	0.88 (0.03)
GA	*days*	73.3 (5.6)	72.9 (3.6)

Values are shown in mean (SD).

Figure S1. CD34 expression in early placental CD34 scRNA-seq data.

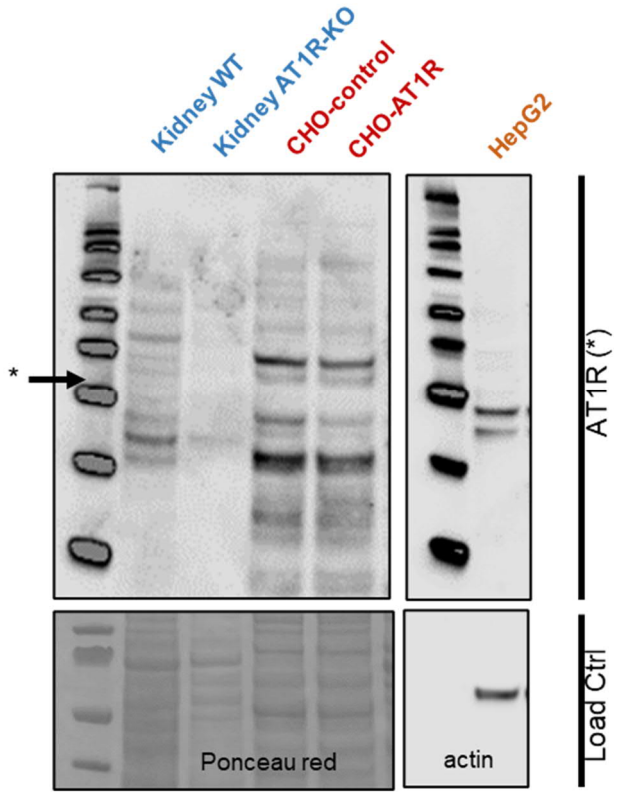
A

B



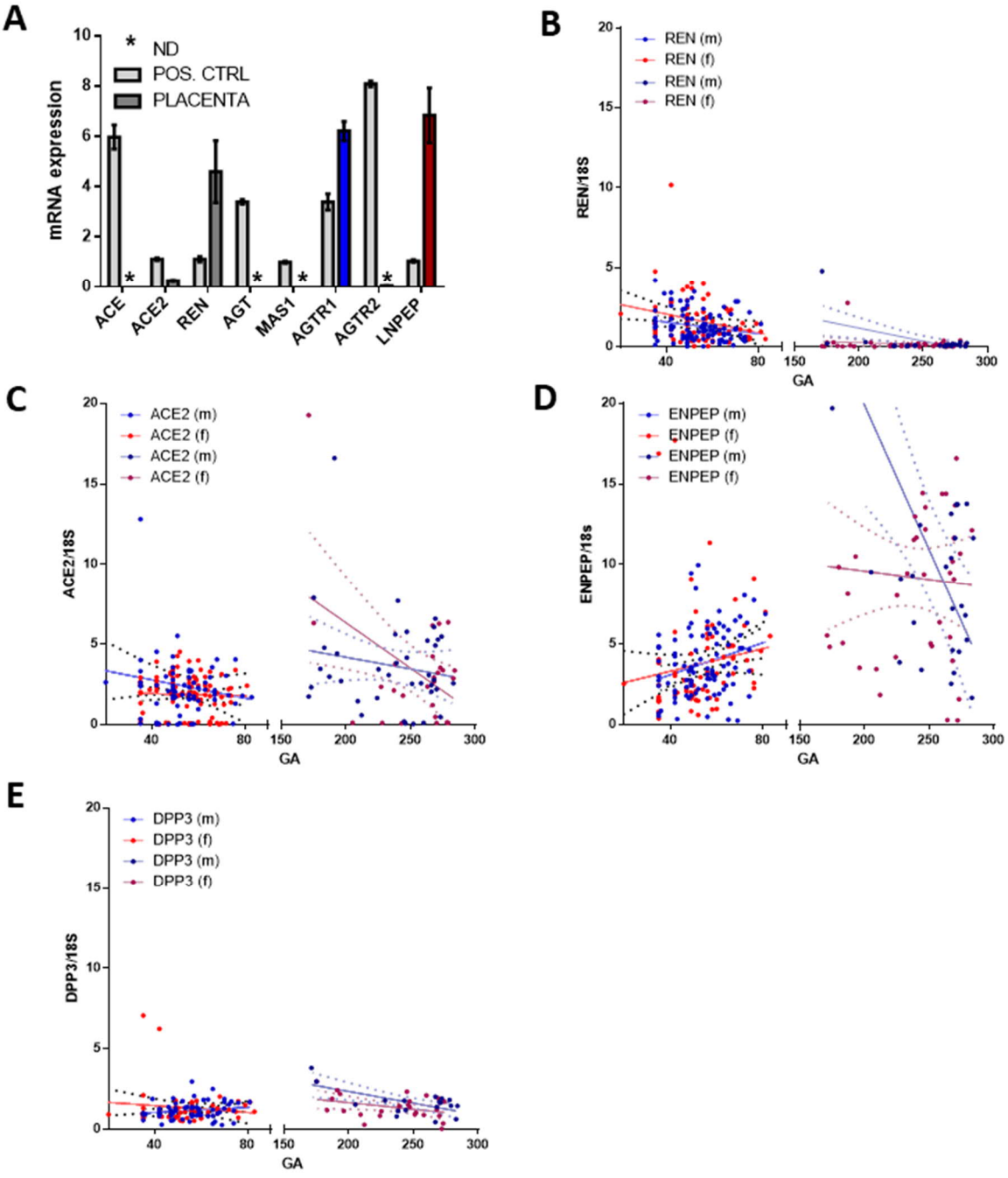
(A) *CD34* as an endothelial marker is expressed in “fEndo” (foetal endothelial cells), while it is not expressed in “fFB2” (arrow) (B) that in turn expresses *ACTA2* and can thus be assumed to represent villous myofibroblasts. (See full cell annotation as published by Vento-Tormo in Figure 7).

Figure S2. Non-specific AT1R Antibody



According to product datasheet a band of approximately 43kDa* (predicted molecular weight 41kDa; Anti-Angiotensin II Type 1 Receptor antibody ab124734 by abcam); pos. control band size (HepG2 cell lysate, marked brown) differs from data sheet; marked red are AGTR1 expression of control CHO and AT1R expressing CHO validated in qPCR (see Figure 14). Marked blue are mouse kidney lysates with AT1R knocked out and as wildtype, respectively.

Figure S3. 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 S4. Amplification products of extended RAS

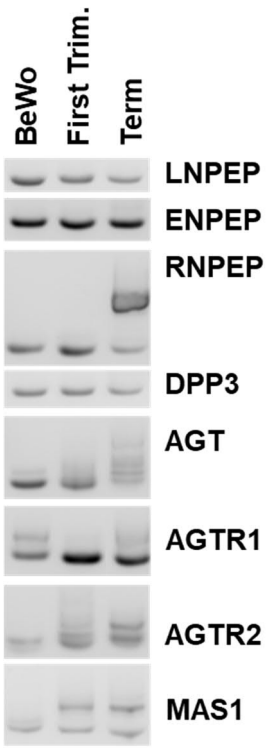
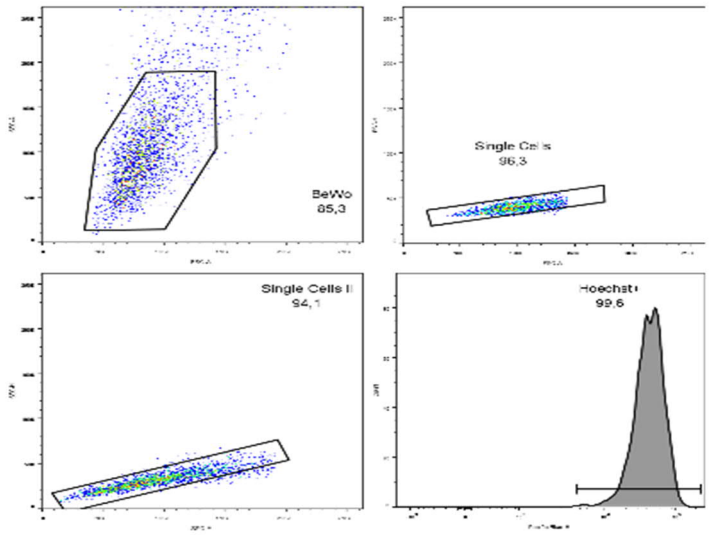
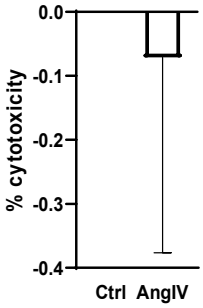


Figure S5. Gating strategy for normalizing Seahorse XF metabolic assays to cell number.



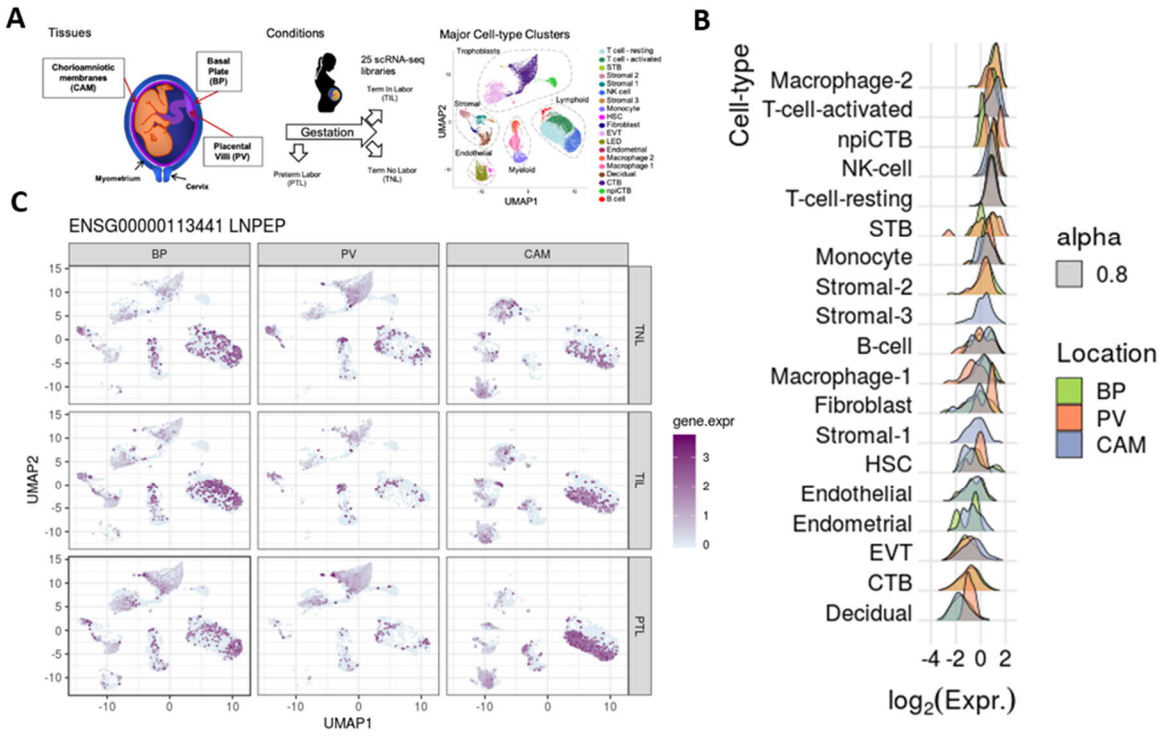
Cells were stained with Hoechst and only positive cells were sorted. BeWo cells from experiments were analysed with the BD FACSCanto II flow cytometer and BD FACSDiva software (BD Bioscience). Data analysis was performed with FlowJo v.10 (FlowJo LLC).

Figure S6. Cytotoxicity of AngIV



Cytotoxicity of AngIV was measured with an LDH assay, explants were incubated for 24h with 4nmol AngIV and showed no significant difference to the control condition.

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 labour (TNL), term in labour (TIL) and preterm labour (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 labour (TNL), term in labour (TIL) and preterm labour (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).

Appendix on single cell RNA sequencing.

This appendix aims to clarify methodological details that may be required to understand results in more detail.

1. *Pre-platform phase*

While it is cutting edge technology that actually sequences RNA derived from single cells, there are several steps before actual sequencing occurs (Figure S8). There are for instance multiple possibilities to achieve the necessary single cell solution that is then used for sequencing. Some authors use enzymatic digestion, sometimes with or without mechanical fragmentation beforehand (needle aspiration or scalpel use (Liu *et al.*, 2018b; Suryawanshi *et al.*, 2018; Vento-Tormo *et al.*, 2018; Tsang *et al.*, 2017). If certain cells are normally underrepresented, they can be enriched using FACS *(f*luorescence-activated cell sorting) or Percoll-gradients to achieve a better characterisation via scRNA-seq protocols.

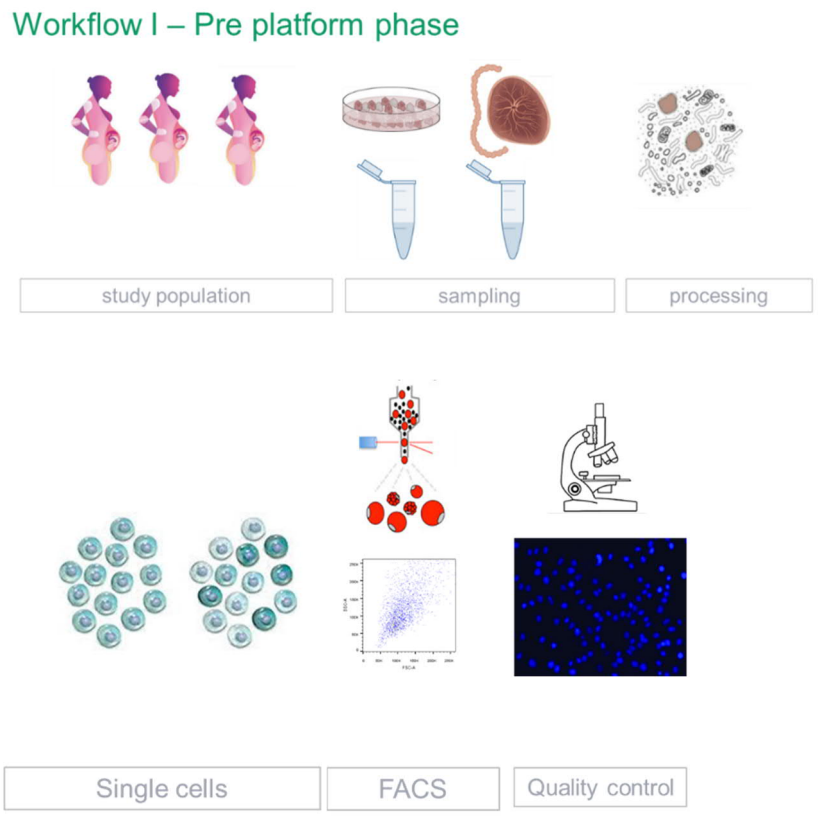


Figure S8. Summary of the first part of scRNA-seq workflow. *Summary of pre-platform processing steps, starting with defining a study population suitable to collect fresh tissue samples then proceeding with the type of sampling and tissue or cell processing to achieve a*

solution of viable single cells. This might be followed by quality control of viability and non-attachment of several cells by FACS and microscope.

Roughly, the steps after having created a single cell suspension that has passed quality control are to load this onto chips where the droplets containing a single cell or single nucleus are created. So called barcoding, that is required to attribute mRNA to its cell of origin, is followed by 3' reverse transcription, to achieve more stable cDNA with sequencing adapters which is then amplified in order to sequence. After library preparation for the according sequencing platform, the reads can be post-processed (Figure S9).

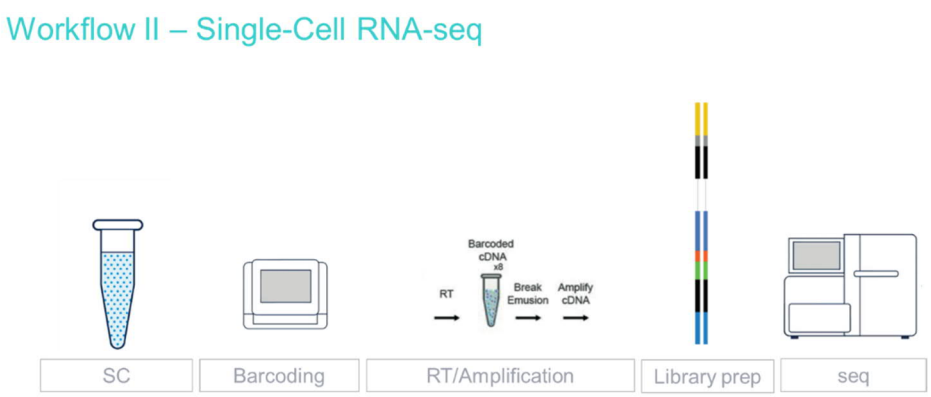


Figure S9. Second part of the workflow in scRNA-seq.

To achieve single cells being barcoded, in droplet based sequencing techniques a microfluidics partitioning approach is used. Cells are mixed with the reagents needed to lyse cells and for reverse transcription (RT), a gel bead is used for providing barcode sequences. Each droplet, also called GEM (gel beads in emulsion), thus ideally contains a gel bead with unique barcodes, a single cell, RT reagents, lysis buffer.

The oligonucleotide primer that are introduced via the gel bead surface contain different sections. The sequencing adapter depends upon the next generation sequencing (NGS) platform used, the barcode is the unique molecular address for each GEM, thus a single cell. The unique molecular identifier (UMI) on the other hand is used to later be able to accurately quantify each transcript. It only exists once in a given run. The poly(dT) tail is used to capture the free mRNA in the droplet that was released after addition of the lysis buffer.

After mRNA capture at the 3' end via the poly(dT) tail of the GEM oligonucleotide, reverse transcription occurs within the droplet as the reagents are already provided during the microfluidics partitioning. The resulting cDNA needs to be cleaned up whereby reagents from RT or remaining oil from the microfluidic emulsion is removed. Amplification of cDNA follows, and the library is prepared, containing specific sequencing adapters (such as "TruSeq" adapters for the commonly used Illumina platform). Enzymatic fragmentation and size selection are used to optimize the cDNA amplicon size. Sample identifiers are added, and sequencing occurs identically to bulk samples. Deconvolution of data to gain transcriptomic data is achieved by post-processing and bioinformatical analyses (Figure S10).

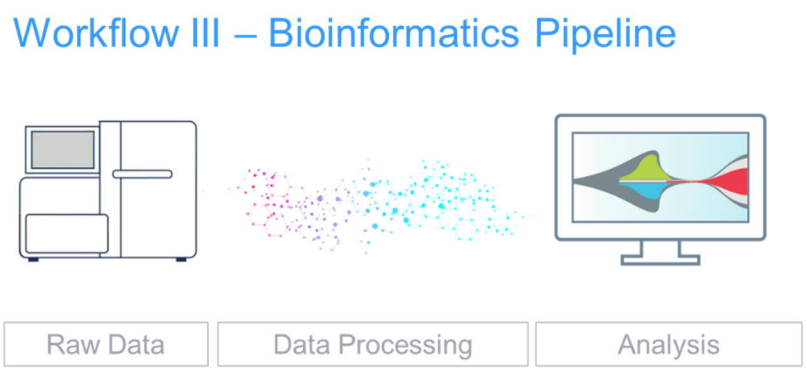


Figure S10. Post-sequencing workflows in scRNA-seq.

Firstly, sequenced data from single cells needs aligning reads. Reads are realigned and quantified against the GRCh37 human reference genome using for instance the Cell Ranger Single-Cell Software (as did Vento-Tormo *et al.*, 2018a).

The technical problem that needs bioinformatical adjustment is the possibility to have not only empty droplets with ambient RNA reads, but also multiplets. This is the case when in Drop-Seq techniques two or more nuclei or cells are enclosed into one droplet and therefore are barcoded with the same code and seemingly derive from one cell.

This is not resolved by using a relatively easy approach of defining cut-offs but by bioinformatical tools that are able to de-convolve droplet-based scRNA-seq experiments in which cells are pooled from multiple genetically distinct individuals (Vento-Tormo *et al.*, 2018).

This is achieved by inferring the most probable genetic identity via estimation of the likelihood of overlapping known single nucleotide polymorphisms in observed scRNA-seq reads.

After evaluation of detected genes per cell for all libraries, we concluded that the cut-off at 300 genes per cell is appropriate. We also looked at the distribution of cells in respect to their percentage of mitochondrial reads. A cut-off above 10% is suggested for human samples (Osorio & Cai, 2020), we evaluated that a standard cut-off above 20% is stricter and appropriate to avoid biased analysis.

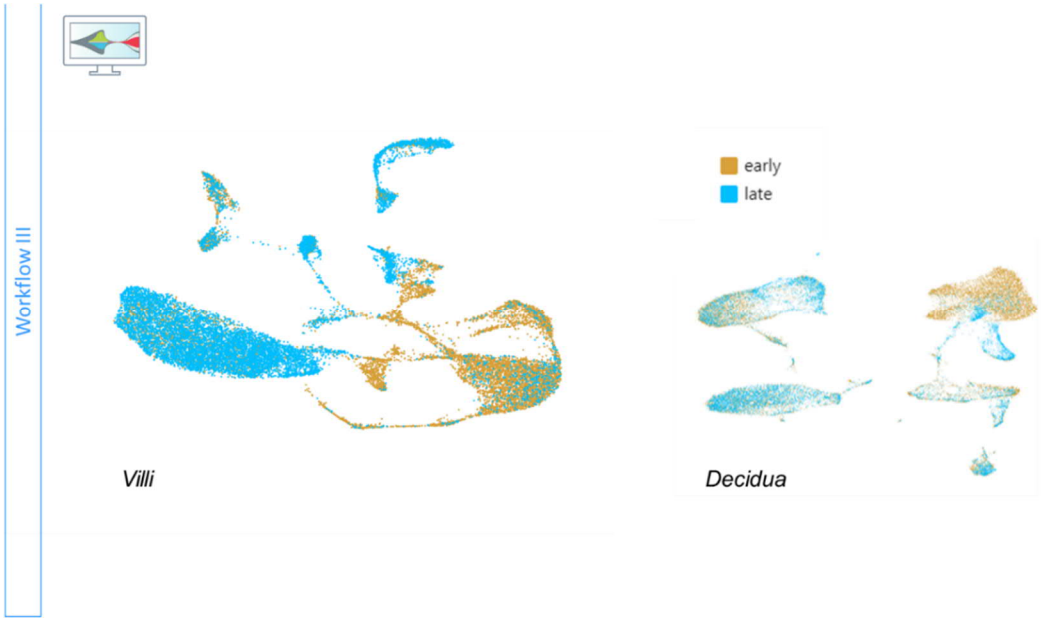


Figure S11. Integrating different data sets. *Integrated scRNA-seq data from different samples, deriving not only from different tissues but also reflecting different time points which is visualised in a UMAP projection that underlies all data entities.*

After unambiguously defining cells and corresponding transcripts, the data can be integrated again on the same projection to find similarity between various samples. As seen in Figure S30 (own unpublished data), early and late villous and decidual samples partly overlap when using an integrated UMAP projection of different data sets.