Uterine immunovascular adaptations in early pregnancy

Voor mijn ouders

Colophon

Uterine immunovascular adaptations in early pregnancy

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Uterine immunovascular adaptations in early pregnancy

Uteriene immuno-vasculaire aanpassingen tijdens de vroege zwangerschap
(met een samenvatting in het Nederlands)

Proefschrift

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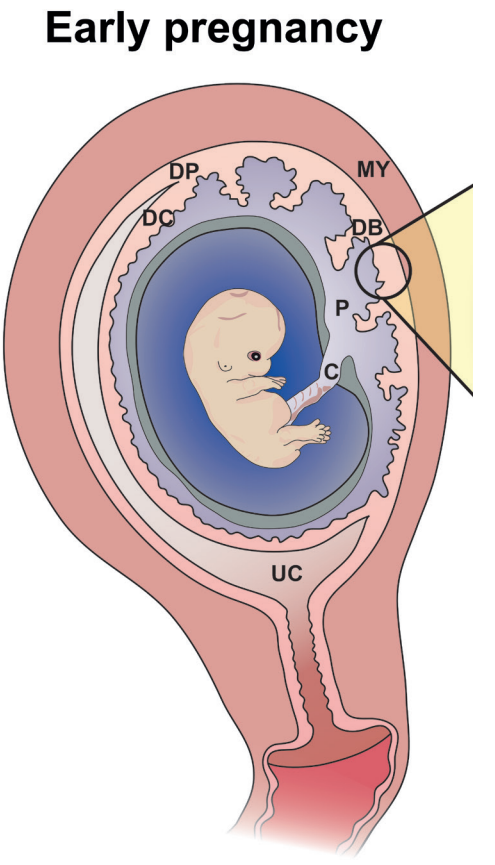
Chapter 1

GENERAL INTRODUCTION

Human pregnancy represents a well-coordinated partnership between the mother and the fetus, which requires extensive maternal adaptations to secure optimal conditions for fetal growth and development.[1] In the human, changes in structure, cellular distribution and function of the endometrium begin in the luteal phase of the menstrual cycle, in anticipation of embryo implantation. This process is termed *decidualization* and is orchestrated by circulating maternal hormones.[2] Endometrial decidualization is accompanied by accumulation of *immune cells*.[3] In the event of embryo implantation decidualization intensifies and the endometrium is characterized by invasion of fetal extravillous trophoblast (EVT) cells originating from anchoring villi of the developing placenta (**Figure 1**).

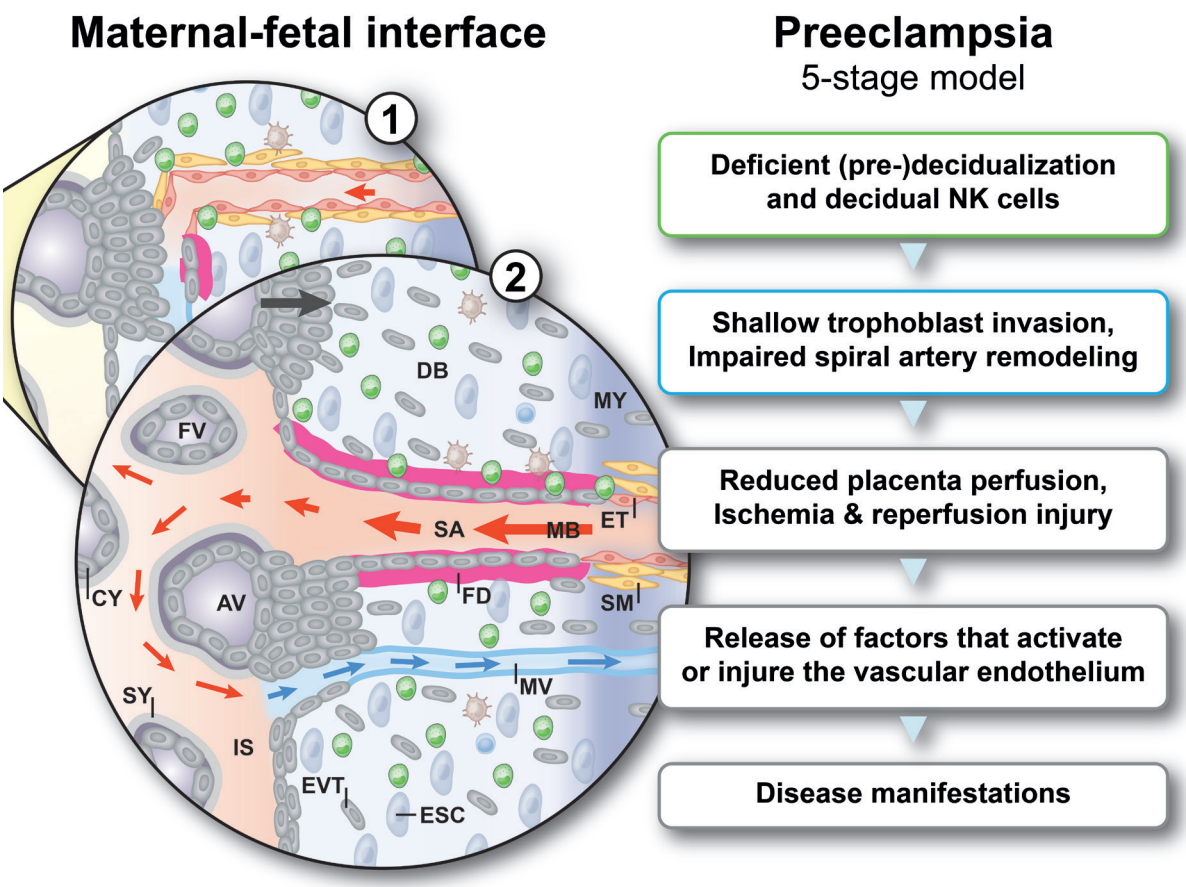


↓ Figure 1

Uterine immunovascular adaptations in early pregnancy and preeclampsia.

Schematic overview of the immunovascular (1st and 2nd trimester) changes in the uterus during early pregnancy, and a five-stage model for the placental syndrome preeclampsia. Endometrial decidualization together with regulatory functions of uterine natural killer (uNK) cells emerge as important prerequisites for successful placentation. Perturbations in either or both of these biological processes may lead to development of placental dysfunction and the maternal vascular manifestations of preeclampsia.

AV=anchoring villus. C=umbilical cord. CY=cytotrophoblast. DB=decidua basalis. DC=decidual capsularis. DP=decidua parietalis. ET=endothelial cell. EVT=extravillous trophoblast. FD=fibrinoid deposition. FV=floating villus. IS=intervillous space. MB=maternal blood. MV=maternal vein. MY=myometrium. SA=spiral artery. SM=smooth muscle cell. ESC=endometrial stromal cell. SY=syncytiotrophoblast. UC=uterine cavity. Gray arrow indicates direction of trophoblast differentiation/invasion.



Next, maternal (immune) and fetal EVTs act in concert to remodel the uterine spiral arteries that deliver maternal blood to the placenta.[4,5] Failure of EVT invasion and spiral artery remodeling are associated with common pregnancy complications including fetal growth restriction and *preeclampsia*.[6,7] Preeclampsia complicates 3-5% of pregnancies, and is an important cause of both maternal and perinatal morbidity and mortality.[8] Although impaired uterine immunovascular adaptations are implicated, we lack clear understanding of preeclampsia etiology. Moreover, prophylactic and curative measures for this clinical syndrome are conspicuously missing.[9] The World Health Organization (WHO) estimated that each year about 1.4 million women worldwide experience a pregnancy complicated by preeclampsia, resulting in an annual mortality rate of 63,000 women.[10] However, the molecular mechanisms underlying impaired immunovascular adaptation in early pregnancy are still largely unknown. As preeclampsia continues to be a worldwide health problem,[11] better insights into the mechanisms regulating the immunovascular adaptations in *early* pregnancy is important to aid a breakthrough in the development of early predictive biomarkers and preventive strategies for preeclampsia and related pregnancy complications.

DECIDUALIZATION AND EXTRAVILLOUS TROPHOBLAST INVASION

Decidualization of the endometrium occurs in all species with placentation and is accompanied by invasion of fetal trophoblast cells into the uterine wall. The extent of endometrial decidualization correlates with the depth of trophoblast invasion in different species.[12] Interestingly, in menstruating mammals such as humans, apes, Old World monkeys, some bats, and the elephant shrew, decidualization is initiated in the second half of each menstrual cycle.[13-15] In menstruating mammals placental trophoblast cells invade deeply into the endometrial spiral arteries and differentiate into EVTs. In humans this EVT invasion extends as far as the spiral artery sections located in the inner third of the myometrium.[13,14,16] Decidualization not only consists of extensive transformation of the endometrial stroma and glands, but also of remodeling of the uterine arterial vasculature. Obviously, these processes require close and continuous cooperation between endometrial vascular, epithelial, stromal and immune cells.[17]

Human decidualization is associated with the endometrial accumulation of immune cells of the macrophage and natural killer cell lineage.[18] Together these cells account for more than 90% of total uterine leukocytes, where they cluster in and around the spiral artery vascular wall.[19] During the secretory phase of the menstrual cycle, especially uterine natural killer cells (uNK) increase dramatically in number

in the endometrium, accounting for 30-40% of all cells in the decidualizing stromal cell compartment at the end of the first trimester of pregnancy. Uterine NK cell numbers decline in the second half of pregnancy, suggesting that these cells are particularly important during early gestation.[3,20,21] In rodents, increasing numbers of uNK cells are also a characteristic feature of the decidual reaction. More specifically, uNK cells have been shown to play a key role in remodeling the rodent uterine vasculature.[22,23] In humans the exact role of uNK cells in these uterine adaptations to pregnancy remains enigmatic, but several lines of evidence support the view that spiral artery remodeling requires a shared effort of uNK cells and invading EVTs.[5,24,25]

Decidualization is induced in the estrogen-primed endometrium by the post-ovulatory rise in circulating progesterone levels.[2] However, the very first morphological signs of decidualization in cells surrounding the terminal spiral arteries adjacent to the luminal epithelium only occur after the ± 9th post-ovulatory day.[26] This lag period in the initiation of decidualization indicates that other cues, such as the circulating peptide hormone relaxin that starts to circulate with the onset of decidualization,[27] are required to initiate this differentiation process.

RELAXIN AND ITS RECEPTOR RXFP1

The circulating hormone relaxin is produced by the corpus luteum.[28] This 6kDa peptide hormone is detectable in the circulation beginning ~7 days after ovulation, and peaks at 14 days post-ovulation,[27,29] followed by a rapid decline if pregnancy fails. During pregnancy, circulating relaxin levels have been observed in several mammals including humans, non-human primates, rats and mice.[30] In humans, circulating relaxin concentrations further increase rapidly up to ~12 weeks' gestation in parallel with human chorionic gonadotropin (hCG). Although the level of relaxin declines modestly in the second trimester, it continues to circulate until term. Relaxin can be detected in decidual stromal cells and fetal trophoblast cells suggesting local production at the maternal-fetal interface.[31,32] The hormone exerts its actions mainly through binding to the G protein-coupled receptor RXFP1, that activates several downstream signal transduction pathways including an intracellular rise in cAMP, release of nitric oxide (NO), or both depending on the cell type.[33] Relaxin contributes to the regulation of maternal cardiovascular adaptations to pregnancy in the rat and is a potent vasodilator.[34] Relaxin-RXFP1 signaling stimulates a rise in cardiac output, renal blood flow, glomerular filtration rate (GFR) and global arterial compliance in conscious rats.[35] Moreover, relaxin stimulates decidualization of human endometrial stromal cells,[36-38] and decidualization by progesterone increases relaxin expression by decidualizing endometrial stromal cells *in vitro*.[39,40] Studies in non-pregnant ovariectomized

rhesus monkeys revealed that administration of relaxin in addition to estrogen and progesterone not only stimulates endometrial vascular changes characteristic of early pregnancy, but also induces a rise in the endometrial number of macrophages and NK cells.[41] Thus, besides systemic vascular actions, relaxin may be involved in the immunovascular adaptations observed at the maternal-fetal interface in early human pregnancy.

UTERINE SPIRAL ARTERY REMODELING

The endometrium is vascularized by spiral and basal arteries both originating from radial arteries that emerge from the arcuate plexus in the myometrium deep in the uterine wall.[42] The basal arteries provide the blood supply to the inner myometrium and basal layer of the endometrium. Meanwhile, the spiral arteries supply the endometrium and form the uteroplacental blood supply during pregnancy.[4] Sufficient uteroplacental blood supply during advanced pregnancy can only be secured by proper remodeling of the spiral arteries in early pregnancy.[43] First signs of spiral artery remodeling occur during endometrial decidualization in the absence of local EVTs. The endothelial cells undergo vacuolization and the smooth muscle cells in the vascular tunica media become slightly larger.[4,44] Uterine NK cells and macrophages are thought to contribute to this "trophoblast-independent" stage of spiral artery remodeling.[19,25,45] Further structural changes of the spiral artery wall occur after the appearance of EVTs in and around the vessels, and include disorganization of the endothelial and vascular smooth muscle cell layers. After 8 weeks of pregnancy invading EVTs reach the lumen of the terminal endings of the endometrial spiral arteries. At 15 weeks of gestation EVTs have reached the myometrial sections of the spiral arteries. EVTs incorporate into the vessel wall and will completely replace the muscular layer of the spiral artery.[4,46,47] As a consequence of this VSMC replacement, vasomotor control of the spiral artery is completely abolished to ensure an increase in uterine blood flow from 20-50 mL/min in the pre-pregnancy state to 450-800 mL/min in late pregnancy.[48] Spiral artery remodeling not only allows for an increase in perfusion of the intervillous space to secure sufficient exchange of nutrients, oxygen and waste products for the growing fetus, but also for perfusion with reduced flow velocity and shear, important for preventing damage to the villous structure of the developing placenta. [42] The importance of adequate spiral artery remodeling by maternal immune cells and EVTs during early pregnancy is exemplified by pregnancy complications such as miscarriage, fetal growth restriction and preeclampsia.

THE PLACENTAL SYNDROME PREECLAMPSIA

The diagnosis of preeclampsia is based on *de novo* hypertension together with either new-onset proteinuria or end-organ dysfunction developing after 20 weeks pregnancy. Clinical manifestations include maternal vascular dysfunction, enhanced systemic inflammation, renal dysfunction and fetal growth restriction. If left unmanaged, preeclampsia may become complicated by eclamptic seizures, cerebral hemorrhage, pulmonary edema, renal failure, hepatic failure, and death.[7,49] Fetal complications are a direct consequence of placental hypoperfusion and often necessitate elected preterm delivery. Preeclampsia is often subdivided into an early-onset (delivery <34 weeks) and a late-onset (delivery >34 weeks) disorder.[50,51] The perinatal and maternal risks for severe complications are more prevalent when preeclampsia develops before 34 weeks of pregnancy.[9]

Primigravidas have a 3- to 5-fold higher *risk* of developing preeclampsia than multiparas. The likelihood to develop preeclampsia is also raised in women with a family history of preeclampsia (mother or sister). Moreover, women with a history of preeclampsia have a ~14% or greater chance to develop a recurrence in their next pregnancy.[52] Other important risk conditions include infertility, multiple pregnancy, chronic hypertension, chronic vascular and/or renal disorders, dyslipidemia, systemic lupus erythematosus, antiphospholipid syndrome, diabetes, obesity, relative young or old age (<20 years or >35 years) and thrombophilia.[7,53]

Since removal of the placenta is currently the only cure of preeclampsia, placental dysfunction is generally believed to play a key role in triggering the development of the disease.[7] It is thought that shallow EVT invasion and impaired spiral artery remodeling lead to a relative reduction in uteroplacental blood flow and hypoxia-reperfusion injury in the placenta. The resulting placental stress causes the release of factors into the maternal circulation that activate or injure the vascular endothelium. The injury of endothelial cells is likely responsible for the development of hypertension and other disease manifestations (**Figure 1**). However, the underlying mechanisms that result in placental stress are currently only partly understood. Several placenta-derived factors with either anti-angiogenic or pro-inflammatory properties have been proposed to trigger maternal systemic endothelial dysfunction.[54] The human endothelium, with a surface area of ~350 m^2, not only provides a physical barrier between the muscular layer of the vessel wall and its lumen, but is also actively involved in many vascular functions such as blood pressure regulation, control of vascular tone, volume homeostasis, hemostasis and inflammatory responses.[55] These endothelial functions play an important role in both enabling and maintaining the early-pregnancy cardiovascular adaptations

and are disturbed during preeclampsia.[56-58] In this context, it should be emphasized that this maternal vascular response to placental dysfunction also depends on the preexisting maternal constitution, as genetic, behavioral and metabolic factors are important determinants of vascular homeostasis.[59,60]

SCOPE OF THE THESIS

Pregnancy is a challenging condition for maternal health. Pregnant women undergo a wide range of structural and functional adaptations in early pregnancy to efficiently accommodate placental and fetal development. In this thesis we describe studies of the mechanisms of maternal immune and vascular adaptations contributing to the development of an adequately functioning placenta. We also investigate how aberrations in these processes may contribute to the development of the placental syndrome preeclampsia.

PART I
THE DECIDUALIZING ENDOMETRIUM AND UTERINE NK CELLS

In **Chapter 2** the transcriptional and functional changes in human uNK cells during early pregnancy, are identified. **Chapter 3** gives a comprehensive overview of the literature with focus on how the 'uterine niche' directs the development and dynamic functions of uNK cells to regulate endometrial and spiral artery remodeling, EVT invasion and maintenance of homeostasis at the maternal-fetal interface. Moreover, aberrations in uNK cell number and the degree of maturation within the uterine niche are discussed in the context of pregnancy complications across the spectrum of early pregnancy loss, fetal growth restriction, and preeclampsia. Taking a bioinformatics approach **Chapter 4** explores the possibility of deficient or defective maturation of the endometrium (decidualization) and/or the abundant endometrial accumulation of NK cells to compromise EVT invasion in preeclampsia.

PART II
THE HORMONE RELAXIN AND ITS RECEPTOR RXFP1 IN PREGNANCY

Important adaptive changes in the systemic cardiovascular system accompany uterine spiral artery remodeling during early pregnancy. The circulating pregnancy hormone relaxin contributes to these changes. In **Chapter 5**, we explored the localization and action of RXFP1 in the vasculature of various vascular beds and on endothelial cells in rats and humans. Circulating relaxin not only plays a role in the systemic maternal vascular adaptation to pregnancy but has also been implicated in the local preparation of the uterus to accommodate placentation. In **Chapter 6**, we used RXFP1 transgenic mice to explore the role of RXFP1 in the immunovascular adaptations at the maternal-fetal interface during early pregnancy. In **Chapter 7**, we tested the hypothesis that subnormal circulating relaxin levels in early human pregnancy predispose to the development of preeclampsia.

Finally, **Chapter 8** provides a summary of the findings presented in this thesis and gives an integral discussion of the implications for future research and opportunities for clinical translation.

SPECIFIC AIMS

PART I
THE DECIDUALIZING ENDOMETRIUM AND UTERINE NK CELLS

A Identifying developmental transcriptional changes in human uNK cells derived from 1st or 2nd trimester human decidual tissues to identify pathways involved in the timely regulation of uNK cell function during human early pregnancy (**Chapter 2**);

B Describe how the 'uterine niche' directs the development and function of NK cells to regulate endometrial and vascular remodeling, as well as maintenance of homeostasis at the maternal-fetal interface supporting human pregnancy (**Chapter 3**).

C To elucidate if preeclampsia and related disorders are antedated by disturbances in decidualization of the endometrium and the uNK cells therein before and after embryo implantation (**Chapter 3** and **4**);

PART II
THE HORMONE RELAXIN AND ITS RECEPTOR RXFP1 IN PREGNANCY

D To identify if vascular effects of relaxin may be influenced by the cellular localization of RXFP1 within different blood vessels (**Chapter 5**);

E To elucidate the role of the relaxin receptor RXFP1 in immunovascular adaptations of the uterus during early mouse pregnancy (**Chapter 6**);

F To explore whether first trimester circulating relaxin is a suitable biomarker for the early prediction of preeclampsia (**Chapter 7**).

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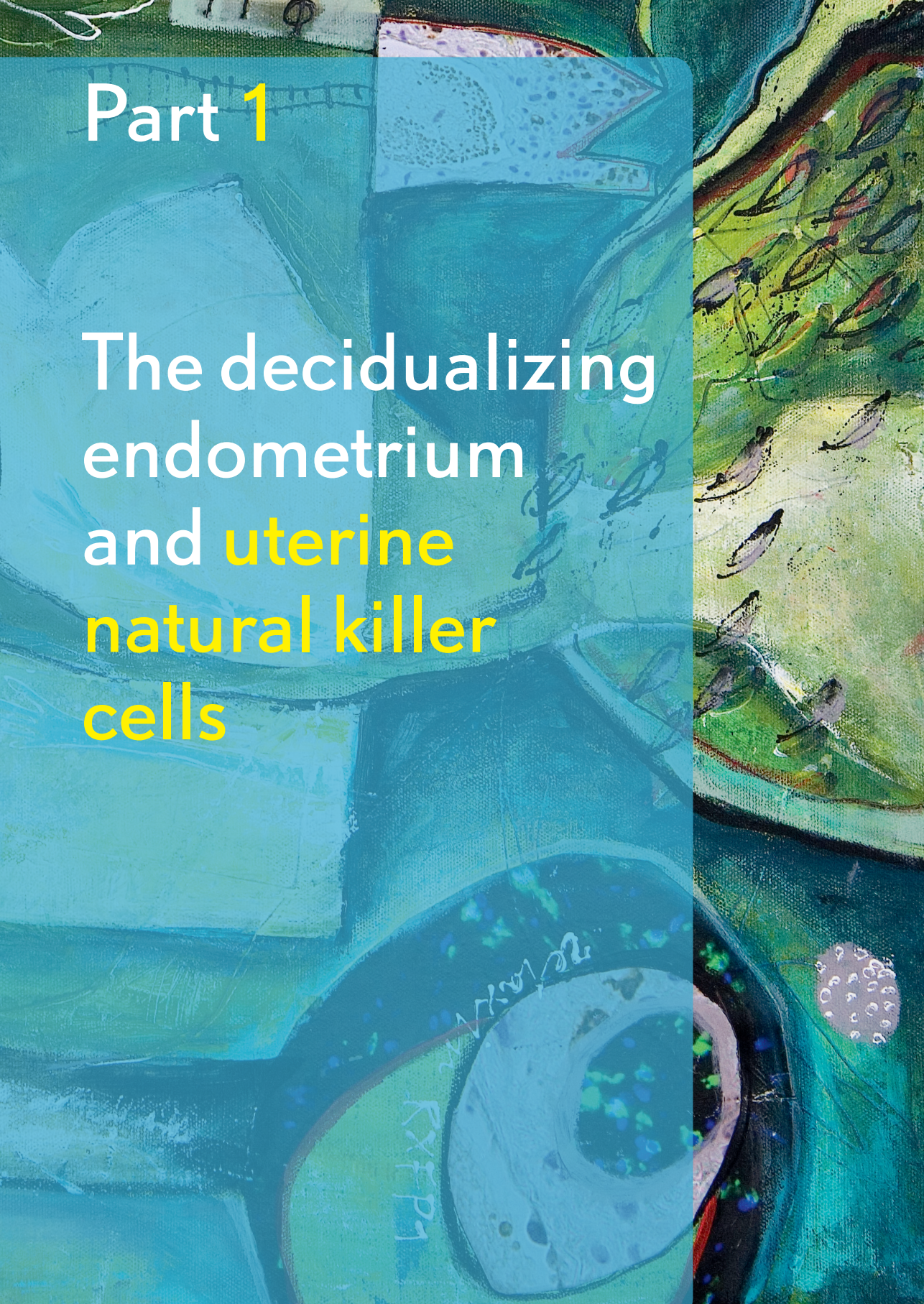

Part 1

The decidualizing endometrium and uterine natural killer cells

Chapter 2

THE INTERFERON TRANSCRIPTOME IN UTERINE NK CELLS IS UPREGULATED DURING THE SECOND TRIMESTER OF PREGNANCY ALLOWING EXTRAVILLOUS TROPHOBLAST INVASION

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ABSTRACT

Uterine natural killer (uNK) cells regulate essential developmental processes at the maternal-fetal interface during early pregnancy. Uterine NK cell functions are tightly regulated during early placentation to stimulate trophoblast invasion and remodel uterine spiral arteries. Access to human 1st and 2nd trimester uterine tissues allowed us to investigate the transcriptional changes in uNK cells during the early stages of early human pregnancy. Microarray analysis identified 97 upregulated genes in 2nd compared to 1st trimester purified uNK cells of which the majority (61%) clustered as interferon-stimulated-genes (ISG), with ISG15 and ISG20 being upregulated profoundly. Type I interferons (IFNα/β), but not type II interferon (IFN) increased expression of the identified interferon target genes ISG15 and ISG20 in uNK cells in vitro. Moreover, the cytokine-like protein ISG15 stimulated in vitro trophoblast invasion. Second trimester uNK cells promoted trophoblast invasion in vitro, whereas both 1st and 2nd trimester uNK cells stimulated endothelial tube formation. IFNα but not IFNβ stimulation of 1st trimester uNK cells enhanced their capacity to promote trophoblast invasion. In conclusion, the uNK cell interferon transcriptome is upregulated during the 2nd trimester allowing uNK cells to promote trophoblast invasion. Type I interferon signaling regulates uNK cell-induced trophoblast invasion via induction of effector molecules like ISG15. First trimester uNK cells can be induced to act like second trimester uNK cells by IFNα with respect to promotion of trophoblast invasion.

Key words: Gene expression profiling • uterine natural killer cells • extravillous trophoblast invasion • interferon alpha • ISG15

SIGNIFICANCE

Uterine natural killer (uNK) cells are regulators at the human maternal-fetal interphase. Relative inaccessibility of the maternal-fetal interface during pregnancy and diversity in placentation among species hamper our understanding of the timely regulation and function of human uNK cells during pregnancy. Aberrant uNK cell interactions with placental trophoblasts are implicated in the etiology of pregnancy complications including fetal growth restriction and preeclampsia. To understand the role of uNK cells in normal and complicated pregnancies the sequential changes in uNK cells during early pregnancy need to be elucidated. In this manuscript we identify a significant transcriptional change in uNK cells induced by type I interferon signaling to promote trophoblast invasion, an essential feature of human placentation.

INTRODUCTION

At the time of conception, the secretory endometrium undergoes a maturation process in anticipation of human embryo implantation and placentation. A hallmark of this maturation process is enrichment of a specialized $CD56^{bright}CD16^{-}$ NK (uNK) cell population in the decidualizing endometrium.[1,2] Decidualization continues during early pregnancy and by the end of the 1st trimester uNK cells constitute 30-40% of all endometrial stromal cells.[3,4]

The endometrium of the gravid uterus forms a unique microenvironment that regulates NK cell function.[5] Uterine NK cells differ functionally and phenotypically from their blood circulating counterparts and have a distinct transcriptional profile.[6] Uterine NK cells markedly change when the human endometrium of the non-gravid uterus decidualizes and matures.[7,8] During early pregnancy major developmental changes occur in the decidualized endometrium to accommodate pregnancy.[9] How these developmental processes affect uNK cell function is poorly understood.

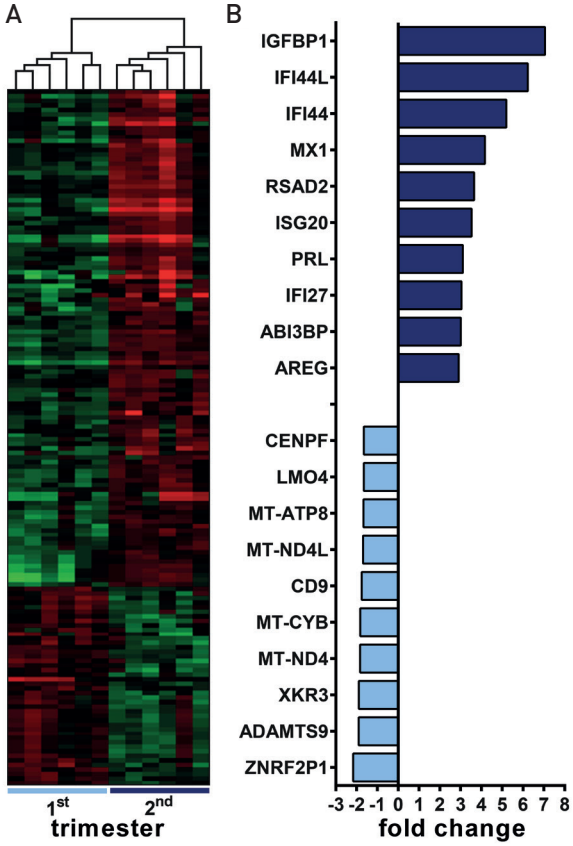
During the 1st trimester of pregnancy uNK cells are thought to contribute to the initial phase of spiral artery remodeling by producing matrix metalloproteinases, vasoactive cytokines and growth factors.[9-11] Towards the end of the 1st trimester (10-12 wks), the maternal arterial circulation of the intervillous space of the placenta is initiated.[12] Now uNK cells stimulate invasion of fetal extravillous trophoblasts (EVT) to complete the remodeling of the spiral arteries.[13,14] These EVT invade deeply into the uterine wall and spiral arteries, replacing the musculo-elastic coat of these blood vessels.[15,16] The resulting low-resistance, high capacitance vessels ensure adequate perfusion of the placental intervillous space.[17] Thus, during early pregnancy uNK cells are programmed in a timely manner in the uterine microenvironment to accommodate pregnancy. Nonetheless, the exact molecular changes in uNK cells that allow the timely regulated functions are poorly understood.

The aim of this study was to investigate the developmental transcriptional differences of human uNK cells derived from 1st or 2nd trimester human decidual tissues to identify pathways involved in the timely regulation of uNK cell function during human pregnancy.

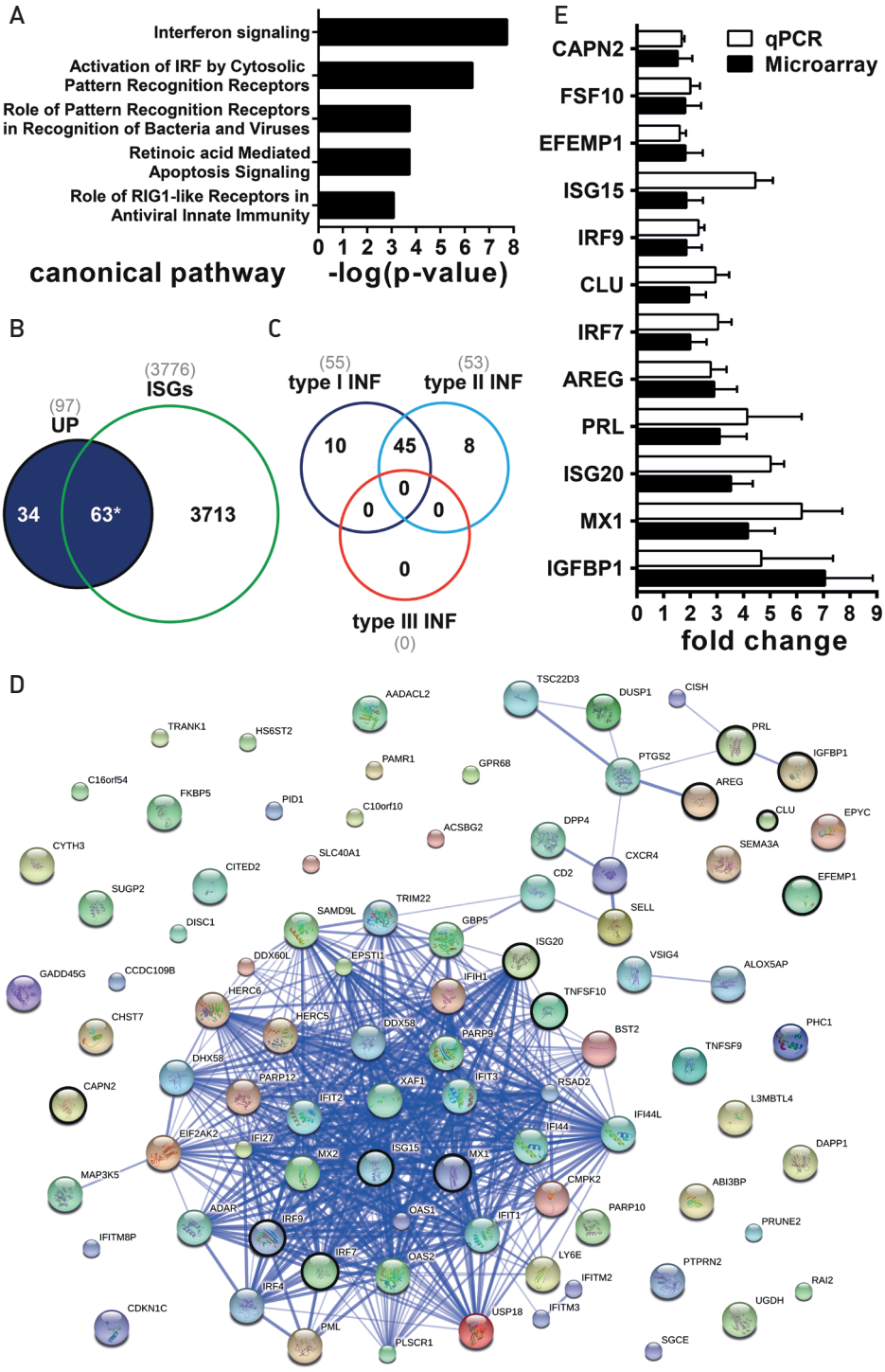
RESULTS

UTERINE NK CELL TRANSCRIPTIONAL CHANGES DURING THE PROGRESSION OF EARLY PREGNANCY

First (7-9 wks) and 2nd trimester (13-15 wks) human uNK cells were isolated from decidual tissues obtained during elective pregnancy terminations of apparently normal pregnancies. We isolated uNK cells on the basis of $CD56^{+}CD16^{-}CD3^{-}$ expression by flow assisted cell sorting (FACS) and analyzed these cells by whole genome gene-expression profiling (**Figure S1**). Uterine NK cells from the 1st and 2nd trimester were indistinguishable at the level of membrane expression of the activation antigens CD69 and HLA-DR, which showed low expression (data not shown).[18] MicroArray Analysis Of Variance (MAANOVA) of 1st and 2nd trimester uNK cells revealed differential expression of 135 known genes with at least a significant 1.4-fold change. Hierarchical clustering of the genes with differential expression clustered the individual uNK samples on the basis of the trimester (1st vs. 2nd) (**Figure 1A**). Of the 135 differentially expressed genes, 97 genes were upregulated, whereas 38 genes were down-regulated in 2nd trimester uNK cells compared to 1st trimester uNK cells (**Figure 1B, Table S1**). For subsequent analysis we focused on genes with increased expression during the 2nd trimester versus the 1st trimester.



← Figure 1
Transcriptional differences between 1st trimester and 2nd trimester uterine NK cells. (**A**) Relative intensity profiles of 135 genes differentially expressed with at least a 1.4-fold change at $P<0.05$ by MAANOVA after false discovery rate determination. Each row represents relative hybridization intensities of a particular gene across different samples. Each column represents one sample and the colors reflect the magnitude of relative expression of a particular gene. Green means lower expression, black average expression and red increased expression across samples. Samples cluster together in two groups based on the trimester of pregnancy they were isolated in; 1st (7-8w) or 2nd (13-14w) trimester. (**B**) List of fold-changes of the 10 genes with the highest fold up- or down-regulation in 2nd (7-8w) trimester compared to 1st (13-14w) trimester.


A
Interferon signaling
Activation of IRF by Cytosolic Pattern Recognition Receptors
Role of Pattern Recognition Receptors in Recognition of Bacteria and Viruses
Retinoic acid Mediated Apoptosis Signaling
Role of RIG1-like Receptors in Antiviral Innate Immunity
0 1 2 3 4 5 6 7 8
canonical pathway
-log(p-value)
E
qPCR
Microarray
CAPN2
FSF10
EFEMP1
ISG15
IRF9
CLU
IRF7
AREG
PRL
ISG20
MX1
IGFBP1
0 1 2 3 4 5 6 7 8 9
fold change
B
(3776)
ISGs
(97)
UP
34
63*
3713
C
(55)
type I INF
(53)
type II INF
10
45
8
0
0
0
type III INF
(0)
D
AADACL2
TSC22D3
DUSP1
CISH
TRANK1
HS6ST2
PRL
PAMR1
GPR68
PTGS2
IGFBP1
C16orf54
FKBP5
PID1
C10orf10
AREG
CLU
EPYC
ACSBG2
DPP4
CYTH3
SLC40A1
CXCR4
SEMA3A
CITED2
CD2
EFEMP1
SUGP2
TRIM22
SAMD9L
SELL
DISC1
GBP5
ISG20
DDX60L
EPSTI1
VSIG4
ALOX5AP
GADD45G
CCDC109B
IFIH1
TNFSF10
HERC6
CHST7
HERC5
DDX58
PARP9
BST2
PHC1
TNFSF9
DHX58
CAPN2
PARP12
XAF1
IFIT3
RSAD2
IFIT2
L3MBTL4
IFI44L
EIF2AK2
IFI27
IFI44
DAPP1
MX2
ISG15
MX1
MAP3K5
CMPK2
ABI3BP
ADAR
IRF9
OAS1
IFIT1
PARP10
PRUNE2
IFITM8P
IRF7
OAS2
LY6E
PTPRN2
IFITM2
RAI2
IRF4
CDKN1C
USP18
IFITM3
UGDH
PML
PLSCR1
SGCE

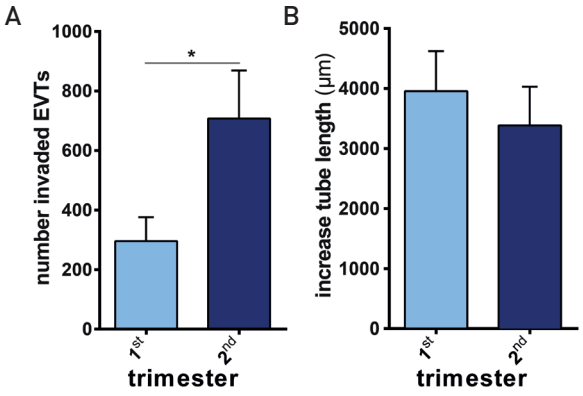
↑ Figure 2

← **Figure 2**
Pathway analysis and validation of uNK cell genes that increased in expression between the 1st and 2nd trimester of pregnancy. (**A**) Canonical pathways expressed as levels of significance by Ingenuity® Pathway Analysis (IPA). (**B**) Venn diagram of the identified upregulated genes in 2nd trimester uNK cells and genes known as 'interferon stimulated genes' (ISGs) (*P<1.0x10^{-22} by hypergeometric test). (**C**) Venn diagram of genes known to be regulated by type I, II and III interferons. (**D**) Clustering of protein-protein interactions of upregulated genes in 2nd trimester uNK cells based on the STRING 9.1 database. Black encircled nodes were selected for validation of the microarray data. Thickness of blue lines represents strength of association. (**E**) Twelve upregulated genes were selected for validation of the microarray data (n=6) by qPCR (n=7). Expression levels of the selected genes were normalized against the expression of the GAPDH and expressed as fold-change of 2nd compared to 1st trimester uNK cells.

PATHWAY ANALYSIS OF UPREGULATED TRANSCRIPTS IN uNK CELLS

In order to map the upregulated transcripts in 2nd trimester uNK cells into known signaling pathways, we employed Ingenuity® Pathway Analysis. The top canonical pathways identified are interferon signaling (P=1.9x10^{-8}) and activation of interferon regulated factors (IRFs; P=4.8x10^{-7}; **Figure 2A**). Next we performed a transcription factor target analysis by WebGestalt software to predict which transcription factors are upstream of the upregulated genes. The upregulated genes were significantly associated with 10 transcription factor binding sites (P<0.00017) of which 8 are known to bind transcription factors involved in interferon signaling (**Table S2**). These transcription factors include the interferon stimulated response element (ISRE), interferon regulatory factors (IRF) and signal transducers and activators of transcription (STAT; see for complete list **Table S2**). Importantly, 63 of the upregulated genes (61%; P<1.0x10^{-22}) are interferon responsive genes (Interferome v2.0), representing type I and type II 'interferon stimulated genes' (ISGs) (**Table 1A, Figure 2B,C**). A protein-protein interaction network was constructed with the Search Tool for the Retrieval of Interacting Genes (STRING) (**Figure 2D**). Fourty-one of the 97 upregulated genes clustered and all these genes are known as interferon stimulate genes (**Table S1**). Quantification of expression of 12 upregulated genes (12%) selected from within and outside the ISG protein-protein interaction network confirmed the magnitude of upregulation of the genes in 2nd trimester uNK cells (**Figure 2E**). In contrast, validation of 4 down-regulated genes (~10% of down-regulated genes) with qPCR did not confirm down-regulation (**Figure S2**). Overall, these bioinformatical analyses indicate that increased type I and/or type II interferon signaling plays an important role in the regulation of uNK cells during the progression of 1st trimester pregnancy towards 2nd trimester pregnancy.

Figure 3 →
Functional differences of 1st and 2nd trimester uNK cells. (**A**) 1st (7-9w; n=6) and 2nd (13-15w; n=6) trimester uNK cells were cultured for 24h and the supernatant was added to the lower well of a transwell Matrigel invasion assay to stimulate extravillous trophoblast (EVT) invasion. After 24h incubation the number of invaded EVTs was determined. (**B**) The capacity of 1st and 2nd trimester uNK cells to promote endothelial tube formation was assessed by adding HMEC-1 cells to the wells of a µ-Slide angiogenesis coated with growth factor reduced Matrigel. The increase in total tube length after 8h incubation with 1st (7-9w; n=6) and 2nd (13-15w; n=6) trimester uNK cell-conditioned medium compared to control medium was determined.

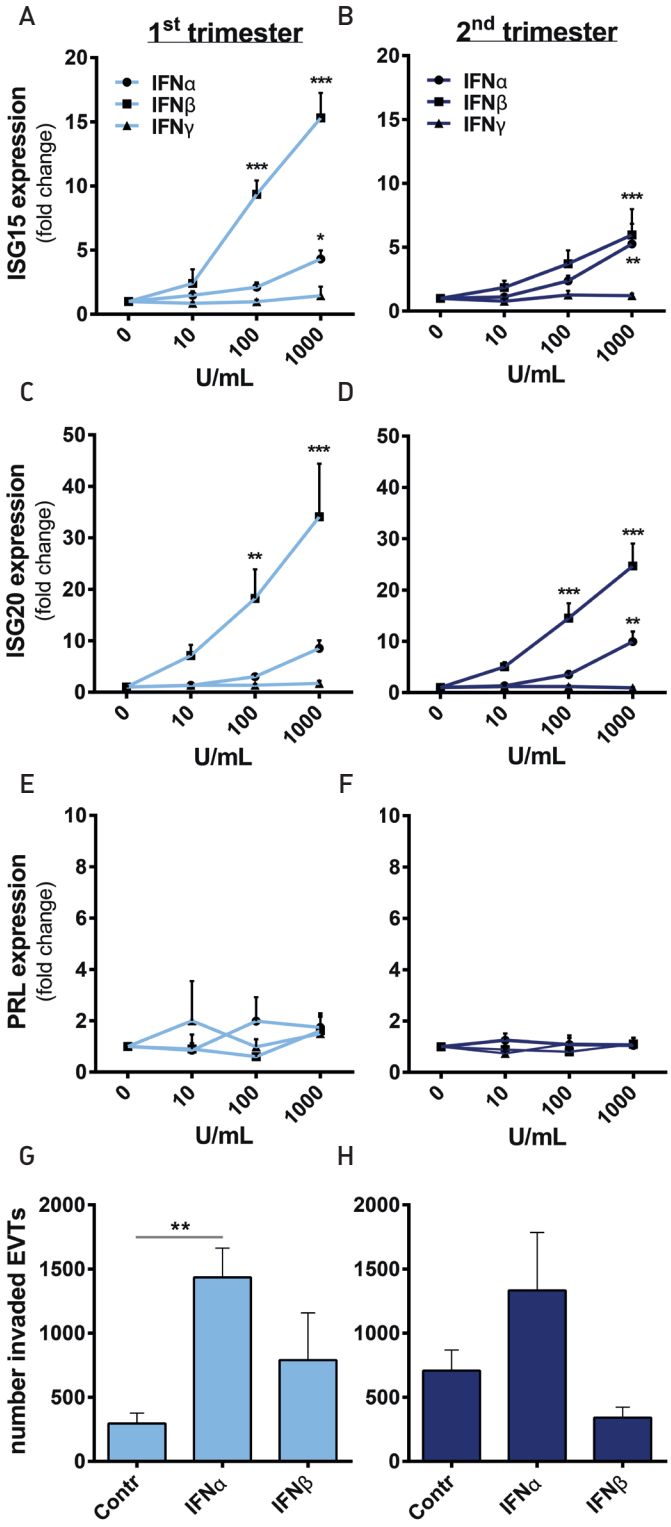


FIRST AND 2ND TRIMESTER uNK CELLS HAVE DIFFERENT FUNCTIONAL CAPACITIES

Next we assessed if the transcriptional differences of 1st and 2nd trimester uNK cells were associated with altered functional capacity to promote extravillous trophoblast (EVT) invasion and to stimulate vascular growth in vitro. First and 2nd trimester uNK cells were cultured for 24 hours and the supernatant was used for functional analysis. Second trimester uNK cells stimulated the invasion of EVTs compared to 1st trimester uNK cells (**Figure 3A**). Uterine NK cells also increased endothelial tube formation compared to control medium (**Figure 3B**). However, 1st and 2nd trimester uNK cells did not differ in their capacity to stimulate endothelial tube formation.

TYPE I BUT NOT TYPE II INTERFERONS UPREGULATE ISGs IN uNK CELLS

On the basis of the microarray analysis we identified expression of downstream targets of interferon signalling in 2nd trimester uNK cells. To investigate if these downstream targets of interferon signaling are induced in uNK cells by stimulation with interferons we tested the sensitivity of 1st (7-9 wks) and 2nd (13-15 wks) trimester human uNK cells to type I and type II interferon stimulation *in vitro*. Expression of the interferon regulated genes *ISG15* and *ISG20* and the non-interferon regulated gene for prolactin (*PRL*) and insulin-like growth factor binding protein (*IGFBP1*) were selected as read out. The type I interferons, IFNα and IFNβ, dose-dependently increased expression of *ISG15* (**Figure 4A,B**) and *ISG20* (**Figure 4C,D**) but not *PRL* (**Figure 4E,F**) and *IGFBP1* (data not shown) in both 1st and 2nd trimester uNK cells.



← **Figure 4**

Type I Interferon stimulation of 1st and 2nd trimester uNK cells induces transcriptional and functional changes. (**A**), (**C**), (**E**) First trimester (7-9w; n=4-6) and (**B**), (**D**), (**F**) 2nd trimester (13-15w, n=4-6) uNK cells were stimulated with 0, 10, 100 and 1000 U/mL IFNα, IFNβ and IFNγ and after 24h mRNA expression for the genes ISG15 (**A**), (**B**), ISG20 (**C**), (**D**) and PRL (**E**), (**F**) was determined to calculated the fold-induction compared to vehicle stimulation. (**G**) 1st trimester (n=5) and (**H**) 2nd trimester (n=6) uNK cell were stimulated with IFNα and IFNβ for 24h, and supernatants were added to the lower well of a transwell Matrigel invasion assay to stimulate extravillous trophoblast (EVT) invasion. After 24h of culture the number of invaded EVT was determined. IFNα and IFNβ did not stimulate EVT invasion. *P<0.01, **P<0.01, and ***P<0.001.

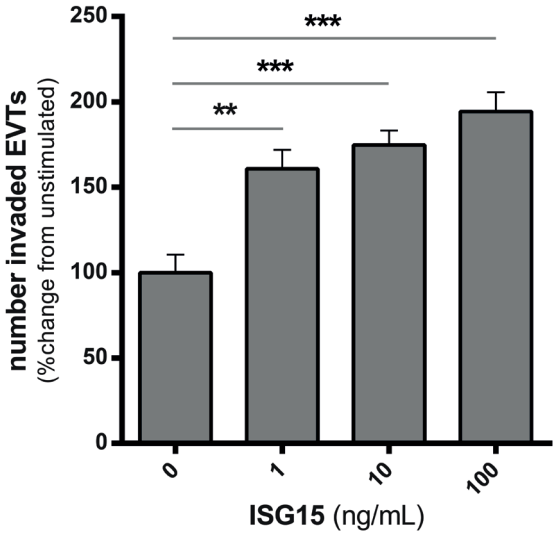
IFNα and IFNβ increased ISG15 expression in 1st trimester uNK cells by 4.3-fold and 15.3-fold respectively. Expression of ISG20 increased by 8.5-fold and 34.1-fold in 1st trimester uNK cells after respectively IFNα and IFNβ stimulation. IFNα stimulation of 2nd trimester uNK cells increased ISG15 and ISG20 expression to the same extent as 1st trimester uNK cells. However, the magnitude of the IFNβ-induced increase in ISG15 expression in 1st trimester uNK was approximately 2.5-fold higher than in 2nd trimester uNK cells (1st trimester 15.3-fold ±1.9 vs. 2nd trimester 6.0-fold ±2.0, $P<0.01$) (**Figure 4A,B**). In response to IFNβ the magnitude of increase in ISG20 expression was not significantly different when 1st trimester uNK cells where compared to 2nd trimester uNK cells (**Figure 4C,D**). Interestingly, stimulation with the type II interferon IFN did not affect expression of *ISG15*, *ISG20* and *PRL* in 1st or 2nd trimester uNK cells (**Figure 3A-F**). These data show that type I interferon but not type II interferon stimulation of 1st trimester uNK cells shifts the expression of interferon regulated genes towards a 2nd trimester uNK cell profile.

TYPE I INTERFERON STIMULATED uNK CELLS PROMOTE EXTRAVILLOUS TROPHOBLAST (EVT) INVASION

Uterine NK cells of the 2nd trimester acquire the capacity to promote EVT invasion compared to uNK cells from the 1st trimester. On the basis of the transcriptional differences between 1st and 2nd trimester uNK cells, we explored whether type I IFNs dictate the functional differences between 1st and 2nd trimester uNK cells. To this end we stimulated 1st trimester uNK cells with type I IFN and investigated whether 1st trimester uNK cells acquire the capacity to stimulate EVT invasion. Supernatant from IFNα-treated 1st trimester uNK cells increased EVT invasion by 4.9-fold compared with unstimulated 1st trimester uNK cell medium (**Figure 4G**). In contrast, stimulation of 1st trimester uNK cells with IFNβ did not induce a significant change in EVT invasion. In 2nd trimester uNK cells, IFNα and IFNβ stimulation did not significantly change the capacity of these uNK cells to promote EVT invasion compared to unstimulated 2nd trimester uNK cells (**Figure 4H**). Overall, these data demonstrate that IFNα promotes 1st trimester uNK cells to stimulate EVT invasion.

INTERFERON STIMULATED GENE 15 (ISG15) PROMOTES EVT INVASION

Our findings indicate that IFNα stimulates uNK cells to produce factors to promote EVT invasion. On the basis of our microarray data and our in vitro data we identified ISG15 as a potential type I interferon-induced protein in uNK cells that can be secreted. As such we tested whether ISG15 could act as an effector of uNK cell mediated stimulation of EVT invasion. Addition of ISG15 to the lower well of a transwell EVT invasion assay stimulated EVT invasion in a dose-dependent fashion. At



← **Figure 5**
ISG15 stimulates extravillous trophoblast (EVT) invasion. To identify if ISG15 promotes trophoblast invasion, 1, 10 and 100 ng/mL recombinant human ISG15 (n=6) was added to the lower well of a transwell Matrigel invasion assay to stimulate EVT invasion. After 24h stimulation the number of invaded EVT was determined. $^{**}P<0.01$ and $^{***}P<0.001$).

a concentration of 1 ng/mL EVT invasion was increased by 59% compared to control medium. Stimulation of EVT with 10 and 100 ng/mL ISG15 further stimulated trophoblast invasion with respectively 73% and 91% (Figure 5).

DISCUSSION

Uterine NK cells populate the dynamic maternal-fetal interface favoring the well-timed process of human placentation. We found that uNK cells undergo prominent transcriptional changes in early pregnancy and acquire functions to allow extravillous trophoblast (EVT) invasion. Analysis of the genome-wide transcriptional changes identified type I interferon signaling as the important upstream pathway in uNK cells especially in the 2nd trimester. We identified upregulation of several type I interferon target genes in particular ISG15. ISG15 is a cytokine-like protein and we show here that ISG15 is a potent stimulator of EVT invasion. ISG15 expression and the capacity to promote EVT invasion were the highest in uNK cells of the 2nd trimester. However, ISG15 expression could be more strongly induced in 1st trimester uNK cells by type I interferons. Moreover, the type I interferon IFNα stimulated uNK cells of the 1st trimester to promote EVT invasion, leading to our conclusion that an increase in type I interferon signaling, most likely by IFNα, is critical for the functional transition of uNK cells with respect to EVT invasion. Overall these findings highlight type I interferons as important regulators of human uNK cells at the maternal-fetal interface during the progression from the 1st to the 2nd trimester of pregnancy.
On the basis of the identified transcriptional changes, we discovered a clear

interferon signature in uNK cells from the 2nd trimester of pregnancy. The interferon signature consisted of clustering of the presence of transcription factor binding sites in the upregulated genes recognized by ESRE, IRF and STAT transcription factors. Moreover, 61% of the upregulated genes were recognized as type I or type II interferon responsive. Predominantly type II interferon is known to regulate developmental changes in the decidua during early pregnancy. For example, uNK cells produce IFNγ that is essential for the transformation of the uteroplacental spiral arteries in mice.[19] Similarly in humans IFNγ may contribute to spiral artery remodelling.[10] We show here that expression of ISGs in human uNK cells is not responsive to IFNγ. These data suggest that uNK cells are not regulated by type II but rather by type I interferons.

Our findings showing an important role of type I interferons in regulating uNK cells raises the question which cells in the uterine niche produce these cytokines. Type I interferons are produced by the placenta.[20-22] In humans, IFNα and IFNβ are detected in chorionic villous syncytiotrophoblast. IFNα was also detected in EVT in the endometrium and maternal decidual leukocytes, as well as fetal Hofbauer cells in the villous mesenchyme.[22,23] In this study we show that 2nd trimester uNK cells have an enhanced interferon stimulated gene profile. Thus, it is likely that during the transition from the 1st to the 2nd trimester the production of type I interferons in the decidua increases through increased numbers of trophoblasts in the decidualizing endometrium to regulate uNK cell functions in a paracrine or endocrine fashion.

During the 1st and 2nd trimester of pregnancy, differentiation of trophoblast cells into an invasive phenotype is of vital importance for adequate remodeling of the uterine spiral arteries.[15,16] Shallow trophoblast invasion and defective spiral artery remodeling accompany pregnancy complications such as fetal growth restriction and preeclampsia.[24] Interestingly, an earlier study has shown that addition of supernatant from 2nd trimester, but not of 1st trimester uNK cells to EVTs from the same gestational age induced their invasion.[13] However, whether either the EVTs or the uNK cells had acquired differential functional capacities has not been studied. In this study we show that 2nd trimester uNK cells are better at promoting EVT invasion than uNK cells of 1st trimester pregnancies. Moreover, earlier studies showed that 2nd trimester EVTs intrinsically do not invade more, but are rather less invasive compared to 1st trimester EVTs.[25,26] Thus, we propose that the increased EVT invasion in the 2nd trimester is due to changes in uNK cell function and not by EVTs.

An important question is which downstream effectors of interferon signaling are changed in uNK cells to promote EVT invasion. Among the upregulated ISGs in 2nd compared to 1st trimester uNK cells, the ISG15 is also known as a secreted protein with cytokine-like properties.[27,28] More importantly, we identified that ISG15 expression in uNK cells is regulated by type I interferons and identified a novel

function of ISG15 in that ISG15 promotes trophoblast invasion. Thus, on the basis of our data we suggest that ISG15 might be a central effector protein of uNK cells to stimulate trophoblast invasion, that is regulated through the 1st and 2nd trimester of human pregnancy by type I interferons. ISG15 has originally been suggested to play a role in decidualization, implantation and placentation in mice. In mice deficient of ISG15, 50% of the embryos die between 7.5 to 12.5 days of pregnancy.[29,30] In humans, primates, ruminants and mice uterine ISG15 expression increases in response to pregnancy.[31,32]

Next to ISG15, our microarray analysis revealed upregulation of other factors in uNK cells during the 2nd trimester, which are known as stimulators of EVT invasion. These factors included prolactin (PRL) and IGF-binding protein-1 (IGFBP-1) and are known to be strongly upregulated in endometrial stromal cells during decidualization.[33,34] However, stimulation of uNK cells with interferons did not change PRL and IGFBP-1 expression. Possibly, factors that induce or are associated with endometrial decidualization regulate expression of these non-ISGs in uNK cells between the 1st and 2nd trimester.

In summary, we have identified a developmental transcriptional change in uNK cells during the progression of early pregnancy. Importantly, these transcriptional changes contribute to the capacity of uNK cells to stimulate EVT invasion and are induced by type I interferon signaling. Especially IFNα converts uNK cells into promoters of EVT invasion. The changes in uNK cell regulatory capacity are reflected by increased ISG15 expression, a cytokine-like protein that we here present as a novel candidate for an uNK cell effector of EVT invasion.

METHODS

TISSUES

Fresh tissue was obtained from apparently healthy women undergoing elective surgical termination of confirmed viable pregnancies (ultrasound) with a gestational age of 7-9 weeks (1st trimester) and between 13-15 weeks (2nd trimester). Gestational age was defined as weeks since the first day of the last menstrual period and if uncertain, adjusted on the basis of embryonic crown-rump length measurements (ultrasound). All patients had given informed consent according to the Declaration of Helsinki and the study was approved by the University Medical Center Utrecht Institutional Review Board (protocol number: 11-181/C).

ISOLATION OF HUMAN uNK CELLS

Decidual tissue was identified macroscopically by its grey-white solid appearance and directly dissociated by mechanic and enzymatic digestion for 30 minutes with a

combination of collagenase type IA (1 mg/mL; Sigma-Alderich, Saint Louis, MO) and DNSase type I (Sigma-Alderich) in RPMI1640 medium (Gibco, Life Technologies, Carlsbad, CA), containing 2 mM L-glutamine, 100 U/mL penicillin and 100 ug/mL streptomycin (RPMI+) supplemented with 10% heat-inactivated fetal calf serum (RPMI++). The decidual homogenate was washed and filtered through 100, 70 and 40 μm cell strainers (BD Biosciences, Franklin Lakes, NJ). After lysing of erythrocytes (FACS lysing solution, BD Biosciences), the decidual cell suspension was labeled with anti-CD56 (clone B159), anti-CD16 (**Figure S1**) and anti-CD3 (clone SK7) (all from BD biosciences). Uterine NK cells were isolated with a BD FACSAria III cell sorter (BD Biosciences) based on $CD56^{bright}CD16^{-}CD3^{-}$ phenotype (**Figure S1**). Uterine NK cell purity was confirmed to be higher than 99% immediately after cell sorting. For *in vitro* studies the filtered decidual cell suspension was cultured overnight in RPMI++ supplemented with 2 ng/mL rhIL15 (Sanquin, Amsterdam, the Netherlands), to improve cell viability. After Ficoll-Paque™ Plus (GE Healthcare, Uppsala, Sweden) gradient separation of the non-adherent decidual cell fraction, cells were labeled and sorted as detailed above.

TOTAL RNA ISOLATION

RNA of $2.0x10^6$ uNK cells was isolated by RNeasy Micro Kit according to the manufacturer's protocol (Qiagen). RNA integrity was assessed with an A2100 Bio-analyser (Agilent Technologies, Waldbronn, Germany). All RNA samples used had a RIN value of 8.2-9.4.

MICROARRAY AND PATHWAY ANALYSIS

Microarrays were performed on an Agilent whole genome microarray platform (Agilent, Santa Clara, CA) as previously described.[35] Biological canonical pathways attributed to the upregulated genes were identified using Ingenuity Pathway Analysis (IPA; Qiagen, Redwood City, CA). Transcription factor target analysis was performed with the WEB-based Gene SeT AnaLysis Toolkit (WebGestalt; http://bioinfo.vanderbilt.edu/webgestalt/). Interferon stimulated genes were identified by Interferome v2.01 (http://interferome.org). Protein-protein interaction networks were assessed by the Search Tool for the Retrieval of Interacting Genes v.9.05 (STRING; www.string-db.org). All microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) under accession number GSE64898. See for detailed description on pathway analysis *Supporting Information*.

IN VITRO uNK CELL STIMULATION

$1.0x10^5$ freshly isolated uNK cells were cultured in 96-well U-bottom plates in 100 μL X-VIVO 15 medium (Lonza, Basel, Switzerland) supplemented with 10% FCS and

20 ng/mL rhIL-15 (Sanquin). Cells were stimulated with 0, 10, 100 and 1000 U/mL IFNα, IFNβ and IFNγ (PeproTech, Rocky Hill, NJ). After 24 h medium was harvested and uNK cells were lysed for RNA analysis in Buffer RLT (Qiagen, Hilden, Germany). Control medium was harvested similarly, though after incubation without uNK cells.

REAL-TIME QUANTITATIVE PCR

Total RNA was isolated as described above and 130 ng was used for reverse transcription reactions with Oligo(dT) T7 MLu VN primers (custom mix) and SuperScript Reverse Transcriptase (Invitrogen, Paisley, UK) according to the manufacturer's protocol. The resulting cDNA was diluted 1:40 for analysis by real-time qPCR performed with the iQ5 Real-Time PCR Detection System (Bio-Rad Laboratories, Veenendaal, the Netherlands). Data obtained by qPCR were individually normalized to the mean expression of housekeeping gene GAPDH. See *Supporting Information* **Table S3** for primer list.

TRANSWELL EXTRAVILLOUS TROPHOBLAST INVASION ASSAY

In short, 3.0×10^4 HTR-8/SVneo cells were serum starved overnight,[36] and placed in the insert of a 96-well format BioCoat Tumor Invasion System (Corning, Bedford, MA) according to the manufacturer's instructions. In the lower well either uNK cell conditioned medium diluted 1.7-fold in RPMI+, or RPMI+ with recombinant human ISG15 (12729-HNAE1, Sino Biological Inc., Beijing, China) or control RPMI+ medium was added. After 26h cells that had reached the lower site of the insert-membrane were labeled with the nuclear dye SYTOX Green (Invitrogen) at a concentration of 1.0 μM. Photographs were taken with an EVOS fluorescent microscope (AMG, Mill Creek, WA). Cell invasion was quantified by counting all nuclei of invaded cells with the Nucleus Counter plugin for ImageJ (http://rsb.info.nih.gov/ij/).

ENDOTHELIAL TUBE FORMATION ASSAY

5.0×10^3 HMEC-1 cells were cultured on 10 μL growth factor reduced Matrigel (concentration 8.0 mg/mL, BD Biosciences) in a μ-Slide angiogenesis (Ibidi, Martinsried, Germany) and incubated in 50 μL uNK cell conditioned media diluted 1:1 in MCDB131 medium (Gibco).[37] After 8 hours at 37°C, 5% CO_2, phase-contrast images were taken of the μ-Slides on a Zeiss Observer.Z1 equipped with AxioCam MRm camera (Zeiss, Jena, Germany) and analyzed by the Angiogenesis Analyzer plugin for ImageJ (http://rsb.info.nih.gov/ij/).

STATISTICS

Data were analyzed for statistical significance using (unpaired) Student *t* test or one-way or two-way analysis of variance (ANOVA) with Bonferroni correction where

appropriate. All values are expressed as mean ± standard error of the mean (SEM). *P*-values <0.05 were considered statistically significant. Data handling of the microarray experiment is detailed in *Supporting Information*.

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DISCLOSURES

The authors declare no conflict of interest or affiliations that might be perceived as influencing the objectivity of this work.

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CHAPTER 1
SUPPORTING INFORMATION (SI)

SI METHODS

MICROARRAY ANALYSIS

For the microarray experiment we used a common-reference design employing the universal Human Reference RNA (Stratagene) as a common reference. All donor samples were labeled with cy5, reference cRNA was labeled with cy3. Microarrays used were human whole genome gene expression microarrays V2 (Agilent, Belgium) representing 34127 *H.sapiens* 60-mer probes in a 4x44K layout (probe sequences from this array were re-annotated by BLAST-searching against genomebuild version 76_38 at ENSEMBL). cDNA synthesis, cRNA amplification, labeling, quantification, quality control and fragmentation were performed with an automated system (Caliper Life Sciences NV/SA, Belgium), starting with 1 μg total RNA from each sample, all as previously described in detail.[1] Microarray hybridization and washing was with a HS4800PRO system with QuadChambers (Tecan, Benelux) using 1000 ng, 1.3-3% Cy5/Cy3 labeled cRNA per channel as described.[1] Slides were scanned on an Agilent G2565BA scanner at 100% laser power, 30% PMT. After automated data extraction using Imagene 8.0 (BioDiscovery), Loess normalization was performed[2] on mean spot-intensities. Data were further analysed by MAANOVA,[3] modeling sample, array and dye effects in a fixed effect analysis. *P*-values were determined by a permutation F2-test, in which residuals were shuffled 10,000 times globally. Gene probes with $P<0.05$ after false discovery rate determination (FDR by Benjamini-Hochberg) were considered significantly changed and fold-change of 1.4 was used as a cutoff. In cases of multiple probes per gene, the values from the most 3' probe were used.

The microarray data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus[4] and are accessible through GEO Series accession number GSE64898 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE64898).

INGENUITY PATHWAY ANALYSIS (IPA)

We used IPA to identify enriched Canonical Pathways among the upregulated genes. *P*-values are calculated by Fisher's exact test right-tailed (http://ingenuity.force.com/ipa/articles/Feature_Description/Canonical-Pathways-for-a-Dataset).

WEBGESTALT TRANSCRIPTION FACTOR TARGET ANALYSIS

The WEB-based GEne SeT AnaLysis Toolkit (WebGestalt) was used for enrichment analysis of upregulated genes. WebGestalt is a web-based integrated gene set analysis

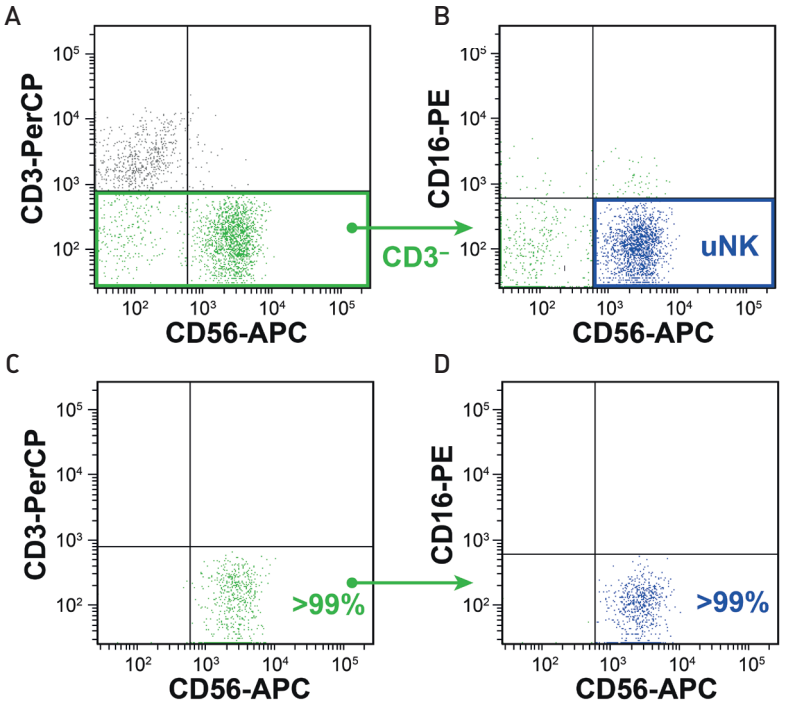
tool to explore large sets of genes. We used the transcription factor target analysis module that is based on comparative genomic analysis made available through MSigDB.[5] The software allows identification of sets of genes sharing common transcription factor target binding sites and was based on the hypergeometric distribution algorithm with multiple testing correction using the Benjamini-Hochberg method ($P<0.01$).[6]

PATHWAY ANALYSIS OF INTERFERON STIMULATED GENES

Interferon stimulated genes were identified by Interferome v2.01 (http://interferome.org).[7]

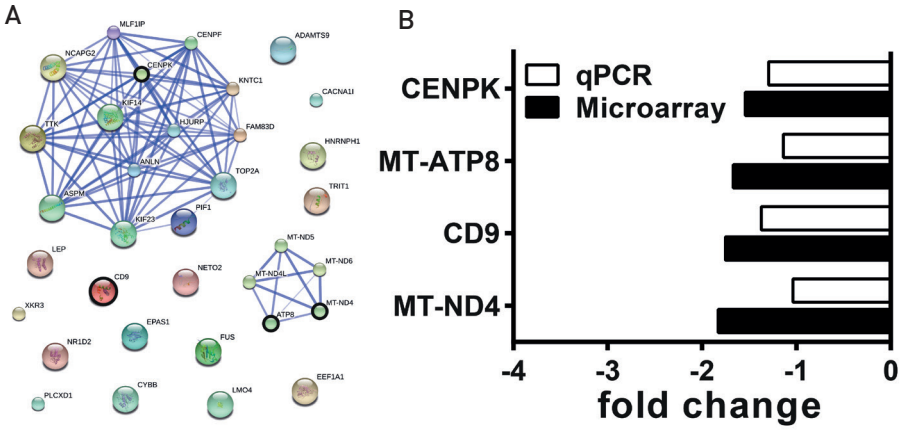
PROTEIN-PROTEIN INTERACTION NETWORK

The Search Tool for the Retrieval of Interacting Genes (STRING) v.9.05 (www.string-db.org)[8,9] was used to annotate interactions of upregulated genes (see **Table 1A**). To cast a wide net we combined information from all prediction methods (neighborhood, gene fusion, co-occurrence, co-expression, experiments, databases and text-mining), with a confidence score of 0.400 as a cut-off, and no limitations to the number of interactors, to generate the protein-protein interaction network.



↑ **Figure S1**

Gating strategy for isolation of uNK cells. (**A**) Uterine NK cells were selected on the basis of a CD_3-CD_{56}+CD_{16}- phenotype and sorted for the microarray or functional analysis. (**B**) Flow cytometric analysis of sorted uNK cells to assess the purity of the selected uNK cell population on the basis of CD_{56} and CD_{16} expression.



↑ **Figure S2**

Pathway analysis and validation of uNK cell genes that decreased in expression between the 1st and 2nd trimester of pregnancy. (**A**) Clustering of protein-protein interactions of down-regulated genes in 2nd trimester uNK cells based on the STRING 9.1 database. Black encircled nodes were selected for validation of whole genome microarray data. Thickness of blue lines represents strength of associations. (**B**) We selected 4 downregulated genes for validation with qPCR. Expression was normalized against GAPDH and expressed as fold-change of 2nd compared to 1st trimester uNK cells.

Table S1A. Up-regulated genes in 2nd compared 1st trimester uNK cells

Agilent ID	HUGO approved symbol	HUGO approved name	HGNC ID	Location	Fold-change	IFN I	INF II	INF III
A_23_P42868	IGFBP1	insulin-like growth factor binding protein 1	HGNC:5469	7p12.3	7.054			
A_23_P45871	IFI44L	interferon-induced protein 44-like	HGNC:17817	1p22.3	6.230	X	X	
A_23_P23074	IFI44	interferon-induced protein 44	HGNC:16938	1p31.1	5.196	X	X	
A_23_P17663	MX1	MX dynamin-like GTPase 1	HGNC:7532	21q22.3	4.169	X	X	
A_24_P28722	RSAD2	radical S-adenosyl methionine domain containing 2	HGNC:30908	2p25.2	3.652	X	X	
A_23_P32404	ISG20	interferon stimulated exonuclease gene 20kDa	HGNC:6130	15q26	3.528	X	X	
A_32_P65616	PRL	prolactin	HGNC:9445	6p22.3	3.109			
A_24_P270460	IFI27	interferon. alpha-inducible protein 27	HGNC:5397	14q32.12	3.044	X	X	
A_23_P218858	ABI3BP	ABI family. member 3 (NESH) binding protein	HGNC:17265	3q12.2	3.001			
A_23_P259071	AREG	amphiregulin	HGNC:651	4q13.3	2.902			
A_23_P72737	IFITM2	interferon induced transmembrane protein 2	HGNC:5413	11p15.5	2.863	X	X	
A_23_P102000	CXCR4	chemokine (C-X-C motif) receptor 4	HGNC:2561	2q21	2.669	X	X	
A_23_P406025	PRUNE2	prune homolog 2 (Drosophila)	HGNC:25209	9q21.32	2.537		X	
A_33_P3401826	CMPK2	cytidine monophosphate (UMP-CMP) kinase 2. mitochondrial	HGNC:27015	2p25.2	2.530	X		
A_23_P110196	HERC5	HECT and RLD domain containing E3 ubiquitin protein ligase 5	HGNC:24368	4q22.1-q23	2.454	X	X	
A_24_P329795	C10orf10	chromosome 10 open reading frame 10	HGNC:23355	10q11.21	2.386		X	
A_23_P428129	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57. Kip2)	HGNC:1786	11p15.5	2.369	X	X	
A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	17p13.2	2.342	X	X	
A_24_P38081	FKBP5	FK506 binding protein 5	HGNC:3721	6p21.31	2.245	X	X	
A_24_P16124	IFITM4P	interferon induced transmembrane protein 4 pseudogene	HGNC:21669	6p22.1	2.212			
A_33_P3400374	HELZ2	helicase with zinc finger 2. transcriptional coactivator	HGNC:30021	20q13.33	2.132	X		
A_23_P102391	SLC40A1	solute carrier family 40 (iron-regulated transporter). member 1	HGNC:10909	2q32	2.117		X	
A_24_P105191	HS6ST2	heparan sulfate 6-O-sulfotransferase 2	HGNC:19133	Xq26.2	2.091			
A_24_P347378	ALOX5AP	arachidonate 5-lipoxygenase-activating protein	HGNC:436	13q12	2.072	X		
A_23_P87545	IFITM3	interferon induced transmembrane protein 3	HGNC:5414	11p15.5	2.028	X	X	
A_33_P3283611	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	HGNC:5411	10q23.31	2.000	X	X	
A_24_P378019	IRF7	interferon regulatory factor 7	HGNC:6122	11p15.5	1.996	X	X	
A_23_P64828	OAS1	2'-5'-oligoadenylate synthetase 1. 40/46kDa	HGNC:8086	12q24.2	1.984	X	X	
A_23_P250358	HERC6	HECT and RLD domain containing E3 ubiquitin protein ligase family member 6	HGNC:26072	4q22	1.976	X	X	
A_23_P215913	CLU	clusterin	HGNC:2095	8p21-p12	1.958			
A_33_P3237634	TSC22D3	TSC22 domain family. member 3	HGNC:3051	Xq22.3	1.952			
A_24_P931443	GPR68	G protein-coupled receptor 68	HGNC:4519	14q31	1.951			
A_33_P3400273	SELL	selectin L	HGNC:10720	1q23-q25	1.917	X	X	
A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1p36.33	1.863	X	X	
A_23_P52266	IFIT1	interferon-induced protein with tetratricopeptide repeats 1	HGNC:5407	10q23.31	1.863	X	X	
A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	14q11.2	1.859	X	X	
A_23_P145874	SAMD9L	sterile alpha motif domain containing 9-like	HGNC:1349	7q21.2	1.828	X	X	
A_23_P501007	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	HGNC:3218	2p16	1.818			
A_23_P121253	TNFSF10	tumor necrosis factor (ligand) superfamily. member 10	HGNC:11925	3q26	1.815	X	X	
A_23_P127911	PAMR1	peptidase domain containing associated with muscle regeneration 1	HGNC:24554	11p13	1.808			
A_23_P69109	PLSCR1	phospholipid scramblase 1	HGNC:9092	3q23	1.789	X	X	
A_24_P332926	SUGP2	SURP and G patch domain containing 2	HGNC:18641	19p13	1.773			
A_23_P105794	EPSTI1	epithelial stromal interaction 1 (breast)	HGNC:16465	13q13.3	1.768	X		
A_23_P131050	ACSBG2	acyl-CoA synthetase bubblegum family member 2	HGNC:24174	19p13.3	1.743			
A_23_P254165	RAI2	retinoic acid induced 2	HGNC:9835	Xp22	1.726			
A_23_P144096	CISH	cytokine inducible SH2-containing protein	HGNC:1984	3p21.3	1.725		X	

↑ → **Table S1**

(**A**) Lists of upregulated genes in 2nd compared to 1st trimester uNK cells. (**B**) Lists of down-regulated genes in 2nd compared to 1st trimester uNK cells. The lists of differentially expressed genes include Agilent ID, HUGO approved symbol, HUGO approved name, HGCN ID, gene location, fold-change in gene expression and genes recognized as type I, type II and type III 'interferon stimulated genes' by Interferome.

A_24_P172481	TRIM22	tripartite motif containing 22	HGNC:16379	11p15	1.725	X	X	
A_24_P317762	LY6E	lymphocyte antigen 6 complex. locus E	HGNC:6727	8q24	1.724	X	X	
A_24_P15502	IFITM8P	interferon induced transmembrane protein 8 pseudogene	HGNC:32202	8q12.3	1.706			
A_24_P287043	IFITM2	interferon induced transmembrane protein 2	HGNC:5413	11p15.5	1.706	X	X	
A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	19p13.2	1.693	X	X	
A_23_P217269	VSIG4	V-set and immunoglobulin domain containing 4	HGNC:17032	Xq12-q13.3	1.690	X	X	
A_33_P3392560	RP11-1A16.1	long intergenic non-protein coding RNA 871	HGNC:47038	14q21.2	1.680			
A_23_P142750	EIF2AK2	eukaryotic translation initiation factor 2-alpha kinase 2	HGNC:9437	2p22-p21	1.679	X	X	
A_33_P3271051	CYTH3	cytohesin 3	HGNC:9504	7p22.1	1.665			
A_33_P3252394	GADD45G	growth arrest and DNA-damage-inducible. gamma	HGNC:4097	9q22.1-q22.2	1.662		X	
A_23_P38346	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	HGNC:29517	17q21.2	1.647	X	X	
A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	22q11.2	1.632	X	X	
A_33_P3397763	TNFSF9	tumor necrosis factor (ligand) superfamily. member 9	HGNC:11939	19p13.3	1.631	X	X	
A_33_P3287223	DPP4	dipeptidyl-peptidase 4	HGNC:3009	2q24.2	1.626	X	X	
A_23_P204087	OAS2	2'-5'-oligoadenylate synthetase 2. 69/71kDa	HGNC:8087	12q24.2	1.622	X	X	
A_23_P111804	PARP12	poly (ADP-ribose) polymerase family. member 12	HGNC:21919	7q34	1.617	X	X	
A_23_P255376	CCDC109B	coiled-coil domain containing 109B	HGNC:26076	4q25	1.616	X	X	
A_23_P69383	PARP9	poly (ADP-ribose) polymerase family. member 9	HGNC:24118	3q13-q21	1.613	X	X	
A_33_P3333960	LINC00426	long intergenic non-protein coding RNA 426	HGNC:42761	13q12.3	1.611			
A_23_P110712	DUSP1	dual specificity phosphatase 1	HGNC:3064	5q35.1	1.601	X	X	
A_23_P62967	DISC1	disrupted in schizophrenia 1	HGNC:2888	1q42.1	1.575			
A_24_P192301	SEMA3A	sema domain. immunoglobulin domain (Ig). short basic domain. secreted. (semaphorin) 3A	HGNC:10723	7p12.1	1.571			
A_24_P362317	ADAR	adenosine deaminase. RNA-specific	HGNC:225	1q21.3	1.567	X	X	
A_23_P6263	MX2	MX dynamin-like GTPase 2	HGNC:7533	21q22.3	1.561	X	X	
A_24_P68079	TRANK1	tetratricopeptide repeat and ankyrin repeat containing 1	HGNC:29011	3p22.2	1.555	X	X	
A_23_P91970	AADACL2	arylacetamide deacetylase-like 2	HGNC:24427	3q25.1	1.553			
A_23_P214360	IRF4	interferon regulatory factor 4	HGNC:6119	6p25-p23	1.543	X		
A_23_P87678	EPYC	epiphycan	HGNC:3053	12q21	1.534			
A_23_P23924	CAPN2	calpain 2. (m/II) large subunit	HGNC:1479	1q41-q42	1.528		X	
A_23_P21485	PID1	phosphotyrosine interaction domain containing 1	HGNC:26084	2q36.3	1.521	X		
A_33_P3378644	PHC1	polyhomeotic homolog 1 (Drosophila)	HGNC:3182	12p13	1.519			
A_23_P134125	MAP3K5	mitogen-activated protein kinase kinase kinase 5	HGNC:6857	6q22.33	1.518			
A_33_P3227443	C16orf54	chromosome 16 open reading frame 54	HGNC:26649	16p11.2	1.517			
A_23_P326474	L3MBTL4	l(3)mbt-like 4 (Drosophila)	HGNC:26677	18p11.31-p11.23	1.508			
A_23_P319617	CHST7	carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 7	HGNC:13817	Xp11.3	1.497			
A_33_P3398448	PARP10	poly (ADP-ribose) polymerase family. member 10	HGNC:25895	8q24	1.497	X		
A_33_P3396607	UGDH	UDP-glucose 6-dehydrogenase	HGNC:12525	4p14	1.485			
A_32_P79434	PTPRN2	protein tyrosine phosphatase. receptor type. N polypeptide 2	HGNC:9677	7q36	1.480			
A_33_P3229617	SGCE	sarcoglycan. epsilon	HGNC:10808	7q21.3	1.480		X	
A_24_P207139	PML	promyelocytic leukemia	HGNC:9113	15q24.1	1.477	X	X	
A_23_P68155	IFIH1	interferon induced with helicase C domain 1	HGNC:18873	2q24.2	1.469	X	X	
A_23_P74290	GBP5	guanylate binding protein 5	HGNC:19895	1p22.2	1.458	X		
A_23_P161076	CD2	CD2 molecule	HGNC:1639	1p13	1.434			
A_33_P3421626	KIAA1147	KIAA1147	HGNC:29472	7q34	1.432			
A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	4q25-q27	1.429		X	
A_33_P3387566	FAM215A	family with sequence similarity 215. member A (non-protein coding)	HGNC:17505	17q21.31	1.423			
A_23_P24004	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	HGNC:5409	10q23.31	1.421	X	X	
A_23_P30069	DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	HGNC:26429	4q32.3	1.419	X		
A_23_P214969	CITED2	Cbp/p300-interacting transactivator. with Glu/Asp-rich carboxy-terminal domain. 2	HGNC:1987	6q23.3	1.415	X		
A_24_P250922	PTGS2	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	HGNC:9605	1q25.2-q25.3	1.414			
A_23_P404821	KIAA1147	KIAA1147	HGNC:29472	7q34	1.401			

Table S1B. Down-regulated genes in 2nd compared 1st trimester uNK cells

Agilent ID	HUGO approved symbol	HUGO approved name	HGNC ID	Location	Fold-change
A_24_P145009	ZNRF2P1	zinc and ring finger 2 pseudogene 1	HGNC:42792	7p14.3	-2.162
A_32_P196263	ADAMTS9	ADAM metallopeptidase with thrombospondin type 1 motif. 9	HGNC:13202	3p14.1	-1.905
A_23_P420431	XKR3	XK. Kell blood group complex subunit-related family. member 3	HGNC:28778	22q11.1	-1.894
A_23_P315252	MT-ND4	mitochondrially encoded NADH dehydrogenase 4	HGNC:7459	mitochondria	-1.829
A_24_P551842	MT-CYB	mitochondrially encoded cytochrome b	HGNC:7427	mitochondria	-1.827
A_23_P76364	CD9	CD9 molecule	HGNC:1709	12p13	-1.752
A_33_P3336696	MT-ND4L	mitochondrially encoded NADH dehydrogenase 4L	HGNC:7460	mitochondria	-1.694
A_33_P3410700	MT-ATP8	Mitochondrially Encoded ATP Synthase 8	HGNC:7415	mitochondria	-1.669
A_33_P3298425	LMO4	LIM domain only 4	HGNC:6644	1p22.3	-1.656
A_24_P96780	CENPF	centromere protein F. 350/400kDa	HGNC:1857	1q41	-1.652
A_24_P365767	CYBB	cytochrome b-245. beta polypeptide	HGNC:2578	Xp21.1	-1.643
A_33_P3226542	SNORD3B-2	small nucleolar RNA. C/D box 3B-2	HGNC:33190	17p11.2	-1.630
A_23_P210210	EPAS1	endothelial PAS domain protein 1	HGNC:3374	2p21-p16	-1.625
A_32_P47701	EEF1A1	eukaryotic translation elongation factor 1 alpha 1	HGNC:3189	6q14.1	-1.617
A_23_P118834	TOP2A	topoisomerase (DNA) II alpha 170kDa	HGNC:11989	17q21-q22	-1.589
A_23_P155989	CENPK	centromere protein K	HGNC:29479	5q12.3	-1.541
A_23_P3681	NETO2	neuropilin (NRP) and tolloid (TLL)-like 2	HGNC:14644	16q11.2	-1.540
A_23_P317056	MT-ND6	mitochondrially encoded NADH dehydrogenase 6	HGNC:7462	mitochondria	-1.538
A_23_P416468	PIF1	PIF1 5'-to-3' DNA helicase	HGNC:26220	15q22.1	-1.536
A_23_P406928	MT-ND5	mitochondrially encoded NADH dehydrogenase 5	HGNC:7461	mitochondria	-1.532
A_23_P254733	MLF1IP	centromere protein U	HGNC:21348	4q35.1	-1.531
A_33_P3807062	HJURP	Holliday junction recognition protein	HGNC:25444	2q37.1	-1.530
A_33_P3288159	ASPM	asp (abnormal spindle) homolog. microcephaly associated (Drosophila)	HGNC:19048	1q31	-1.529
A_23_P259586	TTK	TTK protein kinase	HGNC:12401	6q14.1	-1.518
A_24_P350200	MT-CO3	mitochondrially encoded cytochrome c oxidase III	HGNC:7422	mitochondria	-1.502
A_23_P323751	FAM83D	family with sequence similarity 83. member D	HGNC:16122	20q11.23	-1.500
A_23_P106887	FUS	FUS RNA binding protein	HGNC:4010	16p11.2	-1.489
A_33_P3230548	KIF14	kinesin family member 14	HGNC:19181	1q32.1	-1.484
A_23_P12062	TRIT1	tRNA isopentenyltransferase 1	HGNC:20286	1p34.2	-1.477
A_33_P3659876	NCAPG2	non-SMC condensin II complex. subunit G2	HGNC:21904	7q36.3	-1.466
A_33_P3311755	KIF23	kinesin family member 23	HGNC:6392	15q23	-1.454
A_33_P3350566	CACNA1I	calcium channel. voltage-dependent. T type. alpha 1I subunit	HGNC:1396	22q13.1	-1.443
A_24_P320254	HNRNPH1	heterogeneous nuclear ribonucleoprotein H1 (H)	HGNC:5041	5q35.3	-1.441
A_24_P397817	LEP	leptin	HGNC:6553	7q31	-1.438
A_23_P136817	KNTC1	kinetochore associated 1	HGNC:17255	12q24.31	-1.438
A_23_P356684	ANLN	anillin. actin binding protein	HGNC:14082	7p15-p14	-1.429
A_33_P3354569	GPD2	glycerol-3-phosphate dehydrogenase 2 (mitochondrial)	HGNC:4456	2q24.1	-1.427
A_23_P61180	PLCXD1	phosphatidylinositol-specific phospholipase C. X domain containing 1	HGNC:23148	Xp22.33 and Yp11.32	-1.424

→ **Table S2**
WebGestalt transcription factor target analysis of genes that that were upregulated in uNK cells from 2nd trimester compared to 1st trimester human pregnancy.

Table S2. WebGestalt - Transcription factor target analysis

C: the number of reference genes in the category					
O: the number of genes in the gene set and also in the category					
E: the expected number in the category					
R: ratio of enrichment					
rawP: p value from hypergeometric test					
adjP: p value adjusted by the multiple test adjustment					
Database:Transcription Target	** Name:hsa_V$ISRE_01**	** ID:DB_ID:2029**			
C=246; O=15; E=0.54; R=27.98; rawP=8.92e-18; adjP=1.80e-15					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_33_P3418170	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	HGNC:19102	1.420
2	A_23_P24004	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	HGNC:5409	1.421
3	A_23_P102000	CXCR4	chemokine (C-X-C motif) receptor 4	HGNC:2561	2.669
4	A_23_P38346	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	HGNC:29517	1.647
5	A_23_P105794	EPSTI1	epithelial stromal interaction 1 (breast)	HGNC:16465	1.768
6	A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	1.632
7	A_23_P23074	IFI44	interferon-induced protein 44	HGNC:16938	5.196
8	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
9	A_24_P207139	PML	promyelocytic leukemia	HGNC:9113	1.477
10	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859
11	A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	1.429
12	A_33_P3283611	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	HGNC:5411	2.000
13	A_23_P68155	IFIH1	interferon induced with helicase C domain 1	HGNC:18873	1.469
14	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863
15	A_33_P3258346	XAF1	XIAP associated factor 1	HGNC:30932	2.342
16	A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	2.342
Database:Transcription Target	** Name:hsa_V$IRF_Q6**	** ID:DB_ID:2256**			
C=240; O=13; E=0.52; R=24.85; rawP=7.16e-15; adjP=7.23e-13					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P24004	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	HGNC:5409	1.421
2	A_23_P102391	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	HGNC:10909	2.117
3	A_23_P102000	CXCR4	chemokine (C-X-C motif) receptor 4	HGNC:2561	2.669
4	A_23_P38346	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	HGNC:29517	1.647
5	A_23_P111804	PARP12	poly (ADP-ribose) polymerase family, member 12	HGNC:21919	1.617
6	A_23_P23074	IFI44	interferon-induced protein 44	HGNC:16938	5.196
7	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
8	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859
9	A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	1.429
10	A_33_P3400273	SELL	selectin L	HGNC:10720	1.917
11	A_24_P362317	ADAR	adenosine deaminase, RNA-specific	HGNC:225	1.567
12	A_24_P192301	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	HGNC:10723	1.571
13	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863

Database:Transcription Target Name:hsa_V$IRF7_01 ID:DB_ID:2110					
C=250; O=13; E=0.54; R=23.86; rawP=1.21e-14; adjP=8.15e-13					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_33_P3418170	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	HGNC:19102	1.420
2	A_23_P24004	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	HGNC:5409	1.421
3	A_23_P102000	CXCR4	chemokine (C-X-C motif) receptor 4	HGNC:2561	2.669
4	A_23_P111804	PARP12	poly (ADP-ribose) polymerase family, member 12	HGNC:21919	1.617
5	A_23_P105794	EPSTI1	epithelial stromal interaction 1 (breast)	HGNC:16465	1.768
6	A_24_P105191	HS6ST2	heparan sulfate 6-O-sulfotransferase 2	HGNC:19133	2.091
7	A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	1.632
8	A_33_P3271051	CYTH3	cytohesin 3	HGNC:9504	1.665
9	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
10	A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	1.429
11	A_24_P362317	ADAR	adenosine deaminase, RNA-specific	HGNC:225	1.567
12	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863
13	A_33_P3258346	XAF1	XIAP associated factor 1	HGNC:30932	2.342
14	A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	2.342

Database:Transcription Target Name:hsa_STTTCRNTTT_V$IRF_Q6 ID:DB_ID:2426					
C=186; O=11; E=0.41; R=27.13; rawP=3.60e-13; adjP=1.82e-11					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_33_P3418170	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	HGNC:19102	1.420
2	A_23_P24004	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	HGNC:5409	1.421
3	A_23_P38346	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	HGNC:29517	1.647
4	A_23_P105794	EPSTI1	epithelial stromal interaction 1 (breast)	HGNC:16465	1.768
5	A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	1.632
6	A_23_P23074	IFI44	interferon-induced protein 44	HGNC:16938	5.196
7	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
8	A_23_P214969	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	HGNC:1987	1.415
9	A_33_P3283611	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	HGNC:5411	2.000
10	A_33_P3258346	XAF1	XIAP associated factor 1	HGNC:30932	2.342
11	A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	2.342
12	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863

Database:Transcription Target Name:hsa_V$ICSBP_Q6 ID:DB_ID:2210					
C=246; O=11; E=0.54; R=20.52; rawP=7.56e-12; adjP=3.05e-10					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P102000	CXCR4	chemokine (C-X-C motif) receptor 4	HGNC:2561	2.669
2	A_23_P111804	PARP12	poly (ADP-ribose) polymerase family, member 12	HGNC:21919	1.617
3	A_23_P255376	CCDC109B	coiled-coil domain containing 109B	HGNC:26076	1.616
4	A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	1.632
5	A_23_P23074	IFI44	interferon-induced protein 44	HGNC:16938	5.196
6	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
7	A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	1.429
8	A_24_P362317	ADAR	adenosine deaminase, RNA-specific	HGNC:225	1.567
9	A_33_P3283611	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	HGNC:5411	2.000
10	A_23_P68155	IFIH1	interferon induced with helicase C	HGNC:18873	1.469
11	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863

Database:Transcription Target Name:hsa_V$IRF1_01 ID:DB_ID:1880					
C=247; O=7; E=0.54; R=13.00; rawP=1.24e-06; adjP=4.17e-05					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_33_P3418170	DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	HGNC:19102	1.420
2	A_23_P87678	EPYC	epiphycan	HGNC:3053	1.534
3	A_23_P132159	USP18	ubiquitin specific peptidase 18	HGNC:12616	1.632
4	A_23_P819	ISG15	ISG15 ubiquitin-like modifier	HGNC:4053	1.863
5	A_33_P3258346	XAF1	XIAP associated factor 1	HGNC:30932	2.342
6	A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	2.342
7	A_33_P3220911	BST2	bone marrow stromal cell antigen 2	HGNC:1119	1.693
8	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859

Database:Transcription Target Name:hsa_TTGTTT_V$FOXO4_01 ID:DB_ID:2416					
C=2037; O=16; E=4.44; R=3.60; rawP=8.06e-06; adjP=0.0002					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P102391	SLC40A1	solute carrier family 40 (iron-regulated transporter), member 1	HGNC:10909	2.117
2	A_23_P255376	CCDC109B	coiled-coil domain containing 109B	HGNC:26076	1.616
3	A_23_P428129	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	HGNC:1786	2.369
4	A_23_P42868	IGFBP1	insulin-like growth factor binding protein 1	HGNC:5469	7.054
5	A_33_P3237634	TSC22D3	TSC22 domain family, member 3	HGNC:3051	1.952
6	A_33_P3258346	XAF1	XIAP associated factor 1	HGNC:30932	2.342
7	A_24_P557479	XAF1	XIAP associated factor 1	HGNC:30932	2.342
8	A_23_P501007	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	HGNC:3218	1.818
9	A_23_P214360	IRF4	interferon regulatory factor 4	HGNC:6119	1.543
10	A_24_P105191	HS6ST2	heparan sulfate 6-O-sulfotransferase 2	HGNC:19133	2.091
11	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859
12	A_24_P38081	FKBP5	FK506 binding protein 5	HGNC:3721	2.245
13	A_23_P214969	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	HGNC:1987	1.415
14	A_23_P127911	PAMR1	peptidase domain containing associated with muscle regeneration 1	HGNC:24554	1.808
15	A_23_P68155	IFIH1	interferon induced with helicase C domain 1	HGNC:18873	1.469
16	A_24_P192301	SEMA3A	sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3A	HGNC:10723	1.571
17	A_23_P110712	DUSP1	dual specificity phosphatase 1	HGNC:3064	1.601

Database:Transcription Target Name:hsa_V$STAT5B_01 ID:DB_ID:2113					
C=239; O=6; E=0.52; R=11.52; rawP=1.47e-05; adjP=0.0004					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P144096	CISH	cytokine inducible SH2-containing protein	HGNC:1984	1.725
2	A_23_P214360	IRF4	interferon regulatory factor 4	HGNC:6119	1.543
3	A_23_P215913	CLU	clusterin	HGNC:2095	1.958
4	A_23_P69109	PLSCR1	phospholipid scramblase 1	HGNC:9092	1.789
5	A_33_P3378644	PHC1	polyhomeotic homolog 1 (Drosophila)	HGNC:3182	1.519
6	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859

Database:Transcription Target Name:hsa_RTAAACA_V$FREAC2_01 ID:DB_ID:2417					
C=907; O=10; E=1.98; R=5.06; rawP=2.96e-05; adjP=0.0007					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P501007	EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	HGNC:3218	1.818
2	A_23_P214360	IRF4	interferon regulatory factor 4	HGNC:6119	1.543
3	A_23_P428129	CDKN1C	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	HGNC:1786	2.369
4	A_23_P218858	ABI3BP	ABI family, member 3 (NESH) binding protein	HGNC:17265	3.001
5	A_33_P3237634	TSC22D3	TSC22 domain family, member 3	HGNC:3051	1.952
6	A_23_P255444	DAPP1	dual adaptor of phosphotyrosine and 3-phosphoinositides	HGNC:16500	1.429
7	A_23_P214969	CITED2	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	HGNC:1987	1.415
8	A_23_P68155	IFIH1	interferon induced with helicase C domain 1	HGNC:18873	1.469
9	A_23_P32404	ISG20	interferon stimulated exonuclease gene 20kDa	HGNC:6130	3.528
10	A_23_P110712	DUSP1	dual specificity phosphatase 1	HGNC:3064	1.601
Database:Transcription Target Name:hsa_TTCYNRGAA_V$STAT5B_01 ID:DB_ID:2455					
C=328; O=6; E=0.71; R=8.39; rawP=8.58e-05; adjP=0.0017					
Index	**Agilent ID**	**HUGO approved symbol**	**HUGO approved name**	**HGNC ID**	**Fold-change**
1	A_23_P144096	CISH	cytokine inducible SH2-containing protein	HGNC:1984	1.725
2	A_23_P214360	IRF4	interferon regulatory factor 4	HGNC:6119	1.543
3	A_23_P215913	CLU	clusterin	HGNC:2095	1.958
4	A_23_P69109	PLSCR1	phospholipid scramblase 1	HGNC:9092	1.789
5	A_33_P3378644	PHC1	polyhomeotic homolog 1 (Drosophila)	HGNC:3182	1.519
6	A_23_P65442	IRF9	interferon regulatory factor 9	HGNC:6131	1.859

Table S3. Primer sequences RT-qPCR

HUGO approved symbol	Forward (5' > 3')	Reverse (5' > 3')	Product Size
ACTB	CCTGGCACCCAGCACAAT	GGGCCGGACTCGTCATACT	144
AREG	GAGAAGCTGAGGAACGAAAGA	GGCAGTGACTCCAATGTGATA	103
CAPN2	CCTTATCTCTTGGCTCTGTTTCT	AGCTTAGTCCTTGGCTGATTT	93
CD9	CTGTTCTTCGGCTTCCTCTT	CTCCTGGACTTCCTTAATCACC	99
CENPK	CACCTGAAACAATTCCCTTGAC	TCCTTAGTGGACAGTACCATTTC	103
CLU	AGTCTCCAGGAAGAACCCTAA	CAACATCCACATCTCACTCCTC	98
EFEMP1	GTCACAGGACACCGAAGAAA	TACAAGCGTCTGGGACAATG	119
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226
IGFBP1	CTCTCCATGTCACCAACATCAA	GTGCCTTGGCTAAACTCTCTAC	84
IRF7	TCTTCGACTTCAGAGTCTTCTTC	GAAGCCCAGGTAGATGGTATAG	92
IRF9	CTCTTCAGAACCGCCTACTTC	GGCTCTCTTCCCAGAAATTCA	103
ISG15	GAGGCAGCGAACTCATCTTT	CCAGCATCTTCACCGTCAG	99
ISG20	ATGAGCGGCTACACAATCTAC	GGCTGTTCTGGATGCTCTT	133
MT-ATP8	CACTAAAGGACGAACCTGATCTC	GATAGTTGGGTGGTTGGTGTAA	111
MT-ND4	ACAGCTATCCATTGGTCTTAGG	GTCAGGGTTAGGGTGGTTATAG	95
MX1	GCCTGCTGACATTGGGTATA	GTGGCGATGTCCACATTACT	105
PRL	CTGATAGTCAGCCAGGTTCATC	GAGACTCTTCATCAGCCATCTG	97
TNFSF10	TCAAGACCATAGTGACCAACATAG	TCTCAAGTAGCTGGGACTACA	101

↑ **Table S3**
Sequences of primers used for validation of microarray data with real-time qPCR.

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Chapter 3

SPECIALIZED NATURAL KILLER CELLS AS A KEY COMPONENT OF THE DYNAMIC UTERINE NICHE IN HUMAN REPRODUCTION

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ABSTRACT

The enrichment of natural killer (NK) cells in the uterine mucosa, the endometrium, is a hallmark of maternal preparation for pregnancy. The endometrium is emerging as a unique and dynamic 'uterine niche' for NK cell development. The majority of circulating NK cells are innate $CD56^{dim}CD16^{+}$ cytotoxic effector cells. In contrast, NK cells in the endometrium are a phenotypically and functionally different $CD56^{bright}CD16^{-}$ subset. The developing endometrium that is under control of ovarian hormones regulates changes in NK cell receptor expression and secretory products to allow specialized immunoregulatory and innate immune functions. Uterine NK cells play a role in host defense, immune tolerance of the conceptus, and regulation of spiral artery remodeling including guidance of invading trophoblasts in the endometrium. In this review, we focus on how the 'uterine niche' directs the development and function of NK cells to regulate endometrial and vascular remodeling, as well as maintenance of homeostasis at the maternal-fetal interface supporting human pregnancy. We also discuss how aberrations in NK cell numbers and maturation within its environment impact pregnancy outcomes across the spectrum of early pregnancy loss, fetal growth restriction, and preeclampsia.

Key words: Decidualization • maternal-fetal interface • placentation • pregnancy • uNK cell

NON-STANDARD ABBREVIATIONS

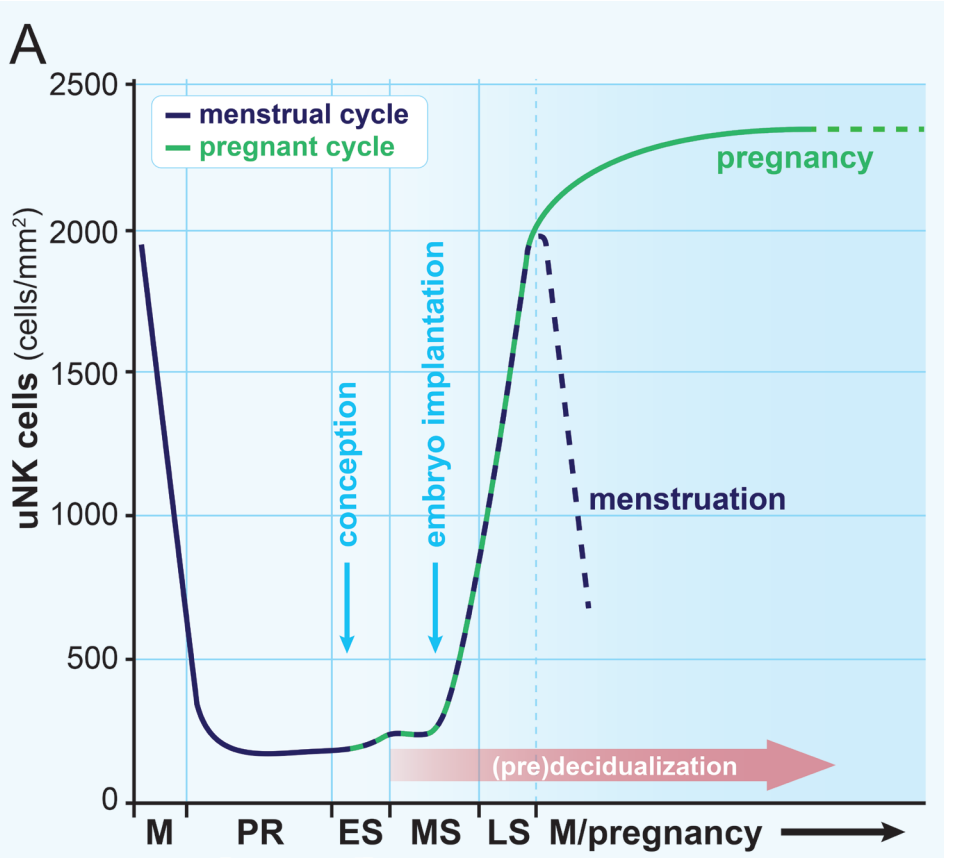
Ang	Angiopoietin
CD16	Cluster of differentiation 16, Fcγ receptor III
CD3	Cluster of differentiation 3, T cell co-receptor
CD56	Cluster of differentiation 56, neural cell adhesion molecule
CD9	Cluster of differentiation 9, member of tetraspanin family
DSC	Decidualizing stromal cell
ECM	Extracellular matrix
ESC	Endometrial stromal cell
EVT	Extravillous trophoblast
hCG	Human chorionic gonadotropin
HGF	Hepatocyte growth factor
HLA	Human leukocyte antigen
HPC	Hematopoietic precursor cell
HSC	Hematopoietic stem cell
IL	Interleukin
IFN	Interferon
KIR	Killer-cell immunoglobulin-like receptor
LGL	Large granular lymphocyte
MHC	Histocompatibility complex
MMP	Matrix metalloproteinase
NCAM	Neural cell adhesion molecule
NCR	Natural cytotoxicity receptor
NK cell	Natural killer cell
NKG2	Natural killer group protein 2
PBMC	Peripheral blood mononuclear cell
pbNK cell	Peripheral blood natural killer cell
PlGF	Placental growth factor
uNK cell	Uterine natural killer cell
VEGF	Vascular endothelial growth factor
VSMC	Vascular smooth muscle cell

INTRODUCTION

Human reproduction is characterized by a high incidence of early pregnancy loss and other adverse outcomes such as fetal growth restriction and preeclampsia arising from placentation defects. Both maternal and embryonic factors underlie these pathologic pregnancy outcomes.[1,2] The exact etiology of these pathologic pregnancy outcomes remains elusive, hampering the development of prophylactic and curative measures. In recent years the role of the local *maternal immune system* in the uterus during embryo implantation and placentation has gained much interest.

Human reproduction requires a well-prepared *uterine microenvironment* that orchestrates selection, implantation and support of healthy embryos.[3] The uterine niche regulates trophoblast invasion into the uterine wall that is prerequisite for placental development.[4] The invasion of these genetically foreign extravillous trophoblasts (EVTs) poses a challenge to maternal tissues and immune system. The uterine mucosa prepares for these challenges by undergoing precisely timed adaptations to accommodate pregnancy.[5] During the extensive endometrial remodeling process (decidualization) that starts in the secretory phase endometrium in non-conceptive and in conceptive cycles prior to embryo implantation, uterine NK (uNK) cells increase rapidly in number (**Figure 1**).

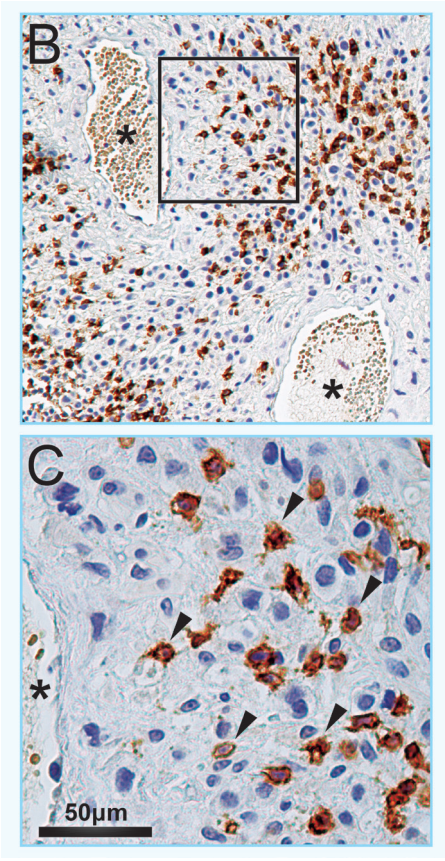
NK cells were first recognized as cytotoxic effectors of innate immunity, representing an initial line of host defense against invading pathogens.[6] However, NK cells that belong to the class of innate lymphoid cells manifest immunoregulatory phenotypes in various environmental 'niches' such as mucosal sites including the uterus.[7] In the uterus, NK cells exert important *regulatory* functions during decidualization, embryo implantation, invasion of EVTs, and remodeling of the uterine vascular bed.[8] Moreover, during pregnancy the maternal immune system, including uNK cells becomes tolerant towards the semi-allogeneic conceptus.[9,10] Finally, uNK cells are thought to have a role in *host defense*, protecting both the host and fetus from pathogens, yet their contribution to the innate immune defense are unclear at present. Therefore, NK cells are innate lymphoid cells that are a critical element in establishing a functional and dynamic maternal-fetal interface within the 'uterine niche'**.** This review focuses on uterine NK cells as a specific group of innate lymphoid cells (ILCs) with dynamic roles within the changing uterine niche in the context of human reproduction. Uterine NK cells (uNK) also have been named endometrial and decidual NK cells (eNK and dNK), for respectively NK cells residing in the endometrium of the non-gravid uterus and those in the decidualizing endometrium of pregnant women. As decidualization already starts prior to embryo implantation



(pre-decidualization) continuing throughout early stages of pregnancy we use the general term uNK cells to refer to NK cells in the uterus during the menstrual cycle or pregnancy.

1 THE UTERINE NICHE

The development of NK cells and their acquisition of specialized functions are guided by the local uterine microenvironment.[6,7] The specialized tissue microenvironment, or 'niche' determines cell fate and guides the capacity of these cells to participate in tissue generation, maintenance, and repair.[11] To discuss the unique features and functions of uNK cells we will define the 'uterine niche' in which uNK cells reside to support their function.

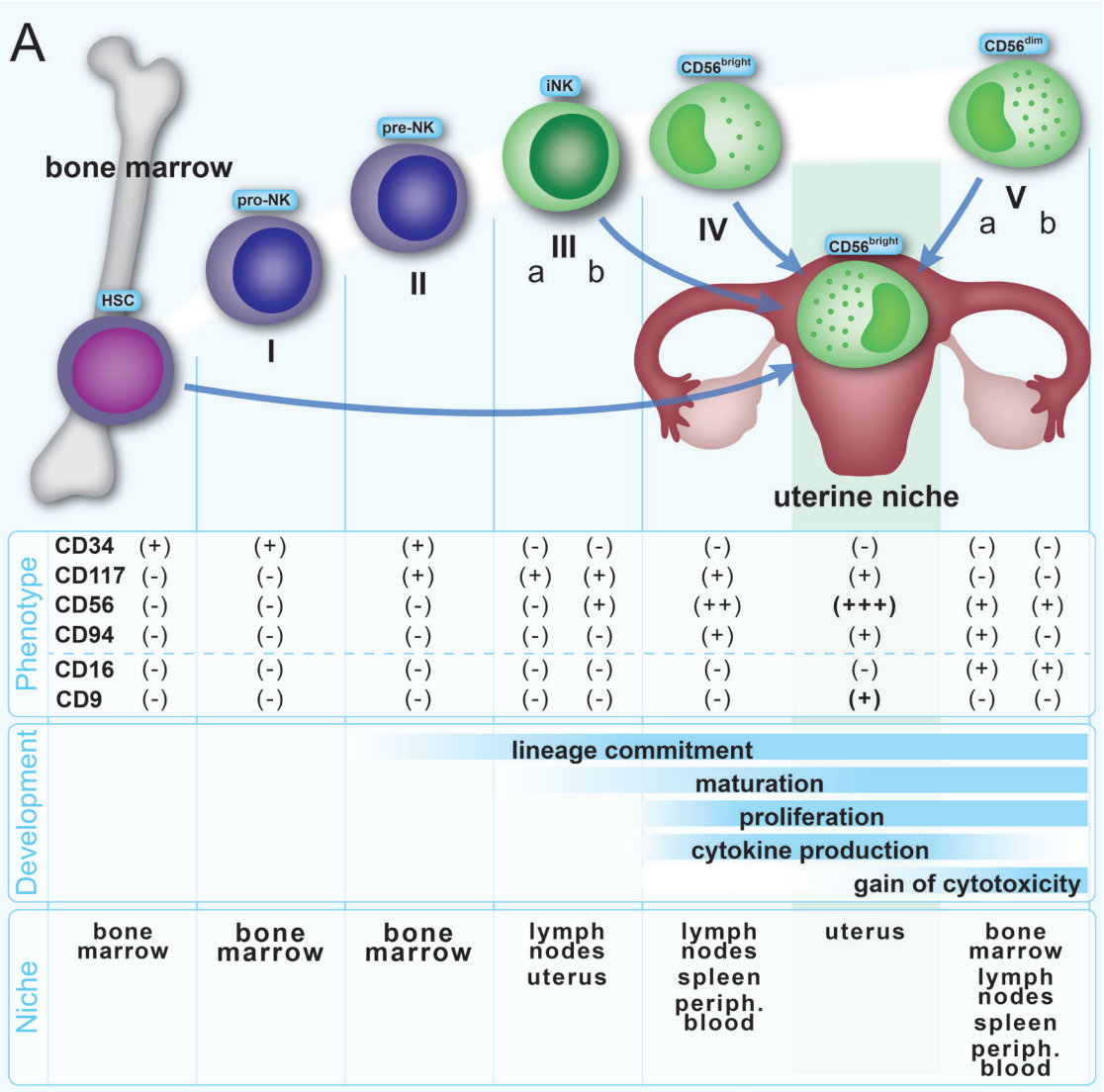


← **Figure 1**

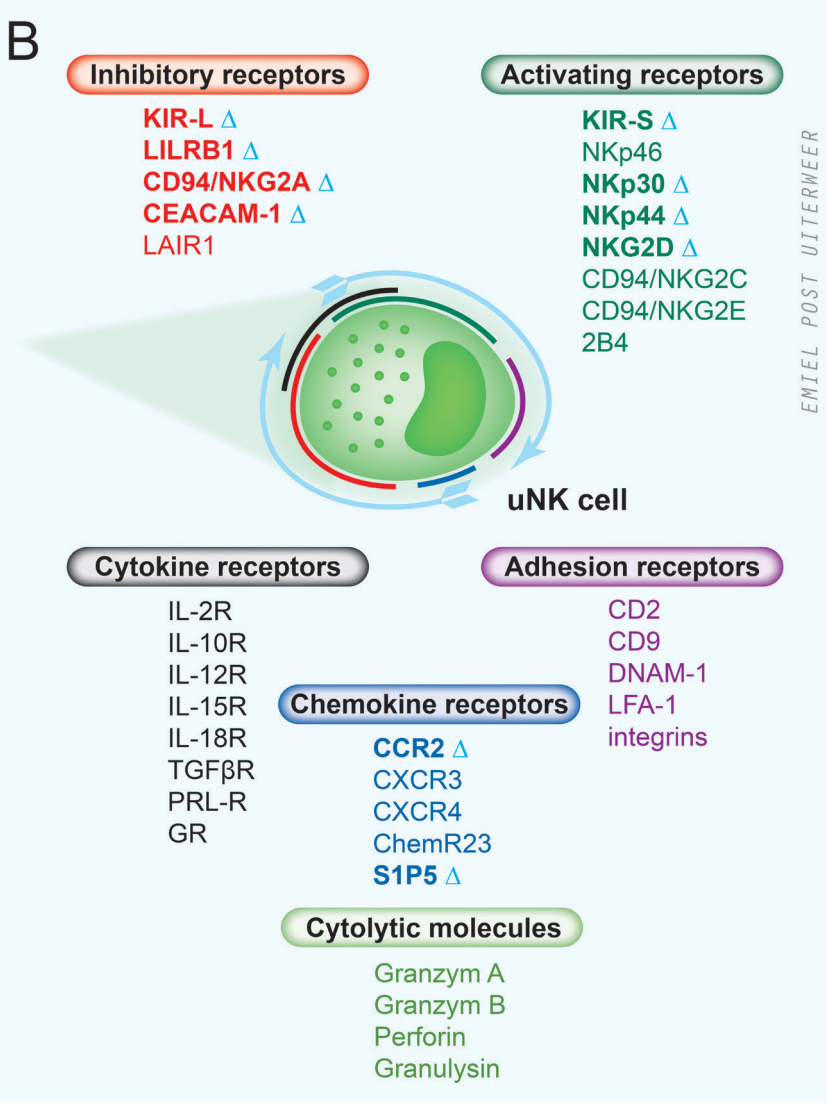
Cyclic changes in uNK cell number in the 'uterine niche'. (**A**) Graphical representation of the number of uNK cells (cells/mm²) in the endometrial stroma over the course of the menstrual cycle (blue line) and early pregnancy (green line) based on Russell et al.[15] and Bulmer et al.[13,14] Uterine NK cell numbers rapidly increase during (pre)decidualization, which starts in the mid-secretory phase (MS). NK cell numbers continue to rise during early pregnancy until the end of the first trimester accounting for ~30% of total stromal cells. When embryo implantation does not occur, menstruation is initiated, and uNK cell numbers will rapidly decline. (**B**) Representative image of the decidua basalis at 8 weeks gestation labeled for CD56 (brown) and counterstained with haematoxylin (blue). (**C**) High-power magnification of area indicated in panel B. In early pregnancy uNK cells (arrows) are abundant in the proximity of remodeling spiral arteries (*).

M: menstruation phase; PR: proliferative phase; ES: early secretory phase; MS: mid-secretory phase; LS: late secretory phase.

The endometrium is part of a continuous mucosa that lines the upper and lower female reproductive tract. In this mucosal lining of the lower female reproductive tract (ectocervix and vagina), the majority of NK cells have a cytotoxic effector ($CD56^{dim}CD16^{+}$) phenotype. The endometrium is part of the mucosa that lines the upper reproductive tract (fallopian tubes, uterus and endocervix) and harbors NK cells with a regulatory ($CD56^{bright}CD16^{-}$) phenotype. In the endometrium the percentage of leukocytes being NK cells markedly fluctuates throughout the menstrual cycle, but not in the other parts of the reproductive tract.[12] The NK cell density in the endometrium is low after menstruation, but increases sharply in the mid- and late- secretory phase of the menstrual cycle and early pregnancy (**Figure 1**).[13-15] In this review we consider the endometrium as a distinct niche that is dynamically populated by NK cells.



The uterine niche is composed of several elements that include the extracellular matrix, stromal support cells (endometrial stromal cells; ESCs), blood vessels (basal and spiral arteries), and some sporadic neurons, particularly nearby the myometrial-endometrial junction and - in approximately half of the women - some small nerve endings extend into the basal layer of the endometrium.[16] During pregnancy the uterine niche also contains invading EVTs. Uterine NK cells are found scattered throughout the endometrial stroma with a higher density around



← **Figure 2**
Origin, developmental stages, and receptor repertoire of uNK cells. (**A**) Seven NK cell developmental stages are identified based on CD34, CD117, CD56 and CD94 surface marker expression (adapted from Eissens et al.[60] and Freud et al.[61]). Stage I *NK cell progenitor* and stage II *Pre-NK cells*, have multi-lineage potential (towards NK cells, T cells and dendritic cells) and originate from the bone marrow. Stage IIIa cells are committed to the NK cell lineage and are primarily found in lymph nodes, where they develop into stage IIIb *immature NK cells*. Stage IV *$CD56^{bright}$ NK cells* are mature NK cells found in relatively high numbers in lymph nodes. Developmental stage Va and Vb *$CD56^{dim}$ NK cells* are the most prominent NK cell subsets in peripheral blood. The uterine $CD56^{bright}$ NK cell subset could be considered as a unique developmental stage and is situated between stage IV and V on the basis of high CD56 expression and absence of CD16. Several NK cell developmental stages have been suggested to traffic to the uterine niche (blue arrows), thereby contributing to rapidly increasing uNK cell numbers during (pre) decidualization. (**B**) Human uNK cells also express a unique set of cell surface receptors that can be divided into activating, inhibitory, cytokine, adhesion and chemokine receptors (adapted from Vivier[6]). In addition uNK cells are equipped with the machinery to execute cytolytic processes, nevertheless uNK cells show relatively reduced cytolytic activity. The pattern of uNK cell receptors is not restricted to those illustrated in figure B and many of these receptors are known to change in expression during development of the uterine niche (indicated with Δ).

the uterine glands, spiral arteries and in the vicinity of the EVT invasion front.[13,17]. The various extracellular and cellular constituents of the uterine niche undergo marked changes during ovarian hormone-dependent endometrial development in the menstrual cycle and pregnancy.

The uterine niche is also populated by T-cells, macrophages and innate lymphoid cells (ILCs) other than uNK cells all of which are subject to regulation provided by

the unique environment of the endometrium.[18] About 10% of endometrial leukocytes are CD14+ and have a gene profile that is skewed towards alternatively activated macrophages, suggesting that these cells are immunosuppressive.[19,20] During pregnancy, macrophage numbers increase to 25% of the total leukocyte population. T cell proportions vary from 10-20%, whilst dendritic cells, B cells and NKT cells are more rare.[21] Finally, non-NK ILCs are present in the non-pregnant uterine mucosa and in the decidua of the gravid uterus.[22] Interactions between these immune cells and uNK cells may contribute to the regulation of embryo implantation and placentation. For example, decidual macrophages restrain uNK cell mediated killing in a TGFb dependent manner.[23] However, knowledge about the role of these immune cell interactions is limited. For more information on the presence, function and interactions of immune cells in the uterus we refer to some excellent reviews.[21,24,25]

2 UNIQUE NK CELL PHENOTYPE IN THE UTERINE NICHE

NK cells are morphologically recognized as large granular lymphocytes, because of their large size (10-12 μm) compared to other circulating lymphocytes, and contain characteristic secretory vesicles filled with lytic enzymes. In humans, NK cells comprise ~10-15% of all peripheral blood leukocytes. These peripheral blood NK (pbNK) cells can be subdivided into two subsets with distinct functions on the basis of their surface expression of CD56. The majority (~90%) of pbNK cells are $CD56^{dim}$, express high levels of CD16 (Fcγ receptor III), and behave functionally as cytotoxic effectors. $CD56^{bright}$ pbNK cells (~10%) express little or no CD16, are mainly agranular,[26] and are a regulatory counterpart that are skewed towards lower cellular cytotoxicity[27] and that secrete high levels of immunoregulatory cytokines such as IFNγ, VEGF, PlGF, IL8, IP10, Ang-1/2, and TGFβ1.[28-31] Human uNK cells were originally designated as endometrial *granulocytes* or *granulated* lymphocytes.[32] In contrast to peripheral blood, the 'uterine niche' harbors NK cells with an almost exclusively $CD56^{bright}$ phenotype that is further defined by the absence of CD16 and expression of CD9 (**Figure 2**).[33] The high CD56 expression is one of the few features in common between uNK cells and the $CD56^{bright}$ pbNK cell subset. Uterine NK cells are more granular and larger (~30-50%) compared to both peripheral blood counterparts.[12,34] Although having a $CD56^{bright}CD16^{-}$ phenotype, uNK cells express high levels of perforin, granzyme and KIRs. More specifically, uNK cells express similar levels of the lytic enzymes perforin and granzyme B as cytotoxic $CD56^{dim}$ pbNK, but even higher levels of granzyme A.[33,35] Moreover, uNK cells also produce the cytolytic and pro-inflammatory substance granulysin.[36] Thus, uNK cells are a

specific granulated NK cell subset different from those in the peripheral blood compartment and equipped with the machinery to execute cytolytic functions.

The unique phenotype of uNK cells is further defined by the specific expression of 3 families of *activating and inhibitory receptors*:[6] The killer immunoglobulin-like receptors (KIRs), the C-type lectin family of NKG2 receptors, and natural cytotoxicity receptors (NCRs) (**Figure 2**). Next we will discuss the expression of these 3 receptor families. The functional consequences of these receptors for uNK cells will be discussed in "The dynamic uterine NK cell niche".

The *KIR family* consists of both activating and inhibitory receptors that are differentially expressed by NK cell subsets. KIRs interact with polymorphic MHC class I molecules and are thought to determine pregnancy success by recognition of the semi-allogeneic invading EVT.[9] KIRs interact with HLA and play a key role in placentation. Several studies have shown that uNK cells express higher levels of several inhibitory (KIR2DL1-4, KIR3DL1 and KIR3DL2), activating (KIR2DS1 and KIR2DS2), and other KIRs with more diverse or unknown activating or inhibitory roles (KIR2DL4, KIR2DS2) at the level of mRNA or protein compared to their pbNK counterparts.[33,37,38] Yet others did not observe differences in e.g. KIR3DL1.[39] The relative proportion of uNK cells not expressing KIRs (KIR2DL1, KIR3DL1 and KIR3DL2) is much smaller than in the circulating NK cell pool.[33] Interestingly, uNK cell KIR expression decreases markedly within the first trimester of pregnancy, whilst circulating pbNK have stable KIR expression patterns.[37] These observations indicate that the uNK cell KIR repertoire in the uterine 'niche' is dynamically regulated, underscoring the unique and specialized nature of uNK cells.

NKG2 receptors regulate NK cell cytotoxic activity. The percentage of uNK cells expressing inhibitory NKG2A is higher compared to pbNK cells, whilst the expression rate of activating NKG2C is similar. In addition, more uNK cells appeared to co-express these inhibitory and activating receptors compared to pbNK cells.[40] NKG2A and NKG2C/E interact with the non-classical MHC class I ligand HLA-E on fetal EVTs,[41] and during early pregnancy their expression is significantly higher in uNK cells than pbNK cells.[39,40,42] In contrast to pbNK cells, most uNK cells express NKG2D with the proportion of NKG2D positive uNK cells increasing with advancing (early) pregnancy.[43,44]

NCRs mediate NK cell cytotoxicity and are specific for non-HLA ligands. Uterine NK cells express NCRs; however, the ligands for these NCRs in the uterine niche remain unknown. Possibly NCR ligands are expressed by endometrial stromal cells and

trophoblasts.[28] The NCR family is comprised of 3 members, NKp46 (NCR_1), NKp44 (NCR_2) and NKp30 (NCR_3) that trigger 'natural cytotoxicity' but also induce immunoregulatory NK cell functions.[45,46] NKp44 is exclusively expressed in activated NK cells, whereas NKp30 and NKp46 are expressed by activated and resting NK cells.[47] During early pregnancy the majority of uNK cells have a higher membrane expression of NKp30 and NKp46 compared to pbNK cells.[44] NCR expression is remarkably stable in pbNK cells, whilst uNK cells express NKp30 and NKp44 dynamically. In the proliferative and mid-secretory phases of the menstrual cycle expression of NKp30 and NKp44 is low, but expression increases in the 1st trimester of pregnancy.[48,49] Some authors were unable to demonstrate NKp44 expression in uNK cells during pregnancy, possibly due to different culture conditions.[39] Towards the end of the first trimester, the proportion of NKp44+ uNK cells declines.[44] These dynamic changes in NCR expression may reflect the activation state of uNK cells,[49] and may allow their active contribution in regulating placentation.

Overall, uNK cells express a unique contingent *of classic NK cells receptors* and at higher level compared to pbNK cells. Uterine NK cells also express chemotactic, cytokine and adhesion molecule receptors (**Figure 2**). However, knowledge on the temporal pattern of expression of these non-classical NK cell receptors in uNK cells and how expression levels compare to pbNK cells is scarce. Freshly isolated uNK cells from the proliferative or secretory phase do not express $CXCR_3$ or $CXCR_4$,[49] but uNK cell clones express these receptors after IL2 stimulation *in vitro*.[50] During pregnancy uNK cells express chemokine receptors including CCR6, CCR_7, $CXCR_1$, $CXCR_3$, $CXCR_4$ and CX_3CR_1, as was evaluated by measuring the percentage of cells expressing the receptors. Importantly, the percentage of cells expressing these receptors differed from that in pbNK cells.[51] The expression of the chemokine receptor CCR_2 is significantly higher in uNK cells from the cycling endometrium compared to uNK cells from the gravid uterus.[52] Uterine NK cell production of cytokine, chemokine and angiogenic growth factor production, and their lytic capacity are regulated by a plethora of soluble factors secreted by uterine stromal cells and glands, such as IL8, IL10, IL12, IL15, IL18, transforming growth factor beta 1 ($TGF\beta_1$) and prolactin that act on the cognate receptors that are likely expressed in uNK cells.[53-58] Until now potential dynamic expression of cytokine and chemokine receptors or changes in responses to cytokine and/or chemokines by uNK cells during pregnancy has not been studied extensively. Nevertheless, one report showed dynamic expression of the sphingosine-1-phosphate receptor 5 (S_1PR_5) in uNK cells during the 1st and 2nd trimester of pregnancy with the highest expression during the 1st trimester.[59] Overall, uNK cells express specific receptor profiles that render these cells receptive for dynamically expressed cues from the uterine niche

that tune uNK cell function. The functional consequences are further discussed in the section: "The dynamic uterine NK cell niche".

3 DEVELOPMENTAL PATHWAY OF UTERINE NK CELLS

The prominence and unique receptor profile of NK cells in the uterine niche has led to questions about their development. Several different concepts about the origin of uNK cells have been proposed none of which are mutually exclusive (**Figure 2**).[60,61] The rapidly evolving knowledge on innate lymphoid subsets has raised important questions on the developmental relationships between NK cells, uNK cells and other ILCs.[62,63]

One concept is that the uterine niche attracts mature NK cells from the peripheral blood, and induces differentiation and specialization of pbNK cells into the unique uNK cell phenotype. However, studies by Koopman et al. and Kopcow et al. have clearly shown that uNK cells markedly differ from their pbNK counterparts.[33,64] Nevertheless, circulating pbNK cells have an increased migratory capacity during pregnancy.[65] Moreover, the homing of pbNK cells to the uterine niche could be facilitated by progesterone- and estrogen-induced chemokine expression in the endometrium during pregnancy (e.g., CX_3CL_1/fractalkine, $CXCL_{10}$/IP10, $CXCL_{11}$/I-TAC).[50,65,66] EVTs produce chemokines such as $CXCL_{12}$/SDF-1 that may contribute to the homing of uNK cells to the uterine niche.[17,67] However, uNK cells numbers also increase in parts of the endometrium that lack EVTs such as decidua parietalis, and endometrium of tubal ectopic pregnancies,[68] indicating EVT independent homing of uNK cells. Moreover, uNK cells increase in number during the late secretory phase of the menstrual cycle when EVTs are absent (**Figure 1A**),[15] indicating that possible attraction of pbNK cells is an EVT-independent event.
Based on the surface marker profile of pbNK cells,[69] the $CD56^{bright}$ pbNK cell subset (stage IV) would be the most likely blood-derived source of uNK cells, despite their clearly different transcript signature.[33] However, cues provided by the uterine niche skew the $CD56^{dim}$ pbNK cell subset (stage V) towards an uNK cell-like surface marker profile (**Figure 2**).[51] Thus the $CD56^{dim}$ pbNK population could also contribute to the uNK cell population. Secreted factors produced by the uterine niche, such as IL-8, IL-10, IL-12, IL-15, and TGFβ1 change the cell surface receptor profile of NK cells.[50,56,65,70] In addition, hypoxia, TGFβ1 and the demethylating agent, 5-aza-2′-deoxycytidine, promote the transition of pbNK cells to an uNK cell phenotype that can stimulate trophoblast invasion.[51] Importantly most of these studies only investigated the phenotype on the basis of cell membrane markers. Whether these cells truly differentiate toward uNK cells with a similar transcriptional and functional

profile remains to be determined. In summary, some evidence exists supporting the concept that circulating $CD56^{bright}$ (stage IV) and possibly $CD56^{dim}$ (stage V) pbNK cells are recruited to and modified by the uterine niche to comprise to a portion of the uNK cell population in early pregnancy (**Figure 2**). Yet true direct evidence showing migration of mature circulating pbNK cells to the endometrium is still lacking.

Immature NK cells (stage III) or their NK cell precursors originating from the bone marrow (HSC, stage I and II) traffic to peripheral tissues for terminal differentiation *in situ*.[60,61] The second concept proposes that uNK cells originate from bone marrow-derived NK cell *progenitors* that traffic to the uterine niche for further differentiation. Male and colleagues isolated immature $CD34^{-}CD117^{+}CD94^{-}$ (stage III) uNK cells that produced IL-22 and expressed RORC and LTA possibly as part of the ILC_3 subset. These cells differentiated to stage IV-like cells when stimulated *in vitro* with IL-15.[22] However, these authors did not identify stage I/II ($CD34^{+}$) progenitors. Other groups have identified stage I/II $LIN^{-}CD34^{+}CD45^{+}$ and stage I $CD34^{+}CD117^{-}$ NK cell progenitors in the endometrium during the menstrual cycle and pregnancy. The hematopoietic origin of this CD34+CD117- population was further confined by absence of the endothelial cell marker VEGF-R2 and on basis of the size of the CD34+CD117- population.[71,72] *In vitro* multipotent hematopoietic progenitor/precursor ($LIN^{-}CD34^{+}CD45^{+}$) cells differentiate into $CD56^{bright}$ NK cells when cultured in conditioned media from decidualizing endometrial stromal cells containing IL-15 and stem cell factor (SCF).[56] These data indicate that the uterine niche contains NK cell progenitors that can differentiate towards mature uNK cells and that endometrial stromal cells likely play a role in uNK cell differentiation (**Figure 2A**). The exact uterine triggers that direct homing of immature NK cell and NK cell progenitors to the uterine niche are currently unknown.

A third possibility is that the rapidly increasing number of uNK cells during endometrial decidualization is derived from proliferation of a *tissue-resident cell population* during the secretory phase and early pregnancy.[49] Indeed, evidence points to uNK cell proliferation as the expression of the proliferation marker Ki-67 is observed in uNK cells. These mitotic uNK cells are primarily observed in the late secretory phase when uNK cell numbers strongly increase. In the proliferative phase mitotic uNK cells are virtually absent and early in pregnancy, the proportion of Ki-67+ uNK cells is relatively low.[73,74]

Although there is likely a considerable overlap in the mechanisms that contribute to the increase in uNK cells, *in situ* proliferation of uNK cells may well contribute to the

rise in uNK cells number during the mid- and late-secretory phase of the cycle. The recruitment of mature pbNK cells and their immature progenitors to the uterine niche may contribute to the rise in uNK cells at the end of the menstrual cycle when the uterine niche starts to mature (pre-decidualize), a process that continues in the first trimester of pregnancy (decidualization) (**Figure 1**).

4 THE DYNAMIC UTERINE NK CELL NICHE

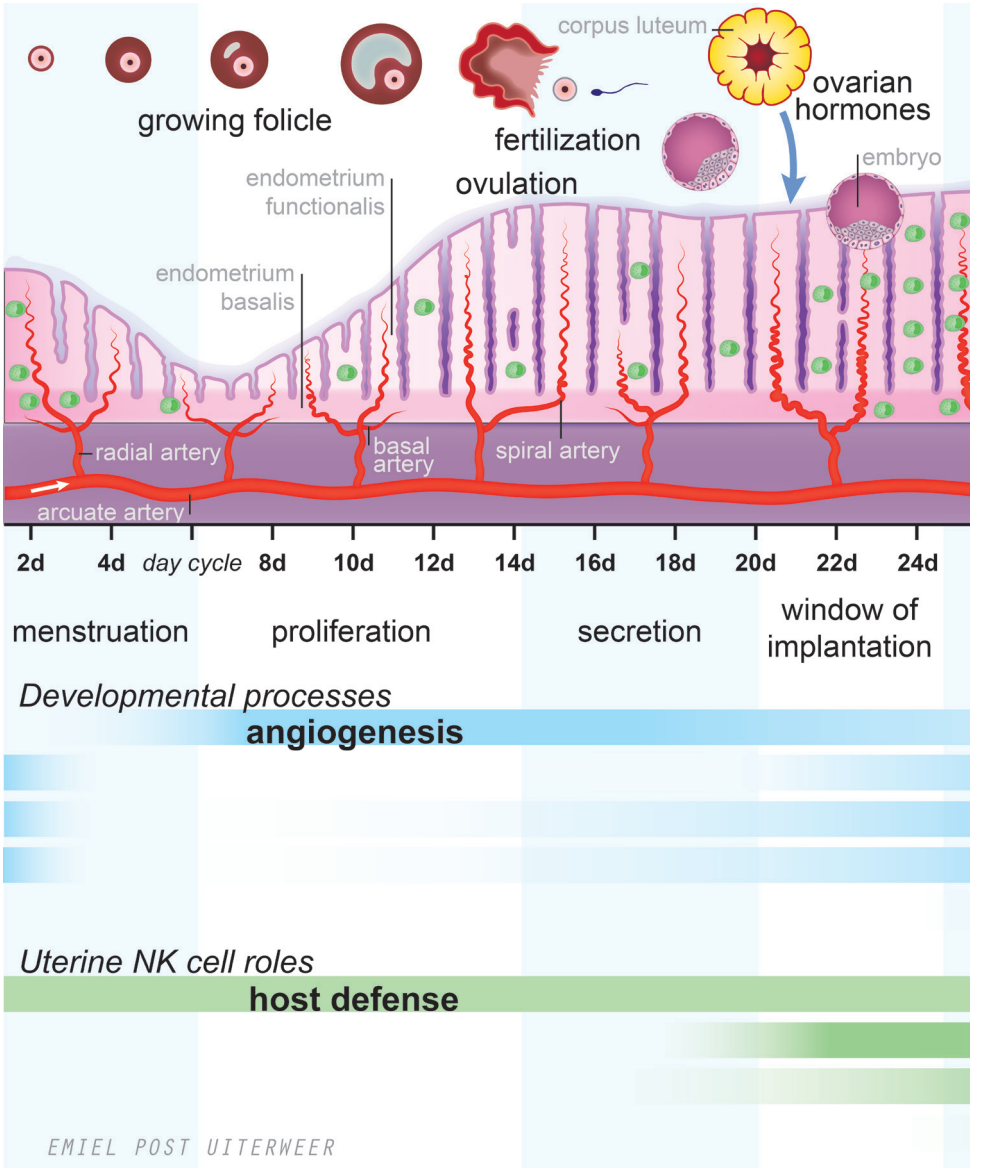
Over the reproductive lifespan of women, the uterine niche is remodeled over 400 times and forms one of the fastest growing tissues of the female body.[75] The menstrual cycle is a hormonally regulated rebuilding process that prepares the human endometrium for an invasive type of hemochorioendothelial placentation.[4] Interestingly, uNK cell and uterine macrophage (CD163+ cells) abundance follows this cyclic pattern of tissue remodeling, whilst other immune cells such as B- and T-cells are less abundant in the uterine niche and remain more stable in numbers (**Figure 3**).[13-15]

4.1 UTERINE NK CELLS IN THE CYCLING NICHE

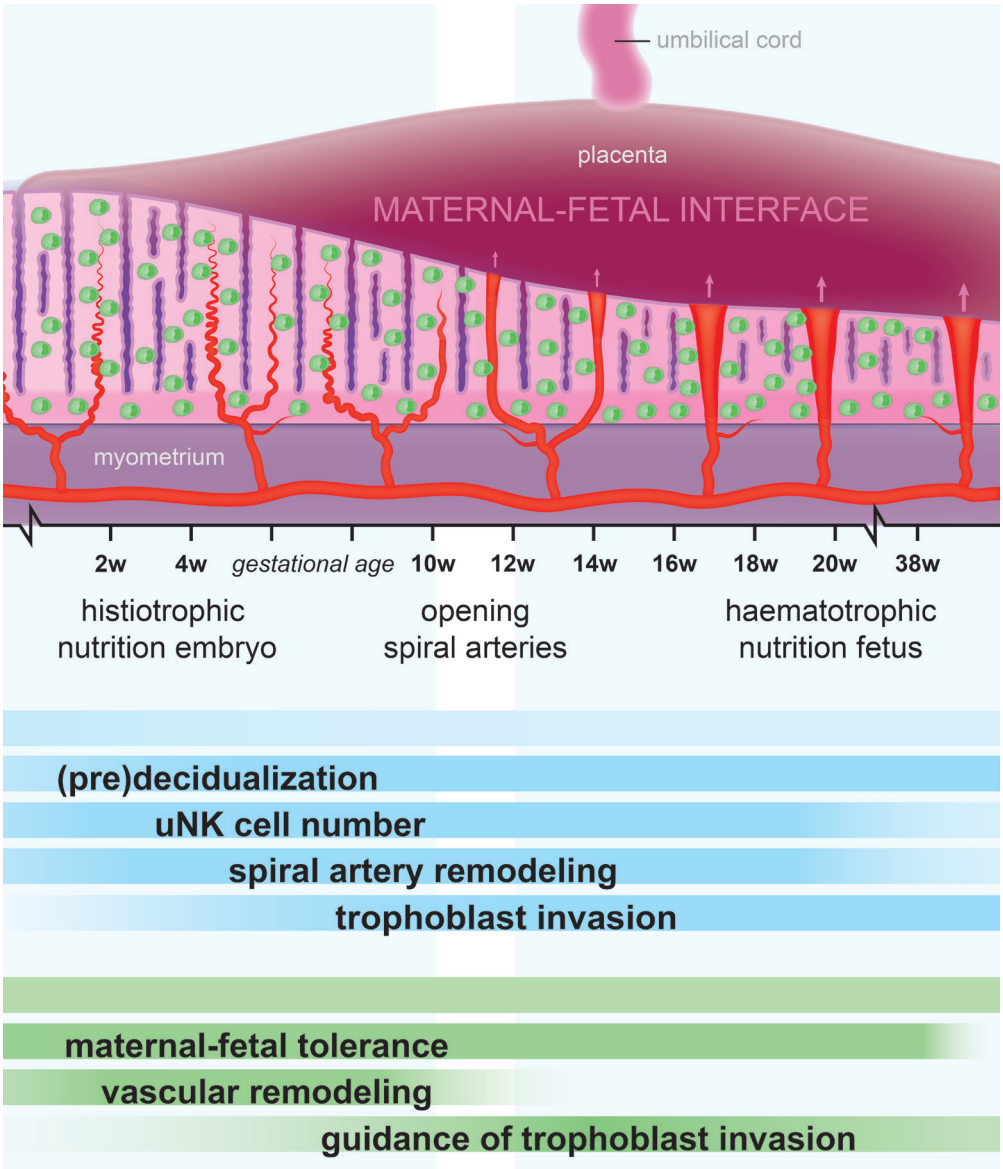
The menstrual cycle starts at the first day of *menstruation*, when regression of the corpus luteum leads to a fall in circulating and endometrial progesterone levels.[76] Nuclear shrinkage and fragmentation of uNK cells is one of the first endometrial signs of progesterone withdrawal in the uterine niche during the late secretory phase.[77] Thus, uNK cells herald the shedding of the functional layer of the endometrium. Menstrual blood is enriched in uNK cells compared to peripheral blood. Uterine NK cells remain present in the remainder of the (partially) shed functional and the basal layer of the endometrium. The role of remaining uNK cells in endometrial regeneration at the start of the new cycle is unknown. However, NK cells derived from menstrual tissue debris produce cytokines and cytolytic products including IFNγ granzyme B and perforin when stimulated with IL-2 and IL-15.[77,78] Thus, uNK cells, accounting for 15-25% of the total number of stromal cells in the perimenstrual endometrium,[13] could play an as of yet undefined role in endometrial breakdown during menstruation.

The *proliferative phase* of the menstrual cycle is characterized by proliferation of epithelial, stromal, and vascular cells. Estrogen is key in regulating the endometrium in this phase, but there is no evidence that estrogen modulates proliferation, cytolytic activity or cytokine secretion by uNK cells.[79] In this phase of the menstrual cycle, the uterine niche is relatively devoid of uNK cells (**Figure 1**) accounting for only ~5-10% of total stromal cell numbers.[13, 77]

The proliferative endometrium is characterized by angiogenesis that is initiated in



↑ **Figure 3**
Dynamic changes in the 'uterine niche' and uNK cell function. Schematic representation of the dynamic changes in the 'uterine niche' during the menstrual cycle and pregnancy that determine uNK cell numbers, maturation and functions at the maternal-fetal interface. The different, temporally regulated phenotypes of uNK cells, in turn, dictate precisely timed developmental processes such as embryo implantation, spiral artery remodeling and extravillous trophoblast invasion.


umbilical cord
placenta
MATERNAL-FETAL INTERFACE
myometrium
2w
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gestational age
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16w
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38w
histiotrophic nutrition embryo
opening spiral arteries
haematotrophic nutrition fetus
(pre)decidualization
uNK cell number
spiral artery remodeling
trophoblast invasion
maternal-fetal tolerance
vascular remodeling
guidance of trophoblast invasion

the basal layer during menstruation and which continues during the proliferative phase. During the proliferative and early-secretory phase of the cycle, spiral arteries are rebuilt by intussusceptive angiogenesis and vessel elongation.[80] Ex vivo studies have shown that uNK cells isolated from the *decidua of early pregnancies* produce angiogenic factors such as vascular endothelial growth factor A and C (VEGF-A/C), placental growth factor (PlGF), angiopoietin 2 (Ang-2), and IL-8 after stimulation with for example IL-15.[28] Studies on the production of angiogenic factor by uNK cells during the proliferative phase are very limited. Nevertheless, one study showed that mRNA transcripts for VEGF-C and PlGF are present in uNK cells in the *secretory phase endometrium,* and Ang2 in the *late secretory phase*.[29,81] On the basis of these data we postulate that uNK cells are likely not involved in angiogenesis during the proliferative phase but rather in vessel development and remodeling in the endometrium during the secretory phase and the implantation period. During the proliferative phase, other cells such as VEGF producing intravascular neutrophils likely participate in endometrial angiogenesis.[82]

In the *secretory phase* of the menstrual cycle ovarian hormones, in particular progesterone, prepare the uterine niche for embryo implantation through the process of "decidualization" that continues throughout early pregnancy (**Figure 3**).[5] At the initiation of decidualization in the mid-secretory phase uNK cells slightly increase in number.[15] The first morphological signs of stromal decidualization occur nearby the spiral arteries accompanied with an increase of uNK cell number in that same region.[15,83] In early pregnancy, decidualization continues and the uNK cell number rises until the end of the first trimester when they account for ~30% of total stromal cells and 70-80% of total uterine leukocytes.[13] The rise in steroid hormones and uNK cell numbers during decidualization has raised the possibility of uNK cell regulation by ovarian hormones. However, several studies indicate that uNK cells lack the progesterone receptor and progesterone does not alter uNK cell proliferation, cytolytic activity and cytokine secretion *in vitro*.[79,84,85]

In the early-secretory endometrium, IL-15 expression increases in concert with the number of uNK cells.[86,87] IL-15 is an essential cytokine for uNK cell differentiation and growth,[88] and is expressed by endometrial stromal, epithelial and endothelial cells.[86] Importantly, ovarian steroids, e.g. progesterone, stimulate IL-15 production by ESC *in vitro*.[57,86] The endothelium of the spiral arteries also expresses a membrane bound form of IL-15, which attracts pbNK cells and stimulates their adhesion to the vascular wall.[89,90] Finally, IL-15 stimulates uNK cells to produce cytokines and angiogenic factors.[30,49]

Overall, uNK cells numbers and function change in the hormonally regulated

maturation of the uterine niche during the menstrual cycle. We postulate that IL-15 is a central mediator to trigger cytokine and angiogenic factors in the secretory phase of the menstrual cycle.

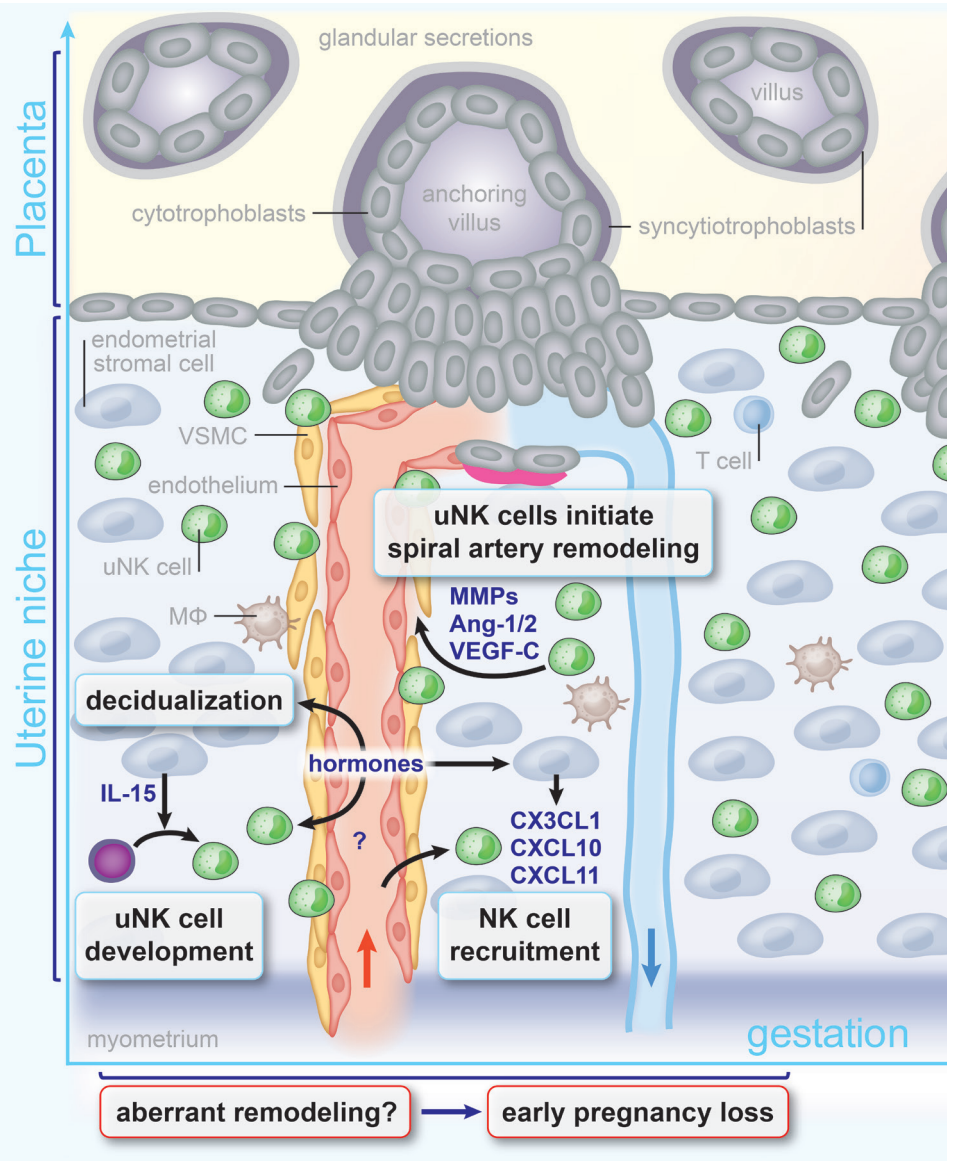
4.2 UTERINE NK CELLS AND EMBRYO IMPLANTATION

In successful human pregnancies embryo implantation occurs around 6-10 days after ovulation.[91] During this 'window of implantation' the decidualizing stromal cells actively participate in embryo implantation.[92] Maternal uNK cells are in close contact with the syncytiotrophoblast of the implanting embryo and are thought to modulate endometrial receptivity.[93,94] In mice, IFNγ produced by uNK cells is essential for initiating decidual vascular modifications.[95] Uterine NK cells also produce a wide range of cytokines and growth factors that are known to prepare the uterine niche for embryo implantation.[94] For example, uNK cells stimulate uterine epithelial cells to produce IL-10, IL-15 and CXCL10 (IP10).[96] CXCL10 facilitates embryo implantation by attracting blastocyst trophectoderm cells.[97] IL-10 is an anti-inflammatory cytokine that restrains potential inflammatory responses at the implantation site.[98] IL-15 is a positive feedback for uNK cells to mature and promote their cell responses to regulate embryo implantation.[57] Uterine NK cells regulate EVT invasion by providing pro- and anti-invasive signals such as IL8 and IFNγ respectively.[99,100] Thus uNK cells fine-tune the endometrium to regulate embryo implantation.

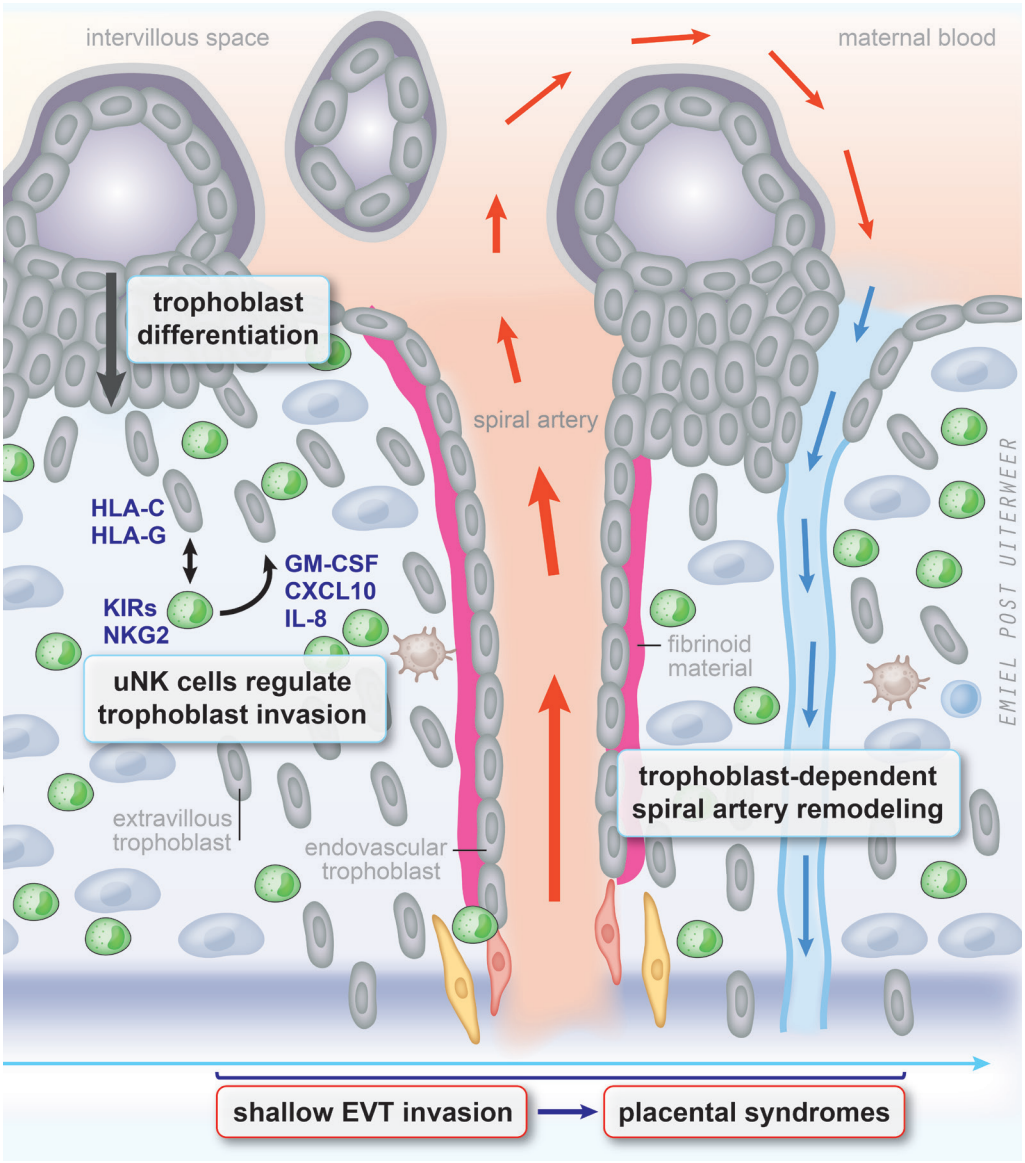
4.3 UTERINE NK CELLS DURING PLACENTATION

The growing conceptus requires formation of a functional placenta to guarantee maternal-fetal exchange of nutrients, oxygen and metabolic waste products. The formation of a placenta requires modification of the uterine niche, which is characterized by ongoing decidualization and ultimately the onset of blood flow across the intervillous space of the developing placenta that is maximized by invasion of fetal trophoblast interacting with uNK cells.[52,101]

During pregnancy the maternal uterine spiral arteries undergo essential adaptations known as spiral artery remodeling.[102] In mice, uNK cells modify spiral arteries, a process which is dependent on IFNγ production by uNK cells.[30,95] For example, systemic IFNγ administration to NK cell-deficient mice completely restores uNK cell mediated spiral artery modifications.[103] In humans invading EVTs remodel the spiral arteries by completely destroying their musculo-elastic coat. Whether the loss of cells from the vascular wall results from apoptosis, re-differentiation, or migration, is still unclear.[104] The resulting large diameter vessels that lack vasomotor control lead to a fall in arterial resistance, thereby maximizing



↑ **Figure 4**
Schematic overview of uNK cells and their role in spiral artery remodeling at the maternal-fetal interface. Spiral arteries (in red) spiral through the placental bed and are remodeled in a trophoblast-independent and a trophoblast-dependent fashion resulting in a high capacitance, low-resistance blood supply to the intervillous space in the early second trimester. The complex process of spiral artery remodeling requires time dependent functions of uNK cells at the maternal-fetal



interface. Uterine NK cells are involved in trophoblast independent changes of the spiral arteries but also regulate the invasion of extravillous trophoblasts (EVTs) into the uterine niche, thereby contributing to the later stages of spiral artery remodeling. Temporal dysregulation of uNK cell mediated initiation of vascular adaptations in the decidua during pregnancy has been implicated in early pregnancy loss whereas shallow extravillous trophoblast invasion is linked to placental syndromes such as fetal growth restriction and preeclampsia.

blood flow into the intervillous space.[2] Further, by increasing arterial diameter especially of the distal portion of the spiral artery, blood flow velocity is reduced, thus protecting the delicate free floating villi from potential damage by high blood flow velocity. The first modifications of the spiral arteries occur in the absence of local trophoblasts,[105] indicating that spiral artery remodeling is likely to be initiated by maternal cells present in the uterine niche with uNK cells being likely candidates because of their abundance around these blood vessels (**Figure 4**).[106]

In humans, uNK cells shape the uterine niche by inducing the first transformations of the spiral arteries, during the *trophoblast-independent phase*. Uterine NK cells initiate early vascular changes in vascular smooth muscle cells (VSMCs) and endothelial cells.[107,108] First trimester (8-10 weeks) uNK cells produce higher amounts of Ang1, Ang2 and VEGF-C *in vitro* compared to the second trimester uNK cells (12-14 weeks). The increased production of these vasoactive factors corresponds with the period of pregnancy when uNK cells destabilize the VSMC layer.[29,107] The uNK cells that infiltrate the VSMC layer also produce matrix metalloproteinases (MMPs) that degrade ECM components thereby contributing to the loss of VSMC integrity in the first trimester increasing the diameter of the arterial lumen.[106,107,109] In this period when uNK cells encircle the spiral arteries, endothelial cells become larger and more vacuolized.[105] In addition, NK cells secrete VEGF-A/C, PlGF and IL-8 that stimulate endothelial reorganization and remodeling *in vitro* and *in vivo*.[28,110] Overall during the first trimester of pregnancy the uterine niche triggers uNK cells to secrete vasoactive factors to initiate spiral artery remodeling, in order to prepare the vascular wall for subsequent "trophoblast-dependent" remodeling (**Figure 4**). The question remains which triggers stimulate uNK cells to produce these vasoactive factors? As discussed before, IL-15 might be a central mediator as it stimulates the production of vasoactive factors by uNK cells, and it is produced by multiple cells in the decidualizing endometrium. Interactions between trophoblast and uNK cell could potentially trigger uNK cells to produce vasoactive substances. Although studies using direct co-culture of uNK cells and primary trophoblast are almost non-existent, one study showed that co-culture of uNK cells with primary EVTs actually reduced production of angiogenic factors by uNK cells such as VEGF and angiotensin.[101]

As a next step in spiral artery remodeling, EVTs invade the uterine wall to the inner one-third of the myometrium in the so-called *trophoblast-associated remodeling* phase.[102,111] Interstitial EVTs further disrupt the uNK cell primed VSMC layer and EVTs incorporate into the vessel wall to fully replace VSMCs. The endothelium is transiently replaced by EVTs but is 're-endothelialized' later in pregnancy.[2] The importance of this vascular remodeling process is underscored by several serious pregnancy complications associated with impaired EVT invasion and abnormal

spiral artery remodeling.[112] Uterine NK cells regulate EVT invasion into the uterine niche by direct cell-to-cell and paracrine interactions. Recently a maternal mechanism to control trophoblast invasion has emerged that involves the interaction of KIR2DS1 on uNK cells with HLA-C2 on fetal EVTs that stimulates GM-CSF production by uNK cells to promote EVT invasion.[113] In addition, interactions of inhibitory KIR2DL4 with HLA-G on EVT promotes EVT invasion through induction of IL-1β, IL-6 and IL-8 production by uNK cells.[114] For more in depth information of KIR-HLA-C interaction in the uterine niche we refer to detailed reviews on this subject.[115,116]

Several uNK cell secreted factors may stimulate or inhibit EVT invasion. Crosslinking NKG2D promotes interferon gamma-induced protein 10 (IP10) and IL-8 production by uNK cells. These chemokines stimulate primary EVT invasion through CXCR3 and CXCR4.[28,117] Other chemokines, cytokines and growth factors including GM-CSF, IL-6 and IL-1β also stimulate EVT invasion.[113,118] Interestingly, uNK cell secreted factors from the second trimester (12-14 weeks) enhance primary EVT invasion, whilst first trimester (8-10 weeks) uNK cell factors do not affect trophoblast invasion.[119] These second trimester uNK cells produce significantly more EVT invasion-stimulating cytokines *in vitro* compared to first trimester uNK cells (**Figure 4**).[29]

Besides the stimulation of trophoblast invasion, the extent of invasion of trophoblast into the endometrium needs to be restricted to prevent invasion too deeply into the myometrium. As such in human pregnancy trophoblasts migrate approximately into one-third of the myometrium. Uterine NK cells are capable of inhibiting primary 1st trimester EVT invasion through IFNγ and TNFα production,[120] however it is still unclear whether this inhibition is dependent of the stage of pregancy. Uterine NK cells interaction with HLA-G expressing EVTs stimulates uNK cells to produce IFNγ. Uterine NK cells also produce anti-inflammatory cytokines such as IL10 and IL4, yet their role in the regulation of EVT invasion, if any, is currently unknown.[54,121] Thus, uNK cells undergo a functional switch between the first and second trimester to stimulate EVT invasion in the second trimester that is linked with increased production of factors that stimulate EVT invasion. It remains to be determined whether uNK cells also contribute to the restriction of trophoblast invasion later in pregnancy.

4.4 UTERINE NK CELLS AND ADVANCING PREGNANCY

The endometrium markedly thins with advancing pregnancy. Nevertheless, CD56+ cell numbers in the uterine niche remain stable during the second trimester, and decrease slightly in the third trimester of pregnancy.[122] The number of granule-containing lymphocytes, and perforin and granzyme B expressing cells declines considerably during gestation, indicating that uNK cells lose cytotoxic machinery compromising their capacity as cytotoxic effectors in local host defense.[14] Critical

developmental processes at the human maternal-fetal interface mainly occur in the first half of pregnancy,[2,111] yet placentation continues throughout pregnancy; the placental weight increases fourfold and placental blood flow increases exponentially in the second half of pregnancy.[123,124] The continuous presence of uNK cells at the maternal-fetal interface during pregnancy suggests that uNK cells continue to play a role in maintaining placental homeostasis. Most studies investigating uNK cells are performed using first trimester and to a lesser extent early second trimester abortion material. The lack of human tissues to study these developmental processes during advancing gestation have hindered the study of the role of the uterine niche in shaping uNK cell responses in advanced gestation (>15 weeks). Despite these limitations, uNK cells have been postulated to prevent excessive myometrial invasion beyond the decidual-myometrial junction, a process that is impaired in placenta accreta.[123,125] As such patients with placenta accreta may have reduced uNK cells numbers.

5 THE UTERINE NK CELL NICHE IN HOST DEFENSE AND MATERNAL-FETAL TOLERANCE

Circulating NK cells target malignant and pathogen-infected cells for destruction. Similarly, uNK cells can play a role in host defense in the uterine niche. Contrary to previous belief the maternal-fetal interface may not be completely sterile, as bacterial DNA of non-pathogenic commensal microbiota was recently isolated from the villous placenta.[126] Yet these results should be interpreted with caution because DNA kits used could be contaminated with bacterial DNA.[127] In some cases the uterine niche is colonized by pathogens causing inflammation and pregnancy complications such as fetal growth restriction, preterm birth and fetal demise.[128,129] Although uNK cells are likely to be of vital importance to protect the host and prevent transplacental transmission of pathogens (congenital infection), the role of uNK cells in host defense in the uterine niche has received little attention.

During normal pregnancy uNK cells are relatively devoid of cytolytic activity. Nevertheless, uNK cells display a range of activating receptors (**Figure 1B**), express the essential molecules required for lysis (**Figure 1C**) and are capable of forming an immunologic synapse with a target cell. Yet they fail to polarize their microtubule organizing centers to release lytic granules into the immunologic synapse.[64] In addition, activating NK cell receptors have different functions in the uNK cells, i.e. the activating NK cell receptor 2B4 (CD244) reduces uNK cell lytic capacity and production of IFNγ.[130,131] Moreover, NKp30 triggers cytotoxicity in pbNK cells but does not mobilize the lytic machinery in uNK cells. Even though uNK cells are

intrinsically less cytotoxic than their peripheral counterparts, inhibitory NK cell receptors (KIR2DL, NKG2A) expressed on uNK cells can interact with fetal ligands on EVTs (HLA-G, HLA-E) to prevent uNK cells from 'naturally' killing trophoblast cells.[48,132] The uterine niche also expresses several secreted factors that can contribute to reduced uNK cell cytotoxicity including progesterone-induced blocking factor (PIBF),[133] VEGF-C,[110] the granzyme B inhibitor protease inhibitor 9 (PI-9),[134] CEACAM1,[135] and soluble HLA-G.[136]

However, infections of the uterine epithelia or the fetus may possibly trigger uNK cells to acquire cytolytic activity or secrete pro-inflammatory cytokines. *In vitro*, NKp46 ligation has been shown to induce potent cytotoxicity by uNK cells. Ligation of NKp30, or cytokine stimulation with IL-12 and IL-15 robustly induced IFN-γ and TNF-α production.[12,54,137] Finally, uNK cells mediate anti-viral immune responses as uNK cells, but not peripheral blood NK cells, were shown to limit HIV-1 replication *in vitro* through their secretion of CXCL12.[138] In the case of a viral infection at the maternal-fetal interface, e.g. human cytomegalovirus (HCMV), crosstalk between HLA-E on infected EVTs and NKG2C/E receptors on uNK cells transforms these cells in cytotoxic effectors.[139] Virus-infected cells produce stress ligands that trigger intracellular calcium mobilization, polarization of perforin containing granules, granule exocytosis and target cell lysis through activation of NKp46 and NKG2D. NK cell mediated cellular cytotoxicity could thereby prevent viral spreading to the conceptus.[28,48] Hence, it is conceivable that uNK cells serve as guardians of the uterine niche to maintain homeostasis in case of an infection.

6 UTERINE NK CELLS, THE 'UTERINE NICHE' AND ADVERSE PREGNANCY OUTCOMES

Normal development of the uterine niche in reciprocal communication with uNK cells is essential for a healthy pregnancy. Aberrations in this communication can have profound effects on placentation and the growing conceptus potentially leading to a wide range of adverse pregnancy outcomes.[140] Below we discuss potential aberrations in the uterine niche and uNK cells in reproductive disorders.

6.1 EARLY PREGNANCY LOSS

Only ~25% of fertilized human oocytes result in live births, which in part can be explained by insufficient preparation of the uterine niche.[141] The majority of embryos are 'lost' in the peri-implantation period.[142] In recent years uNK cells have received special attention in the context of recurrent implantation failure,[143] and recurrent miscarriage.[144] According to this hypothesis, increased cytotoxicity of uNK cells would create a hostile environment for the implanting embryo.

Uterine NK (CD56+) cell numbers are increased in the secretory endometrium of women with a history of recurrent implantation failure or miscarriage.[145-147] Other studies, however, failed to identify changes in total uNK cell numbers,[148] but rather observed a skewing from a $CD56^{bright}CD16^{-}$ towards a $CD56^{+}CD16^{+}$ uNK cell population.[149] The pbNK cell subset that expresses the surface antigen CD16 is associated with cytotoxic effector functions. Therefore, an increased number of CD16 expressing uNK cells could indicate higher cytotoxic activity. Another hypothesis regarding the role of uNK cells in recurrent miscarriage has been postulated that elevated uNK cell numbers increase angiogenesis and peri-implantation blood flow, leading to early maternal circulation and excessive oxidative stress[150] Some evidence exists that aberration in uNK cell numbers or phenotype are associated with recurrent miscarriage, but two recent independent meta-analyses showed that uNK cell analysis has no prognostic or diagnostic value in the management of early pregnancy loss.[151,152]

6.2 THE 'GREAT OBSTETRICAL SYNDROMES' OR PLACENTAL SYNDROMES

A spectrum of human pregnancy complications is associated with defects in the process of spiral artery remodeling. Aberrations in this process have first been described in preeclampsia and fetal growth restriction and are thought to result in poor uteroplacental perfusion.[153,154] More recently, defects in spiral artery remodeling have also been linked to preterm labor, premature rupture of membranes, mid-trimester pregnancy loss, and placental abruption.[112,155] The recognition of uNK cells as key regulators of both spiral artery remodeling and EVT invasion raises the possibility that dysregulation of uNK cell number or function in the uterine niche might be a proximal event in the etiology of placental syndromes.

Most studies have been performed with postpartum placental bed biopsies of pregnancies complicated by preeclampsia and/or fetal growth restriction hampering true insights into the role of uNK cells in the etiology of preeclampsia during early pregnancy. Reports of both increased and decreased numbers of uNK cells in the placental bed exist.[156-160] In addition, conflicting evidence exists on potential changes in the relative skewing of the uNK cell subset towards a more cytotoxic effector phenotype (CD56+CD16+) in preeclampsia.[161-163] Differences in patient selection or donor material (e.g., deep placental bed biopsy or superficial decidua, and with or without spiral arteries) may contribute to these differences. Regardless, the relevance of these studies may be questioned, because uNK cell number and activity during early pregnancy, when trophoblasts invade and spiral arteries remodel, were not examined.

Studies on the crosstalk between uNK cells and EVTs have provided new insights into the potential role of uNK cells in the pathogenesis of placental syndromes.[115] Interaction of KIRs on uNK cells with human leukocyte antigens (HLAs) on EVTs may affect reproductive outcome. Immunogenetic studies showed that mothers homozygous for the inhibitory KIR haplotype AA in combination with trophoblast HLA-C2 derived from the father are at increased risk for developing preeclampsia and fetal growth restriction.[164] Women with a KIR A haplotype lack the activating uNK cell receptor KIR2DS1 and have impaired production of GM-CSF. GM-CSF promotes human trophoblast invasion *in vitro,* and in mutant mice lacking GM-CSF, structural changes occur in the placenta associated with decreased litter sizes, reduced fetal body weight, and increased number of reabsorptions and malformed fetuses.[113,165] These observations indicate that an imbalance of HLA-C2 mediated activation of KIRs on uNK cells may interfere with trophoblast invasion resulting in defective spiral artery remodeling and "shallow placentation".[113,166]

It is challenging to gain insight into the potential role of uNK cells and the uterine niche during early pregnancy in women who develop placental syndromes. Animal models, notably murine models, show differences in reproductive biology and uNK cell function. Moreover, the relative inaccessibility of the relevant early gestational human tissue and the ethical concerns that go along with it, the long latent phase – often more than 4 months – prior to the onset of clinical signs of fetal growth restriction and/or preeclampsia, and the relatively low disease incidence (2-5%) complicate the elucidation of the role of uNK cells in the early phase of these pregnancy disorders. Wallace and colleagues studied first trimester uNK cells derived from decidua of women at low (<1%) and high (>21%) risk to develop preeclampsia to circumvent some of these practical issues.[167,168] The risk assessment was based on uterine-artery Doppler resistance-index measurements to predict development of preeclampsia.[169,170] Uterine NK cells isolated from high-risk subjects produced less pro-invasive factors, such as hepatocyte growth factor (HGF), and more anti-invasive factors including endostatin and agiogenin. The uNK cells from high-risk pregnancies had a reduced capacity to induce EVT motility *in vitro*.[168,171,172] The altered growth factor production and regulatory function of first trimester uNK cells in women at high risk of developing preeclampsia supports the view that uNK cells could be involved in the etiology of preeclampsia. However, the fact still remains that pregnancy termination obviously precluded knowledge of the actual pregnancy outcome. With this perspective the use of Doppler to assess uterine artery blood flow as proxy of placental syndromes should be discussed. Recently, the use of 3D power Doppler of the *placenta bed* has been shown to be a safe, relatively easy and fairly reliable first trimester assessment to predict the risk to develop

preeclampsia later in pregnancy.[173] Therefore this technique may prove to provide a better proxy of placental development.

There also seems to be a discrepancy in the anatomical localization of uNK cells and the defects seen in trophoblast invasion and spiral artery remodeling in placental syndromes. How can uNK cells in de endometrium affect trophoblast invasion and spiral artery remodeling in the inner one-third of the myometrium, as uNK cells are thought to be absent in this anatomical layer of the uterus? Particularly this deeper section of the spiral arteries seems to lack remodeling in placental syndromes.[174] Possibly aberrant uNK cell regulation of EVT invasion in the endometrium affects EVT to reach the inner myometrium. Moreover uNK cell secreted factors may diffuse into the inner myometrium to control remodeling. Nevertheless, a possible role of other immune cells such as macrophages present in the myometrium could contribute to the lack of deep spiral artery remodeling in placental syndromes.[175]

Recent work supports the concept of aberrations in uNK cell number and/or function in the uterine niche during early pregnancy in women destined to develop preeclampsia.[176] Whole genome gene expression profiling of first trimester placental tissues collected by chorionic villous sampling in women who developed preeclampsia ~6 months later showed that the expression of a multitude of decidualization marker genes including insulin-like growth factor-binding protein 1, prolactin, glycodelin and IL-15, as well as genes associated with uNK cell maturation and function such as granulysin, granzyme B and IL-2RB, was significantly reduced. Thus, impairment in the establishment of a functional uterine niche in early pregnancy critical to uNK cell recruitment and maturation may play a role in the etiology of preeclampsia.

7 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

In reciprocal communication with the dynamic uterine niche uNK cells perform a major role in the complex process of human placentation. As the most prominent immune cell in the uterus during early pregnancy, uNK cells not only guarantee maternal-fetal tolerance, but also protect the host from invading pathogens. Uterine NK cells are possibly recruited both as progenitor or differentiated NK cells and may modify the uterine vasculature independently of trophoblast helping to ensure unimpeded maternal blood flow into the intervillous space. The different uNK cell functions within the uterine niche correspond with a unique pattern of cell surface receptors. The uterine mucosa forms a particularly interesting non-lymphoid microenvironment to study NK cell biology as it is regenerated during each

menstrual cycle, and undergoes an intricate and extensive process of remodeling in the secretory phase and during placentation. Infection, endometrial shedding during menstruation, and placentation trigger uNK cells to quickly adapt to maintain homeostasis at the maternal-fetal interface. Aberrations in the uterine NK cell niche may play a role in the development of a broad spectrum of reproductive disorders, which indicate that the uNK cell niche may potentially be a therapeutic target to improve pregnancy outcome. Some fertility clinics offer tests to assess pbNK cell number and activity.[177] Moreover it has been suggested that targeting uNK cell numbers might benefit women with early pregnancy loss.[178,179] However, such approaches should be interpreted with caution as uNK cells have unique and constantly changing functions that we are only beginning to understand and that differ markedly from pbNK cells. Human uNK cells are challenging to study in the early pregnancy uterine niche, particularly in the context of pregnancy complications, but efforts to overcome these challenges could aid in the development of urgently needed strategies to reduce human reproductive disorders. Future studies should therefore include further study of the time specific changes in NK cell phenotype and function during normal and complicated pregnancy.

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Chapter 4

BIOINFORMATICS APPROACH REVEALS EVIDENCE FOR IMPAIRED ENDOMETRIAL MATURATION BEFORE AND DURING EARLY PREGNANCY IN WOMEN WHO DEVELOPED PREECLAMPSIA

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ABSTRACT

Impaired uterine invasion by extravillous trophoblast in early gestation is implicated in the genesis of preeclampsia, a potentially lethal malady of human pregnancy. However, reasons for extravillous trophoblast dysfunction remain unclear because of virtual inaccessibility of early placental and uterine tissues from women who develop preeclampsia, and the absence of animal models in which the disease spontaneously occurs. Consequently, the possibility that deficient or defective maturation of the endometrium (decidualization) may compromise extravillous trophoblast invasion in preeclampsia remains unexplored. Using a bioinformatics approach, we tested this hypothesis identifying 396 differentially expressed genes (DEG) in chorionic villous samples from women at ≈11.5 gestational weeks who developed severe preeclampsia symptoms 6 months later compared with chorionic villous samples from normal pregnancies. A large number, 154 or 40%, overlapped with DEG associated with various stages of normal endometrial maturation before and after implantation as identified by other microarray data sets (P=4.7×10-14). One-hundred and sixteen of the 154 DEG or 75% overlapped with DEG associated with normal decidualization in the absence of extravillous trophoblast, i.e., late-secretory endometrium (LSE) and endometrium from tubal ectopic pregnancy (EP; P=4.2×10-9). Finally, 112 of these 154 DEG or 73% changed in the opposite direction in microarray data sets related to normal endometrial maturation (P=0.01), including 16 DEG unregulated in decidual (relative to peripheral blood) natural killer cells that were down-regulated in chorionic villous samples from women who developed preeclampsia (P<0.0001). Taken together, these results suggest that insufficient or defective maturation of endometrium and decidual natural killer cells during the secretory phase and early pregnancy preceded the development of preeclampsia.

Key words: Decidualization • endometrial cycle • natural killer cell • pregnancy • trophoblast

INTRODUCTION

Preeclampsia, a multiorgan disease affecting 3% to 5% of human pregnancies, is associated with significant maternal, fetal, and neonatal, morbidity and mortality.[1-4] In addition, preeclampsia increases the risk of lifelong cardiovascular and metabolic diseases for both mother and offspring.[5-7] Clear understanding of preeclampsia etiology is lacking, which hampers the identification of early predictive biomarkers and development of specific prophylactic and treatment measures.

Although knowledge of preeclampsia pathogenesis has markedly improved during the past decade, etiology remains less certain and has been only infrequently addressed, largely because of formidable investigative challenges.[8] It is widely thought that insufficient extravillous trophoblast (EVT) invasion of uterine spiral arteries starting in early pregnancy is a causal factor.[9] Consequently, there has been considerable investigation of the cellular and molecular mechanisms of EVT in this biological event. In contrast, little attention has been given to the uterine niche in which EVT invades. Perhaps the "soil," rather than or in addition to the "seed" is aberrant in women destined to develop preeclampsia.[10,11]

A major stumbling block to finding etiological factors in preeclampsia is that the disease is thought to begin in early pregnancy related to inadequate EVT invasion (vide supra). Accordingly, etiology is widely separated in time from the onset of disease symptoms, which does not occur until late-pregnancy.[12] Presently, we are not certain about who will develop preeclampsia because of lack of predictive biomarkers,[13] although these are being actively pursued in numerous laboratories using discovery-based approaches. This ignorance precludes identification of women for prospective exploration of disease etiology in early pregnancy. Nevertheless, even if we knew who would develop preeclampsia, we cannot readily obtain the relevant tissue in which to investigate potential causes, i.e., first trimester placenta and decidua, nor can we study third trimester placentas and basal plate decidua, and necessarily gain insight into disease etiology because one cannot really discern cause from effect at this late stage. Finally, preeclampsia is considered to be a disorder peculiar to human pregnancy, which makes investigation of etiological factors in animal models potentially problematic.[14]

In an attempt to overcome these challenging hurdles, we undertook a unique discovery-based approach to study the etiology of preeclampsia.[11,15] By whole-genome gene expression profiling of a collection of surplus chorionic villous sampling (CVS) tissue obtained for prenatal genetic screening, we unexpectedly noted putative decidualization marker genes, *IGFBP1*, *PAEP,* or glycodelin and *PRL* to be down-regulated in women who developed preeclampsia 6 months later.

Decidualization is a process of endometrial maturation that begins in the secretory phase of the menstrual cycle (predecidualization) and continues after conception and implantation. An important part of this biological process is the enrichment of decidual natural killer (dNK) cells starting in the secretory endometrium.[16] In essence, predecidualization and decidualization are a biological continuum in the preparation of the soil for the seed (EVT and conceptus).[17] Dysregulated endometrial maturation is emerging as an important precursor of recurrent pregnancy loss and infertility.[18,19] By analogy, we asked whether insufficient or defective endometrial maturation might also contribute to the etiology of preeclampsia.

The objective of the present work was to use a bioinformatics approach,[20] to test the hypothesis rigorously that preeclampsia is antedated by disturbances in endometrial maturation before and after implantation. In turn, accumulating evidence links impaired decidualization and deficient dNK cell number and function to compromised extravillous trophoblast invasion, spiral artery remodeling, and placentation.[10,21–23] We reasoned that, if genes upregulated in the endometrium and dNK cells during the biological processes of (pre) decidualization[24–28] are down-regulated in CVS from women destined to develop preeclampsia,[11] then this would provide critical missing, prospective evidence needed to underpin the concept of endometrial antecedents of preeclampsia.

METHODS

We reanalyzed publically available microarray data sets to determine differentially expressed genes (DEG), which increase expression in LSE (predecidualization) and during endometrial maturation after implantation (decidualization); the latter in the presence or absence of extravillous trophoblast. In addition, we investigated DEG upregulated in dNK relative to peripheral blood NK cells by reanalyzing other microarray data sets. These upregulated DEG were then compared with DEG down-regulated in CVS obtained at ≈11.5 gestational weeks from 4 women who developed severe, late onset preeclampsia 6 months later matched to 8 women with normal pregnancy (results below and **Table S1** in the Supporting Information). This overall approach was chosen because our hypothesis was that genes which increased expression during the process of normal endometrial maturation before and after implantation will be decreased in the endometrium of women destined to develop preeclampsia (detailed Materials and Methods are presented in the Supporting Information).

RESULTS

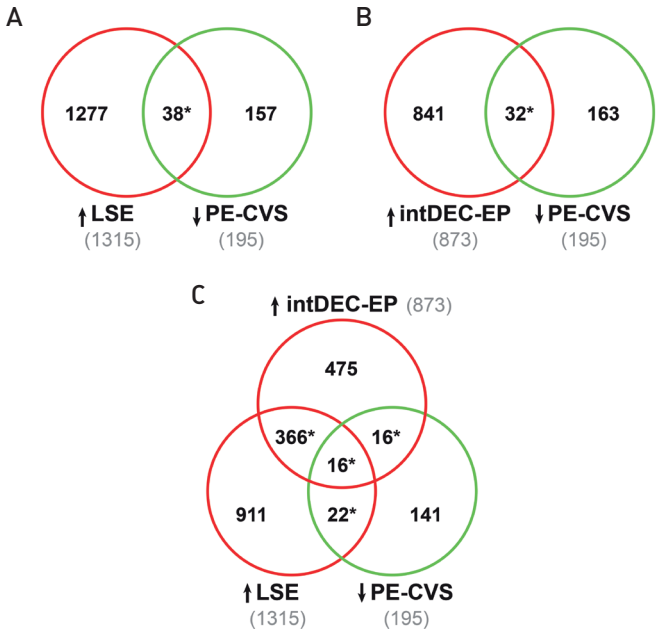
DIFFERENTIALLY EXPRESSED GENES BETWEEN CHORIONIC VILLOUS SAMPLES OBTAINED FROM PREECLAMPTIC AND NORMAL PREGNANT WOMEN

CVS obtained at ≈11.5 gestational weeks from 4 women who developed preeclampsia 6 months later were matched to CVS from 8 women with normal pregnancy.[11] Each of the 4 CVS specimens from women who developed preeclampsia was matched for parity, gestation age at CVS within 3 days, and race with 2 unaffected control specimens.[11] In addition to fetal chorion, CVS invariably contains maternal tissue that is derived mainly from the adherent decidual basal plate with another potential source being placental septae projecting upward from the basal toward the chorionic plate containing an admixture of decidual and uterine NK cells, and EVT.[29,30] Because surplus CVS was frozen within 10 minutes of extraction in this study, they were not cleaned of maternal decidual tissue.[11] The presence of decidua in the CVS is corroborated by the transcriptomics as revealed in this work being consistent with the molecular signature of decidua (see below). Women with preeclampsia met criteria for severe disease,[4,31] and all delivered >34 weeks. There were no comorbidities except the women with preeclampsia tended to have higher body mass index (**Table S1**). RNA integrity was evaluated on an Agilent Bioanalyzer (RIN ≥6.0; Agilent, Santa Clara, CA[11]). Casting a wide net, we established DEG by *t* test ($P<0.05$); fold change, and J5 analysis (Supporting Information). There was a total of 396 DEG between CVS obtained from women with preeclampsia compared with CVS from women with normal pregnancy outcome of which 201 were upregulated and 195 down-regulated in preeclampsia (**Table S2** for gene lists).

DIFFERENTIALLY EXPRESSED GENES DOWN-REGULATED IN CVS FROM PREECLAMPTIC WOMEN ARE UPREGULATED DURING NORMAL ENDOMETRIAL MATURATION IN THE LATE-SECRETORY PHASE AND EARLY PREGNANCY

We analyzed gene expression in normal endometrium from different phases of the menstrual cycle (GSE4888[28] and GSE6364[24]) to identify the cluster of coexpressed genes strongly increasing expression in the endometrium throughout the menstrual cycle and peaking in the LSE. There was a significant overlap of 38 genes between the LSE cluster of 1315 upregulated DEG and the 195 down-regulated DEG in CVS from preeclamptic compared with normal pregnant women (preeclampsia-CVS; $P<0.0001$ by Pearson $\chi 2$ test; **Figure 1A**; **Table S3A**).

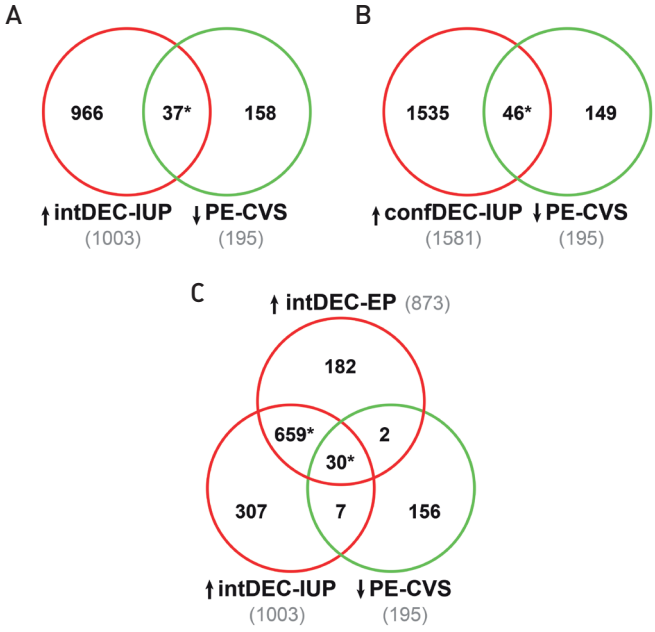
Gene expression in endometrium from tubal EP showing intermediate-decidualization morphology on H&E stained sections as described by Duncan et



→ **Figure 1**
Clusters of genes induced during pre-decidualization and decidualization in ectopic pregnancy (EP) are down-regulated in chorionic villous samples (CVS) from preeclamptic women. The Venn Diagrams show significant overlap (*P<0.0001 by Pearson $\chi 2$ test) between differentially expressed genes (DEG) down-regulated in CVS from preeclamptic (PE) women (PE-CVS; relative to CVS from women with normal pregnancy) and DEG upregulated in (**A**) late-secretory endometrium (LSE; relative to proliferative endometrium; 38 DEG, Table S3A) and (**B**) EP endometrium with intermediate-decidualization (intDEC) changes (relative to EP endometrium without decidualization changes), which lacks extravillous trophoblast (32 DEG, Table S3B). (**C**) There is significant overlap (*P<0.0001) between DEG down-regulated in PE-CVS and DEG upregulated in LSE and EP endometrium with intermediate-decidualization changes (16 DEG, Table S3C).

al.25 was first compared with gene expression in nondecidualized endometrium obtained from women with EP (E-MTAB-680). The upregulated differentially regulated genes in this decidualized endometrium (873 DEG) were then compared with the down-regulated DEG in CVS from preeclamptic compared with unaffected control women. There was significant overlap of 32 genes (P<0.0001; Figure 1B; Table S3B). There was also a large overlap of 382 differentially regulated genes increasing in LSE with DEG upregulated in intermediate-decidualized endometrium (intDEC) from EP (P<0.0001). Of these, 16 significantly overlapped with the DEG down-regulated in CVS from preeclamptic women (P<0.0001; Figure 1C; Table S3C). These results suggest an impairment of endometrial maturation in the late-secretory phase and early pregnancy in the women who developed preeclampsia. This impairment is independent of extravillous trophoblast because they are absent in LSE and EP endometrium (the latter verified by histology and cytokeratin immunohistochemistry).25

Gene expression in both intDEC and confluent-decidualized endometrium (confDEC) from women with intrauterine pregnancy (IUP) was initially compared



← Figure 2
Clusters of genes induced during decidualization in intrauterine (IUP) and ectopic pregnancy (EP) are down-regulated in chorionic villous samples (CVS) from preeclamptic (PE) women. The Venn Diagrams show significant overlap (*P<0.0001 by Pearson $\chi 2$ test) between differentially expressed genes (DEG) down-regulated in PE-CVS relative to CVS from women with normal pregnancy and DEG upregulated in (**A**) intermediate-decidualized endometrium (intDEC; 37 DEG, Table S4A) and (**B**) confluent-decidualized endometrium (confDEC; 46 DEG, Table S4B) both from IUP (relative to EP endometrium without decidualization changes) and containing extravillous trophoblast (EVT). (**C**) There are 32 DEG in common between DEG down-regulated in PE-CVS and DEG upregulated in EP endometrium with intermediate-decidualized changes and without EVT (Figure 1B) and 37 DEG in common between DEG down-regulated in PE-CVS and DEG upregulated in IUP endometrium with intermediate-decidualized changes (EVT present). The majority of these DEG, in turn, are overlapping (30 DEG, Table S4C; *P<0.0001) suggesting minimal EVT contribution to the overlap.

with gene expression in nondecidualized endometrium (E-MTAB-680).[25] The upregulated DEG in IUP endometrium with intermediate (1003 DEG) and confluent (1581 DEG) decidualized changes were next compared with the 195 down-regulated DEG in CVS from preeclamptic compared with normal pregnant women. Thirty-seven and 46 DEG upregulated in intDEC and confDEC from IUP, respectively, overlapped with DEG down-regulated in preeclampsia-CVS (for both P<0.0001; Figure 2A and 2B; Tables S4A and S4B, respectively).

Because the decidua from intrauterine but not from EP was populated by extravillous trophoblast, we were able to estimate the potential EVT contribution to the overlap of DEG down-regulated in preeclampsia-CVS and upregulated in EP and IUP endometrium matched for the extent of decidualization (intermediate). There was large overlap of 689 DEG upregulated in intDEC from EP and IUP (relative to nondecidualized endometrium; P<0.0001; Figure 2C). As illustrated in Figure 2C, 30 of these 689 DEG overlapped significantly with DEG down-regulated in CVS from preeclamptic women relative to normal pregnant women (P<0.0001; Table S4C). The majority of overlapping DEG between those upregulated in intDEC

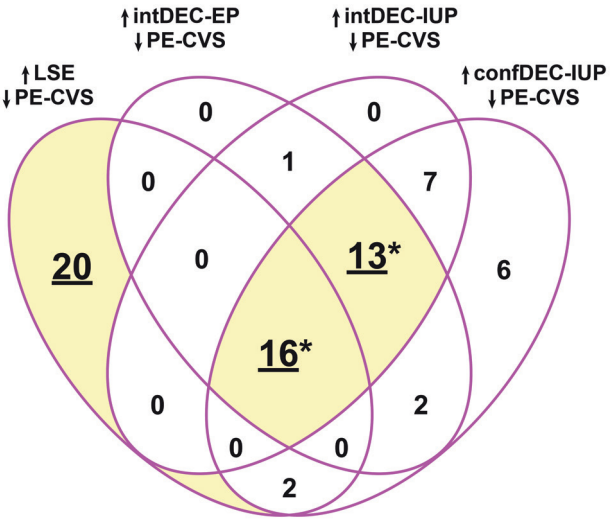


Figure 3 →
Confluence of gene clusters induced during (pre) decidualization is down-regulated in chorionic villous samples (CVS) from preeclamptic (PE) women. There are 20 differentially expressed genes (DEG) down-regulated in CVS from women that developed PE (PE-CVS; relative to CVS from women with normal pregnancy) uniquely upregulated in late-secretory endometrium (LSE, relative to proliferative endometrium). There is also significant overlap (*P<0.0001 by Pearson $\chi 2$ test) between DEG down-regulated in PE-CVS and DEG upregulated in ectopic pregnancy (EP) and intrauterine pregnancy (IUP) endometrium both with intermediate-decidualized (intDEC) changes, and IUP endometrium with confluent-decidualized (confDEC) changes, but not LSE (13 DEG); and in all 4 of the data sets related to different degrees of endometrial maturation (16 DEG). See Table S5 for individual genes and Figure 4 for average expression levels of these genes.

from intrauterine and ectopic pregnancies and down-regulated in preeclampsia-CVS were the same genes (30 of 37 for intDEC from IUP and 30 of 32 for intDEC from EP). These results reinforce the notion that the impaired endometrial maturation during early pregnancy in women who developed preeclampsia was a primary event, largely independent of extravillous trophoblast.25

DIFFERENTIALLY EXPRESSED GENES DOWN-REGULATED IN CVS FROM PREECLAMPTIC WOMEN ARE NOT UPREGULATED IN DECIDUALIZED ENDOMETRIAL STROMAL CELLS BY TROPHOBLAST CONDITIONED MEDIUM

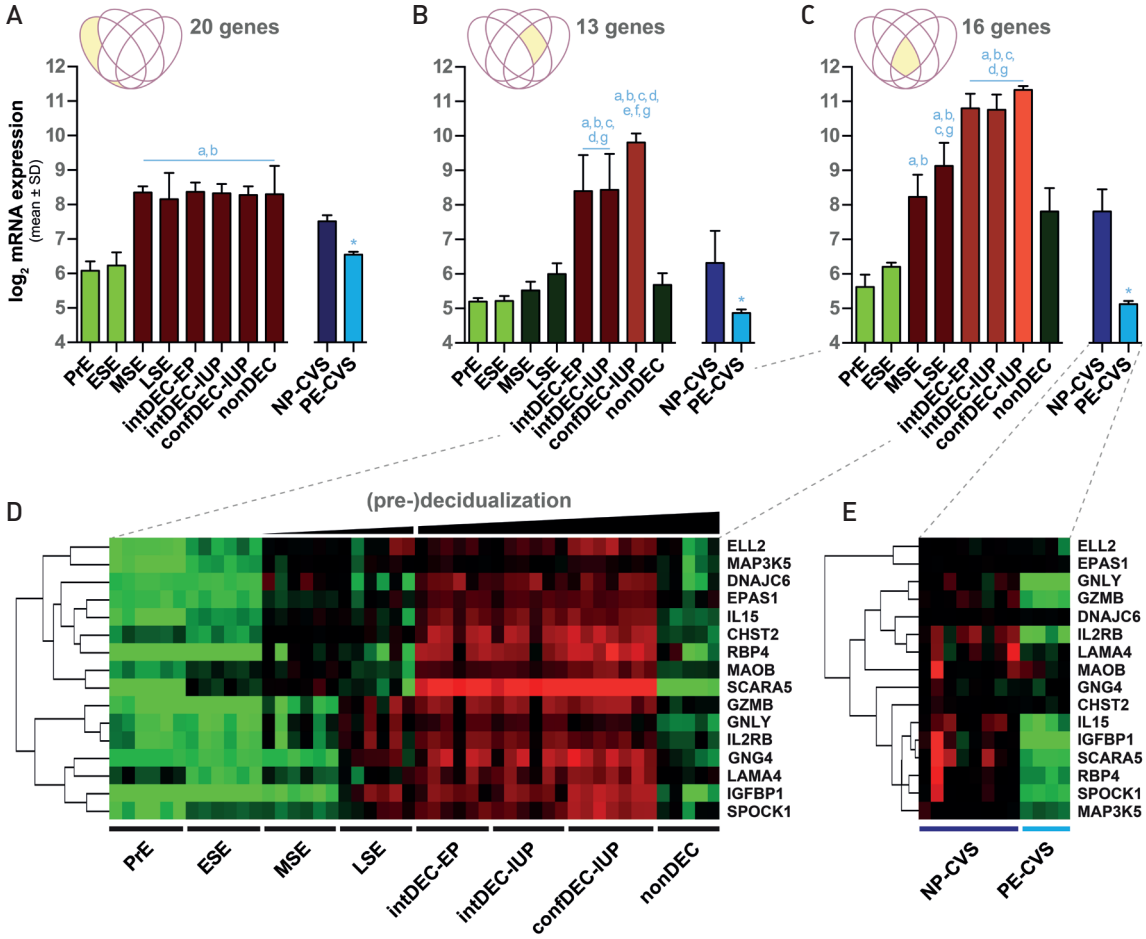
There was no significant overlap of endometrial genes increasing in expression after treatment of decidualized stromal cells in culture with trophoblast conditioned medium and DEG down-regulated in CVS from women who developed preeclampsia compared to CVS from women with normal pregnancy outcome, with only 4 in common (**Figure S1**; P=0.5). These results further support the idea that impaired decidualization in the women who developed preeclampsia may be mostly independent of trophoblast influence (results in the Supporting Information and **Figure S1**).

CONFLUENCE OF OVERLAPPING GENES

We next investigated the confluence of DEG down-regulated in CVS from women with preeclampsia relative to normal pregnancy and upregulated in LSE, intDEC

Figure 4 ↓

Average gene expression levels (log_2) in endometrium from different stages of endometrial maturation and chorionic villous samples (CVS) from preeclamptic women. (**A**) Average expression of 20 DEG down-regulated in CVS obtained from women who developed PE (PE-CVS; relative to CVS from women with normal pregnancy) and upregulated in mid- and late-secretory endometrium (MSE and LSE, respectively; relative to proliferative endometrium, PrE). (**B**) Average expression of 13 DEG down-regulated in PE-CVS and upregulated in IUP and EP endometrium with intermediate-decidualized (intDEC) changes, and IUP endometrium with confluent-decidualized (confDEC) changes, but not LSE. (**C**) Average expression for 16 DEG down-regulated in PE-CVS and upregulated in in all 4 of the datasets related to different degrees of endometrial maturation. (**D**) & (**E**) Heat maps corresponding to Figure 4C as indicated by the dotted lines. The individual DEG in Figure 4A, B and C are listed in Table S5. EP, ectopic pregnancy; IUP, intrauterine pregnancy; nonDEC, non-decidualized endometrium from EP; ESE, early-secretory endometrium. Significantly different ($P<0.05$) from: a, PrE; b, ESE; c, MSE; d, LSE; e, intDEC-EP; f, intDEC-IUP; g, nonDEC; *$P<0.0001$ vs. NP-CVS.



from IUP and EP, and confDEC from IUP. As portrayed by the Venn diagram in Figure 3, there were 20 down-regulated DEG in preeclampsia-CVS, which were upregulated in LSE but not in intDEC or confDEC; 13 DEG down-regulated in preeclampsia-CVS and upregulated in intDEC and confDEC, but not in LSE ($P<0.0001$), and 16 DEG down-regulated in preeclampsia-CVS and upregulated in all data sets related to endometrial maturation ($P<0.0001$). Individual DEG are presented in Table S5, and mean expression values are illustrated in Figure 4.

Figure 4 depicts $\log_2$ mean expression values for the DEG down-regulated in CVS from preeclamptic compared with unaffected control women and upregulated in various states of normal endometrial maturation (Figure 3). Twenty DEG were identified as uniquely upregulated in LSE and down-regulated in preeclampsia-CVS; therefore, their average expression did not increase further with decidualization in early pregnancy (Figure 4A). Average $\log_2$ gene expression of these 20 DEG was significantly less in PE-CVS compared to NP-CVS (6.55 ± 0.09 vs. 7.51 ± 0.18 SD, $P<0.0001$). The 13 DEG down-regulated in preeclampsia-CVS and uniquely upregulated in intDEC and confDEC, only increased slightly in LSE (by definition), and mostly rose during decidualization in early pregnancy (Figure 4B). Average $\log_2$ gene expression of these 13 DEG was also less in PE-CVS compared to NP-CVS (4.87 ± 0.11 vs. 6.32 ± 0.93 SD, $P<0.0001$). Finally, the 16 DEG down-regulated in preeclampsia-CVS and upregulated in LSE, and intDEC and confDEC increased expression beginning in MSE and rose thereafter progressively (Figure 4C). Average $\log_2$ gene expression of these 16 DEG was markedly less in PE-CVS compared to NP-CVS (5.12 ± 0.09 vs. 7.81 ± 0.64 SD, $P<0.0001$). The heat maps shown in Figure 4D and E correspond with the bar graph in Figure 4C. These observations reveal that endometrial maturation was not only impaired in early pregnancy but also during the secretory phase in the women who developed preeclampsia.

DIFFERENTIALLY EXPRESSED GENES UPREGULATED IN DECIDUAL NK CELLS ARE DOWN-REGULATED IN CVS FROM PREECLAMPTIC WOMEN

16 DEG upregulated in dNK relative to CD56dim and CD56bright peripheral blood natural killer cells were down-regulated in CVS from women who developed preeclampsia relative to women who experienced a normal pregnancy ($P<0.0001$; Figure 5; comprehensive results in the Supporting Information, Figure S2, and Table S6).

SYSTEMATIC LITERATURE SEARCH

Because the biological process of decidualization is not available in public bioinformatic databases for pathway analysis, we conducted a systematic and

comprehensive literature search of all 195 DEG down-regulated in CVS from preeclampsia relative to normal pregnancy. Thirty-one were previously associated with decidualization/decidua in the literature. Of these 31 DEG, 18 were in common with the 67 DEG identified by the bioinformatics approach (**Figure S3**, **Tables S2B** and **S5**, and comprehensive results in the Supporting Information).

DISCUSSION

In the present work, we asked whether the expression of genes down-regulated in early placenta (CVS) obtained from women who developed preeclampsia symptoms 6 months later significantly overlaps with expression of genes upregulated during the normal biological processes of predecidualization in the LSE, during decidualization after implantation, and in isolated decidual NK cells (relative to peripheral blood NK cells). If so, then deficient predecidualization, decidualization and dNK cell number and function in women destined to develop preeclampsia may be instrumental in disease etiology. The overall methodology was to capitalize on unique genomics data sets in the public domain, including our own data set from the first trimester placental tissue of women who developed preeclampsia, and to analyze these data sets using a bioinformatics approach to shed light on possible cause(s) of preeclampsia.

With regards to our own microarray data set,[11] we cast a wide net deliberately and identified 396 total upregulated and down-regulated DEG in CVS from women who developed preeclampsia compared with those from a normal pregnancy (**Table S2**). Remarkably, 154 or 40% of these 396 DEG significantly overlapped with DEG associated with various stages of normal endometrial maturation before and after implantation.[24–28] Second, 75% or 116 of these 154 DEG significantly overlapped with DEG associated with normal endometrial maturation in the absence of extravillous trophoblast, i.e., LSE and decidualized endometrium from ectopic tubal pregnancy. Finally, 73% or 112 of the 154 DEG either upregulated or down-regulated in CVS from preeclamptic women changed in the opposite direction in the microarray data sets related to normal endometrial maturation. These findings implicate impairment of predecidualization and decidualization in the women who developed preeclampsia. Because the overlap of differentially expressed genes in CVS from preeclamptic relative to normal pregnant women with DEG linked to normal endometrial maturation was mostly preserved regardless of the presence or absence of extravillous trophoblast, the results further imply that impaired endometrial maturation may be a primary event. This conclusion is underscored by the observation that DEG uniquely upregulated during normal endometrial maturation in the late-secretory phase were significantly down-regulated in CVS from women

who developed preeclampsia relative to normal pregnancy suggesting that the impairment of decidualization actually began before implantation (Figure 4).

As discussed earlier, we primarily focused on those DEG down-regulated in CVS from preeclampsia relative to normal pregnancy, which were upregulated during the biological process of predecidualization in the late-secretory phase (GSE488828 and GSE636424) and decidualization after implantation in women with EP (E-MTAB-68025) (Figure 1). Notably, 54 of the 195 DEG down-regulated in CVS from preeclamptic women were upregulated in LSE or decidualized endometrium from EP (Figure 1C). These results bolster the notion that there is impairment of endometrial maturation in the late-secretory phase and during early pregnancy in the women destined to develop preeclampsia. Included among the genes with diminished expression in decidua of CVS from the women who developed preeclampsia are those classically associated with the biological process of decidualization in the literature, including IGFBP1, PAEP or glycodelin, and PRL (Tables S3A–S3C and S5; Figure S3). The results also point to a primary defect in predecidualization and decidualization rather than in extravillous trophoblast because EVTs are lacking altogether in the late-secretory phase and EVTs were absent from decidualized endometrium of EP.[25]

Further inspection of the microarray analyses from decidualization in early pregnancy revealed >1000 genes each upregulated in endometrium characterized morphologically as being intDEC or confDEC from IUP compared with nondecidualized endometrium. Thirty-seven and 46 of these upregulated DEG, respectively, overlapped significantly with the 195 DEG down-regulated in preeclampsia-CVS (Figure 2A and 2B; Tables S4A and S4B). Of further note, the vast majority of DEG upregulated in intDEC from EP and down-regulated in CVS from preeclamptic compared with normal pregnant women (32 DEG), and those upregulated in intDEC from IUP and down-regulated in CVS from preeclampsia women (37 DEG) overlapped themselves (30 DEG; Figure 2C; Table S4C). These observations suggest that there may have been little, if any, contribution of extravillous trophoblast to the overlap of DEG down-regulated in preeclampsia-CVS and upregulated in intrauterine, and ectopic pregnancy endometrium matched for the degree of decidualization (intermediate) because the vast majority of DEG down-regulated in preeclampsia-CVS were upregulated in intDEC regardless of the presence (IUP) or absence (EP) of EVT.

To scrutinize the potential contribution of EVT to impaired decidualization in preeclampsia further, we compared DEG down-regulated in CVS from preeclamptic women relative to normal pregnant women with DEG upregulated in endometrial stromal cells decidualized in culture after exposure to trophoblast conditioned medium (TrCM; GSE580932) There was only a nonsignificant overlap of

4 genes (results in the Supporting Information and Figure S1). This finding is supportive of a minimal EVT contribution to the overlap observed between DEG down-regulated in CVS from preeclamptic women and DEG upregulated in either intDEC or confDEC from IUP (Figure 2C and S1, respectively), which is consistent with the concept that there may have been a primary defect of endometrial maturation in the women destined to develop preeclampsia. In fact, there were 47 DEG uniquely down-regulated in preeclampsia-CVS, which were upregulated in intDEC or confDEC from IUP; however, the majority, 34 or 72%, were also increased in LSE or decidualized endometrium from EP without extravillous trophoblast (Figure 3).

We examined the confluence of DEG down-regulated in CVS from preeclamptic women relative to normal pregnant women with DEG upregulated in LSE, intDEC from intrauterine and ectopic pregnancies, and confDEC from IUP (i.e., intersection of all 4 data sets; Figure 3; Table S5). By definition, the mean expression value of 20 DEG down-regulated in preeclampsia-CVS and uniquely upregulated in secretory relative to proliferative endometrium was significantly increased in MSE, maintained in LSE, but not increased further during decidualization in early pregnancy (Figure 4A). Of note, the mean expression of these 20 genes was significantly lower in CVS from preeclamptic compared to normal pregnant women by 2.0-fold. Taken together, this analysis reinforces the idea that the impairment of endometrial maturation in the women destined to develop preeclampsia may actually have begun before pregnancy in the secretory phase, a rather startling possibility that we are currently investigating.

The mean expression of the 13 DEG down-regulated in CVS from preeclamptic women and upregulated in intDEC from EP and IUP and in confDEC from IUP, but not in LSE, is shown in Figure 4B. Once again, the mean expression level of these 13 genes in CVS from preeclamptic compared to normal pregnant women was significantly reduced by 2.7-fold. These results provide further evidence that, in addition to a defect in predecidualization (vide supra), there was also impairment of decidualization during early pregnancy in the women who developed preeclampsia. Finally, a core set of 16 overlapping DEG was down-regulated in CVS from preeclamptic women compared with normal pregnant women and upregulated in LSE, intDEC from EP and IUP, and confDEC from IUP (Figure 4C-E). Relative to proliferative endometrium, the average expression level of the 16 DEG increased progressively beginning with the midsecretory phase peaking in confDEC from IUP. It is noteworthy that mean expression of these 16 genes in CVS of preeclamptic women was markedly lower compared to CVS from normal pregnant women by 6.5-fold. On balance, these data present a composite picture of Figure 4A and B, underscoring the notion that both predecidualization and decidualization

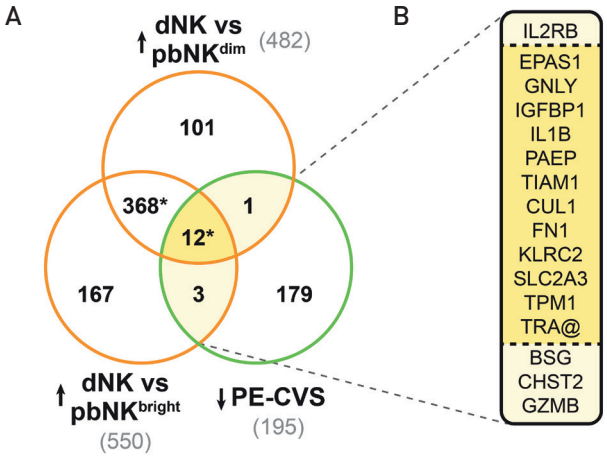


Fig. 5 →
Clusters of genes induced in decidual natural killer cells are down-regulated in chorionic villous samples (CVS) from preeclamptic (PE) women. (**A**) The Venn diagram shows significant overlap (*P<0.0001 by Pearson's chi-square test) between differentially expressed genes (DEG) down-regulated in PE-CVS relative to CVS from women with normal pregnancy and DEG upregulated in decidual natural killer cells (dNK) relative to peripheral blood $CD56^{dim}$ or $CD56^{bright}$ NK cells. The official symbols of the overlapping genes are listed in (**B**); pbNK indicates peripheral blood natural killer cells.

were compromised in the women who developed preeclampsia.

Because DEG known to be involved in dNK function emerged from the aforementioned analyses (e.g., *IL15* and *IL2RB*), we explored the overlap of DEG upregulated in isolated dNK (relative to $CD56^{dim}$ or $CD56^{bright}$ pbNK) cells and down-regulated in CVS from women who developed preeclampsia relative to women with normal pregnancy outcome (**Figures 5; Figure S2**). Our bioinformatics analysis implicates deficient dNK cell number and function in the women who developed preeclampsia because 16 DEG upregulated in dNK were down-regulated in preeclampsia-CVS. This finding is reassuring because dNK cells are an important component of the biological process of (pre) decidualization.[16]

In addition to the evidence provided by bioinformatics approaches linking the DEG down-regulated in CVS from women who developed preeclampsia to deficient endometrial maturation, we pursued a different tack to marshal further evidence associating these down-regulated DEG to inadequate predecidualization and decidualization. To this end, we found that 31 of the 195 down-regulated DEG had been previously linked to decidualization/decidua in the literature (**Figure S3**; **Tables S2B** and **S5**). Thus, the systematic literature search further strengthens the argument that endometrial maturation is inadequate in the women destined to develop preeclampsia.

Aberrant endometrial maturation has been previously linked to infertility,[19] and recurrent pregnancy loss.[18] Women with polycystic ovary syndrome (PCOS) have infertility and increased miscarriage rates. One potential explanation is that PCOS is associated with defective endometrial function possibly mediated through insulin resistance, hyperinsulinemia and androgen excess.[33] Dehydroepisandrosterone,

which is elevated in PCOS, has been shown to inhibit the pentose phosphate pathway, thereby impairing decidualization.[34] By analogy, one might predict that defective (pre)decidualization could also predispose to other adverse pregnancy outcomes, including placental syndromes, such as preeclampsia. In fact, women with PCOS and insulin resistance are at increased risk of developing preeclampsia.[35,36] However, direct evidence to support this linkage is more difficult to obtain in the case of preeclampsia because we do not know in early pregnancy who will develop disease, first trimester chorionic and decidual tissue is not easily accessible for investigation, and molecular interrogation of delivered placenta, basal plate decidua and cells derived thereof, cannot discern between causes or consequences of the disease. The latter is highlighted by further bioinformatics analysis (data not shown), which revealed only 5 DEG in common between the 396 DEG in CVS from preeclamptic women relative to normal pregnant women and 457 DEG in decidua basalis of third trimester placentas obtained from preeclampsia compared with normal pregnant women (E-TABM-682[37]).

Nevertheless, recent evidence is consistent with the concept of endometrial antecedents of preeclampsia. First, women with high uterine vascular resistance as determined by ultrasound before elective termination in early pregnancy demonstrated impairment of dNK cell function, which is considered to be critical for spiral artery remodeling. However, pregnancy termination obviously precluded knowledge of pregnancy outcome in this study. Second, trophoblast isolated from placentas of severe preeclamptic women demonstrated increased expression of *SEMA3B*, a cytokine that impairs trophoblast invasion in vitro. After 48 hours in culture, *SEMA3B* expression spontaneously returned to normal levels suggesting that the in vivo milieu was responsible for the elevation.[38] Although consistent with the idea that defective decidualization may perturb EVT *SEMA3B* expression during early pregnancy in women destined to develop preeclampsia, once again, this interpretation is problematic because trophoblasts were isolated from delivered placentas. As such, it is not possible to discern whether the reported findings caused or resulted from the disease. Finally, women with endometriosis may have decreased risk of preeclampsia,[39] although not all agree.[40,41]

POTENTIAL STUDY LIMITATIONS

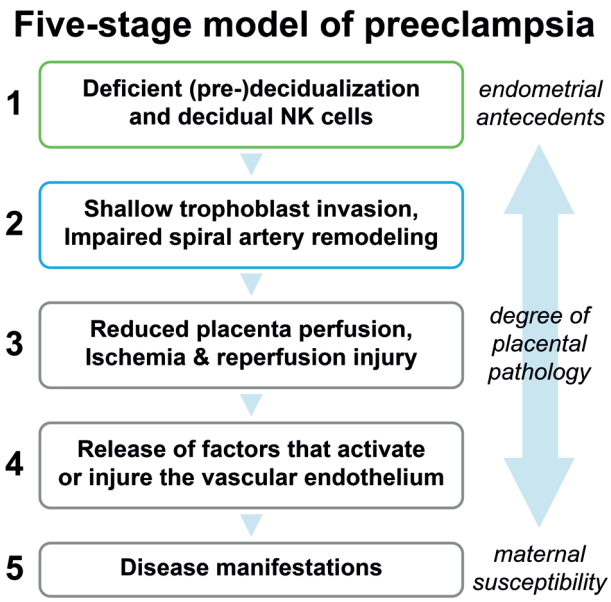
The validity of our conclusions mainly rests on the reliability of the original investigations, which generated the DNA microarray data sets analyzed herein. Whenever possible, we built-in redundancy or overlap by incorporating more than one data set, e.g., GSE4888[28] and GSE6364[24] for endometrial gene expression in the menstrual cycle. Similarly, we tested the potential contribution of extravillous trophoblast using 3 approaches: (1) analysis of LSE obviously devoid of EVT

(GSE4888[28] and GSE6364[24]), (2) comparison of endometrium from IUP and EP matched for the degree of decidualization with and without EVT influence, respectively (E-MTAB-680[25]), and (3) incubation of endometrial stromal cells decidualized in culture with trophoblast conditioned medium [GSE5809 (GEO database) or E-GEOD-5809 (EMBL-EBI database)[32]].

To our knowledge, our genome-wide gene expression study on chorionic villous sampling of women who developed preeclampsia is the only one available study in the public domain (GSE1276711). Surplus villi were snap frozen within 10 minutes of extraction from women undergoing CVS for prenatal genetic screening. The tremendous labor and time involved in CVS collection and the rigorous inclusion criteria affected our sample size; however, this potential limitation may be at least partially offset by the validation methods of class predictions through cross-validation (leave-one-out cross-validation) and by the rigorous statistical methods specifically designed to mitigate the potential limitation of small sample size frequently encountered in microarray studies by scarcity of tissue used (Materials and Methods in the Supporting Information).[42]

Other potential limitations to the study of CVS are detailed in the original publication.[11] However, to date, CVS is the only approach to obtain first trimester chorionic tissue and decidua in women with known pregnancy outcome. Therefore, the specimens for this study provided a rare glimpse into the transcriptomics of early placenta in women who developed severe preeclampsia. Moreover, CVS is decreasing dramatically in the United States because of the emerging practice of noninvasive prenatal screening.

We cannot exclude the possibility of less decidua in CVS from the preeclamptic women compared with normal pregnant women as an etiological factor in the disease, ie, a quantitative difference rather than a qualitative difference or both; conceivably, this deficiency could also compromise placentation. An assumption built into this work is that extravillous trophoblast invasion and spiral artery remodeling depend on normal decidualization and dNK function (vide supra). Although, this linkage is not proven, there is growing evidence supporting the concept.[10,21–23] Moreover, because histiotrophic nutrition of the placenta and fetus in early pregnancy depends on healthy, optimally decidualized endometrial gland epithelium,[43] it seems possible that this physiological process could also have been compromised in the women who developed preeclampsia. As a final cautionary note, preeclampsia is likely to be a disease of heterogenous etiology; consequently, the evidence revealed herein of inadequate or defective (pre)decidualization may only pertain to a subset of women who develop the disease.



← **Figure 6**
Five-stage model of placental preeclampsia. Based on a bioinformatics approach, the findings of this study raise the possibility that impaired endometrial maturation and deficient decidual natural killer (NK) cell number and function in the secretory phase (predecidualization) and during early pregnancy (decidualization) precede the development of preeclampsia. As (pre)decidualization and associated decidual NK cell function are emerging as important players in successful placentation, perturbation of these biological processes may contribute to the etiology of preeclampsia at least in a subset of women who develop the disease. Preeclampsia may arise in some women with little or no endometrial or placental pathology.

PERSPECTIVES

A bioinformatics approach implicates deficient or defective (pre)decidualization and decidual NK cells in the secretory phase and early pregnancy in women who developed severe preeclampsia (Figure 6). Maternal constitutional factors, such as PCOS, obesity, diabetes mellitus, and poor cardiovascular health etcetera, may compromise endometrial maturation before and during early pregnancy, thereby predisposing to preeclampsia. Conceivably, aberrant endometrial gene expression could inform targeted investigation and discovery of protein biomarkers in blood, urine or uterine fluid for women at increased risk of preeclampsia even before conception. Ultimately, designing interventions that improve endometrial maturation to facilitate normal placentation and reduce preeclampsia risk might be a logical therapeutic course of action. At the least, the present study should motivate further inquiry into the concept that deficiency or defects in (pre)decidualization and uterine NK cells antedate preeclampsia.

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DISCLOSURES

None.

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CHAPTER 4
SUPPORTING INFORMATION (SI)

SI MATERIALS AND METHODS

MICROARRAY DATASETS

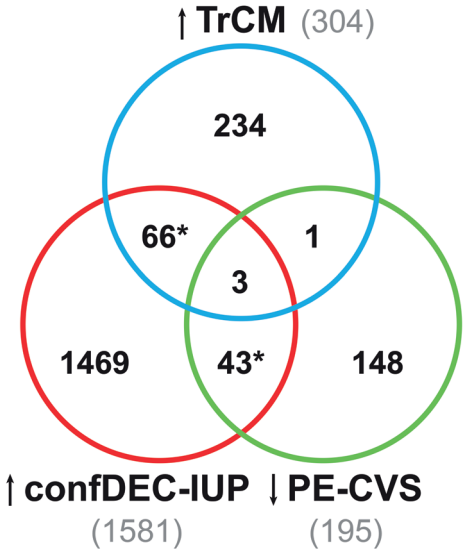
Microarray dataset searches were performed in two public functional genomics data repositories: Gene Expression Omnibus (GEO) from the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/geo/) and the European Bioinformatics Institute from the European Molecular Biology Laboratory (EMBL-EBI) (http://www.ebi.ac.uk/arrayexpress/). Both data repositories support MIAME-compliant data submissions.

An important pre-condition for microarray dataset searches was the selection of microarray data in which RNA was hybridized to the Affymetrix Human Genome U133 Plus 2.0 Array (GPL570 for GEO; A-AFFY-44 for EMBL-EBI), the same platform used for the interrogation of CVS from PE and NP women (GSE12767[1]). This pre-condition is necessary because it enables the direct comparison of the microarray data in the present work.

To search for pre-decidualization data, the Key words entered were "endometrium" AND "menstrual cycle" AND GPL570 (or A-AFFY-44). Two datasets were selected from the GEO database: GSE4888[2] and GSE6364[3]. The dataset GSE4888 consisted of 27 samples obtained from women with normal ovulatory cycles. Twenty-one had histologic phenotypes of proliferative (PrE; n=4), early-secretory (ESE; n=3), mid-secretory (MSE; n=8) or late-secretory (LSE; n=6) endometrium, while 6 had ambiguous histological reading. The dataset GSE6364 consisted of 37 endometrial biopsies obtained from women without pathology (n=16) or diagnosed with some degree of endometriosis (n=21). Biopsy samples of the former were from PrE (n=5), ESE (n=3), and MSE (n=8). The 21 and 16 normal endometrial samples from GSE4888 and GSE6364, respectively, were pooled. The LSE phase was only represented by the 6 samples from GSE4888. To maintain equal number of replicates per stage of the endometrial cycle, 3 samples were randomly selected from each dataset for PrE, ESE and MSE. Thus, each of the 4 menstrual cycle phases was comprised of 6 endometrial samples (n=24 total).

To search for decidualization data, the Key words employed were "endometrium" AND "decidualization" AND GPL570 (or A-AFFY-44). One dataset was selected from EMBL-EBI database: E-MTAB-680.[4] This dataset consisted of 24 endometrial samples collected at approximately 59 days of gestation. Of these, 13 were obtained from intrauterine pregnancies (IUP) and 11 from ectopic tubal pregnancies (EP). As

Figure S1 →
Lack of significant association (n=4 DEG, *P*=0.5) between differentially expressed genes (DEG) down-regulated in PE-CVS and DEG upregulated by exposure of decidualized stromal cells in culture to trophoblast conditioned medium (TrCM).



reported by the authors, these samples presented different degrees of endometrial decidualization as assessed by morphology in H&E stained sections. The IUP samples were classified as confluent-decidualization (confDEC-IUP, n=7) or intermediate-decidualization (intDEC-IUP, n=6), while the EP endometrial samples were intermediate-decidualization (intDEC-EP, n=6) or without decidualization changes (nonDEC, n=5). The presence or absence of trophoblast was determined by cytokeratin staining.

The keyword employed to evaluate the trophoblast influence on the decidualization process were "trophoblast" AND "decidualization" AND "endometrium" AND GPL570 (or A-AFFY-44). One dataset met the search criteria GSE5809 (GEO database) or E-GEOD-5809 (EMBL-EBI database).[5] Human endometrial stromal cells were decidualized in culture or left untreated serving as a control. The decidualized and non-decidualized cells were then incubated with conditioned media from human trophoblast (TrCM) for 0 (n=3), 3 (n=6) and 12 (n=5) hours. Cytotrophoblasts were isolated from placentae obtained after elective pregnancy termination (6-22 gestational weeks), and they were cultured on Matrigel-coated matrix for 48 hours before harvesting of the conditioned media.

To approximate DEG upregulated in NK cells during the pre-decidualization or decidualization process, we wanted to compare gene expression between decidual (d)NK or endometrial (e)NK cells and peripheral blood (pb)NK cells. To this end, Koopman et al. generously provided the microarray datasets performed on dNK

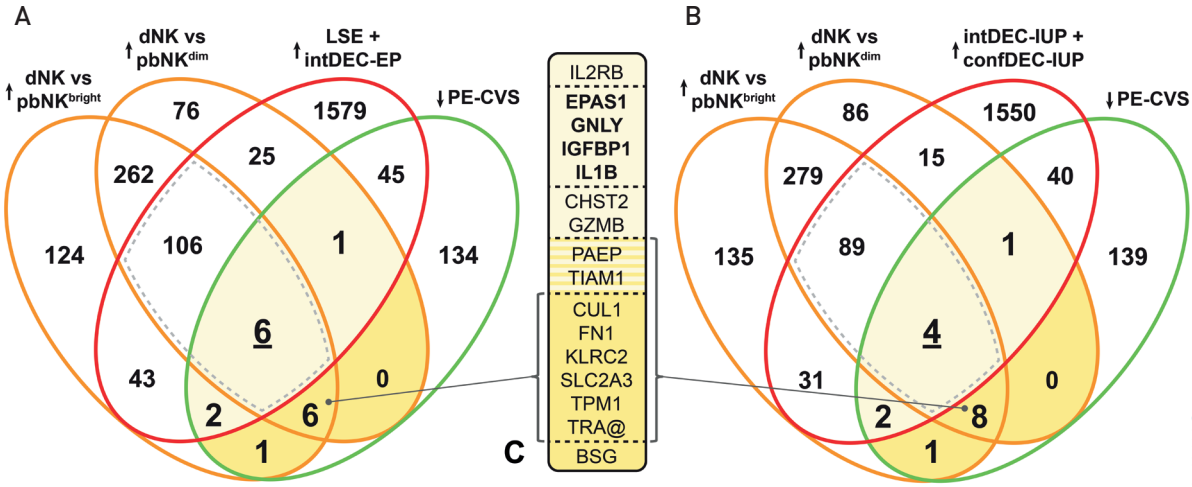


Figure S2 ↑
Clusters of genes induced in decidual natural killer cells are down-regulated in CVS from preeclamptic women. In (**A**) 112 differentially expressed genes (DEG) upregulated in decidual natural killer cells (dNK; relative to peripheral blood $CD56^{dim}$ or $CD56^{bright}$ NK cells), late-secretory endometrium (LSE, relative to proliferative endometrium) and ectopic pregnancy (EP) endometrium with intermediate-decidualization changes (intDEC; relative to EP endometrium without decidualization changes) are overlapping (highlighted by dotted line). In (**B**) 93 DEG upregulated in dNK, and intra-uterine pregnancy (IUP) endometrium with intermediate- and confluent-decidualization (intDEC and confDEC) changes are overlapping (highlighted by dotted line). Seventy-four of the 112 and 93 DEG are in common (P<0.0001, Table S6). In (**A**) and (**B**) (yellow shading), 16 DEG upregulated in dNK are down-regulated in PE-CVS (P<0.0001). The gene symbols of the 16 DEG are listed in panel (**C**).

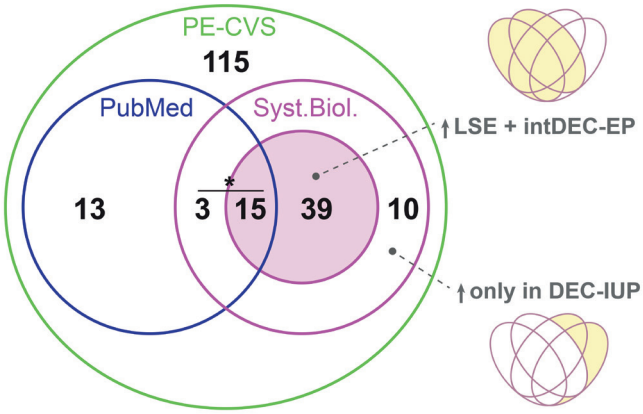


Figure S3 ↑
Systematic literature search of decidual genes. Because there is no "biological process of decidualization" available in public bioinformatic databases for pathway analysis, a systematic literature search was conducted to identify genes, which have previously been associated with decidua or the biological process of decidualization. A literature search of all 195 down-regulated differentially expressed genes (DEG) in PE-CVS revealed 31 previously identified to be related to decidua or decidualization (Table S2B). Of these 31 genes, 18 (3+15) genes were also identified by the systems biology approach (*P=0.001). The majority (15, P=0.03) is upregulated in late-secretory endometrium (LSE, relative to proliferative endometrium) or in ectopic pregnancy (EP) endometrium with intermediate-decidualized changes (intDEC, relative to EP endometrium without decidualization changes), in which extravillous trophoblast influence is absent.

cells (n=9) and pbNK cells (n=10) cells.[6] In this study, decidual samples were collected from pregnant woman between 6 to 12 weeks of gestation after elective termination, and dNK cells were isolated by fluorescence-activated cell sorting. The same technique was employed to isolate pbNK cells from peripheral blood mononuclear cells of healthy donors (n=5 $CD56^{bright}$ pbNK and n=5 $CD56^{dim}$ pbNK). The isolated RNA was amplified, labeled and hybridized to the Affymetrix Human Genome U95 Version 2 Array (GPL8300). This platform is not the same as the others employed for the datasets described above. However, results from the analysis of this dataset are comparable, insofar as data imputation, normalization and transformation are the same.

SI DATA ANALYSIS

DATA INPUT

The Bioconductor software (http://www.bioconductor.org) for the R software environment (http://www.r-project.org) was employed for all the analyses. The gcRMA package[7] was employed to import the raw data into R, perform background correction, as well as normalize and summarize the data. Then, rows of each data set were collapsed, in order to retain the microarray probe with the highest mean value (Max mean) from the group of the genes with the same official symbol. The function applied was the "collapseRows" from the WGCNA package.[8] The purpose of row collapsing is to obtain unique identifiers for each gene in the working data set. Thus, from the original platform GPL570, containing 54675 probes, 21049 probes belonging to unique genes were retained for further analysis. For the platform GPL8300 employed in the NK cell dataset, 9127 probes related to unique genes were retained from 12625 probes.

STATISTICAL ANALYSIS

Statistical analysis of microarray data is challenging, because often there are very few replicates per gene and thousands of genes in an experiment, which can result in high false positive (Type I error) and false negative (Type II error) rates[9] with multiple comparisons.[10] Thus, several authors adapted or developed statistical methods to overcome this potential deficiency in microarray data analysis. In this work, we applied well-known and proven statistical methods to identify DEG in the datasets. For two group comparisons, we employed moderated t-statistics, a variation of the classical t-test, developed by Smyth.[11] This algorithm has robust behavior even for small numbers of arrays. Also, compared to classical tests, the method results in more stable inference when the number of arrays is limited. For the time course experiments, we applied the Bayesian estimation of temporal

regulation method, developed by Aryee et al.[12] This algorithm uses the time dependent structure of the data and employs an empirical Bayes procedure to stabilize estimates derived from the small sample sizes. This method is also suitable for cross sectional time course experiments with one or more conditions, comparable to the datasets analyzed in this report. Thus, by choosing the right statistical methods for the analysis of microarray data, the potential limitation of small sample size frequently encountered in microarray studies by scarcity of tissue, is mitigated.

LSE (or predecidualization)

Data from biopsy samples in GSE4888 and GSE6364 (n=24) were analyzed using time as an ordinal variable. The Bayesian Estimation of Temporal Regulation (BETR)[12] algorithm was used to identify the DEG at a False Discovery Rate (FDR) of <0.05. The first phase of the endometrial cycle (PrE) was considered as the baseline measurement and was compared to subsequent stages of the endometrial cycle, in order to correlate the differential expression among the various stages. This method, which is applied with the BETR package, provides the probabilities of differential expression for each gene in the data set. Genes with a probability higher than 99.99% were considered as differentially expressed genes (DEG).

Next, DEG selected by the BETR analysis were subjected to a supervised weighted gene co-expression network analysis employing the WGCNA package. The automatic method was employed for block-wise network construction and module detection. The co-expression similarity was raised to a soft thresholding power (β) of 12 to calculate adjacency. The adjacency for the signed network is defined as $a_{ij}=|(1+cor(xi,xj))/2|^{\beta}$.[13] The resulting modules for each network were related to the phase of the endometrial cycle in order to identify modules or clusters of co-expressed genes showing increasing expression pattern with progression through the endometrial cycle and peaking in the late secretory phase. Gene significance (GS) was defined as the correlation of i-th gene with a temporal pattern. Module membership (MM) was defined as the correlation of the i-th gene with respect to its corresponding module (the higher the MM the more connected is the i-th gene with the other genes of the corresponding modules). The correlation coefficient of MM and GS was measured for each module, plotting MM versus GS. Higher correlation between MM and GS indicates that genes that are highly associated in a temporal pattern are also the central elements of a given module.[13] The module with the highest positive correlation between MM and GS was selected for further comparison with DEG down-regulated in PE- vs. NP-CVS.

Decidualization

Data from the intDEC-EP (n=6), intDEC-IUP (n=6) and confDEC-IUP (n=7) endometrium were compared to nonDEC endometrium (n=5) from EP, all from the E-MTAB-680 database, to determine DEG upregulated during the biological process of decidualization. The limma package was used for the statistical analysis, applying the empirical Bayes method proposed by Smyth.[11] This method calculates a moderated t-statistic for differential expression of each gene by performing a linear model fit of the data. Then, an empirical Bayes step is applied to moderate the standard errors of the estimated log-fold changes in order to produce more stable estimates, especially when the number of replicates is small. A gene was considered to be significantly differentially expressed, if both of the following conditions were met: 1) the ratio of the normalized intensity of the intermediate- or confluent-decidualization to normalized intensity of the non-decidualized endometrial samples was higher than a 2-fold change; and 2) differences were considered statistically significant at $P \leq 0.05$.

Potential influence of extravillous trophoblast

Data from cultured endometrial stromal cells in GSE5809 (n=14) were analyzed over time (0, 3 and 12 hours incubation with TrCM) and by two conditions (decidualized and non-decidualized). The BETR algorithm[12] was used to identify DEG between decidualized endometrial cells treated with TrCM and non-decidualized endometrial stromal cells treated with TrCM at a FDR <0.05 as a function of TrCM incubation time (0, 3 and 12 hours). This method yields the probability of differential expression for each gene in the data set. Genes with a probability of 99.9% were considered as DEG. Co-expressed genes as determined by WGCNA (see above) increasing in expression by 12 hours of incubation with TrCM were selected for further comparison with DEG down-regulated in CVS from PE women relative to CVS from NP women.

Decidual NK cells

Data from dNK (n=9) were compared to $CD56^{dim}$ pbNK (n=5) or $CD56^{bright}$ pbNK (n=5) by the empirical Bayes method[11] as described above. A gene was considered to be significantly differentially expressed if both of the following conditions were met: (1) the ratio of the normalized intensity of the dNK to normalized intensity of the pbNK samples was higher than a 2-fold change; and (2) differences were considered statistically significant at $P \leq 0.05$.

Chorionic villous samples

Microarray data of CVS from PE (n=4) and NP (n=8) women in the dataset GSE12767 were compared by the empirical Bayes method.[11] The DEG were considered if both

of the following conditions were met: (1) the ratio of normalized intensity in PE-CVS to normalized intensity in NP-CVS samples exceeded a 1.5-fold change; and (2) differences were considered statistically significant at $P \leq 0.05$. To expand the number of genes, down-regulated DEG determined by J_5 and FC analysis were also included from Founds and coworkers (see **Table 2** and **Table S1** in [1]).

The results of the J_5 analysis were taken from our original publication[1], those from t-test were obtained by re-analyzing the original Affymetrix data GSE12767, and FC data stemmed from both the original (< or > 2.0) and re-analysis (< or > 1.5).

CLASS PREDICTION

In order to evaluate the performance of the selected DEG in each dataset, we performed class prediction applying the k-Nearest Neighbors (kNN) algorithm for classification and the K-fold cross validation method as classifier. The methodology was performed with the RWeka package for R.[14] Specifically, after gene selection by the corresponding statistical method, we evaluated if each sample for that dataset would be able to predict to which class it belongs according the Euclidean distance to its kNN. For this, the K-folds number was set to the n samples for each dataset, known as leave-one-out cross validation (LOOCV). The k number for KNN was set as n_i-1, for n_i being the number of samples in the class of interest. The corresponding K-fold and k numbers, and the number of correct classifications for each dataset are shown below:

Dataset	LSE	intDEC-EP	intDEC-IUP	confDEC-IUP	PE-CVS	dNK
Classes	PrE, ESE, MSE, LSE (n=24)	intDEC-EP, NonDEC (n=11)	intDEC-IUP, NonDEC (n=11)	confDEC-IUP, NonDEC (n=13)	PE-CVS, NP-CVS (n=8)	dNK, pbNK (n=19)
Class of interest	LSE (n_i = 6)	intDEC-EP (n_i = 6)	intDEC-IUP (n_i = 6)	intDEC-IUP (n_i = 7)	PE-CSV (n_i = 4)	dNK (n_i = 9)
K-fold	24	11	11	13	12	17
kNN	5	5	5	6	3	9
Number of correct classifications	24 (100%)	11 (100%)	11 (100%)	13 (100%)	12 (100%)	13 (100%)

Data comparison

The DEG down-regulated in CVS from PE relative to NP women were compared to: (i) the cluster of co-expressed endometrial genes increasing expression by the late-secretory phase of the menstrual cycle (predecidualization); (ii) DEG upregulated in intermediate decidualization endometrium from IUP or EP with and without extravillous trophoblast, respectively, and confluent decidualization from IUP; (iii) the cluster of co-expressed genes increasing expression in decidualized endometrial stromal cells in culture after 12 hours of incubation with TrCM; and (iv) DEG upregulated in decidual relative to peripheral blood NK cells. Statistical comparisons were made by the test of independence (Pearson's chi-square test) to determine the relatedness between down-regulated DEG in CVS from PE relative to NP women, and upregulated DEG in LSE, intermediate decidualized (IUP or EP) and confluent decidualized endometrium (IUP), decidualized stromal cells in culture treated with TrCM, and decidual NK cells.

SYSTEMATIC LITERATURE SEARCH

Systematic review of the literature was undertaken on June 1, 2014 by electronic searches in Medline through PubMed without language or publication date restrictions. The goal was to identify all publications related to decidualization that also reported one or more of the DEG *down-regulated* in CVS from PE compared to NP women (195 down-regulated DEG). To enable identification of all relevant publications, Human Genome Organization (HUGO) approved gene symbols were searched, as well as previous symbols and synonyms as listed by HUGO (http://www.genenames.org/cgi-bin/hgnc_downloads). The electronic search strategy was based on the Medical Subject Heading (MeSH) for each gene name, and when applicable, combined with title/abstract searches with all gene symbol synonyms. Synonyms that were not specific for a gene and generated too many irrelevant abstracts were omitted from the search string. By the use of Boolean operators individual gene searches (n=195) were combined with a search strategy identifying titles/abstracts related to "decidua/decidualization" based on the MeSH "decidualization" or a title/abstract search for "decidualization*". Retrieved references reporting DEG(s) down-regulated in PE-CVS and "decidua/decidualization" in the title or abstract were selected by two reviewers (EPU and KPC) who independently scrutinized the titles and abstracts. Full-text articles of any ambiguous references were selected by one reviewer (EPU) and further scrutinized by two reviewers (EPU and KPC) to determine whether there was a clear relationship between the DEG(s) down-regulated in PE-CVS with decidua/decidualization. As a reference we included the PubMed identifier (PMID) of one of the most relevant publications for each gene related to decidualization (**Table S2B**). For all the DEG

down-regulated in CVS from PE relative to NP women (n= 195), we applied the test of independence (Pearson's chi-square test) to determine the relatedness between DEG identified by the system biology approach (n= 67, **Figure 3**), and genes identified by the literature search in Pubmed (n=31).

SI RESULTS

DIFFERENTIALLY EXPRESSED GENES DOWN-REGULATED IN CVS FROM PREECLAMPTIC WOMEN ARE NOT UPREGULATED IN DECIDUALIZED ENDOMETRIAL STROMAL CELLS BY TROPHOBLAST CONDITIONED MEDIUM (TRCM)

Differentially expressed genes down-regulated in CVS from preeclamptic women are not upregulated in decidualized endometrial stromal cells by trophoblast conditioned medium (TrCM)

DEG down-regulated in PE-CVS were compared to genes induced by TrCM in cultured endometrial stromal cells decidualized *in vitro*. TrCM was obtained from cytotrophoblasts isolated from placentae between 6 and 22 weeks of gestation after elective termination and cultured on Matrigel-coated substrate for 48 hours (GSE5809[5]). As expected, there was significant overlap between the cluster of 304 DEG increasing expression in decidualized endometrial cells incubated with TrCM and 1581 DEG upregulated in confluent-decidualized endometrium from IUP containing EVT (69 DEG, $P<0.0001$; **Figure S1**). There was also significant overlap of DEG down-regulated in CVS from PE compared to NP women with DEG upregulated in confluent-decidualized IUP endometrium (46 DEG, $P<0.0001$; **Figure S1**). However, there was no significant intersection of endometrial genes increasing in expression after treatment with TrCM with DEG down-regulated in PE-CVS with only 4 in common (**Figure S1**, p=0.5). These results further support the idea that impaired decidualization in the women who developed PE may be mostly independent of trophoblast influence.

DIFFERENTIALLY EXPRESSED GENES DOWN-REGULATED IN CVS FROM PREECLAMPTIC WOMEN ARE UPREGULATED IN DECIDUAL NK CELLS

In contrast to published gene expression of peripheral blood (pb) and endometrial NK cells derived from different microarray platforms[6,15], we were able to compare gene expression between dNK and $CD56^{dim}$ pbNK or $CD56^{bright}$ pbNK cells[6], because the same microarray platform was employed. As expected, there was a large confluence of 380 DEG upregulated in dNK relative to $CD56^{dim}$ and $CD56^{bright}$ pbNK cells ($P<0.000001$, **Figure S2**). There was also high overlap (112 DEG;

demarcated by dotted line in **Figure S2A**) between DEG upregulated in dNK relative to $CD56^{dim}$ and $CD56^{bright}$ pbNK cells, and DEG upregulated in LSE (relative to proliferative endometrium) plus intermediate-decidualized endometrium from EP (relative to non-decidualized endometrium) in the *absence* of EVT influence ($P<0.00001$); and a high number of overlapping DEG (93 DEG; demarcated by dotted line in **Figure S2B**) upregulated in dNK relative to $CD56^{dim}$ and $CD56^{bright}$ pbNK cells, and intermediate- plus confluent-decidualized endometrium from IUP (relative to non-decidualized endometrium) in the *presence* of EVT influence ($P<0.00001$). The majority of these 112 and 93 DEG were the same (74 DEG, $P<0.00001$, **Table S6**) suggesting little contribution of EVT to the overlap. Of particular note, 16 DEG upregulated in dNK relative to $CD56^{dim}$ and $CD56^{bright}$ pbNK cells were down-regulated in CVS from PE relative to NP women ($P<0.0001$; **Figure S2A-B** and **Figure 5**). Further, there was a significant overlap of 14 differentially expressed genes between DEG upregulated in pbNK relative to dNK cells and DEG upregulated in PE-CVS compared to NP-CVS ($P<0.001$; AOAH, ARFGEF2, CCL_3, DDX_3Y, DGKD, DHX_{30}, GPR_{183}, ISG_{20}, $RFTN_1$, RPGR, RPS_4Y_1, $SCML_2$, SRF, ZNF_{101}). This finding suggests that, if dNK cells wholly or partly derive from differentiation of pbNK cells, this process may not be optimal in the women who developed preeclampsia, because non-decidualized endometrium lacks the cues necessary for dNK differentiation.

SYSTEMATIC LITERATURE SEARCH

Because the biological process of "decidualization" is not available in public bioinformatic databases for pathway analysis, we conducted a systematic and comprehensive literature search of all 195 DEG down-regulated in CVS from PE compared to NP women. Thirty-one were previously associated with decidualization/decidua in the literature (**Figure S3**, **Table S2B** and **Table S5**). We also evaluated the overlap of these 31 DEG identified by literature search with the overlapping DEG determined by systems biology approach, i.e., those down-regulated in PE-CVS and upregulated in: LSE (38 DEG; **Figure 1A** in the text), intermediate-decidualized endometrium from EP (32 DEG; **Figure 1B**) and IUP (37 DEG, **Figure 2A**) and confluent-decidualized endometrium from IUP (46 DEG; **Figure 2B**), all together yielding 67 unique genes (refer to **Figure 3**). We found that 18 of the 31 DEG identified in the literature were in common with 67 DEG identified by systems biology ($P=0.001$). The majority (15 of 18, $P=0.03$) was upregulated in LSE or intermediate- decidualized endometrium from EP in which EVT influence is absent.

ONLINE SUPPORTING INFORMATION

The Supporting Tables S1-S6 are available online only: http://hyper.ahajournals.org/lookup/suppl/doi:10.1161/HYPERTENSIONAHA.114.04481/-/DC1.

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Part 2

The hormone relaxin and its receptor RXFP1 in pregnancy


RXF P1

Chapter 5

LOCALIZATION OF RELAXIN RECEPTORS IN ARTERIES AND VEINS, AND REGION-SPECIFIC INCREASES IN COMPLIANCE AND BRADYKININ-MEDIATED RELAXATION AFTER IN VIVO SERELAXIN TREATMENT

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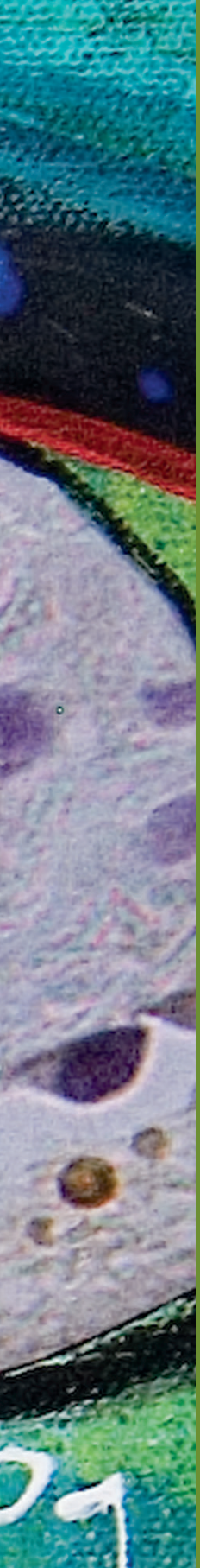

ABSTRACT

Relaxin is a potent vasodilator of small resistance arteries and modifies arterial compliance in some systemic vascular beds, yet receptors for relaxin, such as RXFP1, have only been localized to vascular smooth muscle. This study first aimed to localize RXFP1 in rat arteries and veins from different organ beds and determine whether receptors are present in endothelial cells. We then tested the hypothesis that region-specific vascular effects of relaxin may be influenced by the cellular localization of RXFP1 within different blood vessels. The aorta, vena cava, mesenteric artery, and vein had significantly higher ($P<0.05$) RXFP1 immunostaining in endothelial cells compared with vascular smooth muscle, whereas the femoral artery and vein and small pulmonary arteries had higher ($P<0.01$) RXFP1 immunostaining in the vascular smooth muscle. Male rats were treated subcutaneously with recombinant human relaxin-2 (serelaxin; 4 μg/h) for 5 d; vasodilation and compliance in mesenteric and femoral arteries and veins were compared with placebo controls. Serelaxin significantly ($P=0.04$) reduced wall stiffness and increased volume compliance in mesenteric arteries but not in the other vessels examined. This was associated with changes in geometrical properties, and not compositional changes in the extracellular matrix. Serelaxin treatment had no effect on acetylcholine-mediated relaxation but significantly ($P<0.001$) enhanced bradykinin (BK)-mediated relaxation in mesenteric arteries, involving enhanced nitric oxide but not endothelium-derived hyperpolarization or vasodilatory prostanoids. In conclusion, there is differential distribution of RXFP1 on endothelial and smooth muscle across the vasculature. In rats, mesenteric arteries exhibit the greatest functional response to chronic serelaxin treatment.

Key words: RXFP1 • vasodilation

INTRODUCTION

It is well established that the peptide hormone relaxin plays an important role in the profound maternal vascular adaptations that occur in pregnancy, specifically targeting the renal and uterine vasculature.[1–3] Beneficial cardiovascular effects of relaxin, which are attributed to alterations in the renal and systemic vasculature, have also been demonstrated in congestive and acute heart failure patients in preclinical studies and phase II/III clinical trials.[4–6] In conscious normotensive and hypertensive male and female rats, acute intravenous and chronic subcutaneous administration of relaxin increases cardiac output and global arterial compliance and reduces systemic vascular resistance, without affecting mean arterial pressure.[7,8] Relaxin has also been shown to reduce mean arterial pressure in rat models of hypertension[9,10] and increase coronary flow in rat and guinea pig hearts.[11]

These beneficial cardiovascular effects of relaxin in normotensive rats occur in parallel with increases in renal plasma flow and glomerular filtration rate,[12] and are underpinned by a reduction in myogenic constriction of the small renal arteries.[13] Vasodilatory responses to *in vivo* relaxin treatment have also been demonstrated in rat mesenteric arteries,[13-15] whereas *ex vivo* relaxin treatment induces vasodilation in isolated rodent small renal and mesenteric arteries[16-18] and human small gluteal and subcutaneous arteries but not pulmonary resistance arteries.[18-19] Only one study to date has assessed veins; incubation of mesenteric veins with relaxin causes relaxation but does not affect myogenic reactivity.[17] In small resistance arteries, vascular tone is modulated by endothelium-derived factors, including nitric oxide (NO), prostacyclin (PGI_2), and non-NO/PGI_2 endothelium-derived hyperpolarization (EDH).[20] Relaxin appears to act through an endothelium-dependent NO-mediated vasodilator pathway.[13-15] In small renal arteries, rapid relaxin-induced vasodilation involves phosphatidylinositol-3-kinase (PI3K; ref. 18). Relaxin also directly stimulates NO production from cultured human coronary artery and aortic endothelial cells, with endothelial NO synthase (eNOS) phosphorylation *via* PI3K activation and phosphorylation of protein kinase B (Akt; Ser473).[18] Currently, it is not known whether PGI_2 and EDH contribute to the vasodilator effects of relaxin.

The vascular actions of relaxin extend to modification of passive wall compliance. Chronic subcutaneous relaxin infusion in rats and mice increases arterial compliance in small renal,[7,21] mesenteric,[17] uterine,[22] brain parenchyma,[23,24] and carotid arteries.[25] In mice administered relaxin, the relative increase in small renal artery compliance is mediated by both geometric and compositional (decreased collagen) remodeling.[21] Specifically, there is an increase in unpressurized wall area and wall-thickness-to-lumen area ratio, increased smooth muscle cell density and a decrease in collagen-to-total-protein. Relaxin infusion in nonpregnant rats also

increases wall thickness and inner diameter of brain parenchymal arterioles.[23] The effects of relaxin on arterial compliance appear to be region specific, as relaxin treatment has no effect on the external iliac[21] and middle cerebral arteries,[23] or mesenteric veins.[17]

One explanation for the region-specific vascular effects of relaxin might be differential expression of relaxin/insulin-like family peptide receptor 1 (RXFP1) in arteries and veins and/or between endothelial and smooth muscle cells. RXFP1 gene and protein are expressed in the thoracic aorta and small renal and mesenteric arteries, but not in the cerebral vasculature, of male and female rodents,[14,24,26,27] but these studies did not localize RXFP1 within the artery wall. We recently colocalized RXFP1 with α-smooth muscle actin (α-SMA) muscle-positive cells in the media of uterine arteries,[3] demonstrating expression of relaxin receptors in the vascular smooth muscle. Localization of RXFP1 in endothelial cells is less convincing, despite evidence of a direct action of relaxin on aortic and coronary artery endothelial cells to evoke NO release.[18,28] In summary, the specific localization of RXFP1 in vascular smooth muscle cells is consistent with the hypothesis that relaxin acts directly on blood vessels to influence arterial remodeling and compliance, but RXFP1 is yet to be localized to endothelial cells in intact arteries or veins.

Therefore, in this study we tested the hypothesis that region-specific vascular effects of relaxin may be influenced by the distribution of RXFP1 within different blood vessels in males. The main objectives of this study were to localize RXFP1 in a variety of arteries and veins from different organ beds and determine whether receptors are present in smooth muscle or endothelial cells; and compare the effects of chronic subcutaneous *in vivo* relaxin treatment on specific vasodilator pathways and passive mechanical wall properties in mesenteric and femoral arteries and veins.

MATERIALS AND METHODS

ANIMAL PREPARATION

All experimental protocols were approved by The University of Melbourne Faculty of Science Animal Experimentation Ethics Committee. Male Wistar rats aged 12–14 wk (~300 g body weight) were purchased from Monash Animal Services (Clayton, VIC, Australia). Male rats were used in all experiments because nonpregnant females produce estrogen from the ovaries, which could confound the experimental outcomes. Rats were housed in the Animal Facility (Department of Zoology, The University of Melbourne, VIC, Australia) on a 12/12-h light-dark cycle and had access to standard rat chow (Specialty Feed, Glen Forrest, WA, Australia) and water *ad libitum*.

EXPERIMENTAL PROTOCOLS

Localization of RXFP1 in arteries and veins

Rats were anesthetized with isofluorane and euthanized by cervical dislocation. Thoracic and abdominal aortae, pulmonary, mesenteric, femoral arteries, and mesenteric and femoral veins were dissected from each animal (n=3–4). Blood vessels were then placed in ice-cold 0.1 M phosphate buffered saline (PBS), and excess fat and loose connective tissue were removed before fixing in 10% neutral buffered formalin (NBF). The kidneys were dissected, sectioned bilaterally, and fixed in PLP fixative (8% paraformaldehyde, 0.2 M lysine, 0.01 M periodate in 0.1 M PBS) for 24 h, and then transferred to 0.1 M PBS.

Human mesenteric artery segments were also obtained from 3 male patients, ages 58, 60, and 64. None were smokers, had diabetes, or were on medications that influenced vascular function; all had normal blood pressure and were undergoing cancer-related surgery. Immediately after surgery, the mesenteric arcade was excised from noncancerous gut and cleaned of fat and loose connective tissue. The arteries were washed in ice-cold physiological saline solution (PSS; 120 mM NaCl, 5 mM KCl, 25 mM $NaHCO_3$, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11 mM glucose, and 2.5 mM calcium; bubbled with 5% carbon dioxide, 95% nitrogen gas) to remove excess blood and anesthetic and fixed overnight in 4% paraformaldehyde. Procedures were performed in accordance with the ethics guidelines of the National Health and Medical Research Council of Australia, with approval from the South Eastern Sydney and Illawarra Area Health Service Human Research Ethics Committee and the University of New South Wales Human Research Ethics Committee.

Immunohistochemistry and immunofluorescence

Vessels fixed in PLP were embedded in polyester wax (poly-ethylene glycol 400 distearate; Polysciences, Warrington, PA, USA), whereas those fixed in NBF and paraformaldehyde were embedded in paraffin wax. Sections (5 μm) were cut and mounted on SuperFrost PLUS slides (Menzel-Gläser, Braunschweig, Germany). Procedures for brightfield immunohistochemistry are detailed in Vodstrcil *et al*.[3] Rat tissues were incubated overnight at 4°C with 3 μg/ml rat RXFP1 antiserum (#107, raised against amino acid residues 107–119 of the rat RXFP1 protein) or preimmune serum (rabbit IgG), kindly supplied by Prof. O. David Sherwood (Department of Physiology and Biophysics, University of Illinois, Urbana, IL, USA). An additional negative control was primary antiserum (3 μg/ml) preabsorbed overnight with 3μg/ml rat relaxin. Immunoreactivity was detected using the MACH 2 system (Biocare Medical, Concord, CA, USA) and 3, 3' diaminobenzidine (DAB) as the chromagen substrate (Vector Laboratories, Burlingame, CA, USA). Cross-reactivity of the rat RXFP1 antibody with another relaxin/insulin-like family peptide receptor, RXFP2, was tested on sections of rat gubernaculum testis (kindly provided by Prof, John Hutson, Royal Children's Hospital, University of Melbourne) which only expresses RXFP2.[29] Human arteries were incubated in Background Sniper (Biocare Medical) for 20 min to block nonspecific binding and then with 0.36 μg/ml monoclonal anti-human RXFP1 antiserum (H00059350-M01; Abnova, Novus Biologicals, Littleton, CO, USA) or mouse IgG (diluted to 0.36 μg/ml) overnight at 4°C. Antibody specificity for RXFP1 was determined in previous studies.[30] Immunoreactivity was detected with the MACH 4 Universal HRP detection kit (Biocare Medical) and DAB (Biocare Medical). Relative RXFP1 immunostaining was semiquantified by analyzing the percentage area occupied by DAB in each vessel. Using Photoshop CS6 (Adobe Systems, San Jose, CA, USA), relative pixel density as the proportion of area occupied as brown pixels (reflecting DAB label) was taken to reflect the intensity of labeling according to a method modified from Tse *et al*.[31] In brief, uniform color range was set, and areas of intima and media of the same size were selected at random, and the intensity of signal was recorded using the integrated pixel density function. Equivalent regions of intima and media from negative control tissue were taken as baseline. Comparisons were made between cell types in each vessel (n=6 replicates).

All procedures for immunofluorescence were the same as described above, except different secondary antibodies and detection systems were used.[3] To identify rat RXFP1, goat anti-rabbit Dylight 488 (1:100) was applied for 1 h. Slides were briefly rinsed in 1X TBS containing 1% Tween-20 (TTBS) and then a 1:1000 dilution of monoclonal antibody against α-SMA conjugated to Cy5 (Sigma-Aldrich Pty. Ltd. Sydney, NSW, Australia) was applied for 40 min. Control samples were incubated

with a 1:1000 dilution of anti-mouse IgG conjugated to Cy5. Nuclei were counterstained with DAPI (Invitrogen, Life Technologies, Grand Island, NY, USA). Sections were coverslipped using fluorocare mounting medium (Biocare Medical) and imaged under 488 and 594 wavelengths on a Zeiss imager D1 with AxioCam mrC5 camera (Carl Zeiss Pty. Ltd., North Ryde, NSW, Australia).

EFFECTS OF CHRONIC INFUSION OF SERELAXIN ON VASCULAR FUNCTION IN MALE RATS

Twelve-week old male Wistar rats were implanted with a 7-d Alzet osmotic minipump (Model 2001; Bioscientific, Gymea, NSW, Australia) to infuse recombinant human H2 relaxin (serelaxin; Novartis Pharma AG, Basel, Switzerland; n=23) or placebo (20 mM sodium acetate, pH 5.0; n=21) subcutaneously. The animals were anesthetized using isofluorane, and the osmotic minipumps were inserted under the skin between the shoulder blades using aseptic techniques. Serelaxin was infused at a dose of 13.33 µg/h/kg. This dose was predicted to yield concentrations of circulating serelaxin similar to those measured on gestational d 12–14 in pregnant rats (20 – 40 ng/ml; ref. 32). Between 09:00 and 12:00 on d 5 after pump insertion, rats were anesthetized with isofluorane and euthanized by cervical dislocation. Cardiac puncture was performed to collect 1 ml blood to verify plasma serelaxin levels. The femoral arteries and veins (diameter ~240 µm and 370 µm, respectively), and small second order mesenteric arteries and veins (diameter ~200 and 250 µm, respectively) were dissected from each animal and placed in sterile 0.1 M PBS solution on ice to clear fat and loose connective tissue. These four vessels were selected because they represent arteries and veins in different vascular beds. A section of each vessel was used either for the *in vitro* analysis of passive mechanical wall properties or vascular reactivity studies. Sections of arteries were also snap frozen for additional analyses (see below). The plasma concentration of serelaxin was measured using the Human Relaxin-2 Quantinine ELISA Kit (R&D Systems, Minneapolis, MN, USA) following manufacturer's instructions. Plasma samples were diluted 1:500 in the assay, with a 15.6 pg/ml limit of detection.

PASSIVE MECHANICAL WALL PROPERTIES

Mesenteric and femoral arteries and veins from serelaxin- and placebo-treated rats were transferred to a Ca^{2+}-free PSS (14.9 mM NaCl, 4.7 mM KCl, 1.7 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.7 mM $MgSO_4$, 5 mM glucose, 10 mM HEPES, and 2 mM EGTA). Leak-free segments of vessels were mounted on a pressure myograph system (Living Systems Instrumentation, Burlington, VT, USA), and arterial data [vessel length, outer diameter (OD), inner diameter (ID), and wall thickness (WT)], as well as wall stress and strain were acquired and calculated as described previously.[3,33] For

normalization of ID and OD, values were expressed as (value at pressure – value at baseline)/(value at baseline). Volume compliance was calculated for each pressure increment using the following calculation: volume compliance = (Δ volume)/(Δ pressure), where Δ volume = (Δ cross sectional area) × (Δ length), and cross sectional area = $(\pi \times ID^2)/4$. Because wall thickness could not be defined in veins (it was below the limit of detection for the magnification we used), only OD was measured, and vascular stiffness was evaluated by calculating change in OD/pressure.

COLLAGEN AND ELASTIN ASSAY

Collagen content was measured in abdominal aorta, mesenteric, and femoral arteries using the Sircol collagen dye binding assay (Biocolor, Carrickfergus, UK) as described previously.[21] Elastin content was only determined in mesenteric and femoral arteries (Fastin elastin assay kit; Biocolor). Arteries were lyophilized, and elastin was solubilized using the hot oxalic acid digestion method. Samples and standards (ranging from 0–50 μg) were then incubated in elastin precipitating reagent at 4°C overnight. Final pellets were resuspended in 250 μl of dye dissociation reagent. Absorbance was determined at 540 nm. and relative elastin content was quantified as the ratio of elastin concentration (μg/ml) to dry weight (mg).

VASCULAR REACTIVITY

Rings (1–2 mm in length) of mesenteric and femoral arteries and veins from serelaxin (n=15) and placebo (n=14) treated rats were mounted on a four-channel Mulvany-Halpern myograph (model 610M; Danish Myo Technology, Aarhus, Denmark). The vessels were then stretched in increments to a tension equivalent to ~70 mmHg for arteries and ≈20 mmHg for veins. Vascular smooth muscle and endothelial cell function were tested as described previously.[33,34] Blood vessels were submaximally (50 – 60% maximal constriction) preconstricted with phenylephrine (PE; ~10^{-9} to 10^{-6} M), and endothelium-dependent relaxation was assessed with increasing concentrations of acetylcholine (ACh; 10^{-10} to 10^{-5} M; all arteries and veins) or bradykinin (BK; 10^{-10} to 10^{-6} M; mesenteric arteries and veins because BK caused contraction in the other vessels) applied cumulatively. Responses to ACh and BK were recorded before and after NOS and cyclooxygenase blockade with N′ -nitro-l-arginine methyl ester (L-NAME; 2×10^{-4} M) and/or indomethacin (Indo; 10^{-6} M), respectively. The relaxation remaining in the presence of both inhibitors was attributed to EDH.[35] Smooth muscle contraction was examined in vessels exposed to increasing concentrations of PE (10^{-9} to 10^{-4} M) applied cumulatively. The relative contribution of NO, PGI_2, and EDH to relaxation evoked by BK was determined by analyzing the area under curve (AUC) of the BK-response curves. The responses

attributed to each component were calculated using the following equations: PGI_2 contribution = AUC (L-NAME alone) - AUC (L-NAME+Indo); NO contribution = AUC (no inhibitors) – AUC (L-NAME alone); EDH contribution = AUC (L-NAME+Indo).

QUANTITATIVE PCR

RNA was extracted from individual arteries, and cDNA was synthesized from 0.5 μg of RNA in a 20 μl reaction containing random hexamers (50 ng/μl) and 200 U of Superscript III (Invitrogen, Mulgrave, VIC, Australia; ref. 3). The comparative cycle threshold ($2^{-\Delta Ct}$) method of quantitative real-time polymerase chain reaction (qPCR) was used to quantify eNOS III (*Nos3*) gene expression in the mesenteric arteries and veins of serelaxin and placebo treated male rats. Rat-specific forward/reverse primers and 6-carboxyl fluorescein (FAM)-labeled TaqMan probes were designed and purchased from Biosearch Technologies (Novato, CA, USA). qPCR was performed on the Opticon 3 PCR machine (Bio-Rad, West Ryde, NSW, Australia) using 96-well reaction plates with 20-μl reactions in triplicate containing SensiMix (Bioline, Alexandria, NSW, Australia) and 10 μM of primers and FAM-labeled probe. Ribosomal 18S (*Rn18s*) was the reference gene. Negative template controls substituting cDNA with water or RT negative controls substituting the reverse transcriptase in the cDNA synthesis were included on each plate. For each sample, the mean *Rn18s* CT triplicate value was subtracted from the mean gene of interest triplicate CT value to normalize gene of interest expression to the reference gene. These normalized data were then presented as a relative value (means ± SE).

STATISTICAL ANALYSIS

Data are expressed as means ± SE with *n* representing the number of rats. The stress-strain and volume compliance data were analyzed with repeated measures 2-way ANOVA (treatment vs. strain) with Bonferroni *post hoc* analysis using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Independent *t* tests assessed statistical differences in collagen and elastin concentrations between placebo- and serelaxin-treated rats. Concentration-response curves were constructed using GraphPad Prism and sigmoidal curves were fitted using the least squares method and analyzed using repeated measures 2-way ANOVA (treatment vs. concentration). Contractions to PE were expressed as percentage contraction to high KPSS. ACh- and BK-evoked relaxation was expressed as percentage of precontraction evoked by PE. The concentration of drug required to evoke a half-maximal response (EC_{50}) was determined, and the pD2 (-log EC_{50}), maximum contraction (E_{max}), and maximum relaxation (R_{max}) were compared using independent *t* tests.

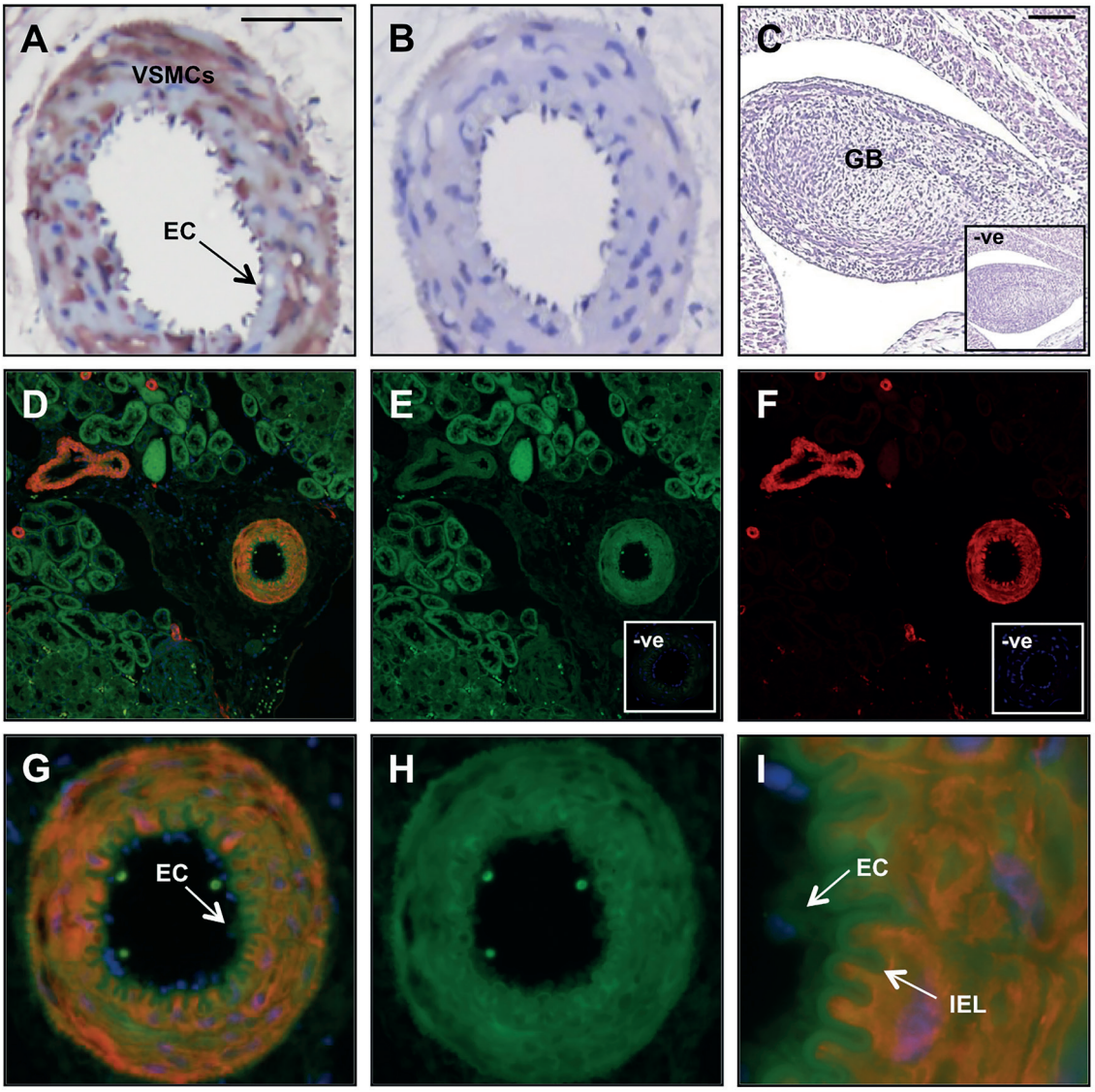

Figure 1 ↑
Localization of RXFP1 protein in rat small renal arteries by immunohistochemistry (**A–C**) and immunofluorescence (**D–I**). Immunoreactive RXFP1 is predominantly localized to the vascular smooth muscle of the media (**A**). Immunofluorescent RXFP1 (**E**) & (**H**) and α-SMA (**F**), with the overlay (**D**), (**G**) & (**I**) demonstrate colocalization. Higher magnification illustrates RXFP1 in endothelial cells that did not stain positive for α-SMA ((**I**), arrow). There is no RXFP1 staining in the preimmune serum negative controls ((**B**) and (**C**), (**E**) & (**F**) insets) and rat gubernaculum testis (GB; (**C**)). VSMC = vascular smooth muscle cells, EC = endothelial cell, panel A scale bar = 50 μm, panel C scale bar = 500 μm. Note the high level of autofluorescence in the internal elastic lamina (IEL).

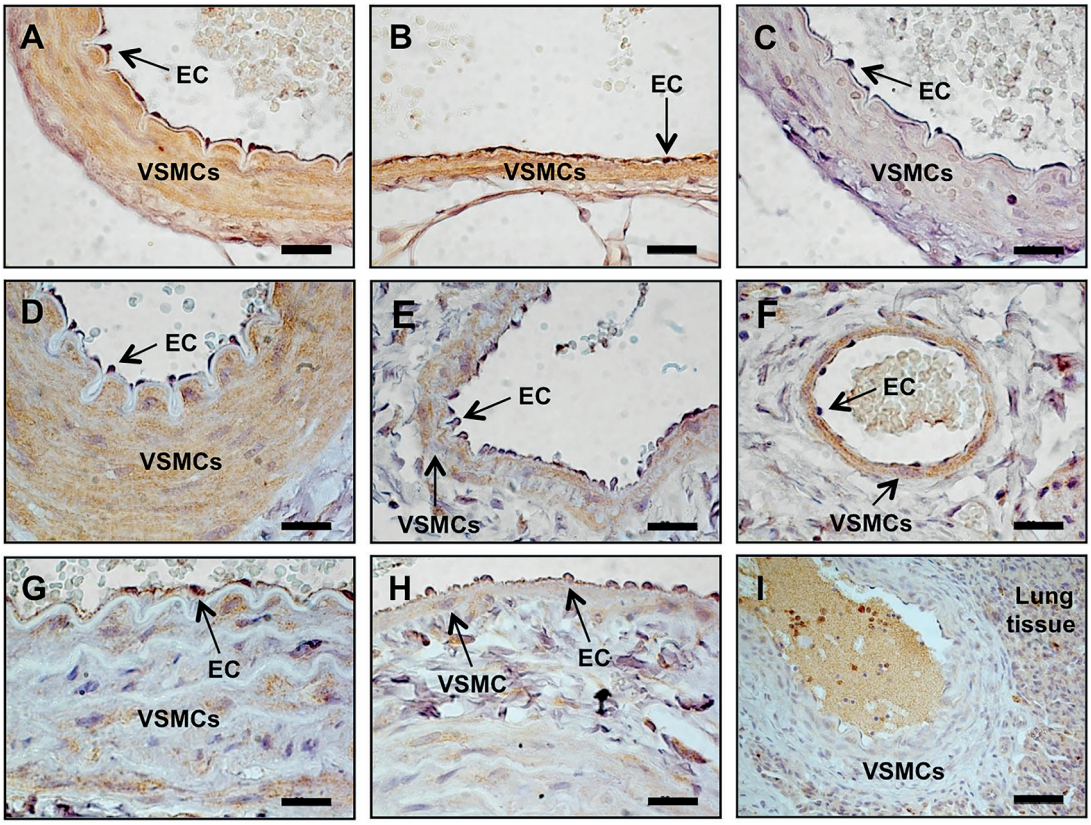

Figure 2 ↑
Localization of RXFP1 protein by immunohistochemistry in endothelial cells (EC) and vascular smooth muscle cells (VSMC) in the mesenteric artery (**A**), mesenteric vein (**B**) and abdominal aorta (**G**). In the femoral artery (**D**) and vein (**E**), and small pulmonary artery (**F**), RXFP1 is predominantly localized to the VSMC. RXFP1 staining in the vena cava (**H**) and large pulmonary artery (**I**) is considerably lower than other blood vessels. There is no RXFP1 staining in the preimmune serum negative control (**C**). Scale bars = 20 μm (A-H); 50 μm (I).

RESULTS

LOCALIZATION OF RXFP1 IN ARTERIES AND VEINS

Immunoreactive RXFP1 was localized in small renal arteries, abdominal aorta, vena cava, large and small pulmonary arteries, mesenteric and femoral arteries, and veins of rats, but the cellular distribution of RXFP1 differed between vessel types (**Figures 1** and **2** and **Table 1**). In the kidney, RXFP1 was predominantly localized to the small renal artery and renal tubules (**Figure 1A,D**). There was no RXFP1 staining in the preimmune serum negative controls (**Figure 1B**) or in sections that were

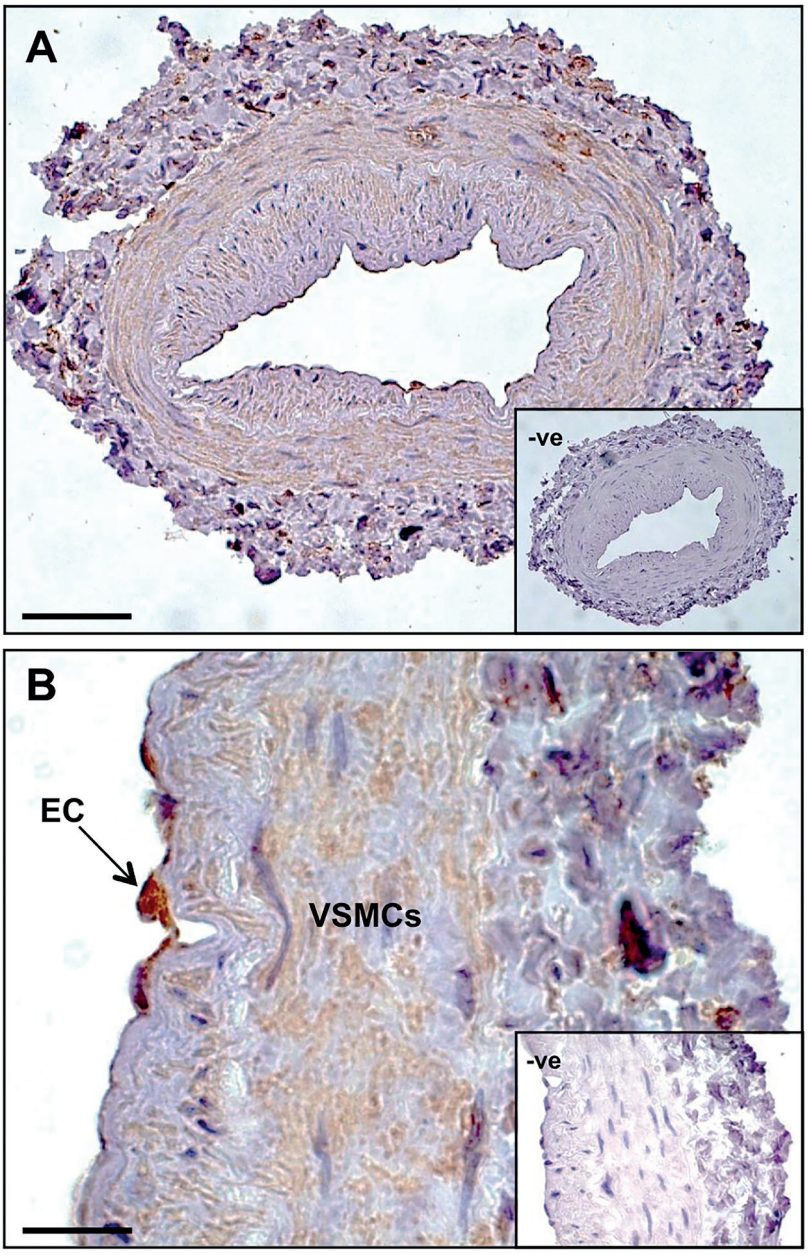

Figure 3 ↑
(**A**) Localization of RXFP1 protein by immunohistochemistry in a human mesenteric artery (representative of arteries from all 3 patients). (**B**) RXFP1 immunostaining is detected in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) but not in the mouse IgG negative controls (insets). Scale bars = 100 μm (A), 20 μm (B).

incubated with antiserum preabsorbed with rat relaxin (data not shown). It was important to demonstrate specificity to RXFP1, as the *Rxfp2* gene is expressed in blood vessels.[26] The absence of RXFP1 immunostaining in rat gubernaculum testis (**Figure 1C**), which only expresses RXFP2,[29] is evidence that the rat RXFP1 antibody used in this study did not cross-react with RXFP2. Double-labeling immunofluorescence confirmed colocalization of RXFP1 with α-SMA in vascular smooth muscle cells within the media of small renal arteries and not veins (**Figure 1D,G**). When one of the primary antisera was replaced with preimmune serum, there was only positive staining for RXFP1 (**Figure 1E,H**) or α-SMA (**Figure 1F**). The negative controls with preimmune rabbit serum revealed little background fluorescence (**Figure 1E,F**; insets). Endothelial cells were difficult to identify in these small renal artery cross- sections but at higher magnification, there appeared to be RXFP1 immunostaining in cells lining the lumen that did not stain positive for α-SMA (**Figure 1I**).

Comparisons between endothelial and vascular smooth muscle cells revealed differences in relative RXFP1 immunostaining in rat arteries and veins (**Figure 2** and **Table 1**). In the mesenteric artery and vein, RXFP1 was localized to both endothelial cells and the smooth muscle (**Figure 2A,B**) but with significantly ($P<0.05$) higher immunostaining in endothelial cells compared with smooth muscle cells (**Table 1**). There was no RXFP1 staining in the preimmune serum negative controls (**Figure 2C**). RXFP1 immunostaining in endothelial cells was also significantly ($P<0.05$) higher in the aorta and vena cava (**Table 1, Figure 2G,H**). In the femoral artery and vein and

Artery or vein	Endothelium	Smooth muscle
Aorta	32.9 ± 0.3*	22.3 ± 0.3
Femoral artery	3.2 ± 0.4	39.7 ± 1.7*
Femoral vein	3.2 ± 0.6	34.6 ± 1.7*
Mesenteric artery	84.3 ± 1.1*	56.5 ± 1.0
Mesenteric vein	78.2 ± 3.4*	68.4 ± 1.7
Pulmonary artery (large)	4.2 ± 0.8	5.4 ± 0.6
Pulmonary artery (small)	11.6 ± 1.0	66.1 ± 4.1*
Vena cava	25.4 ± 2.1*	4.6 ± 0.3

Table 1 ↑
Quantification (% area of DAB labeling) of RXFP1-positive staining in the intima and media of the aorta, vena cava, femoral artery and vein, mesenteric artery and veins, and large and small pulmonary arteries. Data are means ± SE of 6 uniform areas for endothelium and smooth muscle. * $P < 0.05$ vs. other cell type.

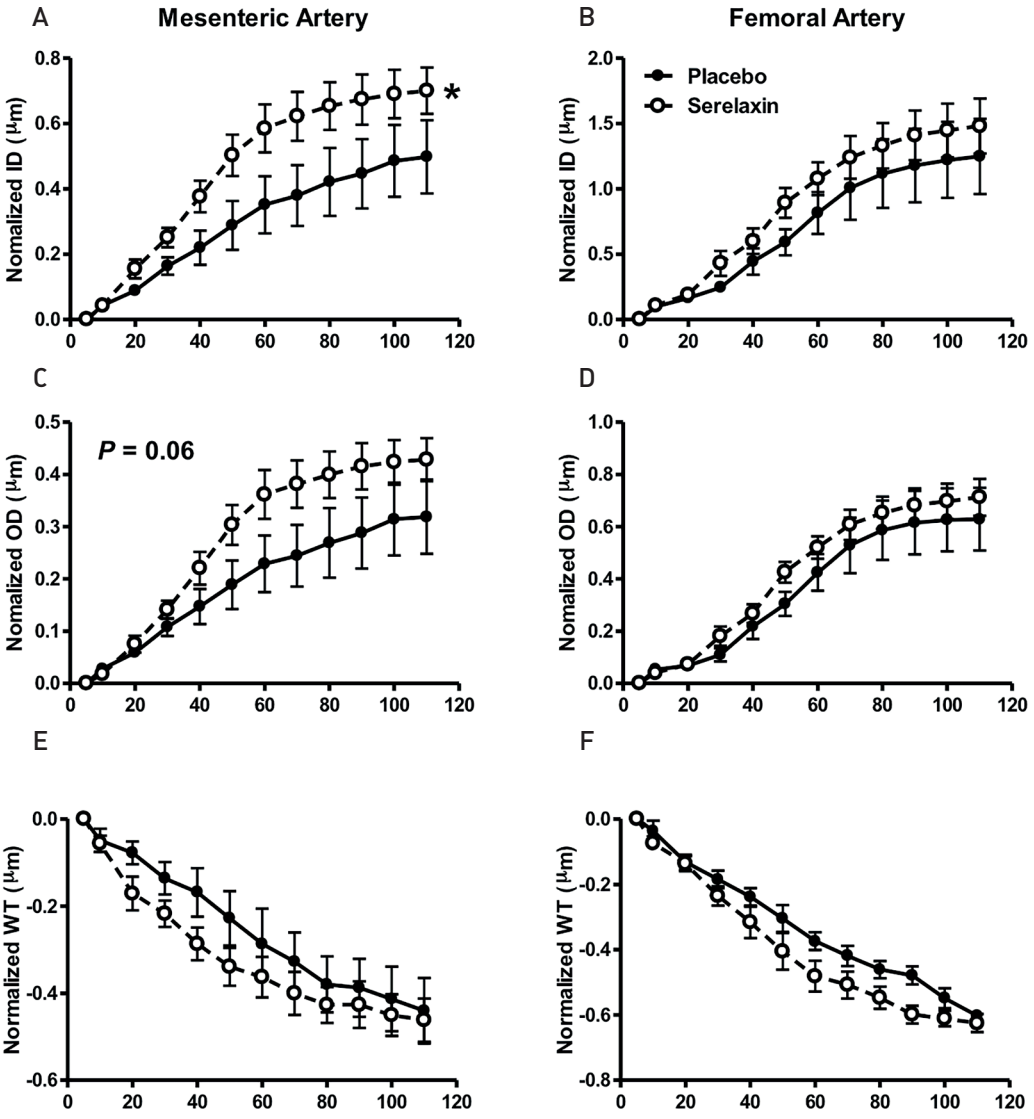

Figure 4 ↑
Normalized passive ID (**A**), (**B**), (OD; (**C**), (**D**)), and WT (**E**), (**F**) against intraluminal pressures in the mesenteric (**A**), (**C**), (**E**) and femoral arteries (**B**), (**D**), (**F**) from placebo-treated (solid circles, n=6) and serelaxin-treated (open circles, n=8) rats. Values are means ± SE. *P<0.05 vs. placebo controls.

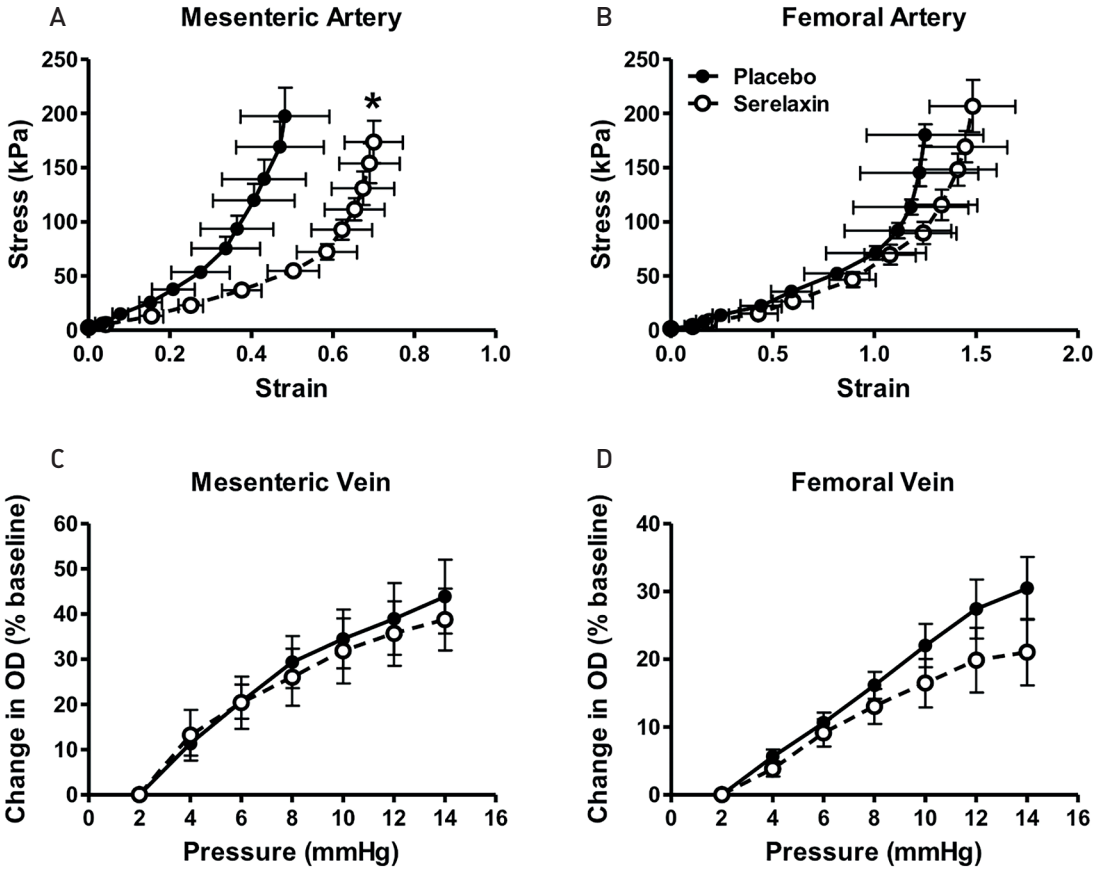

Figure 5 ↑
Stress-strain relationships in mesenteric (**A**) and femoral (**B**) arteries, and pressure-OD relationships in mesenteric (**C**) and femoral (**D**) veins from placebo-treated (solid circles, n=6–7) and serelaxin-treated (open circles, n=7–8) male rats. Values are means ± SE. *P<0.05 vs. placebo controls.

small pulmonary artery, RXFP1 was predominantly localized to smooth muscle (Figure 2D–F) and significantly (P<0.05) higher compared with endothelial cells (Table 1). RXFP1 immunostaining was relatively low in the large pulmonary artery (Figure 2I).

In human mesenteric arteries, immunoreactive RXFP1 was clearly localized in endothelial cells, with less intense immunostaining in the vascular smooth muscle and adventitia (Figure 3). There was no RXFP1 staining in the negative control with mouse IgG (Figure 3, insets).

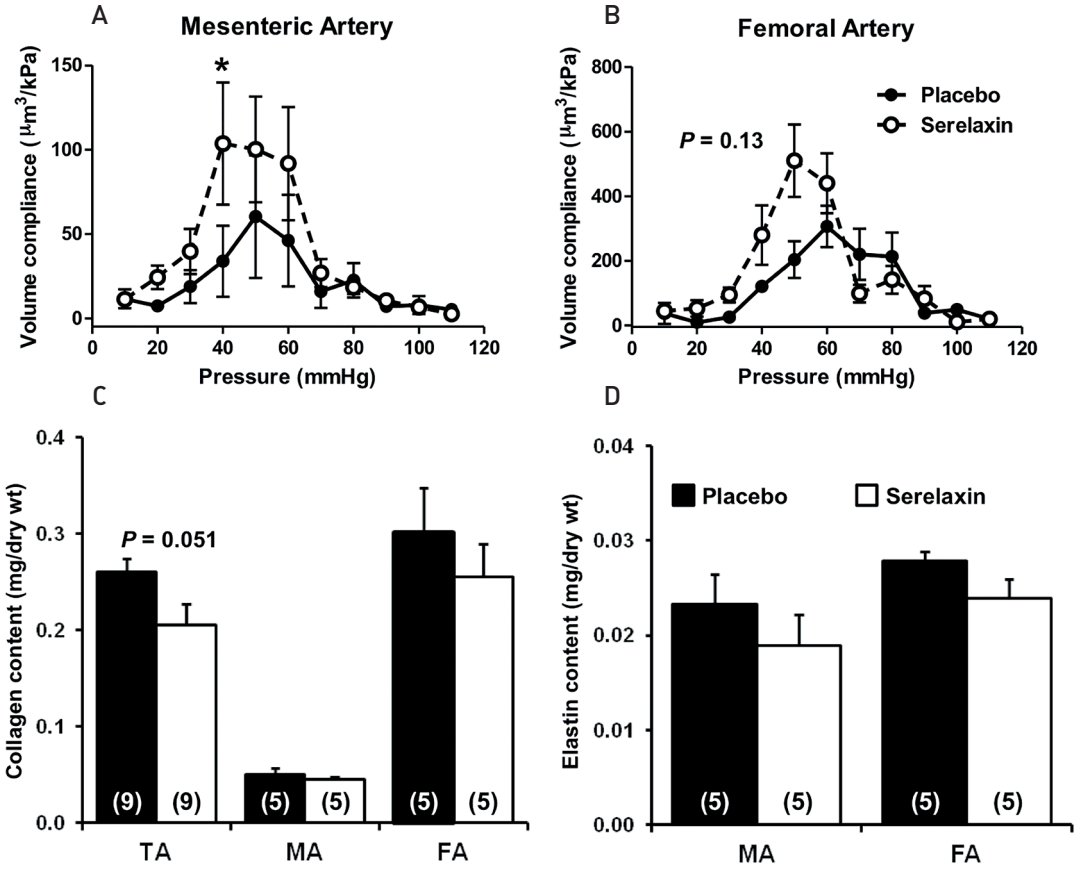

Figure 6 ↑
(**A**), (**B**) Volume compliance throughout the pressure range (5–110 mmHg) in the mesenteric (**A**) and femoral (**B**) arteries from placebo-treated (solid circles, n=6–7) and serelaxin-treated (open circles, n=7–8) rats. Values are means ± SE. *P<0.05 vs. placebo controls. (**C**), (**D**) Quantitative analysis of total soluble collagen (**C**) and elastin (**D**) content in the thoracic aorta (TA), mesenteric artery (MA) and femoral artery (FA) of placebo-treated (solid squares) and serelaxin-treated (open squares) rats. Values are expressed as mean ± SE ratio of total dry weight. Sample sizes are indicated in parentheses.

EFFECTS OF CHRONIC INFUSION OF SERELAXIN ON PASSIVE MECHANICAL WALL PROPERTIES

Rats treated with serelaxin had significantly increased plasma recombinant human serelaxin concentrations (39.93±4.06 ng/ml) compared with placebo-treated rats, in which no human serelaxin was detected. There was no significant difference in WT, OD, and ID in any vessel analyzed at baseline (5 mmHg; **Tables 2** and **3**). Over the

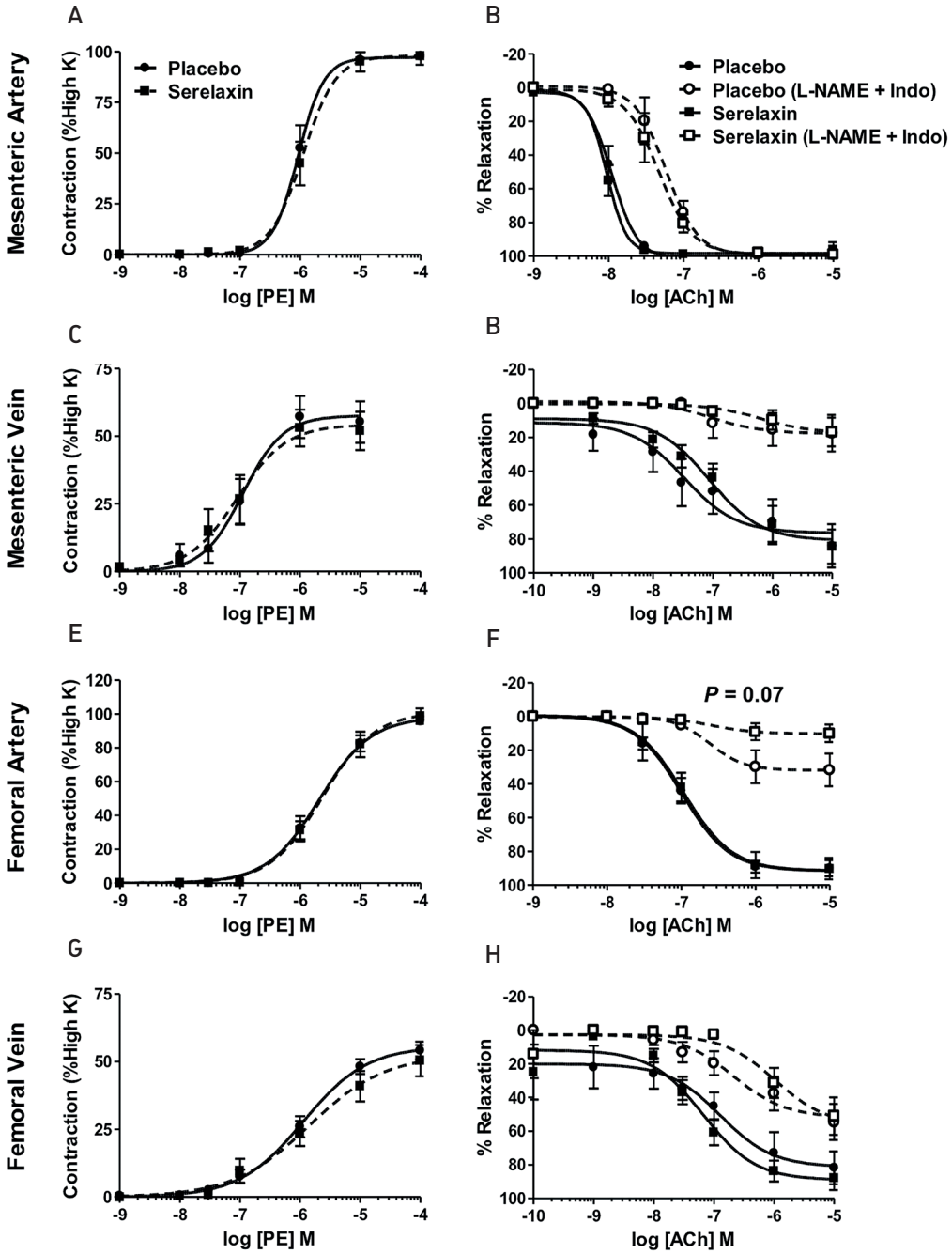

Figure 7 ↑
Concentration-response curves to PE (**A**), (**C**), (**E**), (**G**) and ACh (**B**), (**D**), (**F**), (**H**) in endothelium-intact mesenteric arteries (**A**), (**B**), mesenteric veins (**C**), (**D**), femoral arteries (**E**), (**F**), and femoral veins (**G**), (**H**) isolated from placebo-treated (circles) and serelaxin-treated (squares) rats (n=8–11/group). ACh-evoked relaxation was compared in the absence (continuous line) and presence (dotted line) of L-NAME + Indo. Values are means ± SE.

pressurization range, ID of mesenteric arteries increased to a significantly greater extent (P=0.03) in the serelaxin-treated animals compared with controls (Figure 4A). There was no significant difference in OD and WT responses to increasing pressure between the two groups (Figure 4C,E). There was a significant shift to the right of the stress-strain curve of mesenteric arteries from serelaxin-treated rats ($F_{1,10}$=5.55, P=0.04) compared with controls (Figure 5A), indicative of an overall reduction in passive wall stiffness. Furthermore, overall volume compliance was significantly ($F_{1,10}$=5.04, P=0.03) increased in the mesenteric arteries of serelaxin-treated rats compared with controls (Figure 6A).

In femoral arteries, treatment with serelaxin had no significant effect on normalized OD, ID, and WT (Figure 4B,D,F) or wall stiffness compared with controls (Figure 5B).

	Mesenteric artery				Mesenteric vein			
Parameter	**Placebo**	**n**	**Serelaxin**	**n**	**Placebo**	**n**	**Serelaxin**	**n**
Stress-strain (*K*)	7.09 ± 1.67	6	6.08 ± 0.53	8	N/M	-	N/M	-
Baseline OD (μm)	150.4 ± 6.8	6	149.4 ± 9.5	8	251.3 ± 37.2	7	251.3 ± 34.59	6
Baseline ID (μm)	123.5 ± 5.3	6	116.6 ± 9.2	8	N/M	-	N/M	-
Baseline WT (μm)	13.44 ± 1.37	6	16.39 ± 0.81	8	N/M	-	N/M	-
Hi K E_{max} (mN/mm)	9.59 ± 0.37	13	9.95 ± 0.55	13	3.79 ± 0.35	14	3.88 ± 0.48	13
PE pD2	5.93 ± 0.13	8	5.81 ± 0.14	8	N/D	10	N/D	8
PE E_{max} (% Hi K)	99.74 ± 4.01	8	97.85 ± 4.37	8	60.38 ± 7.39	10	54.83 ± 7.22	8
ACh pD_2	7.97 ± 0.09	8	8.00 ± 0.06	8	7.15 ± 0.52	8	6.71 ± 0.22	8
ACh R_{max} (%)	101.57 ± 0.78	8	99.85 ± 0.82	8	84.06 ± 12.71	8	84.06 ± 10.07	8
ACh (+L-NAME+Indo) pD_2	7.25 ± 0.09	6	7.49 ± 0.13	6	N/D	5	N/D	7
ACh (+L-NAME+Indo) R_{max} (%)	99.17 ± 2.40	6	99.92 ± 2.50	6	17.67 ± 10.82	5	16.98 ± 8.52	7
BK pD2	7.73 ± 0.10	9	9.24 ± 0.61*	9	8.88 ± 0.14	8	8.65 ± 0.12	9
BK R_{max} (%)	92.87 ± 2.28	9	98.45 ± 1.51	9	109.1 ± 1.66	8	105.5 ± 1.62	9
BK (+L-NAME) pD2	7.45 ± 0.25	5	7.22 ± 0.44	7	8.15 ± 0.21	5	8.15 ± 0.24	7
BK (+L-NAME) R_{max} (%)	85.11 ± 7.62	7	92.94 ± 6.15	8	55.99 ± 4.21	7	59.08 ± 5.48	9
BK (+L-NAME+Indo) pD_2	7.46 ± 0.17	4	7.20 ± 0.15	6	8.23 ± 0.26	6	7.69 ± 0.24	7
BK (+L-NAME+Indo) R_{max} (%)	85.84 ± 7.32	7	92.94 ± 9.49	6	58.87 ± 9.21	7	59.06 ± 5.21	7

Table 2 ↑
Mesenteric artery and vein dimensions at baseline (5 and 2 mmHg, respectively). OD = outer diameter; ID = inner diameter; WT = wall thickness. Vascular reactivity data: PE = phenylephrine; ACh = acetylecholine; BK = bradykinin; Hi K = 100 mM potassium PSS; L-NAME = nitric oxide synthase inhibitor; Indo = indomethacin; E_{max} = maximum response; R_{max} = maximum relaxation; pD2 = sensitivity (−log EC50); N/M = not measurable; N/D = not defined. All values are expressed as mean ± SE; n = sample size. * P < 0.05 vs. placebo controls.

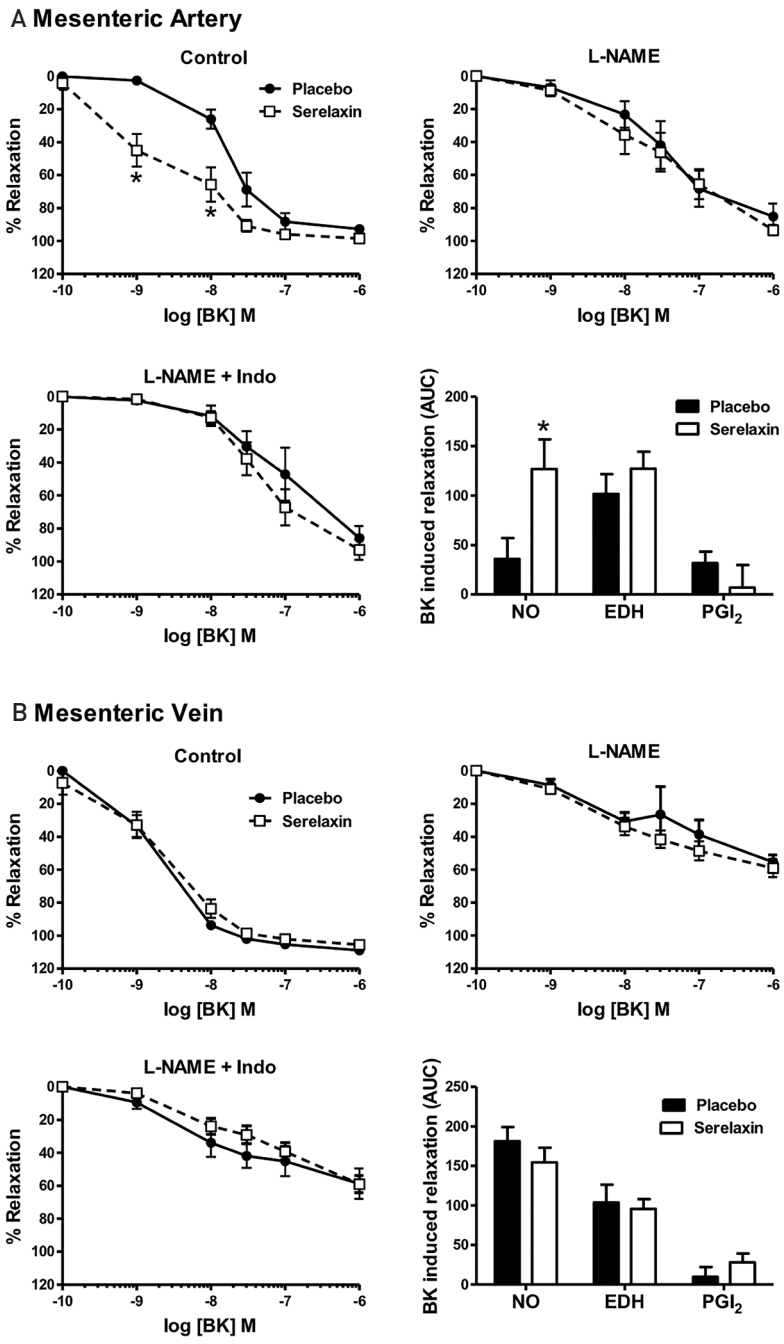

Figure 8 ↑

Concentration-response curves for BK in endothelium-intact mesenteric arteries (**A**) and veins (**B**) isolated from placebo-treated (solid circles) and serelaxin-treated (open squares) rats (n=6–9/group). BK-evoked relaxation was compared between groups in the presence of L-NAME alone and L-NAME + Indo. AUC of BK response curves were analyzed to reveal the relative contribution of NO, EDH, and PGI_2. Values are means ± SE. *P<0.05 vs. placebo controls.

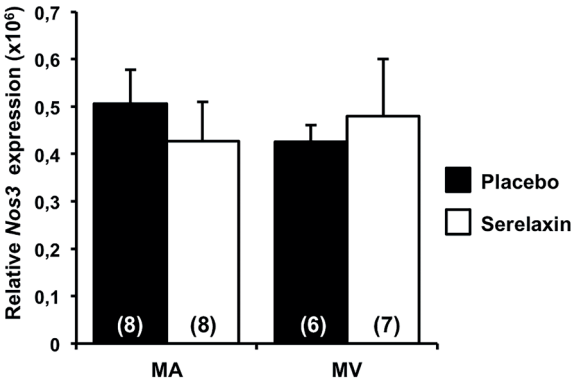

Figure S1 ↑
Quantitative analysis of endothelial nitric oxide synthase (*Nos3*) expression in mesenteric arteries (MA) and veins (MV) of rats treated with placebo (solid squares) and serelaxin (open squares). Values are $2^{-\Delta Ct}$ ± SEM. Sample sizes are indicated in brackets.

Furthermore, there was no significant difference in volume compliance across the pressurization range. Serelaxin treatment also had no significant effect on the OD of the mesenteric and femoral veins when pressurized between 5 and 15 mmHg (**Figure 5C,D**), suggesting no change in vascular remodeling.

We then assessed total soluble collagen and elastin content to establish whether the reduction in wall stiffness in serelaxin-treated rats was associated with compositional remodeling. There was no significant effect of serelaxin treatment on either collagen or elastin content (**Figure 6C,D**) or on the collagen: elastin ratio (data not shown) in the mesenteric and femoral arteries compared with controls. In the thoracic aorta, there was a decrease in collagen content, but it did not reach significance (P=0.051).

EFFECTS OF CHRONIC INFUSION OF SERELAXIN ON VASCULAR FUNCTION

Smooth muscle function

PE evoked concentration-dependent (10^{-9} to 10^{-4} M) contraction in mesenteric and femoral arteries and veins but there was no significant difference in the sensitivity (pD_2) or maximal response (E_{max}) between serelaxin-treated rats and controls (**Figure 7** and **Tables 2** and **3**). Serelaxin treatment had no effect on 100 mM potassium PSS-induced contraction in any blood vessel examined (**Tables 2** and **3**).

Endothelium-dependent relaxation

Stimulation of the endothelium with ACh resulted in concentration-dependent (10^{-10} to 10^{-5} M) relaxation in mesenteric and femoral arteries and veins but serelaxin treatment had no significant effect on either the sensitivity (pD_2) or R_{max} compared with controls (**Figure 7** and **Tables 2** and **3**). The effects of serelaxin treatment on EDH-mediated relaxation evoked by ACh were also evaluated; 75% of femoral and mesenteric veins, but not arteries, developed spontaneous tone after L-NAME + Indo incubation, but this did not differ between serelaxin and placebo-treated rats. L-NAME + Indo shifted the concentration-relaxation curves to ACh to the right in all blood vessels, indicating the involvement of both NO and/or vasodilator

Parameter	Femoral artery Placebo	n	Serelaxin	n	Femoral vein Placebo	n	Serelaxin	n
Stress-strain (*K*)	3.81 ± 0.52	6	3.34 + 0.45	8	N/M	-	N/M	-
Baseline OD (µm)	243.3 ± 16.3	6	234.2 + 13.5	8	373.3 + 26.9	7	375.3 + 31.5	6
Baseline ID (µm) 1	69.2 ± 16.4	6	157.2 + 16.8	8	N/M	-	N/M	-
Baseline WT (µm)	37.08 ± 1.23	6	44.83 + 5.80	8	N/M	-	N/M	-
Hi K E_{max} (mN/mm)	15.07 ± 0.97	13	15.01 + 0.87	12	3.56 + 0.11	14	3.30 + 0.23	13
PE pD_2	5.72 ± 0.12	8	5.63 + 0.12	8	6.03 + 0.15	11	6.05 + 0.20	10
PE E_{max} (% Hi K)	95.57 ± 2.96	8	98.79 + 4.54	8	54.07 + 3.31	11	50.39 + 5.75	10
ACh pD_2	6.98 ± 0.09	10	6.852 + 0.13	9	7.26 + 0.15	11	7.14 + 0.21	8
ACh R_{max} (%)	91.91 ± 3.40	10	91.15 + 5.94	9	96.95 + 2.24	11	95.16 + 3.75	8
ACh (+L-NAME+Indo) pD_2	N/D	9	N/D	8	6.34 + 0.32	9	5.72 + 0.31	7
ACh (+L-NAME+Indo) R_{max} (%)	66.86 ± 10.09	9	89.09 + 5.08	8	45.31 + 10.70	9	48.93 + 11.15	7

Table 3 ↑
Femoral artery and vein dimensions at baseline (5 mmHg). OD = outer diameter; ID = inner diameter; WT = wall thickness. Vascular reactivity data: PE = phenylephrine; ACh = acetylecholine; Hi K = 100 nM potassium PSS; L-NAME = nitric oxide synthase inhibitor; Indo = indomethacin; E_{max} = maximum response; pD2 = sensitivity (−log EC50); N/M = not measurable; N/D = not defined. All values are expressed as mean ± SE; n = sample size.

prostanoids (Figure 7). In the femoral artery, blockade with L-NAME ■ Indo reduced maximum relaxation to ACh (to 10.91±5.08%) in serelaxin-treated rats compared with controls (to 33.14± 10.09%), but this was not significant (P=0.07; Figure 7F). In all other vessels examined, the EDH-mediated relaxation evoked by ACh was unaffected by serelaxin treatment (Figure 7).

We expanded our analysis of endothelium-dependent relaxation in the mesenteric arteries and veins using BK. In mesenteric arteries of serelaxin-treated rats the concentration-dependent relaxation curve was significantly augmented compared with controls (P<0.001; Figure 8A). *Post hoc* analysis revealed that 10^{-9} and 10^{-8} M BK caused significantly (P<0.01) greater relaxation in serelaxin-treated animals compared with controls. Sensitivity (pD_2) to BK was also significantly (P=0.03) greater in serelaxin-treated animals (9.24±0.6) compared with controls (7.73±0.10) (Figure 8A, Table 2). Analysis of the area under the curve indicated that this was due to a significant (P=0.02) increase in the component attributed to NO. Conversely, in the presence of L-NAME and L-NAME + Indo there was no significant difference in BK-mediated relaxation between serelaxin and placebo-treated rats (Figure 8A), indicating the absence of a PGI_2 component. There was no significant difference in BK-evoked relaxation in mesenteric veins of serelaxin and placebo-treated rats (Figure 8B, Table 2).

To investigate whether the serelaxin-enhanced BK-mediated vasodilation involves an increase in total eNOS, we analyzed *Nos3* gene expression in mesenteric arteries and veins. There was no significant difference in *Nos3* expression between placebo and serelaxin-treated animals in these two vessels (**Figure S1**).

DISCUSSION

The major findings of this study were that receptors for relaxin, $RXFP_1$, were differentially localized in endothelial and smooth muscle cells of arteries and veins. $RXFP_1$ was predominantly expressed in endothelial cells in the mesenteric artery and vein, and also in the aorta and vena cava. In contrast, the femoral artery and vein and small pulmonary arteries had higher $RXFP_1$ immunostaining in the vascular smooth muscle. Chronic infusion with serelaxin for 5 d reduced vessel wall stiffness in the mesenteric arteries but not in the other vessels examined. This was primarily associated with vascular remodeling and changes in geometrical properties, and not compositional changes in the extracellular matrix (ECM). Serelaxin treatment had no effect on ACh-mediated relaxation. However, BK-mediated relaxation in mesenteric arteries, but not veins, was enhanced following serelaxin treatment. This was due to an increase in the NO component of BK-mediated relaxation and not to changes in EDH or vasodilator prostanoids. Overall, our data demonstrate increases in compliance and BK-mediated relaxation but only in the mesenteric artery of healthy serelaxin-treated rats.

Previous studies have shown *Rxfp1* gene expression in small renal and mesenteric arteries, as well as thoracic aortae isolated from mice and rats[26,27] and in human subcutaneous arteries.[18] In the rat uterine artery, $RXFP_1$ protein is predominantly localized to the vascular smooth muscle. Our data now demonstrate $RXFP_1$ in endothelial cells of intact mesenteric arteries of rats and humans, and veins, aorta and the vena cava of rats. Importantly, the large pulmonary and femoralartery and femoral vein had few $RXFP_1$-positive endothelial cells illustrating differences between vessel types. These data represent a significant advance in our understanding of serelaxin's vascular actions because they illustrate the diversity in $RXFP_1$ expression be- tween arteries and veins, and suggest that the effects of serelaxin vary across the vasculature.

To test our hypothesis that region-specific vascular responses to serelaxin are due, in part, to differential localization of $RXFP_1$ in endothelial and vascular smooth muscle cells, we first analyzed passive mechanical wall properties in mesenteric and femoral arteries and veins because the cellular distribution of $RXFP_1$ differed between these two vascular beds. In the mesenteric artery, serelaxin treatment reduced passive arterial wall stiffness, which is consistent with previous studies.[7,21] This was associated

with a significant increase in ID, without a change in WT. Serelaxin treatment also increased volume compliance in the low pressure range, which reflects the ability of the artery to lengthen. Conversely, there were no changes in circumferential wall stiffness or volume compliance in the femoral artery. We suggest that this is not related to RXFP1 localization because both vessel types have RXFP1 in the vascular smooth muscle. An alternative explanation for the lack of a response in the femoral artery is that the dose of serelaxin and duration of infusion may not have been sufficient to reach threshold levels to induce substantial vascular remodeling. The media of the femoral artery is thicker and consists of more ECM than a resistance artery, so it is also possible that a greater extent of arterial remodeling is required to influence wall stiffness and compliance. Another hypothesis is related to the known effects of shear stress on the vascular wall, which activates vascular remodeling pathways.[36] Relaxin decreases vascular resistance [37] and increases blood flow velocity.[3] It is therefore possible that the differential effects of serelaxin on vascular remodeling between resistance and capacitance arteries are related to differences in blood flow (or shear stress). Only one study has reported improvements in elasticity in a large capacitance artery (carotid artery) after relaxin treatment but this was in senescent, spontaneously hypertensive rats.[25] The duration of relaxin treatment in that study was also considerably longer (2 wk) and the dose higher (~20 μg/kg/h). Consistent with previous studies,[17] serelaxin treatment had no effect on wall stiffness in mesenteric and femoral veins. However, this was not explained by the absence of RXFP1 in the vascular smooth muscle, and is more likely related to differences in venous wall structure or the relationship between shear stress and vascular remodeling.

The consensus view is that relaxin promotes vascular remodeling and compositional changes in the ECM of arteries. Our study showed increases in pressurized ID, but no significant change in OD or WT of mesenteric arteries in serelaxin-treated rats. Relaxin treatment increased ID and WT of brain parenchymal arterioles[23] and increased unpressurized wall area, wall-to-lumen area ratio and smooth muscle cell density in small renal arteries.[21] Conversely, Xu *et al.*[25] reported decreases in the OD, ID and media thickness in the aorta of relaxin-treated, aged hypertensive rats. Vascular wall compliance is also dependent on the relative contribution and organization of collagen and elastin.[38] In mice administered relaxin, the increase in small renal artery compliance was associated with a decrease in collagen but no change in elastin.[21] Similarly, in aged relaxin-treated hypertensive rats there was a reduction in collagen content, but not elastin, in the aorta resulting in an increase in elastin: collagen ratio.[25] This is thought to be a key factor in altering vascular stiffness. In our study, there was no change in total soluble collagen or elastin, or the elastin:collagen ratio in the mesenteric or femoral arteries of serelaxin-treated rats. However, we did observe a

trend toward a reduction in collagen in the aorta, which suggests that serelaxin may act on some arteries to induce compositional changes in the ECM.

We also investigated the potential differential effects of 5 d *in vivo* serelaxin administration on smooth muscle function and endothelium-dependent relaxation in arteries and veins. As shown by others,[13,17] we found no change in PE-evoked contraction in arteries and veins from serelaxin-treated rats. Other studies in female rats reported a reduction in the sensitivity to PE in mesenteric arteries after *in vivo* relaxin treatment[14,15] but the arteries were bathed in PSS containing 30 ng/ml relaxin in the organ bath and plasma relaxin levels were 60–70 ng/ml. Therefore, it is possible that differences between studies are associated with different concentrations of relaxin in the mesenteric vessel bed. We found that 5 d *in vivo* serelaxin had no effect on ACh-evoked relaxation in arteries and veins. Previous studies have shown that ACh-mediated relaxation of rat aortic rings was impaired by treatment with tumor necrosis factor α (TNF-α), and subsequently improved by coincubation with relaxin *in vitro* for 48 h.[39] This study also demonstrated that *in vivo* serelaxin treatment enhanced BK- evoked relaxation in mesenteric arteries, and involved NO pathways.

The contribution of NO to relaxin-mediated dilation in small renal, mesenteric and human subcutaneous arteries has been demonstrated previously by endothelium removal and NOS inhibitors.[13,14,18] Further analysis in cultured human aortic and coronary artery endothelial cells revealed that relaxin rapidly stimulates phosphorylation of Akt (Ser473) and eNOS (Ser1177) through activation of PI3K, resulting in generation of NO.[18] Similarly, 48 h of relaxin treatment had no effect on eNOS protein expression but augmented eNOS activity *via* enhanced phosphorylation at Ser1177 and Ser633 and dephosphorylation at Thr495 in rat aortic rings with endothelial dysfunction after exposure to TNF-α.[39] We confirmed no effects of serelaxin on *eNOS* expression and suggest that serelaxin enhanced BK-mediated vasodilation through phosphorylation of eNOS which increases NO production in endothelial cells.

Previous studies on mesenteric veins showed that chronic administration of relaxin had no effect on myogenic reactivity but addition of relaxin to the perfusate resulted in a concentration-dependent relaxation in veins preconstricted with PE.[17] Relaxin was also reported to antagonize adrenergic and cholinergic vascular contractions in human saphenous veins.[40] Although there are relaxin receptors in rat mesenteric and femoral veins, our pharmacological approach showed that serelaxin treatment did not alter ACh-mediated endothelium-dependent relaxation or PE-evoked contraction in either vein. Unlike the mesenteric artery, serelaxin also had no effect on total BK-mediated relaxation in mesenteric veins.

In summary, RXFP1 is differentially localized to both endothelial and vascular smooth muscle cells in arteries and veins. Specifically, the aorta, vena cava, mesenteric artery,

and vein had significantly higher RXFP1 immunostaining in endothelial cells compared with vascular smooth muscle, whereas the femoral artery and vein and small pulmonary arteries had higher RXFP1 immunostaining in the vascular smooth muscle. All measures of vascular function (arterial stiffness and volume compliance, BK-mediated relaxation) were significantly improved in mesenteric arteries after serelaxin treatment, demonstrating that this major resistance vascular bed is a key target for serelaxin. The serelaxin-enhanced BK-evoked relaxation involved a significant contribution from NO but not EDH or vasodilatory prostanoids. Despite the localization of RXFP1 in femoral arteries and veins, and mesenteric veins, we showed no significant functional effects of serelaxin in these blood vessels.

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Chapter 6

THE RELAXIN RECEPTOR RXFP1 REGULATES VASCULAR DEVELOPMENT BY ALTERING UTERINE NK CELL AND MACROPHAGE SUBSETS IN EARLY MOUSE PREGNANCY

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ABSTRACT

The hormone relaxin signals through the relaxin family peptide receptor 1 (RXFP1) and plays an important role in maternal systemic vascular adaptation in pregnancy. In non-pregnant ovariectomized Rhesus monkeys relaxin administration increases endometrial vascularization, and macrophage and NK cell numbers. However, the role of RXFP1 signaling in the uterus during early pregnancy is unknown. Here we aimed at elucidating the role of RXFP1 in the immunovascular developments in the mouse decidua during early gestation. In RXFP1-/- mice the abundance of newly developing vessels was unexpectedly higher compared to WT mice. RXFP1-/- mice expressed significantly more endothelial cell marker CD31 in the mesometrial decidua at gd6.5 compared to WT mice. At gd12.5 the number of spiral artery cross- was higher compared to WT mice. In vitro relaxin did not directly affect endothelial tube formation by HUVEC or HMEC-1 endothelial cells. Uterine NK cells and macrophages contribute to early decidual angiogenesis. In RXFP1-/- mice the total number of decidual uNK cells (PAS+) was indistinguishable from WT mice at all time points tested. However, the decidual uNK cell population in RXFP1-/- mice was skewed towards an increase (+75%) in the PAS+DBA+ uNK cells known to produce angiogenic factors and a decrease (-34%) in PAS+DBA- cells at gd6.5 known to produce interferon. In the mesometrial lymphoid aggregate of pregnancy (MLAp), the number of PAS+DBA+ uNK cells was increased (+21%) in RXFP1-/- at gd10.5, whereas decidual PAS+DBA+ uNK cell numbers were comparable to the number in WT mice. At the gd6.5, the decidua of RXFP1-/- mice contained more macrophages with an alternatively activated (F4/80+CD206+), anti-inflammatory phenotype compared to WT mice. In conclusion, deletion of RXFP1 enhances decidual CD31 expression, skews uNK cells towards a DBA+ phenotype, and increases the number of alternatively activated macrophages during early pregnancy. We propose that early in mouse pregnancy RXFP1 signaling controls NK and macrophage cell function thereby affecting decidual vascular development.

Key words: Vascular development • angiogenesis • uterine natural killer cell • macrophage • pregnancy

INTRODUCTION

Relaxin is a peptide hormone recognized for its activities in the maternal cardiovascular system and reproductive tract during pregnancy. In humans, circulating relaxin is produced by the corpus luteum starting at the late-menstrual cycle continuing through pregnancy.[1,2] In rodents, relaxin concentrations do not start to rise in the blood before mid-gestation.[3,4] Relaxin exerts its main functions through binding to the relaxin family peptide receptor 1 (RXFP1), and induces systemic and renal vasodilation.[5]

In addition to the corpus luteum, relaxin is expressed by placental trophoblasts and decidualizing endometrial stromal cells in most mammals.[6-8] The relaxin receptor RXFP1 is expressed in several cell types including vascular smooth muscle cells, endothelial cells, trophoblasts, and macrophages.[9-11] Relaxin binds endometrial stromal cells with high affinity,[12,13] and induces decidualization of stromal cells *in vitro*.[14-16] Studies in monkeys and rats showed that administration of relaxin promotes endometrial thickening and uterine vascular growth.[17-20] Interestingly, in the ovariectomized non-pregnant rhesus monkey, relaxin administration promotes uterine vascular development and is accompanied by increases in uterine natural killer (uNK) cells and macrophages.[18,21]

Pregnancy involves an intricate relationship between conceptus-derived trophoblasts, maternal decidual vessels and leukocytes including uNK cells and macrophages.[22] In mice, adaptations following implantation include decidualization, neoangiogenesis, and trophoblast invasion; events that are associated with an increase in uNK cells and macrophages. Murine uNK cells coordinate gestation-dependent phases of decidual angiogenesis early in pregnancy.[23] These cells are heterogeneous in nature as identified by staining with Periodic Acid Schiff (PAS) and with or without binding by lectin Dolichus Bifloris Agluttine (DBA).[24] PAS+DBA+ uNK cells produce angiogenic growth factors such as vascular endothelial growth factor (VEGF) and stimulate neovascularization,[25,26] whereas PAS+DBA- uNK cells produce high amounts of IFNγ and are involved in spiral artery remodeling.[27,28]

Next to uNK cells, macrophages are the second most abundant leukocyte population in the decidua and contribute to placental development.[29,30] Decidual macrophages acquire an alternatively activated, or M2 (regulatory) phenotype, that is associated with tissue remodeling and formation of vascular networks.[31-33] Macrophages can release pro-angiogenic factors such as VEGF and angiopoietin, and promote angiogenesis.[34]

Until now the role of RXFP1 in the regulation of uterine immune cells and vascular developments during pregnancy are unknown. In this study we aimed to elucidate if the receptor for relaxin RXFP1 confers immunovascular adaptations of the uterus during early pregnancy.

MATERIALS & METHODS

MICE

Mice were maintained at the animal facility of the University of Utrecht and University of Florida. All experiments were performed in accordance with international guidelines and approved by the University Medical Center Utrecht experimental animal committee and the Institutional Animal Care and Use Committee (IACUC) of the University of Florida. Female RXFP1-/- mice on a C57BL/6J background and their wild type (WT) littermates were used.[35] The morning of plug detection was designated gestational day (gd) 0.5.

TISSUE PROCESSING

At gd6.5, 10.5 and 12.5 pregnant RXFP1-/- and WT mice were deeply anesthetized with sodium pentobarbital (50 mg/kg i.p.) and transcardially perfused with PBS followed by 4% paraformaldehyde. Individual implantation sites were isolated, post-fixed and processed into paraffin blocks. Two or three implantation sites were used from each pregnant mouse. Three to six 7 µm mid-saggital sections with a minimal distance of 50 µm apart were cut and mounted on glass slides.

IMMUNOHISTOCHEMISTRY

Tissue sections were deparaffinized, rehydrated and blocked before overnight incubation (4˚C) with rat-anti-CD31 (IgG2a; Abcam, Cambridge, UK), biotin conjugated DBA lectin (Sigma-Aldrich, St. Louis, MI, USA), rat-anti-F4/80 ($IgG_{2b;}$ Cederlane, Burlington, ON, Canada) or goat-anti-CD206 (R&D Systems, Minneapolis, MN). Tissue sections were then washed with PBS and incubated with Alexa Fluor® 488/594-fluorochrome-conjugated secondary antibodies for 1h at room temperature (all from Life technologies, Grand Island, NY). Nuclei were stained with DAPI.

Uterine NK cell subsets were identified based on Periodic Acid Schiff (PAS) and DBA lectin reactivity.[36] In brief, sections were incubated overnight with biotin conjugated DBA lectin (Sigma-Aldrich) and visualized by staining with the ABC nickel-enhanced diaminobenzidine method (Vector Laboratories, Burlingame, CA, USA). Slides were subsequently incubated in 1% periodic acid Schiff's reagent (Sigma-Aldrich) and 0.5% sodium bisulfite solution (Sigma-Aldrich). Slides were counterstained with Harris's Hematoxylin (Sigma-Aldrich).

For quantitative analysis tissues sections were either photographed with a Zeiss Axio Lab.A1 equipped with AxioCam ECc5 camera (Zeiss, Jena, Germany) or a Zeiss Axio Observer Z1 fluorescence microscope equipped with AxioCam MRm camera and AxioVision software where appropriate. Uterine NK cell (PAS+DBA- and

PAS+DBA+) and macrophage (CD_{206}+) numbers were counted manually in microphotographs covering complete cross-sectional areas of the mesometrial decidua, as well as the anti-mesometrial decidua. Quantification of CD_{31} was performed with ImageJ software (http://rsb.info.nih.gov/ij/, ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA). Positive staining area was identified by measuring the area with a staining intensity above a set threshold. All analyses were performed in a blinded fashion.

MORPHOLOGIC ANALYSIS OF BLOOD VESSELS

Hematoxylin and Eosin stained tissues sections were used to determine vessel-to-lumen ratios of the main decidual arteries. Vessel-to-lumen ratios were calculated by dividing the luminal cross-sectional area by the cross-sectional area of the whole vessel. The number of total spiral arteries in decidua was counted manually in 6 sections for each implantation site. Gross morphology of the implantation site was analyzed by measuring the thickness of the mesometrial decidua and MLAp.

EFFECT OF HUMAN RECOMBINANT RELAXIN (rhRLX) ON ENDOTHELIAL CELL TUBE-FORMATION ASSAY

Primary human umbilical vein endothelial cells (HUVECs; Millipore, Billerica, MA, USA) were maintained in EndoGRO™-LS complete medium (Millipore). The microvascular endothelial cell line HMEC-1[37] was maintained in $MCDB_{131}$ medium (Life Technologies) with 10% fetal calf serum, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Life Technologies), 50 nM hydrocortisone (Sigma), and 10 ng/mL epidermal growth factor (PeproTech, Rocky Hill, NJ). HUVECs (passage 4-6) or HMEC-1 (passage 14) were seeded on 10 µL growth factor reduced Matrigel (concentration 8.0 mg/mL, BD Biosciences, Franklin Lakes, NJ, USA) in a µ-Slide angiogenesis (Ibidi, Martinsried, Germany) in 50 µL serum and growth factor free medium. Cells were incubated for 6-h with different doses of rhRLX (Corthera, Inc., San Mateo, CA). The extent of network formation was quantified by determining the total tube length with ImageJ software and the Angiogenesis Analyzer plugin for ImageJ (http://rsb.info.nih.gov/ij/).

STATISTICS

Data are expressed as mean ± SEM. Differences between $RXFP_1$-/- and WT were compared using Student's *t* test, one-way ANOVA followed by Dunnett's post-hoc test or two-way ANOVA followed by Sidak's multiple comparison analysis where appropriate using GraphPad Prism V6 (GraphPad, La Jolla, CA, USA). $P<0.05$ was considered to be statistically significant.

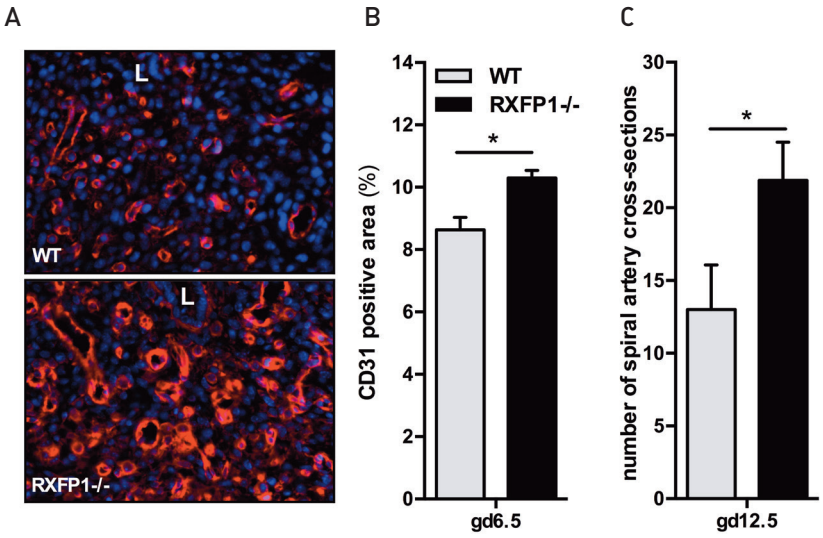
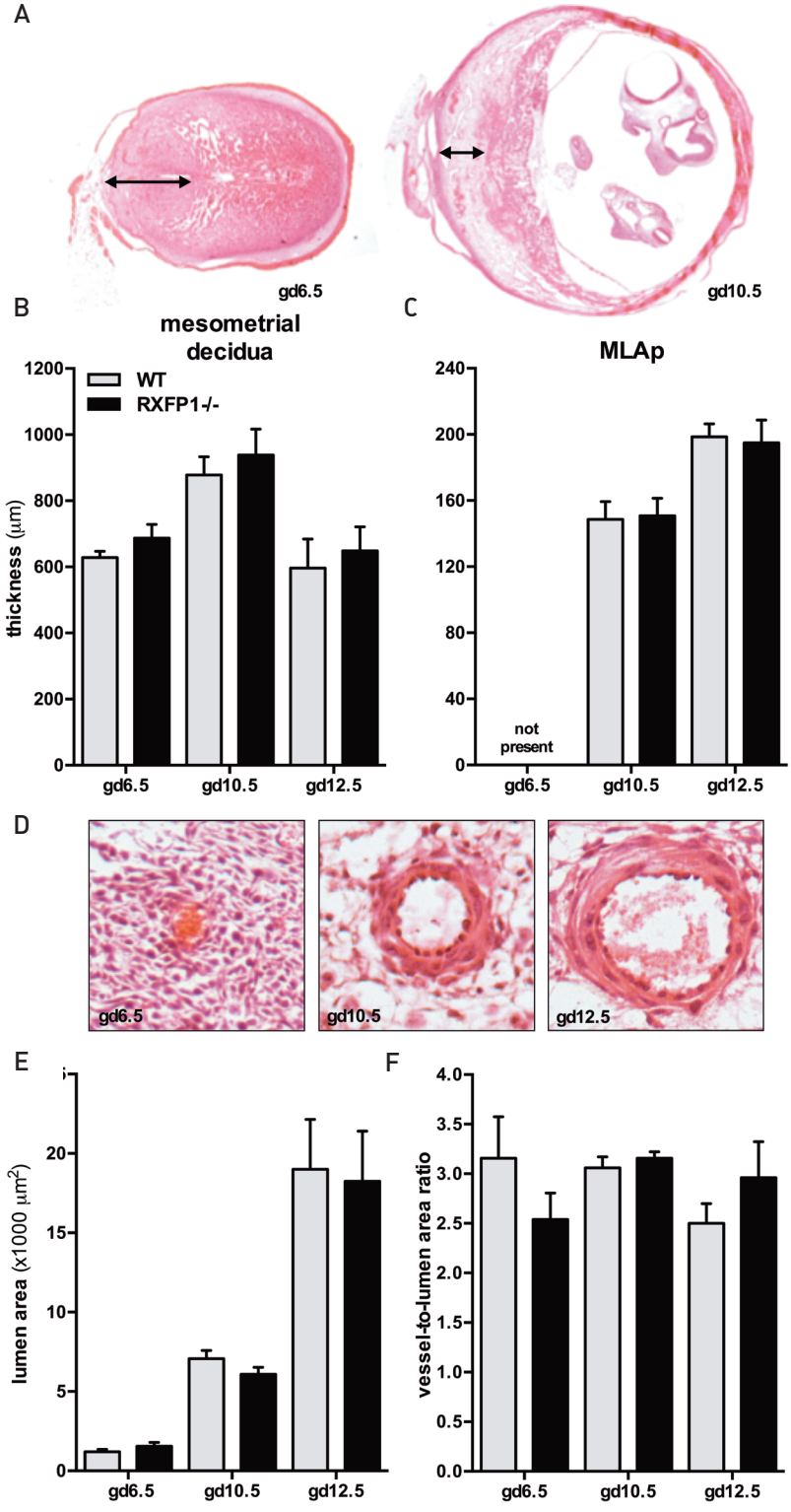


Figure 1 ↑
Decidual vascular development is impaired in RXFP-/- mice in early pregnancy. (**A**) Representative images of CD_{31} expression in the mesometrial decidua of $RXFP_1$-/- and WT mice at gd6.5. L: uterine lumen. (**B**) Percentage CD_{31}+ area in the mesometrial endometrium at gd6.5 (*P<0.05). (**C**) The number of spiral artery cross-sections in the mesometrial decidua at gd12.5 (*P<0.05).

RESULTS

VASCULAR DEVELOPMENT IN DECIDUA OF RXFP1-/- MICE

To assess early decidual vascular development in $RXFP_1$-/- and WT mice, we identified endothelial cells by CD_{31} expression at gd6.5. The structure of the vascular plexus in the mesometrial decidua of $RXFP_1$-/- mice exhibits a different morphology, characterized by larger external diameter, lumen area and wall thickness at gd6.5 (**Figure 1A**). The total area of the mesometrial decidua expressing CD_{31} was increased by 20% compared to WT mice at gd6.5 (**Figure 1B**). To address whether the increase CD_{31} expression affected the vascularization later in pregnancy, we quantified the number of vessels in the decidua at gd12.5 At gd12.5 the number of spiral artery cross-sections was 68% higher in $RXFP_1$ deficient mice (**Figure 1C**). These vessels did not show differences in morphology in the $RXFP_1$-/- (data not shown).



← Figure 2

Gross morphology of implantation site and spiral arteries in RXFP1-/- compared to their congenic controls. (**A**) Representative picture of the morphology of mouse implantation site at gd6.5 and gd10.5. The arrows indicate the distinct anatomical layers (mesometrial decidua and lymphoid aggregate of pregnancy; MLAp). (**B**) Thickness of the mesometrial decidua at gd6.5, gd10.5 and gd12.5. (**C**) Thickness of the MLAp at gd6.5 and gd12.5. (**D**) Representative pictures of spiral artery morphology at gd6.5, gd.10.5 and gd.12 in WT mice. (**E**) Vessel lumen cross-sectional area of spiral arteries in the mesometrial decidua at the different gestational ages. (**F**) Extent of spiral artery outward remodeling in the mesometrial decidua determined by the vessel-to-lumen area ratio.

GROSS MORPHOLOGY OF THE IMPLANTATION SITE AND SPIRAL ARTERY REMODELING

To investigate whether RXFP1 deficiency affects gross morphology of the implantation site, we measured the thickness of the mesometrial decidua and MLAp (**Figure 2A**). The size of these placental structures did not differ significantly between RXFP1-/- and WT mice at each time point studied (**Figure 2B, C**). To determine whether deletion of RXFP1 affects the extent of spiral artery remodeling, the cross-sectional area of the spiral arteries in the mesometrial decidual was measured (**Figure 2D**). The cross-sectional area of the vessel lumen increased over time. However, at all gestational ages tested the cross-sectional lumen area was comparable between RXFP1-/- and WT mice (**Figure 2E**). The vessel-to-lumen area ratios were similar between genotypes and did not change with advancing gestational age (**Figure 2F**).

RELAXIN DOES NOT ALTER ANGIOGENESIS IN VITRO

To determine whether increased CD31 expression in the mesometrial decidua of RXFP1-/- at gd6.5 and the higher abundance of vessels at gd12.5 could be the results of direct relaxin-RXFP1 effects on endothelial cells. We determined whether recombinant human relaxin (rhRLX) stimulates endothelial cell tube formation in a Matrigel angiogenesis assay (**Figure 3A**). HUVECs or HMEC-1 cells were stimulated with increasing doses of rhRLX, and tube formation was followed over time. Relaxin at a dose range of 0.1-100 ng/mL did not affect HUVECs or HMEC-1 endothelial cells tube formation (**Figure 3B,D**).

UTERINE NK CELLS IN RXFP1-/- MICE

Next we determined whether differences in vascular development in in RXFP1-/- mice were associated with alterations in uNK cell numbers and uNK cell subsets (**Figure 4A**). Over the course of pregnancy the total number of uNK cells (PAS+) increased, with the highest numbers observed at gd10.5 after which numbers declined. At gd6.5, the mesometrial decidua of RXFP1-/- contained 75% more PAS+DBA+ uNK cells, whilst the number of PAS+DBA- uNK cells was reduced with 34% compared to wild type mice (**Figure 4B**). At gd10.5 and gd12.5 the PAS+DBA- uNK cell subset contributed only 3-5% to the total uNK cell numbers and at these time points the number of PAS+DBA+ uNK cell population was indistinguishable between WT and RXFP1-/- mice. Similarly, the PAS+DBA+ numbers did not differ between genotypes at gd10.5 and gd12.5. The total uNK cell number in the mesometrial decidua did not differ between genotypes at gd6.5, gd10.5 and gd12.5 (**Figure 4B**). From mid-gestation onwards (>gd10.5) a large population of uNK cells resided in the mesometrial lymphoid aggregate of pregnancy (MLAp). Interestingly,

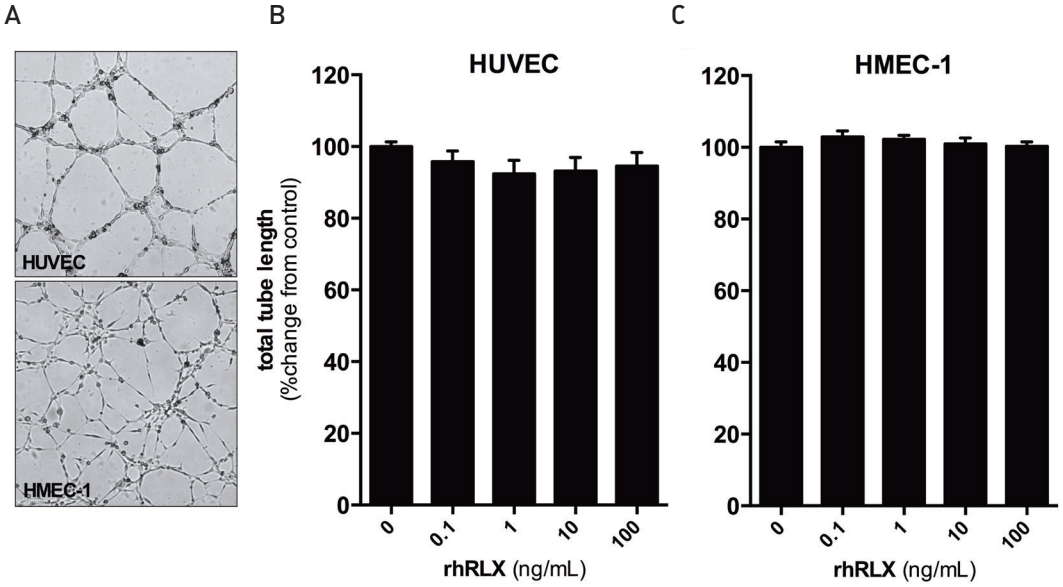


Figure 3 ↑

Recombinant human relaxin (rhRLX) does not directly affect endothelial tube formation *in vitro*. (**A**) Representative image of HUVEC and HMEC-1 tube formation at 6h. (**B**) Total tube length of HUVECs after 6h incubation with control or rhRLX supplemented media at different concentrations (n=12 combined from 3 independent experiments). (**C**) Total tube length of HMEC-1 after 6h incubation with control or rhRLX supplemented media at different concentrations (n=6).

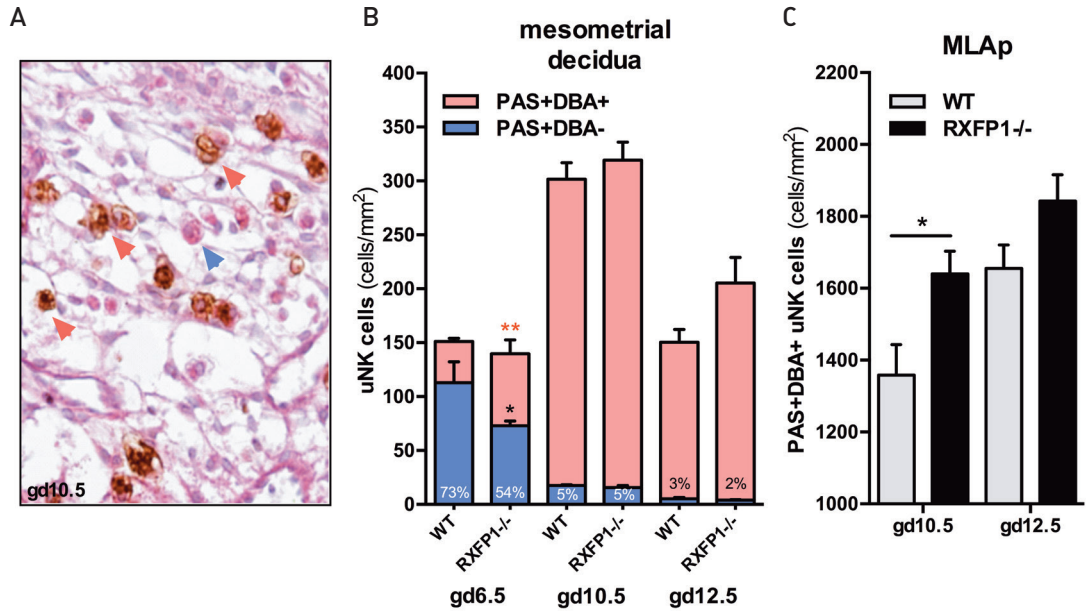


Figure 4 ↑

Uterine NK cells in RXFP1-/- mice are skewed towards a PAS+DBA+ phenotype. (**A**) PAS/DBA lectin double staining. Blue arrowhead indicates PAS+DBA- uNK cell; red arrowheads indicate PAS+DBA+ uNK cells. (**B**) Number of PAS+DBA- and PAS+DBA+ uNK cells in the mesometrial decidua at gd6.5, gd10.5 and gd12.5 (*P<0.05, **P<0.01 for RXFP1-/- vs. wild type). (**C**) Number of DBA+ cells in the mesometrial lymphoid aggregate of pregnancy (MLAp) at gd10.5 and gd12.5 (*P<0.05).

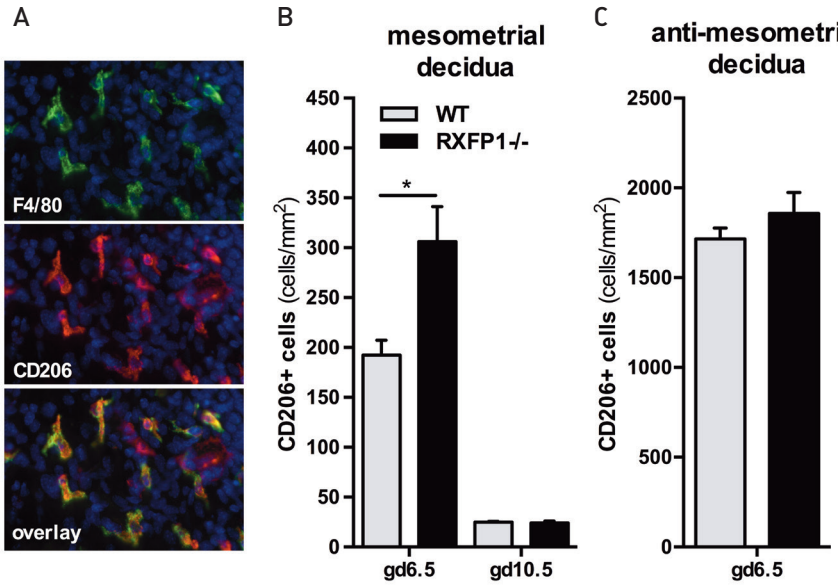


Figure 5 ↑
RXFP1 signaling regulates F4/80+CD206+ macrophages in the early mouse implantation site. (**A**) Exemplar image of F4/80 (green) and CD206 (red) double staining. Almost all macrophages in the mouse implantation site are F4/80+CD206+ (~98%). (**B**) Number of F4/80+CD206+ cells in the mesometrial decidua at gd6.5 and gd10.5 (*P<0.05). (**C**) Number of F4/80+CD206+ cells in the anti-mesometrial decidua at gd6.5.

in the MLAp of RXFP1-/- mice, the number of PAS+DBA+ uNK cells was increased by 20% compared to WT mice at gd10.5 (**Figure 4C**). At gd12.5, the number of PAS+DBA+ uNK cells in the MLAp was not statistically different between RXFP1-/- and WT.

DECIDUAL MACROPHAGES WITH AN ALTERNATIVELY ACTIVATED PHENOTYPE IN RXFP1-/- MICE

Next, we investigated the role of RXFP1 in the regulation of decidual macrophages in early pregnancy. In RXFP1-/- and WT mice the majority (~98%) of decidual F4/80+ macrophages expressed the M2 marker CD206 (**Figure 5A**). In the decidua of RXFP1-/- mice the number of F4/80+CD206+ macrophages was significantly increased by 60% in comparison to WT mice at gd6.5 (**Figure 5B**). At the same time point, F4/80+CD206+ cell density in the anti-mesometrial decidua was similar in WT and RXFP1-/- mice (**Figure 5C**). Between gd6.5 and gd10.5 mesometrial decidual F4/80+CD206+ macrophage numbers declined and at gd10.5 macrophage numbers in the decidua basalis and MLAp were similar in both genotypes.

DISCUSSION

Relaxin signaling through RXFP1 is thought to stimulate angiogenesis, vasculogenesis and arterial remodeling.[38-40] In this study, we show that the relaxin receptor RXFP1 is involved in the regulation of decidual vascular changes and immune cell subsets in the early phases of murine pregnancy. In RXFP1 deficient mice CD31 expression was increased in the mesometrial decidua during early gestation (gd6.5), which was accompanied by an increase in PAS+DBA+ uNK cell numbers and a decrease in PAS+DBA- uNK cells. Moreover, the early mesometrial decidua of RXFP1-/- mice contained more alternatively activated (CD206+) macrophages. During mid-gestation (gd12.5) an increased number of cross sections of vessels was present in the mesometrial decidua of RXFP1-/- mice. Importantly, relaxin did not affect HUVEC and HMEC-1 endothelial tube formation *in vitro* suggesting that regulation of decidual vascular development is not under direct control of relaxin signaling. Therefore, we hypothesize that the relaxin receptor RXFP1 is involved in the regulation of early vascular development in the murine decidua by regulating the number of PAS+DBA+ uNK cells and CD206+ macrophages in the mesometrial decidua.

In non-pregnant ovariectomized rhesus monkeys systemic administration of relaxin increased the number of blood vessels and immune cells in the endometrium.[18,25] On the basis of these observations we expected that absence of the relaxin receptor RXFP1 would attenuate blood vessel formation in association with a decrease in the density of uNK cells and macrophages in the decidua during pregnancy. Our results may indeed suggest a role for RXFP1 signaling in the regulation of vascular development in the gravid murine uterus, since we observed an increased abundance of spiral artery cross sections at gd12.5 and increased CD31 expression at gd6.5 in RXFP1-/- mice. Currently we do not know if the increased CD31 expression at gd6.5 is a consequence of increased angiogenesis or a thicker vessel wall of the vascular structures. The increased number of vascular cross sections at gd12.5 may be the result of increased vascular development but may also be caused by more tortuous vessels in RXFP1 deficient mice.

During the early phases of murine gestation, RXFP1 deficiency increased the DBA+ subset of uNK cells and the CD206+ macrophage subset. RXFP1 is the native receptor for relaxin.[41] Relaxin may also bind RXFP2 but only in humans and at supraphysiological concentrations.[3] Therefore it is unlikely that the effects observed in RXFP1-/- mice are due to effects of relaxin acting on RXFP2 receptors.[42] Species differences may explain divergence in the pattern and function of relaxin.[43] In rodents relaxin starts to circulate in the second half of pregnancy (>gd10.5),[8] whereas in humans relaxin is already present at the start of pregnancy.[1,2] Thus, at the time points when we observed changes in

vascularization and leukocyte numbers between genotypes, levels of circulating relaxin are undetectable in mice. Relaxin is also produced locally by decidual stromal cells and trophoblast cells.[7,12] Currently the course and magnitude of relaxin expression in the decidua remains to be established. We propose that RXFP1 is involved in mediating the effects of relaxin produced locally in the endometrium during early pregnancy.

An important question is how RXFP1 controls decidual vascular development during pregnancy. The literature shows conflicting evidence on the role of relaxin in angiogenesis. In wound sites relaxin stimulates vascular endothelial growth factor expression and angiogenesis.[44] However, in vivo relaxin administration did not induce de novo angiogenesis.[45] Early studies have shown that relaxin promotes HUVEC endothelial tube formation;[46] We show here that in vitro relaxin does not promote endothelial tube formation in embryonic as well as adult endothelial cells. Thus the observed changes in vascular development in the decidua of RXFP1-/- mice during early gestation is likely not mediated via direct relaxin-RXFP1 effects on endothelial angiogenesis, but it might be mediated through indirect effects on immune cells. Indeed, at gd6.5 we did observe an increase in in PAS+DBA+ uNK cells that are known to have angiogenic properties.[26] Moreover, we observed an increase in CD206 expressing decidual macrophages that have also been implicated in the regulation of uterine vascular adaptations during early pregnancy.[47] In particular M2-like macrophages are thought to promote the formation of vascular networks.[31,32] Thus, contrary to our original hypothesis, we now propose that RXFP1 negatively regulates the number of uNK cell subsets and macrophages with angiogenic properties, thereby controlling early vascular development in the gravid mouse uterus.

How does RXFP1 control leukocyte populations in the uterus?[48] We have preliminary evidence that RXFP1 is expressed in DBA+ uNK cells and early studies have shown that uterine macrophages express RXFP1 and respond to relaxin.[49] In mice, the majority of DBA+ cells arise from progenitors in the bone marrow that home via the circulation to the uterus; whereas DBA- uNK cells are not derived from progenitor bone marrow cells that home to the uterus.[24,48] Possibly, activation of RXFP1 restricts this PAS+DBA+ uNK cell subset from homing to the uterus.
Similar to uNK cells, we observed an increase in F4/80+CD206+ macrophages. Relaxin has been shown to inhibit macrophage migration directly.[50] Moreover, endothelial CD31 promotes extravasation of leukocytes,[51] and relaxin reduces TNFα-induced CD31 expression in endothelial cells.[52] In RXFP1-/- mice we observed increased CD31 expression at gd6.5. Thus, RXFP1 deficiency may stimulate recruitment of monocytes through promoting macrophage migration and endothelial CD31 expression. Thus, we speculate that RXFP1 signaling impairs uterine monocyte and DBA+ uNK cell homing from the circulation into the uterus.

In conclusion, we show for the first time that RXFP1 regulates uNK cell and macrophage subsets during early gestation. Absence of RXFP1 signaling is associated with increased expression of CD31 early in pregnancy and higher abundance of vascular cross sections. Our data suggest that deficiency in RXFP1 results in a change in the composition of decidual immune cell subsets in the mouse decidua during early pregnancy. Circulating relaxin was already known for regulating the systemic vascular adaptations during pregnancy and our data may support a role for RXFP1 signaling in the local regulation of immunovascular changes in the early gravid uterus. Thus, relaxin-RXFP1 signaling may play an important role in the timely organization of pregnancy-compatible immune-mediated vascular adaptations. Further studies are needed to unravel the exact role of RXFP1 signaling in the decidua during early pregnancy.

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Chapter 7

CIRCULATING PREGNANCY HORMONE RELAXIN AS A FIRST TRIMESTER BIOMARKER FOR PREECLAMPSIA

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ABSTRACT

Objective: Preeclampsia, a multi-system hypertensive disorder, is associated with perturbations in the maternal cardiovascular system during early pregnancy. The corpus luteal hormone relaxin, a potent vasodilator, may contribute to physiological circulatory changes especially in early gestation when circulating levels are highest. This study investigated whether first trimester circulating relaxin may be a suitable biomarker for the early prediction of preeclampsia.

Methods: Relaxin was initially measured in first-trimester samples of women who developed late-onset preeclamptic (LO-PE; delivery ↘34 weeks; n=33) and uncomplicated pregnancies (n=25) in Pittsburgh, USA. Subsequently, to expand the cohort numbers, relaxin was measured in women who developed LO-PE (n=95), early-onset preeclamptic (EO-PE; delivery <34 weeks; n=57), and uncomplicated pregnancies (n=469) in Utrecht, the Netherlands.

Results: In the Pittsburgh subjects, low relaxin levels (lowest centile: <p10) showed an adjusted odds ratio (OR) of 5.29 (95%CI 1.10-25.5) for LO-PE. In the Utrecht population, low relaxin levels (<p10) demonstrated adjusted ORs of 1.45 (95%CI 0.54-3.90) and 2.03 (95%CI 1.06-3.88) for EO-PE and LO-PE respectively, the latter increasing to an adjusted OR of 3.18 (95%CI 1.41-7.20) with newborn weight <10%. Serum relaxin concentrations improved the detection rate of a previously derived prediction model for LO-PE from 42.5% to 45.1% at a fixed 10% false-positive rate.

Conclusion: These results suggest a pathophysiologic role for low relaxin levels in pregnancies that later develop LO-PE, perhaps through compromise of maternal cardiovascular adaptation. However, relaxin only shows modest improvement in the performance of prediction models, which does not support its clinical implementation as a biomarker.

Key words: Relaxin • preeclampsia • small for gestational age infant • corpus luteal hormone • prediction

INTRODUCTION

Preeclampsia, a gestation-specific hypertensive syndrome affecting 3-5% of all pregnant women, is a leading cause of maternal and perinatal morbidity and mortality.[1,2] The exact etiology of preeclampsia remains to be elucidated, although it is widely believed that preeclampsia is a consequence of an inadequate maternal vascular response to placentation during early pregnancy.[3-6] Due to impaired placental function, women with preeclampsia often give birth to a small for gestational age (SGA) infant.[7,8] In most cases (>75%) preeclampsia symptoms have a late-onset, developing after 34 weeks of gestation (LO-PE).[9] Early-onset preeclampsia (EO-PE) is associated with more severe placental pathology, a higher rate of fetal growth restriction and increased risk of cardiovascular disease later in life.[10-12] Therefore, major challenges of modern obstetrics include the *early identification* of pregnancies at high risk for preeclampsia and subsequent optimization of *maternal vascular adaptations* to reduce the prevalence of disease or prevent development of severe maternal complications and assure normal fetal development.[3,13,14]

Relaxin, a peptide hormone secreted by the corpus luteum into the maternal circulation during the late secretory phase of the menstrual cycle and in pregnancy is emerging as an important regulator of the maternal vascular adaptations to pregnancy.[15-19] Moreover, relaxin holds promise as a novel therapeutic intervention for hypertensive pathologies, such as reduction of vascular wall stiffness.[20-22] A low first trimester serum relaxin concentration is associated with increased blood pressure in the third trimester of pregnancy.[23] Moreover, pregnancies achieved by oocyte donation that lack a corpus luteum, have no detectable circulating relaxin and are at increased risk for hypertensive disorders of pregnancy including preeclampsia.[24-26] Locally at the maternal-fetal interface relaxin may be important in the vascular preparation of the endometrium for placentation. In a non-human primate model of early pregnancy relaxin was shown to stimulate vascularization as demonstrated by a significantly higher number of arterioles in the endometrium.[27-29] In vitro studies corroborate these findings as relaxin was shown to be a potent inducer of human endometrial maturation (decidualization) and increases expression of angiogenic factors such as vascular endothelial growth factor in endometrial cells.[30-33] We therefore hypothesized that women with low concentrations of circulating relaxin could experience defects in the establishment of a functional maternal-fetal interface or fail to adequately vasodilate in early pregnancy, predisposing them to develop preeclampsia. If so, relaxin may be a suitable first trimester biomarker in the early prediction of preeclampsia.

MATERIALS AND METHODS

STUDY POPULATIONS AND ETHICS STATEMENT

We first performed a prospective pilot study at Magee-Womens Research Institute at the Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh, Pittsburgh, PA, USA ('Pittsburgh cohort'). To corroborate the findings of this initial study we used a large Dutch nested case-control cohort collected at the Center for Infectious Disease Research, Diagnostics and Screening at the National Institute for Public Health and the Environment, Bilthoven, The Netherlands ('Dutch cohort'). The University of Pittsburgh Institutional Review Board (protocol 0404159) and the Medical Ethical Committee of the University Medical Center Utrecht (protocol 11-002) approved the research protocols for the Pittsburgh and Dutch studies respectively.

The Pittsburgh population was a matched nested case-control study with samples derived from a longitudinal, prospective cohort of pregnant women studied for the development of preeclampsia and adverse pregnancy outcomes. The cohort consisted of 37 women who developed LO-PE and 25 normotensive women with uncomplicated pregnancies matched for gestational age of sample collection, parity, and race. Blood samples were collected at 5^{+0}-12^{+1} gestational weeks and plasma was aliquoted and stored at -80°C until analysis. Maternal characteristics were obtained through interviews and detailed medical record abstraction and review. Pregnancy outcomes, including the diagnosis of preeclampsia, were determined retrospectively based on medical chart review by a jury of research and clinical investigators.

The Dutch population was a nested case-control cohort with serum samples derived from a large national cohort of women participating in the routine first trimester Down syndrome screening and has been used for previous studies by our group.[34,35] In short, blood samples were collected at 9^{+1}-13^{+6} gestational weeks and serum was aliquoted and stored at -80°C until analysis. Pregnancy outcomes including chromosomal disorders, date of birth, birth weight and hypertensive disorders (preeclampsia, HELLP syndrome pregnancy induced hypertension) were collected through self-reporting of participating women. By follow-up of self-reported cases diagnosis of 57 EO-PE, 95 LO-PE and 469 uncomplicated pregnancies was confirmed by consultation with the participating clinics, where also maternal characteristics were obtained (i.e. medical history, parity, weight, height, first trimester mean arterial pressure [MAP], smoking status) that were recorded by a midwife or gynecologist.

OUTCOME MEASURES

Preeclampsia was defined as the new onset of hypertension (≥ 140/90 mmHg) after 20 weeks of gestation measured on at least two separate occasions at least four hours apart, combined with the presence of proteinuria (a 24-hour collection of urine with ≥ 300 mg/24h or at least 2+ by dipstick on a spot urinalysis), according to the criteria of the International Society for the Study of Hypertension in Pregnancy.[36] EO-PE was defined as preeclampsia necessitating delivery <34 weeks gestational age, and LO-PE as preeclampsia in pregnancies delivering ≥34 weeks.[9] To calculate birth weight z-score, growth charts from the Dutch Perinatal Registry were used (http://perinatreg.nl).[37] SGA was defined as a birth weight under the 10th centile.

MAP was calculated by adding 1/3 of the pulse pressure (difference between diastolic and systolic blood pressure) to the diastolic pressure: *MAP = 1/3 (SBP-DBP) + DBP*.

SAMPLE ANALYSIS

Sample analysis for the Pittsburgh cohort was performed at R&D Systems, Minneapolis, MN, USA. The Dutch cohort was analyzed at the Laboratory for Prenatal Screening, National Institute for Public Health and the Environment, The Netherlands. Samples were analyzed blinded for outcome in duplicate by the Human relaxin-2 Quantikine enzyme-linked immuno-sorbent assay (ELISA), according to the manufacturer's instructions (DRL200, R&D Systems, Abingdon, United Kingdom). All measurements were above the detection limit of the ELISA kit (assay range 7.81-500 pg/mL). The ELISA kit has an intra- and inter-assay coefficient of variance of 3.2% and 7.3% respectively. Values with an intra-assay coefficient of variance above 15% were excluded from analysis (Pittsburgh cohort n=0; Dutch cohort n=2).

STATISTICAL ANALYSIS

Study population characteristics were expressed as numbers and percentages for categorical variables and median and interquartile ranges (IQR) for continuous variables and were compared between preeclampsia cases and controls using Fisher's Exact and Mann-Whitney U tests, respectively. Bonferroni corrections for multiple testing were applied when both EO-PE and LO-PE were compared to controls. Relaxin concentrations were also expressed as median and IQR. Based on the centiles of the relaxin concentrations in the control population, two cut-off points were determined at the 25th (p25) and the 10th (p10) centile, respectively. Subsequently, to study the association between relaxin concentrations and preeclampsia, unadjusted and adjusted odds ratios (OR) were calculated using multivariate logistic regression analysis. In the Dutch cohort, the LO-PE group was

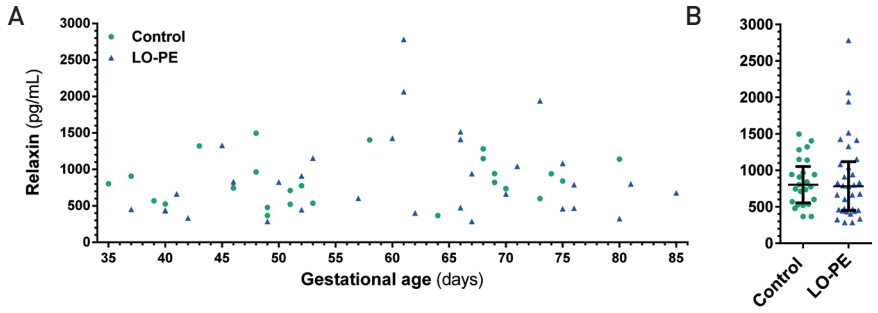

Figure 1 ↑
Distribution of relaxin concentrations in unaffected (green dots) and LO-PE pregnancies (blue triangles) of the Pittsburgh cohort. (**A**) Presented as a function of gestational age. (**B**) Summary plot per outcome group, indicated are median concentrations with interquartile range.

divided into cases with and without a small for gestational age infant. Again, multivariate logistic regression analysis was used to study associations between relaxin and preeclampsia among these subgroups. Based on the predefined cut-off point of relaxin <p10, detection rates (sensitivity) were calculated for relaxin as a single biomarker, as well as in combination with maternal characteristics (prior risk based on: age, BMI, nulliparity) and MAP. Statistical analyses were performed using SPSS (release 20.0; Chicago, IL, USA) and SAS software package (release 9.2; SAS Institute, Cary, NC, USA).

RESULTS

Demographic and clinical characteristics of the study populations are presented in **Table 1**. Data of a total of 25 control pregnant, and 33 LO-PE women were analyzed in the Pittsburgh cohort. The Utrecht cohort consisted of 469 control pregnant, 57 EO-PE and 95 LO-PE women. The LO-PE group was selected to have a SGA infant in half of the cases (n=47), the EO-PE consisted of 21% SGA cases (n=12). Women who developed preeclampsia had a higher MAP in both cohorts. In the Pittsburgh cohort, other characteristics did not reach statistical significance. In the Dutch cohort there were several statistically significant differences between the study groups. Preeclamptic women had a higher BMI, were more often nulliparous and were sampled on average three days earlier than the control group. Furthermore, preeclamptic women had more often a history of hypertensive pregnancy disorders, and reported more often to smoke. They also delivered earlier, their infants had a lower birth weight, and were smaller for gestational age.

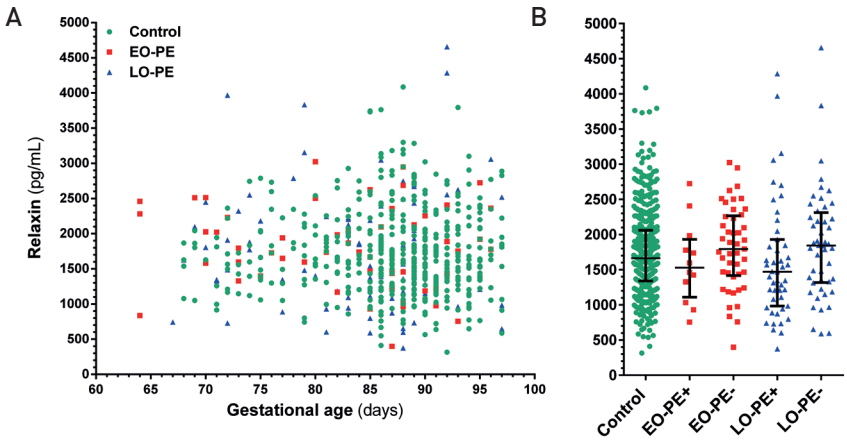

Figure 2 ↑
Distribution of relaxin concentrations in unaffected (green dots), EO-PE (red squares) and LO-PE pregnancies (blue triangles) of the Dutch cohort. (**A**) Presented as a function of gestational age. (**B**) Summary plot per outcome group, (+) with SGA infant (-) without SGA infant. Indicated are median concentrations with interquartile range.

PILOT STUDY IN 'PITTSBURGH COHORT'

In the Pittsburgh cohort relaxin concentrations did not prove to be significantly different in LO-PE from controls (median with IQR for controls 802 [571-966] pg/mL and LO-PE 783 [452-1081] pg/mL; *P*=0.561). Moreover, relaxin concentrations showed no correlation with gestational age in the control group (**Figure 1**). However, significantly more women in the LO-PE group had a relaxin concentration below the 10th centile (p10; <495.4 pg/mL) of control pregnancies, OR 4.19 (1.03-17.0) (**Table 2**). When adjusted for gestational age at sampling, BMI and smoking this OR increased to 5.29 (1.10-25.5). A cut-off value at the p25 of the control group (<570.8 pg/mL) showed a similar trend, which did not reach statistical significance (OR unadjusted 1.81 and adjusted 2.12, respectively).

VALIDATION IN 'DUTCH COHORT'

The same analysis strategy was applied for the independent data of the Dutch cohort. Relaxin concentrations were not different between EO-PE, LO-PE and controls (median with IQR for controls 1663 [1342-2061] pg/mL, EO-PE 1739 [1398-2230] pg/mL and LO-PE 1604 [1198-2198] pg/mL; *P*=0.543 for EO-PE and *P*=0.456 for LO-PE). Again there was no change in relaxin concentration with gestational age (**Figure 2**). Relaxin concentrations were higher in the Dutch cohort compared to the

Pittsburgh cohort; the latter was sampled on average 4-5 weeks earlier in pregnancy. As in the Pittsburgh cohort, a relaxin concentration below the p10 (<1054.4 pg/mL) was associated with LO-PE, OR 2.1 (P=0.015) and adjusted OR 2.03 (P=0.032) (**Table 2**). A cut-off value at the p25 (<1342 pg/mL) resulted in an OR of 1.60 (P=0.05), which was not significant after adjustment (OR 1.50; P=0.124). There was no significant association with EO-PE.

In the LO-PE group we performed a sub-analysis to assess the influence of an SGA infant (**Table 3**). In LO-PE women who delivered a SGA infant (LO-PE with SGA), the correlation with a relaxin concentration below the p25 and p10 was stronger, with adjusted ORs of 2.17 (P=0.034) and 3.18 respectively (P=0.005). In LO-PE women without a SGA infant (LO-PE without SGA), the correlations with a relaxin concentration below the p25 and p10 were lost (adjusted OR's of 1.06, P=0.871; and 1.30, P=0.582 respectively). We did not include a SGA sub-analysis for EO-PE because of low case numbers and lack of correlation between low relaxin concentrations (<p10) with EO-PE.

SCREENING PERFORMANCE IN 'DUTCH COHORT'

In the Dutch cohort, prediction models for the complete LO-PE group (n=95) and for the subgroup of LO-PE with SGA (n=47) were fitted based on maternal characteristics (prior risk), MAP and relaxin <p10 (**Table 4**). Comparison of the performance of these models showed that the highest detection rate (DR) for the complete LO-PE group was obtained for the model combining maternal characteristics, MAP and relaxin <p10, with a DR of 45.1% at a false positive rate (FPR) of 10% (16.6% gain in DR compared to the model using prior risk only; 2.6% gain in DR compared to the model using prior risk + MAP). For the prediction of LO-PE with SGA the best performing model combined maternal characteristics with relaxin <p10 only, with a DR of 37.1% at a FPR of 10% (2.7% gain in DR compared to the model using prior risk only).

DISCUSSION

To the best of our knowledge, this well-characterized two-stage study in two separate cohorts is the first to investigate relaxin as a predictor for women destined to develop preeclampsia. We observed a clear association of low (lowest centile: <p10) first trimester relaxin concentrations with LO-PE (Adjusted Odds Ratio 2.0-5.3) but not with EO-PE. However, a relaxin concentration below the 10th centile only showed a ~2.5% gain in detection rate of prediction models for LO-PE, which combined maternal characteristics (prior risk based on: age, BMI, nulliparity) with or without MAP.

Preeclampsia in combination with a SGA infant is suggestive of a distinct and more severe (placental) pathophysiology,[38,39] which generally results in different performance of biomarkers for this subgroup.[34,40] A low relaxin concentration (<p10) indeed showed a stronger association with LO-PE in combination with SGA, compared to LO-PE without SGA. This, as well as the association between low relaxin concentration with LO-PE but not with EO-PE, underscores the heterogenic nature of the preeclampsia syndrome and is in line with accumulating evidence for differences in the underlying etiology. EO-PE is thought to result from placental stress (placental preeclampsia),[12] whereas LO-PE is more likely to result from poor maternal systemic cardiovascular adaptations to pregnancy (maternal preeclampsia).[41,42] However, logic dictates that this pathophysiologic division is likely to be graded and not absolute with no definite gestational age break point. Nevertheless, the association of low relaxin with LO-PE but not EO-PE may indicate that adequate circulating relaxin levels are more important for the maternal *systemic* vascular adaptations to pregnancy, but less so for processes at the maternal-fetal interface that guarantee the establishment of a functional placenta.
The finding of a stronger association between low circulating relaxin and LO-PE in combination with SGA may at first glance seem paradoxical. However, given the higher occurrence of LO-PE compared to EO-PE,[9] SGA is more commonly associated with LO-PE. On a pathophysiological basis, low circulating relaxin in early human pregnancy may impair maternal vascular adaptation, thereby compromising uterine perfusion and fetal growth. In support of this hypothesis, relaxin was shown to increase uterine blood flow velocity in conscious rats, and to increase uterine arterial compliance.[43,44]

There was no significant correlation between relaxin and placenta-derived biomarkers measured in our previous study in the 469 control samples of the Dutch cohort: pregnancy-associated plasma protein A (PAPP-A), free beta human chorionic gonadotrophin (fb-hCG), A Disintegrin And Metalloprotease 12 (ADAM-12) and placental growth factor (PlGF) (data not shown).[34] Relaxin concentrations showed a slight inverse correlation with BMI and MAP (data not shown).

Overall strengths of the present study are the two well-characterized study populations, with assays run in duplicate and blinded fashion and the consistency in resulting findings of both cohorts adding to the generalizability. The strengths of the Pittsburgh cohort are the very well characterized population with a jury of investigators reviewing and confirming the diagnosis of preeclampsia. Limitations of the Pittsburgh cohort include the relatively small sample size and the selection of participants in a tertiary clinical setting and the lack of EO-PE cases. Furthermore,

preeclampsia cases in this cohort had a relatively high BMI adding to the stronger association with low relaxin concentrations than found in the Dutch cohort. Strengths of the Dutch cohort include the large size and recruitment from an unselected general population, as samples derived from surplus material of the national Dutch first trimester down syndrome screening program. Consequently this population has a relatively high maternal age (~33 years) compared to the Pittsburgh cohort, which may affect study comparison although both studies were internally controlled. Moreover, the gestational age at sampling was on average 4-5 weeks later in pregnancy in the Dutch cohort than the Pittsburgh cohort, which may also partly explain differences in absolute relaxin concentrations between these studies. Furthermore, other differences in study population characteristics of the Pittsburgh compared to the Dutch cohort (predominately Caucasian) may also have contributed. Another advantage of the Dutch cohort was the distinction between EO-PE and LO-PE with and without SGA infants enabling analysis of subgroups likely to have a more homogeneous underling etiology.

In conclusion, relaxin only provides a slight improvement in performance of prediction models for LO-PE, which does not support clinical implementation. Nevertheless, the association between low relaxin concentrations (<p10) with LO-PE and particularly when complicated by a SGA infant suggests a potential pathophysiologic role for deficient circulating relaxin in a subtype of preeclampsia in which there may be compromise of maternal vascular adaptations, such as systemic, renal and uterine vasodilation and the increase in global arterial compliance.

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DISCLOSURES

K.P.C. is an inventor or co-inventor of use patents for relaxin, and has served as a paid or unpaid consultant to Connetics, Corthera and Novartis. D.R.S. was a co-founder and employee of Corthera and currently employed by Novartis with equity interest in each. The other authors have no conflict of interest to disclose.

Portions of this work were presented at the Society for Gynecologic Investigation Annual Meeting 2009 (*Reprod Sci*. 16(3 Suppl): 101A, 2009).

Characteristics	*Pittsburgh cohort*	
	LO-PE	**Controls**
Controls n = 25	n = 33	n = 469
Pregnancy characteristics:		
Maternal age (y)	25.5 (20-32)	23.2 (20-31)
Maternal BMI at sampling (kg/m²)	23.1 (21.3-26.1)	26.5 (22.6-31.0)
Nulliparity	20 (80.0%)	25 (75.8%)
Gestation at sampling (d)	52 (48-69)	61 (49-71)
First trimester MAP (mmHg)	80 (77.0-86.3)	**87** (82.7-90.3)*
Smoking	7 (28%)	12 (36.4%)
Birth characteristics:		
Gestation at delivery (d)	272 (267-277)	272 (260-279)
Sex, n female (%)	8 (32%)	15 (45%)
Birthweight (gr)	3164 (2979-3413)	2948 (2638-3590)
Birthweight centile	55.5 (25.2-76.5)	36.8 (19.0-68.2)

***Pittsburgh cohort* - LO-PE**			**Unadjusted**
	controls (n=25)	**cases** (n=33)	**OR**
n < p25 (< 570.8 pg/mL)	6	14	1.81
n < p10 (< 495.4 pg/mL)	3	13	4.19
***Dutch cohort* - EO-PE**			**Unadjusted**
	controls (n=469)	**cases** (n=57)	**OR**
n < p25 (< 1342 pg/mL)	117	14	0.98
n < p10 (< 1054 pg/mL)	47	8	1.47
***Dutch cohort* - LO-PE**			**Unadjusted**
	controls (n=469)	**cases** (n=95)	**OR**
n < p25 (< 1342 pg/mL)	117	33	1.60
n < p10 (< 1054 pg/mL)	47	18	2.10

Dutch cohort		
EO-PE	**LO-PE**	
n = 57	n = 95	
33 (30-35)	33 (30-36)	32 (30-36)
22.8 (20.9-24.9)	**24.7** (21.9-28.9)*	**23.6** (21.2-26.3)*
217 (46.3%)	**46** (80.7%)*	**69** (72.6%)*
88 (85-91)	**85** (77-89)*	**85** (81-89)*
80 (73.3-86.7)	**88.2** (83.3-96.7)*	**86.7** (81.8-91.7)*
19 (4.1%)	4 (7.0%)	6 (6.3%)
283 (277-288)	**222** (213-230)*	**265** (256-275)*
238 (50.7%)	29 (50.9%)	44 (46.3%)
3560 (3250-3820)	**1300** (1040-1610)*	**2650** (2155-3065)*
57.9 (33.7-79.1)	**26.4** (11.9-52.8)*	**13.8** (4.1-45.6)*

← **Table 1**
Study population characteristics in unaffected and preeclampsia pregnancies of the Pittsburgh and Dutch cohort. Values are presented as median (IQR) or number (%).

Fisher's exact tests and Mann-Whitney U tests, both with *post hoc* Bonferroni correction were used for statistical analysis. BMI: Body Mass Index; MAP: Mean Arterial Pressure; EO-PE: early-onset preeclampsia; LO-PE: late-onset preeclampsia. * $P<0.05$.

		Adjusted		
95% CI	***p*-value**	**OR**	**95% CI**	***p*-value**
0.57-5.77	0.316	2.12	0.58-7.77	0.157
1.03-17.0	**0.045**	5.29	1.10-25.5	**0.038**
		Adjusted		
95% CI	***p*-value**	**OR**	**95% CI**	***p*-value**
0.52-1.89	0.949	0.79	0.36-1.76	0.567
0.66-3.28	0.352	1.45	0.54-3.90	0.465
		Adjusted		
95% CI	***p*-value**	**OR**	**95% CI**	***p*-value**
1.00-2.57	**0.05**	1.50	0.90-2.52	0.124
1.16-3.81	**0.015**	2.03	1.06-3.88	**0.032**

← **Table 2**
Association of relaxin concentration with EO-PE and LO-PE at different cut-off values (p25, p10 of the control population) in the Pittsburgh and Dutch cohort.

Odds ratios were adjusted for gestational age at sample collection, Body Mass Index (BMI) and smoking. OR: Odds ratio; CI: confidence interval; EO-PE: early-onset preeclampsia; LO-PE: late-onset preeclampsia.

Dutch cohort - LO-PE - SGA+			Unadjusted			Adjusted		
	controls (n=469)	**cases** (n=47)	**OR**	**95% CI**	***p*-value**	**OR**	**95% CI**	***p*-value**
n < p25 (< 1342 pg/mL)	117	20	2.23	1.21-4.12	**0.011**	2.17	1.06-4.45	**0.034**
n < p10 (< 1054 pg/mL)	47	12	3.08	1.50-6.34	**0.002**	3.18	1.41-7.20	**0.005**
Dutch cohort - LO-PE - SGA-			Unadjusted			Adjusted		
	controls (n=469)	**cases** (n=48)	**OR**	**95% CI**	***p*-value**	**OR**	**95% CI**	***p*-value**
n < p25 (< 1342 pg/mL)	117	13	1.12	0.57-2.18	0.745	1.06	0.53-2.13	0.871
n < p10 (< 1054 pg/mL)	47	6	1.28	0.52-3.18	0.591	1.30	0.51-3.31	0.582

↑ **Table 3**
Association of relaxin concentration with LO-PE pregnancies with and without a small for gestational age (SGA) infant at different cut-off values (p25, p10 of the control population) in the Dutch cohort

Odds ratios were adjusted for gestational age at sample collection, Body Mass Index (BMI) and smoking. OR: Odds ratio; CI: confidence interval; LO-PE: late-onset preeclampsia; SGA+: with small for gestational age infant; SGA-: without small for gestational age infant.

Table 4 →
Screening performance of relaxin for LO-PE. Model predicted LO-PE detection rate for a false positive rate of 10% with prior risk, MAP and relaxin <p10 in the complete LO-PE group and in the LO-PE group with a SGA infant.

Prior risk of preeclampsia was based on: maternal age, BMI and nulliparity.
MAP: mean arterial pressure; DR: detection rate; AUC: area under the curve; LO-PE: late-onset preeclampsia; SGA+: with small for gestational age infant.

Dutch cohort	**LO-PE - all** (n=95)		**LO-PE - SGA+** (n=47)	
	DR (%)	**AUC**	**DR (%)**	**AUC**
Prior risk	28,5	0,722	34,4	0,765
Prior risk + MAP	42,5	0,798	31,0	0,751
Prior risk + Relaxin <p10	30,9	0,729	37,1	0,791
Prior risk + MAP + Relaxin <p10	45,1	0,801	31,0	0,760

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Chapter 8

SUMMARY, GENERAL DISCUSSION AND FUTURE PERSPECTIVES

SUMMARY

Pregnancy represents a complex partnership between mother and fetus that requires carefully timed immunovascular adaptations in the uterine wall to provide optimal conditions for fetal development without jeopardizing maternal wellbeing. To accommodate pregnancy the gravid uterus transforms into one of the most active sites of tissue remodeling during adult life. Aberrations in maternal adaptations during early pregnancy contribute to the development of pregnancy disorders such as fetal growth restriction and preeclampsia. Pregnancy depends on the maturation of the uterine mucosa or endometrium to create optimal conditions for embryo implantation and placentation. The maturation of the endometrium is strongly regulated by maternal ovarian hormones, such as estrogen and progesterone. These hormones also contribute to other maternal adaptations during pregnancy including systemic vascular and metabolic changes. Importantly, the corpus luteal hormone relaxin is produced during pregnancy but its role in the maternal adaptation to pregnancy is still obscure.

Endometrial maturation, or decidualization, is initiated in the secretory phase of the menstrual cycle and is characterized by the exponential increase in endometrial immune cells. During early pregnancy 30-40% of total endometrial stromal cells are immune cells and therefore the decidualizing endometrium can be considered a specializing lymphoid tissue of pregnancy.[1] Natural killer (NK) cells are the most prominent maternal immune components constituting 70-80% of decidual immune cells during early pregnancy, with macrophages being the second most prominent constituting ~20% of total decidual immune cells.[2,3] Emerging evidence supports the concept that NK cells develop in the decidualizing endometrium into unique regulators of key developmental processes essential for placentation.[4] However, until now neither the regulation of these immune cells in the developing endometrium, nor the role of relaxin in this process is understood. As a consequence, it is still unclear whether a defect in these physiologic changes predisposes to complications later on in pregnancy.

The overall purpose of the projects described in this thesis was to study the regulation of uterine changes that are essential for the developmental processes of placentation. We explored the role of relaxin along with its receptor RXFP1 in the maternal adaptation to pregnancy. Moreover, we identified a novel type I interferon pathway that regulates the uterine NK (uNK) cell transcriptome and function in human pregnancy. Finally, we characterized the progressive transcriptional changes of the endometrium over the secretory phase of the menstrual cycle and first weeks of pregnancy. To this end, we defined which genes are involved in endometrial maturation and determined, whether the transcriptional regulation of these

decidualization-associated genes is disturbed early in pregnancies later on complicated by preeclampsia.

In **Chapter 2-4** we focused on the developmental and transcriptional changes in the human endometrium with emphasis on uNK cells to elucidate how the uterine niche changes during early pregnancy to provide optimal conditions for placentation. Moreover, we tested the concept that insufficient or defective endometrial and uNK cell maturation during the secretory phase of the menstrual cycle and early pregnancy precede the development of preeclampsia. In these studies we presented evidence for:

- Marked transcriptional changes in uNK cells between the first and second trimester of pregnancy (**Chapter 2**);
- Type I interferons to be important regulators of uNK cell function regarding their role in facilitating trophoblast invasion (**Chapter 2**);
- The secreted protein ISG15 to represent a novel effector of uNK cells, which promotes trophoblast invasion in a gestational age-dependent fashion (**Chapter 2**);
- Functions of uNK cells to be strictly temporal and regional regulated during human endometrial development over the course of the menstrual cycle and pregnancy (**Chapter 3**);
- Impaired endometrial maturation and/or uNK cell dysfunction in the peri-implantation period and early pregnancy in women destined to develop preeclampsia (**Chapter 4**);

In **Chapter 5-7** we focused on the pregnancy hormone relaxin and its receptor RXFP1 as contributor to systemic and uterine adaptations during pregnancy. Moreover, we tested the hypothesis that relaxin is a potential biomarker for the early prediction of preeclampsia. From these studies we learned that:

- The relaxin receptor RXFP1 is differentially expressed in endothelial cells and the adjacent vascular smooth muscle in different vascular beds, causing different effects of relaxin on vascular wall properties (**Chapter 5**);
- The relaxin receptor RXFP1 is involved in the regulation of both vascular development and the enrichment of uNK cell and macrophage subsets in decidualizing mouse implantation sites (**Chapter 6**);
- Low first trimester relaxin concentrations ($p<10$) correlate with the development of late-onset but not early-onset preeclampsia (**Chapter 7**);
- First trimester circulating relaxin has no clinically relevant potential in predicting preeclampsia (**Chapter 7**);

In this chapter we will discuss the key findings of this thesis on the importance of

decidualization and associated uNK cells, relaxin and its receptor RXFP1 for the uterine adaptation to pregnancy. First we focus on developments in the endometrium and uterine immune cells, which are essential for uneventful pregnancy. Afterwards, we comment on the impact of impaired early uterine adaptation on pregnancy outcome, with emphasis on preeclampsia. We will conclude the discussion with suggestions for future research to decipher the importance of the uterine adaptations in early pregnancy.

GENERAL DISCUSSION

UNIQUE INSIGHTS IN UTERINE IMMUNOVASCULAR ADAPTATION TO PREGNANCY

Recent studies provide convincing evidence for uNK cells to populate the uterus during early pregnancy for the purpose of regulating placental development.[4] In **Chapter 2** we provide evidence for uNK cells to acquire the capacity to stimulate placental extravillous trophoblast (EVT) invasion during early pregnancy. EVT invasion into the uterine spiral arteries is a crucial step in their remodeling to secure sufficient uteroplacental blood supply throughout pregnancy.[5] Defective EVT invasion during early pregnancy is thought to predispose to fetal and maternal placental syndromes such as preeclampsia later on in pregnancy.[6] As such, most research so far has focused on elucidating the role of EVTs in the development of a functional placenta. However, human placentation requires more than EVTs. As highlighted in **Chapter 3** a well-prepared *uterine microenvironment* that orchestrates selection, implantation and support of the developing conceptus, is another pre-requisite for the maintenance of pregnancy.[7] Hence, as postulated in **Chapter 4**, perhaps it is as much the development of the "soil" as the "seed" that determines pregnancy success.

If we envision the endometrium as the "soil" of pregnancy than endometrial decidualization can be considered as the biological process that "fertilizes" this uterine lining to prepare for placentation.[8,9] As reviewed in the context of human reproduction in **Chapter 3,** NK cells are highly responsive to changes in their local tissue microenvironment.[10] In **Chapter 4** we assembled our own Gene Ontology for the biological process of endometrial (pre)decidualization, as this endometrial maturation process is not represented in databases such as the one from the Gene Ontology Consortium (http://geneontology.org). We observed a gradual increase in the endometrial expression of regulatory cytokines, chemokines and growth factors in the course of the secretory phase (pre-decidualization) of the menstrual cycle and accelerating in first weeks of pregnancy (decidualization). These factors

are known to regulate uNK cell function. Decidualization is for example associated with increasing levels of IL-15 expression by endometrial stromal cells (ESCs) (**Chapter 3**), a key cytokine for uNK cell development (**Chapter 4**). Moreover, in line with a previous report,[11] the transcriptional changes in the endometrium during decidualization are associated with the top canonical pathway natural killer Cell Signaling. Indeed, pre-decidualization of the endometrial stroma during the secretory phase of the menstrual cycle is accompanied by the progressive increase in uNK cell numbers.[12] The latter accelerates during the subsequent decidualization, when uNK cells also undergo substantial transcriptional and functional changes, such as increased production of cytokines and angiogenic factors, to become active regulators at the maternal-fetal interface.[13,14] Taken together, these observations suggest that transcriptional and functional changes of uNK cells are indicative of the developments in the uterine microenvironment, in a period when the pre-implantation embryo adheres to the decidua, nidates and implants to start development of the placenta.

During placentation the implantation site, or placental bed, changes markedly in conjunction with the invasion of EVT cells. In **Chapter 2** we provide experimental evidence for a transcriptional change in uNK cells during this period to stimulate EVT invasion. Uterine NK cells cluster around uterine glands, spiral arteries and the trophoblast invasion front.[12,15] The accumulation of uNK cells around the uterine glands suggests a role of these cells in the regulation of uterine gland function which is essential for histiotrophic nutrition of the embryo during the first trimester.[16] In the first weeks of pregnancy, before massive invasion of EVTs, the decidualizing endometrium is characterized by initial stages of spiral artery remodeling.[5,17] Recent studies strongly support a central role of uNK cells in these early vascular adaptations by contributing to the timely switch to hematotrophic nutrition of the conceptus at the end of the first trimester of pregnancy.[17,18] Presumably, by that time the importance of this specific function of uNK cells has declined, since early second trimester uNK cells seem to have lost their capacity to induce vascular remodeling.[18] Pregnancy progression may now require the uNK cells to deliver an extra stimulus to secure ongoing EVT invasion,[4,19] as EVT cells are critical for the final stages of spiral artery remodeling.[20] Although superficial trophoblast invasion begins with embryo implantation, deeper penetration of EVT into the endometrium and inner one-third of the myometrium is a typical feature of the late first and early second trimester,[21,22] when uNK cell stimulation of this process is most profound (**Chapter 2**). The importance of the crosstalk between EVTs and uNK cells for human placentation is further illustrated by unfavorable natural killer cell receptor (KIR) combinations with HLA-C on EVTs, leading to insufficient stimulation of EVT invasion by factors

such as GM-CSF.[23,24] Thus, uNK cells enrich the endometrium during (pre) decidualization to fulfill time-dependent regulatory functions during placentation (**Chapter 3**), and with progression of early pregnancy these abundant maternal immune cells progressively interact with fetal EVTs to secure ongoing spiral artery remodeling and placentation.

How is the uterine niche regulated during early pregnancy? The majority of transcriptional changes in uNK cells that we identified with increasing gestational age (**Chapter 2**) were annotated as interferon stimulated genes (ISGs), suggesting that uNK cells are exposed to rising concentrations of interferons in the uterine niche with progressing early pregnancy. More recently we found additional evidence for increased regulation of immune cells by interferons. By comparing the transcriptome of first and second trimester macrophages (purified $CD14^+$ decidual cells) we identified a large cluster of ISGs to be up-regulated in the second relative to the first trimester, similar to what we observed in uNK cells (**Chapter 2**) (>70% of total up-regulated genes). Interestingly, the genes up-regulated by uNK cells and macrophages between the first and second trimester show strong overlap (**Figure 1A**). Interestingly, 86% of the genes in common between both cell types were recognized as ISGs (**Figure 1B**). The study presented in **Chapter 2** provides evidence for transcriptional and functional changes in uNK cells in first and second trimester pregnancy to predominantly result from stimulation by type I interferon INFα. In particular ISG15 was markedly regulated by type I interferons and we identified the potential of this cytokine-like protein to stimulate EVT invasion.
Thus, type I interferons are key regulators in the uterine niche modulating functional changes in uNK cells and macrophages in favor of placentation. However, the cells in the uterine niche responsible for their *production* are yet to be identified. Interferons were not among the secreted factors up-regulated during endometrial decidualization (**Chapter 4**), suggesting that not decidualization but rather the invading EVTs are the most likely candidates to induce up-regulation of the type I interferon pathway in decidual leukocytes. Type I interferons are produced by the placental trophoblasts in various species.[25-27] Histological analysis suggested that IFNα is also produced by EVTs in the endometrium,[27,28] further supporting the concept that in early pregnancy INFα acts as an EVT effector to regulate uterine leukocyte function.
The decidualization process that determines the uterine immune environment during early pregnancy is regulated by maternal pregnancy hormones.[9] The pregnancy hormone relaxin is known to induce changes which are typical for endometrial decidualization in several species and circulates in the human during the decidualization period.[29,30] The substantial hormonal changes characteristic for

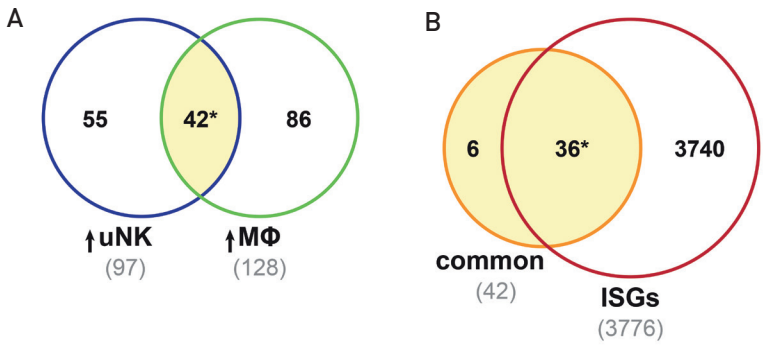

Figure 1
Transcriptional change in uterine NK cells and macrophages from the first to the second trimester of pregnancy shows strong overlap and is associated with the interferon pathway. (**A**) The Venn diagram shows significant overlap between the identified upregulated genes in uNK cells and macrophages in the 2nd trimester. (**B**) The majority of the 42 upregulated genes in second relative to first trimester uNK cells and macrophages (common) represent interferon stimulated genes (ISGs). * $P<0.0001$ by Pearson $\chi 2$ test.

pregnancy are thought to be important for the uterine immunovascular adaptations to create a uterine niche favorable for pregnancy (**Chapter 3**).

Relaxin is emerging as an important regulator of the systemic maternal vascular adaptations, which are characteristic for early pregnancy.[31] In **Chapter 5** we showed that the relaxin receptor RXFP1 is differentially expressed in the vascular smooth muscle and adjacent endothelial cells in different vascular beds, causing the differential effects of circulating relaxin on the vascular wall. Vessel wall properties such as vascular remodeling and changes in geometrical composition significantly improved in mesenteric arteries after relaxin treatment. Even though femoral arteries and veins, and mesenteric veins expressed RXFP1, the latter was not accompanied by similar functional effects in these blood vessels in response to circulating relaxin. These diverse actions of relaxin on RXFP1 across the vasculature aroused our interest in how relaxin and RXFP1 are involved in the vascular remodeling emanating at the maternal-fetal interface during early pregnancy. Our work presented in **Chapter 6** revealed that deletion of RXFP1 interferes with the early vascular and immune adaptations in the mouse uterus. How these findings relate to the role of relaxin and RXFP1 in human pregnancy requires further study, but these observations are in line with studies in a non-human primate model of early pregnancy that showed that relaxin may regulate uterine

vascular development and uNK cell and macrophage numbers.[32,33] Thus, relaxin and its receptor RXFP1 are likely to play a role in defining the uterine niche during early pregnancy.

NEW INSIGHTS IN THE ETIOLOGY OF PREECLAMPSIA

For many years clinicians and scientists with different backgrounds have attempted to elucidate the pathophysiology of the clinical syndrome preeclampsia. As described in **Chapter 3** and **4** the etiology of this potentially lethal pregnancy disorder is complex, firstly due to its presumed onset in early pregnancy and secondly, in conjunction with the generally accepted view that this clinical syndrome represents the common endpoint of various different pathophysiologic pathways. Unraveling the etiology of preeclampsia is further complicated by the absence of a suitable animal model and the lack of access to tissues of the first and second trimester maternal-fetal interface from ongoing pregnancies. It goes without saying that data generated from postpartum placental or uterine samples only provide information on aspects of this disorder in its final stages (**Chapter 4**). Lack of clear understanding of the plausible multiple etiological pathways eventually culminating into the clinical syndrome obstructs discovery of targeted early predictive and/or diagnostic biomarkers, and with it, the development of preventive and curative measures for the disease as delineated in our study in **Chapter 7**.

In **Chapter 4**, we used the experimental evidence generated in the studies of this thesis to propose that - at least in part of preeclamptic pregnancies - placental dysfunction results from defective decidualization and/or inadequate rise and/or dysfunction of uNK cells, already in the post-conceptional luteal phase of the menstrual cycle and the following weeks of pregnancy. We employed systematic bioinformatics to explore the possibility that deficient or defective maturation of the endometrium (decidualization) interferes with placentation in general and placental vascular development in particular. Obviously, the focus of our studies was the dynamics of the uNK-cell in the uterine niche. These results do not allow far-fetched conclusions about the complex etiology of preeclampsia. To generate a more comprehensive picture of the events relevant for early placental development, we require more information about temporal and regional crosstalk between the different cells at the fetal-maternal interface (e.g. uNK cells, macrophages, T cells, ESCs, EVTs, glandular epithelial cells, etc.).

TOWARDS CURATIVE STRATEGIES FOR PREECLAMPSIA

Our evidence for the endometrial antecedents of preeclampsia (**Chapter 4**) highlights immunovascular adaptations to early pregnancy, such as (pre) decidualization and uNK cell regulation of EVT invasiveness, as potential targets to

prevent placental dysfunction and systemic maternal and fetal sequelae later in pregnancy. Evidence provided in **Chapter 2** indicates that IFNα is important in the regulation of uNK cell functions by its modulation of EVT invasiveness and uterine vascular development. On the basis of these findings stimulation of the type I interferon pathway at the maternal-fetal interface could be a target to reverse or limit impairment of EVT invasion by manipulating uNK cell function. However, it should be noted that pharmacologic interventions to treat pregnancy complications bear the potential of teratogenicity and the risk for damage of the developing embryo. Indeed, case reports indicate that INFα treatment during pregnancy for myeloproliferative diseases and chronic viral illnesses is associated with an increased risk of premature birth and fetal growth restriction but not with congenital malformations.[34] Although circulating relaxin has no relevant predictive power for preeclampsia (**Chapter 7**), recombinant human relaxin may have potential as a therapeutic to prevent or delay the onset of preeclampsia, in particular in the subgroup of women becoming pregnant by transfer of a donor embryo as they lack a normally functioning corpus luteum, and are known to be at increased risk of preeclampsia.[31] Even if we had effective therapeutic options to treat disturbances in early uterine and placental development, we currently lack predictive and diagnostic tools in our clinics to identify those pregnant women that could benefit from such early interventions (**Chapter 7**). Thus, until the etiological pathways culminating into preeclampsia are better defined, it remains elusive to develop effective interventions to prevent and treat disease manifestations of preeclampsia.

FUTURE PERSPECTIVES

In this thesis we provided first evidence that defects in endometrial decidualization and the concomitant changes in uNK cells occur already in the *secretory phase* of the menstrual cycle (pre-decidualization), opening up new avenues to study the etiology of preeclampsia. Conceivably, retrospective examination of endometrium, endometrial secretions and/or uNK cells in the mid- to late-secretory phase of the menstrual cycle, in former preeclamptic women, would allow us to better define the contribution of early derangements in the decidualization process to disease etiology. Such defects in the development of the 'uterine niche' may apply to only a subset of preeclamptic women, as preeclampsia is generally accepted to be a pregnancy disorder with highly heterogeneous etiology.[35] Women with known risk factors for preeclampsia, such as polycystic ovary syndrome (PCOS), insulin resistance,[36] obesity,[37] and poor cardiovascular health,[6] are of particular interest in this context, as these underlying conditions may hamper the maturation of the endometrium and cyclic changes in uNK cells, thereby impairing pregnancy

success. Thus, study of endometrial pre-decidualization in women with a history of preeclampsia may provide an opportunity to further define the endometrial antecedents of the disease.

Chorionic villous sampling (CVS) for fetal aneuploidy forms one, if not the only, source of tissue to study the *early pregnancy* maternal-fetal interface in ongoing pregnancies. Unfortunately with the introduction of non-invasive prenatal testing (NIPT), CVS is becoming clinically outdated (**Chapter 4**). An alternative strategy to CVS sampling becomes more appealing with improvement of the early prediction of preeclampsia by discovery of new biomarkers and development of new imaging modalities, such as 3D-power-Doppler of the placenta.[38] By adopting these techniques to detect women at increased risk for preeclampsia prior to elective pregnancy termination, we can obtain unique placental and decidual tissues of the maternal-fetal interface during early pregnancy for mechanistic studies. Although pregnancy outcome will not be certain, this strategy would allow us to further explore the gestational age-dependent regulation of uterine NK cells and macrophages in early pregnancies at-risk for preeclampsia and other fetal and maternal complications.

In conclusion, the uterus forms a fascinating organ that undergoes extensive cyclic and hormonally regulated immunovascular adaptations to optimize placenta development. Uterine immune cells, in particular uNK cells, synchronize uterine and placental developments by temporal and regional changes in regulatory functions to secure optimal fetal development. Deviations in the different pathways resulting in a suboptimal 'uterine niche' and uNK cell functions, may contribute to reproductive disorders such as preeclampsia and form a challenging target for research to elucidate causing mechanisms of these major contributors to perinatal morbidity and mortality.

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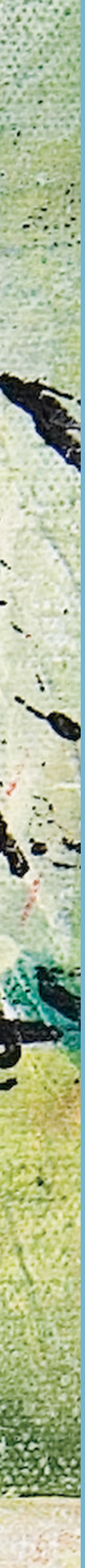

Chapter 9

DUTCH SUMMARY

NEDERLANDSE SAMENVATING

INTRODUCTIE

Een normale zwangerschap wordt gekenmerkt door een dynamische, goed gecoördineerde symbiose tussen moeder en foetus. De zwangerschap vereist voortdurende aanpassingen in het moederlijk lichaam die zorgen voor optimale omstandigheden tijdens ieder stadium van de foetale groei en ontwikkeling. Bij de mens beginnen veranderingen in structuur, distributie en functie van cellen in het baarmoederslijmvlies, het "endometrium", al in de menstruele cyclus, nog vóór het embryo zich innestelt in de wand van de baarmoeder (uterus). Deze voorbereiding van de uterus op de zwangerschap, ook wel "decidualisatie" genoemd, wordt gereguleerd door de ovariële (eierstok) hormonen. Decidualisatie van het endometrium gaat vergezeld met een toename van cellen afkomstig uit het immuunsysteem. Zodra het embryo innestelt, intensiveert de decidualisatie van het endometrium en zullen trofoblastcellen, die van foetale afkomst zijn, vanuit de placenta het endometrium binnen dringen (invaderen). Moederlijke immuuncellen en foetale trofoblastcellen werken dan nauw samen om de bloedvaten (vascularisatie) in de baarmoederwand te remodelleren. Deze vaatremodellering is nodig om de snel groeiende placenta gedurende de gehele zwangerschap van voldoende bloed te kunnen voorzien. Een verstoord verloop van deze aanpassingen in de baarmoederwand verhoogt de kans op veel voorkomende zwangerschapscomplicaties, zoals een té langzame groei van de foetus en pre-eclampsie (zwangerschapsvergiftiging). Pre-eclampsie ontwikkelt zich in 3-5% van alle zwangerschappen en vormt een belangrijke oorzaak voor zowel blijvende schade als sterfte bij moeder en kind. Hoewel er sterke aanwijzingen zijn dat verstoring van de immuno-vasculaire aanpassingen betrokken zijn bij het ontstaan van voornoemde zwangerschapscomplicaties, is het huidig inzicht in de mechanismen die leiden tot pre-eclampsie beperkt.

In dit proefschrift beschrijven we onderzoek naar de mechanismen van moederlijke immunologische en vasculaire aanpassingen in de vroege zwangerschap, die bijdragen aan een normaal functionerende placenta. Tevens bestuderen we hoe verstoringen van deze processen in de uterus kunnen bijdragen aan de ontwikkeling van het placenta-geassocieerde syndroom pre-eclampsie.

DEEL 1

HET DECIDUALISERENDE ENDOMETRIUM EN UTERIENE NK CELLEN

De decidualisatie wordt gezien als het proces dat het endometrium ontvankelijk maakt voor de innesteling van het embryo en de vorming van de placenta. Dit rijpingsproces wordt gekenmerkt door biochemische en morfologische veranderingen van stromale (steuncellen), epitheliale (dekweefsel), vasculaire en immuuncellen. Decidualisatie gaat gepaard met een grote verschuiving in de cellulaire samenstelling van het endometrium. Opvallend daarbij is de sterke toename van een bepaald soort immuuncellen, de zogenaamde "uteriene natural killer" (uNK) cellen. Deze uNK cellen omvatten 70-80% van alle immuuncellen in het endometrium gedurende de vroege zwangerschap en zijn nauw betrokken bij de regulering van essentiële ontwikkelingsprocessen op het scheidingsvlak tussen baarmoederslijmvlies en placenta.

In **Hoofdstuk 2** doen we verslag van onderzoek naar het verschil in genexpressie en functie tussen eerste en tweede trimester uNK cellen, om zo beter inzicht te krijgen in hoe hun rol evolueert gedurende deze belangrijke fase van de placenta-ontwikkeling. We isoleerden uNK cellen uit endometriumweefsel en analyseerden deze met een zogenaamde gen-microarray. Met deze methode, die het mogelijk maakt genoom-breed te kijken naar verschillen in genexpressie niveaus, werd duidelijk dat uNK cellen tussen het eerste en tweede trimester sterk veranderen. Met name de expressie van "interferon-gestimuleerde genen", zoals ISG15 en ISG20, nam sterk toe. Deze veranderingen tussen eerste en tweede trimester op gen niveau gingen in ons experimentele trofoblastinvasie-model gepaard met een toename in de functionele capaciteit van uNK cellen om trofoblastinvasie te stimuleren. Nieuwvorming van bloedvaten werd ook gestimuleerd door uNK cellen, maar deze functie verschilde niet tussen het eerste en tweede trimester. Interferonen zijn signaaleiwitten die door allerlei lichaamscellen kunnen worden uitgescheiden en bestaan uit 3 types, namelijk type I, II en III interferonen. In vervolg experimenten bleek dat met name type I interferonen (IFNα/β) maar niet type II interferonen (IFN) de genexpressie van ISG15 en ISG20 in uNK cellen verhoogde. IFNα stimuleerde het vermogen van eerste trimester uNK cellen om trofoblastinvasie te promoten. Interessant was de ontdekking dat het signaaleiwit ISG15 niet alleen op gen niveau veranderde in uNK cellen maar ook de invasie van trofoblastcellen verhoogde. Kortom, in het verloop van de vroege zwangerschap veranderen uNK cellen zowel qua genexpressie als qua functie. Op basis van onze bevindingen denken wij dat type I interferonen een belangrijke rol spelen bij het in staat stellen van uNK cellen om trofoblasten aan te trekken in het endometrium. Daarnaast hebben wij ISG15 geïdentificeerd als een nieuw signaaleiwit dat trofoblastinvasie stimuleert.

Het dynamische micromilieu van het baarmoederslijmvlies lijkt in belangrijke mate de ontwikkeling van uNK cellen te bepalen. In deze "uteriene niche" verschillen NK cellen dan ook qua fenotype (uiterlijk) en functie van circulerende NK cellen in de bloedbaan. **Hoofdstuk 3** geeft een overzicht van de huidige kennis ten aanzien van de ontwikkeling van het endometrium en de functie van uNK cellen, die de zwangerschap van de mens ondersteunen. Daarnaast bespraken we hoe verstoring van uNK cellen in hun aantal dan wel hun functie in hun uteriene omgeving, de voortplanting van de mens negatief kan beïnvloeden. In hun interactie met de dynamische uteriene niche spelen uNK cellen een belangrijke rol in het complexe proces van placentatie. Uteriene NK cellen zijn niet alleen belangrijk om te voorkómen dat de voor de moeder lichaamsvreemde placentacellen worden afgestoten, aangezien hun genetisch materiaal voor de helft van de vader afkomstig is, zij hebben ook een beschermende rol tegen infecties. Gerekruteerd als voorlopers van of als volwassen NK cellen dragen ze mogelijk ook bij aan de initiatie van vasculaire veranderingen in de baarmoeder, die een onbelemmerde bloedvoorziening naar de placenta moeten garanderen. Het endometrium is een bijzonder weefsel om de biologie van NK cellen te bestuderen, omdat dit slijmvlies tijdens iedere menstruele cyclus opnieuw wordt opgebouwd en uitgebreide remodellering ondergaat, zowel tijdens de secretiefase als bij de placenta-aanleg. Infectie, het opruimen van het endometrium tijdens de menstruatie en de placentatie prikkelen de uNK cellen zich snel aan te passen zodat het fysiologisch evenwicht op het scheidingsvlak tussen moeder en foetus bewaard blijft. Verstoringen in de uNK cel niche spelen mogelijk een belangrijke rol bij het ontstaan van een breed spectrum aan zwangerschapscomplicaties, zoals miskramen, foetale groeivertraging en pre-eclampsie.

Het optreden van pre-eclampsie – een in potentie levensgevaarlijke complicatie tijdens de zwangerschap – lijkt samen te hangen met een verminderde invasie van trofoblastcellen in de baarmoederwand in het begin van de zwangerschap. De reden waarom trofoblastcellen moeilijker invaderen is echter nog onduidelijk, met name vanwege het feit dat de placenta en de baarmoeder van vrouwen die later in de zwangerschap pre-eclampsie ontwikkelen nagenoeg niet toegankelijk zijn voor onderzoek. Daarnaast is er geen geschikt diermodel beschikbaar waarin pre-eclampsie spontaan optreedt. In **Hoofdstuk 4** toetsen we met een bio-informatica benadering de hypothese dat onvolledige of gebrekkige decidualisatie van het baarmoederslijmvlies voorafgaat aan verminderde trofoblastinvasie en het ontstaan van pre-eclampsie. In totaal zijn 396 genen geïdentificeerd die verschillend tot expressie kwamen in vlokkentestweefsel van ≈11,5 week zwangere vrouwen die 6 maanden later in de zwangerschap een ernstige pre-eclampsie ontwikkelden,

vergeleken met die van gezonde zwangerschappen. Maar liefst 154 van deze genen (40%) kwamen overeen met genen die over de verschillende fasen van de normale decidualisatie in het endometrium toenamen in expressie, zowel vóór als ná de embryo-implantatie. Van deze 154 genen bleken 116 (75%) genen betrokken te zijn bij de decidualisatie in de afwezigheid van lokale trofoblastcellen, zoals tijdens de late secretie fase van het endometrium of in het endometrium van buitenbaarmoederlijke zwangerschappen. Tot slot veranderden 112 van deze 154 genen (73%) in tegengestelde richting dan gezien werd in datasets van de normale uitrijping van het endometrium, inclusief 16 genen waarvan de expressie in uteriene NK cellen toegenomen was (ten opzichte van NK cellen in de bloedbaan). Deze bevindingen suggereren dat pre-eclampsie voorafgegaan wordt door een verstoorde of gebrekkige rijping van het endometrium en van de uNK cellen tijdens de secretie fase en de vroege zwangerschap.

DEEL 2
HET HORMOON RELAXINE EN DE RELAXINE RECEPTOR RXFP1 TIJDENS DE ZWANGERSCHAP

Het corpus luteum, ofwel "gele lichaam", in het ovarium produceert tijdens de zwangerschap het hormoon relaxine. Relaxine is een sterke vaatverwijder die mogelijk nauw betrokken is bij het tot stand komen van de fysiologische aanpassingen in het vaatbed, met name in de vroege zwangerschap wanneer de bloedspiegels van dit hormoon het hoogste zijn.

Het effect van relaxine op de bloedvaten wordt gemedieerd door receptoren in de vaatwand, $RXFP_1$ receptoren genaamd. $RXFP_1$ werd tot voor kort alleen geïdentificeerd in de tunica media (gladde spiercellaag) van de vaatwand. In **Hoofdstuk 5** onderzoeken we de lokalisatie van $RXFP_1$ in arteriën (slagaders) en venen (aders) van verschillende vaatbedden in de rat. Daarbij werd vastgesteld dat deze receptor ook aanwezig is in de binnenste bekleding van de bloedvaten, het "endotheel". Met name in de aorta (grote lichaamsslagader), de vena cava (grote holle ader) en de mesenteriale vaten (darmvaten) kwam $RXFP_1$ meer voor in het endotheel dan in de omringende tunica media. In de arteria en vena femoralis (dijbeenslagader en ader) en arteria en vena pulmonalis (longslagader en ader) kwam $RXFP_1$ echter relatief meer voor in de tunica media dan in het endotheel. Vervolgens werd gekeken of deze twee groepen vaten met verschillende $RXFP_1$ distributie ook verschillend reageerden op relaxine. Hiertoe werden vaatverwijding en volume-rekbaarheid van mesenteriale en femorale vaten bestudeerd na behandeling met relaxine. Relaxine verminderde de vaatstijfheid en verhoogde de volume-rekbaarheid in de mesenteriale slagaderen maar niet in de andere

bestudeerde vaten. Kortom, een verschil in RXFP1 distributie tussen het endotheel en de tunica media leidt tot een andere relaxine respons in de wand van verschillende bloedvaten. Tevens impliceert deze studie dat relaxine, dat circuleert tijdens de zwangerschap, de vaatweerstand in het mesenteriale vaatbed reguleert.

Het biologisch effect van relaxine wordt gemedieerd via de RXFP1 receptor, die onder andere betrokken is bij de moederlijke vaataanpassingen tijdens de zwangerschap. In niet-zwangere resusapen zorgt behandeling met relaxine voor meer bloedvaten en meer immuuncellen in het baarmoederslijmvlies, met name macrofagen en NK cellen. In **Hoofdstuk 6** bestuderen we de rol van de relaxine receptor RXFP1 tijdens immuno-vasculaire aanpassingen in het baarmoederslijmvlies van de vroeg-zwangere muis. Hiervoor maakten we gebruik van een genetisch gemodificeerde muis waarin de RXFP1 receptor ontbreekt en vergeleken deze muizen met "wild-type" muizen die deze receptor wel hebben. In RXFP1 deficiënte muizen zagen we dat de vaatontwikkeling vroeg in de zwangerschap verschillend verloopt (dag 6.5). Op dag 12.5 van de zwangerschap, die 19.5 dag duurt, vonden we tevens meer doorsneden van bloedvaten in het baarmoederslijmvlies terug. Om te onderzoeken of relaxine mogelijk zorgt voor versnelde vaatnieuwvorming (angiogenese), onderzochten we vervolgens in een zogenaamd reageerbuis-experiment (in vitro) of relaxine de vaatnieuwvorming in twee types endotheelcellen (HUVEC en HMEC-1) stimuleert. Relaxine leek niet direct een aantoonbaar effect op de angiogenese te sorteren. In de RXFP1 receptor-deficiënte muizen zagen we ook geen verschillen in het totaal aantal uNK cellen op 6.5, 10.5 en 12.5. Aanvullende gedetailleerdere karakterisering van de uNK cellen toonde echter aan dat er wél duidelijk verschillen waren in uNK cel eigenschappen. We zagen op zwangerschapsdag 6.5 dat de uNK cellen in het endometrium meer vaatgroei stimulerende eigenschappen hadden (PAS+DBA+) in muizen zonder RXFP1, en dit ging ten koste van het aantal uNK cellen die bekend staan om hun vaatremodellerende eigenschappen (PAS+DBA-). Op dag 10.5 zagen we ook meer van deze PAS+DBA+ NK cellen in het myometrium (de spierlaag van de baarmoeder). Macrofagen met anti-inflammatoire en weefsel-herstellende eigenschappen (F4/80+CD206+) waren op dag 6.5 groter in aantal in het endometrium van de RXFP1 deficiënte muizen. Dus het ontbreken van de relaxine receptor RXFP1 heeft een effect op de ontwikkeling van de bloedvaten en de lokale afweercellen in de baarmoeder. Wij denken dan ook dat de RXFP1 receptor de lokale afweercellen in de baarmoeder reguleert tijdens de vroege zwangerschap en zo bijdraagt aan de vaatontwikkeling in het baarmoederslijmvlies.

In **Hoofdstuk 7** presenteren we een onderzoek naar de betekenis van de perifere relaxine spiegels als vroege voorspeller van pre-eclampsie. In eerste instantie werd relaxine gemeten in eerste trimester bloedmonsters van vrouwen die laat in de zwangerschap (bevalling ná 34 weken) pre-eclampsie ontwikkelden, dan wel een normale zwangerschap doormaakten. Deze studie werd in eerste instantie uitgevoerd in Pittsburgh, USA, maar later werd de studiepopulatie aangevuld met een grote groep zwangeren uit Nederland, in samenwerking met het Rijks Instituut voor Volksgezondheid en Milieu (RIVM). Hierbij werden zowel eerste trimester bloedmonsters van vrouwen met een late of vroege pre-eclampsie (bevalling vóór 34 weken) vergeleken met ongecompliceerde zwangerschappen. Een lage relaxine spiegel in de vroege zwangerschap was geassocieerd met de ontwikkeling van late pre-eclampsie. Deze correlatie was nog sterker wanneer een late pre-eclampsie vergezeld ging met de geboorte van een baby met een té laag geboortegewicht. Dit suggereert dat een lage relaxine-spiegel in de vroege zwangerschap ongunstig is en kan bijdragen aan het ontwikkelen van een late pre-eclampsie, mogelijk vanwege een suboptimale aanpassing van het cardiovasculaire systeem van de vrouw in de vroege zwangerschap. Opvallend was dat er geen correlatie was tussen de relaxine spiegel in de vroege zwangerschap en het optreden van vroege pre-eclampsie. De waarde van het meten van relaxine spiegels in de vroege zwangerschap ter predictie van zowel vroege als late pre-eclampsie was beperkt. Het breed toepasbaar maken van relaxine bepalingen tijdens de vroege zwangerschap als test voor pre-eclampsie lijkt dan ook geen meerwaarde te hebben.

TOT BESLUIT

De bevindingen in dit proefschrift onderstrepen dat immuno-vasculaire aanpassingen in de uterus een belangrijke voorwaarde vormen voor een normaal verloop van de zwangerschap. Onder invloed van ovariële hormonen zoals relaxine en de receptor $RXFP_1$, wordt tijdens de decidualisatie van het endometrium een 'uteriene niche' gecreëerd die gekenmerkt wordt door een groot aantal uNK cellen. Deze uNK cellen hebben daarbij de taak de ontwikkelingen in de baarmoeder en de placenta in het verloop van de zwangerschap voortdurend op elkaar af te stemmen. Verstoringen in de rijping van de uteriene niche en uNK cel functies vroeg in de zwangerschap, of zelfs al in de secretiefase van de menstruele cyclus, lijken bij te dragen aan de ontwikkeling van zwangerschapscomplicaties zoals pre-eclampsie. Het verbeteren van ons inzicht in de uteriene immuno-vasculaire aanpassingen in de vroege zwangerschap vormt dan ook een belangrijke uitdaging voor verder onderzoek.

Chapter 10

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CURRICULUM VITAE

Emiel Post Uiterweer was born on February 9, 1984 in Geldrop, the Netherlands. During his training at the Medical School of the University of Maastricht (2004-2010), he completed an elective internship at the Department of Obstetrics and Gynecology, Makerere University, Kampala Uganda and performed a six-month research elective in the D.H. Barron Reproductive and Perinatal Biology Research Program and the Departments of Physiology and Functional Genomics and of Obstetrics and Gynecology, University of Florida, Gainesville, Florida, USA. For this research project under the supervision of Dr. Kirk P. Conrad, he received an award for best thesis at the University of Maastricht during its 35th Dies Natalis. After graduating from Medical School in 2010, Emiel worked as an intern at the Atrium Medical Center Parkstad, Department of Cardiology in Heerlen for six-months. In 2011 he started his Ph.D. research at the University Medical Center of Utrecht, under supervision of Dr. Louis L.H. Peeters and Dr. Arie Franx of the Department of Obstetrics as well as Dr. Niels Eijkelkamp and Dr. Cobi J. Heijnen of the Laboratory for Neuroimmunology and Developmental Origins of Disease. During the following four years, Emiel performed the research delineated in this thesis while continuing to collaborate with his mentor Dr. Conrad. He presented his work at several international conferences, including the annual meetings of the Society for Reproductive Investigation and the congress for the International Society for the Study of Hypertension in Pregnancy. Emiel has served on the In Training Member Committee and the Career Development & Diversity Committee of the Society for Reproductive Investigation and currently holds a position as In-training *ad hoc* Council Member.

In April 2015 Emiel started a post-doctoral research fellowship in Reproductive Immunology, under supervision of Dr. Adrian Erlebacher in the Department of Pathology at the New York University School of Medicine, New York City, New York, USA. For this post-doctoral fellowship on tissue-resident memory T cells at the maternal-fetal interface, he was awarded a Rubicon grant by the Netherlands Organization for Scientific Research (NWO).