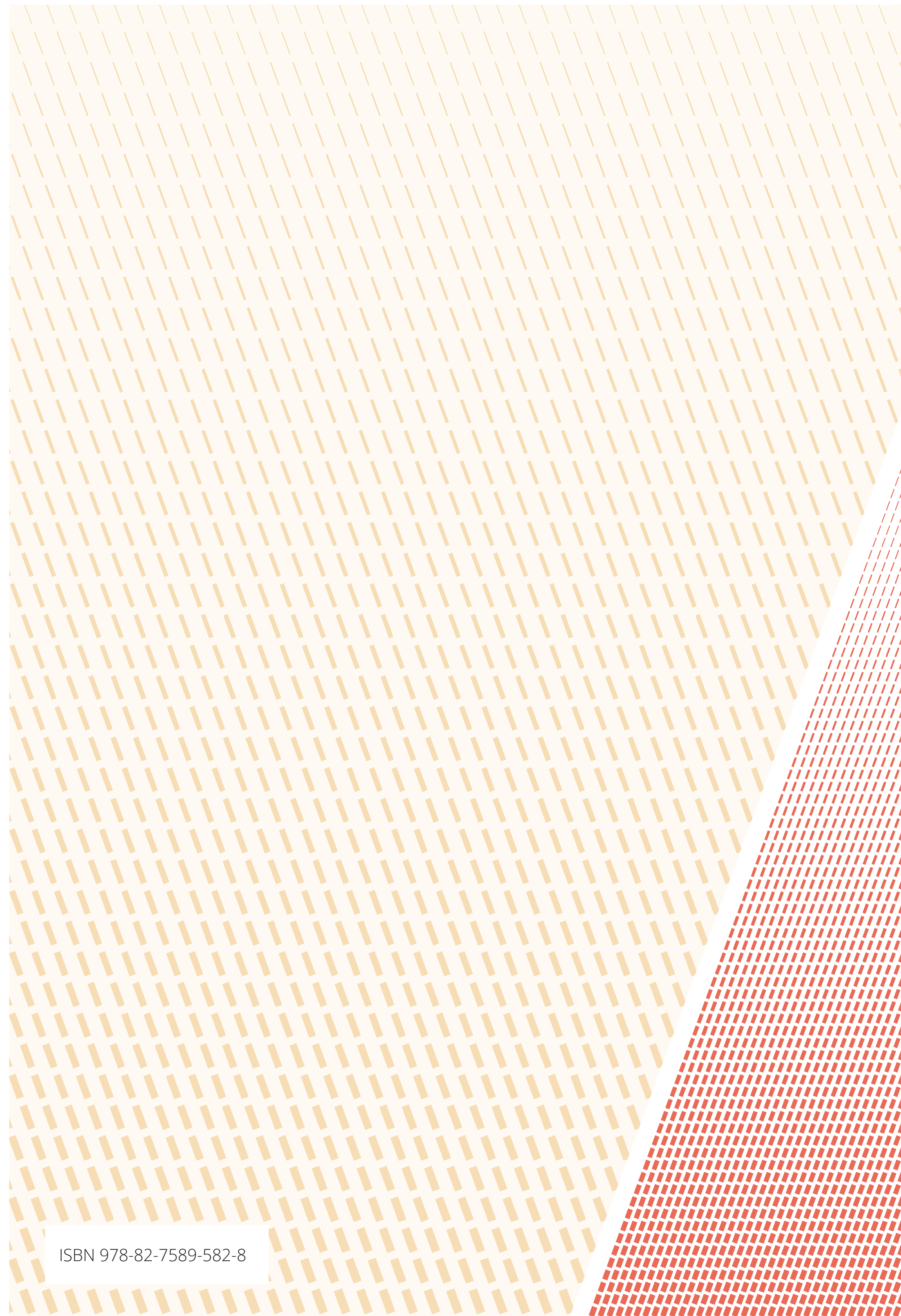

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Role of laeverin in the pathophysiology of preeclampsia - Mona Nystad

Faculty of Health Sciences
Department of Clinical Medicine

# Role of laeverin in the pathophysiology of preeclampsia.

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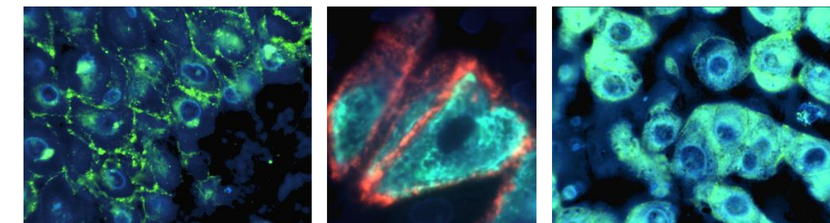
**Mona Nystad**

*A dissertation for the degree of Philosophiae Doctor, May 2018*



# ROLE OF LAEVERIN IN THE PATHOPHYSIOLOGY OF PREECLAMPSIA

**Mona Nystad**

*A dissertation for the degree of Philosophiae Doctor – May 2018*

**Women's Health and Perinatology Research Group**
**Department of Clinical Medicine**
**Faculty of Health Sciences**
**UiT – The Arctic University of Tromsø**

**Cover photos**

Picture of the baby and the placenta on the front page is reproduced with the courtesy of the photographer and midwife Emma Jean (Emma Jean Photography, Brisbane, Australia) and the parents of the baby. Pictures in the lower column are immunofluorescence pictures of trophoblast cells, which are the major constituents of the placenta. Left picture is of cytotrophoblast cells from normal placenta stained with green fluorescence antibodies against laeverin protein (in plasma membrane). Middle picture is of extravillous trophoblast cells of normal placenta stained with red fluorescence antibodies against laeverin (in plasma membrane) and green fluorescence antibodies against cytokeratin 7 protein (in cytoplasma). Right picture is of cytotrophoblast cells from placenta of a preeclampsia patient stained with green fluorescence antibodies against laeverin protein (in cytoplasma). Cells are counterstained with DAPI II (blue) to show the nuclei.

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*Dedicated to my Father*

*What is our life? The play of passion.*

*WHAT is our life? The play of passion.*
*Our mirth? The music of division:*
*Our mothers' wombs the tiring-houses be,*
*Where we are dressed for life's short comedy.*
*The earth the stage; Heaven the spectator is,*
*Who sits and views whosoe'er doth act amiss.*
*The graves which hide us from the scorching sun*
*Are like drawn curtains when the play is done.*
*Thus playing post we to our latest rest,*
*And then we die in earnest, not in jest.*

*-Sir Walter Raleigh (1612)*[1]

## ACKNOWLEDGEMENTS

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I thank Ganesh for all scientific discussions and always having time for me in his busy life. I am very grateful to Ganesh for teaching me the skills of scientific writing. Ganesh and my co-supervisor Vasilis Sitras have raised my potential with gentle pushing and most importantly been discussion partners always believing in me and my ideas. I am very thankful to Ganesh for sharing his friends and colleagues in the interesting scientific field of preeclampsia. Vasilis has also been a leading star, always effective and willing to discuss anything related to the project. His knowledge, supportive, enthusiastic and visionary mind has given me an inspiration to fulfill the thesis.

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I want to express my deepest gratitude to my closest and dearest. Thank you Dad for always being patient. Your encouragement always made me take that extra step forward in all areas of life. You introduced me to nature and the wonders of life. Thank you, Mum and Dad, for giving me the opportunity of having a creative childhood, never punishing all my crazy doodads. I am very grateful to my daughters Ida Amalie and Solveig for their support, giving me lots of inspiration, cheering on me when my motivation has been low and reminded me of the meaning of life. At last I will thank my soul-mate and husband Eirik Reierth for his patience, very good dinners and encouragement during this intense period.

Mona Nystad

Mona Nystad, February 12th 2018.

## ABBREVIATIONS

**ACTB** β-actin

**ADAM-12** a disintegrin and metalloprotease 12

**AGT** angiotensinogen

**AGTR1** angiotensin II receptor type 1

**AGTR2** angiotensin II receptor type 2

**AP-A/APA** aminopeptidase A (ENPEP)

**AP-N** aminopeptidase N

**APQ/AP-Q/AQPEP** aminopeptidase Q

**AT1** type-1 angiotensin II receptor

**AV** anchoring villi

**BMP4** bone morphogenic protein 4

**CAM** cell adhesion molecules

**CC** cell column

**CHL2** chorion laeve 2 antibody

**CK7** cytokeratin 7

**CMV** cytomegalovirus

**COL1A1** collagen α1 chain

**CT** cytotrophoblast

**CTB** cytotrophoblast

**DZ** dizygotic

**EC** endothelial cell

**ECM** extracellular matrix

**eCTB** endovascular cytotrophoblast

**EDTA** ethylenediamine tetraacetic acid

**eEVT** endovascular extravillous cytotrophoblasts

**ELISA** enzyme-linked immunosorbent assay

**ENG** endoglin

**ER** endoplasmic reticulum

**EVT(s)** extravillous cytotrophoblast(s)

**F5** factor V Leiden

**FITC** fluorescein isothiocyanate

**FFPE** formalin fixed paraffin embedded

**Flt-1** fms-like tyrosine kinase 1 (vascular endothelial growth factor/vascular permeability factor receptor)

**FSTL3** follistatin-like 3

**FV** floating villi

**GAPDH** glyceraldehydephosphate dehydrogenase

**GC** trophoblast giant cells

**GOPEC** Genetics Of Pre-Eclampsia

**GWAS** genome-wide association studies

**hCG** human chorionic gonadotropin

**HELLP** hemolysis-elevated liver enzymes-low platelets

**HIV** human immunodeficiency virus

**HLA-G** histocompatibility complex class I G

**HTRA1** high-temperature requirement A serine peptidase 1

**ICH** intracerebral haemorrhage

**iCTB** interstitial cytotrophoblast

**iEVT** interstitial extravillous cytotrophoblasts

**IF** immunofluorescence

**IGS** immunogold staining

**IHC** immunohistochemistry

**IL1A** interleukin-1α

**INHA** inhibin Alpha Subunit

**INHBA** inhibin Beta A Subunit

**IUGR** intrauterine growth restriction

**Kp-10** kisspeptin-10

**LEP** leptin

**LVRN** laeverin

**MAP** mean arterial pressure

**MC** mesenchymal core

**MMPs** matrix metalloproteinases

**MTHFR** methylenetetrahydrofolate reductase

**MZ** monozygotic

**NMR** nuclear magnetic resonance

**NOS3** nitric oxide synthase 3

**PAPP-A** pregnancy-associated protein A

**PDYN** dynorphin A1

**PGH** placental growth hormone

**PI** pulsatile index

**PIGF** placental growth factor (PGF)

**PL** placental lactogen

**P-LAP** placental leucine aminopeptidase

**PLAUR** urokinase plasminogen activator surface receptor

**PP13** placental protein 13

**PSG11** pregnancy-specific β1-glycoprotein 11

**ROS** reactive oxygen species

**SA** spiral arteries

**SEM** scanning electron microscopy

**sENG** soluble endoglin

**SERPINE1** plasminogen activator type 1 (serine proteinase inhibitor)

**sFlt-1** soluble fms-like tyrosine kinase

**SLE** systemic lupus erythematosus

**SNP** single nucleotide polymorphism

**ST** syncytiotrophoblast (also known as STB)

**STOX1** storkhead box 1

**sVEGFR-1** soluble vascular endothelial growth factor receptor 1

**TAC4** endokinin C

**TEM** transmission electron microscopy

**TGFα1** transforming growth factor α1

**TGFβ2** transforming growth factor β2

**TMA** tissue microarray

**TNF** tumor necrosis factor

**vCTB** villous cytotrophoblast (also known as vCT)

**VEGF** vascular endothelial growth factor

**VEGFR-1** vascular endothelial growth factor receptor 1

# ABSTRACT

## English version

Preeclampsia is a pregnancy-specific disease, affecting 5-10% of human pregnancies worldwide. It is defined as new-onset hypertension after 20 weeks of gestation with either proteinuria and/or organ failure. Laeverin is a membrane-bound aminopeptidase exclusively expressed in the placenta. Our previous studies revealed a significant upregulation of laeverin mRNA in preeclamptic placentas, building the background for this doctoral thesis. Therefore, we adopted a translational approach aiming to investigate the possible role of laeverin in the pathophysiology of preeclampsia. First, we used immunofluorescence microscopic analysis of healthy third trimester placentas showing expression of laeverin protein in cell membrane of villous trophoblasts. In preeclamptic placentas however, laeverin was expressed ectopically in the cytoplasm, especially in microvesicles. Immunoelectron microscopy showed laeverin leakage into the fetal capillaries and abundant expression in microvesicles in preeclamptic placentas. Laeverin gene-silencing showed a reduction in migration and invasion capacity of first trimester immortalized trophoblast (HTR-8/SVneo) cells by 11.5% (P=.023) and 56.7% (P=.001), respectively. Pathway analysis using PCR-array and laeverin-silencing demonstrated significant downregulation of integrin A2 (39-fold), integrin B3 (5-fold), and matrix metalloprotease 1 (36-fold), indicating possible downstream effects of laeverin at the molecular level. We further investigated the expression of laeverin in placenta tissue, and maternal and fetal blood samples. Enzyme-linked immunosorbent assay analysis of laeverin protein in maternal blood showed decreasing laeverin concentration with advancing gestation during the second half of normal pregnancy. Moreover, we found that lower levels of laeverin in the maternal plasma measured at 22–24 weeks might be associated with the development of preeclampsia later in gestation. Tissue microarray analysis of 77 placentas (from 36 preeclamptic and 41 uncomplicated pregnancies) was performed to investigate laeverin protein expression differences in healthy and preeclamptic placentas. Immunohistochemistry showed that laeverin was expressed in syncytiotrophoblasts, cytotrophoblasts and extravillous trophoblasts in all placentas. All placental samples that showed cytoplasmic expression of laeverin were obtained from women delivered before 34 weeks of gestation (early-onset preeclampsia). Further, immunofluorescence studies showed laeverin expression in the cytoplasm of six preeclamptic (three early-onset and three late-onset) and one normal placenta. In summary, we demonstrated that laeverin has a role in human placentation and possible implications in the pathophysiology and clinical manifestation of preeclampsia.

## Norwegian version

Preeklampsi (svangerskapsforgiftning) er en sykdom som oppstår i 5-10% av humane svangerskap. Den defineres som nyoppstått hypertensjon med enten proteinuri og/eller organsvikt fra svangerskapsuke 20. Laeverin er en membranbundet aminopeptidase som bare uttrykkes i placenta (morkaken). Våre tidligere studier viste en 10 ganger oppregulering av laeverin mRNA i preeklamptiske placenta, som dannet grunnlaget for denne doktorgradsavhandlingen. Vi valgte derfor en translasjonell tilnærming for å undersøke mulige roller for laeverin i patofysiologien til preeklampsi. Først gjorde vi immunfluorescens-mikroskopisk analyse av tredje trimester placenta som viste membranuttrykk av laeverinprotein i villøse trofoblaster. I preeklamptiske placenta fant vi imidlertid laeverin uttrykt i cytoplasma, spesielt i mikrovesikler. Immunoelektronmikroskopi viste laeverinlekkasje i føtale kapillærer og rikelig uttrykk i mikrovesikler i preeklamptiske morkaker. Migrasjon- og invasjons-studier av første trimester immortaliserte trofoblastceller (HTR-8/SVneo) celler viste reduksjon på 11.5% (P=.023) og 56.7% (P=.001), respektivt i laeverin «gene-silencing»-studier. Pathway-analyse ved bruk av PCR-array og laeverin-silencing påviste nedregulering av integrin A2 (39 ganger), integrin B3 (5 ganger) og matrix metalloprotease 1 (36 ganger); som antydet nedstrømseffekter av laeverin på molekylært nivå. Vi gikk deretter videre med undersøkelser av laeverinuttrykk i kliniske prøver (placenta, maternelt- og føtalt blod). «Enzyme-linked immunosorbent assay»-analyse av laeverinprotein i mors blod viste synkende laeverinkonsentrasjon gjennom andre halvdel av normalt svangerskap. I tillegg fant vi lavere nivå av laeverin i maternelt plasma målt i uke 22-24, som kan være assosiert med utvikling av preeklampsi senere i svangerskapet. «Tissue microarray»-analyse av 77 placenta (fra 36 preeklamptiske- og 41 normale svangerskap) ble gjort for å sammenligne forskjeller i laeverinproteinuttrykk i normale- og preeklamptiske placenta. Immunohistokjemiske studier viste laeverinuttrykk i syncytiotrofoblaster, cytotrofoblaster og extravilløse trofoblaster i alle placenta. Cytoplasmisk laeverinproteinuttrykk ble funnet hos kvinner som hadde tidlig oppstått preeklampsi (fødsel før svangerskapsuke 34). Immunfluorescens-studier viste cytoplasmisk ekspresjon i seks preeklampsi - (tre tidlig- og tre sent oppstått preeklampsi) og i en normal placenta. Vi har demonstrert at laeverin har en mulig rolle i patofysiologien bak preeklampsi og i klinisk uttrykk av sykdommen.

## LIST OF PAPERS

Present thesis is based on the following articles, which are referred in the text by their respective Roman numbers:

I. Nystad M, Sitras V, Larsen M and Acharya G. Placental expression of aminopeptidase-Q (laeverin) and its role in the pathophysiology of preeclampsia. *American Journal of Obstetrics and Gynecology*. 2014 **211**(6):686.e1-31.

II. Nystad M, Sitras V, Flo K, Widnes C, Vårtun Å, Wilsgaard T and Acharya G. Longitudinal reference ranges for maternal plasma laeverin, and its role as a potential biomarker of preeclampsia. *BMC Pregnancy and Childbirth.* 2016 **16**(1):377.

III. Nystad M, Sitras V, Nordbakken CV, Pedersen MI and Acharya G. Laeverin protein expression in normal and preeclamptic placentas using tissue microarray analysis. *Acta Obstet et Gynecol Scand* 2018; https://doi.org/10.1111/aogs.13304.

# INTRODUCTION

Hippocrates described the clinical symptoms of preeclampsia already at 400 BC[2], however despite centuries of intense research efforts the etiology of preeclampsia is still unknown. Preeclampsia runs in families, indicating a heritable pattern for the development of the disease.[3] The placenta plays a crucial role in the development of the fetus and in maintaining maternal well-being during pregnancy. It is proposed that defective placentation leads to different pregnancy-related disorders including preeclampsia.[4] Trophoblast cells are essential determinants of placental development.[5] They remodel the decidua and invade the maternal spiral arteries in order to establish adequate utero-placental circulation. Therefore, shallow trophoblast invasion and defective angiogenesis are considered to play a major role in the development of preeclampsia.[6]

## Genes and the human genome

The human genome contains all the information needed to determine the human phenotype. It is a complex resource of information containing 3.2 billion nucleotides on 23 pairs of homologous chromosomes, or a total of 46 chromosomes in each cell.[7] Each chromosome contains thousands of genes which are parts of the deoxyribonucleic acid (DNA). The genes are split in exons and introns, which are transcribed into messenger RNA (mRNA)(Figure 1).[7] Both ends of the transcript are modified and introns are cut out of the final protein-coding mRNA, which is comprised only of exons. The mature mRNA is transported out of the nucleus to the cytoplasm where the information from the gene is translated into amino acids chains forming functional proteins.[7]

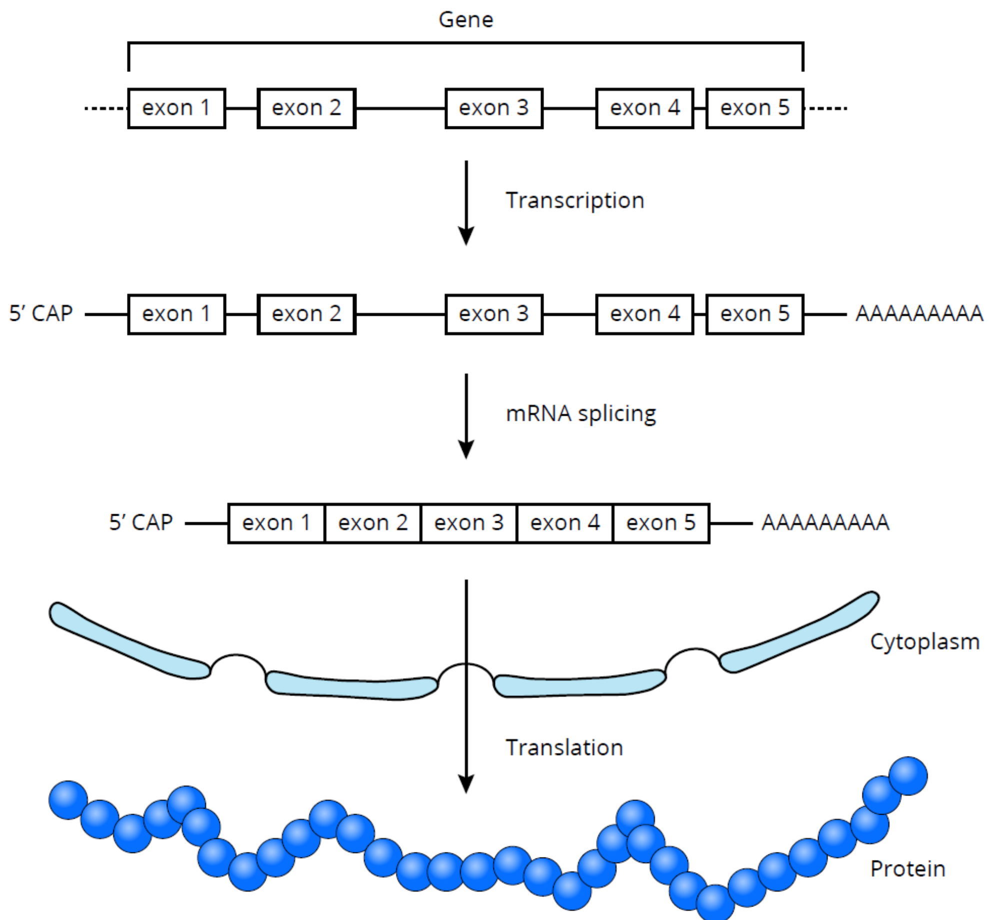


**Figure 1 Simplified illustration of transcription, mRNA splicing and translation in a eukaryotic cell.** The gene in the nucleus contains exons and introns. The gene is transcribed to mRNA, which is modified with a 5'CAP and a 3'poly-A tail. The mRNA is then spliced, where introns are removed and exons spliced together to form a mature mRNA, which is transported out to the cytoplasm where translation takes place. The end product is a protein made of many amino acid chains (blue circles).

The human genome project (HUGO) gave full access to our genome[8-10] demonstrating that only 1.5 % of the genome (approximately 20,000 genes) are protein coding.[8] The rest of the genome are non-coding regulatory sequences (promoters, enhancers/silencers, 5'UTRs and 3'UTRs) and non-coding RNA (ncRNA) which was previously considered junk DNA of unknown function.[7]

## Fertilization and early embryo development

Life starts with the fertilization of a haploid oocyte by a haploid sperm cell forming a zygote, containing 46 chromosomes with all information necessary to form a human organism. The fertilized egg cleaves in two and four by the process of mitosis (Figure 2). Each blastomere interacts with its neighbors through cell-surface adhesion molecules mainly epithelial cadherin (E-cadherin). This process is called compaction and is the first incident of morphogenic and cellular differentiation, creating two different cell populations (blastomeres and trophoectodermal lineage) giving polarity to the embryo.[11] After further mitotic divisions of the blastomeres, the morula (16-24 cells) and later the blastocyst (32-64 cells) are formed.[11] At the blastocyst stage (fifth day after conception) cell specification and lineage segregation occurs with the inner cell mass developing into the embryo proper and the outer cells into the placenta (trophoectoderm).[12]

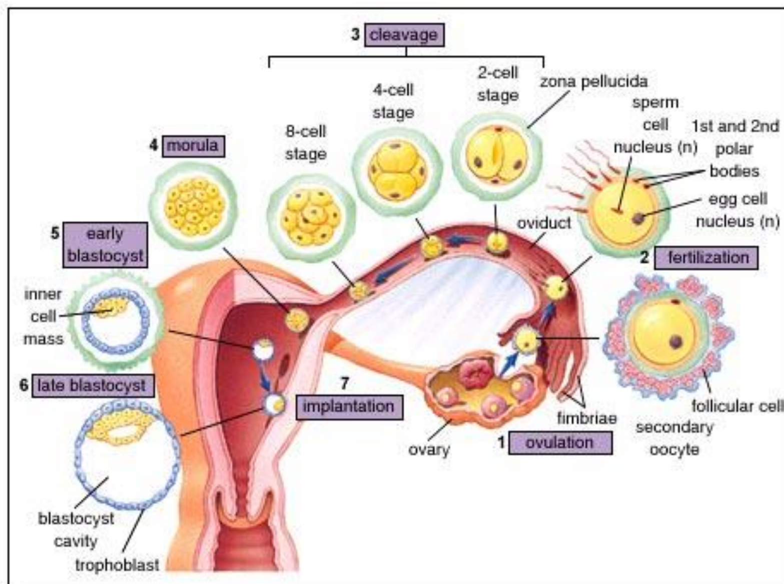


**Figure 2 Fertilization and early embryo development.** Many eggs, arrested in prophase I of meiosis, are stored in the follicles of the ovaries until puberty where follicles mature and release the mature egg (ovulation) (1) into the Fallopian tube. Fertilization (2) of the egg with the sperm triggers maturing of the oocyte and thus the rest of the meiotic divisions I and II. The haploid number of chromosomes are contained in the first and second polar bodies. The mature oocyte contains 23 chromosomes and lots of nutrients and metabolites needed for the development of the embryo. The outer layer of the egg develops into a thick capsule called the zona pellucida (Latin; transparent zone). In the cleavage stages (3) the zygote divides by mitosis to form 2-, 4,- and 8-cells. A morula is formed (4) and after this the early blastocyst (5) with the inner cell mass is formed. The inner cell mass gives rise to the embryo, while the trophoblast cells (blue) develop into the placenta. In the late blastocyst stage (6) the early embryo implants into the endometrium. Reproduced with permission from McGraw-Hill Education.

The blastocyst attaches and implants into the endometrial lining of the uterine wall, which is called decidua during gestation. Trophoblast cells are major actors in the process of implantation giving rise to the placenta and extraembryonic membranes.[13]

# TROPHOBLAST CELL BIOLOGY

A.A.W. Hubrecht was the first to use the term trophoblast (Gk *trophy*, nourishment)(Hubrecht, 1899, cited by Boyd and Hamilton, 1970[5]) indicating the cells of the blastocyst that are necessary to nourish the embryo. When these cells are implanted in the uterus they are called cytotrophoblasts.[11]

The intrinsic capability of trophoblast cells to attach and invade the decidua leads to the development of a mature placenta. Trophoblast invasion and migration involves extracellular matrix (ECM) components, ECM receptors and ECM degrading enzymes.[14] Trophoblast proliferation, migration and invasion have one major goal, i.e. to sustain the developing fetus.[15]

## Blastocyst implantation

"*Implantation in the human is unique. This uniqueness is characterized on the maternal side by a spontaneous and massive decidualization of the endometrium and on the embryonic side by an almost unlimited invasive potential.*"[16]

The quote above describes the first feto-maternal crosstalk. The feto-maternal crosstalk between the embryo and the uterus takes place in a restricted period of time called "the window of implantation"[17] at day 20-24 of the menstrual cycle.[18] This process is regulated by autocrine and paracrine factors.[19] Paracrine signals from the endometrial tissue to the embryo are important for a successful implantation of the blastocyst.[12] This process is dependent on perfect synchronization between endometrial maturation and embryo development.

In general terms, the cell's ability to change position within tissues is vital and depends on cell migration and invasion. Cell migration is the cell´s ability to move and is involved in different processes such as embryonic development, tissue repair and regeneration, cancer development, atherosclerosis and arthritis.[20] Cellular invasion can be defined as entry of foreign cells into a tissue. An invasive trophoblast phenotype is characterized by a special integrin coating of the trophoblasts, the ability to digest extracellular matrix by matrix metalloproteases (MMPs) and the intrinsic migrating capacity of the cells.[19] Many different molecules are involved in these

processes. Cell adhesion molecules (CAM), extracellular matrix, matrix degrading metalloproteases, among other molecules control trophoblast migration. Cell migration and invasion are dependent mainly on four molecular mechanisms: 1) cytoskeleton activation in order to move the cell, 2) modulation of cell-adhesive molecules to provide traction, 3) clearing of the physical barrier of the extracellular matrix (ECM), and 4) chemoattractants in order to direct movement.[20]

In the implantation process different molecular mediators are important. Many CAMs on the trophoblast and the endometrium control human implantation. Among these molecules, integrins play an important role in the implantation process.[21] They are the functional markers determining the time of maximal uterine receptivity. The endometrium responds to implantation and transforms into decidua influenced by progesterone.[22] Implantation begins six to seven days after fertilization[23] and consists of three stages[19,24]: 1) Apposition of the blastocyst to the uterine wall 2) adhesion of the blastocyst to the uterus and 3) invasion of the syncytiotrophoblasts into the uterine epithelium, reaching the inner thirds of the myometrium.

## Trophoblast differentiation and function

Bone morphogenic protein 4 (BMP4) induces differentiation of human embryonic stem cells to form trophoblasts.[25] Villous cytotrophoblast (CT) stem cells differentiate to follow the extravillous and the villous pathway (Figure 3).[26] The extravillous trophoblasts invade the maternal decidua, while the villous trophoblasts form the villous tree that is responsible for the nutrient and gas exchange between the mother and the fetus. Several factors control this process, including transcription factors, hormones, growth factors, cytokines and $O_2$ level.[27]

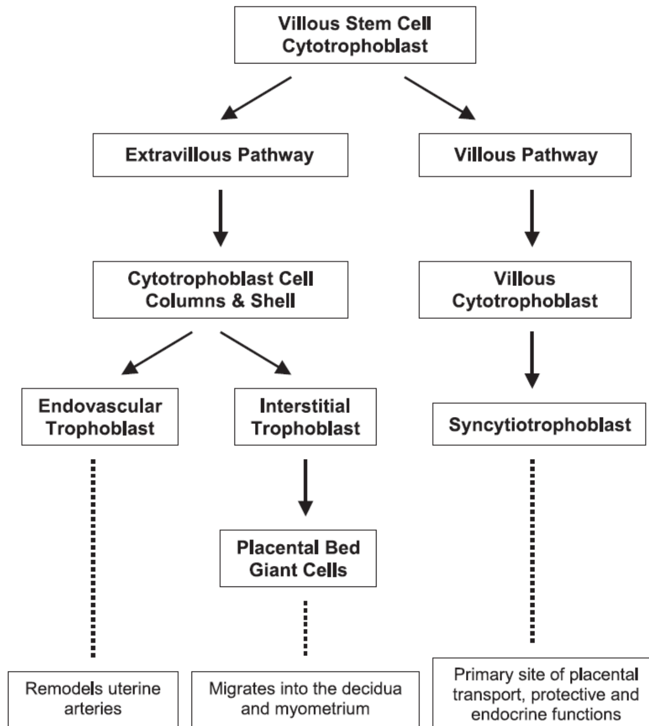


**Figure 3 Trophoblast differentiation and function**. Villous stem cell cytotrophoblasts give rise to extravillous and villous pathway leading to villous cytotrophoblasts, syncytiotrophoblasts, cytotrophoblasts in the cell columns, endovascular trophoblasts and interstitial trophoblasts.[26]. Reproduced with permission from Elsevier.

Villous cytotrophoblasts (Langhan's layer) fuse to form a multinucleated non-proliferative cell layer called syncytiotrophoblast (ST) which is the primary site of maternal gas and nutrient exchange. Moreover, STs have protective and endocrine functions.[26,27] In particular, STs secrete hormones such as human chorionic gonadotropin (hCG), placental growth hormone (PGH) and human placental lactogen (PL), which are vital for placental and fetal growth. [28-31]
Extravillous trophoblasts (EVTs) invade the lumen of spiral arteries replacing the maternal endothelium.[32] This process is referred to as "pseudovasculogenesis" or "vascular mimicry".[26] EVTs first invade the decidua, then the myometrial stroma.[33] Membrane-bound peptidases, regulate human extravillous trophoblast invasion.[34]
The different trophoblast cells (Figure 4 and Table 1) are major constituents of the placenta.

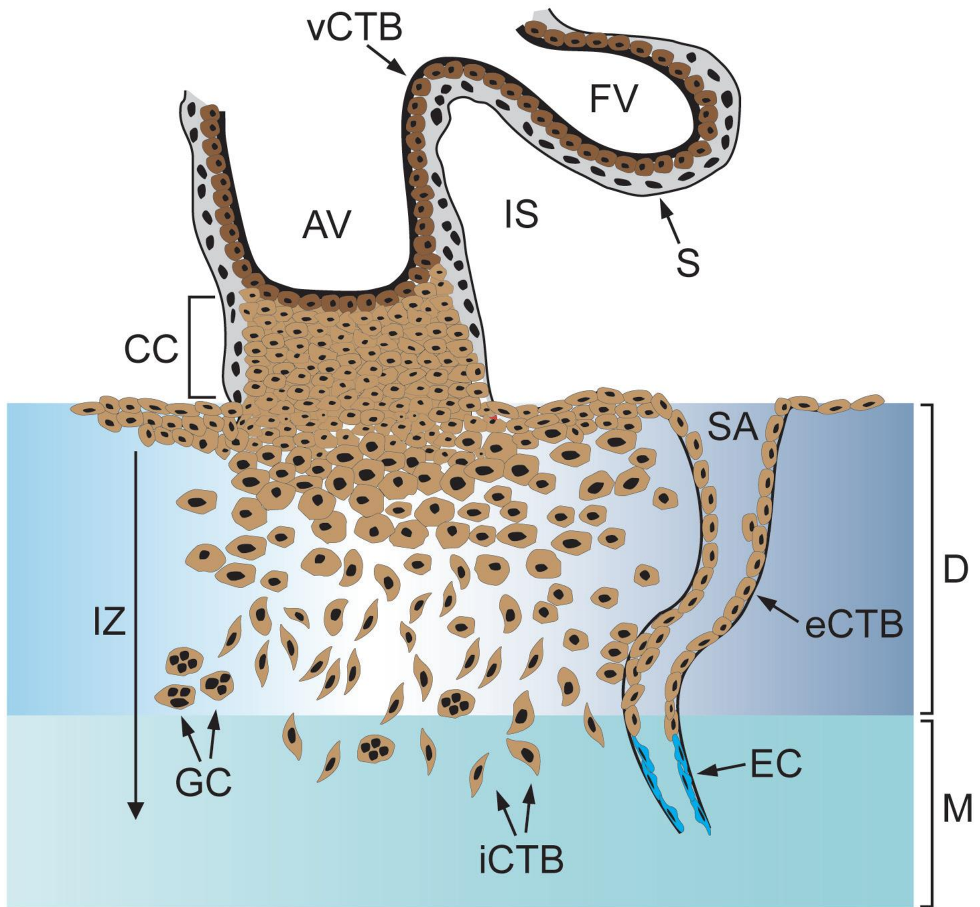


**Figure 4 Differentiation of the trophoblasts as a consequence of invasion.** When the anchoring villi (AV) contact the uterine basement membrane, the villus cytotrophoblasts (vCTB) transform into proliferative cell columns (CC). At the edge non-proliferating extravillous trophoblasts detache from the CC and migrate into the maternal decidua (D), forming interstitial cytotrophoblasts (iCTB). Sometimes the iCTB differentiate into giant cells (GC). Endovascular cytotrophoblasts (eCTB) migrate into the spiral arteries (SA) replacing maternal endothelial cells (EC). ECTBs also migrate into the inner third of the myometrium (M). Floating villi (FV) are bathed in maternal blood in the intervillous space (IS). Cytotrophoblast progenitors forms the multinucleated syncytium, or syncytial trophoblasts (S). The invasion zone (IZ) of EVTs are shown in the left part of the figure (black arrow). Reproduced with permission from Elsevier.[35]

**Table 1 Trophoblast cell subtypes and their function in the placenta.**

| Trophoblast (subtype) | Function |
|---|---|
| **Trophoblast stem cells** | Precursors of the differentiated cells of the placenta.[36] |
| **Cytotrophoblast (CTB)** | Progenitor of CC, iCTB, eCTB, syncytiotrophoblasts and placental giant cells.[11,35] |
| **Cell column (CC)** | Give rise to iCTB and eCTB.[11,26] |
| **Interstitial cytotrophoblast (iCTB)** | The extravillous trophoblasts that invade fibrin deposits on the villi and those that invade decidua and myometrium are collectively called interstitial cytotrophoblasts. They may differentiate into multinucleate giant cells.[11,26] |
| **Villous cytotrophoblast (vCTB)** | Immotile polarized epithelial stem cells that differentiate into syncytia. vCTBs give rise to proliferative cell columns.[11,26] |
| **Intermediate trophoblast** | Equivalent to extra-villous trophoblast.[11] |
| **Extravillous trophoblasts (EVTs)** | Two populations of EVTs are formed after differentiation: endovascular and interstitial trophoblasts.[26] |
| **Interstitial extravillous trophoblasts (iEVTs)** | Invade the decidua. Migrate into the decidual arteries and differentiate into multinucleate cells.[26,37] |
| **Endovascular cytotrophoblasts (eCTB) = Endovascular trophoblasts (eEVTs)** | Migrate into the maternal spiral arteries forming plugs replacing maternal endothelial cells.[11] |
| **Syncytiotrophoblasts (ST)** | CTB progenitors fuse to build the multinucleated, non-proliferative, syncytium called syncytiotrophoblasts. They are involved in maternal gas and nutritional exchange. STs have endocrine functions (secrete chorionic gonadotropin, placental growth hormone, human chorionic gonadotropin among others).[11,26,27] |
| **Trophoblast giant cells (GC)** | Multinucleate EVT-cells.[11] |
| **Hofbauer cells** | Placental macrophages of fetal origin which may have multiple origins depending on gestational stage.[38] In early pregnancy they may originate from villous mesenchymal stem cells of the stroma or monocyte progenitor cells from the hypoblast-derived yolk-sac. Later in pregnancy Hofbauer cells may originate from fetal hematopoietic stem cells. |

# PLACENTAL MORPHOLOGY AND FUNCTION

During evolution of the species, the transition from an aquatic to a terrestrial environment had major implications in fetal development. Therefore, some animals called eutherian (Gk *Eutheria*, good true) developed the placenta (Gk *plakuos*, flat cake) in order to sustain the developing fetus.[39] The placenta has always been a mysterious and powerful organ. In some cultures the placenta symbolizes the tree of life.[40] Since the groundbreaking work of Page[41] describing the placental origin of preeclampsia, more emphasis has been put towards placental research. The placenta is the "gateway" to the fetus and is a vital organ supplying the fetus with oxygen and nutrients, in addition to transporting $CO_2$ and metabolic waste away from the fetus. Placental dysfunction can put maternal and fetal lives at risk. One such potentially life-treatening condition is preeclampsia.

## Normal placental development

Placental development starts in the first trimester of pregnancy.[5] At day 14-20 post-conception, the trophoblasts are very proliferative and result in the identifiable placental structure.[42] Placental development continues throughout pregnancy and at term a normal placenta weighs on average 670 g.[43] The human placenta has a discoid shape and two main parts which have fetal (chorionic plate) and maternal origin (basal plate)(Figure 5).[13] It is approximately 2.5 cm thick and gradually tapers towards the periphery. The umbilical cord at the chorionic plate connects the fetus to the placenta.

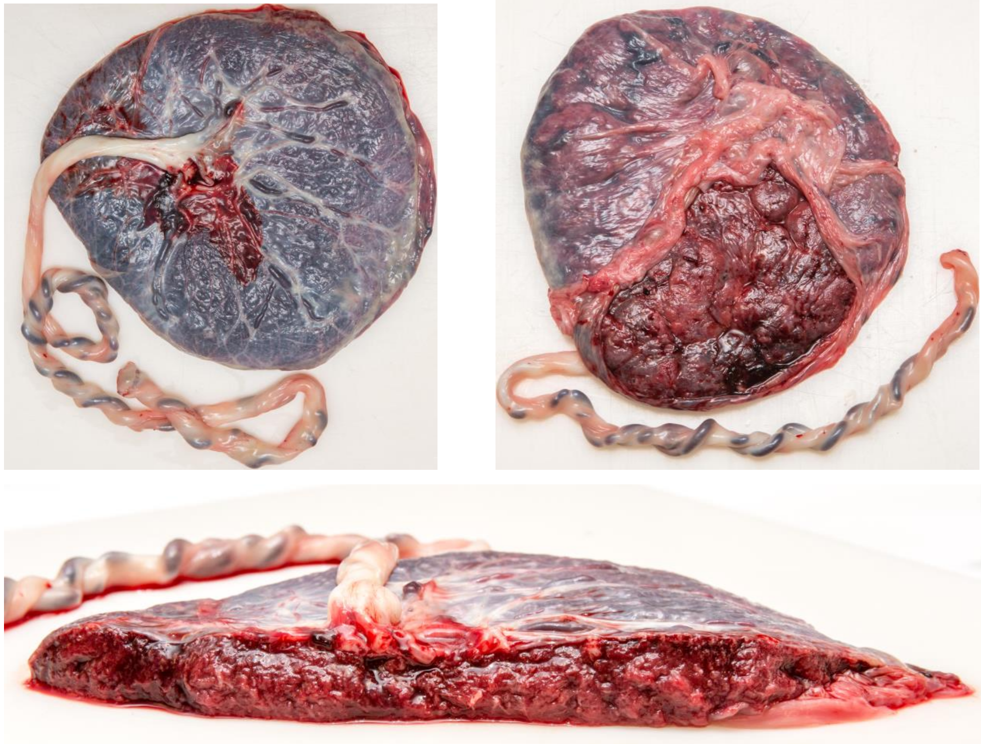

**Figure 5 Normal placenta at term.** Placenta delivered from a healthy pregnant woman at term. A) Fetal side of the placenta showing amnion, chorionic vessels and the chorionic plate with the umbilical cord. B) Maternal side of the placenta where decidua basalis is partly removed showing the cotyledons. C) Transverse section near the umbilical cord insertion showing fetal chorion villi submerged in maternal blood. Photo Bjørn-Kåre Iversen, UiT-The Arctic University of Tromsø, Norway.

A microscopic cross section through the placenta reveals the functional structures called the chorionic villi (Figure 6). These seaweed-like structures are of fetal origin made of trophoblast cells, mesenchymal cells and capillaries. At 20-25 weeks gestation, placental decidual septa form the smallest fetal functional units of the placenta, called cotyledons[44] that contain 15-25 groups of villi. A placenta at term consists of approximately 15-25 cotyledons.[44] A cotyledon consists of a main stem of a chorionic villus with branches and sub-branches. These are submerged into maternal blood and contribute to gas and nutrition exchange between the mother and the fetus.

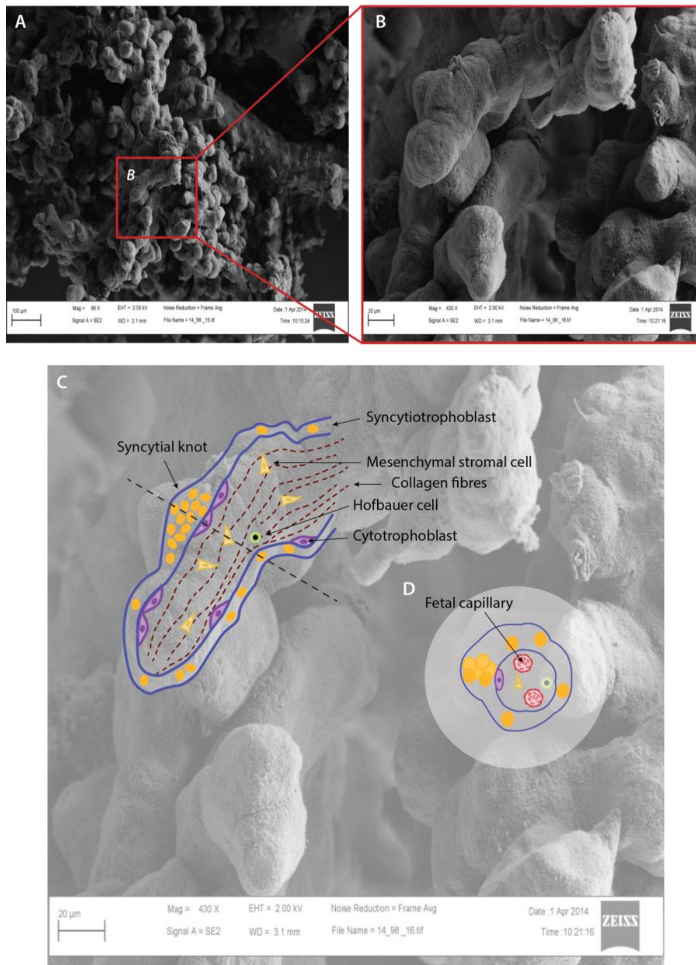


**Figure 6 Scanning electron microscopy (SEM) picture of a dissected chorion villus sample from a healthy placenta at term**. Many chorion villi and cotyledons (A) and close up of some chorion villi (B). Longitudinal section of one villus with different cells depicted (C). Syncytiotrophoblasts (yellow circles), cytotrophoblasts (purple), mesenchymal stromal cells (yellow triangles), collagen fibres (brown hatched lines) and Hofbauer cells (green). Dashed black line shows the transverse plane. Cross section of the villus (D) showing two fetal capillaries with red blood cells and other constituents of the mesenchymal core (MC) and syncytiotrophoblast layer.[1]

[1] Term placental biopsy samples of the fetal side were dissected with scalpels in cubes of approximately 1 $cm^3$. Samples were collected in 1xPBS and washed several times to avoid red blood cells. Chorion villus samples were identified in a Leica MZ6 stereomicroscope (Leica microsystems GmbH, Germany. Maternal material and amnion membranes were discarded, separating the villus tree by microdissection. Placental samples were cut in 1 mm pieces and fixed in McDowell's solution over night, then washed in Sorensens PBS, postfixed in 1% Osmium tetroxyde (OsO4) for 1 h and 30 minutes at room temperature. Samples were critical point died in Balzers CPD 020 Critical Point Dryer (BAL-TEC GmbH, Germany) and mounted on aluminium stabs with silver glue and coated with gold/palladium in a Polaron Range Sputter Coater (Ringmer, UK). Pictures were taken in a Jeol JSM 6300 Scanning electron microscope (Tokyo, Japan).

Connective tissue and fetal capillaries comprise the core of the villi. Placental vascular development is of critical importance for normal development, gas exchange and nutrition of the developing fetus. Both vasculogenesis (i.e. *de novo* process of blood vessel generation *de novo* from angioblast precursor cells) and angiogenensis (i.e. neovascular sprouting of pre-existing blood vessels) are involved in these processes.[45] Branching of the fetal villi occurs throughout pregnancy providing a large surface area for exchange of gas and nutritients.

## Placental function

The placenta is a specialized organ present only during pregnancy, which is crucial for normal growth and development of the fetus.[26] The main functional units of the placenta are the chorionic villi containing the fetal capillaries. Maternal blood surrounds the villi, where nutrients and waste products are exchanged between the mother and fetus through the three layers of trophoblast cells and stroma.[26] Moreover, the placenta is a hormone-producing organ influencing pregnancy, metabolism, fetal growth, parturition etc.[26] Endocrine control of intrauterine development is of vital importance for normal development.[46] Furthermore, the placenta has a protective role for the fetus against xenobiotic molecules, maternal disease and infections.[26] The placenta is indeed impermeable to most microorganisms, but some bacteria and viruses can cross the placental barrier and reach the fetus leading to infections (e.g. *toxoplasma gondii*, *varicella-zoster virus*, *herpes-simplex virus*, *human immunodeficiency virus* (HIV) and *cytomegalo virus* (CMV)).[13] Some non-genetic environmental or pharmacological substances (teratogens), such as drugs and alcohol, may also cross the placenta and can cause birth defects.[13]

## Placenta-related diseases

The fascination for pregnancy-related diseases has been prominent for decades.[2] Two of the most common disorders of human pregnancy, which are placenta-related, are miscarriage and preeclampsia.[4] However, preterm labour (i.e. birth <37 weeks of gestation), occurring in 10% of all pregnancies[47] might also be related to placental ischemia or dysfunction.[48] Poor villous development, abnormal stromal cells and reduced vascular branching[49] may lead to missed abortions or miscarriage. Intrauterine growth restriction (IUGR) is a condition where the fetus does not reach its genetic growth potential and is associated with higher risk of prenatal mortality and morbidity.[13] Placental insufficiency is a major cause of IUGR and early-onset preeclampsia.

# PREECLAMPSIA

## Historical perspectives

Preeclampsia has been known since ancient times. For example, aphorism XXXI 507 in the Coan Prognosis states that a headache accompanied by heaviness and convulsions during pregnancy is considered bad (Hippocrates, 400 BCE/1950) for the pregnant woman.[50] The treatment was to obtain "a balance" in the body with diets, cleansing of the body and blood-letting.[2]

In the middle of the 18th century the classical symptoms of preeclampsia were identified. These are headache, visual disturbances, upper abdominal pain and edema. It was not until 1896, when Scipione Riva-Rocci's mercury manometer was introduced, that blood pressure measurements could be performed and preeclampsia was identified as a disease with elevated blood pressure.[51] Still the cardinal symptoms of preeclampsia are hypertension and proteinuria, and are used for screening to identify risk pregnancies.

In 1914 Young[52] postulated that toxins from the placenta released into the maternal circulation may induce eclampsia (Gk *eklampsis*, lightning). In 1939 E.W. Page was the first to suggest that the placenta plays a central role in preeclampsia.[53]

Preeclampsia is a potential lethal disease affecting previously healthy women, but can also be superimposed on pre-existing diseases, such as hypertension. It is difficult to understand why as many as 5% of human pregnancies are affected by preeclampsia[54], leading to the speculation that preeclampsia might constitute an evolutionary advantage of the human species that we have not elucidated yet. Definitive treatment of preeclampsia is delivery of the placenta, leading to the assumption that defective placentation might be one of the causes of preeclampsia.[55,56]

## Definitions

Pregnancy-related hypertensive disorders are defined in different ways depending on the time of onset and severity of the condition. Chronic or preexisting hypertension refers to a condition that is not related to pregnancy and occurs usually before pregnancy or before week 20 of gestation.[57] Hypertension developing after week 20 is called gestational hypertension (pregnancy related hypertension) and usually relieves 12 weeks postpartum.[57] Preeclampsia is defined as new onset hypertension (systolic ≥140 mm Hg/diastolic ≥90 mm Hg) and proteinuria after 20 weeks gestation. In the absence of proteinuria, preeclampsia is defined as the onset of hypertension with any of the following: thrombocytopenia, renal insufficiency, impaired liver function, pulmonary edema and cerebral or visual symptoms.[58] Eclampsia is a severe form of

preeclampsia characterized by new-onset grand mal seizures. Approximately 20-30 cases of eclampsia occur in Norway every year.[59] Preeclampsia is one of the main direct causes of maternal death in Norway.[60] HELLP syndrome is a severe variant of preeclampsia characterized by hemolysis, elevated liver enzymes and low platelets.[61]

## Risk factors

Preeclampsia is a leading cause of maternal mortality. Worldwide it is estimated that 500,000 mothers die every year, of which 10-15% (50,000-75,000) is believed to be caused by hypertensive disorders of pregnancy.[54] Preeclampsia occurs in 5-10% of pregnancies worldwide[54] and in 3-4% of pregnancies in Norway.[62] Maternal risk factors are presented in table 2.

**Table 2 Maternal risk factors for preeclampsia.[58,63,64]**

| Maternal risk factor |
| --- |
| Maternal age >35 years and <20 years |
| Multiple gestation |
| Nulliparity |
| Renal disease |
| Systemic lupus erythematosus (SLE) |
| Diabetes mellitus |
| Obesity |
| Previous pregnancy with preeclampsia |
| Mutations in Factor V (F5) Leiden-, angiotensinogen- and prothrombin genes |
| Antiphospholipid antibody syndrome |

Women with a normal first pregnancy have a slightly increased risk of developing preeclampsia in the second pregnancy, especially if the woman has a new partner.[65] The etiology of this “dangerous” father[66] is not yet known, but immunologic mechanisms are suggested.[65,67] A prolonged time interval of unprotected intercourse (i.e. without barrier contraceptives) significantly decreases the risk of preeclampsia.[65,67] Repeated exposure of sperm from the same partner in the maternal genital tract seems to induce tolerance to paternal antigens, which may be lost in women changing partners.[65] However, the time interval between pregnancies may be more important than a new partner.[68]

## Pathophysiology and clinical manifestations

Preeclampsia may occur in rare cases of complete hydatidiform mole, which is a pregnancy without the embryo.[13] This finding implies that the placenta is required for development of preeclampsia, not the fetus.[69] The pathogenesis of preeclampsia is suggested to evolve in two stages: the first stage is asymptomatic with abnormal placentation, possibly related to

ischemia.[70] The incidence of placenta infarction is indeed higher in preeclamptic pregnancies compared to uncomplicated pregnancies.[71-73] Traditionally the ischaemic placenta is thought to release soluble factors (previously called toxins) in the maternal circulation leading to maternal endothelial dysfunction causing the clinical manifestations of preeclampsia.[74,75] Recent hypotheses propose additional stages for the pathogenesis of preeclampsia.[76]

In normal pregnancy, trophoblast cells invade the myometrium and spiral arteries, establishing high-perfusion, low resistance blood flow to the placenta. However, in preeclampsia, trophoblast invasion is shallow and spiral artery remodeling is impaired. This results in decreased blood flow and low oxygen uptake in the placenta, leading to the release of inflammatory and anti-angiogenic molecules in the maternal circulation.[45,77]

## Molecular basis

A diversity of pathways and molecules are thought to be involved in the pathogenesis of preeclampsia such as genetic factors, abnormal trophoblast-decidual interaction, oxidative stress and increased type-1 angiotensin II receptor ($AT_1$) autoantibodies (Figure 7).[78] Oxidative stress (in the placenta and in the maternal circulation) is a particularly important component in the pathophysiology of preeclampsia.[79] Defective spiral artery transformation and defective placentation leads to endoplasmic reticulum oxidative stress, release of proinflammatory cytokines, increased $AT_1$ autoantibodies and release of micro- and nanoparticles. Syncytiotrophoblast fragments and microparticles are of special importance[79], leading to inflammatory cytokine release.[80] Soluble antiangiogenic factors, such as endoglin (sEng) and vascular endothelial growth factor-1 (sVEGFR-1) are elevated. In contrast, the pro-angiogenic factors placental growth factor (PIGF) and vascular endothelial growth factor (VEGF) are decreased giving rise to leukocyte and endothelial cell activation leading to the classical symptoms of preeclampsia.

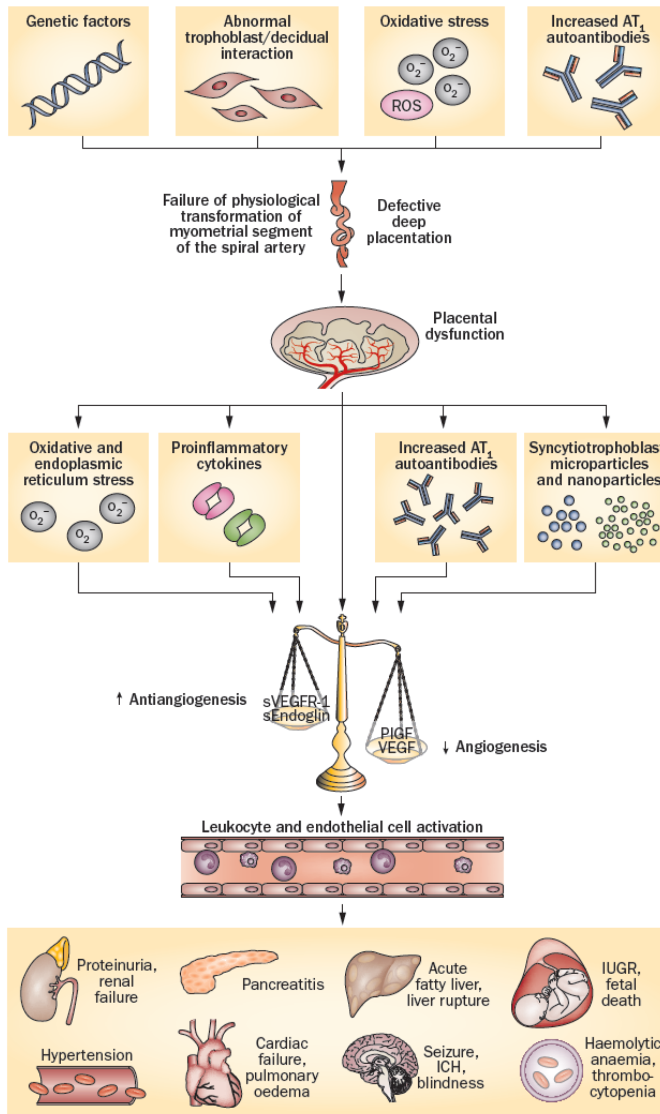


**Figure 7 Model of the molecular basis and pathophysiology of preeclampsia.** Several factors (genetic, abnormal trophoblasts/decidua, oxidative stress and increased $AT_1$ autoantibodies) lead to failure of transformation of spiral arteries and placental dysfunction. This in turn causes oxidative- and endoplasmic stress, release of proinflammatory cytokines, syncytiotrophoblast microparticles and nanoparticles. In this disease process antiangiogenic biomarkers (e.g., sVEGFR-1 and sEndoglin) are elevated in contrast to angiogenic factors (e.g., VEGF and PIGF) which are decreased, causing endothelial cell dysfunction and excessive thrombin generation. Preeclampsia can result in multiorgan failure. Abbreviations: AT1, type-1 angiotensin II receptor; ER, endoplasmic reticulum; ICH, intracerebral haemorrhage; IUGR, intrauterine growth restriction; PIGF, placental growth factor; ROS, reactive oxygen species; s, soluble; VEGF, vascular endothelial growth factor; VEGFR-1, vascular endothelial growth factor receptor 1.[78] Reproduced with permission from Nature Publishing Group.

## Genetic predisposition

Preeclampsia runs in families suggesting a genetic cause of the disease. Genetic susceptibility may be simple with one or more common alleles; or complex, involving many alleles. It is also possible that there are private familial mutations, predisposing to preeclampsia in one particular family. The search for the genes involved in preeclampsia has been diverse including a candidate gene approach, linkage studies, single nucleotide polymorphism (SNP)-based approach and recently exome and genome wide studies. In addition, several whole transcriptome studies revealed different preeclampsia patterns of up- and down regulated genes. The feto-maternal interactions further complicate the search for predisposing placental or maternal factors. Genetic studies of preeclampsia are difficult because families with many affected family members are rare, the mode of inheritance is unknown, the definition of preeclampsia phenotype may vary and animal models are sparse.[81]

### Family studies and twin studies

Women born of a mother with preeclampsia are three times more likely to develop preeclampsia compared to the general population.[82] The first family studies were performed by Leon Chesley from 1935 to 1974.[3,83] A familial clustering of preeclampsia was found in 1968, which was the first suggestion of a genetic predisposition.[3] These data fit with a single gene model.[83] Many studies in Australia, Iceland, Scandinavia, Scotland and USA confirmed a familial predisposition of a 2-5 fold increase in risk of first-degree relatives of women affected by preeclampsia.[3,84-87] Segregation analysis suggested maternal recessive or maternal dominant mode of inheritance with partial penetrance.[86] Four different inheritance models were tested by Arngrimsson *et al.*, who suggested one or more common genetic variants and many “private” aberrant genes.[88]

Twin studies are one common method of testing heritability (the proportion of phenotypic variability attributable to genetic causes) of a genetic trait or disease.[89] Genetically identical monozygotic (MZ) twins are of special interest. A big study including 917 MZ and 1199 dizygotic (DZ) female twin pairs revealed that 25% of women with a monozygotic twin sister who had preeclampsia would develop the disease.[90] Dizygotic twin sisters, in comparison, gave only 6% recurrence.[90] The heritability of preeclampsia ranges from 22% to 47% based on twin studies.[91] Heritability estimates in the study by Thornton *et al.*,[91] showed that the genetic and the environmental effects are of equal importance in the development of preeclampsia.

Another study by Cnattingius *et al*.,[92] stated that genetic factors contributed to preeclampsia in more than half of the cases and that the maternal genes contributed more than the fetal genes.

### Paternal impact

Paternal genes of the fetus may contribute to preeclampsia according to a population study in Norway. Mothers who were pregnant with a partner who had fathered a preeclamptic pregnancy in another woman had approximately twice the risk in the new pregnancy.[84] An extensive study in Utah confirmed these results.[82] Paternally imprinted genes, where only the maternal inherited allele is active, could be a model explaining preeclampsia.[93]

### Genes involved in preeclampsia

Many genetic mechanisms are proposed as the underlying mechanism of preeclampsia. The first genetic studies of preeclampsia started out as candidate gene studies[81], which are genetic association studies looking for genetic variation within pre-specified genes of interest and phenotypes or disease states.[7] To test for genetic polymorphism (i.e. the variation in a DNA sequence that is found at least in 1% of the population and occurs once every 500-1000 base pairs)[94] in a candidate gene, may be a good approach to pinpoint differences between healthy individuals and patients. Many of the studies performed until 2006 used a single polymorphism in a single candidate gene, while a minority of studies tested several genes or many polymorphisms in one or more genes.[95] Haplotype analysis with multiple markers would be a better choice to get additional power in detecting or excluding association. A large study of 775 SNPs and 190 genes performed in 2007 revealed an association with preeclampsia for collagen α1 chain (*COL1A1*), interleukin-1α (*IL1A*) in the maternal genome and urokinase plasminogen activator surface receptor (*PLAUR*) in the fetal genome.[96] There are different specific DNA variants associated with preeclampsia that include the Factor V Leiden mutation, mutations in endothelial nitric oxide synthase, human leucocyte antigen and angiotensin-converting enzyme.[67] A meta-analysis of 11 studies performed in 2013 found a modest, but statistically significant association between preeclampsia and the SNP rs1799889 in plasminogen activator inhibitor type 1 (*SERPINE1*) (serine proteinase inhibitor).[97] Genome-wide association studies (GWAS) with evenly spaced microsatellites throughout the genome gave some promising results and linkage to chromosome bands.[81] Different studies in Scotland, Australia, Iceland, Australia and New Zealand, Netherlands and Finland (including

many families and markers) revealed linkage to chromosomal loci on 2p13, 2p25, 2q23, 4q34, 9p13, 9q33, 10q22, 11q23 and 22q12 using general and strict criteria (strict criteria included women with new onset hypertension and proteinuria, or eclampsia, whilst general criteria included women with non-proteinuric new onset hypertension).[81,95,98,99] The reproduction of these results has in some of the populations been difficult. Indeed, in the Dutch population, a parent-of-origin effect of the storkhead box 1 (*STOX1)*-gene at chromosome band 10q22 has been identified, which suggests imprinting as a possible mechanism.[100] Oudejans and coworkers identified identical missence mutations in the gene in affected sisters co-segregating with preeclampsia.[101] Linkage analysis has not proven however to be the best method for identification of genes or chromosome regions of complex traits as preeclampsia.[81]

On the other hand, gene expression studies of the transcriptome gave more promising results and identified expression profiles specific for preeclamptic placentas. Altered profile of the placental genome may reflect defects in implantation, placental development and maturation that may lead to complications and abnormal pregnancy outcome. From 2002 to 2005 nine studies showed few overlapping genes.[95] These discouraging results are probably due to the variation in microarray design, tissue and use of different mRNA labeling and extraction methods. However, with the advances in gene-array engineering, the studies gave overlapping and interesting results. Amongst the most promising findings was the aberrant expression of the anti-angiogenic factors: *sENG* and soluble fms-like tyrosine kinase 1 (s*Flt*-1).[102,103] Many other genes investigated were identified in microarray studies as potential biomarkers of preeclampsia, for example leptin (*LEP*), high-temperature requirement A serine peptidase 1 (*HTRA1*), inhibin alpha subunit (*INHA*), inhibin beta A subunit (*INHBA*), pappalysin 2 (*PAPPA2*) and follistatin like 3 (*FSTL3*).[104-107] These genes are involved in different processes such as apoptosis, cell signaling, lipid response, hypoxia, immune, inflammation, and oxidative stress.[108-115] In HELLP syndrome similar gene expression patterns have been found using transcriptome microarray analysis.[116] Meta- and integrative analysis may yield more information than small studies.[117] Kleinrouweler *et al.*,[105] and Vaiman *et al.*,[107] performed a meta-analyses showing 40-98 consistently differentially expressed genes across the datasets. Among these were *LEP*, *Flt-1*, *ENG*, *INHA* and *INHBA*. In a study by Moslehi *et al.*, (2013) multiple preeclampsia datasets were included in a larger database, which confirmed previous results involving hypoxia and angiogenesis in the pathophysiology of preeclampsia.[118]

Preeclampsia is a multifactorial, polygenic condition.[67] Many common variants contribute to the individual susceptibility to the disease and may have different penetrance (i.e. percentage of individuals with a given genotype who exhibit the phenotype associated with that genotype).

Therefore, a genome-wide analysis would be the most reasonable method to investigate the genetic etiology of preeclampsia. The Genetics Of Pre-Eclampsia (GOPEC) was a collaborative study among 10 British universities that included 1000 women.[119] The first goal was to try to verify earlier conflicting results in the genes angiotensinogen (*AGT)*, angiotensin II receptor type 1 (*AGTR1)*, angiotensin II receptor type 2 (*AGTR2)*, nitric oxide synthase 3 (*NOS3)*, methylenetetrahydrofolate reductase (*MTHFR)*, tumor necrosis factor (*TNF)* and factor V Leiden (*F5*) variant. However, the study did not confirm these hypotheses; neither in maternal nor in fetal haplotypes. In contrast, a genome-wide association study of 177 preeclampsia cases did not reveal any disease associated variants, but association with four SNPs and a copy number variant (deletion) in pregnancy-specific β1-glycoprotein 11 (*PSG11*).[120] Recently McGinnis and co-workers performed GWAS and identified variants in the fetal genome near *Flt-1* which are associated with risk of preeclampsia.[121]

In conclusion, since the early family studies it has been evident that there is a genetic influence in the pathophysiology of preeclampsia. Nevertheless, no study has so far been able to separate the effects of fetal and maternal genetic factors from environmental and epigenetic influence.

## Biomarkers of preeclampsia

Hulka *et al*. have in 1990 defined biological markers (biomarkers) as "*cellular, biochemical or molecular alterations that are measurable in biological media such as human tissues, cells or fluids.*"[122] The National Institute of Health Biomarkers Definitions Working Group has, on the other hand, defined a biomarker as "*a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses to a therapeutic intervention*".[123] Biomarkers may thus be cells, proteins, small chemical compounds, enzymes, DNA and RNA. Mutations (gene-, chromosome- and genome mutations) may also be used as biomarkers of preeclampsia. These are collectively called genetic-based biomarkers or genomic biomarkers.[124]

Bioactive molecules secreted by the placenta are required for physiological adaptations and a successful pregnancy.[125] These bioactive peptides are known to change before disease onset and may have a strong diagnostic potential, but limited predictive capacity.

Research on predictive tests for preeclampsia has been done for more than 50 years with some success. In 2004, Conde-Aguledo *et al*.[126] performed a large systematic review on behalf of the World Health Organization (WHO). Of the 50 tests examined, many had low predictive value and were thus discarded. Among those included in the meta-analysis only uterine artery

Doppler, antiphospholipid antibodies and kallikrein were found to have moderate predictive value in low-risk women. Since the incidence of preeclampsia is low (3-4%) in developed countries, the authors advised that the positive likelihood ratio should be of satisfactory predictive value. Moreover, a prediction test should also be simple, rapid, non-invasive, reliable and reproducible.[126,127]

Several screening tests for preeclampsia are available, which are based on physical measurements or biomarkers of either the fetoplacental unit or the mother. Most attention has been given to protein markers, probably because they are easily detectable in urine.

### Pro-and anti-angiogenic factors

Pro- and anti-angiogenic proteins are the most promising biomarkers based on current understanding of the disease. A study by Maynard *et al*., (2003) paved the way for more studies of angiogenic proteins involved in normal and abnormal placentation.[128] In normal pregnancy the pro-angiogenic proteins VEGF and PIGF are bound to the receptor VEGF-R, while transforming growth factor α1 (TGFα1) is bound to endoglin (Eng).[129] Transforming growth factor β2 (TGFβ2) is circulating in the maternal spiral arteries leading to vasodilation and adequate perfusion of the placenta. On the other hand, in placentas of mothers with preeclampsia the situation is shifted to massive expression of the anti-angiogenic factor sFlt-1, which binds to VEGF and PIGF; while sEng binds both TGFα1 and TGFβ2.[129] This binding leads to vasoconstriction of the spiral arteries, reduction of placental blood flow and eventually development of preeclampsia.

However, circulating maternal angiogenic factors, as a single test is not proven to be clinically useful to predict preeclampsia.[130] On the contrary, it seems that the ratio (or the combination) of angiogenic and antiangiogenic factors might be used to predict early-onset preeclampsia.[130] The best combinations are the ratios: sEng/sFlt-1[131], sFlt-1/PIGF[132], PIGF/sEng[133] and slope PIGF/sEng[133].

Moreover, multivariable prediction models for preeclampsia using combinations of maternal characteristics and biomarkers gave promising results for predicting early-onset preeclampsia.[130] The most promising studies are using a combination of maternal characteristics, uterine artery Doppler pulsatility index (PI), mean arterial pressure (MAP) and different biomarkers (PIGF, pregnancy-associated protein A (PAPP-A), activin A, P-selectin, inhibin A, sEng, placental protein 13 (PP13), pentraxin, P-selectin, a disintegrin and metalloprotease 12 (ADAM-12) and β-hCG).[134-149]

# LAEVERIN

In a previous study we showed that laeverin mRNA is ten-fold upregulated in preeclamptic placentas, compared to placentas obtained from healthy pregnancies.[112] Therefore, we hypothesized that laeverin protein might be involved in the pathophysiology and/or clinical manifestation of preeclampsia.

## History of laeverin identification

Laeverin was first identified by Fujiwara *et al*, 2004[150] using monoclonal antibodies (mAbs) against chorion laeve and found one mAb, named chorion leave antibody 2 (CHL2), reacting with an EVT-bound molecule. This protein was named laeverin, as it originates from the chorion laeve. Laeverin was expressed in migrating EVTs in maternal decidual tissues. It was not found in fetal amniotic epithelial cells or maternal decidual cells nor in villous trophoblasts at implantation site at 12 weeks gestation.[150] In 2003, one year before the publication by Fujuwara *et al*., Puente *et al*. predicted a novel aminopeptidase using a comparative genomic approach and named this aminopeptidase Q[151], which is an alias to laeverin.

## Laeverin gene

Laeverin synonymous names are *LVRN*, aminopeptidase Q (*APQ*, *AP-Q* or *AQPEP*) and *CHL2* antigen.[152] The gene is mapped to chromosome 5q23.1 based on an alignment of the laeverin sequence (GenBank AK075131) with the genomic sequence (GRCh37). Fluorescence *in situ* hybridization (FISH) experiments performed on healthy human metaphase chromosomes with *AQPEP* probe (Figure 8) show the localization of the laeverin gene on chromosome band 5q23.1.

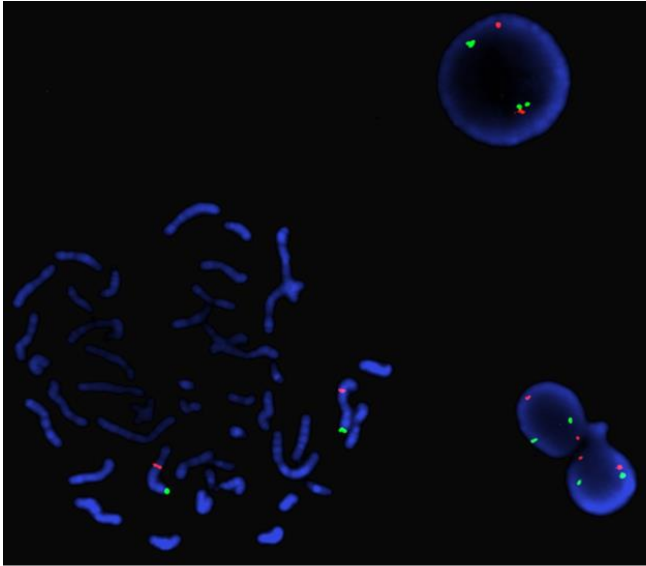

**Figure 8 Fluorescence *in situ* hybridization (FISH) picture of laeverin gene on chromosome 5q23.1.** Healthy human female peripheral blood lymphocyte metaphase chromosomes and interphase nuclei labelled with *AQPEP* probe in locus 5q23.1 (orange) and 5p subtelomere as control probe (green). Counter colouring with DAPI II (blue).[2]

The laeverin gene has 20 exons and 19 introns and the coding region or cDNA sequence is 2970 bp long.[150] The gene has 10 transcripts (splice variants), only 6 of which are protein-coding and 4 of these are subjected to nonsense-mediated decay (i.e. a process which detects nonsense mutations and prevents the expression of truncated or erroneous proteins).[153] Placenta-specific transcripts of 4.0 kb and 3.0 kb have been identified by Northern blot analysis (tissue tested: brain, heart, skeletal muscle, colon, thymus, spleen, kidney, small intestine, lung and peripheral mononuclear cells).[150] Two transcripts are protein-coding, giving rise to two proteins of 990 amino acids and 978 amino acids.

[2] Fluorescence in situ hybridization (FISH) analysis was performed to visualize the laeverin gene on healthy human female peripheral blood lymphocyte metaphase chromosomes and interphase nuclei. Standard lymphocyte cell culturing, harvesting and slide making were performed. Hybridization with laeverin probe locus 5q23.1 (AQPEP; Empire Genomics, Buffalo, NY 14203) and locus specific probe for 5p subtelomere (5p TelVysion probe; Abbott Molecular, Illinois, USA) was performed on sides with interphase nuclei and metaphase chromosomes. Slides were counter coloured blue (DAPI II; Abbott Molecular, Illinois, USA). Images were obtained with CytoVision digital system (Applied Imaging, Grand Rapids, MI) that was equipped with a charge-coupled device camera (Cohu Inc, Poway, CA). A total 30 metaphases and 100 interphase nuclei were inspected.

## Laeverin protein

The MEROPS database has listed 1088 known and putative human proteases and 359 homologues.[154] Some of these are restricted to the placenta. They are classified according to their site of hydrolysis of peptides. Three classes are known: Exopeptidases (consist of aminopeptidases), carboxypeptidases and endopeptidases.[155] Aminopeptidases cleave peptides from the N-terminal aminoacid, while carboxypeptidases cleave aminoacids from the C-terminal end. Endopeptidases hydrolyze internal peptide-bonds. [34]

Laeverin is a transmembrane metalloprotease in the gluzincin group [150] M1 family of aminopeptidases.[156] The peptidase family M1 contains primarily aminopeptidases and is named family type M01.001 in the MEROPS database.[157,158] In mammals this family consists of 9 different proteins of which 5 are integral membrane proteins.[159] Laeverin is a membrane-bound gluzincin metalloprotease/metallopeptidase[150], which is an exopeptidase hydrolyzing peptide bonds from the N-terminus of the substrate protein or peptide.

Laeverin protein is predicted to have 990 amino acids[150] and a predicted molecular mass from cDNA of 113 kDa.[150] Initiator methionine in position 1 of laeverin protein is removed in the mature protein.[150] Laeverin has a short cytoplasmic tail at amino acid position 2-13 (Figure 10).[156] At position 14-34 is transmembrane helical and signal-anchor for type II membrane protein, while position 35-990 is luminal.[160] The long region from amino acid 98-506 corresponds to the peptidase M1 motif.[150] Substrate binding positions are 379-383 (by similarity in UniProt). Peptide binding or exopeptidase motif GXMEN are in amino acid position 379-395[160]. A highly conserved zinc-binding HEXXH$(X)_{18}$E motif (E-glucicine motif) is found in position 415-438 of the laeverin protein.[160] This is shared by many enzymes and is called gluzicin aminopeptidase.[161] Zinc-binding is thought to occur on the two histidines and the glutamate accompanying the first histidine.[162] The outer domain has an active site consisting of GAMEN and HEXXH$(X)_{18}$E motifs (Figure 9).[34,156] The two histidines in the HEEXH-motif are within a long helix with the glutamate on another antiparallel helix.[158,163] Glutamate in position 416 in the HEXXH motif is known to be important for catalysis.[163] The catalytic pocket of laeverin is in position 238 of the protein.[164]

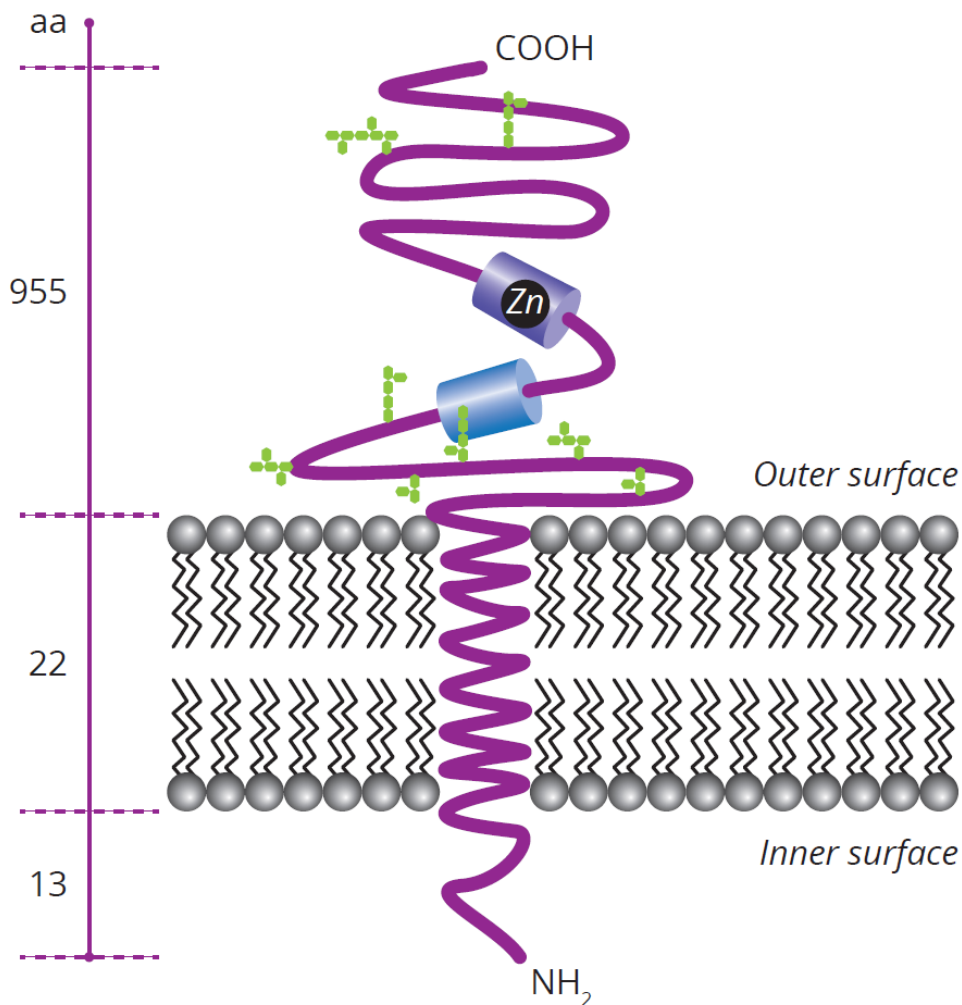


**Figure 9 Model of the laeverin protein.** The protein (purple) is membrane (grey) bound. Laeverin protein consists of different parts: a short cytoplasmic tail, a transmembrane helix and an extracellular signal-anchor. The signal-anchor part consists of different motifs: The zinc-binding motif (violet), the peptide-binding motif (light blue) and N-glycosylation residues (green diamonds) at 8 different positions of the protein. A zinc atom is shown (black). Position 238 is the catalytic pocket of the protein. At the left of the figure a vertical purple line indicates number of aminoacids in each part of the protein. Cystein disulfide bonds or bridges are not depicted in the figure. Laeverin has 15 potential glycoslation sites; 8 sites are shown to be heavily glycosylated.

Four different laeverin protein isoforms are known (Table 3).[165] Isoform 1 is the longest consisting of 990 aminoacids.

**Table 3 Laeverin protein isoforms.**[165]

| Isoform | Aminoacid changes reative to isoform 1 (position: aminoacids changed) | Identifier (UniProt) | Length (aminoacids) | Mass (kDa) |
|---|---|---|---|---|
| 1 | | Q6Q4G3-1 | 990 | 113 |
| 2 | 698-701: KNNY→GKTY<br>702-990 are missing | Q6Q4G3-2 | 701 | 79 |
| 3 | 1-483 are missing<br>698-709:<br>KNNIEETALE→NLQDFGHLKVPN<br>710-990 are missing. | Q6Q4G3-3 | 226 | 26 |
| 4 | 1-483 are missing<br>680-681: AI→MR | Q6Q4G3-4 | 198 | 23 |

The last two isoforms may be produced in very low levels due to a premature stop codon in mRNA, which may lead to nonsense-mediated decay.

Maruyama *et al*., 2007[156] found that most of human laeverin expressed in insect cells is secreted in the medium. Further characterization of these proteins revealed that the N-terminus of the protein started at lys65, indicating processing of the protein before secretion.[156] This soluble laeverin protein has a molecular mass of 120 kD (SDS-PAGE under reducing and non-reducing conditions).

Membrane-bound M1 family of aminopeptidases are often forming homodimers. Indeed, Maruyama *et al*., 2007 measured recombinant human soluble laeverin by gelfiltration column chromatography and found that the purified enzyme eluted between 440 kDa and 232 kDa, suggesting a non-linked homodimeric protein (homodimer without a intermolecular disulfide linkage).[156]

SDS-PAGE gel separation of purified laeverin from term placenta using the CHL2 monoclonal antibody identified a band of 160 kDa.[150] The estimated molecular mass is 113 kDa, based on the cDNA sequence, which is smaller than the size of the isolated protein from placenta. A process of protein modification such as glycosylation may explain this discrepancy. Studies by Maruyama *et al*., 2007 showed that laeverin protein is post-translationally N-glycosylated and has 15 potential N-glycosylation sites.[156] Laeverin protein is heavily glycosylated in position 132, 168, 261, 288, 319, 346, 607 and 653 (Figure 9).[156,165]

## Function of laeverin

The M1 family of zinc aminopeptidases is conserved and found in many animals, fungi, bacteria and plants.[162,166] The family consists of 11 enzymes in humans.[162,163,166,167] Aminopeptidases are involved in maintenance of homeostasis, maintenance of normal pregnancy, memory retention, blood pressure regulation and antigen presentation.[166]

### Enzyme function

Membrane-bound peptidases, in general, are considered as local regulators of peptide signaling systems. The most prominent feature of membrane-bound peptidases are that they have their catalytic activity extracellularly.[168] Membrane-bound peptidases regulate the concentration of biologically active peptides (Figure 10).[34]

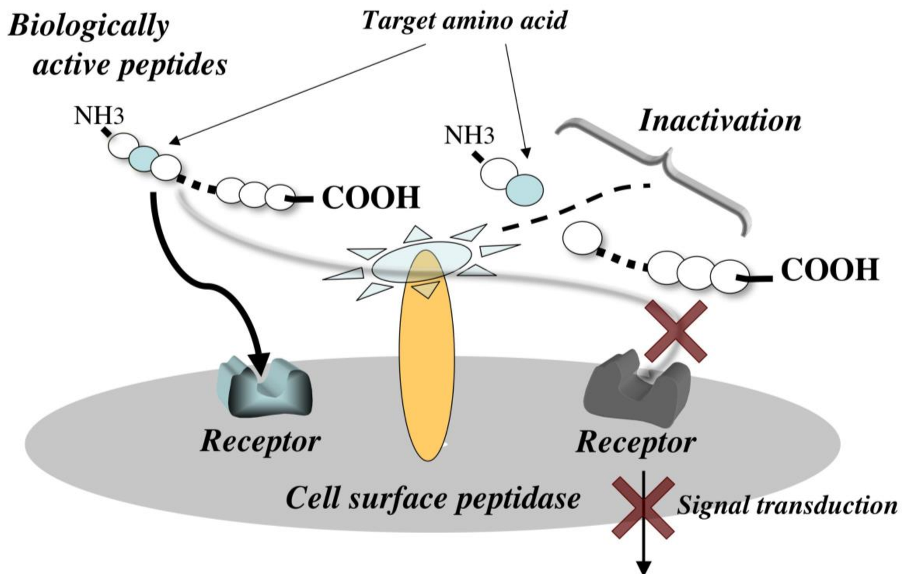


**Figure 10 Cleavage of biologically active peptides by membrane-bound peptidases on the cell surface.** Peptidases regulate the concentration of biologically active peptides before these peptides access their specific receptors. [34] Reproduced with permission from Elsevier.

Many of these control cell differentiation and growth.[169] In the process of removal of one of two aminoacids from the substrate, peptidases reduce the stability of the substrate, sometimes leading to further degradation by other enzymes, or reduce the substrate's receptor-binding capacity. Membrane-bound peptidases are important local regulators for cell function and critical for maturation of proteins.[167,169] Laeverin is part of house-hold enzymes and may be a part of ubiquitin-dependent pathways of degradation, on the basis of their removal of $NH_2$-terminal residues involved in regulation of hydrolysis of proteins.[167]

Aminopeptidases may be secreted as a soluble form, which is produced by cleavage at the plasma membrane.[159] The aminopetidases placental leucine aminopeptidase (P-LAP), aminopeptidase A (APA) and aminopeptidase N (APN), are expressed initially as a type II membrane integral protein shedded by a proteolytic protein as a disintegrin and metalloproteinase-12 (ADAM-12) and secreted into plasma.[170-173] Maruyama et al., 2007 showed that soluble laeverin (sLaeverin) has the enzyme function.[156]

Recombinant soluble laeverin has a broad substrate specificity with highest aminopeptidase activity against leucine-4-methylcoumaryl-7-amide (leu-MCA) (Supplementary table I).[156] Laeverin cleaves the N-terminal amino acid of several peptides such as angiotensin III (AGT), dynorphin A(1-8) (PDYN), endokinin C (TAC4) and kisspeptin-10 (Kp-10) (Supplementary table I).[154,156] Laeverin's aminopeptidase activity is inhibited by bestatin, bradykinin and angiotensin IV (Supplementary table II).[156]

### Role of laeverin in trophoblast proliferation and invasion

Horie *et al*., in 2012 demonstrated laeverin's involvement in trophoblast invasion.[174] Invasion assays of isolated EVTs with siRNA-silenced laeverin showed reduced cell invasion[174] Moreover, the soluble form of recombinant laeverin enhances EVT in invasion assays in a dose dependent manner.[174] Horie *et al*., 2012[174] also demonstrated stimulation of invasion by addition of soluble recombinant laeverin in laeverin negative human choriocarcinoma cell line (BeWo) cells, which don't express laeverin constitutively. Primary villous explant culture silenced with siRNA against laeverin reduced laeverin mRNA expression and migration on the distal site of the cell sheet.[174]

### Expression of laeverin in trophoblast cells and tissues

Laeverin has resemblance to aminopeptidase N (AP-N), which is expressed in reproductive organs (ovaries and endometrium).[150,168] In chorion tissue at 4- and 11 weeks of gestation laeverin was found in distal parts of cell columns using the CHL2 antibody.[174] Laeverin mRNA was detected by RT-PCR analysis in placental samples at 4-, 9- and 38 weeks gestation; and in isolated EVTs (6 weeks gestation).[174] Laeverin mRNA was not detected in BeWo cells.[174] Experiments with primary villous explant culture showed laeverin expression in EVTs grown from attached villous tips.[174] Laeverin was detected in invading EVTs in maternal decidua in early pregnancy and in EVTs in placental bed in term pregnancy.[174] Laeverin is further found to be overexpressed in synovial fluid of patients with rheumatoid arthritis.[175]

### Role of laeverin in blood pressure regulation

Many placental proteases are involved in controlling fetal and maternal blood pressure through regulation of vasoactive peptides.[176] In general, aminopeptidases are known to have a role in blood pressure regulation by metabolism of bioactive peptides at the feto-maternal interface.[177] Laeverin has 7 paralogues, 84 ortologues and one protein family (Family ID: PTHR11533_SF31).[153] One paralogue, aminopeptidase A (ENPEP), is a metalloprotease which appears to have a role in the renin-angiotensin system.[178,179] Aminopeptidase A (AP-A, APA) regulates blood pressure through angiotensin II metabolism, creating angiotensin III.[177] Its subcellular location is in extracellular-, lysosome-, plasma membrane- and vacuole compartments.[180] Laeverin is known to cleave angiotensin III (the precursor of angiotensin IV), kisspeptin-10 (Kp-10), endokinin C (TAC4) and dynorphin A1 (PDYN).[152,156] AP-N hydrolyses different peptides as oxytocin, bradykinin, angiotensin II and interleukin.[168] AP-N and P-LAP degrades angiotensin II to angiotensin IV.[177]

## AIMS OF THE STUDY

The overall aim of this thesis was to elucidate the role of *laeverin* in human placentation and pathophysiology of preeclampsia. The specific objectives were to investigate:

- The protein expression pattern of laeverin in healthy- and preeclamptic placentas at term.
- The role of *laeverin* gene in trophoblast cell migration and invasion in first trimester trophoblast cells.
- Plasma laeverin protein expression in preeclamptic and healthy pregnancies during the second and third trimesters of pregnancy and postpartum.
- The expression of laeverin protein in placenta using tissue microarray.

# MATERIALS AND METHODS

Materials and methods related to specific studies (I-III) are described in detail in the articles included in the appendix. This part of the thesis describes the principles of the methods and an overview of all methods used.

## Clinical evaluation of preeclampsia

The Norwegian guidelines for the diagnosis and treatment of preeclampsia were followed.[181] Pregnant women with a suspicion or diagnosis of preeclampsia were evaluated by an obstetrician and generally hospitalized for close observation/monitoring. An ultrasound examination and cardiotocography (CTG) were performed to assess fetal wellbeing. During the study period, quantification of proteinuria in the urine sample collected for 24-hours was a routine at the Department of Obstetrics and Gynecology, University Hospital of North Norway (UNN), when the urine dipstick was ≥2+. In case of severe preeclampsia hemoglobin, hematocrit, platelets, uric acid and liver enzymes (alanine aminotransferase and aspartate aminotransferase), coagulation status, electrolytes, creatinine, serum albumin, and haptoglobin were analyzed. Biochemical analyses were performed on a Cobas 8000 machine (Roche Diagnostics, Indianapolis, USA) or Sysmex XN 1000/2000 (Sysmex America Inc., IL, USA). Histologic macro- and microscopical examinations were also performed on preeclamptic placentas after delivery[182] using a novel classification system developed in Oslo University.[183]

## Sample collection and conservation

Maternal- and fetal blood samples and placental tissue samples were used. An overview of samples is presented in Table 4.

**Table 4 Samples used in the different studies indicated with different Latin numbers**.

| Paper | Material | Number of samples | Gestational stage |
|---|---|---|---|
| I | Placental tissue | 12 (6 N and 6 PE) | Delivery |
| II | Maternal serum/plasma longitudinal study | 53 N patients (243 samples) | 22-40 weeks (42 of these 2-6 days postpartum) |
| II | Umbilical venous blood | 38 | Immediately after birth |
| II | Serum samples of negative controls | 7 healthy men, 10 menopausal women, 10 non-pregnant premenopausal women | NA |
| II | Umbilical arterial and venous serum samples | 10 neonates of healthy pregnancies | Immediately after birth |
| II | Placental tissue | 11 N and 13 PE | Delivery |
| III | Placental tissue | 77 (41 N and 36 PE) | Delivery |

## Blood samples

Maternal venous blood samples were collected from the pregnant women participating in the studies (Paper I-III). Blood samples were used for clinical evaluation of the women or for laeverin monitoring during pregnancy. Umbilical venous and arterial blood samples were obtained immediately after delivery and sampled separately. Blood bank donors and other healthy volunteers were used as negative controls (Paper II). Samples were processed and frozen at -70°C until analysis.

Peripheral blood was collected by venipuncture using a BD Vacutainer® Safety-Lok™ Blood Collection Set (Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, USA) and vacuum tubes.

### Plasma preparation

Peripheral whole blood was collected in a covered anticoagulant-treated tube (BD Vacutainer® K2E ethlenediamineteraacetic acid (EDTA)-treated; Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, USA). Cells were removed from plasma by centrifugation for 10 minutes at 1,250 x *g* using a centrifuge at room temperature. The supernatant containing the plasma was transferred immediately to clean polypropylene tubes (Sigma Aldrich Gmbh, Munich, Germany) and stored at –70°C until use.

### Serum preparation

Peripheral whole blood was collected in a covered tube containing no anticoagulant (Vacuette® Z Serum Sep Clot Activator-treated; Greiner bio-one, Kremsmünster, Austria). The tubes were left undisturbed at room temperature for 15-30 min before centrifugation at 1,000–2,000 x *g* for 10 minutes at room temperature. The supernatant containing the serum was transferred immediately to clean polypropylene tubes (Sigma Aldrich GmbH, Munich, Germany) and stored at –70°C until use.

## Placental samples

Placental samples from preeclampsia patients were used for macro- and microscopic histopathological analysis. Both normal and preeclamptic placentas were used in Paper I, II and III. Placental samples were obtained immediately after delivery and chorionic tissue was dissected approximately 2 cm from the umbilical cord insertion, the margin of the placenta and the most abnormal part of the placenta. All samples were collected from macroscopically normal areas excluding sites of infarction, fibrin deposition and hemorrhage. The specimen (~2 $cm^3$) was washed briefly in physiological saline to get rid of maternal blood and amniotic fluid. Each cube was snap frozen at -70°C.

All placentas were immersed in 4% formalin and sent for histopathological examination. All preeclamptic placentas were examined by a single pathologist, without prior knowledge of clinical diagnosis. Five different parts of the placenta were collected (umbilical cord, membranes, central portion of placenta, tangential section from basal plate and transmural central section). All sections were evaluated for inflammation and graded[184] using standard hematoxylin and eosin stained slides. Evaluation for any evidence of atherosclerosis, thrombosis or infarction was also performed.

Placental samples for transmission electron microscopy (TEM) were collected in 1xPBS and washed several times to avoid maternal red blood cells and prepared for TEM immediately.

## First trimester trophoblast cell line

The HTR-8/SVneo p69 trophoblast cell line, produced by immortalization of HTR-8 cells with SV40 virus obtained from primary cultures of human trophoblast cells[185] was used for migration, invasion and PCR array studies (Paper I). Cells were maintained in RPMI Medium 1640 (GIBCO®, Invitrogen, USA) supplemented with 5% FBS (GIBCO®, Invitrogen, USA) in a 37°C incubator (Forma Scientific Water-Jacketed Incubator, USA) with 5% $CO_2$. Trypsin-EDTA (Sigma, USA) was used for harvesting and for sub-culturing cells.

## Laeverin protein expression analysis

Western blot analysis with placental protein extracts was performed with different antibodies against laeverin (Table 5) searching for differences in laeverin molecular weight between uncomplicated and preeclamptic pregnancies.

Protein localization studies with anti-laeverin antibodies (Table 5) were performed on formalin fixed paraffin embedded (FFPE) tissue sections of preeclamptic- and healthy placentas in order to elucidate any differences in laeverin protein expression. Sub-cellular localization studies were done on ultrathin sections of frozen samples freeze substituted and infiltrated in Lowicryl HM20 in transmission electron microscopic analysis (TEM).

### Total protein isolation from placenta

Placental tissue was cut in pieces and homogenized using MagNA Lyser Green Beads on MagNA Lyser (Roche, Indianapolis, IN). Total protein isolation was performed using lysis buffer for tissue protein (Tissue Protein Extraction Reagent (T-PER™) from Pierce Chemical Co, Rockford, IL) with Complete Mini (EDTA)-free protease inhibitor cocktail (Roche, Indianapolis, IN) in combination MagNA Lyser Green Beads for homogenizing on MagNA Lyser (Roche, Indianapolis, IN). Total protein concentration was measured using DC Protein Assay kit (Bio-Rad Laboratories, Hercules, CA) in a ThermoMax Microplate Reader (Molecular Devices, Downington, PA).

## Antibodies

Five different antibodies for laeverin protein detection were used in different studies (Table 5).

**Table 5 Laeverin antibodies.**

| Name | Aminoacid sequence | Localization in laeverin domain | Company | Paper |
|---|---|---|---|---|
| EP073418 (rabbit polyclonal anti-Laeverin) | CRVHANLQTIKNENLK | COOH-end of the protein | Eurogentec, Seraing, Belgium | I |
| EP073419 (rabbit polyclonal anti-Laeverin) | CERAEVRGPLSPGTG | External surface | Eurogentec, Seraing, Belgium | I |
| sc-164825 (goat polyclonal anti-Laeverin) | Not specified by the manufacturer | | Santa Cruz Biotechnology Inc., Santa Cruz, CA. | I |
| AP-Q | Not specified by the manufacturer | | EIAab Science co., Ltd, Wuhan, China | II |
| ab185345 (rabbit polyclonal anti-Laeverin) | IPYPIKDVVLCYGIALGSDKEWDILLNTYTNTTNKEEKIQLAYAMSCSKDPWILNRYMEYAISTSPFTSNETNIIEVVASSEVGRYVA | External surface | AbCam, Cambridge, UK | III |

Several other antibodies were used in the Paper I and III (Table 6).

**Table 6 Other antibodies used.**

| Name | Company | Paper |
|---|---|---|
| Actin | Santa Cruz Biotechnology Inc., Santa Cruz, CA. | I |
| Goat anti-mouse immunoglobulin G-alkaline phosphatase conjugated antibody | Santa Cruz Biotechnology Inc., Santa Cruz, CA. | I |
| Secondary goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate | Santa Cruz Biotechnology Inc., Santa Cruz, CA. | I |
| Golgi apparatus specific antibody (MG160 ab58826) | Abcam, Cambridge, UK | I |
| Endoplasmic reticulum specific antibody (RL90 ab2792) | Abcam, Cambridge, UK | I |
| Secondary antibody gold conjugated goat F(ab)2 anti-rabbit IgG (5 nm) | British Bio Cell International, UK | I |
| Secondary antibody gold conjugated goat F(ab)2 anti-mouse IgG (10 nm) | British Bio Cell International, UK | I |
| Cytokeratin 7 (CK7)(rabbit monoclonal anti-CK7, SP52, code 05986818001) | Roche, Tucson, USA | III |

Some additional secondary antibodies were used for laeverin and CK7 detection by immunohistochemistry and immunofluorescence in tissue microarray analysis (Paper III).

### Western blot analysis

Western blot analysis is a widely used technique to detect specific proteins in a given tissue homogenate or extract. Gel electrophoresis is used to separate native or denatured proteins by the length of the polypeptide (denaturing conditions) or by the 3-D structure of the protein (native/non-denaturing conditions). The proteins are then transferred to a membrane (typically

nitrocellulose or PVDF), where they are detected using antibodies specific to the target protein.[186] Western blot analysis was used to test the specificity of laeverin antibody and to detect laeverin protein in different placental samples (Paper I).

Reduced and denatured proteins (5 mg) that had been isolated from 8 placentas (4 preeclamptic and 4 normal) were separated by sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) on 4-12% NuPAGE (Invitrogen, Carlsbad, CA, USA). Electrophoresis and blotting (polyvinylidene difluoride nylon membrane, pore size 0.45 mm; Invitrogen) were run on Novex Mini Cell XCell Sure Lock (Invitrogen) including MagicMark™XP Standard (Invitrogen) and SeeBlue® Plus Pre-Stained Standard (Invitrogen). Blots were cut under a 49-kDa protein band to provide 2 blots; 1 for laeverin and another for the housekeeping protein actin. Labeling was done with primary antibodies against laeverin (our antibody [0.42mg/ ml] and commercial antibody [1 mg/mL; Roche Biotechnology Inc] or actin [1 mg/mL; Santa Cruz Biotechnology Inc]). Detection was performed with goat anti-mouse immunoglobulin G-alkaline phosphatase conjugated antibody (0.2 mg/mL; Santa Cruz Biotechnology Inc) and CDP-Star (Roche). Pictures were taken on Image-Quant LAS 4000 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Experiments were run in triplicate.

### Immunofluorescence

This technique uses the specificity of antibodies to their antigen in order to visualize specific cell molecules by fluorescent dyes (Figure 11).[187] Immunofluorescence was used initially to investigate the expression of laeverin in some healthy and preeclamptic placentas (Paper I); and later by tissue microarray analysis on a larger series of healthy and preeclamptic placentas (Paper III).

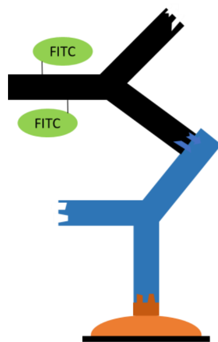


**Figure 11 Principle of immunofluorescence detection with antibodies.** Specimen (orange) with epitope is detected by primary antibody (blue). Secondary antibody (black) coupled to fluorophore (green; fluorescein isothiocyanate (FITC)) are detected in a fluorescence microscope.

Slides with tissue sections of formalin fixed and paraffin embedded placentas were made according to standard procedures. Placental tissue samples were fixed in formalin, embedded in paraffin blocks, cut (4-6 μ sections), and mounted on SuperfrostTM Plus glass slides (Thermo Fischer Scientific, Boston, MA, USA). Tissue sections were deparaffinised (xylene three times 5 minutes immersion), hydrated (100% ethanol twice 10 min/95% ethanol twice 10 min) and washed in deionized water for 1 min before antigen unmasking and blocking. Slides were placed in prewarmed 95°C 10 mM sodium citrate buffer, pH 6.0 for 5 minutes and repeated once more before cooling at room temperature for 20 min. Slides were then washed in deionized water with stirring for 2 min and repeated 3 times. Blocking was performed by adding 10% normal goat serum (Santa Cruz Biotechnologies Inc.) diluted in PBS. Slides were incubated in a humidifying chamber for 20 min at room temperature. Reagents were removed and washed in PBS for 2 minutes and repeated twice. Immunofluorescence staining was then performed using primary in-house laeverin antibody (2.1 mg/mL) diluted in 1.5% normal goat blocking serum for 60 min at room temperature in a humidifying chamber. Reagents were removed and washed in PBS for 5 minutes and repeated three times. A secondary fluorescently labelled antibody (goat anti-rabbit immunoglobulin G-fluorescein isothiocyanate (2.5 mg/mL; Santa Cruz Biotechnology Inc) diluted in 1.5% normal goat serum was then added and left at room temperature for 45 minutes in a humidifying chamber. Reagents were removed and washed in PBS for 2 minutes and repeated three times. Slides were air dried and counterstained with DAPI (4',6-diamidino-2-phenylindole) II (Vysis; Abbott Diagnostics, Lake Forest, IL). Images were obtained with CytoVision digital system (Applied Imaging, Grand Rapids, MI) that was equipped with a charge-coupled device camera (Cohu Inc, Poway, CA). A total of ≥200 cells were inspected on each slide. Experiments were run in triplicate. Immunofluorescence labeling was performed with the optimal dilution of primary antibodies.

### Immunohistochemistry

Immunohistochemistry has been performed since 1930s, but it was not reported until 1941.[188] The immunohistochemistry (IHC) principle is based on detection of antigens (e.g. proteins) with a sandwich of different antibodies (Figure 12). Some are coupled to different molecules (e.g. biotin). This method was used for individual staining of placental FFPE slides using hematoxylin and eosin (H&E) stain or other chromogenes in clinical evaluation of preeclampsia. The same principle was used for analysis of tissue microarrays (Paper III).

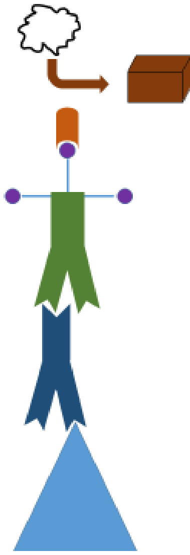

**Figure 12 Principle of immunohistochemistry detection with antibodies.** Specimen (light blue) with epitope is detected by primary antibody (dark blue). Secondary antibody (green) coupled to biotin (violet spikes). Horseradish peroxidase (HRP) coupled to streptavidin (orange) bind with biotin. 3,3'-diaminobenzidine (DAB) (white cloud) is oxidised by HRP giving a brown colour of the target molecule.

### Tissue microarray analysis

Tissue microarray (TMA) analysis is a technique used for simultaneous analysis of many tissue samples in one slide by IHC- or fluorescence (IF) analysis of a specific protein or several proteins (Figure 13).[189] Initially, this method was used in tumor research[190], but has recently been shown to be effective for placental research.[191,192] Protein detection is performed using labelled antibodies. We used a series of healthy- and preeclamptic placentas in duplicate and triplicate. Liver samples were used as a negative control on each TMA slide. A strategy of performing both IHC and IF procedures was chosen, since these methods have different applications. A manual method was first established, before an automatic protocol was later developed. More detailed protocols and description of equipment used are presented in Paper III.

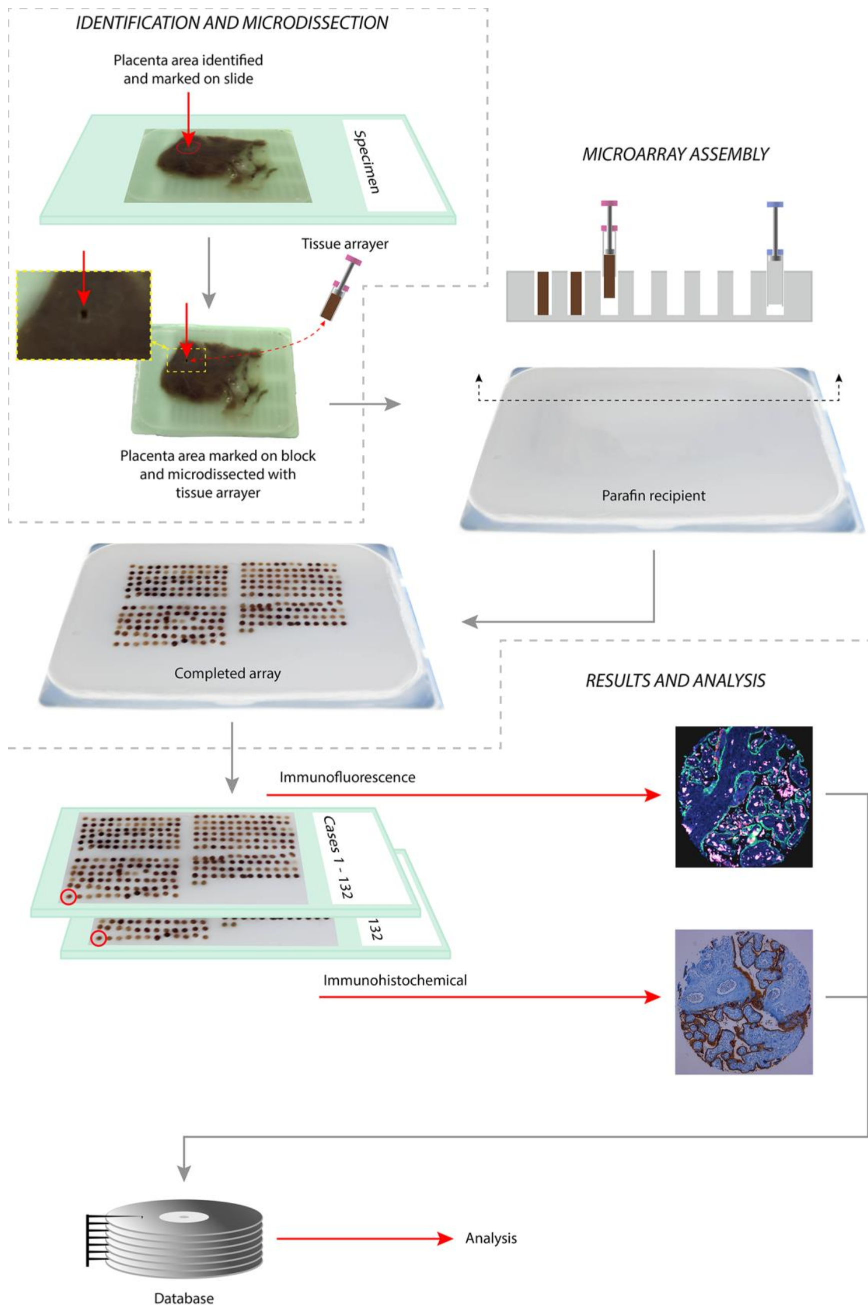


**Figure 13 Tissue microarray (TMA) principle.** Slides of individual formalin fixed paraffin embedded (FFPE) placental specimen were inspected in microscope and the area of interest was marked. A tissue arrayer was then used to microdissect out the area from the original donor FFPE-block and transferred to a paraffin recipient block with slightly bigger holes. The paraffin block was warmed to let the individual cylinders melt into the recipient block to make a complete array. Tissue sections were made from the block and slides for immunofluorescence and immunohistochemistry made for analysis. Data were stored in a database and analyzed.

### Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) was used to measure the amount of laeverin protein in maternal and cord blood serum and plasma in healthy and preeclamptic pregnancies (Paper II). In addition, healthy non-pregnant women, menopausal women and healthy men were used as controls. Placental total protein samples from healthy and preeclamptic patients were used for quantification and as a positive control. In summary the method is a combination of different antibodies and molecules in a sandwich (Figure 14). Serum or plasma was added and laeverin protein in these samples bound to the laeverin primary antibody. Then a secondary polyclonal antibody coupled to biotin was added before avidin conjugated to Horseradish Peroxidase (HRP). Substrate solution (3,3',5,5'-Tetramethylbenzidine or TMB) was then added. The reaction was terminated using sulphuric acid and $OD_{450}$ nm was measured spectrophotometrically.

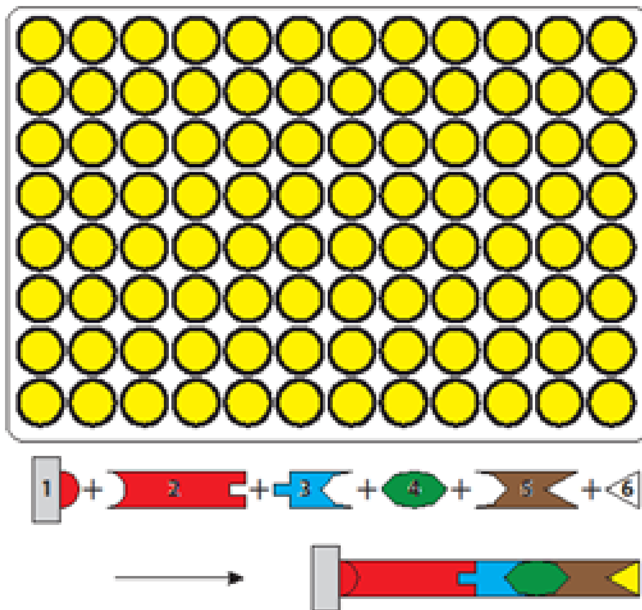


**Figure 14 ELISA-principle of laeverin detection.** 1=laeverin primary antibody, 2=laeverin protein in solution, 3=secondary polyclonal antibody coupled to detection reagent A (biotin), 4=Detection reagent B (Avidin conjugated to Horseradish Peroxidase (HRP)), 5=Substrate solution (TMB) and 6=Stop solution (sulphuric acid).

The ELISA assay for laeverin detection was done using a commercial kit (EIAab Science co., Ltd, Wuhan, China). Serum, plasma and protein samples were adjusted to room temperature. The standard (100 µl), sample (100 µl) or blank (100 µl) were added to wells, the plate covered and incubated at 37°C for 2 h. Liquid were removed and detection reagent A working solution

(100 µl) added in each well. The plate was covered with plate sealer and incubated at 37°C for 1 h. Each well was aspirated and washed with l washing buffer (400 µl) for three times in total leaving the solution for 1-2 minutes at each washing step. The plate was inverted and blotted against clean paper towels to remove liquid in each step. Then detection reagent B working solution (100 µl) was added to each well, the plate covered with plate sealer and incubated at 37°C for 1 h. Washing was performed as above, repeated for five times in total. Substrate solution (90 µl) was added to each well, the plate covered with plate sealer and incubated at 37°C for 15-30 min protected from light. The ELISA reaction was ended by adding stop solution (50 µl) to each well. Measurements were performed in duplicate and optical density was measured using a ThermoMax Microplate Reader (Molecular Devices, USA) at 450 nm. Concentrations were estimated relating it to a standard curve of laeverin protein provided by the manufacturer. A calibration curve was plotted using absorbance (y-axis) against the concentration (x-axis). The mean absorbance was calculated for each set of standards, controls and samples and then subtracted the mean zero STD from each. Unknown concentration was determined by the interpolation method.

### Transmission electron microscopy

Transmission electron microscopy after double immunogold labelling or immunogold staining (IGS)[193] was used to study the subcellular localisation of laeverin (Paper I).

First, an ultrathin section of the sample was cut, using a microtome. The sample was then incubated with a specific antibody designed to bind the molecule of interest. Next, a secondary antibody attached to colloidal gold particles was added, binding to the primary antibody. The electron-dense gold particle was detected in an electron microscope as a black dot, indirectly labelling the molecule of interest. Moreover, immunogold labelling can be used to visualize more than one target simultaneously. This can be achieved in electron microscopy by using two different-sized gold particles. Two markers for the Golgi apparatus (MG160 (ab58826), Abcam, Cambridge, UK) and endoplasmic reticulum (RL90 (ab2792) Abcam, Cambridge, UK) were used in addition to the laeverin antibody.

Immunoelectron microscopy was performed on ultrathin tissue sections of 2 healthy placentas and 2 placentas that were obtained from women with severe preeclampsia. All experiments were run in triplicates. Fresh placental tissue samples were dissected, mounted in membrane carriers, and frozen at high pressure (EMPACT 2 HPF; Leica Microsystems, Vienna, Austria). Frozen samples freeze substituted (EM AFS2, Leica Microsystems) and infiltrated in Lowicryl

HM20 (Electron Microscopy Sciences, Hatfield, UK).10 Ultrathin sections of 70 nm were cut on a Leica EM UC6 ultramicrotome (Leica Microsystems) and mounted on copper grids (Agar Scientific, Stansted, UK) with Formwar and carbon. Immunolabeling was performed with the optimal dilution of primary antibodies.[194,195] Single and double labeling experiments were performed with both locally designed (26.25 mg/mL) and commercially purchased laeverin (5 mg/mL) antibodies. For double labeling, anti-endoplasmic reticulum (ER) mouse monoclonal antibody (RL90) to protein disulphide isomerase (ab2792; 0.1 mg/mL) and anti-Golgi apparatus (GA) mouse monoclonal antibody (AE-6) to MG160 protein (MG160; ab58826; 0.05 mg/mL; Abcam, Cambridge, UK) were used as the specific markers of ER and GA, respectively. Secondary antibodies were gold conjugated goat F(ab)2 anti-rabbit IgG (5 nm) and goat anti-mouse IgG (10 nm) (British Bio Cell International, UK). Microscopy was done with a JEM-1010 transmission electron microscope (JEOL, Tokyo, Japan) at 4000, 10,000, 20,000, 30,000, and 70,000 magnifications. Images were taken and processed in Morada Soft Imaging Camera system with iTEM software (Olympus, Hamburg, Germany). A total of 200 images from each experiment were processed. Image montage was done in Adobe Photoshop and Adobe Illustrator (Adobe Systems Inc, San Jose, CA). Immunoglobulin G conjugated gold particles were used as controls in similar experiments. Possible secondary antibody cross-reactivity was excluded by the omission of primary antibodies in separate experiments.

### Laeverin gene silencing

Small (or short) interfering RNA (siRNA), composed of duplexes of 21 nucleotides, was used for RNA interference (RNAi) to inhibit the expression of laeverin protein coding genes.[196] We investigated the effect on other interaction partners using PCR-array, and migration and invasion capacities of the immortalized trophoblast cell line HTR-8/SV neo p69 (Paper I). Cells were grown and transfected with either siRNA directed against laeverin or controls. The transfected cells were used in the different assays. For each assay the transfection efficiency and laeverin siRNA silencing were measured by real-time PCR.

### Trophoblast cell migration assays

Wounding-healing studies may be used to study cell migration.[197] This is a simple method where cells are grown in monolayer, a scratch wound is made in the cell layer and cell migration is assessed during the wound healing process. We started with this method, but replaced it with a more objective and robust one, namely the xCelligence system.[198] This technique allows automatic count of cells to study migration properties of trophoblast cells. This is a method to monitor cellular responses in real time, without exogenous labels, through impedance-based technology. Several drawbacks (e.g. staining, removing cells of the Matrigel, and laborious cell counting) of the manual method may be overcome using this method.

The Roche xCELLigence Real-Time Cell Analyzer (RTCA) DP instrument provides kinetic information about cell migration by dynamically recording the entire cell migration and invasion process in real time without labelling cells, considerably improving invasion and migration assay quality. The RTCA DP instrument uses the CIM (cellular invasion/migration)-Plate 16, that has microelectronic sensors integrated onto the underside of the microporous polyethylene terephthalate (PET) membrane of a Boyden-like chamber. As cells migrate from the upper chamber through the membrane into the bottom chamber in response to chemoattractant, they contact and adhere to the electronic sensors on the underside of the membrane, resulting in an increase in impedance. The increase of impedance correlates to increasing numbers of migrated cells on the underside of the membrane, and cell-index values reflecting impedance changes are automatically and continuously recorded by the RTCA DP instrument. Therefore, cell migration activity can be monitored via the cell-index profile plotted as a graph. We used this system for to test laeverin silenced trophoblast cells migration capacities (Paper I).

HTR-8/SVneo trophoblast cells ($2x10^5$cells/well) were seeded the day before small interfering RNA (siRNA) transfection with FuGENE transfection reagent (Promega Corp, Madison, WI). SiRNA (10 pmol) against laeverin or scrambled siRNA A or D (control; Santa Cruz Biotechnology Inc.) were used. Plates were incubated at 37°C with 5% $CO_2$ for 5 hours; transfection solution was replaced with fresh RPMI Medium 1640 with 5% FBS, and cells were further incubated for 24 hours; $2x10^5$cells/well were added to each well of the CIM-Plate 16 (ACEA Biosciences Inc, San Diego, CA). Migration assays were performed (for 72 hours, with sweeps of 30 minutes each) in the xCelligence system (ACEA Biosciences Inc). Three different CIMPlates 16 were used. Experiments were run in quadruplicate. Coefficients of variation for siRNA A, D, and laeverin were 3.5%, 1.2%, and 3.5%, respectively. Untransfected cells were

used as controls and were run in duplicates on each plate. Analysis was performed in the RTCA software (version 1.2.1; ACEA Biosciences Inc).

### Trophoblast cell invasion assays

Defective trophoblast invasion and endothelial inflammatory damage precede the clinical manifestation of preeclampsia.[16] Invasion studies in Matrigel in Boyden chambers is a classical technique to investigate the invasiveness of the trophoblast cells (Figure 15).[199]

Silencing studies using siRNA against *laeverin* aimed to elucidate its role of this gene in trophoblast invasion. Cells are grown to semiconfluency and then transfected with siRNA, before the invasion. Cells are added to inserts containing matrigel, the wells inserted into plates with wells containing medium and 2% fetal calf serum which serves as a chemoattactant. Invading cells will migrate through the pores in the Matrigel (a basement membrane-like matrix, mimicking ECM) and will grow on the bottom of the inserts. Cells were counted or measured by MTT-test[200,201] to get a measurement of the amount of cells migrating through the Matrigel. More details are found in Paper I.

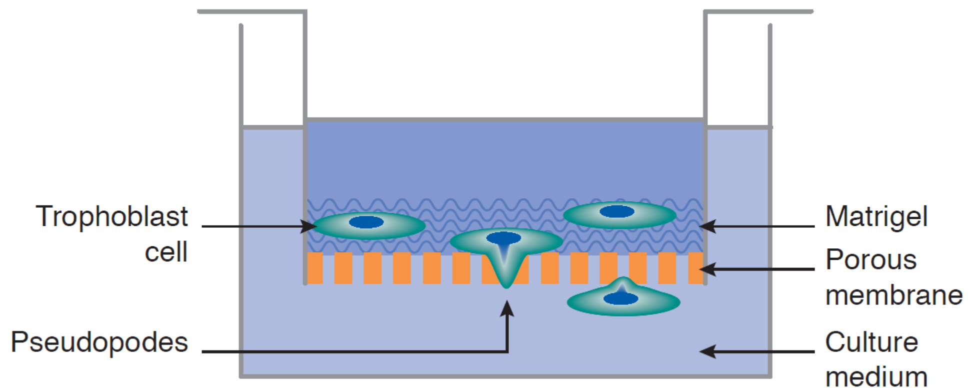


**Figure 15 Boyden chamber used for invasion studies.** Trophoblast cell invasion setup where cells are seeded on extracellular matrix (like Matrigel; wavy lines) in wells in culture medium. Cells will migrate through the Matrigel and the porous membrane to the attractant in the bottom of the well, where the amount of cells can be counted using the spectrophotometric MTT-method. Cells (green), nuclei (blue), porous membrane (orange) and media (blue).

HTR-8/SVneo trophoblast cells ($5 \times 10^5$ cells) were grown in RPMI Medium 1640 with 10% FBS and incubated at 37°C, with 5% $CO_2$ overnight. Medium was replaced by RPMI Medium 1640 with 5% FBS the next day. On day 3, transfection with 50 pmol laeverin siRNA or 50 pmol of siRNA A control (Santa Cruz Biotechnology Inc) with Lipofectamine 2000 (Invitrogen) was performed in separate flasks. Cultures were incubated at 37°C with 5% $CO_2$

for 4 hours and washed with RPMI Medium 1640 without serum before incubation overnight. Invasion studies (1x$10^5$ cells/well; 5% FBS used as chemoattractant) were performed in 48 hours in BD BioCoateBD Matrigel Invasion Chambers (24-well plate 8 µ with control inserts; BD Biosciences, San Jose, CA) and incubated at 37°C with 5% $CO_2$. The noninvading cells were removed from the upper part of the insert's membrane by scrubbing with cotton-tipped swabs that had been moistened with medium. Cell invasion analysis was performed by methylthiazolyldiphenyl-tetrazolium bromide (MTT)-assay.[202] Results were monitored in Thermo Multiscan Ex (ThermoFisher Scientific Inc, Waltham, MA). Experiments were run in triplicate.

### Gene expression profiling using PCR-arrays

We used PCR-array after silencing *laeverin* in order to identify down-stream effects on selected tumorigenesis pathway genes (Paper I). HTR-8/SVneo trophoblast cells (4-5,7 x $10^5$ cells) were transfected with Lipofectamine 2000 and 120 pmol siRNA laeverin or siRNA A (control). Cells were mixed with TRIzol Reagent and RNA isolated by RNeasy Mini kit. Complementary DNA synthesis and quantitative reverse transcription PCR were performed with the use of RT2 Profiler PCR Array Human Cancer Pathway Finder (PAHS-033A; SABiosciences Corporation, Frederick, MD). Actin, beta was used as housekeeping gene. Analysis of fold changes was done by the comparative Ct (ΔΔCt) method with the integrated web-based software package for the PCR array system. This protocol involved transfecting laeverin siRNA to the HTR-8/SVneo trophoblast cell-line to silence the *laeverin* gene. A negative control with scrambled siRNA was added to another cell population of the same cell line. RNA from these cells was isolated, transformed to cDNA and real-time PCR run on the PCR Array system. Genes influenced by the *laeverin* silencing were identified in a plot representing genes in the pathway.

### Validation of laeverin silencing

Total RNA was isolated from three different cell populations of untransfected, siRNA silenced for laeverin and silenced controls (scrambled siRNA A and D) from the HTR-8/SVneo p69 cell line immersed in Trizol Reagent (Invitrogen, Carlsbad, CA, USA) 55 hours after transfection at the migration optimum. Total RNA was extracted with RNeasy Mini Kit (Qiagen, Venio, The Netherlands). RNA was quantified by measuring absorbance at 260 nm, and RNA purity was determined by the ratios OD260 nm/280 nm and OD230 nm/280 nm using the NanoDrop instrument (NanoDrop® ND-1000, Wilmington, USA). The RNA integrity was determined by

electrophoresis using the Agilent 2100 Bioanalyser (Matriks, Norway). Only samples with RNA Integrity Number (RIN) >7.2 were used. Reverse transcription was performed with the High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Complementary DNA samples were profiled for the relative expression of the genes of laeverin (LVRN; Hs 01060572_m1), β-actin (ACTB; Hs99999903_m1) and glyceraldehydephosphate dehydrogenase (GAPDH; Hs02758991_g1) with the Taq Man Gene Expression Assays on 7900HT Fast Real-Time PCR system (Applied Biosystems). Samples for each experiment were run in triplicate and averaged for final quantification, before the fold inductions were calculated.[203]

## Statistical methods

Data were analyzed with IBM SPSS Statistics 21 software (SPSS Inc, Chicago, IL)(Paper I and Paper III). Assessment of normality was performed with the Shapiro-Wilk test. Differences between groups were assessed with the Student t test for parametric variables and the Mann-Whitney U test, $\chi 2$ test, or F-test for nonparametric variables. Analysis of the longitudinal data was performed using SAS v.9.4 (SAS Institute Inc. Cary, NC) (Paper II). Multilevel modeling was used to estimate the reference percentiles[204].

SPSS software (IMB SPSS Statistics 22, Armonk, NY, USA) was used for the analysis of cross-sectional data (Paper II). Sensitivity, specificity, positive and negative predictive values were calculated to evaluate usefulness of laeverin in predicting preeclampsia setting a cut-off value at ≤5 ng/mL. Linear mixed model ANOVA was used to evaluate the differences in laeverin concentrations between different gestational weeks antenatally and post-partum (Paper II). Kappa value[205,206] for inter-rater reliability was calculated for the TMA IHC studies (Paper III). A probability value of < .05 was considered significant.

## Ethical considerations

The study was approved by the Regional Committee for Medical and Health Research Ethics-North Norway (REK Nord) and informed written consent was obtained from all the participants. The research was performed following the principles of the Helsinki declaration.[207] The study was observational, and except for the inconvenience of extra visits and possible discomfort related to blood sampling, participants were not subjected to any intervention and the dignity of each participant was prioritized. Placental sampling and umbilical cord blood sampling were

performed after delivery and had no implication for patient management. None of the research collaborators had any conflicts of interests.

## Synopsis of results

### Paper I

We used a custom-made monoclonal laeverin antibody for immunofluorescence analysis of paraffin embedded tissue sections from third-trimester placentas showing different laeverin expression patterns in healthy and preeclamptic pregnancies. In particular, we found that laeverin was plasma membrane-bound in cytotrophoblasts of the healthy placental villi but was expressed in the cytoplasm of preeclamptic placentas. We further performed Western blot analysis of laeverin protein from healthy- and preeclamptic placentas to investigate if differences in molecular mass could explain the differences in expression pattern. We found that laeverin has a molecular mass of 60 kDa protein in both healthy and preeclamptic placentas. We further investigated the subcellular localization of laeverin protein by immunoelectron microscopy. Laeverin protein was found to leak into fetal capillaries and we found massive expression of laeverin in microvesicles in preeclamptic placentas. We further aimed to elucidate the molecular function of the protein. Therefore, we tested the hypothesis that silencing of the laeverin gene in the trophoblast cell line HTR-8/SVneo p69 could influence cell migration and invasion capabilities. Migration and invasion of HTR-8/SVneo cells were indeed reduced by 11.5% (P =.023) and 56.7% (P=.001), respectively. We then set out to perform a targeted pathway analysis using PCR-arrays. Analysis of downstream pathways affected by laeverin-silencing demonstrated significant downregulation of integrin A2 (39-fold), integrin B3 (5-fold), and matrix metalloprotease 1 (36-fold).

### Paper II

Since we found that laeverin was expressed in preeclamptic and healthy placentas we aimed to test if the laeverin protein was expressed in the maternal and fetal circulations. More specifically we: 1) performed a longitudinal study of plasma levels of laeverin in healthy women during the second half of pregnancy and postpartum, 2) determined whether laeverin is differently expressed at 22-24 weeks in women who later develop preeclampsia compared to controls, 3) compared laeverin protein expression in placenta and umbilical cord blood in healthy and preeclamptic pregnancies at birth. ELISA was chosen for its simplicity and

convenience.

We found that healthy pregnant women at term (37-40 weeks) had significantly higher plasma levels of laeverin (mean 4.95±0.32ng/mL; $p<0.0001$) compared to men (mean 0.18±0.31 ng/mL), non-pregnant premenopausal women (mean 0.77±0.26 ng/mL) and postmenopausal women (mean 0.57±0.40 ng/mL). Maternal plasma laeverin levels decreased with advancing gestation. The reference values ranged from 6.96±0.32 ng/mL at 22-24 weeks to 4.95±0.32 ng/mL at term (p=0.0001) in uncomplicated pregnancies. Half of the women who developed preeclampsia had plasma laeverin levels below the 5th percentile at 22-24 weeks gestation. However, laeverin levels were 1.6 fold higher in preeclamptic compared to healthy placentas (p=0.0071). Umbilical venous samples of healthy neonates (n=38) had higher (p=0.001) mean levels of laeverin (16.63±0.73 ng/mL), compared to neonates of preeclamptic (n=14) mothers (12.02±1.00 ng/mL). Postpartum plasma levels of laeverin decreased in healthy and preeclamptic women with a half-life of 3 and 5 days, respectively.

We concluded that maternal plasma levels of laeverin decrease with advancing gestation during the second half of normal pregnancy and lower levels measured at 22-24 weeks might be associated with the development of preeclampsia later in gestation.

### Paper III

To further investigate the differences in tissue expression of laeverin we investigated a series of healthy and preeclamptic placentas using tissue microarray analysis. Immunohistochemistry- and immunofluorescence analysis of 36 patients with severe preeclampsia and 41 women with uncomplicated pregnancies revealed laeverin expression in all trophoblast cells (syncytiotrophoblasts, cytotrophoblasts and in extravillous trophoblasts). In preeclamptic placentas (n=33) compared to normal placentas (n=39), laeverin was expressed in the cell membrane in 21 (64%) vs 21 (54%) of samples (p=0.726), in the cytoplasm in 3 (9%) vs 2 (5%) of samples (p=0.795) and in both the cytoplasm and membrane in 9 (27%) vs 16 (41%) of samples (p=0.0522). Cytoplasmic placental expression of laeverin was found in women who had early-onset preeclampsia (delivered before 34 weeks of gestation). Immunofluorescence studies showed laeverin expression in the cytoplasm of six preeclamptic (three early-onset and three late-onset) and one normal placenta, but did not reveal any simultaneous cell membrane and cytoplasmic expression of laeverin. We concluded that laeverin might play an important role in human placentation and its expression is heterogenous in normal and preeclamptic placentas.

# Discussion

The results of the studies included in the thesis are discussed in the Papers I-III. Only some selected aspects will be highlighted in the following discussion.

## Microscopic observations of laeverin in healthy and preeclamptic placentas

Pioneering work on the molecular characterization of laeverin was reported by Fujiwara *et al*., in 2004.[150] In our initial IF studies with in-house produced antibody (Paper I) we aimed to investigate possible differences in laeverin protein expression in healthy and preeclamptic placentas near term.[208] We showed membrane staining on cytotrophoblast in healthy placentas, as expected, and a surprising finding of ectopic cytoplasmic expression of laeverin. This led us to speculate that laeverin protein might be modified or damaged in preeclampsia. Preeclampsia is indeed associated with increased cytotrophoblast apoptosis. The exact molecular mechanisms behind this phenomenon are not fully elucidated. However, it is suggested that endoplasmic reticulum stress can lead to cell apoptosis and protein degradation.[209,210] Therefore, we hypothesize that the observed accumulation of laeverin in the cytoplasm of cytotrophoblasts in preeclamptic placentas could be explained by defective protein glycosylation and/or misfolding of the protein occurring normally in the endoplasmic reticulum.[209,211] Indeed our electron microscopic co-localization studies of laeverin and endoplasmic reticulum and Golgi-markers indicated aberrant processing of laeverin in preeclamptic placentas that may have resulted in massive production of microvesicles. The accumulation of laeverin in the cytoplasm, instead of the plasma membrane would suggest that preeclampsia is a conformational disease.[211]

In tissue microarray studies in Paper III we used a commercially available antibody for IHC and IF studies. We showed laeverin protein expression in all subpopulations of trophoblast cells at term; i.e. in syncytiotrophoblasts, cytotrophoblasts and in extravillous trophoblast cells. On the contrary, Horie *et al*., 2012 have shown laeverin expression *only* in EVTs at different stages of healthy pregnancy (at 4-, 11- and 38 weeks of gestation).[174] This discrepancy may be related to methodological differences, choice of antibodies or focus on EVTs.

Subcellular localization studies/ultrastructural studies of laeverin protein by immunogold transmission electron microscopy (TEM) (Paper I) revealed massive expression in microvesicles both in syncytial knots, in the maternal circulation and in the cytoplasm of cytotrophoblasts obtained from preeclamptic patients. Moreover, laeverin was localized in the cytoplasm of syncytial knots of both healthy women and preeclamptic patients in Paper III. This is in accordance with other studies stating that during development of the disease

cytotrophoblasts, which are precursors of syncytiotrophoblasts, divide asynchronously giving rise to syncytial knots.[212] It is known that microvesicles or exosomes are shed from the syncytial trophoblasts and are released into the maternal circulation.[213] Pioneer studies indicated that trophoblast material is released into the maternal circulation, and that equal amount of the syncytial "factor" was found in the blood circulation of healthy and preeclamptic mothers. However increased amounts were present in uterine blood vein blood of women with preeclampsia compared to normal pregnancies.[214-217] Moreover, extracellular vesicles of different size are shed from the syncytiotrophoblast and enter the maternal circulation.[218,219] Interestingly, we found that laeverin was expressed inside microvesicles in placentas from preeclamptic mothers using TEM, indicating an intracellular dysregulation. In healthy placentas, where laeverin is expressed in the plasma membrane of trophoblast cells, an expression of laeverin in membranes of exosomes or microvesicles would be expected. We did not detect any exosomes or microvesicles in healthy placentas using transmission electron microscopy. This finding merits further investigation.

## Molecular mass of laeverin protein in healthy and preeclamptic placentas

We used SDS-PAGE and Western blot analysis of proteins extracted from placentas at term aiming to find possible differences in the molecular mass of laeverin between healthy- and preeclamptic placentas. We found only one laeverin protein isoform with a molecular mass of 60 kDa in both normal and preeclamptic placentas (Figure 2A and 2B in Paper I). However, the predicted molecular mass from the amino acid sequence of laeverin would be 113 kDa.[165] Several reasons can explain this discrepancy. Firstly, native laeverin might be cleaved during the purification process or by natural enzymes resulting in two identical proteins of 60 kDa - with a total mass of 120 kDa. There is evidence of further processing of the protein into a mature form.[154,165,220] Furthermore, alternate splicing of laeverin gene may produce 4 protein isoforms with a mass of 113 kDa, 80 kDa, 26 kDa and 23 kDa.[165] Indeed, Horie *et al*., 2012[174] detected 3 different bands of 200-270 kDa, 160 kDa and 130 kDa in normal placenta (at 4, 9 and 38 weeks gestation). Moreover, this discrepancy could be due to the differences in antibodies and methods used for protein purification and analysis. Previous studies used an antibody that was raised by immunizing rabbits against the whole molecule of recombinant laeverin, which was originally produced by transfection of recombinant Baculoviruses in Sf9 insect cells.[174,221] We immunized rabbits with two small peptides from the N- and C- terminal parts of the human laeverin protein to improve the specificity of our antibodies. We performed experiments using

antibodies against either N-terminal, C-terminal or the commercially available antibody with the same results. Another explanation for higher molecular weight reported by Horie *et al*.,2012[174] could be that laeverin is part of a larger multimeric protein complex or is glycosylated, producing different sized bands.

We investigated placental proteins from healthy placentas and preeclamptic placentas in native gels. We used NativePAGE™ Gel System to maintain native protein conformation and provide high-resolution native electrophoresis for analyzing native protein complexes, as compared to traditional native electrophoresis systems such as the Tris-Glycine system.[222] Western blotting analysis was then performed. We found 4 major bands indicating several 3-D conformations of laeverin protein or subunits. In addition we found surplus high molecular weight bands in two of four preeclampsia patients, compared to protein isolated from healthy pregnancies (results not published). The same results were obtained with in-house antibody and the commercial antibody.

## Laeverin protein expression in the maternal and fetal circulations

The observation of microvesicles containing laeverin protein in syncytial knots of preeclamptic placentas (Paper I) led us to investigate whether laeverin is expressed in the maternal blood circulation.

During preeclampsia trophoblastic cells are degraded and placental tissue fragments are released into the maternal and fetal circulations containing membrane-bound laeverin, laeverin in microvesicles and free laeverin protein. In Paper II we found decreasing laeverin levels from 6.96±0.32 ng/mL to 4.95±0.32 ng/mL (from week $22^{+0}$ to $30^{+6}$; second half of pregnancy), indicating that laeverin might have important role during placental development. In addition we showed higher expression in the plasma of healthy pregnant women compared to healthy non-pregnant, pre- and postmenopausal women, indicating that laeverin can be used as a placenta-specific biomarker.

mRNA levels of laeverin were shown to be significantly higher in placental tissue obtained from preeclamptic compared to healthy women. Accordingly, laeverin protein levels measured by ELISA were 1.6-fold higher in preeclamptic placentas compared to healthy placentas. On the contrary, laeverin levels in maternal serum were significantly lower in preeclamptic compared to healthy women (Paper II). The difference of laeverin protein expression between tissue- and serum samples may be explained by the fact that laeverin was trapped in microvesicles (Paper I). Alternatively, microvesicles could be destroyed in the placental tissue

protein extraction process, thus not detected in the ELISA-method used in Paper II.

The concentration of laeverin in the umbilical vein of neonates of healthy pregnant women was higher compared to neonates of preeclamptic women. We found similar levels of laeverin in the umbilical arteries and umbilical vein, demonstrating no significant production or metabolism of laeverin by the fetus, which is in accordance with previous knowledge of placenta-specific laeverin protein expression.

## Involvement of laeverin in trophoblast migration and invasion

Migration and invasion are important biological processes during placentation. Migration can be investigated performing wound-healing assays or *in vitro* scratch assays of trophoblast cells.[223] We started with wound-healing assays, but we later verified these experiments with a more objective method, namely with the xCelligence system (Paper I). Invasion experiments were performed using Boyden chambers with extracellular matrix in the chambers, simulating the extracellular environment. We further simulated a dysfunctional laeverin gene by using siRNA for laeverin silencing in both migration and invasion experiments (Paper I). The cells used were immortalized trophoblast cell line HTR-8/SVneo p69[185], which originate from first trimester placentas, when migration and invasion occurs. We found significant reduction in migration and invasion indicating that laeverin is involved in these processes *in vitro*. Moreover, we performed similar migration- and invasion assays of cells originating from legal first trimester abortions with similar results (results not shown). Similarly, Horie *et al*, 2012[174] performed siRNA silencing experiments on laeverin in EVTs isolated from first trimester abortion material and found reduced migration and invasion.

In addition, PCR-array analysis of selected pathways involved in tumorigenesis, after laeverin gene silencing, showed a significant down-regulation of 3 genes; integrin A2 ($\alpha 2$), integrin B3 ($\beta 3$) and matrix metalloprotease 1 (Paper I). Integrins are cell surface receptors composed of two subunits, $\alpha$ and $\beta$.[224] Each combination of these subunits is specific for specific cells. Trophoblasts express different subtypes of the subunits in different developmental stages. Extracellular matrix binding to integrins in the membrane of trophoblast cells promotes integrin clustering and positive feedback to the actin cytoskeleton leading to movement.[224] Integrins are involved in human embryo implantation and in trophoblast cell invasion.[225] Extracellular matrix (ECM)-components influence migration, differentiation and adhesion through integrins. Matrix metalloproteases are important regulators of extracellular matrix remodeling during pregnancy.

## Laeverin - a biomarker?

An ideal biomarker should: 1) Be suitable for early diagnosis of the disease; 2) Correlate with disease progression; 3) Be easily obtained and 4) Have high sensitivity and specificity.

Laeverin has a placenta specific expression.[150,174] According to our studies, laeverin is a good histologic biomarker of all trophoblast cells (Paper I and III). Maternal plasma levels of laeverin protein decrease with advancing gestation during the second half of normal pregnancy and lower levels measured at 22–24 weeks might be associated with the development of preeclampsia later in gestation. With a cut-off value for laeverin concentration set at ≤5 ng/mL, the sensitivity, specificity, positive predictive value and negative predictive value for the prediction of preeclampsia were 0.60, 0.79, 0.82 and 0.88, respectively (Paper II). There are no current screening test for early detection of preeclampsia.[226] The most promising models for early prediction of PE are combinations of biochemical and biophysical tests.[227] Laeverin may be used as a biomarker of preeclampsia in combination with other biomarkers, but more research is needed to reveal its potential as a clinical biomarker.

Furthermore, gene-, chromosome or genome mutations may be used as biomarkers. A dysfunctional laeverin protein may originate from a gene- or chromosome mutation and thus might have an implication for the susceptibility of preeclampsia. We found some possible disease causing sequence variants in a limited patient material (unpublished results). Molecular karyotyping by SNP-array analysis and array comparative genomic hybridization have also been performed on samples from healthy and preeclamptic mothers as well as their fetuses (DNA from umbilical cord blood). No copy number variations (CNVs) involving laeverin were found (unpublished results).

## Strengths and limitations

A major strength of our studies is that we used a translational approach to investigate the involvement of laeverin in normal pregnancy and in preeclampsia. Firstly, we used phenotypically well characterized clinical samples. Paired placental tissue sections from uncomplicated pregnancies and preeclamptic women were used for our initial microscopic studies of laeverin protein expression. The second strength of our study was the use of our own well-working antibody against laeverin. Trophoblast membrane expression was detected using a commercial antibody as well. Immortalized trophoblast cells were used as a model system to mimic early pregnancy and to test the hypothesis that laeverin might be involved in migration and invasion. Both wound-healing experiments and the xCelligence method showed that

laeverin-silenced cells reduce their migration, indicating laeverin's role in this process. ELISA was found to be a reliable and easy method in order to detect laverin protein in plasma or serum and to test whether there are laeverin protein concentration differences between normal and preeclamptic pregnancies and between the maternal and fetal circulations. The tissue microarray study gave us the opportunity to study a series of patients and controls in one single experiment, minimizing inter-experimental differences and errors. In general, several different methods used in this study strengthen our finding of laeverin's involvement in placentation process.

A limitation of our study is that the preeclamptic placentas were delivered earlier compared to the normal placentas. However, because term placentas normally express higher levels of laeverin, one would expect to see lower levels of laeverin in preeclamptic placentas that were delivered preterm. Therefore, the observed differences in laeverin expression are likely to be real and suggest that the overexpression of laeverin in preeclamptic placentas is associated with the disease process rather than the differences in gestational age.
Serum and plasma samples were stored for up to 5 years at −70°C and some samples were thawed once. However minimal differences in laeverin levels were found when thawed and non-thawed samples were compared (data not shown). Further, we were able to include only a small number of samples from preeclamptic women. Therefore, this study should be reproduced in a bigger cohort, and preferably first trimester samples should also be included.

## Conclusions

The aim of this thesis has been to elaborate the role of laeverin in pregnancy and in preeclampsia. We used a translational research method with many different methods to look at laeverin's potential role as a biomarker of pregnancy and preeclampsia. From the results of this thesis the following conclusions can be drawn:

- Laeverin is a placenta-specific protein expressed in membranes of cytotrophoblasts, syncytiotrophoblasts and extravillous trophoblasts in healthy pregnancies at term. Laeverin protein can thus be used as a histologic biomarker of trophoblast cells.
- In preeclamptic placentas cytoplasmic expression of laeverin may indicate dysregulated or damaged protein.

- Tissue microarray analysis of placental tissue appears to be a good method for simultaneous analysis of several samples for protein expression.

- Laeverin protein has a molecular mass of 60 kDa (both in healthy and preeclamptic pregnancies).

- Longitudinal reference ranges were established for laeverin in maternal circulation during the second half of pregnancy and postpartum period. Laeverin levels decrease with advancing gestational age.
- About 50% of women who developed preeclampsia had plasma laeverin levels below the 5th percentile at 22-24 weeks gestation.
- Laeverin is involved in migration and invasion capabilities of first trimester trophoblast *in vitro*, indicating a possible role during early placental development.

- PCR-array analysis identified 3 possible interaction partners of laeverin, namely integrin α2, integrin β3 and matrix metalloprotease 1.

## Future perspectives

Further functional studies are needed to investigate closer laeverin's relationship with other interaction-partners, and its involvement in different molecular pathways.
High-resolution nanoscopy studies of laeverin protein expression and major histocompatibility complex class I G (HLA-G) trophoblast specific membrane marker for co-localization are underway. These experiments on placental tissue sections will be used to compare normal with preeclamptic placentas at term. Co-localization experiments with membrane-bound integrins and extracellular matrix metalloproteases will reveal any interactions with laeverin. Moreover, first trimester trophoblast explants could be used to investigate laeverin expression in early stages of placental development, compared to our results from term pregnancy.
Furthermore, detailed knowledge of the molecular structure of laeverin is essential to understand its molecular function and interaction with other molecules. For that scope, X-ray crystallography- and nuclear magnetic resonance (NMR) spectroscopy studies should be performed in order to make an *in vivo* model of the protein. Immune precipitation and co-immune precipitation assays may reveal any homodimerization of laeverin and identify other interaction partners. Traditional cloning of the *laeverin* gene in an expression vector and

transfer into a mammalian recipient cell may reveal if laeverin is secreted as large multimeric protein complexes. Laeverin is normally glycosylated and membrane bound. De-glycosylation may be the reason for erroneous cytoplasmic expression. This could be investigated using de-glycosylation enzymes in combination with Western blot analysis.

It would also be interesting to isolate microvesicles from the maternal circulation and look for differences in content and quantity of laeverin in healthy pregnant women compared to preeclamptic patients.

A potential role of laeverin as a biomarker merits further investigation. Larger prospective studies of laeverin protein expression in maternal serum together with other biophysical and biochemical markers could clarify whether laeverin could be used as an additional biomarker for prediction, triage or stratification of preeclampsia in clinical practice.

## References


1. Raleigh SW. *What is our life? The play of passion.* London: George Bell & Sons; 1892.
2. Bell MJ. A historical overview of preeclampsia-eclampsia. *Journal of obstetric, gynecologic, and neonatal nursing : JOGNN / NAACOG.* 2010;39(5):510-518.
3. Chesley LC, Annitto JE, Cosgrove RA. The familial factor in toxemia of pregnancy. *Obstetrics and gynecology.* 1968;32(3):303-311.
4. Jauniaux E, Poston L, Burton GJ. Placental-related diseases of pregnancy: Involvement of oxidative stress and implications in human evolution. *Human reproduction update.* 2006;12(6):747-755.
5. Boyd JD, Hamilton WJ. *The human placenta.* Cambridge [Eng.]: Heffer; 1970.
6. Fisher SJ, McMaster M, Roberts JM. Chapter 5 - The Placenta in Normal Pregnancy and Preeclampsia. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:81-112.
7. Strachan T, A R. Nucleic Acid Structure and Gene Expression and Chromosome Structure and Function. In: *Human Molecular Genetics.* 4th ed. New York, USA: Garland Science, Taylor & Francis Group; 2011:1-61.
8. Finishing the euchromatic sequence of the human genome. *Nature.* 2004;431(7011):931-945.
9. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. *Nature.* 2001;409(6822):860-921.
10. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. *Science (New York, NY).* 2001;291(5507):1304-1351.
11. Bischof P, Irminger-Finger I. The human cytotrophoblastic cell, a mononuclear chameleon. *The international journal of biochemistry & cell biology.* 2005;37(1):1-16.
12. Aplin JD. The cell biological basis of human implantation. *Bailliere's best practice & research Clinical obstetrics & gynaecology.* 2000;14(5):757-764.
13. Schoenwolf GC, Bleyl, Steven B., Brauer, Philip R. and Francis-West, Philippa H. *Larsen's Human Embryology.* 5th ed: Churchill Livingstone, Elsevier Saunders, Philadelphia, USA; 2015.
14. Murray MJ, Lessey BA. Embryo implantation and tumor metastasis: common pathways of invasion and angiogenesis. *Seminars in reproductive endocrinology.* 1999;17(3):275-290.
15. Soundararajan R, Rao AJ. Trophoblast 'pseudo-tumorigenesis': significance and contributory factors. *Reproductive biology and endocrinology : RB&E.* 2004;2:15.

16. Cohen M, Bischof P. Factors regulating trophoblast invasion. *Gynecologic and obstetric investigation.* 2007;64(3):126-130.
17. Psychoyos A. Hormonal control of ovoimplantation. *Vitamins and hormones.* 1973;31:201-256.
18. Lessey BA. Endometrial receptivity and the window of implantation. *Bailliere's best practice & research Clinical obstetrics & gynaecology.* 2000;14(5):775-788.
19. Staun-Ram E, Shalev E. Human trophoblast function during the implantation process. *Reproductive biology and endocrinology : RB&E.* 2005;3:56.
20. Ridley AJ, Schwartz MA, Burridge K, et al. Cell migration: integrating signals from front to back. *Science (New York, NY).* 2003;302(5651):1704-1709.
21. Lessey BA, Castelbaum AJ. Integrins and implantation in the human. *Reviews in endocrine & metabolic disorders.* 2002;3(2):107-117.
22. Drews U. *Color Atlas of Embryology.* Georg Thieme Verlag, Stuttgart, Thieme Medical Publishers, Inc., New York, USA.; 1995.
23. Vigano P, Mangioni S, Pompei F, Chiodo I. Maternal-conceptus cross talk--a review. *Placenta.* 2003;24 Suppl B:S56-61.
24. Norwitz ER, Schust DJ, Fisher SJ. Implantation and the survival of early pregnancy. *The New England journal of medicine.* 2001;345(19):1400-1408.
25. Xu RH, Chen X, Li DS, et al. BMP4 initiates human embryonic stem cell differentiation to trophoblast. *Nature biotechnology.* 2002;20(12):1261-1264.
26. Gude NM, Roberts CT, Kalionis B, King RG. Growth and function of the normal human placenta. *Thrombosis research.* 2004;114(5-6):397-407.
27. Lunghi L, Ferretti ME, Medici S, Biondi C, Vesce F. Control of human trophoblast function. *Reproductive biology and endocrinology : RB&E.* 2007;5:6.
28. Muyan M, Boime I. Secretion of chorionic gonadotropin from human trophoblasts. *Placenta.* 1997;18(4):237-241.
29. Morrish DW, Dakour J, Li H. Functional regulation of human trophoblast differentiation. *Journal of reproductive immunology.* 1998;39(1-2):179-195.
30. Handwerger S, Freemark M. The roles of placental growth hormone and placental lactogen in the regulation of human fetal growth and development. *Journal of pediatric endocrinology & metabolism : JPEM.* 2000;13(4):343-356.
31. Lacroix MC, Guibourdenche J, Frendo JL, Muller F, Evain-Brion D. Human placental growth hormone--a review. *Placenta.* 2002;23 Suppl A:S87-94.
32. Zhou Y, Fisher SJ, Janatpour M, et al. Human cytotrophoblasts adopt a vascular phenotype as they differentiate. A strategy for successful endovascular invasion? *The Journal of clinical investigation.* 1997;99(9):2139-2151.
33. Brosens JJ, Pijnenborg R, Brosens IA. The myometrial junctional zone spiral arteries in normal and abnormal pregnancies: a review of the literature. *American journal of obstetrics and gynecology.* 2002;187(5):1416-1423.
34. Fujiwara H. Membrane-bound peptidases regulate human extravillous trophoblast invasion. *Placenta.* 2007;28 Suppl A:S70-75.
35. Knofler M. Critical growth factors and signalling pathways controlling human trophoblast invasion. *The International journal of developmental biology.* 2010;54(2-3):269-280.
36. Roberts RM, Fisher SJ. Trophoblast Stem Cells. *Biology of reproduction.* 2011;84(3):412-421.
37. Pijnenborg R, Dixon G, Robertson WB, Brosens I. Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy. *Placenta.* 1980;1(1):3-19.
38. Reyes L, Wolfe B, Golos T. Hofbauer Cells: Placental Macrophages of Fetal Origin. *Results and problems in cell differentiation.* 2017;62:45-60.
39. Cross JC. How to make a placenta: mechanisms of trophoblast cell differentiation in mice--a review. *Placenta.* 2005;26 Suppl A:S3-9.
40. Sylvie E. Paper presented at: International Federation of Placenta Associations2014; Paris.
41. Page EW. On the pathogenesis of pre-eclampsia and eclampsia. *The Journal of obstetrics and gynaecology of the British Commonwealth.* 1972;79(10):883-894.

42. Hamilton WJ, Boyd JD. Development of the human placenta in the first three months of gestation. *Journal of anatomy.* 1960;94:297-328.
43. Dahlstrom B, Romundstad P, Oian P, Vatten LJ, Eskild A. Placenta weight in pre-eclampsia. *Acta obstetricia et gynecologica Scandinavica.* 2008;87(6):608-611.
44. Varney H. KJ, Gegor CL. *Varney's midwifery.* 4th ed. Mississauga, Canada.: Jones and Bartlett Publishers; 2004.
45. Karumanchi SA, Rana S, Taylor RN. Chapter 6 - Angiogenesis and Preeclampsia. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:113-132.
46. Fowden AL, Forhead AJ. Endocrine regulation of feto-placental growth. *Hormone research.* 2009;72(5):257-265.
47. Beck S, Wojdyla D, Say L, et al. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. *Bulletin of the World Health Organization.* 2010;88(1):31-38.
48. Goldenberg RL, Culhane JF, Iams JD, Romero R. Epidemiology and causes of preterm birth. *Lancet.* 2008;371(9606):75-84.
49. Kingdom J, Huppertz B, Seaward G, Kaufmann P. Development of the placental villous tree and its consequences for fetal growth. *European journal of obstetrics, gynecology, and reproductive biology.* 2000;92(1):35-43.
50. Hippocrates. *Hippocrates. The medical works of Hippocrates. (Original work published 5th century B.C.).* England: Blackwell Scientific Publications; 1950.
51. Roguin A. Scipione Riva-Rocci and the men behind the mercury sphygmomanometer. *International journal of clinical practice.* 2006;60(1):73-79.
52. Young J. The AEtiology of Eclampsia and Albuminuria and their Relation to Accidental Haemorrhage: (An Anatomical and Experimental Investigation.). *Proceedings of the Royal Society of Medicine.* 1914;7(Obstet Gynaecol Sect):307-348.
53. E.W. P. The relation between hydatid moles, relative ischemia of the gravid uterus, and the placental origin of eclampsia. *American journal of obstetrics and gynecology.* 1939;37:291-293.
54. Khan KS, Wojdyla D, Say L, Gulmezoglu AM, Van Look PF. WHO analysis of causes of maternal death: a systematic review. *Lancet.* 2006;367(9516):1066-1074.
55. Foidart JM, Schaaps JP, Chantraine F, Munaut C, Lorquet S. Dysregulation of anti-angiogenic agents (sFlt-1, PLGF, and sEndoglin) in preeclampsia--a step forward but not the definitive answer. *Journal of reproductive immunology.* 2009;82(2):106-111.
56. Redman CW, Sargent IL. Latest advances in understanding preeclampsia. *Science (New York, NY).* 2005;308(5728):1592-1594.
57. Lindheimer MD, Taylor RN, Roberts JM, Cunningham FG, Chesley L. Chapter 1 - Introduction, History, Controversies, and Definitions. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:1-24.
58. Hypertension in pregnancy. Report of the American College of Obstetricians and Gynecologists' Task Force on Hypertension in Pregnancy. *Obstetrics and gynecology.* 2013;122(5):1122-1131.
59. Medical birth registry of Norway http://mfr-nesstar.uib.no/mfr/. Accessed 15.09.16.
60. Vangen S, Ellingsen L, Andersgaard AB, et al. Maternal deaths in Norway 2005-2009. *Tidsskrift for den Norske laegeforening : tidsskrift for praktisk medicin, ny raekke.* 2014;134(8):836-839.
61. Weinstein L. Syndrome of hemolysis, elevated liver enzymes, and low platelet count: a severe consequence of hypertension in pregnancy. *American journal of obstetrics and gynecology.* 1982;142(2):159-167.
62. Naimy Z, Grytten J, Monkerud L, Eskild A. The prevalence of pre-eclampsia in migrant relative to native Norwegian women: a population-based study. *BJOG : an international journal of obstetrics and gynaecology.* 2015;122(6):859-865.

63. WHO Guidelines Approved by the Guidelines Review Committee. In: *WHO Recommendations for Prevention and Treatment of Pre-Eclampsia and Eclampsia.* Geneva: World Health Organization World Health Organization.; 2011.
64. Walker JJ. Pre-eclampsia. *Lancet.* 2000;356(9237):1260-1265.
65. Dekker G, Robillard PY, Roberts C. The etiology of preeclampsia: the role of the father. *Journal of reproductive immunology.* 2011;89(2):126-132.
66. Astin M, Scott J, Worley R. PRE-ECLAMPSIA/ECLAMPSIA: A FATAL FATHER FACTOR. *The Lancet.* 1981;318(8245):533.
67. Ward K, Taylor RN. Chapter 4 - Genetic Factors in the Etiology of Preeclampsia/Eclampsia. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:57-80.
68. Ananth CV, Skjaerven R, Klunssoyr K. Change in paternity, risk of placental abruption and confounding by birth interval: a population-based prospective cohort study in Norway, 1967-2009. *BMJ open.* 2015;5(2):e007023.
69. Acosta-Sison H. The relationship of hydatidiform mole to pre-eclampsia and eclampsia; a study of 85 cases. *American journal of obstetrics and gynecology.* 1956;71(6):1279-1282.
70. Stillman IE, Karumanchi SA. The glomerular injury of preeclampsia. *Journal of the American Society of Nephrology : JASN.* 2007;18(8):2281-2284.
71. Brosens I, Renaer M. On the pathogenesis of placental infarcts in pre-eclampsia. *The Journal of obstetrics and gynaecology of the British Commonwealth.* 1972;79(9):794-799.
72. Salafia CM, Pezzullo JC, Lopez-Zeno JA, Simmens S, Minior VK, Vintzileos AM. Placental pathologic features of preterm preeclampsia. *American journal of obstetrics and gynecology.* 1995;173(4):1097-1105.
73. Salafia CM, Pezzullo JC, Ghidini A, Lopez-Zeno JA, Whittington SS. Clinical correlations of patterns of placental pathology in preterm pre-eclampsia. *Placenta.* 1998;19(1):67-72.
74. Mol BWJ, Roberts CT, Thangaratinam S, Magee LA, de Groot CJM, Hofmeyr GJ. Pre-eclampsia. *Lancet.* 2016;387(10022):999-1011.
75. Hod T, Cerdeira AS, Karumanchi SA. Molecular Mechanisms of Preeclampsia. *Cold Spring Harbor perspectives in medicine.* 2015;5(10).
76. Redman C. The six stages of pre-eclampsia. *Pregnancy hypertension.* 2014;4(3):246.
77. Redman CWG, Sargent IL, Taylor RN. Chapter 8 - Immunology of Normal Pregnancy and Preeclampsia. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:161-179.
78. Chaiworapongsa T, Chaemsaithong P, Yeo L, Romero R. Pre-eclampsia part 1: current understanding of its pathophysiology. *Nature reviews Nephrology.* 2014;10(8):466-480.
79. Redman CW, Sargent IL. Placental debris, oxidative stress and pre-eclampsia. *Placenta.* 2000;21(7):597-602.
80. Knight M, Redman CW, Linton EA, Sargent IL. Shedding of syncytiotrophoblast microvilli into the maternal circulation in pre-eclamptic pregnancies. *British journal of obstetrics and gynaecology.* 1998;105(6):632-640.
81. Laivuori H. Genetic aspects of preeclampsia. *Frontiers in bioscience : a journal and virtual library.* 2007;12:2372-2382.
82. Esplin MS, Fausett MB, Fraser A, et al. Paternal and maternal components of the predisposition to preeclampsia. *The New England journal of medicine.* 2001;344(12):867-872.
83. Chesley LC, Cooper DW. Genetics of hypertension in pregnancy: possible single gene control of pre-eclampsia and eclampsia in the descendants of eclamptic women. *British journal of obstetrics and gynaecology.* 1986;93(9):898-908.
84. Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E, Irgens LM. Fetal and maternal contributions to risk of pre-eclampsia: population based study. *BMJ (Clinical research ed).* 1998;316(7141):1343-1347.

85. Sutherland A, Cooper DW, Howie PW, Liston WA, MacGillivray I. The indicence of severe pre-eclampsia amongst mothers and mothers-in-law of pre-eclamptics and controls. *British journal of obstetrics and gynaecology.* 1981;88(8):785-791.
86. Arngrimsson R, Bjornsson S, Geirsson RT, Bjornsson H, Walker JJ, Snaedal G. Genetic and familial predisposition to eclampsia and pre-eclampsia in a defined population. *British journal of obstetrics and gynaecology.* 1990;97(9):762-769.
87. Cincotta RB, Brennecke SP. Family history of pre-eclampsia as a predictor for pre-eclampsia in primigravidas. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics.* 1998;60(1):23-27.
88. Arngrimsson R, Bjornsson H, Geirsson R. Analysis of Different Inheritance Patterns in Preeclampsia/Eclampsia Syndrome. *Hypertension in Pregnancy.* 1995;14(1):27-38.
89. Visscher PM, Hill WG, Wray NR. Heritability in the genomics era--concepts and misconceptions. *Nature reviews Genetics.* 2008;9(4):255-266.
90. Salonen Ros H, Lichtenstein P, Lipworth L, Cnattingius S. Genetic effects on the liability of developing pre-eclampsia and gestational hypertension. *American journal of medical genetics.* 2000;91(4):256-260.
91. Thornton JG, Macdonald AM. Twin mothers, pregnancy hypertension and pre-eclampsia. *British journal of obstetrics and gynaecology.* 1999;106(6):570-575.
92. Cnattingius S, Reilly M, Pawitan Y, Lichtenstein P. Maternal and fetal genetic factors account for most of familial aggregation of preeclampsia: a population-based Swedish cohort study. *American journal of medical genetics Part A.* 2004;130a(4):365-371.
93. Graves JA. Genomic imprinting, development and disease--is pre-eclampsia caused by a maternally imprinted gene? *Reproduction, fertility, and development.* 1998;10(1):23-29.
94. Daly AK, Day CP. Candidate gene case-control association studies: advantages and potential pitfalls. *British journal of clinical pharmacology.* 2001;52(5):489-499.
95. Chappell S, Morgan L. Searching for genetic clues to the causes of pre-eclampsia. *Clinical science (London, England : 1979).* 2006;110(4):443-458.
96. Goddard KA, Tromp G, Romero R, et al. Candidate-gene association study of mothers with pre-eclampsia, and their infants, analyzing 775 SNPs in 190 genes. *Human heredity.* 2007;63(1):1-16.
97. Zhao L, Bracken MB, Dewan AT, Chen S. Association between the SERPINE1 (PAI-1) 4G/5G insertion/deletion promoter polymorphism (rs1799889) and pre-eclampsia: a systematic review and meta-analysis. *Molecular human reproduction.* 2013;19(3):136-143.
98. Cross JC. The genetics of pre-eclampsia: a feto-placental or maternal problem? *Clin Genet.* 2003;64(2):96-103.
99. Lachmeijer AM, Dekker GA, Pals G, Aarnoudse JG, ten Kate LP, Arngrimsson R. Searching for preeclampsia genes: the current position. *European journal of obstetrics, gynecology, and reproductive biology.* 2002;105(2):94-113.
100. Oudejans CB, Mulders J, Lachmeijer AM, et al. The parent-of-origin effect of 10q22 in pre-eclamptic females coincides with two regions clustered for genes with down-regulated expression in androgenetic placentas. *Molecular human reproduction.* 2004;10(8):589-598.
101. van Dijk M, Mulders J, Poutsma A, et al. Maternal segregation of the Dutch preeclampsia locus at 10q22 with a new member of the winged helix gene family. *Nature genetics.* 2005;37(5):514-519.
102. Maynard SE, Venkatesha S, Thadhani R, Karumanchi SA. Soluble Fms-like tyrosine kinase 1 and endothelial dysfunction in the pathogenesis of preeclampsia. *Pediatric research.* 2005;57(5 Pt 2):1r-7r.
103. Venkatesha S, Toporsian M, Lam C, et al. Soluble endoglin contributes to the pathogenesis of preeclampsia. *Nature medicine.* 2006;12(6):642-649.
104. Leavey K, Bainbridge SA, Cox BJ. Large scale aggregate microarray analysis reveals three distinct molecular subclasses of human preeclampsia. *PloS one.* 2015;10(2):e0116508.

105. Kleinrouweler CE, van Uitert M, Moerland PD, Ris-Stalpers C, van der Post JA, Afink GB. Differentially expressed genes in the pre-eclamptic placenta: a systematic review and meta-analysis. *PloS one.* 2013;8(7):e68991.
106. Kleinrouweler CE, Wiegerinck MM, Ris-Stalpers C, et al. Accuracy of circulating placental growth factor, vascular endothelial growth factor, soluble fms-like tyrosine kinase 1 and soluble endoglin in the prediction of pre-eclampsia: a systematic review and meta-analysis. *BJOG : an international journal of obstetrics and gynaecology.* 2012;119(7):778-787.
107. Vaiman D, Calicchio R, Miralles F. Landscape of transcriptional deregulations in the preeclamptic placenta. *PloS one.* 2013;8(6):e65498.
108. Tsai S, Hardison NE, James AH, et al. Transcriptional profiling of human placentas from pregnancies complicated by preeclampsia reveals disregulation of sialic acid acetylesterase and immune signalling pathways. *Placenta.* 2011;32(2):175-182.
109. Meng T, Chen H, Sun M, Wang H, Zhao G, Wang X. Identification of differential gene expression profiles in placentas from preeclamptic pregnancies versus normal pregnancies by DNA microarrays. *Omics : a journal of integrative biology.* 2012;16(6):301-311.
110. Nishizawa H, Ota S, Suzuki M, et al. Comparative gene expression profiling of placentas from patients with severe pre-eclampsia and unexplained fetal growth restriction. *Reproductive biology and endocrinology : RB&E.* 2011;9:107.
111. Nishizawa H, Pryor-Koishi K, Kato T, Kowa H, Kurahashi H, Udagawa Y. Microarray analysis of differentially expressed fetal genes in placental tissue derived from early and late onset severe pre-eclampsia. *Placenta.* 2007;28(5-6):487-497.
112. Sitras V, Paulssen RH, Gronaas H, et al. Differential placental gene expression in severe preeclampsia. *Placenta.* 2009;30(5):424-433.
113. Xiang Y, Cheng Y, Li X, et al. Up-regulated expression and aberrant DNA methylation of LEP and SH3PXD2A in pre-eclampsia. *PloS one.* 2013;8(3):e59753.
114. Song Y, Liu J, Huang S, Zhang L. Analysis of differentially expressed genes in placental tissues of preeclampsia patients using microarray combined with the Connectivity Map database. *Placenta.* 2013;34(12):1190-1195.
115. Enquobahrie DA, Meller M, Rice K, Psaty BM, Siscovick DS, Williams MA. Differential placental gene expression in preeclampsia. *American journal of obstetrics and gynecology.* 2008;199(5):566.e561-511.
116. Varkonyi T, Nagy B, Fule T, et al. Microarray profiling reveals that placental transcriptomes of early-onset HELLP syndrome and preeclampsia are similar. *Placenta.* 2011;32 Suppl:S21-29.
117. Cox B, Leavey K, Nosi U, Wong F, Kingdom J. Placental transcriptome in development and pathology: expression, function, and methods of analysis. *American journal of obstetrics and gynecology.* 2015;213(4 Suppl):S138-151.
118. Moslehi R, Mills JL, Signore C, Kumar A, Ambroggio X, Dzutsev A. Integrative transcriptome analysis reveals dysregulation of canonical cancer molecular pathways in placenta leading to preeclampsia. *Scientific reports.* 2013;3:2407.
119. Disentangling fetal and maternal susceptibility for pre-eclampsia: a British multicenter candidate-gene study. *American journal of human genetics.* 2005;77(1):127-131.
120. Zhao L, Triche EW, Walsh KM, et al. Genome-wide association study identifies a maternal copy-number deletion in PSG11 enriched among preeclampsia patients. *BMC pregnancy and childbirth.* 2012;12:61.
121. McGinnis R, Steinthorsdottir V, Williams NO, Thorleifsson G, Shooter S. Variants in the fetal genome near FLT1 are associated with risk of preeclampsia. 2017;49(8):1255-1260.
122. Hulka BS. Overview of biological markers. In: Hulka BS, Griffith JD, Wilcosky TC, eds. *Biological markers in epidemiology.* New york: Oxford University Press; 1990:3-158.
123. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clinical pharmacology and therapeutics.* 2001;69(3):89-95.

124. Novelli G, Ciccacci C, Borgiani P, Papaluca Amati M, Abadie E. Genetic tests and genomic biomarkers: regulation, qualification and validation. *Clinical Cases in Mineral and Bone Metabolism.* 2008;5(2):149-154.
125. Cuffe JSM, Holland O, Salomon C, Rice GE, Perkins AV. Review: Placental derived biomarkers of pregnancy disorders. *Placenta.* 2017;54:104-110.
126. Conde-Agudelo A, Villar J, Lindheimer M. World Health Organization systematic review of screening tests for preeclampsia. *Obstetrics and gynecology.* 2004;104(6):1367-1391.
127. Ferlini A, Scotton C, Novelli G. Biomarkers in rare diseases. *Public health genomics.* 2013;16(6):313-321.
128. Maynard SE, Min JY, Merchan J, et al. Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia. *The Journal of clinical investigation.* 2003;111(5):649-658.
129. Nissaisorakarn P, Sharif S, Jim B. Hypertension in Pregnancy: Defining Blood Pressure Goals and the Value of Biomarkers for Preeclampsia. *Current cardiology reports.* 2016;18(12):131.
130. Conde-Agudelo A, Romero R, Roberts JM. Chapter 11 - Tests to Predict Preeclampsia. In: *Chesley's Hypertensive Disorders in Pregnancy (Fourth Edition).* San Diego: Academic Press; 2015:221-251.
131. Stepan H, Geipel A, Schwarz F, Kramer T, Wessel N, Faber R. Circulatory soluble endoglin and its predictive value for preeclampsia in second-trimester pregnancies with abnormal uterine perfusion. *American journal of obstetrics and gynecology.* 2008;198(2):175.e171-176.
132. Diab AE, El-Behery MM, Ebrahiem MA, Shehata AE. Angiogenic factors for the prediction of pre-eclampsia in women with abnormal midtrimester uterine artery Doppler velocimetry. *International journal of gynaecology and obstetrics: the official organ of the International Federation of Gynaecology and Obstetrics.* 2008;102(2):146-151.
133. Kusanovic JP, Romero R, Chaiworapongsa T, et al. A prospective cohort study of the value of maternal plasma concentrations of angiogenic and anti-angiogenic factors in early pregnancy and midtrimester in the identification of patients destined to develop preeclampsia. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet.* 2009;22(11):1021-1038.
134. Yu CK, Smith GC, Papageorghiou AT, Cacho AM, Nicolaides KH. An integrated model for the prediction of preeclampsia using maternal factors and uterine artery Doppler velocimetry in unselected low-risk women. *American journal of obstetrics and gynecology.* 2005;193(2):429-436.
135. Akolekar R, Zaragoza E, Poon LC, Pepes S, Nicolaides KH. Maternal serum placental growth factor at 11 + 0 to 13 + 6 weeks of gestation in the prediction of pre-eclampsia. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology.* 2008;32(6):732-739.
136. Onwudiwe N, Yu CK, Poon LC, Spiliopoulos I, Nicolaides KH. Prediction of pre-eclampsia by a combination of maternal history, uterine artery Doppler and mean arterial pressure. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology.* 2008;32(7):877-883.
137. Poon LC, Karagiannis G, Leal A, Romero XC, Nicolaides KH. Hypertensive disorders in pregnancy: screening by uterine artery Doppler imaging and blood pressure at 11-13 weeks. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology.* 2009;34(5):497-502.
138. Poon LC, Akolekar R, Lachmann R, Beta J, Nicolaides KH. Hypertensive disorders in pregnancy: screening by biophysical and biochemical markers at 11-13 weeks. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology.* 2010;35(6):662-670.

139. Poon LC, Stratieva V, Piras S, Piri S, Nicolaides KH. Hypertensive disorders in pregnancy: combined screening by uterine artery Doppler, blood pressure and serum PAPP-A at 11-13 weeks. *Prenatal diagnosis.* 2010;30(3):216-223.
140. Audibert F, Boucoiran I, An N, et al. Screening for preeclampsia using first-trimester serum markers and uterine artery Doppler in nulliparous women. *American journal of obstetrics and gynecology.* 2010;203(4):383.e381-388.
141. Akolekar R, Syngelaki A, Sarquis R, Zvanca M, Nicolaides KH. Prediction of early, intermediate and late pre-eclampsia from maternal factors, biophysical and biochemical markers at 11-13 weeks. *Prenatal diagnosis.* 2011;31(1):66-74.
142. Odibo AO, Zhong Y, Goetzinger KR, et al. First-trimester placental protein 13, PAPP-A, uterine artery Doppler and maternal characteristics in the prediction of pre-eclampsia. *Placenta.* 2011;32(8):598-602.
143. Di Lorenzo G, Ceccarello M, Cecotti V, et al. First trimester maternal serum PIGF, free beta-hCG, PAPP-A, PP-13, uterine artery Doppler and maternal history for the prediction of preeclampsia. *Placenta.* 2012;33(6):495-501.
144. Akolekar R, Syngelaki A, Poon L, Wright D, Nicolaides KH. Competing risks model in early screening for preeclampsia by biophysical and biochemical markers. *Fetal diagnosis and therapy.* 2013;33(1):8-15.
145. Scazzocchio E, Figueras F, Crispi F, et al. Performance of a first-trimester screening of preeclampsia in a routine care low-risk setting. *American journal of obstetrics and gynecology.* 2013;208(3):203.e201-203.e210.
146. Kuc S, Koster MP, Franx A, Schielen PC, Visser GH. Maternal characteristics, mean arterial pressure and serum markers in early prediction of preeclampsia. *PloS one.* 2013;8(5):e63546.
147. Myers JE, Kenny LC, McCowan LM, et al. Angiogenic factors combined with clinical risk factors to predict preterm pre-eclampsia in nulliparous women: a predictive test accuracy study. *BJOG : an international journal of obstetrics and gynaecology.* 2013;120(10):1215-1223.
148. Caradeux J, Serra R, Nien JK, et al. First trimester prediction of early onset preeclampsia using demographic, clinical, and sonographic data: a cohort study. *Prenatal diagnosis.* 2013;33(8):732-736.
149. Rolnik DL, Wright D, Poon LCY, et al. ASPRE trial: performance of screening for preterm pre-eclampsia. *Ultrasound in obstetrics & gynecology : the official journal of the International Society of Ultrasound in Obstetrics and Gynecology.* 2017;50(4):492-495.
150. Fujiwara H, Higuchi T, Yamada S, et al. Human extravillous trophoblasts express laeverin, a novel protein that belongs to membrane-bound gluzincin metallopeptidases. *Biochemical and biophysical research communications.* 2004;313(4):962-968.
151. Puente XS, Sanchez LM, Overall CM, Lopez-Otin C. Human and mouse proteases: a comparative genomic approach. *Nature reviews Genetics.* 2003;4(7):544-558.
152. GeneCards (Human Gene Database). http://www.genecards.org/cgi-bin/carddisp.pl?gene=LVRN. Accessed 10.10.16, 2016.
153. e!Ensembl. EMBL-EBI; 2017. http://www.ensembl.org. Accessed 30.09.2017.
154. MEROPS the Peptidase Database Release 11.0. EMBL-EBIRawlings, N.D., Barrett, A.J. & Finn, R.D. (2016) Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res 44, D343-D350. https://www.ebi.ac.uk/merops/. Accessed 26.08.17.
155. Mizutani S. Physiological roles of placental proteases in feto-placental homeostasis. *Nagoya journal of medical science.* 1998;61(3-4):85-95.
156. Maruyama M, Hattori A, Goto Y, et al. Laeverin/aminopeptidase Q, a novel bestatin-sensitive leucine aminopeptidase belonging to the M1 family of aminopeptidases. *The Journal of biological chemistry.* 2007;282(28):20088-20096.
157. Rawlings ND, Barrett AJ. Evolutionary families of peptidases. *The Biochemical journal.* 1993;290 ( Pt 1):205-218.

158. Rawlings ND, Barrett AJ, & Finn RDTyotMdope, their substrates and inhibitors. . Twenty years of the MEROPS database of proteolytic enzymes, their substrates and inhibitors. *Nucleic Acids Res.* 2016;44:D343-D350.
159. Albiston AL, Ye S, Chai SY. Membrane bound members of the M1 family: more than aminopeptidases. *Protein and peptide letters.* 2004;11(5):491-500.
160. Fujiwara H, Higuchi T, Sato Y, et al. Regulation of human extravillous trophoblast function by membrane-bound peptidases. *Biochimica et biophysica acta.* 2005;1751(1):26-32.
161. Barrett AJ. Classification of peptidases. *Methods in enzymology.* 1994;244:1-15.
162. Hooper NM. Families of zinc metalloproteases. *FEBS letters.* 1994;354(1):1-6.
163. MEROPS the Peptidase Database Release 11.0 2017. https://www.ebi.ac.uk/merops/. Accessed 26.08.2017.
164. Goto Y, Yoshioka R, Arisaka N, Hattori A, Tsujimoto M. Involvement of glutamine-238 in the substrate specificity of human laeverin/aminopeptidase Q. *Biological & pharmaceutical bulletin.* 2011;34(1):24-27.
165. UniProt Knowledgebase (UniProtKB). European Bioinformatics Institute (EMBL-EBI), SIB Swiss Institute of Bioinformatics and Protein Information Resource (PIR); 2017. http://www.uniprot.org. Accessed 27.09.2017.
166. Tsujimoto M, Hattori A. The oxytocinase subfamily of M1 aminopeptidases. *Biochimica et biophysica acta.* 2005;1751(1):9-18.
167. Taylor A. Aminopeptidases: structure and function. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology.* 1993;7(2):290-298.
168. Fujiwara H, Imai K, Inoue T, Maeda M, Fujii S. Membrane-bound cell surface peptidases in reproductive organs. *Endocrine journal.* 1999;46(1):11-25.
169. Kenny AJ, O'Hare MJ, Gusterson BA. Cell-surface peptidases as modulators of growth and differentiation. *Lancet.* 1989;2(8666):785-787.
170. Ito N, Nomura S, Iwase A, et al. ADAMs, a disintegrin and metalloproteinases, mediate shedding of oxytocinase. *Biochemical and biophysical research communications.* 2004;314(4):1008-1013.
171. Yamahara N, Nomura S, Suzuki T, et al. Placental leucine aminopeptidase/oxytocinase in maternal serum and placenta during normal pregnancy. *Life sciences.* 2000;66(15):1401-1410.
172. Okuyama T, Ishiura S, Nojima M, Tsukahara T, Yanagida M, Sugita H. Aminopeptidase A in human placenta and pregnant serum. *Clinica chimica acta; international journal of clinical chemistry.* 1991;196(2-3):207-215.
173. Watanabe Y, Iwaki-Egawa S, Mizukoshi H, Fujimoto Y. Identification of an alanine aminopeptidase in human maternal serum as a membrane-bound aminopeptidase N. *Biological chemistry Hoppe-Seyler.* 1995;376(7):397-400.
174. Horie A, Fujiwara H, Sato Y, et al. Laeverin/aminopeptidase Q induces trophoblast invasion during human early placentation. *Human reproduction (Oxford, England).* 2012;27(5):1267-1276.
175. Haas CS, Creighton CJ, Pi X, et al. Identification of genes modulated in rheumatoid arthritis using complementary DNA microarray analysis of lymphoblastoid B cell lines from disease-discordant monozygotic twins. *Arthritis and rheumatism.* 2006;54(7):2047-2060.
176. Mizutani S, Tomoda Y. Effects of placental proteases on maternal and fetal blood pressure in normal pregnancy and preeclampsia. *American journal of hypertension.* 1996;9(6):591-597.
177. Mitsui T, Nomura S, Itakura A, Mizutani S. Role of aminopeptidases in the blood pressure regulation. *Biological & pharmaceutical bulletin.* 2004;27(6):768-771.
178. Mizutani S. [Possible role of placental proteases via degradation of vasoactive peptides in the maternal and fetal blood pressure]. *Nihon Sanka Fujinka Gakkai zasshi.* 1994;46(8):729-734.
179. Goto Y, Hattori A, Ishii Y, Mizutani S, Tsujimoto M. Enzymatic properties of human aminopeptidase A. Regulation of its enzymatic activity by calcium and angiotensin IV. *The Journal of biological chemistry.* 2006;281(33):23503-23513.

180. GeneCards HUMAN GENE DATABASE. Weizmann Institute of Science.; 2017. http://www.genecards.org/. Accessed 30.09.2017.
181. Norwegian Gynecological Society. Standard in Obstetric Aid 2014. *Hypertensive pregnancy complications and eclampsia Chapter 28* 2014.
182. Norwegian Gynecological Society. Standard in Obstetric Aid 2014. *Placenta Chapter 44.* 2014.
183. Turowski G, Berge LN, Helgadottir LB, Jacobsen EM, Roald B. A new, clinically oriented, unifying and simple placental classification system. *Placenta.* 2012;33(12):1026-1035.
184. Redline RW. Inflammatory responses in the placenta and umbilical cord. *Seminars in Fetal and Neonatal Medicine.* 2006;11(5):296-301.
185. Graham CH, Hawley TS, Hawley RG, et al. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Experimental cell research.* 1993;206(2):204-211.
186. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America.* 1979;76(9):4350-4354.
187. Coons AH, Kaplan MH. Localization of antigen in tissue cells; improvements in a method for the detection of antigen by means of fluorescent antibody. *The Journal of experimental medicine.* 1950;91(1):1-13.
188. Coons AH CH, Jones RN. Immunological properties of an antibody containing a fluorescent group. . *Proc Soc Exp Biol* 1941;47.
189. Dhir R. Tissue microarrays: an overview. *Methods in molecular biology (Clifton, NJ).* 2008;441:91-103.
190. Kononen J, Bubendorf L, Kallioniemi A, et al. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nature medicine.* 1998;4(7):844-847.
191. Richani K, Romero R, Kim YM, et al. Tissue microarray: an effective high-throughput method to study the placenta for clinical and research purposes. *The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet.* 2006;19(8):509-515.
192. Zhang Z, Zhang L, Yang X, Li Y, Duan Z. Construction and validation of a placental tissue microarray from specimens of well-documented preeclampsia patients. *Placenta.* 2013;34(2):187-192.
193. Faulk WP, Taylor GM. An immunocolloid method for the electron microscope. *Immunochemistry.* 1971;8(11):1081-1083.
194. Griffiths GE. *Fine structure immunocytochemistry.*: Heidelberg,Germany: Springer-Verlag; 1993.
195. Webster P, Schwarz H, Griffiths G. Preparation of cells and tissues for immuno EM. *Methods in cell biology.* 2008;88:45-58.
196. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature.* 2001;411:494.
197. Sandquist JC, Swenson KI, Demali KA, Burridge K, Means AR. Rho kinase differentially regulates phosphorylation of nonmuscle myosin II isoforms A and B during cell rounding and migration. *The Journal of biological chemistry.* 2006;281(47):35873-35883.
198. Keogh RJ. New technology for investigating trophoblast function. *Placenta.* 2010;31(4):347-350.
199. Albini A, Noonan DM. The 'chemoinvasion' assay, 25 years and still going strong: the use of reconstituted basement membranes to study cell invasion and angiogenesis. *Current opinion in cell biology.* 2010;22(5):677-689.
200. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of immunological methods.* 1983;65(1-2):55-63.

201. Imamura H, Takao S, Aikou T. A modified invasion-3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay for quantitating tumor cell invasion. *Cancer research.* 1994;54(13):3620-3624.
202. Zhang X, Green KE, Yallampalli C, Dong YL. Adrenomedullin enhances invasion by trophoblast cell lines. *Biology of reproduction.* 2005;73(4):619-626.
203. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic acids research.* 2001;29(9):e45.
204. Royston P, Altman DG. Regression Using Fractional Polynomials of Continuous Covariates: Parsimonious Parametric Modelling. *Journal of the Royal Statistical Society Series C (Applied Statistics).* 1994;43(3):429-467.
205. McHugh ML. Interrater reliability: the kappa statistic. *Biochemia medica.* 2012;22(3):276-282.
206. Karlsson C, Bodin L, Piehl-Aulin K, Karlsson MG. Tissue microarray validation: a methodologic study with special reference to lung cancer. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology.* 2009;18(7):2014-2021.
207. World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects. *The Journal of the American College of Dentists.* 2014;81(3):14-18.
208. Nystad M, Sitras V, Larsen M, Acharya G. Placental expression of aminopeptidase-Q (laeverin) and its role in the pathophysiology of preeclampsia. *American journal of obstetrics and gynecology.* 2014;211(6):686.e681-631.
209. Caramelo JJ, Parodi AJ. A sweet code for glycoprotein folding. *FEBS letters.* 2015;589(22):3379-3387.
210. Liu MQ, Chen Z, Chen LX. Endoplasmic reticulum stress: a novel mechanism and therapeutic target for cardiovascular diseases. *Acta pharmacologica Sinica.* 2016;37(4):425-443.
211. Kitamura A, Nagata K, Kinjo M. Conformational analysis of misfolded protein aggregation by FRET and live-cell imaging techniques. *International journal of molecular sciences.* 2015;16(3):6076-6092.
212. Kingdom JC, Drewlo S. Is heparin a placental anticoagulant in high-risk pregnancies? *Blood.* 2011;118(18):4780-4788.
213. Roberts JM. Pathophysiology of ischemic placental disease. *Seminars in perinatology.* 2014;38(3):139-145.
214. Chua S, Wilkins T, Sargent I, Redman C. Trophoblast deportation in pre-eclamptic pregnancy. *British journal of obstetrics and gynaecology.* 1991;98(10):973-979.
215. Sargent IL, Johansen M, Chua S, Redman CW. Clinical experience: isolating trophoblasts from maternal blood. *Annals of the New York Academy of Sciences.* 1994;731:154-161.
216. Jaameri KE, Koivuniemi AP, Carpen EO. Occurrence of trophoblasts in the blood of toxaemic patients. *Gynaecologia International monthly review of obstetrics and gynecology Revue internationale mensuelle d'obstetrique et de gynecologie Monatsschrift fur Geburtshilfe und Gynakologie.* 1965;160(5):315-320.
217. Smarason AK, Sargent IL, Redman CW. Endothelial cell proliferation is suppressed by plasma but not serum from women with preeclampsia. *American journal of obstetrics and gynecology.* 1996;174(2):787-793.
218. Redman CW, Tannetta DS, Dragovic RA, et al. Review: Does size matter? Placental debris and the pathophysiology of pre-eclampsia. *Placenta.* 2012;33 Suppl:S48-54.
219. Mincheva-Nilsson L, Baranov V. Placenta-derived exosomes and syncytiotrophoblast microparticles and their role in human reproduction: immune modulation for pregnancy success. *American journal of reproductive immunology (New York, NY : 1989).* 2014;72(5):440-457.
220. Rawlings ND, Waller M, Barrett AJ, Bateman A. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. *Nucleic acids research.* 2014;42(1):D503-509.

221. Maruyama M, Arisaka N, Goto Y, et al. Histidine 379 of human laeverin/aminopeptidase Q, a nonconserved residue within the exopeptidase motif, defines its distinctive enzymatic properties. *The Journal of biological chemistry.* 2009;284(50):34692-34702.

222. Schagger H, Cramer WA, von Jagow G. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. *Analytical biochemistry.* 1994;217(2):220-230.

223. Rodriguez LG, Wu X, Guan JL. Wound-healing assay. *Methods in molecular biology (Clifton, NJ).* 2005;294:23-29.

224. Giancotti FG, Ruoslahti E. Integrin signaling. *Science (New York, NY).* 1999;285(5430):1028-1032.

225. Merviel P, Challier JC, Carbillon L, Foidart JM, Uzan S. The role of integrins in human embryo implantation. *Fetal diagnosis and therapy.* 2001;16(6):364-371.

226. Cuckle HS. Screening for pre-eclampsia--lessons from aneuploidy screening. *Placenta.* 2011;32 Suppl:S42-48.

227. Leslie K, Thilaganathan B, Papageorghiou A. Early prediction and prevention of pre-eclampsia. *Best practice & research Clinical obstetrics & gynaecology.* 2011;25(3):343-354.


## Supplementary tables

**Supplementary table I** Substrates of laeverin protein.[156,163]

| Substrate | UniProt reference | Residue range | Cleavage site | Cleavage type* |
|---|---|---|---|---|
| **Ala-Mca** | | | Ala+Mca | S |
| **Angiotensin-3** | P01019 | 35-41 | Arg+Val-Tyr-Ile-His-Pro-Phe | N |
| **Arg-Mca** | | | Arg+Mca | S |
| **Dynorphin A(1-8)** | P01213 | 207-214 | Tyr+Gly-Gly-Phe-Leu-Arg-Arg-Ile | N |
| **Dynorphin A(2-8)** | P01213 | 208-2014 | Gly+Gly-Phe-Leu-Arg-Arg-Ile | N |
| **Dynorphin A(3-8)** | P01213 | 209-2014 | Gly+PPhe-Leu-Arg-Arg-Ile | N |
| **Endokinin C** | Q86UU9 | 82-95 | Lys+Lys-Ala-Tyr-Gln- | N |

| | | | | |
|---|---|---|---|---|
| | | | Leu-Glu-His-Thr-Phe-Gln-Gly-Leu-Leu | |
| **Gln-Mca** | | | Gln+Mca | S |
| **Kisspeptin-10** | Q15726 | 112-121 | Tyr+Asn-Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe | N |
| **Leu-Mca** | | | Leu-Mca | S |
| **Lys-Mca** | | | Lys-Mca | S |
| **Met-Mca** | | | Met-Mca | S |
| **Phe-Mca** | | | Phe-Mca | S |
| **SBzl-Cys-Mca** | | | SBzl-Cys+Mca | S |
| **[des-Lys]endokinin C** | Q86UU9 | 83-95 | Lys+Ala-Tyr-Gln-Leu-Glu-His-Thr-Phe-Gln-Gly-Leu-Leu | N |
| **[des-Tyr]kisspeptin-10** | Q15726 | 113-121 | Asn+Trp-Asn-Ser-Phe-Gly-Leu-Arg-Phe | N |
| **[Lys]-bradykinin (kallidin)** | P01042 | 380-389 | Lys+Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg | N |

*N=Not physiologically relevant; S=Synthetic substrates.

**Supplementary table II** Inhibitors of laeverin protein.[156,163]

| Inhibitor | Ki |
|---|---|
| **Bradykinin** | 21.5 µM |
| **Substance P** | 1.83 µM |
| **Angiotensin IV** | 8.92 µM |
| **Bestatin** | 0.96 µM |
| **Phebestin** | 3.02 µM |
| **1.10→phenanthroline** | |

# Appendix

## Papers I-III

# PAPER I

# PAPER II

# PAPER III