OULU 2024
D 1781

ACTA UNIVERSITATIS OULUENSIS


*Pinja Tissarinen*

# HUMAN PLACENTAL AND SERUM PROTEINS IN SPONTANEOUS PRETERM BIRTH

*UNIVERSITY OF OULU GRADUATE SCHOOL;*
*UNIVERSITY OF OULU,*
*FACULTY OF MEDICINE;*
*MEDICAL RESEARCH CENTER OULU;*
*OULU UNIVERSITY HOSPITAL*

ACTA UNIVERSITATIS OULUENSIS
D Medica 1781

*PINJA TISSARINEN*

# HUMAN PLACENTAL AND SERUM PROTEINS IN SPONTANEOUS PRETERM BIRTH

Academic dissertation to be presented with the assent of the Doctoral Programme Committee of Health and Biosciences of the University of Oulu for public defence in Auditorium 12 of the Department of Paediatrics, on 10 May 2024, at 12 noon

UNIVERSITY OF OULU, OULU 2024

Acta Univ. Oul. D 1781, 2024

Supervised by
Professor Mika Rämet
Docent Antti Haapalainen
Doctor Heli Tiensuu

Reviewed by
Docent Raakel Luoto
Docent Nina Kaminen-Ahola

Opponent
Assistant Professor Samuli Rautava

ISBN 978-952-62-4063-3 (Paperback)
ISBN 978-952-62-4064-0 (PDF)

ISSN 0355-3221 (Printed)
ISSN 1796-2234 (Online)

Cover Design
Raimo Ahonen

PUNAMUSTA
TAMPERE 2024

**Tissarinen, Pinja, Human placental and serum proteins in spontaneous preterm birth**

University of Oulu Graduate School; University of Oulu, Faculty of Medicine; Medical Research Center Oulu; Oulu University Hospital

*Acta Univ. Oul. D 1781, 2024*

University of Oulu, P.O. Box 8000, FI-90014 University of Oulu, Finland

## *Abstract*

Preterm birth is defined as live birth before 37 completed weeks of pregnancy. It is the leading cause of neonatal mortality and morbidity among children under five years of age. Most preterm births are considered spontaneous preterm births (SPTBs), and nearly 50% of those do not have an identifiable etiology. It is thought that the cues initiating parturition at term or too soon derive from the mother, the fetus, and/or the placenta. However, the pathological processes behind SPTB have not been completely elucidated.

We utilized placental proteomics and discovered 24, 27, and six proteins that were associated with prematurity, spontaneity, or both, respectively. Ultimately, we obtained six SPTB-associated proteins. Potentially damaging genetic variants of the genes encoding these proteins were studied using published whole exome sequencing data. Genetic variants of *SERPINA1* and *HSPA5* were associated with SPTB. Hence, the two proteins and their genes, alpha-1 antitrypsin (AAT/*SERPINA1*), and a heat shock protein family A member 5 (HSPA5), were obtained and studied further. AAT levels were also studied in serum samples from participants with preterm or term deliveries.

Placental AAT/*SERPINA1* was decreased in SPTBs. We proposed that this low expression could be due to reduced messenger RNA expression, which could alter the anti-inflammatory state of the placenta by proteolytic degradation of immunosuppressive placental fibrinoids.

Placental HSPA5 was shown to be increased in SPTBs. We concluded that this upregulation could promote the pro-inflammatory state of the placenta and disturb the immunotolerance between mother and fetus.

While higher serum concentrations of AAT in early pregnancy were associated with subsequent SPTBs than with those delivering at term, the difference was too modest to be used in a clinical setting. Thus, serum AAT does not constitute a predictive test for SPTB.

In conclusion, our findings are useful in better understanding the complex pathogenesis of SPTB. Ultimately, these results could help develop preventive strategies and predictive tests for SPTB.

*Keywords:* AAT, HSPA5, preterm birth, proteomics

**Tissarinen, Pinja, Ihmisen istukan ja seerumin proteiinit spontaanissa ennenaikaisessa synnytyksessä**
Oulun yliopiston tutkijakoulu; Oulun yliopisto, lääketieteellinen tiedekunta; Medical Research Center Oulu; Oulun yliopistollinen sairaala
*Acta Univ. Oul. D 1781, 2024*
Oulun yliopisto, PL 8000, 90014 Oulun yliopisto

## *Tiivistelmä*

Ennenaikainen synnytys tapahtuu ennen kuin 37 raskausviikkoa on täynnä, ja se on yleisin alle viisivuotiaiden lasten kuolinsyy ja sairastuvuuden aiheuttaja. Suurin osa ennenaikaisista synnytyksistä käynnistyy spontaanisti. Noin 50 prosentissa spontaaneista ennenaikaisista synnytyksistä ei tunnisteta taustalla olevaa syytä. Täysiaikaisesti tai ennenaikaisesti käynnistyvän synnytyksen ajatellaan johtuvan äidistä, sikiöstä ja/tai istukasta, mutta spontaanin ennenaikaisen synnytyksen patofysiologiaa tunnetaan vielä huonosti.

Hyödynsimme istukan proteomiikkaa ja havaitsimme, että 24 proteiinia assosioitui ennenaikaisuuteen ja 27 proteiinia spontaaniin synnytykseen. Nämä vertailut yhdistämällä jäljelle jäi kuusi proteiinia, jotka assosioituivat sekä ennenaikaisuuteen että spontaaniin synnytykseen. Kun näiden proteiinien geeneistä etsittiin mahdollisesti haitallisia variantteja eksomin sekvensointidatasta, jäljelle jäi kaksi proteiinia ja niitä koodaavat geenit: alfa-1 antitrypsiini (AAT/*SERPINA1*) ja heat shock protein family A member 5 (HSPA5). Lisäksi tutkimme seeruminäytteistä AAT-pitoisuutta sekä ennenaikaisesti että täysiaikaisesti synnyttäneillä.

Istukan AAT/*SERPINA1* -taso oli matalampi spontaanissa ennenaikaisessa synnytyksessä. Vähentynyt mRNA:n tuotto voi johtaa istukassa AAT:n vähentyneeseen määrään. AAT:n vähentynyt määrä saattaa lisätä istukan immunosuppressiivisten rakenteiden hajoamista ja siten muuttaa istukan inflammatorista tilaa.

Havaitsimme, että HSPA5-proteiinin taso oli koholla istukoissa, jotka olivat spontaaneista ennenaikaisista synnytyksistä. HSPA5-proteiinin lisääntynyt määrä saattaa lisätä tulehdusta edistävien sytokiinien ekspressiota ja siten häiritä äidin ja sikiön välistä immuunitoleranssia.

Alkuraskauden seeruminäytteiden AAT-konsentraatio oli koholla ennenaikaisesti synnyttäneillä äideillä verrattuna kontrolliraskauksiin. Ryhmien välinen ero ei kuitenkaan ollut kliinisesti merkittävä, joten alkuraskauden seerumin AAT-pitoisuus ei sovellu ennustavaksi testiksi spontaanille ennenaikaiselle synnytykselle.

Tutkimustemme tulokset auttavat ymmärtämään paremmin spontaanin ennenaikaisen synnytyksen patogeneesiä. Lisäksi tulokset voivat auttaa kehittämään ennenaikaista synnytystä ehkäiseviä menetelmiä sekä spontaanin ennenaikaisen synnytyksen riskiä ennustavia testejä.

*Asiasanat:* AAT, ennenaikainen synnytys, HSPA5, istukka, proteomiikka

*To all who have been born prematurely*

# Acknowledgements

This study was carried out at the Research Unit of Clinical Medicine, Medical Research Center Oulu, University of Oulu, and Oulu University Hospital during the years 2020–2024. The work was supported by grants from the Stiftelsen Alma och K. A. Snellman Foundation, the Foundation for Pediatric Research, the Eemil Aaltonen Foundation, the University of Oulu Scholarship Foundation, and the Finnish Medical Foundation.

I would like to express my deepest gratitude to my supervisors, Professor Mika Rämet, Docent Antti Haapalainen, and Heli Tiensuu, PhD, for their encouraging words and assistance during this journey. As my principal supervisor, Mika Rämet offered me a chance to work as part of this research team and I am grateful for his supportive words. Antti Haapalainen has been ready to offer guidance throughout these years. Heli Tiensuu has been my mentor, especially in the laboratory experiments, and I could not imagine a better person in that role. I am grateful to Professor Mikko Hallman for the opportunity to work in this research group and for his guidance and encouragement.

I am in great debt to the pre-examiners of this doctoral thesis, Docent Raakel Luoto, and Docent Nina Kaminen-Ahola, for their valuable and thorough comments, which have improved my thesis. I warmly thank my opponent, Assistant Professor Samuli Rautava, and am looking forward to our discussion.

I thank Marja Ojaniemi for data collection, valuable comments, and guidance, Eveliina Ronkainen for priceless help with data collection and guidance, Anu Pasanen and Minna Karjalainen for contribution and support, Riitta Vikeväinen for sample and data collection, Marianne Haapea for help with statistical analyses, Emma Koivulehto for support, and Maarit Haarala for support and assistance in the laboratory. All coauthors are acknowledged for their contributions: Ulrich Bergmann, Johanna Huusko, Liisa Laatio, Louis Muglia, Tomi Määttä, Steffen Ohlmeier, Marja Vääräsmäki, and Hanna Öhman. I warmly thank all participants that took part in these studies. My follow-up group members, Professor Terhi Ruuska (former Tapiainen), Kristian Koski, PhD, and Miia Salo, PhD, are also acknowledged for their encouragement and support.

I am also grateful to my friends for their support and encouraging words over these recent years. Rita and Nina, you have always been there for me. Veera has supported me all the way from London with our regularly irregular video calls. I warmly thank the 112 group: Heidi, Julia, and Marianne: you have supported me in my medical studies as colleagues and friends.

I am thankful for the support from ukki and Leena, who have always believed in me. Mummi, you have been my mainstay, and I am grateful for our conversations during the ups and downs. My cats, Kisu and Piki, saw to it that I had a pause once in a while writing this thesis and the manuscripts. I thank my little sister Maija for help with the statistics and all the hilarious moments we have shared. Lastly, I am grateful to my parents, Satu and Juha-Pekka. You have supported and believed in me from the very beginning of my life, as I was born very prematurely. This journey would not have been possible without you.

Oulu, February 2024 Pinja Tissarinen

# Abbreviations

| | |
|---|---|
| AAT | Alpha-1 antitrypsin |
| ACTH | Adrenocorticotropic hormone |
| ALB | Albumin |
| ANXA5 | Annexin A5 |
| aOR | Adjusted odds ratio |
| C | Cysteine |
| CAP | Contraction associated protein |
| cDNA | Complementary DNA |
| CI | Confidence interval |
| cOR | Crude odds ratio |
| CRH | Corticotropin-releasing hormone |
| CRP | C-reactive protein |
| CVF | Cervicovaginal fluid |
| CYC1 | Cytochrome C1 |
| E | Glutamic acid |
| ECM | Extracellular matrix |
| EPTB | Elective preterm birth |
| ER | Endoplasmic reticulum |
| FC | Fold change |
| fFN | Fetal fibronectin |
| FMC | Finnish Maternity Cohort |
| *FN1* | *Fibronectin 1* gene |
| G | Glycine |
| GA | Gestational age |
| GRP78 | 78 kDa glucose-regulated protein |
| GWAS | Genome-wide association study |
| HSP | Heat shock protein |
| HSPA5 | Heat shock protein family A member 5 |
| *HSPA5* | *Heat shock protein family A member 5* gene |
| IBP-4 | Insulin-like growth factor-binding protein 4 |
| IL-6 | Interleukin 6 |
| Immuno-EM | Immunoelectron microscopy |
| ICM | Inner cell mass |
| K | Lysine |
| KRT19 | Keratin type I cytoskeletal 19 |

| | |
|---|---|
| LMP | Last menstrual period |
| mRNA | Messenger RNA |
| NBD | Nucleotide binding domain |
| OR | Odds ratio |
| PAMG-1 | Placental alpha macroglobulin |
| PG | Prostaglandin |
| phIGFBP | Phosphorylated insulin-like growth factor-binding protein 1 |
| PPROM | Preterm prelabor rupture of the fetal membranes |
| PROM | Prelabor rupture of the fetal membranes |
| PR | Progesterone receptor |
| PTB | Preterm birth |
| qPCR | Quantitative PCR |
| R | Arginine |
| SBD | Substrate binding domain |
| SD | Standard deviation |
| SERPIN | Serine protease inhibitor |
| *SERPINA1* | Serpin family A member 1 gene |
| SHGB | Sex hormone-binding globulin |
| siRNA | Small interfering RNA |
| SPTB | Spontaneous preterm birth |
| STB | Spontaneous term birth |
| TE | Trophectoderm |
| VIM | Vimentin |
| WES | Whole exome sequencing |

# List of original publications

This thesis is based on the following publications, which are referred throughout the text by their Roman numerals:

I Tiensuu, H.[#], Haapalainen, A. M.[#], Tissarinen, P., Pasanen, A., Määttä, T. A., Huusko, J. M., Ohlmeier, S., Bergmann, U., Ojaniemi, M., Muglia, L. J., Hallman, M.*, & Rämet, M.* (2022). Human placental proteomics and exon variant studies link AAT/SERPINA1 with spontaneous preterm birth. *BMC Medicine*, *20*(1), 141. https://doi.org/10.1186/s12916-022-02339-8.[1]

II Tissarinen, P., Tiensuu, H., Haapalainen, A. M., Määttä, T. A., Ojaniemi, M., Hallman, M.*, & Rämet, M.* (2023). Elevated human placental heat shock protein 5 is associated with spontaneous preterm birth. *Pediatric Research, 94*(2), 520–529. https://doi.org/10.1038/s41390-023-02501-9.

III Tissarinen, P., Tiensuu, H., Haapalainen, A. M., Ronkainen, E., Laatio, L., Vääräsmäki, M., Öhman, H., Hallman, M.*, & Rämet, M.*. Maternal serum alpha-1 antitrypsin levels in spontaneous preterm and term pregnancies. Submitted.

[#]*Equal contribution

[1]Study I was also used in the doctoral thesis of Heli Tiensuu (University of Oulu, 2022).

# Contents

# 1 Introduction

Preterm birth (PTB; birth before 37 completed weeks of pregnancy) is the top cause of neonatal mortality and morbidity (Frey & Klebanoff, 2016; Ohuma et al., 2023; Romero et al., 2014). Prematurity predisposes to several short-term and lifelong health consequences, such as pulmonary diseases and neurodevelopmental disorders. Globally, the rate of PTBs has not changed much over the years (Ohuma et al., 2023). Several risk factors increasing the likelihood of PTB have been identified. Approximately 70% of PTBs are spontaneous (spontaneous preterm births, SPTBs), and about 50% of these do not have an identifiable etiology (Hallman et al., 2019).

The main problem related to the prediction and prevention of SPTB is the incomplete understanding of its pathophysiology (Smith, 2007). It has been thought that both preterm and term births may share similar pathways (Romero et al., 2006). However, molecular mechanisms behind spontaneous term labor are not completely understood (Haapalainen et al., 2018; Wijaya et al., 2020). This has led to studies regarding timing of birth and the pathogenesis of SPTB. Maternal, fetal, and/or placental involvement play a role in the timing of birth and SPTB. Several studies have explored maternal and fetal genomes in relation to SPTBs. Over a hundred SPTB-associated genes have been identified, although verification is needed (Jain et al., 2022). In addition to gene candidates, several tissues have been studied to identify protein candidates associated with SPTB. Identification of SPTB-associated proteins could lead to plausible pathways to premature delivery. Indeed, over 60 dysregulated proteins have been linked with SPTB (Kacerovsky et al., 2014). However, no specific protein candidate has been demonstrated to be accurate in predicting PTB (Leow et al., 2020).

The overall aim of this thesis is to identify and study the role of potential proteins associated with SPTB. The starting point was the placental proteomes that were used as a hypothesis-free method to obtain protein candidates associated with SPTB. The placental protein candidates obtained were then studied with several biochemical methods to determine their possible roles in SPTB.

# 2 Review of literature

## 2.1 Definition of term and preterm birth

Gestation length and reproductive physiology are highly species-specific (Jenkin & Young, 2004). Term human gestation lasts approximately 280 days (40 weeks) (American College of Obstetricians and Gynecologists, 2013; McLean et al., 1995), whereas in sheep, term gestation lasts 145–147 days (Bezold et al., 2013; Jenkin & Young, 2004), and average gestation length in mice varies from 18 to 22 days (Bezold et al., 2013; Murray et al., 2010).

In humans, gestation can be divided into three trimesters (Fig. 1). Length of pregnancy, or gestational age (GA), is calculated from the last menstrual period (LMP) or based on an ultrasound examination (Spong, 2013; Vogel et al., 2018). Usually, GA is reported in weeks$^{+days}$, and delivery of the baby between $37^{+0}$ and $41^{+6}$ weeks from the LMP is considered term birth. More precisely, a full-term birth occurs between $39^{+0}$ and $40^{+6}$ weeks, whereas post-term takes place after $42^{+0}$ weeks (American College of Obstetricians and Gynecologists, 2013). Thus, PTB is a live birth before $37^{+0}$ weeks of pregnancy or less than 259 days after the LMP (Barfield, 2018; Blencowe et al., 2013). The World Health Organization recommended "a viability criterion" of the fetus to classify live births and fetal deaths (1970), but no minimum GA criterion has been defined (Morgan et al., 2022). The viability of extremely preterm babies, which depends on the level of perinatal care, varies between countries; thus, no official GA cut-off has been determined (Blencowe et al., 2013; Morgan et al., 2022). In Finland, a birth is defined as GA of $\geq 22^{+0}$ weeks or as birth weight of at least 500 g (Preterm birth: Current Care Guidelines 2018).

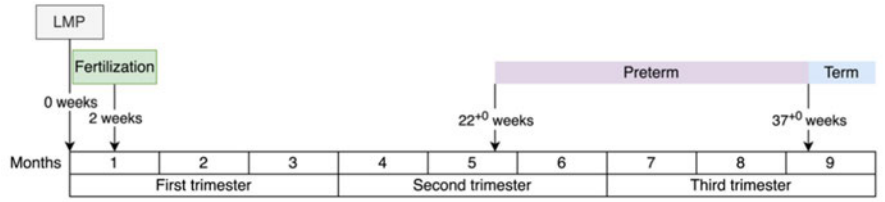


**Fig. 1. Human pregnancy trimesters illustrated on a timeline. Times of preterm and term births are also indicated. LMP: last menstrual period. Modified from Häggström, Mikael (2014), https://en.wikipedia.org/wiki/Gestational_age.**

## 2.2 The human placenta

The placenta serves many vital functions for the fetus during pregnancy (Cindrova-Davies & Sferruzzi-Perri, 2022). It is a species-specific organ (Benirschke, 1998), and contains cells of both maternal and fetal origin (Gude et al., 2004; Shibata et al., 2020).

### *2.2.1 Development*

After the egg is fertilized in the fallopian tube, the zygote is formed and it passively moves toward the uterine cavity. Before implantation, approximately five days after fertilization, the blastocyst (the pre-implantation embryo) is formed; it consists of the blastocyst cavity, the inner cell mass (ICM), and the peripheral trophoblast layer, or the trophectoderm (TE) (Jansen et al., 2020; Shibata et al., 2020; Turco & Moffett, 2019). The TE forms the placenta, whereas the embryo, umbilical cord, and the placental mesenchyme are formed from the ICM (Huppertz, 2008; Jansen et al., 2020). At the same time, the endometrium is prepared for implantation of the blastocyst, and a special tissue layer called the decidua is formed in a process known as decidualization (Jansen et al., 2020; Turco & Moffett, 2019).

Approximately six to seven days after fertilization, the implantation of the blastocyst begins, and the early placenta starts to develop (Cindrova-Davies & Sferruzzi-Perri, 2022; Huppertz, 2008). A simplified depiction of the stages of early human placental development is presented in Fig. 2. The first stage of the formation of the placenta is the prelacunar stage. The part of the TE that is in direct contact with the underlying ICM attaches to the uterine epithelium. After the blastocyst has been attached, the development of the primary syncytium, which consists of oligonucleated syncytiotrophoblasts, begins (Huppertz, 2008; Turco & Moffett, 2019). The remaining trophoblast cells that do not participate in forming the syncytium are cytotrophoblasts (the progenitor cells of the syncytiotrophoblasts) (Cindrova-Davies & Sferruzzi-Perri, 2022; Huppertz, 2008). The cytotrophoblasts will form, together with the syncytium and extraembryonic mesoderm, the early fetal side of the placenta (Cindrova-Davies & Sferruzzi-Perri, 2022; Huppertz, 2008). At this stage, the invasive phenotype of the syncytiotrophoblasts enables the embedding of the blastocyst into the endometrium (Huppertz, 2008).

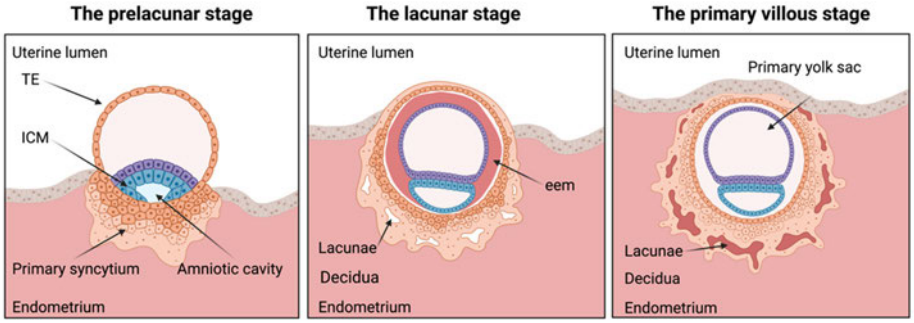


**Fig. 2. A simplified depiction of the three stages of early human placental development: the prelacunar stage, the lacunar stage, and the primary villous stage (eem is not shown in the primary villous stage). TE, trophectoderm; ICM, inner cell mass; eem, extraembryonic mesoderm. Modified from Turco & Moffett (2019). Created with BioRender.com.**

The blastocyst is fully embedded within the endometrium, and implantation is finalized at ~12 days post-conception (Huppertz, 2008). The lacunar stage takes place approximately 14 days after conception and is usually observed as a missing menstrual period (Turco & Moffett, 2019). The formation of lacunae begins. Syncytiotrophoblasts between the lacunae are referred to as trabeculae (Huppertz, 2008; Turco & Moffett, 2019). The cytotrophoblasts start to invade the syncytium and reach the decidua (Huppertz, 2008; Turco & Moffett, 2019). These cytotrophoblasts differentiate into extravillous trophoblasts, which can reach the spiral arteries of the endometrium and participate in vascular remodeling of the placenta (Cindrova-Davies & Sferruzzi-Perri, 2022; Huppertz, 2008). Additionally, the cytotrophoblasts form cell columns, which are anchoring structures between the placenta and decidua (Cindrova-Davies & Sferruzzi-Perri, 2022). These cytotrophoblastic cell columns spread and eventually form the early basal plate. The early placenta has now three zones: the early chorionic plate, the lacunar system, and the early maternal side of the placenta (Huppertz, 2008).

Primary villi are comprised of the cytotrophoblasts that have pushed themselves through the syncytium and are covered by the syncytiotrophoblast cell layer (Huppertz, 2008; Turco & Moffett, 2019). Shortly after the formation of the primary villi, secondary villi are formed by extraembryonic mesodermal cells that penetrate the primary villi structures. The first placental vessels appear by ~18 days after fertilization, transforming the secondary villi into tertiary villi (Cindrova-Davies & Sferruzzi-Perri, 2022; Huppertz, 2008; Turco & Moffett, 2019). Those

tertiary villi begin to branch progressively, forming a system of villous trees (Turco & Moffett, 2019), eventually making the tertiary villi the dominant villous type in the placenta (Huppertz, 2008).

Proper vascularization of the placenta is necessary for the growth and function of the placenta and the development and growth of the baby. During the first trimester, placental development takes place in a physiologically hypoxic environment that is thought to be essential for successful pregnancy (Gude et al., 2004). Maternal vascularization is characterized by remodeling of the spiral arteries (Weckman et al., 2019). Vasculogenesis is the formation of new blood vessels from the mesenchymal cells, which differentiate into hemangiogenic stem cells and eventually to endothelial cells (Weckman et al., 2019). Later, development of the placental vascular network is characterized by branching (the formation of new vessels through sprouting) and non-branching (the formation of capillary loops) angiogenesis of the existing blood vessels (Weckman et al., 2019). The basis for uteroplacental circulation is formed approximately 13 days after fertilization and the first placental vessels appear by ~18 days after fertilization. The fetoplacental perfusion is established as the capillaries in the villous core are connected via the umbilical cord to the fetal heart within four weeks of conception (Burton et al., 2009). The intervillous space of the placenta is formed from the lacunae (Turco & Moffett, 2019), and the intervillous space starts to continuously fill with maternal blood by 10–12 weeks of gestation (Cindrova-Davies & Sferruzzi-Perri, 2022; Degner et al., 2017; Gude et al., 2004). Vascular endothelial growth factor and angiopoietins are the key regulators of placental vasculogenesis and angiogenesis, and their signaling is strictly coordinated (Burton et al., 2009; Weckman et al., 2019).

### *2.2.2 Structure*

The essential structures of the placenta are formed by the end of the first trimester (Turco & Moffett, 2019). On average, the mature placenta is 22 cm in diameter, and weighs 500 g (Burton & Fowden, 2015; Huppertz, 2008). The relevant placental structures are illustrated in Fig. 3. The fetal side (chorionic plate) and the maternal side (basal plate) of the placenta merge to form the placental membranes that surround the baby and the amniotic fluid. The placental membranes have three layers: the amnion (the inner layer), the chorion (the outer layer), and the decidua capsularis. The chorionic plate is covered by the amnion (Huppertz, 2008).

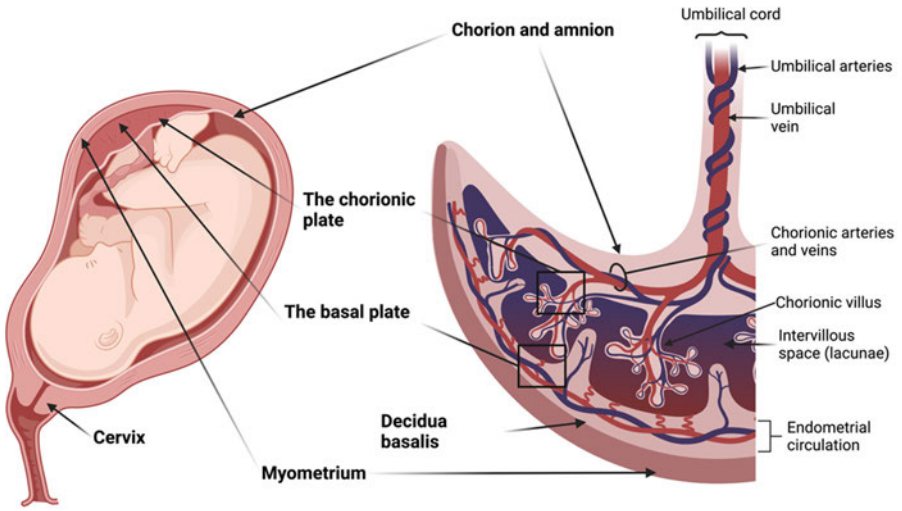


**Fig. 3. Illustration of the human placenta. Adapted from Jansen et al. (2020) and created with BioRender.com.**

The chorionic plate constitutes of the fetal capillaries, the villous core, and the chorionic (i.e., placental) villi (Cindrova-Davies & Sferruzzi-Perri, 2022). An illustration of the chorionic villi appears in Fig 4. The syncytiotrophoblast layer is covered by microvilli on its apical border (Burton & Fowden, 2015; Turco & Moffett, 2019). The syncytiotrophoblasts participate in several functions of the placenta, such as transportation and secretion of molecules and protective and endocrine functions (Burton & Fowden, 2015; Gude et al., 2004). Beneath the syncytiotrophoblast layer are the cytotrophoblasts. Briefly, the cytotrophoblast cells fuse together to form syncytiotrophoblasts (Burton & Fowden, 2015). In addition to trophoblast cells, the villous core contains several other cell types, such as fibroblasts, immune cells, and vascular cells, all of which originate from the extraembryonic mesenchyme (Turco & Moffett, 2019). The fibroblasts produce matrix proteins (e.g., collagens and proteoglycans). Hofbauer cells are a type of macrophages within the villous core and are thought to originate from mesenchymal cells in the villous stroma and from monocytes derived from the embryonic or fetal bone marrow (Huppertz, 2008; Zulu et al., 2019). They are crucial for the placenta's immunological functions and participate in various tasks, such as antigen presentation and cytokine secretion (Zulu et al., 2019). Several

types of extracellular matrix (ECM) components, such as collagens, laminins, and fibronectin, have been described in the villous core (Chen & Aplin, 2003).

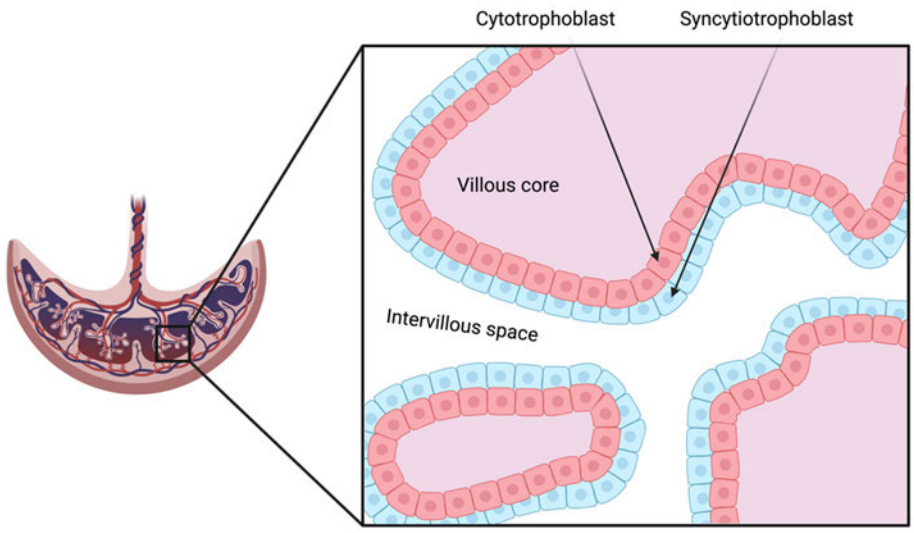


**Fig. 4. A simplified illustration of the cellular structure of the chorionic villus. Microvilli on the apical border of the syncytiotrophoblasts and cells within the villous core not shown. Created with BioRender.com.**

The maternal side of the placenta (the basal plate) consists of extravillous trophoblasts, maternal cells (decidual stromal cells, immune cells), the ECM, and fibrinoids (Huppertz, 2008). The extravillous trophoblasts participate in uterine artery remodeling (Gude et al., 2004), while the stromal cells produce several growth factors that stimulate the endometrial glands (Turco & Moffett, 2019). The major maternal immune cells include natural killer cells, macrophages, and T cells (Bulmer et al., 2010); other immune cells such as B cells and dendritic cells are rare (Erlebacher, 2013). Indeed, decidual natural killer cells constitute most of the leukocytes present in the first-trimester placenta (Arck & Hecher, 2013; Turco & Moffett, 2019). The natural killer cells participate in remodeling the decidual vasculature and secrete angiogenesis-related factors like angiopoietin-1 and vascular endothelial growth factor (Liu et al., 2017). Decidual macrophages that are derived from the circulating monocytes support maternal–fetal immunotolerance and participate in immunomodulation (Bulmer et al., 2010; Liu et al., 2017).

Placental fibrinoids can be divided into two types: fibrin-type fibrinoid and matrix-type fibrinoid (Huppertz et al., 1996; Kaufmann et al., 1996; Zhang, 2021). The former is a product of fibrin deposition, while the latter is secreted by extravillous trophoblasts (Huppertz, 2008; Huppertz et al., 1996). Fibrin-type fibrinoids are in direct contact with maternal blood and the intervillous space as they are a product of maternal blood clotting. By contrast, matrix-type fibrinoids are not in direct contact with maternal blood and contain, for example, collagen IV, laminin, vitronectin, and fibronectins (Huppertz et al., 1996; Kaufmann et al., 1996). Fibrin-type fibrinoids are thought to contribute to many placental functions, such as mechanically stabilizing the placenta and regulating intervillous circulation. Matrix-type fibrinoids have mostly immunological functions and participate in the adhesion of the placenta to the uterine wall (Kaufmann et al., 1996). Placental fibrinoids are present in normal and pathological placentas, but their functions have not been completely identified in their entirety (Zhang, 2021).

### *2.2.3 Function and role during pregnancy*

The placenta plays a pivotal role in enabling the development of the fetus (Burton & Fowden, 2015). It participates in gas (oxygen and carbon dioxide), water, nutrient (carbohydrates, amino acids, lipids, electrolytes, hormones), and waste product exchange between fetus and mother. The connection between fetus and placenta is formed through the umbilical cord. The intervillous space is filled with maternal blood from the spiral arteries, allowing maternal–fetal exchange to occur. The capillaries in the villi carry oxygen rich blood to the fetus via chorionic veins and the umbilical vein. Oxygen-deficient fetal blood returns to the placenta and the villous capillaries through two umbilical arteries (Gude et al., 2004). The exchange between various molecules occurs through maternal to fetal circulation and vice versa. Additionally, the placenta itself is a metabolically active organ (e.g., glycolysis) and has endocrine activity (Gude et al., 2004). The placenta produces a variety of hormones and other placenta-specific proteins, such as progesterone, human chorionic gonadotropin, and insulin-like growth factors. The placenta also acts as a protective barrier, and several enzymes and transporters are present within it (Burton & Fowden, 2015), such as cytochrome P450 enzymes (Gude et al., 2004). Although the placental barrier can prevent transmission of many bacteria, some bacteria and viruses are able to penetrate the placenta and infect the fetus (Gude et al., 2004). The fetus also acquires maternal antibodies via the placenta (Chucri et al., 2010), which is important for the development of passive immunity.

### 2.2.4 Abnormalities and pregnancy complications

Disturbance in placental function and placental abnormalities affect both fetus and mother. Placental pathologies have been linked to several adverse pregnancy outcomes, such as PTB, preeclampsia, intrauterine growth restriction, placental abruption, and unexplained stillbirth (Turco & Moffett, 2019). Placental insufficiency can lead to preeclampsia (Turco & Moffett, 2019), which is one of the indications for medically indicated preterm delivery. Abnormal location of the placenta (such as low-lying placenta and placenta previa) increases the risk of excessive blood loss and preterm delivery (Jansen et al., 2020). Abnormal trophoblast invasion of the uterine wall without a decidual contact between is classified as abnormally invasive placenta, which increases the risk of excessive bleeding and can even be life-threatening (Jansen et al., 2020). Recently, placental malperfusion, high-grade chronic inflammation, and accelerated villous maturation have been linked to SPTB (Brink et al., 2022; Jaiman et al., 2022; Suresh et al., 2022). Infection is one of the most common placental findings in PTB, including chorioamnionitis, infectious villitis (infection of the villous tree), vasculitis of umbilical vessels, and funisitis (infection of the umbilical cord) (Brink et al., 2022).

## 2.3 Physiology of human birth

In addition to gestation length and the physiology behind gestation, parturition displays striking differences between species (Jenkin & Young, 2004; Smith, 2007; Weiss, 2000). Gestation length varies even within the same species, as with full- and post-term pregnancy in humans, indicating the presence of "gestational clocks" (Wijaya et al., 2020). For example, circadian regulation has been proposed to play a role in timing of birth (Waddell et al., 2012). Despite extensive research, a complete understanding of the physiology determining the length of pregnancy and the triggers that initiate labor remains lacking (Golightly et al., 2011; Rokas et al., 2020; Smith, 2007; Wijaya et al., 2020).

### 2.3.1 Phases of human parturition

Human parturition is thought to consist of four phases (Fig. 5) (Challis, 2000; Vannuccini et al., 2016). During phase 0, known as the quiescent phase, myometrial activity is inhibited by several hormones and substances, such as progesterone. These substances increase the intracellular level of cyclic nucleotides, which

eventually inhibit the release of calcium or reduce the activity of myosin light-chain kinase. The reduced amount of intracellular calcium and the inhibition of myosin light-chain kinase inhibit myometrial contractility. Initiation of parturition is thought to take place between phase 0 and phase 1 (Challis, 2000; Challis et al., 2002; Ilicic et al., 2020).

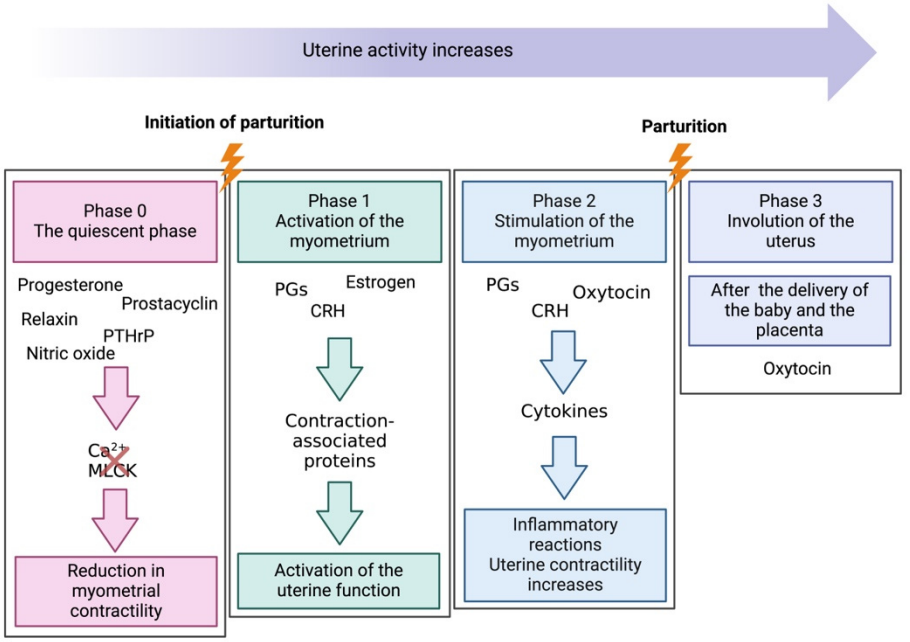


**Fig. 5. A simplified depiction of the phases of human parturition, adapted from Vannuccini et al. (2016) and Challis (2000). PTHrP, parathyroid hormone-related peptide; MLCK, myosin light-chain kinase; PG, prostaglandin; CRH, corticotropin-releasing hormone. Created with BioRender.com.**

Phase 1, the activation phase, involves elevation in estrogen and corticotropin-releasing hormone (CRH) levels and in prostaglandin (PG) production. These stimulate the production of contraction-associated proteins (CAPs), which include connexin 43, PG receptors, oxytocin receptors, and ion channel encoding proteins. The inhibition of myometrial contractility is disturbed by the CAPs, thus leading to activation of the uterine function and phase 2 of parturition (Challis et al., 2002; Ilicic et al., 2020), in which the uterine muscle is stimulated by several uterotonics (e.g., PGs, oxytocin, and CRH) that generate coordinated contractions in the uterine muscle. After the delivery of the baby and placenta, the uterus contracts and shrinks,

mainly due to oxytocin (phase 3) (Blanks & Thornton, 2003; Ilicic et al., 2020; Vannuccini et al., 2016).

Clinically, parturition can be divided into three stages (Challis, 2000). The first (stage 1) is characterized by uterine contractions and a softening and shortening of the cervix that leads to its full dilation. The baby is delivered during stage 2; finally, the placenta is expelled in stage 3.

### 2.3.2 Hormonal, immunological, and physical factors in the initiation of term and preterm birth

The activation of parturition is highly complex and involves a number of biochemical processes. Several inflammatory, endocrine, paracrine, and autocrine pathways and other physical changes during pregnancy and parturition have been identified (Shynlova et al., 2020; Weiss, 2000). The phases of parturition described above largely represent the "common pathway" of labor (Romero et al., 2014). It is thought that the common pathway is physiologically stimulated at term, whereas pathological processes activate the common pathway too soon in the case of PTB (Romero et al., 2014). The common pathway of birth probably consists of multiple and possibly overlapping pathways (Weiss, 2000) that operate in concert. Different pathways and factors in term births and PTBs are also feasible, as PTBs have multiple etiologies (Weiss, 2000). It is likely that these events originate from the mother, but placental and/or fetal involvement is also plausible (Jenkin & Young, 2004; Weiss, 2000). Some hormonal and immunological factors and physical changes in relation to the initiation of birth are discussed more in detail below.

#### *Progesterone*

At the beginning of pregnancy, progesterone is produced by the corpus luteum of the ovary (Rokas et al., 2020). At an approximate GA of 6–10 weeks, the production of progesterone shifts to the placenta (Challis, 2000; Rokas et al., 2020). Progesterone is a key factor in maintaining myometrial quiescence (Challis, 2000; Shynlova et al., 2020). Syncytiotrophoblasts convert pregnenolone to progesterone and trophoblasts within the fetal membranes (chorion) also produce progesterone (Challis, 2000).

Progesterone withdrawal has been observed in most mammalian species at labor, but maternal plasma progesterone levels remain relatively constant in humans at the end of pregnancy (Golightly et al., 2011; Zakar & Hertelendy, 2007).

However, it is thought that the role of progesterone is essential in parturition in humans via "functional progesterone withdrawal" (Ilicic et al., 2020; Shynlova et al., 2020). The progesterone receptors (PRs) PR-A and PR-B play an important role in this phenomenon (Mendelson et al., 2017; Shynlova et al., 2020). Expression of PR-A is increased at term, and the ratio between these two receptors changes and leads to functional progesterone withdrawal (Kamel, 2010), which has thus been proposed as one possible mechanism participating in the initiation of labor.

### *Estrogen*

Balance between progesterone and estrogen is essential during pregnancy (Shynlova et al., 2020). Estrogen promotes uterine contractility and activation of the myometrium (Shynlova et al., 2020); it also induces production of PGs that stimulate the expression of CAPs (Ilicic et al., 2020). Like progesterone levels, estrogen levels remain high during parturition (Ilicic et al., 2020). This has led to an idea of "a functional estrogen activation" via changes in the estrogen receptors, which could participate in parturition (Ilicic et al., 2020). It has been proposed that functional progesterone withdrawal via PRs leads to functional estrogen activation (Kamel, 2010).

### *Corticotropin-releasing hormone*

CRH originates in the hypothalamus of both mother and fetus and is also produced by the placenta (Weiss, 2000). Maternal circulating CRH increases during pregnancy (Smith, 2007). CRH controls the secretion of maternal adrenocorticotropic hormone (ACTH) from the anterior pituitary gland via a negative feedback loop. ACTH stimulates the adrenal glands, which produce cortisol. That increased cortisol production diminishes the production of CRH, which correspondingly decreases the release of ACTH to complete the negative feedback loop (Weiss, 2000). However, a paradoxical positive feedback loop is observed in the placenta, as increased cortisol promotes secretion of CRH (Weiss, 2000). In fact, the placenta serves as the major source of CRH from the second trimester onward. Fetal circulating CRH also increases as the pregnancy advances (Smith, 2007). CRH stimulates fetal ACTH production, which increases fetal cortisol secretion. Fetal cortisol plays an important role in fetal lung maturation (Smith, 2007).

Higher CRH levels in maternal serum in mid-pregnancy may predict PTB (Weiss, 2000), but administration of cortisol has not been demonstrated to induce labor. In maternal circulation, CRH is bound to CRH-binding protein, leading to biologically inactive CRH. Toward the end of pregnancy, the levels of the binding protein decrease, and CRH is released into the circulation (Ilicic et al., 2020). However, plasma CRH levels have not been demonstrated as a reliable predictive test for PTB (Ilicic et al., 2020). To summarize, a complex maternal–fetal–placental axis using the hypothalamus–pituitary–adrenal gland axis may play a role in the timing of birth.

### *Oxytocin*

Maternal oxytocin levels during pregnancy increase after the cervix has fully dilated. Oxytocin is produced by the hypothalamus and secreted in a pulsatile manner by the posterior pituitary gland. The fetus produces oxytocin during labor. The uterus produces oxytocin, but the contribution of endogenous oxytocin is not clear. Oxytocin receptor expression increases toward the end of pregnancy. Oxytocin increases the release of PGs, which promotes myometrial activation. The release of oxytocin is maximal during the expulsion phase (Ilicic et al., 2020; Kamel, 2010; Weiss, 2000). Intravenous oxytocin infusion is used to induce labor, but oxytocin does not seem to play a critical role in the initiation of labor (Golightly et al., 2011; Weiss, 2000). Instead, oxytocin appears to play an important part in the last stage of parturition, the expulsion of the placenta (Ilicic et al., 2020).

### *Inflammation and infection*

The fetus is a semi-allogenic "graft" that must be tolerated by the maternal immune system to ensure the continuation of pregnancy (Gomez-Lopez et al., 2014; Liu et al., 2017). In fact, extravillous trophoblasts are in direct contact with decidual stroma and maternal immune cells (Arck & Hecher, 2013). Labor is an inflammatory event (Gomez-Lopez et al., 2014; Mendelson et al., 2017), and increased expression of inflammatory factors occurs in both term and preterm labor (Kamel, 2010; Xu et al., 2016). In addition to a balance between progesterone and estrogen, a balance between proinflammatory and anti-inflammatory state is important.

Factors from both the innate and adaptive immune systems are required to maintain pregnancy (Gomez-Lopez et al., 2014). The decidua contains various

immune cells and plays an important role in maternal–fetal tolerance. The placenta and other gestational tissues express cytokines, and it has been postulated that a slight increase in cytokine expression occurs in normal-term parturition (Keelan et al., 2003). In addition to inflammation, infection is linked to parturition and pregnancy complications. Highly elevated levels of cytokines (mostly chemokines) are associated with chorioamnionitis (Keelan et al., 2003). In fact, intra-amniotic infection (chorioamnionitis) is one of the most common causes of PTB (Gomez-Lopez et al., 2022; Hallman et al., 2019; Romero et al., 2014).

### *Physical changes*

Women with uterine anomalies, polyhydramnios, and multiple pregnancies are at higher risk for PTB. It is thought that mechanical stretch induces several factors that promote myometrial contractility (Romero et al., 2006). Premature cervical ripening has been linked to multiple gestation and women with certain uterine anomalies. In multiple pregnancies, uterine distention is believed to result in contractions (Goldenberg et al., 2008). To conclude, increased uterine pressure can lead to expression of CAPs and can also trigger inflammation related pathways, resulting in labor (Hallman et al., 2019).

## 2.4 Preterm birth

Approximately 15 million babies are born prematurely worldwide every year (Barfield, 2018; Romero et al., 2014). Prematurity is the leading cause of neonatal mortality and morbidity, and after infectious diseases, the second leading cause of death among children under five years old (Blencowe et al., 2013; Frey & Klebanoff, 2016; Romero et al., 2014). In 2019, complications of prematurity were the top cause of death in children aged 0–59 months (Perin et al., 2022). PTB rates vary between countries (Blencowe et al., 2013; Ohuma et al., 2023). In Finland, preliminary data indicate that 5.6% of all births in 2022 were preterm deliveries, and the prevalence of PTBs in Finland has remained quite constant (Suomen virallinen tilasto (SVT), 2023). Globally the rate is around 11–13% (Hallman et al., 2019; Ohuma et al., 2023).

### 2.4.1 Classification

PTB can be classified based on several factors. When subgrouping by GA, the majority of PTBs take place in the late preterm period (Table 1) (Ahmed et al., 2023; Frey & Klebanoff, 2016). Additionally, subgroups of PTB can be identified by clinical presentation. SPTB is defined as spontaneous initiation of birth with or without intact fetal membranes (Blencowe et al., 2013; Goldenberg et al., 2008). Preterm prelabor rupture of the membranes (PPROM) is defined as the spontaneous rupture of the fetal membranes at $< 37^{+0}$ weeks of gestation and at least 1 h before contractions begin (Goldenberg et al., 2008). Usually, labor begins within a few days after PPROM, and one common complication related to PPROM is intrauterine infection (Goldenberg et al., 2008). Maternal, fetal, and/or placental conditions may compromise the pregnancy and lead to SPTB or a medically indicated PTB (Blencowe et al., 2013; Goldenberg et al., 2008). In medically indicated PTBs labor is induced by a provider, with or without cesarean section. Some indications for medically induced PTB are preeclampsia, placental abruption, intrauterine growth restriction, and fetal distress (Vogel et al., 2018). A large majority of PTBs are spontaneous (70%, including SPTBs with or without intact membranes), with the remainder consisting of medically indicated PTBs (Vogel et al., 2018). Although some risk factors for and causes of PTB can be identified, approximately 50% of SPTBs have no known etiology and are thus considered idiopathic (Blencowe et al., 2013; Hallman et al., 2019; Moutquin, 2003).

**Table 1. Subgroups of preterm birth based on gestational age. Modified from Frey and Klebanoff (2016).**

| Classification | Gestational age (weeks$^{+days}$) |
|---|---|
| Very early preterm birth | $< 28^{+0}$ |
| Early preterm birth | $28^{+0}$–$31^{+6}$ |
| Moderate preterm birth | $32^{+0}$–$33^{+6}$ |
| Late preterm birth | $34^{+0}$–$36^{+6}$ |

### 2.4.2 Risk factors

Numerous risk factors for PTB have been established. Table 2 summarizes certain known risk factors concerning pre-pregnancy characteristics and risk factors in the current pregnancy. Some factors listed in Table 2 can contribute to SPTB, such as previous PTB, and some may predispose to medically indicated PTB. For example,

hypertension is a risk factor for preeclampsia (Blencowe et al., 2013); thus, an overlap in risk factors between different PTB phenotypes is apparent. The risk of PTB has been associated with cervical insufficiency, which can be congenital or caused by surgery or trauma. However, differential diagnosis between cervical insufficiency and cervical shortening may be difficult (Goldenberg et al., 2008). Even changes in maternal gut microbiota have been suggested to be associated with SPTB (Hiltunen et al., 2022).

**Table 2. Some known risk factors for preterm birth (PTB).**

| Risk factor | Reference |
|---|---|
| Pre-pregnancy | |
| Previous PTB or a family history of PTBs | Parets *et al.,* 2015, Goldenberg *et al.,* 2008, Blencowe *et al.,* 2013 |
| Previous miscarriage | Frey & Klebanoff, 2016 |
| Low body mass index | Goldenberg *et al.,* 2008 |
| Young or advanced maternal age | Barfield, 2018, Vogel *et al.,* 2018 |
| Anomalies of the uterus | Frey & Klebanoff, 2016, Moutquin, 2003 |
| Previous surgical treatment of the cervix | Frey & Klebanoff, 2016 |
| Diabetes, gestational diabetes | Vogel *et al.,* 2018 |
| Hypertension | Blencowe *et al.,* 2013 |
| Current pregnancy | |
| Multiple pregnancy | Goldenberg *et al.,* 2008, Barfield, 2018, Blencowe *et al.,* 2013 |
| Short interpregnancy interval | Goldenberg *et al.,* 2008, Barfield, 2018, Vogel *et al.,* 2018 |
| Stress and depression | Goldenberg *et al.,* 2008, Barfield, 2018 |
| Smoking, substance abuse | Goldenberg *et al.,* 2008, Barfield, 2018, Blencowe *et al.,* 2013 |
| Maternal infections (e.g., urinary tract infections, bacterial vaginosis, syphilis) | Goldenberg *et al.,* 2008, Romero *et al.,* 2014, Blencowe *et al.,* 2013 |
| Infertility treatments | Barfield, 2018, Vogel *et al.,* 2018 |
| Vaginal bleeding | Goldenberg *et al.,* 2008 |
| Short cervical length | Frey & Klebanoff, 2016, Goldenberg *et al.,* 2008 |
| Polyhydramnios, oligohydramnios | Frey & Klebanoff, 2016, Barfield, 2018 |

### *2.4.3 Genetics and preterm birth*

PTB is a complex phenotype that is affected by multiple environmental and genetic factors and their interactions. PTBs tend to aggregate in families; thus, genetics has been suggested to play a role in SPTB, accounting for 25%–40% of the variation

in gestational length (Bezold et al., 2013; Pasanen et al., 2023; Solé-Navais et al., 2023). Both maternal and fetal genomes contribute to the risk of SPTB (Hallman et al., 2019; Zhang et al., 2017). For example, hypothesis-free genome-wide association study (GWAS) analyses and whole exome sequencing (WES) have identified plausible candidate genes and genetic variants that affect gestational duration and could predispose to SPTB (Hallman et al., 2019; Huusko et al., 2018).

GWAS analyses reveal plausible associations between single-nucleotide polymorphisms and complex human phenotypes, like SPTB. For example, a GWAS of maternal genomes of European ancestry revealed several candidate genes (*EEFSEC*, *EBF1*, and *AGTR2*) associated with both gestational length and PTB (Jain et al., 2022; Zhang et al., 2017). Tiensuu et al. (2019) found a variant of *SLIT2* in a fetal GWAS to be associated with length of pregnancy and SPTB. Several loci associated with gestational duration or SPTB (including *ZBTB38*, *HAND2*, *TET3*, and *KCNAB1*) were identified in a recent GWAS meta-analysis by Pasanen et al. (2023). Another GWAS meta-analysis reported various loci linked to gestational duration and birth weight (Solé-Navais et al., 2023). However, most GWAS findings still require verification (Jain et al., 2022), and the heterogenous populations used in GWASs cause population stratification and should be acknowledged in the analyses (Hallman et al., 2019).

While GWAS accounts for the whole genome, WES includes genes that encode proteins (the exome). The exome contains the majority (~85 %) of known disease-related genetic variants, although it represents only 1–2% of the whole genome (Seaby et al., 2016; Strauss et al., 2018). Strauss et al. (2018) summarized that WES is based heavily on the assumption that disease-causing common and rare genetic variants of the exome make a substantial contribution to diseases. Only a few studies have explored WES to identify possible genetic variants in SPTB. McElroy et al. (2013) identified several genes in the complement and coagulation factor pathway, such as *CR1*. They found an association between three variants of *CR1* and SPTB (McElroy et al., 2013). Huusko et al. (2018) linked glucocorticoid receptor signaling pathways and variants of a heat shock protein (HSP), *HSPA1L*, with recurrent SPTBs. Data mining of HSPs and nuclear receptor genes from several datasets, including WES, revealed several possibly damaging gene variants in association with SPTB (Huusko et al., 2021). For example, variants of *HSPA5*, *HSPA1L*, *PGR*, and *AR* were predicted to be possibly damaging.

### 2.4.4 Transcriptomics and preterm birth

Gene expression can be studied using transcriptomics and RNA sequencing (Paquette et al., 2023). Indeed, transcriptomes of several reproductive tissues (e.g., cervical tissue, myometrium, placental tissue) have been studied in relation to PTB (Pique-Regi et al., 2019). Recently, Paquette et al. (2023) characterized over 1700 SPTB-related genes, and Couture et al. (2023) found 331 SPTB-related genes in their transcriptomic analysis of the placenta. Genes in immune or inflammatory pathways were particularly likely to be upregulated in PTB. RNA sequencing of the placental membranes revealed 270 differentially expressed genes associated with SPTB when compared to term controls (Pereyra et al., 2019). Most upregulated genes (e.g., *IL1B*) were involved in immune response. A study focused on HSPs and nuclear receptors in relation to SPTB found several HSP and nuclear receptor genes (e.g., *HSPA1*, *HSPD1*, and *NR6A1*) from the placental transcriptomics associated with prematurity (Huusko et al., 2021). Single-cell transcriptomic analysis showed that extravillous trophoblasts and cytotrophoblasts had the largest number of differentially expressed genes between placentas from preterm and term labors (Pique-Regi et al., 2019). However, a major limitation in several transcriptomic studies is the potential overlapping of genes responsible for PTB and GA, as those genes may reflect both differences related to pathological events of preterm labor and maturation of the placenta (Pereyra et al., 2019).

### 2.4.5 Proteomics and preterm birth

Human placental proteomics have focused on term placentas (Heywood et al., 2017; Mushahary et al., 2013) and pregnancy pathologies such as preeclampsia (Law et al., 2015). Proteomes of normal first-trimester and term placentas revealed different protein expressions. For example, expression of 78 kDa glucose-regulated protein (GRP78) increased (Gharesi-Fard et al., 2015; Khorami Sarvestani et al., 2021) whereas expression of alpha-1 antitrypsin (AAT) decreased (Gharesi-Fard et al., 2015) when comparing first-trimester placentas to term placentas. Haapalainen et al. (2018) studied placental proteome differences between spontaneous and elective uncomplicated term deliveries and identified 10 differentially expressed proteins associating with spontaneous delivery. They found that decreased level of CPPED1 could contribute to spontaneous term delivery. Proteomics of the placental membranes revealed 11 proteins expressed uniquely in preterm or term placentas (Butt et al., 2006). These proteins belonged to cytoskeletal components (e.g.,

vimentin), endoplasmic reticulum (ER) luminal proteins (e.g., GRP78), and proteins with anticoagulant properties (e.g., annexin A4) (Table 3). Another proteomic study identified several proteins from choriodecidual tissue (Shankar et al., 2010); those authors studied placental proteomes of spontaneous preterm and term labors, with placental samples from elective preterm and term labors were used as controls. Proteins associated with GA and labor were identified. For example, levels of annexin A3 and annexin A5 (ANXA5) were increased only in samples from spontaneous preterm labor compared to samples from cesarean sections (GA < 35 weeks) (Shankar et al., 2010).

In addition to proteomes from the placenta, proteomes from other tissues have been determined when focusing on SPTB. Over 60 proteins have been identified as associated with SPTB (Kacerovsky et al., 2014). D'Silva et al. (2018) found altered protein expression of nine phosphorylated proteins and 11 glycosylated proteins in first trimester serum to be associated with SPTB. Altered protein expression of 25 proteins (e.g., IL-6, IL-7, and HSPA8) in serum from the second trimester (GA of 16–17 weeks) was associated with subsequent preterm delivery (Gunko et al., 2016). Over 50 differentially expressed proteins in PTB were identified in the cervicovaginal fluid (CVF), including inflammation-associated proteins, serine-type endopeptidase inhibitors, and cytoskeletal proteins (Kim et al., 2021). The top proteomic findings of selected proteomic studies are listed in Table 3. In sum, protein candidates have been sought from several biological tissues, but the results have been inconsistent.

**Table 3. Proteomics of different tissues in spontaneous preterm births (SPTBs); selected proteomic findings from each study are displayed in the table.**

| Protein | ID | Tissue/sample | GA at sampling | SPTB vs. control | Study |
|---|---|---|---|---|---|
| Vitamin D binding protein (phosphorylated) | VDBP | Maternal serum | From 11 to 13 weeks | ↓ in SPTB | D'Silva et al., 2018 |
| Vitamin D binding protein (glycosylated) | VDBP | Maternal serum | From 11 to 13 weeks | ↑ in SPTB | D'Silva et al., 2018 |
| Apolipoprotein A1 (phosphorylated and glycosylated) | APOA1 | Maternal serum | From 11 to 13 weeks | ↑ in SPTB | D'Silva et al., 2018 |
| Gelsolin | GSN | Maternal serum | First trimester | ↓ in SPTB | Beernink et al., 2023 |
| Fibulin 1 | FBLN1 | Maternal serum | First trimester | ↓ in SPTB | Beernink et al., 2023 |
| C-reactive protein | CRP | Maternal serum | First trimester | ↑ in SPTB | Beernink et al., 2023 |
| Complement C5 | CO5 | Maternal serum | First trimester | ↑ in SPTB | Beernink et al., 2023 |
| Inter-alpha-trypsin inhibitor heavy chain 4 (three peptides) | ITIH4 | Maternal serum | At 24 and 28 weeks | ↓ in SPTB | Esplin et al., 2011 |
| Insulin-like growth factor-binding protein 4 | IBP4 | Maternal serum | From 19 to 21 weeks | ↑ in SPTB | Saade et al., 2016 |
| Sex hormone-binding globulin | SHBG | Maternal serum | From 19 to 21 weeks | ↓ in SPTB | Saade et al., 2016 |
| Annexin A4 | ANXA4 | Placental membranes | Immediately after delivery | Absent in SPTB | Butt et al., 2006 |
| 78 kDa glucose-regulated protein | GRP78 | Placental membranes | Immediately after delivery | Absent in STB | Butt et al., 2006 |
| Vimentin | VIM | Placental membranes | Immediately after delivery | Absent in STB | Butt et al., 2006 |
| Annexin A5 | ANXA5 | Choriodecidua | Immediately after delivery | ↑in SPTB | Shankar et al., 2010 |
| Annexin A3 | ANXA3 | Choriodecidua | Immediately after delivery | ↑in SPTB | Shankar et al., 2010 |
| Galectin 1 | GAL1 | Choriodecidua | Immediately after delivery | ↑in SPTB | Shankar et al., 2010 |
| Protein disulfide isomerase | PDI | Choriodecidua | Immediately after delivery | ↑in SPTB | Shankar et al., 2010 |
| Alpha-1 antitrypsin precursor | AAT | CVF | From 15.8 to 35.9 weeks | ↑ in SPTB | Pereira et al., 2007 |
| Epidermal fatty acid-binding protein | FABP | CVF | From 15.8 to 35.9 weeks | ↓ in SPTB | Pereira et al., 2007 |

CVF, cervicovaginal fluid; GA, gestational age.

### 2.4.6 Health consequences of preterm birth

Prematurity predisposes infants to several short-term complications and lifelong sequelae largely due to the immaturity of several organs (Barfield, 2018). For example, preterm infants are at higher risk for neonatal respiratory complications (e.g., respiratory distress syndrome, bronchopulmonary dysplasia), necrotizing enterocolitis, visual and hearing impairments, and neurological complications like intraventricular hemorrhage, cerebral palsy, and hypoxic ischemic encephalopathy (Vogel et al., 2018). Premature babies are at higher risk for severe viral and bacterial infections like sepsis (Vogel et al., 2018). Nosocomial respiratory virus infections have been associated with increased risk of morbidity and prolonged hospitalization (Luoto et al., 2016). Some respiratory symptoms may progress, and in adults the risk of cardiopulmonary diseases (e.g., chronic lung disease, asthma, COPD, hypertension) is increased (Blencowe et al., 2013; Pulakka et al., 2023). Additionally, increased risk of neurodevelopmental disorders (e.g., motor problems, learning impairments) have been reported (Blencowe et al., 2013). In fact, the risk of multimorbidity increases in those born prematurely (Heikkilä et al., 2023). Preterm infants with very low birth weight are at higher risk for impaired glucose metabolism, such as impaired glucose tolerance (Kaseva et al., 2023). SPTB has been proposed to have an influence on the neonatal gut microbiome, which may contribute to, for example, poor postnatal growth (Hiltunen et al., 2022).

## 2.5 Prediction and prevention of preterm birth

Accurate prediction of PTB remains a challenge, as up to 85% of patients admitted to hospital due to imminent SPTB do not deliver within the subsequent seven days (Melchor et al., 2018). This can lead to unnecessary hospital admissions and potentially harmful treatments (Melchor et al., 2018; Nikolova et al., 2018). As stated above, the etiology of SPTB is heterogenous, and the pathophysiology behind SPTB is not fully understood. Thus, the predictive and preventive methods available may not apply to all women at risk for SPTB. A better understanding of the pathophysiology of SPTB can lead to new potential markers of subsequent SPTB. Moreover, this could help clinicians make more accurate diagnoses of imminent SPTB, which could also prevent unnecessary hospitalization and treatments.

### 2.5.1 Predictive tests for preterm birth

A few predictive tests are available for clinical use, including cervical length measurement and several biochemical tests (Melchor et al., 2018). As labor approaches, the cervix starts to shorten and soften. Cervical length can be measured by transvaginal ultrasound, and short cervical length may predict SPTB (Son & Miller, 2017). However, the ultrasound assessment should preferably be done between 16 and 24 weeks of pregnancy, and the criterion defining short cervical lengths varies from 15 to 30 mm (Glover & Manuck, 2018; Son & Miller, 2017). In addition, the Bishop score is used to assess the maturity of the cervix and the risk of imminent PTB, although it was not developed to predict the risk of preterm delivery but to evaluate the inducibility of the cervix at term (Newman et al., 2008).

To date, the most commonly used biomarkers to predict imminent PTB are fetal fibronectin (fFN), phosphorylated insulin-like growth factor-binding protein 1 (phIGFBP-1), and placental alpha macroglobulin (PAMG-1). A glycoprotein in the ECM (Son & Miller, 2017), fFN can be measured from the CVF due to choriodecidual disruption and leakage of fFN into the CVF (Goldenberg et al., 2008). Both qualitative and quantitative tests are available (Glover & Manuck, 2018; Suff et al., 2019). As to phIGFBP-1, it is synthetized in the decidualized endometrium during pregnancy (Conde-Agudelo & Romero, 2016). It is thought that uterine contractions can lead to the secretion of phIGFBP-1 into cervical secretions, which explains its use as a predictive marker of SPTB. PAMG-1 is a glycoprotein synthetized by the decidua (Nikolova et al., 2018). Amniotic fluid contains high concentration of PAMG-1, whereas concentrations are low in cervicovaginal secretions. In the case of preterm labor, it has been suggested that the amount of PAMG-1 increases in the CVF due to uterine contractions and/or degradation of the ECM of fetal membranes, which enables PAMG-1 molecules to permeate vaginal secretions (Nikolova et al., 2018). All three tests have been studied extensively, but the results are inconsistent (Melchor et al., 2018). To summarize, the above tests are better at excluding imminent SPTB within seven days of testing, although the ranges in negative predictive values also vary (fFN 73.2–100%, phIGFBP-1 61.8–98.4%, PAMG-1 93.0–100%) (Melchor et al., 2018).

Other potential markers to predict the risk of PTB have been proposed. Upregulation of insulin-like growth factor-binding protein 4 (IBP4) and downregulation of sex hormone-binding globulin (SHBG) in maternal serum at 19–20 weeks of pregnancy could contribute to SPTB (Saade et al., 2016). A widely used biomarker of inflammation, C-reactive protein (CRP), has been suggested as

a biomarker for several pregnancy complications, including preeclampsia, intrauterine infections, fetal growth restriction, and PTB (Beernink et al., 2023; Sorokin et al., 2010). Elevated levels of maternal serum interleukin 6 (IL-6) and CRP have been suggested to be associated with preterm delivery and neonatal intraventricular hemorrhage (Sorokin et al., 2010). A recent study proposed that increased levels of IL-6 and CRP in amniotic fluid, maternal blood, and umbilical cord blood indicate a generalized inflammation rather than a specific physiological or pathological process (Menon & Taylor, 2019). Thus, it appears that serum CRP is not a useful marker to predict the risk of PTB, at least on its own.

### *2.5.2 Prevention of preterm birth*

Prevention of PTB can be subdivided into two preventive strategies: primary and secondary (Ahmed et al., 2023). The aim of primary prevention is to reduce or even eliminate risk factors prior to and during pregnancy (Ahmed et al., 2023). As stated above, risk factors for PTB can be identified with a thorough medical and obstetric history. Interventions in primary prevention include screening for and treatment of lower genital tract infections, promoting cessation of smoking, and optimizing interpregnancy intervals (Ahmed et al., 2023; Daskalakis et al., 2019).

Whereas primary prevention can usually be applied to the general population, secondary prevention aims to detect those at risk of SPTB, including interventions to delay preterm labor (Ahmed et al., 2023). Transvaginal ultrasound of the cervix can be used to diagnose cervical shortening. Cervical cerclage (usually a stitch around the cervix with a vaginal approach) offers mechanical support to the cervix, whereas cervical pessary changes the position of the cervix, leading to decreased pressure (Wennerholm et al., 2023). Both cervical cerclage and pessary can be applied in cases of cervical shortening or cervical insufficiency (da Fonseca et al., 2020; Daskalakis et al., 2019).

A few medicinal interventions to delay SPTB are also available. Administration of oral, vaginal, or intramuscular progesterone may inhibit uterine contractions and cervical ripening (da Fonseca et al., 2020; Wennerholm et al., 2023). Vaginal progesterone was suggested as a treatment of choice in singleton pregnancies at high risk of SPTB (Care et al., 2022). However, there is no consensus as to whether progesterone should be offered to women with a history of PTBs and normal cervical length or to asymptomatic women with cervical length of 25 to 30 mm (Wennerholm et al., 2023). Tocolytics (usually nifedipine, a calcium channel blocker, and atosiban, an oxytocin receptor antagonist) can be given to the

mother to suppress contractions (Ahmed et al., 2023; Preterm birth: Current Care Guidelines, 2018). Tocolytics do not significantly delay labor, but they do allow the administration of other medications, such as corticosteroids, that improve the prognosis of preterm neonates (Ahmed et al., 2023; Daskalakis et al., 2019). In conclusion, the prediction and prevention of SPTB are both challenging, as the etiology of SPTB is multifactorial, and treatments available to delay the delivery apply only in certain situations.

# 3 Aims of the study

The understanding of pathogenesis of SPTB is still incomplete. This study was set out to identify potential SPTB-associated protein candidates and to study their role in SPTB. The aims of this doctoral dissertation are as follows:

1. To determine placental protein candidates of SPTB by exploring proteomes from SPTB, elective preterm birth (EPTB), and spontaneous term birth (STB) placentas.
2. To establish the role of the protein candidates AAT and HSPA5 in SPTB and the location of the protein in the placenta.
3. To examine maternal serum AAT and CRP levels in early pregnancy and before delivery and compare levels between preterm and term deliveries.

# 4 Materials and methods

## 4.1 Ethical approval

Experimentation on samples from human tissues and processing of patient information was required for this doctoral thesis. Written informed consent was obtained from all study participants or their guardian(s). The collection and use of human tissue samples and the processing of patient information were approved by the regional medical research ethics committee of the Wellbeing Services County of North Ostrobothnia (79/2003, 14/2010, and 73/2013; amendments). The use of serum samples from the Finnish Maternity Cohort (FMC) was approved by the Biobank Borealis scientific committee (BB22-0093). The study was carried out in accordance with the Declaration of Helsinki.

## 4.2 Placental samples (studies I and II)

Placental samples were collected at Oulu University Hospital between 2010 and 2016. The samples were taken from the basal and chorionic plates of the placentas. Collection of the tissue biopsies took place immediately after delivery of the placenta, and biopsies (one biopsy per placenta) were taken approximately 2 cm from the umbilical cord insertion. A total of four groups of placental samples were collected: SPTBs, EPTBs, elective term births, and STBs. Of these groups, placental samples from SPTBs, EPTBs, and STBs were used in this thesis in studies I and II. The pregnancies' clinical characteristics are presented in Table 4. In addition to the placental proteomics, placental samples from SPTBs and STBs were used also in other experiments with a larger sample size. Samples from EPTBs were used only for proteomics ($n$ = 6). GA was based on ultrasound examination before 16 weeks of pregnancy at a local maternity unit (Haapalainen et al., 2018).

Inclusion criterion of GA for PTB samples ranged from $25^{+0}$ to $36^{+6}$ weeks, whereas the criterion for term samples was from $38^{+0}$ to $41^{+6}$ weeks. EPTB was defined as a delivery with no signs of labor. Additionally, delivery was not initiated by medication, Foley balloon, and/or amniotomy, and the baby was delivered by caesarean section. Some exclusion criteria were applied to preterm and term births (Tiensuu et al., 2019). In SPTBs, pregnancies with certain known risk factors (multiple pregnancy, intrauterine growth restriction, placental abruption, polyhydramnios, and fetal anomalies) were excluded. Preeclampsia and

intrauterine growth restriction affected some EPTBs. Likewise, following exclusion criteria applied to STBs: pregnancies with multiple gestation, intrauterine growth restriction, placental abruption, polyhydramnios, fetal anomalies, or requirements for special care of the newborn (Tiensuu et al., 2019).

**Table 4. Placentas from spontaneous preterm births (SPTBs), elective preterm births (EPTBs), and spontaneous term births (STBs) were used in this thesis. Clinical characteristics of the pregnancies are presented in the table.**

| Variable | SPTB<br>*n* = 22 | EPTB<br>*n* = 6[1] | STB<br>*n* = 24 |
|---|---|---|---|
| Gestational age | | | |
| In days, median (range) | 223 (177–252) | 197 (178–212) | 283 (272–293) |
| In weeks, median (range) | 31.9 (25.3–36.0) | 28.1 (25.4–30.3) | 40.4 (38.9–41.9) |
| Delivery type | | | |
| Vaginal delivery, *n* (%) | 14 (63.6) | 0 (0.0) | 24 (100.0) |
| Caesarean section, *n* (%) | 8 (36.4) | 6 (100.0) | 0 (0.0) |
| PPROM | | | |
| Yes, *n* (%) | 6 (27.3) | - | - |
| No, *n* (%) | 13 (59.1) | - | - |
| NA, *n* (%) | 3 (13.6) | - | - |
| Preeclampsia | | | |
| Yes, *n* (%) | 0 (0.0) | 5 (83.3) | 0 (0.0) |
| No, *n* (%) | 20 (90.9) | 1 (16.7) | 22 (91.7) |
| NA, *n* (%) | 2 (9.1) | 0 (0.0) | 2 (8.3) |
| Clinical chorioamnionitis | | | |
| Yes, *n* (%) | 5 (22.7) | 1 (16.7) | 0 (0.0) |
| No, *n* (%) | 9 (40.9) | 5 (83.3) | 15 (62.5) |
| NA, *n* (%) | 8 (36.4) | 0 (0.0) | 9 (37.5) |
| Birth weight, median (range), g | 2085 (820–3205) | 950 (520–1135) | 3860 (2850–4610) |

EPTB, elective preterm birth; NA, not available; SPTB, spontaneous preterm birth; STB, spontaneous term birth; PPROM, preterm premature rupture of the membranes.

[1]Samples used only in the proteomics.

## 4.3 Placental proteomics (study I)

Placental proteomics was performed, as it is a hypothesis-free method to obtain protein candidates. Placental proteomes were compared to discover proteins associated with short GA (SPTB vs. STB) and spontaneity of labor (SPTB vs. EPTB). The proteomic analysis was performed at Proteomics and Mass Spectrometry Core Facilities, Biocenter Oulu. The samples included in the analysis

were collected in 2010–2012. Proteomes of placental samples from SPTBs ($n$ = 6), EPTBs ($n$ = 6) and STBs ($n$ = 6) were determined. Clinical characteristics of the pregnancies concerning the proteomic samples are detailed in Table 5.

**Table 5. Clinical characteristics of pregnancies. Placentas from spontaneous preterm births (SPTBs), elective preterm births (EPTBs), and spontaneous term births (STBs) were selected for proteomics from the collected placental samples (Table 4).**

| Variable | SPTB | EPTB | STB |
|---|---|---|---|
| | $n$ = 6 | $n$ = 6 | $n$ = 6 |
| Gestational age | | | |
| In days, median (range) | 222 (183–249) | 197 (178–212) | 281 (276–290) |
| In weeks, median (range) | 31.7 (26.1–35.6) | 28.1 (25.4–30.3) | 40.1 (39.4–41.4) |
| Delivery type | | | |
| Vaginal delivery, *n* (%) | 6 (100.0) | 0 (0.0) | 6 (100.0) |
| Caesarean section, *n* (%) | 0 (0.0) | 6 (100.0) | 0 (0.0) |
| PPROM | | | |
| Yes, *n* (%) | 2 (33.3) | - | - |
| No, *n* (%) | 3 (50.0) | - | - |
| NA, *n* (%) | 1 (16.7) | - | - |
| Preeclampsia | | | |
| Yes, *n* (%) | 0 (0.0) | 5 (83.3) | 0 (0.0) |
| No, *n* (%) | 6 (100.0) | 1 (16.7) | 6 (100.0) |
| Clinical chorioamnionitis | | | |
| Yes, *n* (%) | 0 (0.0) | 1 (16.7) | 0 (0.0) |
| No, *n* (%) | 6 (100.0) | 5 (83.3) | 6 (100.0) |
| Birth weight, median (range), g | 1848 (870–2828) | 950 (520–1135) | 3536 (3060–4220) |

PPROM, preterm premature rupture of the membranes; NA, not available.

The proteomes were determined using two-dimensional minimal-difference gel electrophoresis and mass spectrometry. The methods for both have been detailed elsewhere (Haapalainen et al., 2018). Briefly, protein samples were labelled with fluorescent dyes, and the fluorescence signals were detected and analyzed with computer software, after which the peptide masses of both labeled and unlabeled protein spots were measured. The proteins were identified according to their spot-specific peptide mass. In the proteomic analyses, the difference between mean normalized protein spot volumes was considered significant with a fold change (FC) of at least ± 1.5 (i.e., increased or decreased levels of proteins in SPTB) and a $p$-value of $< 0.05$ using Student's $t$-test.

Proteomic results of AAT and HSPA5 were verified by western blot using placental samples from SPTBs and STBs and a larger sample size. All samples were

normalized against the reference protein tubulin α-1B. Appropriate monoclonal antibodies were applied to detect the proteins. Quantitative western blot results were analyzed using normalized protein expression ratios. Statistical analyses were performed with the SPSS Statistics (IBM Corporation).

## 4.4 Whole exome sequencing (study I)

WES data were applied to filter the protein candidates and discover possible damaging genetic variants that may affect the function of the encoded protein. The WES data consisted of Northern Finnish mothers ($n$ = 17) who gave birth preterm (Huusko et al., 2018), Northern Finnish children ($n$ = 23) who were born preterm (Huusko et al., 2021) and Danish WES of mothers of European ancestry ($n$ = 192) who gave birth preterm (Huusko et al., 2018). Corresponding genes of the protein findings from the placental proteomics were compared to the WES data. The WES protocol has previously been detailed (Huusko et al., 2018, 2021). The selected variants met the following criteria:

1. The gene variant is potentially pathogenic (Huusko et al., 2021).
2. The gene variant is rare (minor allele frequency < 1%) or common (minor allele frequency 1% ≤ 10%).
3. The gene variant is found in at least two different affected families.

## 4.5 Quantitative PCR (studies I and II)

Placental samples from basal and chorionic plates were collected as described above. Isolation of the placental RNA has previously been detailed (Karjalainen et al., 2015). Briefly, total placental RNA was isolated with the RNeasy Micro Kit (Qiagen). NanoDrop spectrophotometer (Thermo Fisher Scientific) was used to measure the quality and concentration of isolated RNA at absorbance values of 230 nm, 260 nm, and 280 nm (Becker et al., 2010).

Quantitative PCR (qPCR) was used to determine relative placental messenger RNA (mRNA) level changes of selected genes between SPTB and STB placentas, and qPCR was used with samples from the basal plate. The isolated RNA was reverse transcribed to complementary DNA (cDNA) with the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics) according to the manufacturer's instructions.

Primers and probes were designed using the Assay Design Center (Roche Diagnostics). Primer sequences and Universal Probe Library probe numbers are listed in Table 6. Primers were designed as intron spanning assays, if possible, to minimize the amplification of genomic DNA contaminations during PCR reactions. The cDNA samples were diluted with RNase-free $H_2O$. FastStart Essential DNA Probes Master (Roche Diagnostics) was used in the PCR reaction mixtures. Each measurement was carried out in triplicate, and qPCR was performed with the LightCycler96 instrument (Roche Diagnostics).

**Table 6. Primers and probes used in *SERPINA1* and *HSPA5* qPCR experiments.**

| Gene | Forward primer | Reverse primer | UPL[1] probe |
|---|---|---|---|
| *SERPINA1* | 5′ -GTGGTTTCTGAGCCAGCAG-3′ | 5′ -CCCTGTCCTCGTCCGTATT-3′ | #86 |
| *HSPA5* | 5′ -TGTTACAATCAAGGTCTATGAAGGTG-3′ | 5′ -CAAAGGTGACTTCAATCTGTGG-3′ | #68 |
| *CYC1* | 5′ -ATAAAGCGGCACAAGTGGTCA-3′ | 5′ - GATGGCTCTTGGGCTTGAGG-3′ | #47 |

[1]Universal Probe Library

To evaluate changes in mRNA levels of the selected genes between different groups, the qPCR data were analyzed with the LightCycler96 1.1 software, and mRNA levels were normalized against a reference gene, *cytochrome c1* (*CYC1*) (Karjalainen et al., 2015). The ΔΔ cycle threshold method was applied to calculate the normalized expression ratios. The SPSS software package was used to evaluate statistical differences between SPTB and STB samples using a nonparametric Mann–Whitney *U* test.

## 4.6 Protein localization in placental samples (studies I and II)

### *4.6.1 Immunohistochemical staining (studies I and II)*

To study the location of AAT and HSPA5 in the human placenta, immunohistochemical staining was performed. Paraffin-embedded placental samples from the basal plate from SPTBs and STBs were cut with a microtome into thin slices of 4 μm that were then mounted on a glass slide. Tris-EDTA buffer was used in antigen retrieval to enable the antibodies to access the target protein (AAT or HSPA5) within the placental tissue. Endogenous peroxidase activity was blocked

in blocking solution (Agilent) to prevent non-specific binding of the antibodies. The samples were incubated with the primary antibodies: rabbit anti-human AAT antibody (1:1000 dilution, PA5-16661, Invitrogen) or rabbit anti-human HSPA5 antibody (1:4000 dilution, 3177, Cell Signaling Technology). Non-immune rabbit IgG was used for the negative controls. Detection of bound antibodies was performed with the Envision Kit (Agilent).

### 4.6.2 Immunoelectron microscopy (studies I and II)

To evaluate the extra- and intracellular locations of AAT and HSPA5, placental samples were studied with immunoelectron microscopy (immuno-EM), as was fibronectin. Immuno-EM was performed at the Biocenter Oulu Electron Microscopy Core Facility. The samples used in AAT and fibronectin immuno-EM were from STBs. For HSPA5 immuno-EM, the samples were from SPTBs and STBs.

Fresh human placenta samples were fixed in paraformaldehyde and then frozen in liquid nitrogen. Thin cryosections were cut with a cryomicrotome. The sections were then exposed to the primary antibodies (studies I and II). Bound antibodies were labeled by incubation with protein A-conjugated 10 nm gold particles. In HSPA5 immunostaining, endogenous immunoglobulins were blocked using Fab fragments to reduce background labelling (study II). The immunostained sections were examined with a Tecnai G2 Spirit 120 kV transmission electron microscope and the images were captured by a Quemesa CCD camera (Olympus Soft Imaging Solutions GMBH).

### 4.6.3 Fluorescence colocalization analysis (study I)

To identify the type of vesicle that contained AAT, fluorescence colocalization analysis was performed with AAT and the endosome- and exosome-specific antibodies Rab-7 and CD63, respectively. Additionally, localization between AAT and placental fibrinoid proteins (fibronectin, vitronectin, and collagen IV) was evaluated with colocalization analysis. The procedure of fluorescence colocalization analysis has previously been detailed (Haapalainen et al., 2021); the analysis was carried out on human placental samples. Antibody complexes analyzed were as follows: AAT-fibronectin, AAT-vitronectin, AAT-collagen IV, AAT-Rab7, and AAT-CD63. The antibodies used in the colocalization analysis are described in study I. Primary and secondary antibodies were used to detect the

protein. Negative controls were prepared in the same manner with primary antibodies omitted. A laser scanning confocal microscope was used to observe the samples, and the images were captured with LAS X software.

### 4.7 Gene silencing by small interfering RNAs (studies I and II)

To examine the effect of *SERPINA1* and *HSPA5* silencing on the transcriptome of placenta-derived cells, gene silencing with small interfering RNAs (siRNAs) in a placental cell line was performed. The HTR8/SVneo human trophoblast cells (CRL-3271, ATCC) were cultured under standard culturing conditions in RPMI-1640 growth medium (Thermo Fisher Scientific) supplemented with fetal bovine serum (Sigma-Aldrich) and penicillin/streptomycin (Sigma-Aldrich). The transfection method was similar for both *HSPA5*- and *SERPINA1*-silencing experiments. Reverse and forward transfections were performed with siRNAs targeting *SERPINA1* (sense GUCCAUUACUGGAACCUAU, antisense AUAGGUUCCAGUAAUGGAC) or *HSPA5* (sense GAUAAUCAACCAACUGUUA, antisense UAACAGUUGGUUGAUUAUC). MISSION siRNA Universal Negative Control #1 (Sigma-Aldrich) was transfected in the same way as siRNAs targeting *SERPINA1* or *HSPA5*. Lipofectamine 3000 (Invitrogen) was used as a transfection reagent. Appropriate siRNA concentrations were tested. Cells (100,000/well) were incubated in appropriate siRNA concentrations in both reverse and forward transfection and were harvested with trypsin/EDTA (Sigma-Aldrich).

To determine the transcriptomes of the *SERPINA1*- and *HSPA5*-silenced cells and negative control cells, RNA was isolated with the RNeasy Micro Kit (Qiagen) as described above, and the quality of isolated RNA was determined with an Agilent 2100 Bioanalyzer system at the Biocenter Oulu Sequencing Center. After a quality check, the cells (silenced cells $n = 3$, control cells $n = 3$) were sent to the Finnish Functional Genomics Centre, Turku, Finland, for RNA sequencing. The sequencing data were analyzed by the Bioinformatics Unit Core Service at the Turku Centre for Biotechnology. Student's *t*-test was used to assess statistical significance.

RNA sequencing results were verified using qPCR. *ACTG1*, *CEACAM1*, *FN1*, and *SLIT2* were chosen from the *SERPINA1*-silencing data. From the *HSPA5*-silecing results, *AP2A1*, *TNFRSF9*, *HSP90B1*, *CXCL8*, and *CCL2* were chosen. Verification was performed with a larger number of specimens (silenced cells $n = 6$, control cells $n = 6$). Primers and probes of the selected genes can be found in the

studies I and II; qPCR was performed in the same manner as described above, and a *p*-value was calculated with a nonparametric Mann–Whitney *U* test.

## 4.8 Maternal serum samples (study III)

### *4.8.1 Study populations*

Study III involved two populations; only singleton pregnancies were included. The first population (study population 1) comprised women who gave birth preterm (SPTB group) or term (control group). Enrollment of participants took place between 1998 and 2014 at Oulu University Hospital or at Tampere University Hospital, as has been described in detail elsewhere (Karjalainen et al., 2015; Tiensuu et al., 2019). As stated in Karjalainen et al. (2015), SPTB was defined as GA ≤ $36^{+0}$ weeks of pregnancy, and also cases with preterm premature rupture of the membranes (PPROMs) were included. Exclusion criteria were: multiple gestation, polyhydramnios, acute septic infection of the mother and/or evidence of systemic inflammatory response, diseases of the mother that may affect timing of the onset of delivery, alcohol or narcotic use, severe accidents, and fetal congenital anomalies (detailed in Karjalainen et al. 2015). In term births, GA for term birth ranged from $38^{+0}$ to $42^{+0}$ weeks. Pregnancies with any pregnancy- or labor-associated complication were excluded as same exclusion criteria applied as in placental samples (Karjalainen et al., 2015). Corresponding serum samples of participants were obtained from the FMC serum collection (Biobank Borealis of Northern Finland, Oulu University Hospital, Finland) (Lehtinen et al., 2017). A total of 529 serum samples collected during pregnancy were used in this study. GA thresholds were as follows: first trimester < $13^{+0}$ weeks and $13^{+0}$ to $26^{+6}$ weeks for the second trimester. Preterm cases included births with spontaneous onset. Term births had both spontaneous and medically indicated labors. Additional clinical information was collected retrospectively from birth diaries and is presented in Table 7.

**Table 7. Clinical characteristics of study population 1 (*n* = 529). Modified from study III.**

| Variable | SPTB | Term birth | *p*-value[1] |
|---|---|---|---|
| *n* | 131 (24.8) | 398 (75.2) | |
| GA at sampling < 13+0 | 101 (23.1) | 337 (76.9) | |
| GA at sampling 13+0 – 26+6 | 30 (33.0) | 61 (67.0) | |
| GA at sampling (all samples), weeks | 10.6 (9.43–12.6) | 10.9 (10.1–11.9) | 0.126 |

| Variable | SPTB | Term birth | *p*-value[1] |
|---|---|---|---|
| GA < 13+0, weeks | 10.1 (9.2–10.9) | 10.7 (10.0–11.3) | < 0.001 |
| GA 13+0–26+6, weeks | 15.0 (13.5–16.9) | 14.4 (13.6–15.9) | 0.380 |
| Parity | | | < 0.001 |
| Nullipara and primipara | 89 (70.1) | 142 (40.1) | |
| Multipara | 38 (29.9) | 212 (59.9) | |
| History of preterm births | 16 (13.6) | 2 (0.6) | < 0.001 |
| History of miscarriage | 28 (23.3) | 95 (28.9) | 0.282 |
| Interpregnancy interval < 2 years | 25 (22.5) | 152 (45.9) | < 0.001 |
| Primary diseases[2] | 17 (13.0) | 42 (10.6) | 0.522 |
| Pregnancy | | | |
| *In vitro* fertilization or treated with hormones | 9 (7.3) | 6 (1.7) | 0.005 |
| Smoking during pregnancy | 14 (17.7) | 21 (6.7) | 0.004 |
| BMI before pregnancy, kg/m$^2$ | 23.3 (20.4–27.0) | 22.8 (20.8–25.8) | 0.714 |
| Age of the mother at collection, years | 28.9 (26.5–32.9) | 30.4 (26.7–34.8) | 0.005 |
| Delivery | | | |
| GA at birth, weeks | 30.6 (28.6–33.6) | 40.1 (39.3–40.9) | < 0.001 |
| Onset of delivery | | | < 0.001 |
| Spontaneous | 131 (100.0) | 322 (81.3) | |
| Medically indicated[3] | 0 (0.0) | 74 (18.7) | |
| Mode of delivery | | | < 0.001 |
| Vaginal delivery | 92 (74.8) | 314 (94.6) | |
| Cesarean section[4] | 31 (25.2) | 18 (5.4) | |
| Preeclampsia | 0 (0.0) | 3 (0.9) | 0.566 |
| Birth weight, g | 1505 (1125–2000) | 3630 (3350–3940) | < 0.001 |

BMI, body mass index; GA, gestational age; SPTB, spontaneous preterm birth.

Continuous variables are displayed as medians (interquartile range [IQR]). Categorical variables are displayed as numbers (%).

[1]*p*-value calculated with Mann–Whitney *U* test for continuous variables and with chi-square test for categorical variables.

[2]Primary diseases include cardiovascular diseases, endocrine diseases, pulmonary diseases, gastrointestinal diseases, mental disorders, and rheumatic diseases.

[3]Induction of labor medically (oxytocin, Foley balloon, amniotomy and/or a cesarean section).

[4]Indications for cesarean section were breech position, fetal macrosomia, fear of childbirth, and other fetal or maternal conditions.

Study population 2 comprised pregnant participants ($n$ = 32) and nonpregnant controls ($n$ = 15). The participants were recruited at Oulu University Hospital during 2019–2021. Sera were collected in the delivery room or the operating room at admission. Later, each sample was assigned into a pregnancy group: SPTB ($n$ = 8), EPTB ($n$ = 4), or term birth ($n$ = 20). In the EPTB group, no signs or symptoms of active labor were present, and cesarean sections were performed. GA threshold

for preterm pregnancies was < $37^{+0}$ weeks. GA ranged from $37^{+6}$ weeks to $41^{+0}$ weeks in term pregnancies. The control group consisted of healthy women who were not pregnant at the time of serum collection. The absence of pregnancy was based on a personal interview by the research nurse but pregnancy test was not performed. Additional clinical information about the pregnant participants was collected retrospectively from birth diaries. Clinical characteristics are presented in Table 8.

**Table 8. Clinical characteristics of study population 2 (*n* = 47). Modified from study III.**

| Variable | SPTB | EPTB | Term birth | *p*-value[1] | Nonpregnant |
|---|---|---|---|---|---|
| *n* | 8 | 4 | 20 | | 15 |
| Parity | | | | 0.731 | |
| Nullipara and primipara | 6 (75.0) | 2 (50.0) | 14 (70.0) | | NA |
| Multipara | 2 (25.0) | 2 (50.0) | 6 (30.0) | | NA |
| History of preterm births | 1 (12.5) | 1 (25.0) | 1 (5.0) | 0.310 | NA |
| Interpregnancy interval < 2 years | 1 (12.5) | 0 (0.0) | 0 (0.0) | 0.375 | NA |
| Primary diseases[2] | 5 (62.5) | 3 (75.0) | 6 (30.0) | 0.147 | NA |
| Pregnancy | | | | | |
| *In vitro* fertilization or treated with hormones | 2 (25.0) | 0 (0.0) | 0 (0.0) | 0.073 | NA |
| Smoking during pregnancy | 1 (12.5) | 1 (33.3) | 1 (6.3) | 0.327 | NA |
| BMI before pregnancy, kg/m$^2$ | 24.1 (21.6–26.2) | 22.3 (21.0–28.7) | 22.5 (20.0–26.8) | 0.776 | NA |
| Delivery | | | | | |
| Age of the mother, years | 31.0 (30.0–33.5) | 26.5 (23.5–38.5) | 31.0 (28.0–33.0) | 0.602 | 27.0 (24.0–33.0) |
| GA at sampling/birth, weeks | 34.6 (31.3–35.4) | 34.0 (28.0–35.1) | 39.7 (38.9–40.4) | < 0.001 | NA |
| Onset of delivery | | | | < 0.001 | |
| Spontaneous | 8 (100.0) | 0 (0.0) | 17 (85.0) | | NA |
| Medically indicated[3] | 0 (0.0) | 4 (100.0) | 3 (15.0) | | NA |
| Mode of delivery | | | | < 0.001 | |
| Vaginal delivery | 8 (100.0) | 0 (0.0) | 20 (100.0) | | NA |

| Variable | SPTB | EPTB | Term birth | *p*-value[1] | Nonpregnant |
|---|---|---|---|---|---|
| Cesarean section[4] | 0 (0.0) | 4 (100.0) | 0 (0.0) | | NA |
| Preeclampsia | 0 (0.0) | 1 (25.0) | 0 (0.0) | 0.125 | NA |
| Infection at labor[5] | 1 (12.5) | 1 (25.0) | 0 (0.0) | 0.133 | NA |
| Birth weight, g | 2383 (1805–2781) | 2410 (1030–3790) | 3445 (3147–3833) | 0.002 | NA |

BMI, body mass index; EPTB, elective preterm birth; GA, gestational age; NA, not applicable; SPTB, spontaneous preterm birth.

Continuous variables are displayed as medians (interquartile range [IQR]). Categorical variables are displayed as numbers (%).

[1]*p*-value calculated with Kruskal–Wallis *H* test for continuous variables and with with chi-square test for categorical variables.

[2]Primary diseases include cardiovascular diseases, pulmonary diseases, gastrointestinal diseases, mental disorders, and endocrine disorders.

[3]Induction of labor medically: oxytocin, Foley balloon, amniotomy and/or cesarean section.

[4]Indications for cesarean section were breech position, chorioamnionitis, fear of childbirth, and other fetal or maternal conditions.

[5]Infection at labor was determined as clinical signs and symptoms of infection: fever and a significant increase in infection parameters.

### *4.8.2 Sample preparation*

The samples were diluted with 0.9% NaCl. Samples from study population 1 were analyzed for AAT and CRP at HUSLAB Laboratory, Helsinki, Finland. AAT levels of samples from study population 2 were measured at HUSLAB, and CRP levels were determined at the Nordlab Laboratory, Oulu, Finland. Both laboratories use accredited methods for AAT and CRP measurements.

### *4.8.3 Statistical analyses of study populations 1 and 2*

The baseline and delivery characteristics in study populations 1 and 2 are reported as medians (interquartile range [IQR]) or as numbers (%) as appropriate in Tables 7 and 8. Statistical differences in continuous variables between study population groups were assessed with the Mann–Whitney *U* test (study population 1) or the Kruskal–Wallis *H* test (study population 2). In categorical variables, a chi-square test was applied.

AAT levels in study population 1 were normally distributed and are presented as means and standard deviations (SDs). In study population 2, AAT levels were

interpreted as the median and interquartile ranges because the data were skewed. Appropriate statistical tests were applied to determine *p*-values (Student's *t* test or Mann–Whitney *U* test). Spearman's rank-order correlation was used to evaluate correlation ($r_s$) between serum AAT and CRP levels in both study populations.

Binary logistic regression was performed in study population 1 to investigate the relationship between serum AAT and SPTB. Serum AAT and serum CRP levels were included in the model as explanatory variables. Odds ratios (ORs) of SPTB are reported as crude and adjusted ORs (cOR and aOR, respectively), both with *p*-values and 95% confidence intervals (CIs).

# 5 Results

## 5.1 Placental AAT is decreased and placental HSPA5 increased in SPTB (studies I and II)

Placental proteomes from SPTBs and STBs were compared to obtain protein candidates associated with short GA. Comparison of proteomes from SPTB and EPTB placentas revealed proteins that were affected by spontaneous delivery. When combining both comparisons (SPTB vs. STB and SPTB vs. EPTB), protein candidates associated with SPTB were obtained. Additionally, the basal and chorionic plates were studied to assess differences in protein expression in different sites of the placenta.

A total of 24 and 27 proteins were increased and decreased in the SPTB vs. STB and SPTB vs. EPTB comparisons, respectively. In the former, levels of 10 proteins were increased or decreased in both the basal and chorionic plates. Correspondingly, in the SPTB vs. EPTB comparison, levels of eight proteins were increased or decreased on both sides of the placenta. Some protein expression differences were seen only in either the basal or the chorionic plate, or significant difference were detected in only one of the comparisons. When comparing SPTB vs. STB and SPTB vs. EPTB protein findings, six proteins were expressed differently in both comparisons: alpha-1 antitrypsin (AAT), serum albumin (ALB), annexin A5 (ANXA5), heat shock 70 kDa protein 5 (HSPA5), keratin type I cytoskeletal 19 (KRT19), and vimentin (VIM). Of these, levels of ANXA5 and HSPA5 were increased, and levels of AAT, ALB, KRT19, and VIM were decreased in SPTB placentas (Table 9). AAT, HSPA5, and VIM were expressed differently in both the basal and chorionic plates in SPTB vs. STB comparisons, while significant changes in the expression of ALB, ANXA5, and KRT19 were seen only in either the basal or the chorionic plate. All six proteins were expressed differently only in the basal plate in SPTB vs. EPTB comparison (Table 9).

The qPCR showed that placental (basal plate) expression of *SERPINA1*, which encodes AAT, is downregulated in SPTB (FC −1.9, $p = 0.001$). By contrast, mRNA levels of *HSPA5* did not differ between SPTB and STB placentas (FC −1.1, $p = 0.31$). These findings suggest that placental AAT could be regulated at the mRNA level whereas post-transcriptional regulation may apply to placental HSPA5.

**Table 9. Proteomes of SPTB, EPTB, and STB placentas were determined to obtain proteins that associate with both short GA (SPTB vs. STB) and spontaneity (SPTB vs. EPTB). Levels of six proteins were increased or decreased in both comparisons on either side of the placenta.**

| | SPTB vs. STB | | | | SPTB vs. EPTB | | | |
|---|---|---|---|---|---|---|---|---|
| | Basal plate | | Chorionic plate | | Basal plate | | Chorionic plate | |
| Protein | Ratio | *p*-value | Ratio | *p*-value | Ratio | *p*-value | Ratio | *p*-value |
| AAT | –1.6 | 0.002 | –2.4 | 0.01 | –1.53 | 0.01 | NS | NS |
| ALB | –1.5 | 0.02 | NS | NS | –1.57 | 0.02 | NS | NS |
| ANXA5 | 2.19 | 0.02 | NS | NS | 2.08 | 0.04 | NS | NS |
| HSPA5 | 2.6 | 0.03 | 2.7 | 0.03 | 2.78 | 0.02 | NS | NS |
| KRT19 | NS | NS | –2.3 | 0.002 | –1.76 | 0.03 | NS | NS |
| VIM | –1.69 | 0.004 | –1.77 | 0.03 | –1.54 | 0.05 | NS | NS |

EPTB, elective preterm birth; GA, gestational age; NS, not significant; SPTB, spontaneous preterm birth; STB, spontaneous term birth.

## 5.2 Genetic variants of *SERPINA1* and *HSPA5* may be linked to recurrent SPTBs (studies I and II)

Both rare and common potentially damaging variants of the corresponding genes of the six proteins AAT, ALB, ANXA5, HSPA5, KRT19, and VIM were sought in the WES data. Only gene variants of *SERPINA1* (encoding AAT) and *HSPA5* met the criteria.

Three gene variants of *SERPINA1* were identified in the WES data (study I), of which at least two are considered pathological: rs28929474 (E366K) and rs28929470 (R247C). These variants are also known as the Z variant and the F variant, respectively (Foil, 2021; Seixas & Marques, 2021). The former leads to decreased serum AAT levels (Foil, 2021), whereas the latter appears to alter the conformation of the molecule but does not affect the serum protein level (Okayama et al., 1991). Variant rs121912712 (E387K) has not been linked to AAT deficiency (Foil, 2021). When the locations of these amino acid changes were illustrated with the program PyMOL, they affected the reactive center loop of the protein (study I), which interacts with the target protease (Kim et al., 2001).

One variant of *HSPA5* was identified from the WES data. The variant rs56136100 (E557G) has been predicted to be damaging by several in silico tools (Huusko et al., 2021). Additionally, an alteration in the amino acid may affect the physiochemical properties of HSPA5 in the form of a change from an acidic to a hydrophobic amino acid (Huusko et al., 2021). When illustrating the location of the amino acid change in the protein structure with the program PyMOL, we observed that E557G does not compromise the structure of the protein (study II).

## 5.3 Protein localization in the placenta (studies I and II)

Immunohistochemical staining showed that AAT was present in cyto- and syncytiotrophoblasts (Fig. 6a and b). Staining was strong in the ECM in the basal plate and nonexistent in decidual cells (Fig. 6c and d). AAT appeared to be present in granule-type structures in cyto- and syncytiotrophoblasts, a finding that was also observed by immuno-EM. However, we were not able to clarify the type of these vesicles with colocalization analysis. Additionally, immuno-EM showed that AAT in the ECM was in fibrillar structures that could represent decidual fibrinoid deposits, as we were able to detect fibronectin by immuno-EM in these structures. Fibronectin is one of the components in decidual fibrinoid deposits, along with collagen, laminin, and vitronectin (Huppertz et al., 1996). Fluorescence

colocalization analysis was then performed to assess the relation between AAT and the fibrinoid components. In the colocalization analysis, collagen IV colocalized mainly with AAT.

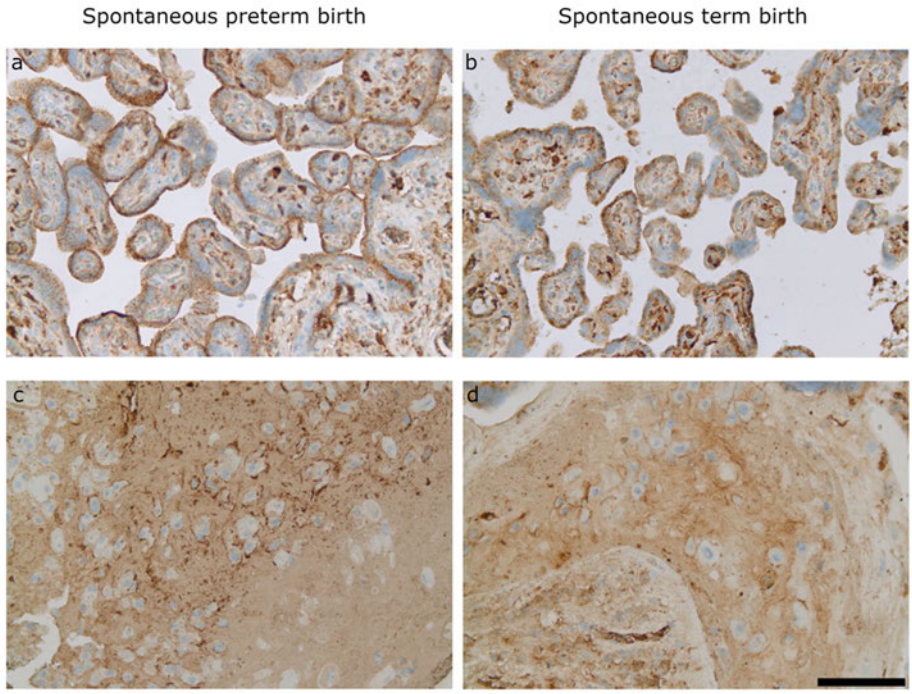


**Fig. 6. Immunohistochemical staining of AAT in placental samples from the basal plate (a and b) and decidua (c and d) that were immunostained with anti-human AAT antibody. Original magnification is ×20 in all images. The scale bar represents 100 µm.**

The immunohistochemical staining of HSPA5 was strong for HSPA5 in villous trophoblasts (Fig. 7a and 7b), weaker in decidual trophoblasts (Fig. 7c and d), and non-existent in the ECM. More precise location of HSPA5 was studied with immuno-EM, which showed that HSPA5 was present mainly in the cytoplasm in placental trophoblasts; no specific locations for cell organelles were observed.

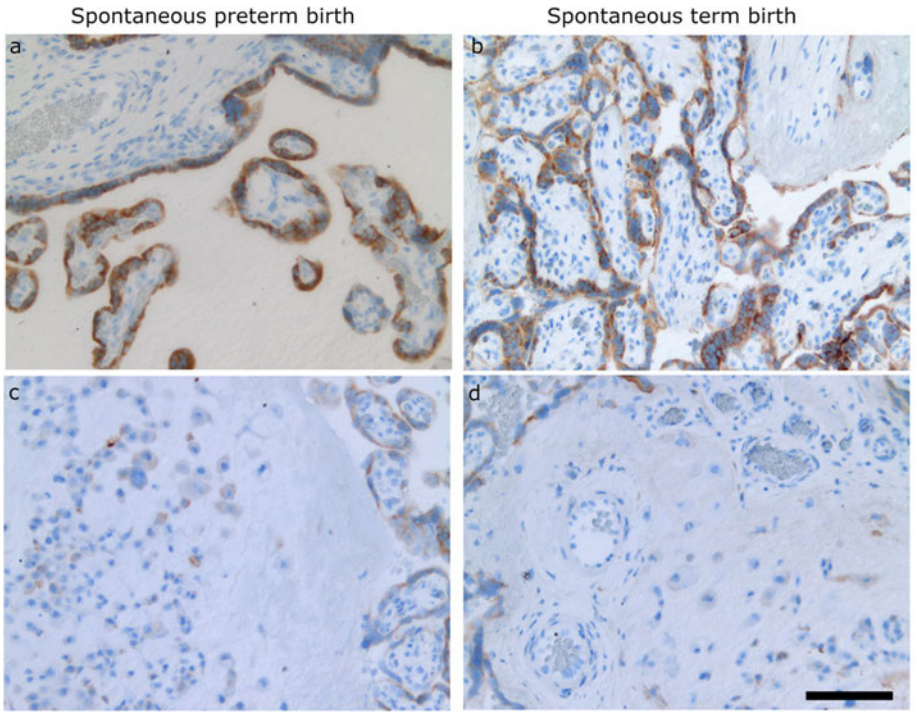


**Fig. 7. Immunohistochemical staining of HSPA5 in placental samples from the basal plate (a and b) and decidua (c and d) that were immunostained with anti-human HSPA5 antibody. Original magnification is ×20 in all images. The scale bar represents 100 µm.**

## 5.4 Gene silencing of *SERPINA1* and *HSPA5* in a placental cell line and mRNA transcriptomics (studies I and II)

The HTR8/SVneo human placental cell line was used in the siRNA-induced gene silencing experiments. Silencing percentages in *SERPINA1* silencing were 94% by qPCR and 89% by RNA sequencing, whereas efficacies of *HSPA5* silencing were 54% (qPCR) and 45% (RNA sequencing).

When *SERPINA1* was silenced in a placental cell line, pathways associated with regulation of actin cytoskeleton (KEGG pathway search, $p = 0.00013$) and positive regulation of cell migration were particularly affected (Gene Ontology biological processes search, $p = 1.8 \times 10^{-6}$). *Fibronectin 1* (*FN1*) was one of the genes affected in the actin cytoskeleton pathway and was upregulated after

silencing (FC 1.8, $p < 0.001$). We verified the result of *FN1* (FC 2.8, $p = 0.004$), among a few other genes, using qPCR (study I).

Gene silencing of *HSPA5* mainly affected pathways and genes related to inflammation. The most affected pathway in the KEGG database search was IL-17 signaling pathway ($p = 2.0 \times 10^{-6}$), and the top biological pathway affected in the Gene Ontology search was inflammatory response ($p = 3.0 \times 10^{-8}$). In both searches, the most affected genes included proinflammatory cytokines such as *CCL2* and *CXCL8*. After *HSPA5*-silencing, downregulation of *CCL2* (FC –1.9, $p < 0.001$) and *CXCL8* (FC –1.9, $p < 0.001$) was observed in the transcriptomics and verified by qPCR (*CCL2* FC –2.4, $p = 0.002$; *CXCL8* FC –2.4, $p = 0.002$). Complete lists of affected pathways can be found in the Supplement files of study I and study II.

In conclusion, gene silencing of *SERPINA1* affects regulation of the actin cytoskeleton, and silencing of *HSPA5* affects the expression of several genes involved in immune response in a placental cell culture model.

## 5.5 Maternal serum AAT and CRP levels in preterm and term pregnancies (study III)

### *5.5.1 AAT and CRP levels in early pregnancy (study population 1)*

Study population 1 comprised a total of 529 serum samples from the first and second trimesters of pregnancy. Baseline characteristics of the study population are shown in Table 7. Parity status was statistically different between the SPTB and control group, as nulliparity or primiparity was more common among those who gave birth prematurely (70.1% vs. 40.1%, $p < 0.001$). History of PTB was more common in mothers belonging to the SPTB group (13.6% vs. 0.6%, $p < 0.001$) whereas short interpregnancy interval was more common among the controls (45.9% vs. 22.5%, $p < 0.001$). Smoking during pregnancy was more common among SPTB group participants than among controls (17.7% vs. 6.7%, $p = 0.004$). In vitro fertilization treatments were more common in the SPTB group (7.3% vs. 1.7%, $p = 0.005$). Mothers in the SPTB group were slightly younger than the controls (28.9 years vs. 30.4 years, $p = 0.005$). GA at sampling was statistically different between the SPTB and control groups in samples from the first trimester. Otherwise, GA at sampling did not show statistically significant difference between the study groups.

Maternal serum AAT levels (mean [SD]) were significantly higher in the SPTB group compared to the controls (1.68 [0.46] g/l vs. 1.52 [0.42] g/l, respectively, $p$

< 0.001). In the further analyses the result did not change: maternal serum AAT levels (mean [SD]) were significantly higher in the SPTB group compared to the control group both in the samples taken during the first trimester (1.61 [0.42] g/l vs. 1.49 [0.42] g/l, $p = 0.01$, Fig. 8) and during the second trimester (1.94 [0.50] g/l vs. 1.70 [0.39] g/l, $p = 0.02$, Fig. 8).

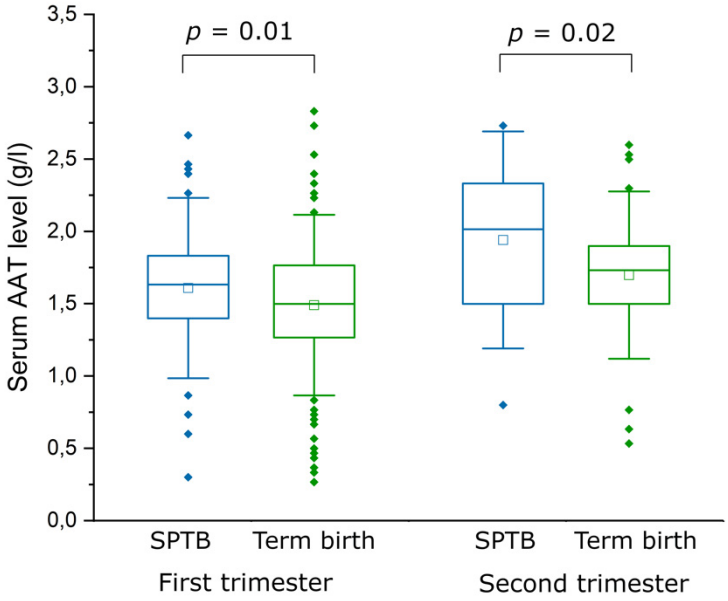


**Fig. 8. Maternal serum AAT concentrations in the first (SPTB *n* = 101, term birth *n* = 337) and second (SPTB *n* = 30, term birth *n* = 61) trimesters. Pregnancies resulted in spontaneous preterm birth (SPTB) or term birth. Quartiles (25th and 75th) are represented by box and 1.5*SD by whiskers. Inside the box, the band indicates the median, and the mean is indicated with a square. Statistical analysis was performed with Student's *t*-test. Modified from study III.**

Statistically significant difference between the SPTB and control groups was observed in following baseline variables (Table 7) which may have an effect on the serum AAT levels: in vitro fertilization, smoking during pregnancy, and age of the mother at sampling. In vitro fertilization/hormonal treatments, smoking during pregnancy, and advanced maternal age did not affect serum AAT levels (Table 10).

**Table 10. Effect of different pre-pregnancy variables on maternal serum AAT levels in study population 1. Modified from study III.**

| | Serum AAT level | | |
|---|---|---|---|
| Variable | Mean (SD), g/l | Mean (SD), g/l | *p*-value[1] |
| In vitro fertilization/treated with hormones | Yes | No | |
| Whole population | 1.59 (0.40) | 1.56 (0.44) | 0.77 |
| Only SPTBs | 1.77 (0.39) | 1.68 (0.47) | 0.58 |
| Only controls | 1.33 (0.28) | 1.52 (0.41) | 0.26 |
| Smoking during pregnancy | Yes | No | |
| Whole population | 1.58 (0.42) | 1.53 (0.42) | 0.46 |
| Only SPTBs | 1.60 (0.60) | 1.67 (0.42) | 0.60 |
| Only controls | 1.57 (0.27) | 1.49 (0.43) | 0.42 |
| Age of the mother at sampling | < 40 years[2] | ≥ 40 years[3] | |
| Whole population | 1.56 (0.44) | 1.63 (0.37) | 0.39 |
| Only SPTBs | 1.67 (0.46) | 2.01 (0.39) | 0.10 |
| Only controls | 1.52 (0.42) | 1.54 (0.30) | 0.81 |

SPTB, spontaneous preterm birth.

[1]*p*-value calculated with Student's *t* test.

[2]Age < 20 years in $n < 5$.

[3]$n = 30$.

Binary logistic regression analysis showed that elevated serum AAT level in early pregnancy associated with SPTB (all-samples cOR 2.5, 95% CI 1.5–4.1, $p < 0.001$; first-trimester cOR 2.0, 95% CI 1.2–3.5, $p = 0.014$; second-trimester cOR 4.1, 95% CI 1.3–12.6, $p = 0.016$). After adjusting for serum CRP, higher serum AAT level increased the odds of SPTB in all samples (aOR 2.4, 95% CI 1.4–4.0, $p = 0.001$) and in first-trimester samples (aOR 2.0, 95% CI 1.1–3.7, $p = 0.019$) but not in second-trimester samples (aOR 2.9, 95% CI 0.7–11.2, $p = 0.13$).

A weak to moderate correlation between maternal serum AAT and CRP was observed. In the first trimester, correlations were as follows: SPTB group ($n = 101$), $r_s = 0.31$, $p = 0.002$; control group ($n = 337$), $r_s = 0.36$, $p < 0.001$. In the second trimester, correlations were $r_s = 0.79$ ($p < 0.001$) in the SPTB group ($n = 30$) and $r_s = 0.36$ ($p = 0.004$) in the controls ($n = 61$).

### 5.5.2 AAT and CRP levels at labor (study population 2)

Study population 2 comprised pregnant and nonpregnant participants. No statistically significant differences were found in the baseline characteristics among pregnant women concerning pre-pregnancy factors (Table 8).

In general, no statistically significant difference in serum AAT levels was observed between SPTBs, EPTBs, and STBs at delivery (Fig. 9a). When SPTBs, EPTBs, and STBs were pooled together, a statistically significant difference was found when comparing that combined group to the nonpregnant group ($p < 0.001$) (Fig. 9b). No correlation between serum AAT and CRP was observed in samples collected at labor ($r_s = -0.025$, $p = 0.89$).

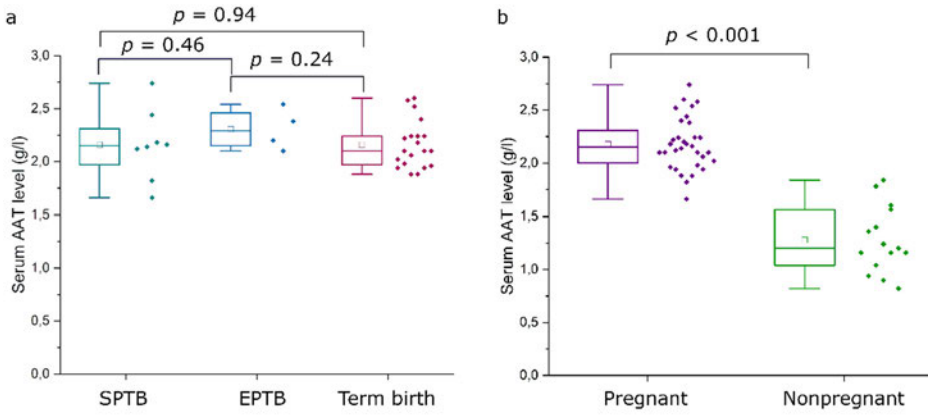


**Fig. 9. Maternal serum AAT levels in study population 2: (a) pregnancy groups (SPTBs, EPTBs, and term births) comparable to each other (nominal association); (b) pregnant women (SPTBs, EPTBs, and term births) compared to nonpregnant participants. The 25th and 75th percentiles are represented by boxes and minimum and maximum values by whiskers. Inside each box, the band and square indicate the median and the mean, respectively. Statistical analysis was performed with the Mann–Whitney *U* test. SPTB, spontaneous preterm birth; EPTB, elective preterm birth. Modified from study III.**

# 6 Discussion

The pathophysiology behind SPTB remains incomplete, but it is believed that maternal, fetal, and/or placental contributions are involved. In order to understand the contribution of the placenta to SPTB, we determined placental proteomes to identify placental proteins that are associated with SPTB (study I). Six proteins were obtained that were associated with both short GA and spontaneous labor. We then used WES data to explore potentially damaging genetic variants of the genes that encode the proteins. Genetic variants of *SERPINA1* (encoding AAT) and *HSPA5* met the criteria. Thus, these two proteins and their genes were chosen for further examination to determine their possible roles in SPTB (studies I and II). In addition, maternal serum AAT levels in preterm and term pregnancies were investigated (study III).

## 6.1 Six placental proteins are associated with spontaneous preterm birth (study I)

A total of six SPTB-associated proteins (AAT, ALB, ANXA5, HSPA5, KRT19, and VIM) were identified. Levels of two proteins were increased (ANXA5 and HSPA5), while levels of the rest were decreased (AAT, ALB, KRT19, and VIM) in SPTB. Some of these proteins have previously been associated with pregnancy complications. Expression of ANXA5 has been shown to be increased in choriodecidual tissue from SPTBs (Shankar et al., 2010), and reduced placental ANXA5 levels have been associated with preeclampsia (Gourvas et al., 2014). Expression of ALB from CVF was increased in samples from PTBs (Liong et al., 2015). However, it should be noted that ALB contamination from blood is plausible (Kruger et al., 2023). Additionally, KRT19 and VIM have previously been detected in placental membrane samples: VIM was detected in samples from PTBs, and KRT19 was detected in samples from term pregnancies (Butt et al., 2006). As VIM and keratins form different types of filaments and participate in cytoskeletal networks, it was suggested that these proteins may participate in regulating the cytoskeletal organization of the placenta, which could contribute to PTB (Butt et al., 2006). Below, placental AAT and HSPA5 are described in greater detail in relation to SPTB.

## 6.2 Downregulation of placental AAT could lead to increased degradation of placental fibrinoid deposits (study I)

AAT is one of the most abundant anti-proteases in human serum (Cazzola et al., 2020) and belongs to the serine protease inhibitor (SERPIN) superfamily (Seixas & Marques, 2021). It is an acute-phase protein produced mainly by the liver (Seixas & Marques, 2021) and to a lesser degree by, for example, monocytes and trophoblasts (Bergman et al., 1993; Cazzola et al., 2020). AAT/*SERPINA1* production at both the protein and mRNA levels in human amnion has been demonstrated (Izumi-Yoneda et al., 2009). AAT plays an important role in the lungs, where it protects the alveolar space by inactivating proteolytic enzymes; it also has immunomodulatory functions (Cazzola et al., 2020; Köhnlein & Welte, 2008). The contribution of placental AAT to pregnancy complications has not been evaluated in detail. One study suggested that in prelabor rupture of the fetal membranes (PROM), inactivation of AAT may lead to decreased anti-proteinase capacity and increased action of proteolytic enzymes (Izumi-Yoneda et al., 2009). We observed that low expression of placental AAT and *SERPINA1* were associated with SPTB. This suggests that low placental AAT levels are due to decreased mRNA expression of *SERPINA1*. For example, micro RNAs can regulate protein expression through repressed mRNA translation (Fabian et al., 2010).

Three potentially harmful gene variants of *SERPINA1* were obtained from the WES data of Finnish and Danish participants: rs28929474 (Z variant), rs28929470 (F variant), and rs121912712. Over 100 genetic variants of *SERPINA1* have been associated with AAT deficiency (Seixas & Marques, 2021; Silva et al., 2016). Those variants can be classified as deficient (decreased AAT serum levels) or dysfunctional (reduced inhibitory activity of AAT) (Seixas & Marques, 2021). The F variant has reduced binding ability to the substrate, leading to dysfunctional anti-protease function of AAT, but it does not necessarily affect serum concentration (Cazzola et al., 2020; Foil, 2021). The Z variant, meanwhile, leads to low AAT serum levels (Foil, 2021; Seixas & Marques, 2021). As both Z and F variants have been linked with reduced AAT levels or impaired anti-protease activity, we propose that these pathological variants may play a role in SPTB.

We observed by immunohistochemical staining and immuno-EM that AAT resided in granule-type structures inside cyto- and syncytiotrophoblasts in SPTB and STB placentas. Fluorescence colocalization analysis did not enable us to determine the type(s) of vesicles containing the granules. We detected AAT in the ECM of the decidua in SPTB and STB placentas by immunohistochemistry. Similar

staining in placental samples has been observed in cases of preeclampsia, in which the amount of fibrinoid deposits increased (Starodubtseva et al., 2020). When we observed the extracellular location of AAT by immuno-EM, it appeared to be located within fibrillar structures in the ECM. Fibronectin also appeared within these structures; consequently, those fibrillar structures may represent decidual fibrinoid deposits.

Moreover, *SERPINA1* silencing revealed ECM organization as one of the affected pathways. The amount of placental fibrinoids increases during pregnancy (Kaufmann et al., 1996), but their role in the placenta and pregnancy more generally is not completely understood (Zhang, 2021). *FN1* (from the actin cytoskeleton pathway) was among the affected genes after the silencing experiment. AAT inhibits serine proteases such as neutrophil elastase, trypsin, chymotrypsin, and thrombin (Jezela-Stanek & Chorostowska-Wynimko, 2019), and plasmin (Churg et al., 2007). The serine proteases degrade ECM components like fibronectin and laminin (Lu et al., 2011). Oncofetal isoforms of fibronectin (i.e., fFN) have been detected in the matrix-type fibrinoid (Huppertz et al., 1996). Indeed, the presence of fFN in cervicovaginal secretions has been proposed to be associated with increased risk of preterm labor (Guller et al., 2003). Remodeling of the ECM has been suggested to contribute to cervical softening, mainly due to the effects of cytokines, estrogen, progesterone, nitric oxide, and PGs (Törnblom et al., 2005). Our results suggest that AAT could also participate in remodeling of the ECM at the feto-maternal site and may play a role in the cervical ripening process.

Others have hypothesized that AAT may regulate inflammatory cytokine expression through ER stress response (Yoshida et al., 2021). Those authors showed that knockdown of *SERPINA1* in the HTR8/SVneo cell line downregulated the mRNA expression of the proinflammatory cytokine *CXCL8*. Similarly, the overexpression of *SERPINA1* promoted the expression of several ER stress markers, including HSPA5, which was upregulated at both the mRNA and protein levels (Yoshida et al., 2021). In our study, *CXCL8* was one of the most downregulated genes after *SERPINA1* gene silencing. Even though ER stress did not arise in our pathway analysis searches, the ER membrane and lumen were both in the top 10 terms. The contribution of AAT to ER stress and whether it is involved in pathogenic processes of SPTB require further investigation.

To conclude, we propose that the downregulation of placental AAT could lead to reduced anti-protease activity due to impaired protein function or decreased protein concentration (Fig. 10). Thus, proteases within the placental tissue are not inhibited properly and could lead to proteolytic degradation of the placental

fibrinoid structures. As fibrinoids are immunosuppressive, their degradation could lead to altered immunological tolerance at the feto-maternal interface. The presence of fFN in the cervicovaginal secretions in imminent SPTB could be explained by degradation of the ECM components at the feto-maternal site that then leak into the CVF (Guller et al., 2003).

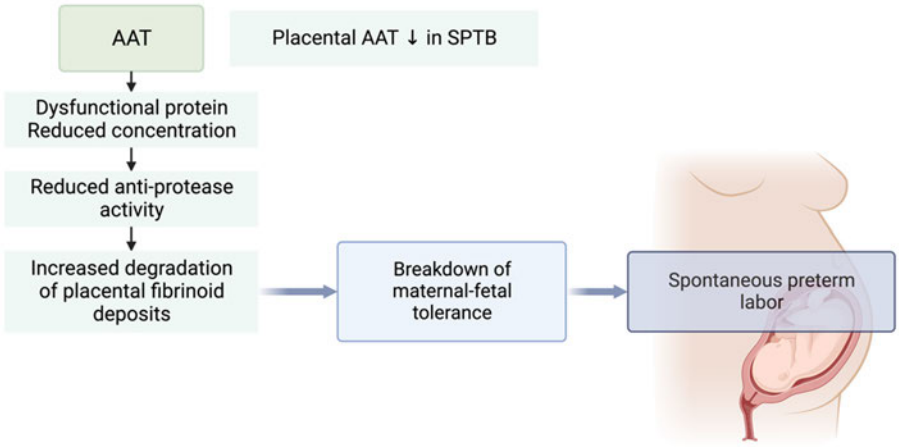


**Fig. 10. The role of AAT in SPTB. Level of placental AAT is decreased in SPTBs. Additionally, the harmful gene variants from the WES analysis may affect the function of the protein or protein synthesis. Thus, we propose that the downregulation of placental AAT could lead to reduced anti-protease activity and increased degradation of placental fibrinoids like fibronectin. This could compromise the immunological tolerance of the placenta and enable AAT to participate in the pathogenesis of SPTB. SPTB, spontaneous preterm birth. Created with BioRender.com.**

### *6.2.1 Maternal serum AAT levels do not have clinically relevant difference between preterm and term pregnancies in early gestation (study III)*

We observed that decreased protein level of placental AAT is associated with SPTB in study I and continued to examine maternal serum AAT levels in spontaneous preterm and term pregnancies to determine whether any dysregulation of AAT could be observed. As AAT is an acute-phase protein, its serum concentration increases during, for example, inflammation, infection, cancer, and pregnancy (Köhnlein & Welte, 2008). Dysregulation of serum AAT levels has been observed

in various diseases. The impaired inhibitory capacity of AAT has been observed in pregnant women with type 1 diabetes (Lisowska-Myjak et al., 2001; Lisowska-Myjak & Pachecka, 2003) and in gestational diabetes mellitus (Yaghmaei et al., 2009). Low concentrations of circulating AAT were detected in women with recurrent and sporadic pregnancy loss (Madar et al., 2013). Women with severe preeclampsia were reported to have decreased AAT levels and enzymatic activity (Jezela-Stanek & Chorostowska-Wynimko, 2019). Serum AAT level correlated inversely with fetal growth restriction in pregnancies complicated by preeclampsia (Nori & Ali, 2021). However, maternal serum AAT levels during early pregnancy and their association with subsequent SPTB have not been studied extensively. Proteomics of the first-trimester serum samples revealed higher serum levels of AAT in SPTBs compared to control pregnancies (D'Silva et al., 2018), but that upregulation was not verified using western blot and a larger sample size (D'Silva et al., 2020). We observed that maternal AAT levels were higher in early-pregnancy serum samples in pregnancies resulting in SPTB, although there was overlapping in concentrations between the SPTB and control groups. This suggests that serum AAT cannot constitute as predictive test for SPTB, at least on its own. Low levels of serum AAT have been suggested to contribute to PPROM (Baron et al., 2012). In our data, PPROM and PROM cases were not distinguished. PPROM cases were included in the original population of SPTBs, but term deliveries with pregnancy- and labor-associated complications were excluded, and it is likely that PROMs were not included in the original population of term deliveries (Karjalainen et al., 2015). We observed that serum AAT levels did not differ between SPTBs, EPTBs and term births, which aligns with results from a previous study (Baron et al., 2012).

CRP is used as a marker for infection and inflammation. Like AAT, CRP is an acute-phase protein produced by the liver (Sorokin et al., 2010). It is not specific enough to diagnose a certain condition; rather, it is used alongside other diagnostic tests (Sanders et al., 2018). Early-pregnancy CRP levels have been studied in relation to risk of PTB with variable results. Elevated serum or plasma CRP levels have been associated not only with preterm delivery (Hvilsom et al., 2002; Lohsoonthorn et al., 2007; Pitiphat et al., 2005) but also with preeclampsia and intrauterine growth restriction (Tjoa et al., 2003). By contrast, Ghezzi et al. (2002) did not observe a difference in maternal blood CRP level between women with preterm (GA < 34 weeks) and term births. At time of delivery, maternal plasma CRP did not differ between preterm and term pregnancies (Menon & Taylor, 2019). To our knowledge, the question of a correlation between serum AAT and CRP has not been studied in samples from early pregnancy and different pregnancy

outcomes (i.e., preterm vs. term delivery). Larsson et al. (2008) estimated lower and upper limits of AAT and CRP during uncomplicated term pregnancies: both proteins showed an increasing trend toward the end of pregnancy, with their highest values in weeks 34–38 (AAT) and 28–31 (CRP). Correlation between serum AAT and CRP was curved in a population with different *SERPINA1* genotypes (Sanders et al., 2018). We observed a weak to moderate correlation between serum AAT and CRP, but at time of delivery, no correlation was found.

To summarize, we found statistically significantly elevated levels of maternal serum AAT in first- and second-trimester serum samples from pregnancies with spontaneous preterm delivery in index pregnancy. However, there was an apparent overlapping of AAT concentrations between the SPTB and control groups. Early-pregnancy serum AAT does not constitute a predictive test for SPTB per se, but we propose that a modest elevation of maternal serum AAT during early pregnancy may be associated with subsequent SPTB. Our results indicate that maternal AAT serum levels do not explain previously observed decreased placental AAT levels in SPTB (Tiensuu et al., 2022). One explanation could be that the tissues studied are from different origins: the serum was from the mother, and the placenta is of fetal origin. We do not know serum AAT levels of the mothers that participated in study I and whether there was any correlation between maternal serum and placental AAT levels. Thus, the role of AAT in SPTB remains to be studied further.

## 6.3 Placental HSPA5 may regulate the inflammatory state of the placenta (study II)

In study I, we identified that increased level of placental HSPA5 is associated with SPTB. HSPA5, which is also known as GRP78 and immunoglobulin heavy chain-binding protein (Wang et al., 2017), belongs to the HSP70 family, which is the most studied HSP family (Chaiworapongsa et al., 2008; Dvorakova et al., 2017). HSPA5 plays many roles: it assists in folding newly synthetized proteins and participates in ER stress response (Lee, 2005) and in unfolded protein response (Wang et al., 2017). We found that *HSPA5* mRNA expression did not differ between SPTB and STB placentas, but HSPA5 protein level was increased in SPTB placentas. Elevated mRNA levels of *HSPA5* have been observed in placental membranes from SPTBs complicated by ongoing infection (Liong & Lappas, 2014), in preeclamptic placentas (Fu et al., 2015; Xiong et al., 2023), and in first-trimester cytotrophoblasts compared to term cytotrophoblasts (Fradet et al., 2012). Increased protein levels of HSPA5 have been observed in the placental membranes from preterm deliveries

(Butt et al., 2006), and in placentas from pregnancies complicated by preeclampsia (Du et al., 2017; Fu et al., 2015). Protein overexpression of HSPA5 has been associated with number of diseases, such as cancer, certain cardiovascular diseases, and Parkinson's disease, mainly via ER stress (Wang et al., 2017). As we observed increased amount of HSPA5 at the protein level but not at the mRNA level, we suggest that this could be explained by post-translational modifications of HSPA5. Others have reported that HSPA5 may be regulated post-translationally by, for example, phosphorylation, acetylation, AMPylation, and ADP ribosylation (Gething, 1999; Nitika et al., 2020). These post-translational modifications may participate in regulating the synthesis of HSPA5 and the protein binding ability of HSPA5 (Gething, 1999). Previously, protein and mRNA level discordance of HSPA5 has been reported in ER stress (increased protein level and downregulated mRNA level), but it was concluded that discordance between protein and mRNA levels is rare (Cheng et al., 2016).

All members of the HSP70 family, including HSPA5, have two domains: the nucleotide-binding domain (NBD) and the substrate-binding domain (SBD) (Yang et al., 2015). We observed that the variant of HSPA5 (rs56136100; E557G) did not compromise the structure of the protein, although several in silico tools predicted the change to be damaging: from an acidic amino acid, glutamic acid (E), to a hydrophobic amino acid, glycine (G) (Huusko et al., 2021). We illustrated the crystal structure of HSPA5 to visualize the location of the amino acid change: it was in the SBD and did not have hydrogen bonds with neighboring amino acids. However, we speculated that as the amino acid change resided in the lid structure of the SBD, it could disrupt the interaction between HSPA5 and its co-chaperone. The co-chaperones can interact with both the SBD and NBD (Yang et al., 2020), and regulate the chaperone activity of the HSP (Yang et al., 2015).

Our immunohistochemistry showed that staining of HSPA5 was strong in both syncytiotrophoblasts and cytotrophoblasts and weaker in decidual trophoblasts. We did not detect significant differences in the staining between SPTB and STB placentas. Others have shown that HSPA5 is present in the placental membranes from term deliveries (Liong & Lappas, 2014). Specifically, the protein was observed in amnion epithelium, chorionic trophoblasts, and decidual cells (Liong & Lappas, 2014). Furthermore, HSPA5 has been detected in syncytiotrophoblasts and cytotrophoblasts in first-trimester placentas (Arnaudeau et al., 2009). We also performed immuno-EM of HSPA5 in placentas from SPTBs and STBs and HSPA5 was found mainly in the cytoplasm of the trophoblasts. However, we were not able to detect any cell organelle-specific location of HSPA5. Others have reported that

HSPA5 is localized mainly in the ER, where it assists folding synthetized proteins (Gething, 1999). Expression of HSPA5 has been detected on the cell surface, mainly under pathological conditions like cancer (Fradet et al., 2012; Wang et al., 2017).

Gene silencing of *HSPA5* in a placental cell line showed that the most affected pathways were the IL-17 signaling pathway and inflammatory response. Recruitment and infiltration of immune cells and change in the inflammatory state of the placenta have been suggested as possible pathway of both term and preterm labors (Gomez-Lopez et al., 2014). We observed by silencing *HSPA5* that several pro-inflammatory cytokines, such as *CCL2* and *CXCL8*, were downregulated. This suggests that HSPA5 may participate in regulation of the inflammatory state of the placenta. CCL2 is one of the important regulators of macrophage migration and activation (Bonney & Johnson, 2019) and trophoblasts have been shown to produce CCL2 (Lin et al., 2023). IL-8 (encoded by *CXCL8*) stimulates migration of several immune cells, including macrophages (Meniailo et al., 2018). Indeed, several immune cells, including neutrophils and macrophages, in reproductive tissues (e.g., uterus, decidua, and fetal membranes) have been reported during labor (Gomez-Lopez et al., 2014).

We propose that in the case of SPTB, dysregulation of placental HSPA5 could promote the expression of several pro-inflammatory cytokines (Fig. 11). These pro-inflammatory cytokines potentially increase the migration of immune cells to the feto-maternal interface, which may disturb the immune tolerance. These events could lead to a shift in the placenta from an anti-inflammatory to a pro-inflammatory state, eventually leading to SPTB. An implication of this is the possibility that HSPA5 participates in the pathogenesis of SPTB.

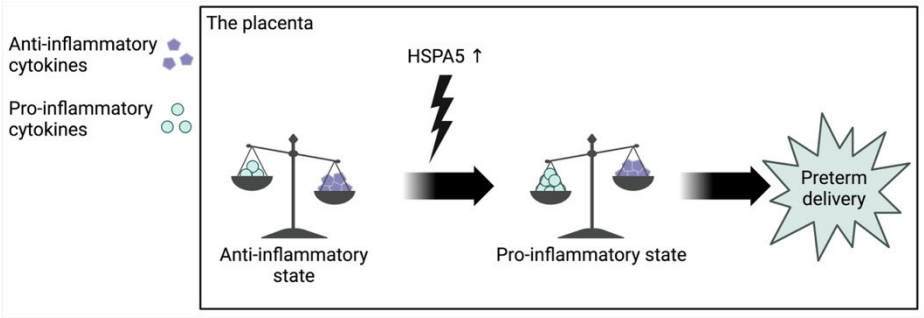


**Fig. 11. The role of HSPA5 in SPTB. It has been suggested that a change in the inflammatory state of the placenta could contribute to the pathogenesis of SPTB. A change from an anti-inflammatory to a pro-inflammatory state could be triggered by, for example, increased protein level of placental HSPA5. Modified from Gomez-Lopez et al. (2014) and created with BioRender.com.**

## 6.4 Limitations and strengths

Our placental and blood samples were of Finnish origin; thus, the results may not apply to other ancestries. In the SPTB cases, the aim was to collect samples from pregnancies with as few risk factors as possible. Therefore, placental samples had exclusion criteria, such as multiple gestation and fetal anomalies. However, chorioamnionitis affected a few SPTB and EPTB placentas. In some cases, an infection may have developed after the initiation of labor. However, this could not be distinguished in placental samples collected. Additionally, data about possible pathological anatomical diagnoses (PAD) of placentas were not collected. That information could have offered more knowledge about the macroscopic and microscopic findings among the placental samples and whether there is, for example, infection suggested by the PAD but not clinically. Although the number of placental samples was low, the samples were collected in the same manner in each case which ensures the quality of samples.

We silenced the gene expression of *SERPINA1* and *HSPA5* using siRNAs in the HTR8/SVneo cell line, which is generated from freshly isolated extravillous cytotrophoblasts from first-trimester placenta. The cell line contains epithelial and mesenchymal cells (Abou-Kheir et al., 2017). Later, it was shown that an epithelial-to-mesenchymal transition did take place in the HTR8/SVneo cell line, which could explain the heterogenous cell population (Msheik et al., 2020). Because of that heterogeneity, the silencing experiment may not necessarily reflect only

trophoblasts but may also include other placental cells. However, the placenta contains a variety of cell types, so the HTR8/SVneo cell line is suitable for studying the effects of gene silencing and applying the knowledge obtained to the human placenta, at least to some extent.

We used the FMC serum bank, which covers over 90% of all pregnant women in Finland from 1987 to 2016. Hence, an adequate amount of serum samples from study population 1 participants was obtained, though power calculations were not performed. One of the challenges regarding retrospective studies is the lack of data. Consequently, the clinical data collected in study III relied on what was available in the birth diaries. Information about possible maternal infection at the time of sampling was not available, which is indeed a major limitation to this study. Because both AAT and CRP are acute-phase proteins, ongoing infection can elevate their levels. We observed that smoking was more common in the SPTB group, which may have affected pregnancy outcomes because smoking during pregnancy is a risk factor for SPTB. It should be noted that quite a large proportion of participants did not make note of intoxicant use (including smoking) before or during pregnancy in their birth diaries. Additionally, questionnaires regarding smoking during pregnancy may not be accurate (Cnattingius, 2004). We observed that smoking did not affect serum AAT levels in our population, which aligns with previous result from a nonpregnant population (Olsen et al., 1975). Although many risk factors for SPTB were excluded from study population 1, it is worth noting that previous studies focused on participants that had previous SPTBs (Karjalainen et al., 2015; Tiensuu et al., 2019). Hence, an overrepresentation of women with a history of SPTB in the SPTB group is possible.

## 6.5 Prospects for future studies

We studied AAT/*SERPINA1* and HSPA5 experimentally in vitro. To validate the involvement of AAT and HSPA5 in the pathogenesis of SPTB, in vivo studies in relevant animal models are required. Several ethical considerations apply to such models; mice are the most commonly used animal model (Bezold et al., 2013), and *SERPINA1*-knockout and *HSPA5*-overexpression mice models could provide insights into the effects of these SPTB-associated proteins and genes.

Serum AAT concentrations may not accurately represent the anti-protease function of AAT because inactive molecules may falsely ascribe concentrations, and the concept of “relative functional AAT deficiency” has been introduced (Lisowska-Myjak & Pachecka, 2003; Madar et al., 2013). Baron et al. (2012) did

not find differences in the serum elastase inhibitory capacity of AAT between preterm and term births. The anti-protease capacity of AAT was not studied in this dissertation in relation to SPTB. It remains to be examined whether a decreased placental anti-protease function of AAT could contribute to the pathogenesis of SPTB, for example in recurrent idiopathic SPTBs. Additionally, it would be interesting to see whether AAT concentration and anti-protease function in umbilical cord blood after delivery of the placenta differ from concentration in maternal blood or between the different delivery phenotypes (e.g., SPTBs, EPTBs, and STBs).

In addition to serum AAT levels, we chose to study serum CRP levels, as CRP is a widely used marker for inflammation and infection. As stated earlier, elevated levels of several inflammatory markers, such as CRP and IL-6, have been associated with preterm delivery (Sorokin et al., 2010). Indeed, combining several suitable SPTB-associated protein markers could lead to more accurate identification and prediction of SPTB (Leow et al., 2020). Thus, in the future, it could be interesting to determine AAT levels among other inflammatory markers, such as different cytokines and chemokines, to see whether any correlation could be observed.

Intravenous AAT augmentation therapy is in clinical use to treat patients with lung diseases (COPD, emphysema, bronchiectasis) caused by AAT deficiency (Brebner & Stockley, 2013; Connolly et al., 2018). Additionally, mRNA therapies have been suggested to treat AAT-deficient patients (Connolly et al., 2018). Perhaps intravenous AAT augmentation and/or *SERPINA1* mRNA therapy could be used to treat AAT-deficient mothers in the future to delay or even prevent PTBs, although this should be studied experimentally in appropriate animal models. To summarize, more research is required to understand the causes and consequences of the maternal–fetal–placental interactions of AAT and HSPA5 and their relationship to SPTB.

# 7 Conclusions

SPTB is a consequence of environmental, biological, and genetic factors and their interactions, and the pathogenesis of SPTB has not yet been fully elucidated. This study set out to examine potential placental protein candidates associated with SPTB. We used hypothesis-free placental proteomics and discovered six SPTB-associated placental proteins: AAT, ALB, ANXA5, HSPA5, KRT19, and VIM. We then explored potentially damaging variants of the genes that encoded these proteins and found that variants of *SERPINA1* (encoding AAT) and *HSPA5* met our criteria. Consequently, these two proteins and their genes were studied further. The studies presented in this thesis have demonstrated that levels of placental AAT and HSPA5 are associated with SPTB, and the results offer insights into the possible pathways in the pathogenesis of spontaneous premature birth. Additionally, an increased concentration of maternal serum AAT in early pregnancy may be associated with subsequent SPTB. The following conclusions can be made based on our results:

1. We showed that placental AAT/*SERPINA1* was downregulated in SPTB cases at both the protein and mRNA levels. Hence, we propose that dysregulation of AAT may compromise a pregnancy due to dysfunctional protein or decreased protein expression. This could impair the anti-protease function or activity of AAT and lead to increased degradation of placental fibrinoid deposits. That degradation may alter the inflammatory balance of the placenta toward a pro-inflammatory state.
2. Placental HSPA5 was increased in SPTBs. We observed that placental HSPA5 may have immunomodulatory functions in the placenta. Therefore, we propose that increased protein level of placental HSPA5 may promote the release of pro-inflammatory cytokines, which could lead to disturbance at the feto-maternal interface and eventually to premature labor.
3. We found statistically significantly higher serum AAT levels in pregnancies with SPTB, but there was substantial overlapping in the concentrations between the SPTB and control groups. Based on our results, serum AAT is not a useful marker to predict SPTB, at least on its own. However, we propose that elevated serum concentration of AAT in early pregnancy could be associated with subsequent SPTB.

# References


Abou-Kheir, W., Barrak, J., Hadadeh, O., & Daoud, G. (2017). HTR-8/SVneo cell line contains a mixed population of cells. *Placenta*, *50*, 1–7. https://doi.org/10.1016/j.placenta.2016.12.007

Ahmed, B., Abushama, M., & Konje, J. C. (2023). Prevention of spontaneous preterm delivery–an update on where we are today. *Journal of Maternal-Fetal and Neonatal Medicine*, *36*(1). https://doi.org/10.1080/14767058.2023.2183756

American College of Obstetricians and Gynecologists. (2013). Definition of term pregnancy. Committee Opinion No. 579. *Obstetrics & Gynecology*, 1139–1140. https://www.acog.org/clinical/clinical-guidance/committee-opinion/articles/2013/11/definition-of-term-pregnancy

Arck, P. C., & Hecher, K. (2013). Fetomaternal immune cross-talk and its consequences for maternal and offspring's health. *Nature Medicine*, *19*(5), 548–556. https://doi.org/10.1038/nm.3160

Arnaudeau, S., Arboit, P., Bischof, P., Shin-ya, K., Tomida, A., Tsuruo, T., Irion, O., & Cohen, M. (2009). Glucose-regulated protein 78: a new partner of p53 in trophoblast. *Proteomics*, *9*(23), 5316–5327. https://doi.org/10.1002/pmic.200800865

Barfield, W. D. (2018). Public health implications of very preterm birth. *Clinics in Perinatology*, *45*(3), 565–577. https://doi.org/10.1016/j.clp.2018.05.007

Baron, J., Sheiner, E., Abecassis, A., Ashkenazi, E., Shahaf, G., Salem, S. Y., Madar, T., Twina, G., Wiznitzer, A., Holcberg, G., & Lewis, E. C. (2012). α1-Antitrypsin insufficiency is a possible contributor to preterm premature rupture of membranes. *Journal of Maternal-Fetal and Neonatal Medicine*, *25*(7), 934–937. https://doi.org/10.3109/14767058.2011.600369

Becker, C., Hammerle-Fickinger, A., Riedmaier, I., & Pfaffl, M. W. (2010). mRNA and microRNA quality control for RT-qPCR analysis. *Methods*, *50*(4), 237–243. https://doi.org/10.1016/j.ymeth.2010.01.010

Beernink, R. H. J., Schuitemaker, J. H. N., Zwertbroek, E. F., Scherjon, S. A., & Cremers, T. I. F. H. (2023). Early pregnancy biomarker discovery study for spontaneous preterm birth. *Placenta*, *139*, 112–119. https://doi.org/10.1016/j.placenta.2023.06.011

Benirschke, K. (1998). Remarkable placenta. *Clinical Anatomy*, *11*(3), 194–205.

Bergman, D., Kadner, S. S., Cruz, Mar. R., Esterman Michael M Tahery, A. L., Young, B. K., & Finlay, T. H. (1993). Synthesis of α1-antichymotrypsin and α1-antitrypsin by human trophoblast. *Pediatric Research*, *34*(3), 312–317.

Bezold, K. Y., Karjalainen, M. K., Hallman, M., Teramo, K., & Muglia, L. J. (2013). The genomics of preterm birth: from animal models to human studies. *Genome Medicine*, *5*(4), 1–11. https://doi.org/10.1186/gm438

Blanks, A. M., & Thornton, S. (2003). The role of oxytocin in parturition. *BJOG*, *110*(SUPPL. 20), 46–51. https://doi.org/10.1016/S1470-0328(03)00024-7

Blencowe, H., Cousens, S., Chou, D., Oestergaard, M., Say, L., Moller, A.-B., Kinney, M., & Lawn, J. (2013). Born too soon : the global action report on preterm birth. *Reproductive Health*, *10(Suppl1)*(S2), 1–15. https://doi.org/doi: 10.1186/1742-4755-10-S1-S2

Bonney, E. A., & Johnson, M. R. (2019). The role of maternal T cell and macrophage activation in preterm birth: cause or consequence? *Placenta*, *79*, 53–61. https://doi.org/10.1016/j.placenta.2019.03.003

Brebner, J. A., & Stockley, R. A. (2013). Recent advances in α-1-antitrypsin deficiency-related lung disease. *Expert Review of Respiratory Medicine*, *7*(3), 213–230. https://doi.org/10.1586/ers.13.20

Brink, L. T., Roberts, D. J., Wright, C. A., Nel, D. G., Schubert, P. T., Boyd, T. K., Hall, D. R., & Odendaal, H. (2022). Placental pathology in spontaneous and iatrogenic preterm birth: Different entities with unique pathologic features. *Placenta*, *126*, 54–63. https://doi.org/10.1016/j.placenta.2022.06.004

Bulmer, J. N., Williams, P. J., & Lash, G. E. (2010). Immune cells in the placental bed. *International Journal of Developmental Biology*, *54*(2–3), 281–294. https://doi.org/10.1387/ijdb.082763jb

Burton, G. J., Charnock-Jones, D. S., & Jauniaux, E. (2009). Regulation of vascular growth and function in the human placenta. *Reproduction*, *138*(6), 895–902. https://doi.org/10.1530/REP-09-0092

Burton, G. J., & Fowden, A. L. (2015). The placenta: a multifaceted, transient organ. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *370*(1663). https://doi.org/10.1098/rstb.2014.0066

Butt, R. H., Lee, M. W. Y., Pirshahid, S. A., Backlund, P. S., Wood, S., & Coorssen, J. R. (2006). An initial proteomic analysis of human preterm labor: Placental membranes. *Journal of Proteome Research*, *5*(11), 3161–3172. https://doi.org/10.1021/pr060282n

Care, A., Nevitt, S. J., Medley, N., Donegan, S., Good, L., Hampson, L., Tudur Smith, C., & Alfirevic, Z. (2022). Interventions to prevent spontaneous preterm birth in women with singleton pregnancy who are at high risk: systematic review and network meta-analysis. *BMJ*, *376*, e064547. https://doi.org/10.1136/bmj-2021-064547

Cazzola, M., Stolz, D., Rogliani, P., & Matera, M. G. (2020). α1-antitrypsin deficiency and chronic respiratory disorders. *European Respiratory Review*, *29*(155). https://doi.org/10.1183/16000617.0073-2019

Chaiworapongsa, T., Erez, O., Pedro Kusanovic, J., Vaisbuch, E., Mazaki-Tovi, S., Gotsch, F., Gabor Than, N., Mittal, P., Mee Kim, Y., Camacho, N., Edwin, S., Gomez, R., Hassan, S. S., & Romero, R. (2008). Amniotic fluid heat shock protein 70 concentration in histologic chorioamnionitis, term and preterm parturition. *Journal of Maternal-Fetal and Neonatal Medicine*, *21*(7), 449–461. https://doi.org/doi: 10.1080/14767050802054550

Challis, J. R. G. (2000). Mechanism of parturition and preterm labor. *Obstetrical and Gynecological Survey*, *55*(10), 650–660. https://doi.org/doi: 10.1097/00006254-200010000-00025.

Challis, J. R. G., Sloboda, D. M., Alfaidy, N., Lye, S. J., Gibb, W., Patel, F. A., Whittle, W. L., & Newnham, J. P. (2002). Prostaglandins and mechanisms of preterm birth. *Reproduction*, *124*, 1–17. https://doi.org/doi: 10.1530/rep.0.1240001

Chen, C. P., & Aplin, J. D. (2003). Placental extracellular matrix: gene expression, deposition by placental fibroblasts and the effect of oxygen. *Placenta*, *24*(4), 316–325. https://doi.org/10.1053/plac.2002.0904

Cheng, Z., Teo, G., Krueger, S., Rock, T. M., Koh, H. W., Choi, H., & Vogel, C. (2016). Differential dynamics of the mammalian mRNA and protein expression response to misfolding stress. *Molecular Systems Biology*, *12*(1). https://doi.org/10.15252/msb.20156423

Chucri, T. M., Monteiro, J. M., Lima, A. R., Salvadori, M. L. B., Junior, J. R. K., & Miglino, M. A. (2010). A review of immune transfer by the placenta. *Journal of Reproductive Immunology*, *87*(1–2), 14–20. https://doi.org/10.1016/j.jri.2010.08.062

Churg, A., Wang, X., Wang, R. D., Meixner, S. C., Pryzdial, E. L. G., & Wright, J. L. (2007). α1-antitrypsin suppresses TNF-α and MMP-12 production by cigarette smoke-stimulated macrophages. *American Journal of Respiratory Cell and Molecular Biology*, *37*(2), 144–151. https://doi.org/10.1165/rcmb.2006-0345OC

Cindrova-Davies, T., & Sferruzzi-Perri, A. N. (2022). Human placental development and function. *Seminars in Cell and Developmental Biology*, *131*, 66–77. https://doi.org/10.1016/j.semcdb.2022.03.039

Cnattingius, S. (2004). The epidemiology of smoking during pregnancy: smoking prevalence, maternal characteristics, and pregnancy outcomes. *Nicotine and Tobacco Research*, *6*(SUPPL. 2). https://doi.org/10.1080/14622200410001669187

Conde-Agudelo, A., & Romero, R. (2016). Cervical phosphorylated insulin-like growth factor binding protein-1 test for the prediction of preterm birth: a systematic review and metaanalysis. *American Journal of Obstetrics and Gynecology*, *214*(1), 57–73. https://doi.org/10.1016/j.ajog.2015.06.060

Connolly, B., Isaacs, C., Cheng, L., Asrani, K. H., & Subramanian, R. R. (2018). SERPINA1 mRNA as a treatment for alpha-1 antitrypsin deficiency. *Journal of Nucleic Acids*, *2018*. https://doi.org/10.1155/2018/8247935

Couture, C., Brien, M. E., Boufaied, I., Duval, C., Soglio, D. D., Enninga, E. A. L., Cox, B., & Girard, S. (2023). Proinflammatory changes in the maternal circulation, maternal–fetal interface, and placental transcriptome in preterm birth. *American Journal of Obstetrics and Gynecology*, *228*(3), 332.e1-332.e17. https://doi.org/10.1016/j.ajog.2022.08.035

da Fonseca, E. B., Damião, R., & Moreira, D. A. (2020). Preterm birth prevention. *Best Practice and Research Clinical Obstetrics and Gynaecology*, *69*, 40–49. https://doi.org/10.1016/j.bpobgyn.2020.09.003

Daskalakis, G., Goya, M., Pergialiotis, V., Cabero, L., Kyvernitakis, I., Antsaklis, A., & Arabin, B. (2019). Prevention of spontaneous preterm birth. *Archives of Gynecology and Obstetrics*, *299*(5), 1261–1273. https://doi.org/10.1007/s00404-019-05095-y

Degner, K., Magness, R. R., & Shah, D. M. (2017). Establishment of the human uteroplacental circulation: a historical perspective. *Reproductive Sciences*, *24*(5), 753–761. https://doi.org/10.1177/1933719116669056

D'Silva, A. M., Hyett, J. A., & Coorssen, J. R. (2018). Proteomic analysis of first trimester maternal serum to identify candidate biomarkers potentially predictive of spontaneous preterm birth. *Journal of Proteomics*, *178*, 31–42. https://doi.org/10.1016/j.jprot.2018.02.002

D'Silva, A. M., Hyett, J. A., & Coorssen, J. R. (2020). First trimester protein biomarkers for risk of spontaneous preterm birth: identifying a critical need for more rigorous approaches to biomarker identification and validation. *Fetal Diagnosis and Therapy*, *47*(6), 497–506. https://doi.org/10.1159/000504975

Du, L., He, F., Kuang, L., Tang, W., Li, Y., & Chen, D. (2017). ENOS/iNOS and endoplasmic reticulum stress-induced apoptosis in the placentas of patients with preeclampsia. *Journal of Human Hypertension*, *31*(1), 49–55. https://doi.org/10.1038/jhh.2016.17

Dvorakova, L., Ivankova, K., Krofta, L., & Hromadnikova, I. (2017). Expression profile of heat shock proteins in placental tissues of patients with preterm prelabor rupture of membranes and spontaneous preterm labor with intact membranes. *American Journal of Reproductive Immunology*, *78*(4), e12698. https://doi.org/10.1111/aji.12698

Erlebacher, A. (2013). Immunology of the maternal-fetal interface. *Annual Review of Immunology*, *31*, 387–411. https://doi.org/10.1146/annurev-immunol-032712-100003

Esplin, M. S., Merrell, K., Goldenberg, R., Lai, Y., Iams, J. D., Mercer, B., Spong, C. Y., Miodovnik, M., Simhan, H. N., Van Dorsten, P., & Dombrowski, M. (2011). Proteomic identification of serum peptides predicting subsequent spontaneous preterm birth. *American Journal of Obstetrics and Gynecology*, *204*(5), 391.e1-391.e8. https://doi.org/10.1016/j.ajog.2010.09.021

Fabian, M. R., Sonenberg, N., & Filipowicz, W. (2010). Regulation of mRNA translation and stability by microRNAs. *Annual Review of Biochemistry*, *79*, 351–379. https://doi.org/10.1146/annurev-biochem-060308-103103

Foil, K. E. (2021). Variants of SERPINA1 and the increasing complexity of testing for alpha-1 antitrypsin deficiency. *Therapeutic Advances in Chronic Disease*, *12*, 33–48. https://doi.org/10.1177/20406223211015954

Fradet, S., Pierredon, S., Ribaux, P., Epiney, M., Ya, K., Irion, O., & Cohen, M. (2012). Involvement of membrane grp78 in trophoblastic cell fusion. *PLoS ONE*, *7*(8), e40596. https://doi.org/10.1371/journal.pone.0040596

Frey, H. A., & Klebanoff, M. A. (2016). The epidemiology, etiology, and costs of preterm birth. *Seminars in Fetal and Neonatal Medicine*, *21*(2), 68–73. https://doi.org/10.1016/j.siny.2015.12.011

Fu, J., Zhao, L., Wang, L., & Zhu, X. (2015). Expression of markers of endoplasmic reticulum stress-induced apoptosis in the placenta of women with early and late onset severe pre-eclampsia. *Taiwanese Journal of Obstetrics and Gynecology*, *54*(1), 19–23. https://doi.org/10.1016/j.tjog.2014.11.002

Gething, M.-J. (1999). Role and regulation of the ER chaperone BiP. *Cell & Developmental Biology*, *10*(5), 465–472.

Gharesi-Fard, B., Zolghadri, J., & Kamali-Sarvestani, E. (2015). Proteome differences in the first-and third-trimester human placentas. *Reproductive Sciences*, *22*(4), 462–468. https://doi.org/10.1177/1933719114549857

Ghezzi, F., Franchi, M., Raio, L., Di Naro, E., Bossi, G., D'Eril, G. V. M., & Bolis, P. (2002). Elevated amniotic fluid C-reactive protein at the time of genetic amniocentesis is a marker for preterm delivery. *American Journal of Obstetrics and Gynecology*, *186*(2), 268–273. https://doi.org/10.1067/mob.2002.119628

Glover, A. V., & Manuck, T. A. (2018). Screening for spontaneous preterm birth and resultant therapies to reduce neonatal morbidity and mortality: A review. *Seminars in Fetal and Neonatal Medicine*, *23*(2), 126–132. https://doi.org/10.1016/j.siny.2017.11.007

Goldenberg, R. L., Culhane, J. F., Iams, J. D., & Romero, R. (2008). Preterm birth: epidemiology and causes of preterm birth. *Lancet*, *371*(9606), 75–84. https://doi.org/doi: 10.1016/S0140-6736(08)60074-4

Golightly, E., Jabbour, H. N., & Norman, J. E. (2011). Endocrine immune interactions in human parturition. *Molecular and Cellular Endocrinology*, *335*(1), 52–59. https://doi.org/10.1016/j.mce.2010.08.005

Gomez-Lopez, N., Galaz, J., Miller, D., Farias-Jofre, M., Liu, Z., Arenas-Hernandez, M., Garcia-Flores, V., Shaffer, Z., Greenberg, J. M., Theis, K. R., & Romero, R. (2022). The immunobiology of preterm labor and birth: intra-amniotic inflammation or breakdown of maternal-fetal homeostasis. *Reproduction*, *164*(2), R11–R45. https://doi.org/10.1530/REP

Gomez-Lopez, N., StLouis, D., Lehr, M. A., Sanchez-Rodriguez, E. N., & Arenas-Hernandez, M. (2014). Immune cells in term and preterm labor. *Cellular & Molecular Immunology*, *11*(6), 571–581. https://doi.org/10.1038/cmi.2014.46

Gourvas, V., Soulitzis, N., Konstantinidou, A., Dalpa, E., Koukoura, O., Koutroulakis, D., Spandidos, D. A., & Sifakis, S. (2014). Reduced ANXA5 mRNA and protein expression in pregnancies complicated by preeclampsia. *Thrombosis Research*, *133*(3), 495–500. https://doi.org/10.1016/j.thromres.2013.12.027

Gude, N. M., Roberts, C. T., Kalionis, B., & King, R. G. (2004). Growth and function of the normal human placenta. *Thrombosis Research*, *114*(5–6), 397–407. https://doi.org/10.1016/j.thromres.2004.06.038

Guller, S., Ma, Y., Raju, U., Kadner, S., Thung, S. F., Colasacco, L., Malek, A., & Schneider, H. (2003). Release of oncofetal fibronectin from human placenta. *Placenta*, *24*(8–9), 843–850. https://doi.org/10.1016/S0143-4004(03)00131-0

Gunko, V. O., Pogorelova, T. N., & Linde, V. A. (2016). Proteomic profiling of the blood serum for prediction of premature delivery. *Bulletin of Experimental Biology and Medicine*, *161*(6), 829–832. https://doi.org/10.1007/s10517-016-3522-z

Haapalainen, A. M., Daddali, R., Hallman, M., & Rämet, M. (2021). Human CPPED1 belongs to calcineurin-like metallophosphoesterase superfamily and dephosphorylates PI3K-AKT pathway component PAK4. *Journal of Cellular and Molecular Medicine*, *25*(13), 6304–6317. https://doi.org/10.1111/jcmm.16607

Haapalainen, A. M., Karjalainen, M. K., Daddali, R., Ohlmeier, S., Anttonen, J., Määttä, T. A., Salminen, A., Mahlman, M., Bergmann, U., Mäkikallio, K., Ojaniemi, M., Hallman, M., & Rämet, M. (2018). Expression of CPPED1 in human trophoblasts is associated with timing of term birth. *Journal of Cellular and Molecular Medicine*, *22*(2), 968–981. https://doi.org/10.1111/jcmm.13402

Hallman, M., Haapalainen, A., Huusko, J. M., Karjalainen, M. K., Zhang, G., Muglia, L. J., & Rämet, M. (2019). Spontaneous premature birth as a target of genomic research. *Pediatric Research*, *85*(4), 422–431. https://doi.org/10.1038/s41390-018-0180-z

Heikkilä, K., Metsälä, J., Pulakka, A., Nilsen, S. M., Kivimäki, M., Risnes, K., & Kajantie, E. (2023). Preterm birth and the risk of multimorbidity in adolescence: a multiregister-based cohort study. *Lancet Public Health*, *8*, e680–e690. www.thelancet.com/

Heywood, W. E., Preece, R. L., Pryce, J., Hallqvist, J., Clayton, R., Virasami, A., Mills, K., & Sebire, N. J. (2017). Proteomic profiling reveals sub proteomes of the human placenta. *Placenta*, *59*, 69–72. https://doi.org/10.1016/j.placenta.2017.09.014

Hiltunen, H., Collado, M. C., Ollila, H., Kolari, T., Tölkkö, S., Isolauri, E., Salminen, S., & Rautava, S. (2022). Spontaneous preterm delivery is reflected in both early neonatal and maternal gut microbiota. *Pediatric Research*, *91*(7), 1804–1811. https://doi.org/10.1038/s41390-021-01663-8

Huppertz, B. (2008). The anatomy of the normal placenta. *Journal of Clinical Pathology*, *61*(12), 1296–1302. https://doi.org/10.1136/jcp.2008.055277

Huppertz, B., Kertschanska, S., Frank, H.-G., Gaus, G., Funayama, H., & Kaufmann, P. (1996). Extracellular matrix components of the placental extravillous trophoblast: immunocytochemistry and ultrastructural distribution. *Histochemistry and Cell Biology*, *106*, 291–301.

Huusko, J. M., Karjalainen, M. K., Graham, B. E., Zhang, G., Farrow, E. G., Miller, N. A., Jacobsson, B., Eidem, H. R., Murray, J. C., Bedell, B., Breheny, P., Brown, N. W., Bødker, F. L., Litterman, N. K., Jiang, P. P., Russell, L., Hinds, D. A., Hu, Y., Rokas, A., … Muglia, L. J. (2018). Whole exome sequencing reveals HSPA1L as a genetic risk factor for spontaneous preterm birth. *PLoS Genetics*, *14*(7), e1007394. https://doi.org/10.1371/journal.pgen.1007394

Huusko, J. M., Tiensuu, H., Haapalainen, A. M., Pasanen, A., Tissarinen, P., Karjalainen, M. K., Zhang, G., Christensen, K., Ryckman, K. K., Jacobsson, B., Murray, J. C., Kingsmore, S. F., Hallman, M., Muglia, L. J., & Rämet, M. (2021). Integrative genetic, genomic and transcriptomic analysis of heat shock protein and nuclear hormone receptor gene associations with spontaneous preterm birth. *Scientific Reports*, *11*(1), 17115. https://doi.org/10.1038/s41598-021-96374-9

Hvilsom, G. B., Thorsen, P., Jeune, B., & Bakketeig, L. S. (2002). C-reactive protein: A serological marker for preterm delivery? *Acta Obstetricia et Gynecologica Scandinavica*, *81*(5), 424. https://doi.org/10.1034/j.1600-0412.2002.810509.x

Ilicic, M., Zakar, T., & Paul, J. W. (2020). The regulation of uterine function during parturition: an update and recent advances. *Reproductive Sciences*, *27*(1), 3–28. https://doi.org/10.1007/s43032-019-00001-y

Izumi-Yoneda, N., Toda, A., Okabe, M., Koike, C., Takashima, S., Yoshida, T., Konishi, I., Saito, S., & Nikaido, T. (2009). Alpha 1 antitrypsin activity is decreased in human amnion in premature rupture of the fetal membranes. *Molecular Human Reproduction*, *15*(1), 49–57. https://doi.org/10.1093/molehr/gan071

Jaiman, S., Romero, R., Bhatti, G., Jung, E., Gotsch, F., Suksai, M., Gallo, D. M., Chaiworapongsa, T., & Kadar, N. (2022). The role of the placenta in spontaneous preterm labor and delivery with intact membranes. *Journal of Perinatal Medicine*, *50*(5), 553–566. https://doi.org/10.1515/jpm-2021-0681

Jain, V. G., Monangi, N., Zhang, G., & Muglia, L. J. (2022). Genetics, epigenetics, and transcriptomics of preterm birth. *American Journal of Reproductive Immunology*, *88*(4), e13600. https://doi.org/10.1111/aji.13600

Jansen, C., Kastelein, A., Kleinrouweler, C., Van Leeuwen, E., De Jong, K., Pajkrt, E., & Van Noorden, C. (2020). Development of placental abnormalities in location and anatomy. *Acta Obstetricia et Gynecologica Scandinavica*, *99*, 983–993. https://doi.org/https://doi. org/10.1111/aogs.13834

Jenkin, G., & Young, I. R. (2004). Mechanisms responsible for parturition; the use of experimental models. *Animal Reproduction Science*, *82–83*, 567–581. https://doi.org/10.1016/j.anireprosci.2004.05.010

Jezela-Stanek, A., & Chorostowska-Wynimko, J. (2019). Beyond the lungs: alpha-1 antitrypsin's potential role in human gestation. *Advances in Clinical and Experimental Medicine*, *28*(9), 1257–1261. https://doi.org/10.17219/acem/103668

Kacerovsky, M., Lenco, J., Musilova, I., Tambor, V., Lamont, R., Torloni, M. R., & Menon, R. (2014). Proteomic biomarkers for spontaneous preterm birth: A systematic review of the literature. *Reproductive Sciences*, *21*(3), 283–295. https://doi.org/10.1177/1933719113503415

Kamel, R. M. (2010). The onset of human parturition. *Archives of Gynecology and Obstetrics*, *281*(6), 975–982. https://doi.org/10.1007/s00404-010-1365-9

Karjalainen, M. K., Ojaniemi, M., Haapalainen, A. M., Mahlman, M., Salminen, A., Huusko, J. M., Määttä, T. A., Kaukola, T., Anttonen, J., Ulvila, J., Haataja, R., Teramo, K., Kingsmore, S. F., Palotie, A., Muglia, L. J., Rämet, M., & Hallman, M. (2015). CXCR3 polymorphism and expression associate with spontaneous preterm birth. *Journal of Immunology*, *195*(5), 2187–2198. https://doi.org/10.4049/jimmunol.1501174

Kaseva, N., Kuula, J., Sandboge, S., Hauta-alus, H., Björkqvist, J., Hovi, P., Eriksson, J. G., Vihervaara, T., Pietiläinen, K. H., & Kajantie, E. (2023). Cardiometabolic health in adults born with very low birth weight—a sibling study. *Pediatric Research*. https://doi.org/10.1038/s41390-023-02828-3

Kaufmann, P., Huppertz, B., Frank, H.-G., Semper, I., & Fischer Verlag lena, G. (1996). The fibrinoids of the human placenta: origin, composition and functional relevance. *Annals of Anatomy*, *178*, 485–501.

Keelan, J. A., Blumenstein, M., Helliwell, R. J. A., Sato, T. A., Marvin, K. W., & Mitchell, M. D. (2003). Cytokines, prostaglandins and parturition - a review. *Placenta*, *24*(SUPPL. A). https://doi.org/10.1053/plac.2002.0948

Khorami Sarvestani, S., Shojaeian, S., Vanaki, N., Ghresi-Fard, B., Amini, M., Gilany, K., Soltanghoraee, H., Arefi, S., Jeddi-Tehrani, M., & Zarnani, A. (2021). Proteome profiling of human placenta reveals developmental stage-dependent alterations in protein signature. *Clinical Proteomics*, *18*(1), 18. https://doi.org/10.1186/s12014-021-09324-y

Kim, S. J., Woo, J. R., Seo, E. J., Yu, M. H., & Ryu, S. E. (2001). A 2.1 Å resolution structure of an uncleaved α1-antitrypsin shows variability of the reactive center and other loops. *Journal of Molecular Biology*, *306*(1), 109–119. https://doi.org/10.1006/jmbi.2000.4357

Kim, Y. E., Kim, K., Oh, H. Bin, Lee, S. K., & Kang, D. (2021). Quantitative proteomic profiling of cervicovaginal fluid from pregnant women with term and preterm birth. *Proteome Science*, *19*(1). https://doi.org/10.1186/s12953-021-00171-1

Köhnlein, T., & Welte, T. (2008). Alpha-1 antitrypsin deficiency: pathogenesis, clinical presentation, diagnosis, and treatment. *American Journal of Medicine*, *121*(1), 3–9. https://doi.org/10.1016/j.amjmed.2007.07.025

Kruger, L., Yue, G., Paquette, A., Sathyanarayana, S., Enquobahrie, D. A., Bammler, T. K., MacDonald, J., Zhao, Q., & Prasad, B. (2023). An optimized proteomics-based approach to estimate blood contamination and cellular heterogeneity of frozen placental tissue. *Placenta*, *131*, 111–118. https://doi.org/10.1016/j.placenta.2022.12.007

Larsson, A., Palm, M., Hansson, L. O., Basu, S., & Axelsson, O. (2008). Reference values for α1-acid glycoprotein, α1-antitrypsin, albumin, haptoglobin, C-reactive protein, IgA, IgG and IgM during pregnancy. *Acta Obstetricia et Gynecologica Scandinavica*, *87*(10), 1084–1088. https://doi.org/10.1080/00016340802428146

Law, K. P., Han, T. L., Tong, C., & Baker, P. N. (2015). Mass spectrometry-based proteomics for pre-eclampsia and preterm birth. *International Journal of Molecular Sciences*, *16*(5), 10952–10985. https://doi.org/10.3390/ijms160510952

Lee, A. S. (2005). The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. *Methods*, *35*(4), 373–381. https://doi.org/10.1016/j.ymeth.2004.10.010

Lehtinen, M., Surcel, H. M., Natunen, K., Pukkala, E., & Dillner, J. (2017). Cancer registry follow-up for 17 million person-years of a nationwide maternity cohort. *Cancer Medicine*, *6*(12), 3060–3064. https://doi.org/10.1002/cam4.1222

Leow, S. M., Di Quinzio, M. K. W., Ng, Z. L., Grant, C., Amitay, T., Wei, Y., Hod, M., Sheehan, P. M., Brennecke, S. P., Arbel, N., & Georgiou, H. M. (2020). Preterm birth prediction in asymptomatic women at mid-gestation using a panel of novel protein biomarkers: the Prediction of PreTerm Labor (PPeTaL) study. *American Journal of Obstetrics and Gynecology MFM*, *2*(2). https://doi.org/10.1016/j.ajogmf.2019.100084

Lin, Z., Shi, J. L., Chen, M., Zheng, Z. M., Li, M. Q., & Shao, J. (2023). CCL2: an important cytokine in normal and pathological pregnancies: a review. *Frontiers in Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.1053457

Liong, S., Di Quinzio, M. K. W., Fleming, G., Permezel, M., Rice, G. E., & Georgiou, H. M. (2015). New biomarkers for the prediction of spontaneous preterm labour in symptomatic pregnant women: a comparison with fetal fibronectin. *BJOG*, *122*(3), 370–379. https://doi.org/10.1111/1471-0528.12993

Liong, S., & Lappas, M. (2014). Endoplasmic reticulum stress is increased after spontaneous labor in human fetal membranes and myometrium where it regulates the expression of prolabor mediators. *Biology of Reproduction* , *91*(3), 1–19. https://doi.org/10.1095/biolreprod.114.120741

Lisowska-Myjak, B., & Pachecka, J. (2003). Antigenic and functional levels of alpha-1-antitrypsin in serum during normal and diabetic pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *106*(1), 31–35. https://doi.org/doi: 10.1016/s0301-2115(02)00201-4

Lisowska-Myjak, B., Sygitowicz, G. Ç., Wolf, B., & Pachecka, J. (2001). Serum alpha-1-antitrypsin concentration during normal and diabetic pregnancy. *European Journal of Obstetrics & Gynecology and Reproductive Biology*, *99*, 53–56. https://doi.org/doi: 10.1016/s0301-2115(01)00356-6

Liu, S., Diao, L., Huang, C., Li, Y., Zeng, Y., & Kwak-Kim, J. Y. H. (2017). The role of decidual immune cells on human pregnancy. *Journal of Reproductive Immunology*, *124*, 44–53. https://doi.org/10.1016/j.jri.2017.10.045

Lohsoonthorn, V., Qiu, C., & Williams, M. A. (2007). Maternal serum C-reactive protein concentrations in early pregnancy and subsequent risk of preterm delivery. *Clinical Biochemistry*, *40*(5–6), 330–335. https://doi.org/10.1016/j.clinbiochem.2006.11.017

Lu, P., Takai, K., Weaver, V. M., & Werb, Z. (2011). Extracellular matrix degradation and remodeling in development and disease. *Cold Spring Harbor Perspectives in Biology*, *3*(12). https://doi.org/10.1101/cshperspect.a005058

Luoto, R., Jartti, T., Ruuskanen, O., Waris, M., Lehtonen, L., & Heikkinen, T. (2016). Review of the clinical significance of respiratory virus infections in newborn infants. *Acta Paediatrica*, *105*(10), 1132–1139. https://doi.org/10.1111/apa.13519

Madar, T., Shahaf, G., Sheiner, E., Brazg, J., Levinson, J., Yaniv Salem, S., Twina, G., Baron, J., Mazor, M., Holcberg, G., & Lewis, E. C. (2013). Low levels of circulating alpha-1 antitrypsin are associated with spontaneous abortions. *Journal of Maternal-Fetal and Neonatal Medicine*, *26*(18), 1782–1787. https://doi.org/10.3109/14767058.2013.801955

McElroy, J. J., Gutman, C. E., Shaffer, C. M., Busch, T. D., Puttonen, H., Teramo, K., Murray, J. C., Hallman, M., & Muglia, L. J. (2013). Maternal coding variants in complement receptor 1 and spontaneous idiopathic preterm birth. *Human Genetics*, *132*(8), 935–942. https://doi.org/10.1007/s00439-013-1304-5

McLean, M., Bisits, A., Davies, J., Woods, R., Lowry, P., & Smith, R. (1995). A placental clock controlling the length of human pregnancy. *Nature Medicine*, *1*(5), 460–463. https://doi.org/doi: 10.1038/nm0595-460

Melchor, J. C., Khalil, A., Wing, D., Schleussner, E., & Surbek, D. (2018). Prediction of preterm delivery in symptomatic women using PAMG-1, fetal fibronectin and phiGFBP-1 tests: Systematic review and meta-analysis. *Ultrasound in Obstetrics & Gynecology* , *52*(4), 442–451. https://doi.org/10.1002/uog.19119

Mendelson, C. R., Montalbano, A. P., & Gao, L. (2017). Fetal-to-maternal signaling in the timing of birth. *Journal of Steroid Biochemistry and Molecular Biology*, *170*, 19–27. https://doi.org/10.1016/j.jsbmb.2016.09.006

Meniailo, M. E., Malashchenko, V. V., Shmarov, V. A., Gazatova, N. D., Melashchenko, O. B., Goncharov, A. G., Seledtsova, G. V., & Seledtsov, V. I. (2018). Interleukin-8 favors pro-inflammatory activity of human monocytes/macrophages. *International Immunopharmacology*, *56*, 217–221. https://doi.org/10.1016/j.intimp.2018.01.036

Menon, R., & Taylor, B. D. (2019). Exploring inflammatory mediators in fetal and maternal compartments during human parturition. *Obstetrics & Gynecology*, *134*(4), 765–773. https://doi.org/10.1097/AOG.0000000000003470

Morgan, A. S., Mendonça, M., Thiele, N., & David, A. L. (2022). Management and outcomes of extreme preterm birth. *BMJ*, *376*, e055924. https://doi.org/10.1136/bmj-2021-055924

Moutquin, J. M. (2003). Classification and heterogeneity of preterm birth. *BJOG*, *110 Suppl*, 30–33. https://doi.org/10.1016/S1470-0328(03)00021-1

Msheik, H., Azar, J., El Sabeh, M., Abou-Kheir, W., & Daoud, G. (2020). HTR-8/SVneo: a model for epithelial to mesenchymal transition in the human placenta. *Placenta*, *90*, 90–97. https://doi.org/10.1016/j.placenta.2019.12.013

Murray, S. A., Morgan, J. L., Kane, C., Sharma, Y., Heffner, C. S., Lake, J., & Donahue, L. R. (2010). Mouse gestation length is genetically determined. *PLoS ONE*, *5*(8), e12418. https://doi.org/10.1371/journal.pone.0012418

Mushahary, D., Gautam, P., Sundaram, C. S., & Sirdeshmukh, R. (2013). Expanded protein expression profile of human placenta using two-dimensional gel electrophoresis. *Placenta*, *34*(2), 193–196. https://doi.org/10.1016/j.placenta.2012.11.015

Newman, R. B., Goldenberg, R. L., Iams, J. D., Meis, P. J., Mercer, B. M., Moawad, A. H., Thom, E., Miodovnik, M., Caritis, S. N., & Dombrowski, M. (2008). Preterm prediction study comparison of the cervical score and Bishop score for prediction of spontaneous preterm delivery. *Obstetrics & Gynecology*, *112*(3), 508–515. www.greenjournal.org/cgi/content/full/112/3/508/DC1.

Nikolova, T., Uotila, J., Nikolova, N., Bolotskikh, V. M., Borisova, V. Y., & Di Renzo, G. C. (2018). Prediction of spontaneous preterm delivery in women presenting with premature labor: a comparison of placenta alpha microglobulin-1, phosphorylated insulin-like growth factor binding protein-1, and cervical length. *American Journal of Obstetrics and Gynecology*, *219*(6), 610.e1-610.e9. https://doi.org/10.1016/j.ajog.2018.09.016

Nitika, Porter, C. M., Truman, A. W., & Truttmann, M. C. (2020). Post-translational modifications of Hsp70 family proteins: expanding the chaperone code. *Journal of Biological Chemistry*, *295*(31), 10689–10708. https://doi.org/10.1074/jbc.REV120.011666

Nori, W., & Ali, A. I. (2021). Maternal alpha-1-antitrypsin as a noval marker for growth restriction in pre-eclampsia. *Journal of Obstetrics and Gynaecology Research*, *47*(12), 4250–4255. https://doi.org/10.1111/jog.15043

Ohuma, E. O., Moller, A.-B., Bradley, E., Chakwera, S., Hussain-Alkhateeb, L., Lewin, A., Okwaraji, Y. B., Mahanani, W. R., Johansson, E. W., Lavin, T., Estevez Fernandez, D., Gatica Domínguez, G., De Costa, A., Cresswell, J. A., Krasevec, J., Lawn, J. E., Blencowe, H., Requejo, J., & Moran, A. C. (2023). National, regional, and global estimates of preterm birth in 2020, with trends from 2010: a systematic analysis. *Lancet*, *402*(10409), 1261–1271. https://doi.org/doi: 10.1016/S0140-6736(23)00878-4

Okayama, H., Brantly, M., Holmes, M., & Crystal, R. G. (1991). Characterization of the molcular basis of the a1-antitrypsin F allele. *American Journal of Human Genetics*, *48*, 1154–1158.

Olsen, G. N., Harris, J., Castle, J. R., Waldman, R. H., & Karmgard, H. J. (1975). Alpha 1 antitrypsin content in the serum, alveolar macrophages, and alveolar lavage fluid of smoking and nonsmoking normal subjects. *Journal of Clinical Investigation*, *55*(2), 427–430. https://doi.org/10.1172/JCI107947

Paquette, A. G., MacDonald, J., Bammler, T., Day, D. B., Loftus, C. T., Buth, E., Mason, W. A., Bush, N. R., Lewinn, K. Z., Marsit, C., Litch, J. A., Gravett, M., Enquobahrie, D. A., & Sathyanarayana, S. (2023). Placental transcriptomic signatures of spontaneous preterm birth. *American Journal of Obstetrics and Gynecology*, *228*(1), 73.e1-73.e18. https://doi.org/10.1016/j.ajog.2022.07.015

Parets, S. E., Knight, A. K., & Smith, A. K. (2015). Insights into genetic susceptibility in the etiology of spontaneous preterm birth. *The Application of Clinical Genetics*, *8*, 283–290. https://doi.org/10.2147/TACG.S58612

Pasanen, A., Karjalainen, M. K., Zhang, G., Tiensuu, H., Haapalainen, A. M., Ojaniemi, M., Feenstra, B., Jacobsson, B., Palotie, A., Laivuori, H., Muglia, L. J., Rämet, M., & Hallman, M. (2023). Meta-analysis of genome-wide association studies of gestational duration and spontaneous preterm birth identifies new maternal risk loci. *PLOS Genetics*, *19*(10), e1010982. https://doi.org/10.1371/journal.pgen.1010982

Pereira, L., Reddy, A. P., Jacob, T., Thomas, A., Schneider, K. A., Dasari, S., Lapidus, J. A., Lu, X., Rodland, M., Roberts, C. T., Gravett, M. G., & Nagalla, S. R. (2007). Identification of novel protein biomarkers of preterm birth in human cervical-vaginal fluid. *Journal of Proteome Research*, *6*(4), 1269–1276. https://doi.org/10.1021/pr0605421

Pereyra, S., Sosa, C., Bertoni, B., & Sapiro, R. (2019). Transcriptomic analysis of fetal membranes reveals pathways involved in preterm birth. *BMC Medical Genomics*, *12*(1). https://doi.org/10.1186/s12920-019-0498-3

Perin, J., Mulick, A., Yeung, D., Villavicencio, F., Lopez, G., Strong, K. L., Prieto-Merino, D., Cousens, S., Black, R. E., & Liu, L. (2022). Global, regional, and national causes of under-5 mortality in 2000–19: an updated systematic analysis with implications for the Sustainable Development Goals. *The Lancet Child and Adolescent Health*, *6*(2), 106–115. https://doi.org/10.1016/S2352-4642(21)00311-4

Pique-Regi, R., Romero, R., Tarca, A. L., Sendler, E. D., Xu, Y., Garcia-Flores, V., Leng, Y., Luca, F., Hassan, S. S., & Gomez-Lopez, N. (2019). Single cell transcriptional signatures of the human placenta in term and preterm parturition. *ELife*, *8*, e52004. https://doi.org/10.7554/eLife.52004

Pitiphat, W., Gillman, M. W., Joshipura, K. J., Williams, P. L., Douglass, C. W., & Rich-Edwards, J. W. (2005). Plasma C-reactive protein in early pregnancy and preterm delivery. *American Journal of Epidemiology*, *162*(11), 1108–1113. https://doi.org/10.1093/aje/kwi323

Pulakka, A., Risnes, K., Metsälä, J., Alenius, S., Heikkilä, K., Nilsen, S. M., Näsänen-Gilmore, P., Haaramo, P., Gissler, M., Opdahl, S., & Kajantie, E. (2023). Preterm birth and asthma and COPD in adulthood: a nationwide register study from two Nordic countries. *European Respiratory Journal*, *61*(6). https://doi.org/10.1183/13993003.01763-2022

Rokas, A., Mesiano, S., Tamam, O., Labella, A., Zhang, G., & Muglia, L. (2020). Developing a theoretical evolutionary framework to solve the mystery of parturition initiation. *ELife*, *9*, 1–22. https://doi.org/10.7554/ELIFE.58343

Romero, R., Dey, S. K., & Fisher, S. J. (2014). Preterm labor: One syndrome, many causes. *Science*, *345*(6198), 760–765. https://doi.org/10.1126/science.1251816

Romero, R., Espinoza, J., Kusanovic, J. P., Gotsch, F., Hassan, S., Erez, O., Chaiworapongsa, T., & Mazor, M. (2006). The preterm parturition syndrome. *BJOG*, *113*(SUPPL. 3), 17–42. https://doi.org/10.1111/j.1471-0528.2006.01120.x

Saade, G. R., Boggess, K. A., Sullivan, S. A., Markenson, G. R., Iams, J. D., Coonrod, D. V., Pereira, L. M., Esplin, M. S., Cousins, L. M., Lam, G. K., Hoffman, M. K., Severinsen, R. D., Pugmire, T., Flick, J. S., Fox, A. C., Lueth, A. J., Rust, S. R., Mazzola, E., Hsu, C., … Hickok, D. E. (2016). Development and validation of a spontaneous preterm delivery predictor in asymptomatic women. *American Journal of Obstetrics and Gynecology*, *214*(5), 633.e1-633.e24. https://doi.org/10.1016/j.ajog.2016.02.001

Sanders, C. L., Ponte, A., & Kueppers, F. (2018). The effects of inflammation on alpha 1 antitrypsin levels in a national screening cohort. *COPD: Journal of Chronic Obstructive Pulmonary Disease*, *15*(1), 10–16. https://doi.org/10.1080/15412555.2017.1401600

Seaby, E. G., Pengelly, R. J., & Ennis, S. (2016). Exome sequencing explained: a practical guide to its clinical application. *Briefings in Functional Genomics*, *15*(5), 374–384. https://doi.org/10.1093/bfgp/elv054

Seixas, S., & Marques, P. I. (2021). Known mutations at the cause of alpha-1 antitrypsin deficiency an updated overview of SERPINA1 variation spectrum. *The Application of Clinical Genetics*, *14*, 173–194. https://doi.org/10.2147/TACG.S257511

Shankar, R., Johnson, M. P., Williamson, N. A., Cullinane, F., Purcell, A. W., Moses, E. K., & Brennecke, S. P. (2010). Molecular markers of preterm labor in the choriodecidua. *Reproductive Sciences*, *17*(3), 297–310. https://doi.org/10.1177/1933719109353454

Shibata, S., Kobayashi, E. H., Kobayashi, N., Oike, A., Okae, H., & Arima, T. (2020). Unique features and emerging in vitro models of human placental development. *Reproductive Medicine and Biology*, *19*(4), 301–313. https://doi.org/10.1002/rmb2.12347

Shynlova, O., Nadeem, L., Zhang, J., Dunk, C., & Lye, S. (2020). Myometrial activation: novel concepts underlying labor. *Placenta*, *92*, 28–36. https://doi.org/10.1016/j.placenta.2020.02.005

Silva, D., Oliveira, M. J., Guimarães, M., Lima, R., Gomes, S., & Seixas, S. (2016). Alpha-1-antitrypsin (SERPINA1) mutation spectrum: Three novel variants and haplotype characterization of rare deficiency alleles identified in Portugal. *Respiratory Medicine*, *116*, 8–18. https://doi.org/10.1016/j.rmed.2016.05.002

Smith, R. (2007). Mechanisms of disease: parturition. *The New England Journal of Medicine*, *356*, 271–283. https://doi.org/doi: 10.1056/NEJMra061360

Solé-Navais, P., Flatley, C., Steinthorsdottir, V., Vaudel, M., Juodakis, J., Chen, J., Laisk, T., LaBella, A. L., Westergaard, D., Bacelis, J., Brumpton, B., Skotte, L., Borges, M. C., Helgeland, Ø., Mahajan, A., Wielscher, M., Lin, F., Briggs, C., Wang, C. A., … Jacobsson, B. (2023). Genetic effects on the timing of parturition and links to fetal birth weight. *Nature Genetics*, *55*(4), 559–567. https://doi.org/10.1038/s41588-023-01343-9

Son, M., & Miller, E. S. (2017). Predicting preterm birth: Cervical length and fetal fibronectin. *Seminars in Perinatology*, *41*(8), 445–451. https://doi.org/10.1053/j.semperi.2017.08.002

Sorokin, Y., Romero, R., Mele, L., Wapner, R. J., Iams, J. D., Dudley, D. J., Spong, C. Y., Peaceman, A. M., Leveno, K. J., Harper, M., Caritis, S. N., Miodovnik, M., Mercer, B. M., Thorp, J. M., O'Sullivan, M. J., Ramin, S. M., Carpenter, M. W., Rouse, D. J., & Sibai, B. (2010). Maternal serum interleukin-6, C-reactive protein, and matrix metalloproteinase-9 concentrations as risk factors for preterm birth <32 weeks and adverse neonatal outcomes. *American Journal of Perinatology*, *27*(8), 631–639. https://doi.org/10.1055/s-0030-1249366

Spong, C. Y. (2013). Defining "term" pregnancy: recommendations from the defining "term" pregnancy workgroup. *JAMA*, *309*(23), 2445–2446. https://doi.org/10.1001/jama.2013.6235

Starodubtseva, N., Nizyaeva, N., Baev, O., Bugrova, A., Gapaeva, M., Muminova, K., Kononikhin, A., Frankevich, V., Nikolaev, E., & Sukhikh, G. (2020). SERPINA1 peptides in urine as a potential marker of preeclampsia severity. *International Journal of Molecular Sciences*, *21*(3). https://doi.org/10.3390/ijms21030914

Strauss, J. F., Romero, R., Gomez-Lopez, N., Haymond-Thornburg, H., Modi, B. P., Teves, M. E., Pearson, L. N., York, T. P., & Schenkein, H. A. (2018). Spontaneous preterm birth: advances toward the discovery of genetic predisposition. *American Journal of Obstetrics and Gynecology*, *218*(3), 294–314.e2. https://doi.org/10.1016/j.ajog.2017.12.009

Suff, N., Story, L., & Shennan, A. (2019). The prediction of preterm delivery: what is new? *Seminars in Fetal and Neonatal Medicine*, *24*(1), 27–32. https://doi.org/10.1016/j.siny.2018.09.006

Suomen virallinen tilasto (SVT). (2023, June 30). *Preliminary data: perinatal statistics – parturients, deliveries and newborns 2022*. THL Tilastoraportti. https://thl.fi/fi/tilastot-ja-data/tilastot-aiheittain/seksuaali-ja-lisaantymisterveys/synnyttajat-synnytykset-ja-vastasyntyneet/perinataalitilasto-synnyttajat-synnytykset-ja-vastasyntyneet

Suresh, S. C., Freedman, A. A., Hirsch, E., & Ernst, L. M. (2022). A comprehensive analysis of the association between placental pathology and recurrent preterm birth. *American Journal of Obstetrics and Gynecology*, *227*(6), 887.e1–887.e15. https://doi.org/10.1016/j.ajog.2022.06.030

Tiensuu, H., Haapalainen, A. M., Karjalainen, M. K., Pasanen, A., Huusko, J. M., Marttila, R., Ojaniemi, M., Muglia, L. J., Hallman, M., & Rämet, M. (2019). Risk of spontaneous preterm birth and fetal growth associates with fetal SLIT2. *PLoS Genetics*, *15*(6), e1008107. https://doi.org/10.1371/journal.pgen.1008107

Tiensuu, H., Haapalainen, A. M., Tissarinen, P., Pasanen, A., Määttä, T. A., Huusko, J. M., Ohlmeier, S., Bergmann, U., Ojaniemi, M., Muglia, L. J., Hallman, M., & Rämet, M. (2022). Human Placental Proteomics and Exon Variant Studies Link AAT/SERPINA1 with Spontaneous Preterm Birth. *BMC Medicine*, *20*(1), 141.

Tjoa, M. L., Van Vugt, J. M. G., Go, A. T. J. J., Blankenstein, M. A., Oudejans, C. B. M., & Van Wijk, I. J. (2003). Elevated C-reactive protein levels during first trimester of pregnancy are indicative of preeclampsia and intrauterine growth restriction. *Journal of Reproductive Immunology*, *59*(1), 29–37. https://doi.org/10.1016/S0165-0378(02)00085-2

Törnblom, S. A., Klimaviciute, A., Byström, B., Chromek, M., Brauner, A., & Ekman-Ordeberg, G. (2005). Non-infected preterm parturition is related to increased concentrations of IL-6, IL-8 and MCP-1 in human cervix. *Reproductive Biology and Endocrinology*, *3*(39). https://doi.org/10.1186/1477-7827-3

Turco, M. Y., & Moffett, A. (2019). Development of the human placenta. *Development*, *146*(22), dev163428. https://doi.org/10.1242/dev.163428

Vannuccini, S., Bocchi, C., Severi, F. M., Challis, J. R., & Petraglia, F. (2016). Endocrinology of human parturition. *Annales d'Endocrinologie*, *77*(2), 105–113. https://doi.org/10.1016/j.ando.2016.04.025

Vogel, J. P., Chawanpaiboon, S., Moller, A. B., Watananirun, K., Bonet, M., & Lumbiganon, P. (2018). The global epidemiology of preterm birth. *Best Practice and Research Clinical Obstetrics and Gynaecology*, *52*, 3–12. https://doi.org/10.1016/j.bpobgyn.2018.04.003

Waddell, B. J., Wharfe, M. D., Crew, R. C., & Mark, P. J. (2012). A rhythmic placenta? Circadian variation, clock genes and placental function. *Placenta*, *33*(7), 533–539. https://doi.org/10.1016/j.placenta.2012.03.008

Wang, J., Lee, J., Liem, D., & Ping, P. (2017). HSPA5 Gene encoding Hsp70 chaperone BiP in the endoplasmic reticulum. *Gene*, *618*, 14–23. https://doi.org/10.1016/j.gene.2017.03.005

Weckman, A. M., Ngai, M., Wright, J., McDonald, C. R., & Kain, K. C. (2019). The impact of infection in pregnancy on placental vascular development and adverse birth outcomes. *Frontiers in Microbiology*, *10*(1924), 1–11. https://doi.org/10.3389/fmicb.2019.01924

Weiss, G. (2000). Endocrinology of parturition. *Journal of Clinical Endocrinology and Metabolism*, *85*(12), 4421–4425. https://doi: 10.1210/jcem.85.12.7074

Wennerholm, U.-B., Bergman, L., Kuusela, P., Ljungström, E., Möller, A. C., Hongslo Vala, C., Ekelund, A.-C., Liljegren, A., Petzold, M., Sjögren, P., Svensson, M., Strandell, A., & Jacobsson, B. (2023). Progesterone, cerclage, pessary, or acetylsalicylic acid for prevention of preterm birth in singleton and multifetal pregnancies – a systematic review and meta-analyses. *Frontiers in Medicine*, *10*. https://doi.org/10.3389/fmed.2023.1111315

Wijaya, J. C., Khanabdali, R., Georgiou, H. M., & Kalionis, B. (2020). Ageing in human parturition: impetus of the gestation clock in the decidua. *Biology of Reproduction*, *103*(4), 695–710. https://doi.org/10.1093/biolre/ioaa113

Working group set up by the Finnish Medical Society Duodecim and the Finnish Society of Obstetrics and Gynaecology. (2018). *Preterm birth: Current Care Guidelines*. Helsinki: The Finnish Medical Society Duodecim. https://www.kaypahoito.fi/hoi50089#s1

World Health Organization. (1970). The prevention of perinatal mortality and morbidity. *WHO Techical Report Series*, *No. 457*.

Xiong, Z., Wang, Q., Pei, S., Zheng, H., & Wang, W. (2023). CircRNA_0088196 regulates trophoblast proliferation and apoptosis in preeclampsia through the miR-379-5p/HSPA5 axis. *Biochemical Genetics*. https://doi.org/10.1007/s10528-023-10506-6

Xu, Y., Romero, R., Miller, D., Kadam, L., Mial, T. N., Plazyo, O., Garcia-Flores, V., Hassan, S. S., Xu, Z., Tarca, A. L., Drewlo, S., & Gomez-Lopez, N. (2016). An M1-like macrophage polarization in decidual tissue during spontaneous preterm labor that is attenuated by rosiglitazone treatment. *Journal of Immunology*, *196*(6), 2476–2491. https://doi.org/10.4049/jimmunol.1502055

Yaghmaei, M., Hashemi, M., Shikhzadeh, A., Mokhtari, M., Niazi, A., & Ghavami, S. (2009). Serum trypsin inhibitory capacity in normal pregnancy and gestational diabetes mellitus. *Diabetes Research and Clinical Practice*, *84*(3), 201–204. https://doi.org/10.1016/j.diabres.2009.03.003

Yang, J., Nune, M., Zong, Y., Zhou, L., & Liu, Q. (2015). Close and allosteric opening of the polypeptide-binding site in a human Hsp70 chaperone BiP. *Structure*, *23*(12), 2191–2203. https://doi.org/10.1016/j.str.2015.10.012

Yang, J., Zhang, H., Gong, W., Liu, Z., Wu, H., Hu, W., Chen, X., Wang, L., Wu, S., Chen, C., & Perrett, S. (2020). S-Glutathionylation of human inducible Hsp70 reveals a regulatory mechanism involving the C-terminal α-helical lid. *Journal of Biological Chemistry*, *295*(24), 8302–8324. https://doi.org/10.1074/jbc.RA119.012372

Yoshida, K., Kusama, K., Tamura, K., Fukushima, Y., Ohmaru-Nakanishi, T., & Kato, K. (2021). Alpha-1 antitrypsin-induced endoplasmic reticulum stress promotes invasion by extravillous trophoblasts. *International Journal of Molecular Sciences*, *22*(7), 3683. https://doi.org/10.3390/ijms22073683

Zakar, T., & Hertelendy, F. (2007). Progesterone withdrawal: key to parturition. *American Journal of Obstetrics and Gynecology*, *196*(4), 289–296. https://doi.org/10.1016/j.ajog.2006.09.005

Zhang, G., Feenstra, B., Bacelis, J., Liu, X., Muglia, L. M., Juodakis, J., Miller, D. E., Litterman, N., Jiang, P.-P., Russell, L., Hinds, D. A., Hu, Y., Weirauch, M. T., Chen, X., Chavan, A. R., Wagner, G. P., Pavličev, M., Nnamani, M. C., Maziarz, J., … Muglia, L. J. (2017). Genetic associations with gestational duration and spontaneous preterm birth. *The New England Journal of Medicine*, *377*(12), 1156–1167. https://doi.org/10.1056/nejmoa1612665

Zhang, P. (2021). CD42b immunostaining as a marker for placental fibrinoid in normal pregnancy and complications. *Fetal and Pediatric Pathology*, *40*(5), 387–394. https://doi.org/10.1080/15513815.2020.1716899

Zulu, M. Z., Martinez, F. O., Gordon, S., & Gray, C. M. (2019). The elusive role of placental macrophages: the hofbauer cell. *Journal of Innate Immunity*, *11*(6), 447–456. https://doi.org/10.1159/000497416

# Original publications

I Tiensuu, H.[#], Haapalainen, A. M.[#], Tissarinen, P., Pasanen, A., Määttä, T. A., Huusko, J. M., Ohlmeier, S., Bergmann, U., Ojaniemi, M., Muglia, L. J., Hallman, M.*, & Rämet, M.* (2022). Human placental proteomics and exon variant studies link AAT/SERPINA1 with spontaneous preterm birth. *BMC Medicine*, *20*(1), 141. https://doi.org/10.1186/s12916-022-02339-8.[1]

II Tissarinen, P., Tiensuu, H., Haapalainen, A. M., Määttä, T. A., Ojaniemi, M., Hallman, M.*, & Rämet, M.* (2023). Elevated human placental heat shock protein 5 is associated with spontaneous preterm birth. *Pediatric Research, 94*(2), 520–529. https://doi.org/10.1038/s41390-023-02501-9.

III Tissarinen, P., Tiensuu, H., Haapalainen, A. M., Ronkainen, E., Laatio, L., Vääräsmäki, M., Öhman, H., Hallman, M.*, & Rämet, M.*. Maternal serum alpha-1 antitrypsin levels in spontaneous preterm and term pregnancies. Submitted.

[#]*Equal contribution.

[1]The original publication I was also used in the doctoral thesis of Heli Tiensuu (University of Oulu, 2022).



Original publications are not included in the electronic version of the dissertation.

[2] https://creativecommons.org/licenses/by/4.0/

1765. Puhakka, Soile (2024) Natural residential environment and physical activity in adolescence and midlife : the role of greenness, landscape diversity, and nature relatedness

1766. Pöyry, Hilla (2024) Pediatric emergency department visits and parental assessment of acutely ill children

1767. Kerimaa, Heli (2024) Leikki-ikäisten lasten ja heidän vanhempiensa päiväkirurgiaan valmistaminen

1768. Valinen, Yrtti (2024) ADAM12 and first trimester trisomy screening

1769. Hanhivaara, Jaakko (2024) Lumbosacral transitional vertebrae : prevalence, associated degenerative changes, and association with low back pain in Finnish population

1770. Lehtovirta, Sami (2024) Utility of quantitative magnetic resonance imaging for articular cartilage assessment in clinical scenarios

1771. Li, Tianqi (2024) Epidemiological applications of quantitative urinary NMR metabolomics

1772. Aitavaara-Anttila, Mia (2024) Clinical pathway of the critically ill patient in Oulu University Hospital district

1773. Lipponen, Joonas (2024) MitAtax : hereditary ataxias in Northern Finland

1774. Pramila-Savukoski, Sari (2024) Hybridikoulutusintervention kehittäminen terveystieteiden geneerisen osaamisen varmistamiseksi

1775. Heikkinen, Anna-Leena (2024) Evaluation of cognitive complaints and neuropsychological performance in early-onset cognitive impairment and dementia

1776. Tervonen, Miikka (2024) Dexmedetomidine sedation in children

1777. Partanen, Terhi (2024) Central nervous system manifestations in Puumala hantavirus infection

1778. Nätynki, Antti (2024) Autoimmunization against collagen XVII in patients with high risk for bullous pemphigoid and the effect of vildagliptin on the mouse skin proteome

1779. Sarfraz, Sonia (2024) Bacterial adhesion on patient-specific implant materials

1780. Heikkilä, Laura (2024) Terveyden edistäminen liikuntaseurassa : nuorten ruokailutottumukset, urheilun tavoitteellisuus ja kehonkuva

Book orders:
Virtual book store
https://verkkokauppa.omapumu.com/fi/

UNIVERSITY OF OULU P.O. Box 8000 FI-90014 UNIVERSITY OF OULU FINLAND

# ACTA UNIVERSITATIS OULUENSIS




ISBN 978-952-62-4063-3 (Paperback)
ISBN 978-952-62-4064-0 (PDF)
ISSN 0355-3221 (Print)
ISSN 1796-2234 (Online)