Biomarkers in mid-trimester amniotic fluid in relation to gestational duration and spontaneous preterm delivery

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"Opportunities don't happen. You create them."

~ Chris Grosser ~

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PREFACE

This thesis is based on a unique cohort of women with singleton pregnancies and intact membranes, without preterm labor or signs of infection, who underwent mid-trimester amniocentesis for genetic testing at Sahlgenska University Hospital/Östra, Gothenburg, Sweden during 2008–2017. Its focus is on the composition of amniotic fluid as a key to gain a deeper understanding of the normal delivery process and the etiology of spontaneous preterm delivery. Fundamental concepts of pregnancy such as the placenta, fetal membranes and amniotic fluid are presented, as well as the delivery, with especial focus on preterm delivery in general and spontaneous preterm delivery in particular. The thesis has a particular emphasis on inflammation, as inflammatory processes are highly involved in pregnancy maintenance and delivery, both at term and at preterm. The thesis further comprises more summaries and discussions than descriptions in order to reflect thoughts and decisions made along the way, but also to minimize repetitions of the constituent papers. For the sake of clarity, the term “gestational age” is used when referring to the pregnancy, while “gestational duration” refers to the timing around delivery.

ABSTRACT

Background: The biological mechanisms and physiological pathways of pregnancy maintenance and timing of delivery are complex and multifactorial. Pregnancy clocks, partly controlled by timing mechanisms linked to fetal development, which regulate the onset of labor has previously been described. These clocks include inflammatory processes, involving endocrine, mechanical and genetic factors. However, the sequence and timing of events preceding the spontaneous onset of labor, both at term and at preterm, are as yet incompletely identified. Spontaneous preterm delivery, defined as delivery before 37 weeks of gestation, is a serious global health problem accounting for the majority of all perinatal deaths and half of the short- and long-term postnatal morbidity. Identifying women at risk of spontaneous preterm delivery is complicated by its heterogeneous etiology and several different sub-phenotypes. Mid-trimester amniocentesis, clinically performed for prenatal genetic testing, provides a unique opportunity to obtain insight into the intrauterine environment in asymptomatic women early in gestation. However, the complex and dynamic composition of amniotic fluid changes continually as pregnancy progresses, making early identification of factors involved in the process of spontaneous preterm delivery and other pregnancy complications, a major challenge.

Objective: The aim of this thesis and its constituent papers was to identify specific biomarkers related to the development of subsequent spontaneous preterm delivery, by examination of mid-trimester amniotic fluid composition in asymptomatic women. During the period of doctoral studies, new data emerged, indicating that a shift to gestational duration as the main outcome might increase the likelihood of finding associations that could assist in the prediction of spontaneous preterm delivery. The aim thus partly shifted toward investigating associations between mid-trimester amniotic fluid composition and gestational duration.

Material and methods: All constituent papers in this thesis are based on subsets of a single cohort of 1,240 amniotic fluid samples collected from asymptomatic women aged over 18 years with a singleton viable pregnancy, intact membranes, without preterm labor or signs of infection, undergoing genetic amniocentesis at gestational weeks 14-19 at Sahlgrenska University Hospital/Östra, Gothenburg, Sweden during September 2008 to December 2017. Demographics and clinical data were obtained from medical records at inclusion and after delivery. Studies investigating inflammatory, immuno-logical and cellular-metabolic markers were designed to contribute to early identification of women with subsequent spontaneous preterm delivery and to

study associations with gestational duration. Amniotic fluid samples were analyzed with targeted hypothesis-driven approaches using multiplex technologies such as Luminex xMAP and Meso-Scale Discovery, as well as with broad, untargeted hypothesis-generating approaches such as proteomics and metabolomics. The proteomics analyses were followed by validation/replication with Enzyme-Linked Immunosorbent Assay, a singleplex technology.

Results: No mid-trimester amniotic fluid biomarkers associated with spontaneous preterm delivery were identified. Thrombospondin-1, macrophage inflammatory protein-1 beta and S100 calcium-binding protein A8, two alarmins and one chemokine, were found to be significantly associated with gestational duration in women with a spontaneous onset of labor at term. Gestational age at sampling was strongly associated with protein concentrations in several of the constituent studies.

Conclusions: I) Biological signals in early mid-trimester amniotic fluid may be of insufficient strength for accurate risk prediction of spontaneous PTD, or the condition may result from acute events not detectable in amniotic fluid as early as at mid-trimester; II) Alarmins and chemokines, which seem to play an essential role in the inflammatory processes preceding the spontaneous onset of labor at term, can be detected in amniotic fluid as early as in the mid-trimester; III) The concept of a pregnancy clock is strengthened by our findings, which also suggest that this is reflected in the amniotic fluid, where deviations from the clock may precede spontaneous preterm delivery; and IV) The results emphasize the importance of adjusting for gestational age at sampling when performing amniotic fluid biomarker studies.

Keywords: amniotic fluid, biomarkers, cytokine, damage-associated molecular pattern, gestation, gestational duration, inflammation, labor, mid-trimester, multiplex, pregnancy clock, proteins, spontaneous preterm delivery, term delivery

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SAMMANFATTNING PÅ SVENSKA

Bakgrund: De biologiska och fysiologiska mekanismer som styr graviditetens fortskridande och som initierar förlossningsstart är komplexa och multifaktoriella. Graviditetsklockor, som reglerar förlossningsstarten och delvis kontrolleras av tidsmekanismer kopplade till fostrets utveckling, har tidigare beskrivits. Dessa klockor involverar inflammatoriska processer med inslag av endokrina, mekaniska och genetiska faktorer. Dock saknar vi idag grundläggande förståelse för de händelser som föregår den spontana förlossningsstarten samt vid vilken tidpunkt och hur dessa uppstår, såväl vid förlossning i fullgången tid som i förtid. Spontan förtidsbörd, förlossning före graviditetsvecka 37+0, är ett allvarligt globalt folkhälsoproblem som står för majoriteten av all neonatal mortalitet och hälften av den neonatala morbiditeten, både på kort och lång sikt. Tillståndets heterogena etiologi och olika subfenotyper försvårar möjligheten att identifiera kvinnor som riskerar att föda spontant för tidigt. En amniocentes i andra trimestern, utförd i enlighet med kliniska rutiner för fosterdiagnostik, skapar en unik möjlighet att få tillgång till biologiskt material som kan ge insikt i den intrauterina miljön hos asymtomatiska kvinnor i tidig graviditet. Fostervattnets komplexa och dynamiska sammansättning förändras dock i takt med att graviditeten fortskrider. Att tidigt identifiera faktorer som är involverade i utvecklingen av spontan förtidsbörd och andra graviditetskomplikationer är därför en stor utmaning.

Syfte: Syftet med denna avhandling och dess ingående delarbeten var att identifiera specifika biomarkörer relaterade till spontan förtidsbörd genom att studera sammansättningen av fostervattnet från asymtomatiska kvinnor i andra trimestern. Under projektets gång har nya forskningsresultat publicerats som indikerar att möjligheten att finna associationer som kan bidraga till att prediktera spontan förtidsbörd ökar om fokus istället läggs på att studera graviditetslängd som huvudsakligt utfall. Vårt fokus skiftades således delvis under tidens gång till att även studera associationer mellan fostervattnets sammansättning och graviditetslängd.

Material och metoder: Samtliga ingående delarbeten i denna avhandling är baserade på subgrupper ur en enda kohort bestående av 1240 fostervattenprover från asymtomatiska kvinnor ≥18 år med en viabel singelgraviditet som genomgick fosterdiagnostisk amniocentes i graviditetsvecka 14-19 vid Sahlgrenska Universitetssjukhuset/Östra, Göteborg, Sverige, under september 2008 till december 2017. Demografiska och kliniska data har samlats in från

medicinska journaler vid inklusion och efter förlossning. Studier som undersökte inflammatoriska och immunologiska markörer samt cellulär metabolism i fostervattnet utformades för att, i ett tidigt skede, kunna identifiera kvinnor som riskerarde att drabbas av spontan förtidsbörd samt för att bidraga med ny kunskap kring de processer som reglerar graviditetslängd. Fostervattenproverna analyserades med riktade hypotesdrivna multiplexmetoder såsom Luminex xMAP och Meso-Scale teknologi, samt med breda hypotesgenererande metoder såsom proteomik och metabolomik. Proteomikanalyserna följdes av validering/replikering med singleplex-analyser (enzymkopplad immunadsorberande analys).

Resultat: Vi fann inga biomarkörer i fostervattenprover från tidig andra trimester som var associerade med spontan förtidsbörd. Trombospondin-1, makrofaginflammatoriskt protein-1 beta och S100 kalciumbindande protein A8, två alarminer och en kemokin, visade sig vara signifikant associerade med tidpunkt för spontan förlossningsstart i fullgången tid. Gestationsålder vid provtagning var starkt associerad med proteinkoncentrationer i flera av de ingående delarbetena.

Slutsatser: I) Biologiska signaler i fostervatten från andra trimestern kan vara av otillräcklig styrka för att kunna förutsäga spontan förtidsbörd, eller så uppstår spontan förtidsbörd som en följd av akuta händelser som kanske inte kan upptäckas i fostervatten så tidigt som i andra trimestern; II) Alarminer och kemokiner, vilka verkar ha en väsentlig funktion i de inflammatoriska processer som föregår en spontan förlossningsstart i fullgången tid, kan identifieras redan i andra trimestern; III) Konceptet om en graviditetsklocka styrks av våra resultat, och de antyder att denna även reflekteras i fostervatten, där avvikelser från klockan skulle kunna föregå en spontan förtidsbörd; och IV) Resultaten av de ingående delarbetena betonar vikten av att justera för gestationsålder vid provtagning i studier där fostervatten analyseras i syfte att finna biomarkörer.

Nyckelord: fostervatten, biomarkörer, cytokin, damage-associated molecular patterns, graviditet, graviditetslängd, inflammation, förlossning, andra trimestern, multiplex, graviditetsklocka, proteiner, spontan förtidsbörd, fullgången tid

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals:

I. Hallingström M, Cobo T, Kacerovský K, Skogstrand K, Hougaard DM, Holst RM, Tsiartas P, Bullarbo M, Carlsson Y, Nilsson S, Jacobsson B. The association between selected mid-trimester amniotic fluid candidate proteins and spontaneous preterm delivery. J Matern Fetal Neonatal Med. 2018 Sep 10:1-10.

II. Hallingström M, Lenčo J, Vajrychová M, Link M, Tambor V, Liman V, Bullarbo M, Nilsson S, Tsiartas P, Cobo T, Kacerovský M, Jacobsson B. Proteomic analysis of early mid-trimester amniotic fluid does not predict spontaneous preterm delivery. PLoS One. 2016 May 23;11(5).

III. Hallingström M*, Zedníková P*, Tambor V, Barman M, Vajrychová M, Lenčo J, Viklund F, Tancred L, Rabe H, Jonsson D, Kachikis A, Nilsson S, Kacerovský M, Adams Waldorf K, Jacobsson B. Mid-trimester amniotic fluid proteome's association with spontaneous preterm delivery and gestational duration. Accepted for publication in PLoS One, April 2020.

IV. Viklund F*, Hallingström M*, Kacerovský M, Cobo T, Skogstrand K, Hougaard D M, Sävman K, Carlsson Y, Tsiartas P, Juodakis J, Nilsson S, Jacobsson B. Protein concentrations of thrombospondin-1, MIP-1β and S100A8 suggest the reflection of a pregnancy clock in mid-trimester amniotic fluid. Revision submitted to Reproductive Sciences, April 2020.

V. Hallingström M*, Barman M*, Savolinen O, Viklund F, Kacerovský M, Brunius C*, Jacobsson B*. Metabolomic profiles of mid-trimester amniotic fluid are not associated with subsequent spontaneous preterm delivery or gestational duration at delivery. Revision submitted to Journal of Maternal-Fetal & Neonatal Medicine, April 2020.

* Equal contribution

CONTENTS

1 Abbreviations

1.1 General abbreviations

AF	Amniotic fluid
BMI	Body mass index
°C	Degrees Celsius
CRL	Crown-rump length
CP	Cerebral palsy
HCA	Histological chorioamnionitis
IAI	Intra-amniotic inflammation
IVF	In vitro fertilization
LMP	Last menstrual period
MIAC	Microbial invasion of the amniotic cavity
NIPT	Non-invasive prenatal testing
PPROM	Preterm prelabor rupture of membranes
PTD	Preterm delivery
PTL	Preterm labor
SSI	Statens Serum Institut

1.2 Abbreviations related to analyses

ANCOVA	Analysis of covariance
ELISA	Enzyme-linked immunosorbent assay
g	Gravity (relative centrifugal force)
IQR	Interquartile range
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAP	Multi-analyte profiling
pg/mL	Picogram per milliliter
pH	Potential hydrogen
QF-PCR	Quantitative fluorescent-polymerase chain reaction
SD	Standard deviation

1.3 Abbreviations related to inflammatory markers

CRP	C-reactive protein
GM-CSF	Granulocyte-macrophage colony stimulating factor
DAMP	Damage-associated molecular pattern
HMG1	High-mobility group protein 1
HSP70	Heat-shock protein 70
IFN	Interferon
IGFBP	Insulin-like growth factor-binding protein
IL	Interleukin
MIF	Macrophage migration inhibitory factor
MIP	C-C motif chemokine [macrophage inflammatory protein]
MMP	Matrix metalloproteinase
NT	Neurotrophin
S100A8	S100 calcium-binding protein A8
WBC	White blood cell

2 Introduction

2.1 General introduction

Preterm delivery (PTD), defined as delivery occurring before 37 weeks of gestation, is the largest problem in current obstetric and neonatal care (1, 2) and a serious global health problem (3). Complications of PTD include severe short- and long-term morbidity (4) and it is the leading cause of neonatal death and of death in children aged under five (5). The prediction of PTD is complicated by its multifactorial and complex heterogeneous etiology, including several different sub-phenotypes (6). The two major ones are spontaneous and iatrogenic (medically indicated delivery due to maternal or fetal conditions) PTD (3), of which the former constitutes the focus of this thesis.

The process of spontaneous PTD is hypothesized to be initiated early in gestation (7). During the last decades, tremendous progress has taken place in neonatal and pediatric care, improving survival and decreasing the severity and frequency of morbidity in preterm-born infants (8). The rate has, however, been relatively stable in Sweden during the last 45 years (9). Few obstetric advances have resulted from the plethora of studies analyzing proteins, bacteria and metabolites in maternal blood, vaginal and cervical fluid and amniotic fluid. This research project therefore studied hitherto asymptomatic women in order to detect underlying processes in their early stages, with the aim of enabling identification of potential prognostic or diagnostic biomarkers before onset of symptoms. Improved understanding of the pathways leading to spontaneous PTD could lead to new insights in the spontaneous PTD etiology which eventually reduce the rate and related fatal consequences.

Amniotic fluid is the biological compartment with the highest likelihood of mirroring the dynamic intrauterine biological environment. This thesis therefore focus on mid-trimester amniotic fluid samples, obtained in conjunction with a clinically indicated ultrasound-guided transabdominal amniocentesis. The procedure is somewhat controversial since it aims at diagnosing fetal chromosomal abnormalities and is associated with an increased risk of miscarriage. However, recent studies suggest that the increase is as low as 0.06% to 0.13% (10-12). Due to the rapidly developing diagnostic landscape, amniocentesis rates have decreased significantly, indicating that samples such as those taken for this research will soon be a rarity.

Efforts in spontaneous PTD research have aimed at identifying a reliable and solid biomarker (13). However, early prediction of spontaneous PTD will most likely not be based on a single marker, but rather on coordinated networks (14). The papers employed hypothesis-driven or hypothesis-generating strategies to contribute to understanding of causal factors triggering the onset of labor. As yet, no biomarker has been identified that accurately predict spontaneous PTD, neither of asymptomatic women in the mid-trimester nor of symptomatic women in the late second or third trimesters. It is apparently challenging to predict spontaneous PTD early in gestation but even more challenging to affect the process later in gestation once symptoms have occurred. It is therefore crucial to identify new strategies to address this difficult situation.

The biological mechanisms and physiological pathways of pregnancy maintenance and the timing of delivery include inflammatory, immunological, endocrine, mechanical and genetic factors and processes, but the sequence and timing of events preceding the spontaneous onset of labor, both at term and at preterm, are as yet incompletely identified. During the last years, several studies have reported on different pregnancy clocks, partly controlled by timing mechanisms, which regulate the onset of labor by synchronizing to the fetal development (15-19). During the course of the research underlying this thesis, it was suggested that treating a variable such as gestational age as a continuous variable, using its full interval, instead of as a dichotomous trait (term/preterm) would increase the statistical power in identifying associations that could also assist in the prediction of spontaneous PTD (20). This is supported by genetic studies by our group in which we have identified several genes associated with gestational duration at term, a few of which are also related to spontaneous PTD (21). Data indicated that the phenotype of pregnancy maintenance and timing of delivery become more homogeneous as pregnancy progresses, causing stronger biological associations at term than at preterm. Our focus thereby expanded to also investigate associations between mid-trimester amniotic fluid composition and gestational duration. We hoped that this strategy would improve the chance of detecting associations that could contribute to the prediction of spontaneous PTD. As decreasing gestational duration within both the term and preterm intervals increases the risk of adverse neonatal outcome, this approach is also clinically relevant (4, 22).

2.2 Pregnancy

The first stage of embryonic development consists of fertilization, preceded by a two-week pre-embryonic development of the conceptus. The embryo devolves, by definition, into a fetus by the ninth gestational week (23). The fetus contains both maternal and paternal genetic material and the paternal genes are foreign to the pregnant woman. Under other circumstances, the immune system would attack and eliminate this foreign body (24). However, during gestation, signals and responses originating from the maternal immune system, in combination with the fetal-placental immune system, result in different immunological phases (25) that together prevent immune-mediated rejection (24, 26).

Pregnancy is characterized by three distinct immunological phases, also referred to as the three trimesters. Each trimesters is represented by different biological processes (24, 27) in pro-inflammatory or anti-inflammatory environments (28, 29). The pregnancy itself also elicits a general inflammatory response, with significantly increased serum concentrations of C-reactive protein (CRP), total white blood cells (WBC), neutrophils, granulocyte-macrophage colony stimulating factor (GM-CSF) and lactoferrin in all trimesters (30).

Inflammation is a key physiological process in pregnancy and delivery (31). The first trimester of pregnancy consists of a pro-inflammatory phase (24, 32) that extends into the early second trimester. Following implantation and placentation, a strong inflammatory response is necessary to adequately repair the uterine epithelium and remove cellular debris (33, 34). The second trimester of pregnancy, also referred to as the mid-trimester, entails rapid fetal growth and development and an anti-inflammatory state, in which the pregnant woman, placenta and fetus are in symbiosis (35, 36). The immune system must, however, still protect the pregnant woman and fetus from external harmful pathogens. This occurs through substantial expression of anti-inflammatory mediators and reduced expression of many cytokines linked to host defense and inflammatory immune capacity (26). During the third trimester, fetal development is completed and all organs are functional. A pro-inflammatory phase recurs (35, 36) in preparation for delivery, leading to a cascade of events that trigger myometrial contractions and cervical remodeling.

Cytokines, low-molecular-weight proteins and small intercellular signal peptides in the immune system (37) are produced by several fetal and maternal

cells (38-41). They are found in varying concentrations in amniotic fluid and in fetal and maternal serum before and during labor (42-46). Cytokines are capable of modulating the behavior of other cells (37) and affecting prostaglandin production (47-50). One cytokine can have different effects, depending on its concentration and its interaction with other cytokines and the type of target cell (37).

2.2.1 The placenta

Early in gestation, the placenta is shaped from embryonic (trophoblastic) and maternal (endometrial) tissues (23). It has unique features, essential for the developing fetus. It is an important tissue barrier, thick in early gestation to protect the fetus from external influences. As the pregnancy progresses and the placenta develops, this barrier attenuates by the second month of gestation (51) in order to facilitate diffusion of nutrients and oxygen from maternal to fetal blood. It also serves as a carrier that removes embryonic metabolic waste from fetal to maternal blood (23). Toxins, such as environmental pollutants, drugs, alcohol, and tobacco (23, 51), may nonetheless enter fetal blood by crossing the placental barriers. This may cause fetal physiological abnormalities or congenital malformations (23). The placenta is thus not impenetrable, but does serve as a natural barrier. Studies suggest that size, material composition and surface characteristics such as solubility are crucial factors determining whether it is permeated by particles (52-55).

2.2.2 The fetal membranes

During the first weeks of gestation, the embryonic membranes, including the internal amnion, yolk sac, allantois and external chorion, are formed. The amnion fills with amniotic fluid and eventually extends all the way around the embryo (23) and lines the uterine cavity (19). The yolk sac produces the earliest blood cells and later forms part of the gut. The allantois constitutes the structural base of the umbilical cord, which contains a core of embryonic connective tissue, the umbilical arteries and the vein. Externally, it is covered by the amniotic membrane which links the fetus to the placenta. The allantois also becomes part of the fetal urinary bladder. Finally, the chorion, involved in

placentation, constitutes the outermost membrane enclosing the embryo and all the other membranes (23). The much thicker trophoblast layer is directly linked to the maternal decidua and consists of trophoblasts procured from the yolk sac and allantois. (19). It forms the chorionic villi that grow on contact with maternal blood. They later form the umbilical arteries and the vein, all protected by the outer layer, the decidua (23).

2.2.3 Amniotic fluid

The amnion, with its content of amniotic fluid, provides a buoyant environment that cushions and protects the developing embryo from physical trauma (23, 56). It also assists in the maintenance of a constant homeostatic temperature, prevents the rapidly growing embryonic parts from adhering (23), reduces the risk of compression between the uterine wall and the fetus (57) and supports fetal growth (56) by allowing the embryo considerable freedom of movement, enabling musculoskeletal development (23, 58) and development and growth of the gastrointestinal and pulmonary systems. The amniotic fluid also has antibacterial properties that protect the fetus from infectious agents (58).

During the first 20 weeks of gestation, amniotic fluid is similar to fetal plasma. It is mainly derived from maternal plasma (23, 56), water and solutes that pass through the fetal membranes or across the placenta. It also consists of organic macromolecules (carbohydrates, proteins, lipids) and hormones (58). The amniotic fluid volume increases significantly in the second trimester, from a total of about 10-25 mL at gestational week 10 to a total of about 400 mL at gestational week 20. Although the fetal kidneys start to produce urine already at gestational week 8, followed shortly after by fetal swallowing, neither fetal urine nor swallowing contribute significantly to the content or volume of amniotic fluid until around gestational week 16, when the fetal kidneys are fully functioning (56, 58, 59). Keratinization of fetal skin usually begins at 19-20 gestational weeks and is completed at gestational week 25. Around gestational week 24-28, the amniotic fluid volume reaches its peak of approximately 800 mL (56, 58). At this stage, the amniotic fluid is mainly derived from the kidneys' excretion of fetal urine, pulmonary excretion of lung fluid (23, 56, 60), fetal breathing and fetal secretion of oral, nasal and tracheal fluid (56). Little change then occurs until near term when the amniotic fluid volume begins to decrease (61). The fetal skin and surfaces of the amnion, placenta and umbilical cord, which are permeable to water and solutes, provide

a rapid bi-directional diffusion between the fetus and the amniotic fluid, in which the removal of amniotic fluid is predominately accomplished by fetal swallowing (56) and intramembranous absorption (60).

The amniotic fluid's complex and dynamic contents, evolving as pregnancy progresses and including nutrients, growth factors (56), enzymes, fetal epithelial cells (62), proteins (58, 61), electrolytes, immunoglobulins and vitamins from the pregnant woman (58), make it a highly interesting matrix for research. The amniotic fluid content and status reflect the current intrauterine environment (63). Researchers and clinicians thus use it to monitor the progression of pregnancy and as a source of biomarkers for the prediction of adverse pregnancy outcomes, such as spontaneous PTD (64-67), and adverse neonatal outcomes (68).

2.3 Gestational age estimations

For centuries, Naegele's rule has been used to estimate pregnancy duration. This is based on a 28-day ovulatory cycle with ovulation on cycle day 14. The estimated date of delivery is calculated in four steps:

1. Determine the first day of the last menstrual period (LMP)
2. Add seven days
3. Count back three calendar months
4. Add one year

Naegele's rule is still used in developing countries, whereas ultrasound measurement has taken over gestational age estimation in the industrialized world. As each woman has her individual menstrual cycle, ultrasound measurement is considered to be more accurate (69) and has remained the most reliable method for gestational age estimation (70) since its introduction in obstetrics over 50 years ago. It is a safe procedure when used appropriately (71), but it should only be performed when clinically indicated. Scan duration should be limited, as data indicate that the energy used to obtain ultrasound images may have an effect on tissues (72) and the fetus.

Gestational age estimation is important for several reasons; it is the basis for the timing of prenatal visits, examinations, screening tests and certain interventions, it is essential for accurate assessment of certain laboratory

results and it is vital when delivery is medically indicated or when elective cesarean section is planned (70). Gestational age estimation is initially based on the LMP, except for pregnancies conceived by in vitro fertilization (IVF), for which it is based on the date of embryo transfer. The golden rule is that the best clinical estimate of gestational age should always serve as the basis for dating the pregnancy. Table 1 presents an overview of the most accurate method for gestational age determination based on the number of days that the ultrasound-determined date differs from the date according to the LMP.

Table 1. The most accurate method for gestational age estimation. Data from the Committee on Obstetric Practice, the American Institute of Ultrasound in Medicine, and the Society for Maternal-Fetal Medicine (70)

Gestational age (weeks+days)	**Difference (days)**	**Most reliable method**
< 9+0	≤ 5 > 5	LMP Ultrasound examination
≥ 9+0 – ≤ 13+6	≤ 7 > 7	LMP Ultrasound examination
≥ 14+0 – ≤ 15+6	≤ 7 > 7	LMP Ultrasound examination
≥ 16+0 – ≤ 21+6	≤ 10 > 10	LMP Ultrasound examination
≥ 22+0 – ≤ 27+6	≤ 14 > 14	LMP Ultrasound examination

It is recommended that all pregnant women undergo a transvaginal or abdominal ultrasound scan before 22+0 gestational weeks to confirm or revise the estimated LMP-based gestational age (70). Pregnancies for which this recommendation has not been followed are considered to be suboptimally dated (73). The most reliable estimate is obtained in the first trimester, at <13+6 gestational weeks, yielding an accuracy of ± 5-7 days (70). This is preferably performed transvaginally at <8+0 gestational weeks and abdominally at ≥ 8+0 gestational weeks (72). This early dating is based on crown-rump length (CRL) (74), preferably an average of three measurements. However, accuracy decreases when CRL >84 mm, equivalent to approximately 14+0 gestational weeks. Thereafter, a full fetal biometry, consisting of

biparietal diameter, head circumference, abdominal circumference and femur length, is recommended for estimating gestational age (70). This is optimally performed at around gestational weeks 18-20. A full fetal anatomic survey can be performed at this time as well (71). However, this second-trimester ultrasound does not provide the basis for gestational age estimation if the estimated date of delivery is established at a first-trimester ultrasound (70).

There is controversy regarding the accuracy of ultrasound dating between gestational weeks 14+0 and 27+6. However, it is generally agreed that accuracy improves with decreasing gestational age within this interval (71). The most inaccurate period for gestational age determination is thus the third trimester, during which the margin of error is up to ± 21-30 days (70).

2.4 Delivery

2.4.1 Timing of delivery

Timing of delivery is biologically regulated (6, 75), and the onset of labor results from coordinated signals, as well as from maternal and fetal endocrine and immune events (35, 76-81). Pregnancies that last for 259-293 days, corresponding to 37+0 to 41+6 gestational weeks (and days), are considered to be at term (82, 83). This is quite a broad interval, but the term pregnancy group has previously been considered to be rather homogeneous, and has constituted the basis for comparison of risks associated with preterm and post-term deliveries (82). However, data increasingly suggest that there is a significant difference in the outcomes of infants delivered in the term pregnancy interval. The group has thus been divided into sub-groups based on gestational duration within the term interval (Table 2). Infants born at early term post-term are suggested to have an increase in neonatal morbidity, compared with those born at full and later term (82, 83). Infants born at full term have the lowest rate of neonatal morbidity (83).

Table 2. Subgroups of gestational duration at term. Modified from Fleischman et al. (82) and Spong et al. (83)

Definition	Gestational duration (weeks+days)
Early term	37+0 – 38+6
Full term	39+0 – 40+6
Late term	41+0 – 41+6
Post-term	≥ 42+0

Fetal maturity and growth are thus a continuous process. At a certain stage at term, the fetus has reached a size equivalent to that of the entire delivery channel. This is referred to as the "obstetric dilemma", a key mechanism from an evolutionary point of view, for the well-being and survival of the fetus and its mother (75). At this stage, the fetus has also reached a certain level of development and is physiologically prepared to maintain homeostasis as a neonate. The conditions for neonatal survival are thus optimal (19).

2.4.2 The delivery process

At the time of delivery, coordinated signals trigger an inflammatory process (35, 36, 84) in several gestational tissues such as the cervix, decidua, fetal membranes and myometrium (19). This creates a pro-inflammatory environment that transforms the myometrium from a quiescent to a highly contractile state (19, 85) with strong, rhythmic muscle contractions (19). It results in edema, neutrophil infiltration and expression of pro-inflammatory cytokines and chemokines (86-88). Several cascade pathways are involved in this process, such as the endocrine and immune systems and prostaglandins (37). Estrogens also play an important role, reaching peak concentrations in maternal blood during the last weeks of gestation, causing the uterus to contract. Oxytocin, produced by the fetus, causes the placenta to release prostaglandins and the two powerful uterine muscle stimulants cause more regular contractions. The hypothalamus is activated and more oxytocin is released. Fetal fibronectin, which has bonded to maternal and fetal tissues

throughout gestation and changes into a lubricant just before labor, also assists in the process (23). Altogether, this leads to increased uterine activity, cervical remodeling and weakening of the fetal membranes, which facilitates the rupture of the chorioamniotic membranes (85, 89). This process is referred to as the common pathway of parturition (19).

Despite partial knowledge of the common pathway of parturition, the exact sequence and synchronization of the coordinated events preceding the spontaneous onset of labor and controlling gestational duration have not as yet been fully characterized, either from a biological or mechanistic standpoint. It has though recently been suggested that each of these local and systemic signals or events constitutes an intrinsic functional clock or alarm with interdependent timing mechanisms. Such a biological clock can function as a device that records events or as a responder monitoring the level and number of specific signals or events (e.g. nutrient availability or concentration of circulating hormones) (19). Corticosteroid hormones are powerful synchronizers of such biological clocks, and they have long been thought to control the timing of delivery. In most mammalian species, increased corticosteroid concentrations are evident in the maternal and fetal circulations and amniotic fluid prior to the onset of labor (90).

The wide variability in human gestational duration at term, the incidence of early and post-term deliveries and the associated neonatal risks ultimately suggest the existence of multiple, redundant clock and monitoring systems. These may be controlled by the extent to which any individual clock dominates over other physiological or pathophysiological signaling mechanisms that stem from infection or metabolic derangement. Such biological pregnancy clocks could count recurring events and/or monitor a threshold of specific signals. Clock-like mechanisms, coordinating fetal maturation with delivery, have been described in sheep and mice. The issue of whether similar systems control the gestational duration of human pregnancies is currently the object of intense research (19).

It has further been hypothesized that the uterus is sensitive to several inflammatory developmental factors such as fetal membrane senescence, as well as to environmental factors such as social stress or infections, and that labor is triggered when an inflammatory load threshold is surpassed. Uterine wall distention is likely involved in a similar feedback mechanism. Senescence (19), the natural and physiological aging processes (91, 92) of the fetal membranes (amnion and chorion) (19), has also been hypothesized as a key contributing factor to the onset of labor (91, 92). It constitutes a counting device, modified by different factors including oxidative stress. Damage-

associated molecular patterns (DAMPs), endogenous mediators, reflect changes in fetal membrane physiology (19). They are released from fetal membranes or by an inflammation in the choriodecidual space (93), transmitted to the decidual layer via leukocyte and natural killer cell activation (94), where they cause immune activation (95), tissue damage (19) and trigger an inflammatory process (96, 97), known as senescence-associated inflammation (94), constituting sterile inflammation (91, 92). This is associated with an overall increase of the fetal membranes' inflammatory load that change the quiescent myometrium to an active state (94), leading to the onset of labor (98). Several DAMPs have been reported in laboring tissue, including high-mobility group box (HMG) 1, heat-shock protein 70 (HSP70), uric acid, single- and double-stranded DNA and RNA, S100 calcium-binding protein A8 (S100-A8) and hyaluronan (81, 93, 99-103). Theses hypotheses that have arisen during the last couple of years, indicating that the intrauterine environment may not be as sterile as previously thought, are though hotly debated (104-106).

2.5 Preterm delivery

2.5.1 Definitions and consequences

The upper limit of PTD is globally agreed (3, 107), while the lower gestational age limit for PTD varies in the world. In Sweden, it is set at 22 gestational weeks, while it is 20 gestational weeks in the United States and Australia (6). Despite the low rate of PTD in Sweden (9, 108), it accounts for over 70% of all perinatal deaths and half of all short- and long-term postnatal morbidity (109-111), such as chronic lung, vision, hearing and neurological problems. About 7-8% of children born at <28 weeks of gestation will develop cerebral palsy (CP). CP is a major international health problem and the most common physical disability in childhood (112-114). More than 50% of children with CP also have other associated neurological conditions, such as mental retardation or epilepsy (115-117).

The single strongest risk factor for severe neonatal morbidity is the degree of immaturity at birth (118). The respective number of neonatal deaths worldwide is equal among deliveries at <28+0 gestational weeks, at 28+0 to 31+6 gestational weeks and at 32+0 to 35+6 gestational weeks. This is because there is a higher rate of PTD in the later gestational intervals, while the individual risk is significantly lower. Data from the Swedish Neonatal Quality Register

indicate an increasing survival rate, even at the earlier gestational weeks (119). Table 3 presents the survival rate of liveborn children in Sweden during 2013-2017.

Table 3. Approximate survival rate of preterm-born neonates in Sweden, 2013-2017. Data from Läkartidningen (6)

Gestational duration (weeks+days)	Survival rate (%)
22+0 – 22+6	53
23+0 – 23+6	64
<28+0	83
28+0 – 31+6	97
32+0 – 36+6	99

2.5.2 Epidemiology

There are several socio-demographic or lifestyle risk factors associated with PTD, among which are ethnicity, low socioeconomic or educational status, inadequate prenatal care, single marital status, maternal age <18 or >40 years, smoking, alcohol and drug abuse, hard working conditions, anxiety and depression, low or high maternal body mass index (BMI) (3, 120, 121), maternal short stature, maternal stress and domestic violence (6). There are also obstetric risk factors, such as previous PTD or previous second-trimester pregnancy loss, multiple pregnancy, an inter-pregnancy interval of <6 months, low maternal gestational weight gain, vaginal bleeding, infection/ inflammation, oligo- or polyhydramnios, abdominal surgery in the second or third trimester, cervical procedures during gestation, uterine anomalies (3, 120, 121), myoma, assisted fertilization, altered vaginal flora, male fetal gender and primiparity. Recent studies also suggest that 25-30% of all PTD have a genetic association and the first data on genes related to PTD and gestational duration have recently been published (21). However, in more than 50% of all cases of PTD cases, known or obvious risk factors are missing (122).

Spontaneous PTD originates from multiple causes such as infection, inflammation, vascular disease and uterine overdistension (3). The most

important risk factors for spontaneous PTD are previous PTD, which increases the risk two- to sixfold (6), and short cervix in the second trimester (23). Other risk factors include black race, periodontal disease and low maternal BMI (3).

2.5.3 Rates

There are large geographical variations in PTD rates, both nationally (123) and internationally (124), and only a minor part of these geographical differences can be explained by known risk factors (6). In Sweden, the PTD rate, currently 5.7%, has been relatively stable during the last 45 years (9, 108). Approximately 2% occur at <34+0 gestational weeks and 0.3% occur at <28+0 gestational weeks (9). Denmark and Norway have slightly higher rates, approximately 6.0-6.5% (108, 124). These Nordic rates are low, compared to reported rates of up to 20% in some parts of the world (Fig.1). The highest rates are reported in India, China, Nigeria, Bangladesh and Indonesia, and these five countries together account for approximately 44.6% of all PTDs globally (125). A general worldwide increase in PTD has been observed over the last 30-40 years, especially in the developing countries, most likely due to the increase in iatrogenic PTDs and the increased number of twin pregnancies after IVF (6).

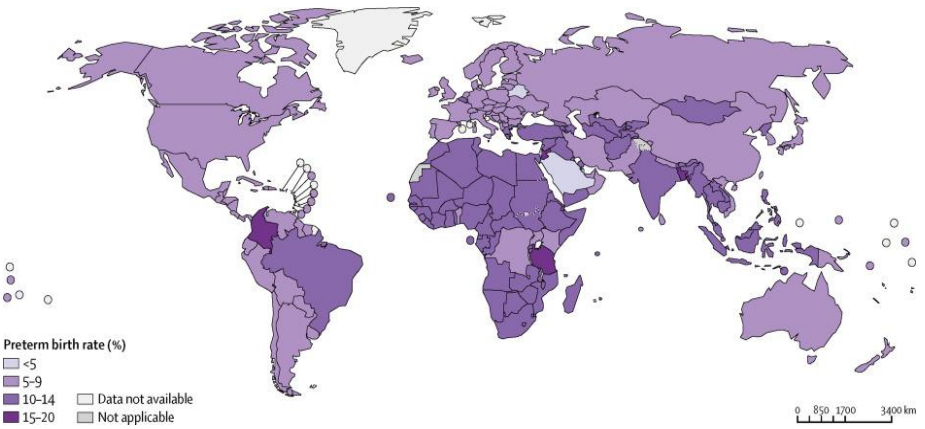

Figure 1. Global estimated rates of PTD in 2014, published in Chawanpaiboon et al. (125).

2.5.4 Subgroups

PTD is divided into subgroups based on gestational duration (Table 4). The majority (approximately 60%) of all PTDs are late or near-term PTD. The rate thereafter decreases by decreasing gestational duration; moderately PTD accounts for 20% of all PTDs while severely and extremely PTD accounts for 15 % and 5%, respectively (3).

Table 4. Subgroups of PTD. Data from Goldenberg et al (3) and Fleischman et al. (82)

Definition	**Gestational duration (weeks+days)**
Extremely PTD	<28+0
Severely PTD	28+0 – 31+6
Moderately PTD	32+0 – 33+6
Late or near-term PTD	34+0 – 36+6

PTD is also divided into subgroups according to origin; multiple pregnancies, malformations and intrauterine fetal deaths, spontaneous and iatrogenic (3). This thesis and its constituent papers focus exclusively on spontaneous PTD (Fig.2).

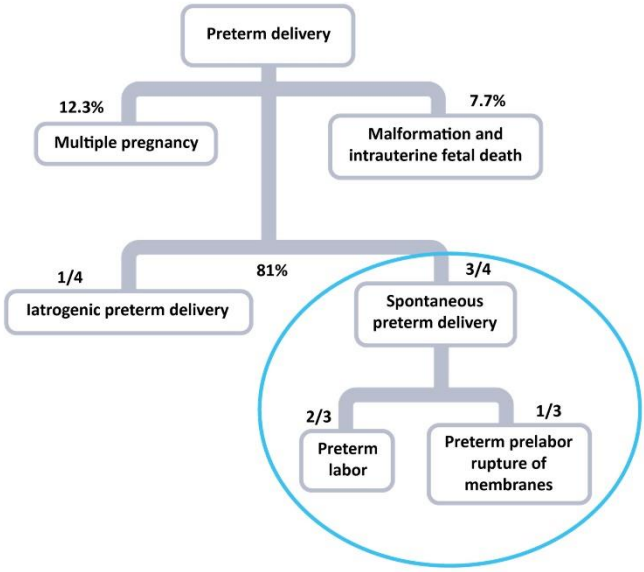

Figure 2. Subgroups of preterm delivery. Data from Morken et al (9). Illustration by Jan Funke.

2.6 Spontaneous preterm delivery

Spontaneous PTD accounts for 75% of all PTDs and constitutes preterm labor (PTL), defined as regular uterine contractions and cervical ripening, and preterm pre-labor rupture of membranes (PPROM), defined as spontaneous rupture of the fetal membranes with leakage of amniotic fluid at least one hour prior to the onset of regular uterine contractions (3) (Fig.3).

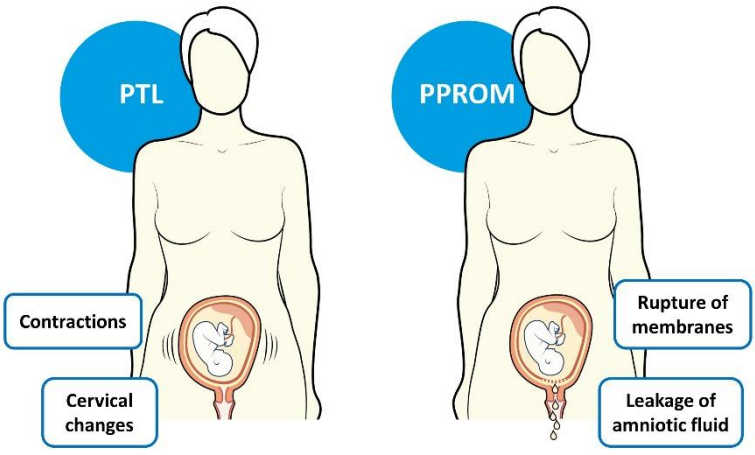

Figure 3. Subgroups of spontaneous preterm delivery. Data from Goldenberg et al (3) and Morken et al (9). Illustration by Jan Funke.

2.6.1 Etiology

Despite decades of research, the multifactorial and complex etiology of spontaneous PTD is incomplete, as is understanding of the sequence and timing of events preceding the condition (9, 84, 126-133). The exact mechanisms that trigger the inflammatory process (35, 89, 134) have not been completely identified but there are some important common pathophysiological processes in spontaneous PTD etiology: maternal stress, maternal systemic or genital tract infections, placental ischemia or vascular lesions, cervical disease and uterine overdistension (135-137). Mechanical distention of the fetal membranes, that induce inflammatory cytokine expression, tissue-level inflammation (138, 139) and fetal membrane senescence, are also associated with spontaneous PTD, the latter especially in cases of PPROM (19) (Fig.4). The processes have different initiators and factors that vary somewhat according to gestational duration within the PTD interval (135).

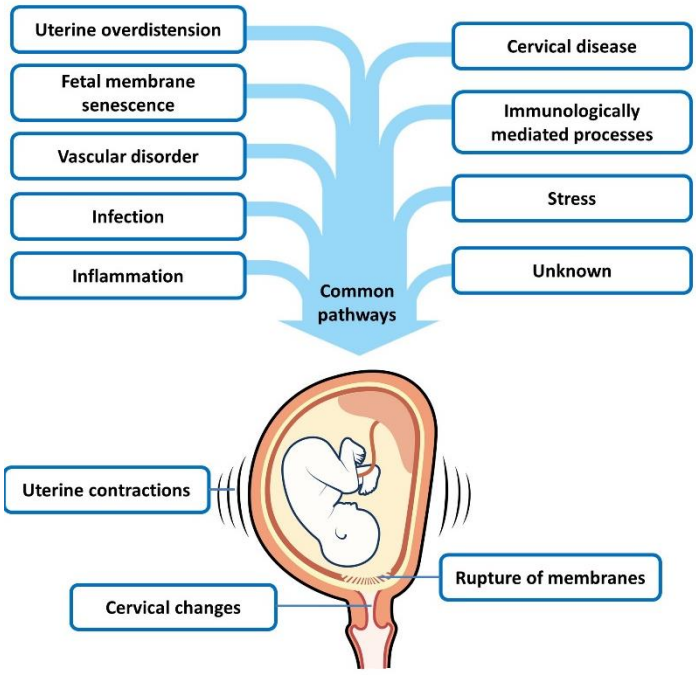

Figure 4. Common pathways in onset of spontaneous PTD. Data from Menon et al. (19), Behrman et al. (135), Goncalves et al. (136), Romero et al. (137), Hua et al. (138) and Kendal-Wright (139), modified from Läkartidningen (7). Illustration by Jan Funke.

In many cases, spontaneous PTD occur as a result of a foreign stimulus, originating from foreign bodies or bacterial pathogens which causes an infection that triggers an inflammatory response in maternal and fetal tissues. These infectious and subsequent inflammatory processes (9, 84, 126-132) are particularly present in cases of extremely PTD. There are several ways for bacteria to reach the amniotic cavity. Among the most common are ascension from the lower genital tract, crossing the cervical barrier and invasion of the decidua, chorioamniotic membranes and amniotic cavity (84). This is defined as microbial invasion of the amniotic cavity (MIAC) (96, 97). Studies suggest that up to 20-60% of women with PTL at <28 weeks of gestation and 10-25% of women with PTL at 28-32 weeks of gestation have MIAC, verified by positive culture for related microorganisms (140-142). The pathogenesis of PTL is, however, not yet completely understood and it may also occur as an early idiopathic activation of the normal labor process. Asymptomatic MIAC is though more common in women with PPROM (3), presumably because the

membranes, which usually act as a barrier, are ruptured. In these cases, MIAC can cause spontaneous labor within a couple of days after membrane rupture, but a minority with PPROM remain undelivered for weeks or months (143).

The subsequent intra-amniotic inflammation (IAI) causes an increased release of several amniotic fluid inflammatory markers, such as interleukin-6 (IL-6) (142, 144-150), other pro-inflammatory cytokines and chemokines (131, 146, 148, 149, 151), tumor necrosis factor alpha (140, 146, 148, 152), or matrix metallo-proteinase-8 (MMP-8) (153-155), as well as a release of prostaglandins (156, 157). This trigger myometrial contractions, membrane rupture and cervical maturation (84, 156-159), leading to spontaneous PTD (84, 156-159). Women with PTL may, however, have IAI if the amniotic fluid culture is negative, referred to as sterile IAI (93).

MIAC and IAI are separated into four subgroups based on the presence or absence of infection or inflammation (96, 97): sterile IAI (IAI is present, MIAC is absent), MIAC-associated IAI (both MIAC and IAI are present), MIAC alone (IAI is absent) and a negative subgroup without MIAC or IAI (Fig.5). However, it has been hypothesized that IAI is not just simply present or absent but rather a continuum, where clinical outcomes are correlated with gradations of the inflammatory response based on the number of biomarkers present in amniotic fluid (160).

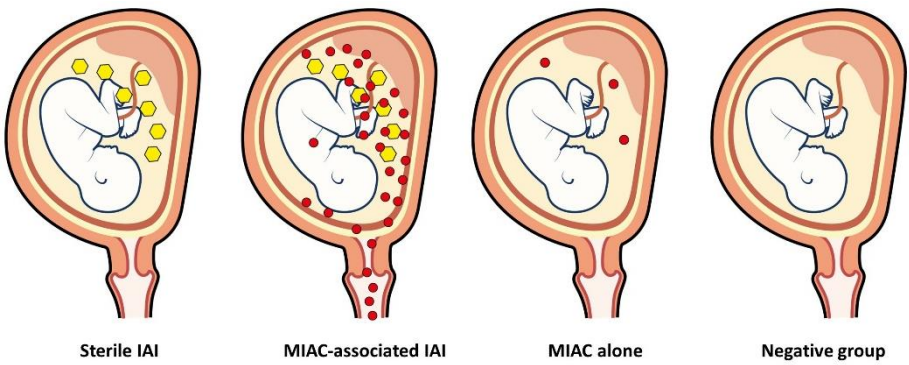

Figure 5. Subgroups of MIAC and IAI. Data from Romero et al (93), Combs et al. (96) and Cobo et al. (161). Illustration by Jan Funke.

Microbiological and histological evidence, and clinical signs of MIAC and/or IAI in the mother may not always coincide. Histological chorioamnionitis

(HCA) is defined as infection of the uterine cavity, fetal membranes and placenta. However, DAMPs or alarmins cause HCA to the same extent as bacteria (162). The prevalence is inversely correlated with gestational duration, and studies suggest that HCA is present in as many as 50% of all cases of spontaneous PTD (163). The later spontaneous PTD occurs in gestation, the more likely that the HCA is of non-infectious origin (6).

The IAI resulting from MIAC is measurable in the choriodecidual and chorioamniotic spaces, amniotic fluid, umbilical cord, fetus and, rarely, the placenta (159), as shown in Fig.6. Other pathways have, however, been discussed, such as via maternal blood or the oral buccal mucosa (6). Both MIAC and IAI are associated with short latency to delivery (131, 140, 142, 145, 147, 150-155, 164) and high rates of perinatal morbidity and mortality (140, 142, 150, 152, 154, 164, 165), especially in infants that showed signs of inflammation already in fetal life.

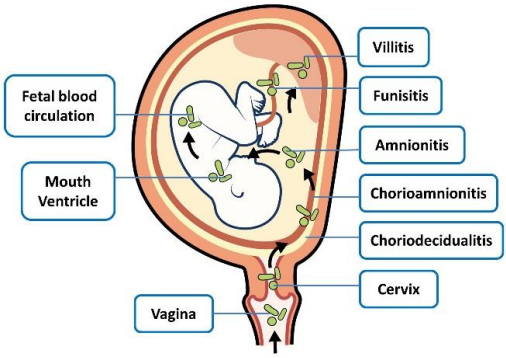

Figure 6. Sites of intrauterine inflammation and infection. Modified from Goldenberg et al. (159) and Läkartidningen (6). Illustration by Jan Funke.

2.6.2 Biomarkers

Biomarkers are measurable biological markers or indicators of normal or pathological processes (166). Since the majority of women with spontaneous PTD lack known risk factors (167) and since the condition is most likely quite advanced once symptoms occur, there is an urgent need to study asymptomatic women for the early detection of prognostic or diagnostic biomarkers that can predict risk. Asymptomatic women identified early in gestation are also those most likely to benefit most from preventive interventions.

The ideal biomarker for spontaneous PTD would be easily tested early in gestation and would identify both high and low risk women with a low false positive rate. However, due to limited understanding of mechanisms involved in spontaneous PTD etiology, time of onset and duration of the asymptomatic state, the identification of reliable biomarkers is a major challenge. As both IAI and MIAC are strongly associated with spontaneous PTD (168), amniotic fluid is an interesting biological matrix for biomarker discovery (169). Attention has primarily been devoted to biomarkers (170, 171) involved in inflammatory pathways associated with spontaneous PTD (172). However, systematic reviews of symptomatic women in the third trimester or shortly prior to delivery, examining a wide range of potential biomarkers, conclude that none of these are clinically useful for the prediction of spontaneous PTD (171, 173).

A meta-analysis and systematic review of the literature concerning women in the first or mid-trimester suggest an association between elevated concentrations of IL-6 and MMP-8 and decreased concentrations of glucose in amniotic fluid, and spontaneous PTD (169). Hus et al. also suggest mid-trimester amniotic fluid concentrations of IL-6 as a potential biomarker for spontaneous PTD prediction (174). However, the results of these two studies are inconsistent with others. No single first or mid-trimester biomarker has neither as yet emerged that can predict which women will delivery spontaneously preterm (175).

In summary, several systematic reviews of both targeted and untargeted approaches at all gestational ages have reached an unanimous conclusion; multiple and intricate pathways are involved in spontaneous PTD etiology and it is therefore unlikely that one single biomarker would serve as a reliable predictor (171, 176-179). Diverse study designs and sampling procedures, as

well as the adoption of different analytical approaches in diverse technological assay platforms, contribute to heterogeneity and variability of results (180).

2.6.3 Prediction strategies

There are a few strategies for the diagnosis of symptomatic women with threatening PTD, especially in cases of PPROM, which is still unpredictable and unpreventable. Prevention is challenged by the fact that the most common symptoms (contractions, back pain, menstruation-like pain, increased vaginal discharge) are frequent during normal pregnancy as well. Effective prediction methods are therefore needed for this group of women. In Sweden, clinical assessment includes evaluation of the pain and frequency of contractions and cervical status (length, consistency and dilation), but there are also several commercially available biochemical tests, the most common of which is the quantitative fetal fibronectin test. Low concentrations of fetal fibronectin indicate low risk of PTD with a good negative predictive value, while high concentrations indicate increased risk of PTD, but with a limited positive predictive value (181). Studies suggest the benefit of combining fetal fibronectin with cervical length measurement (182), but the former is as yet in limited clinical use in Sweden. Tests with high negative predictive value will reduce unnecessary interventions and hospitalization of women with threatening PTD (7), decreasing societal costs. To further improve prediction, biomarkers or predictive tests can be used in combination with epidemiological risk factors (183).

Measuring cervical length by transvaginal ultrasound at gestational weeks 18-23 is one of the most applied methods globally for predicting spontaneous PTD in asymptomatic women. There are, however, several confounders related to cervical structure and development that may bias results. Furthermore, many studies include a high proportion of high-risk women, limiting generalizability. There appears to be clinical value in repeated measurement within a couple of days after the first measurement, as this may indicate progression (7). However, there is little evidence that cervical length measurement predicts spontaneous PTD within 7 days in asymptomatic or symptomatic women. On the other hand, it may be useful in clinical management by facilitating decisions such as hospital admission and antenatal corticosteroid administration.

2.6.4 Preventive strategies and treatments of asymptomatic women

A few preventive strategies and treatments are available, such as progesterone, cervical cerclage and cervical pessary, and it is more or less recommended for different risk groups. Progesterone, produced by the placenta from the second or third month of gestation (23), is secreted into the maternal circulation in large amounts (19). It prepares and maintains the endometrium and is a key factor in suppressing the maternal immunologic response to fetal antigens. Maternal rejection of the trophoblast is thereby prevented, a requirement for pregnancy maintenance (184). Progesterone also plays an important role in maintaining uterine and decidual quiescence, inhibiting myometrial contractility and sustaining cervical closure (185, 186). Administration of progesterone as a preventive strategy has been widely and hotly debated for several years. Current evidence does not support the administration of progesterone, either as intramuscular 17-hydroxiprogesterone or vaginal micronized progesterone, to asymptomatic women with a prior history of PTD (187-189). In asymptomatic women with a short cervix (≤25 mm) in the late midtrimester, vaginal micronized progesterone reduces PTD at <34+0 gestational weeks, although there is no impact on long-term neonatal outcome (190).

Cervical cerclage requires a surgical procedure but the side effects are mild (vaginal irritation and discharge) (7). Cervical cerclage has been postulated to decrease the risk of perinatal mortality and morbidity (191) and is currently indicated for (192, 193):

1. women with a history of an indicated cerclage for one of the following: a) three or more prior early PTDs; b) trachelectomy; c) two or more prior early PTDs

2. women with a prior history of PTD and cervical length ≤25 mm

3. physical exam indicates cerclage, i.e. cervical dilation with visible amniotic membranes at < 24 gestational weeks

Cervical pessary has also been proposed as a preventive strategy in asymptomatic women with a short cervix as it relieves pressure on the internal os by changing the angle of the cervix. However, study results are contradictory (194, 195).

2.6.5 Treatments of symptomatic women

Many interventions, aimed at prolonging pregnancy and improving the outcome in children born preterm, have been applied in women with PTL and PPROM. However, there have been few successes, especially in regard to improved neonatal outcome. In women with PTL and PPROM, the process may already be so advanced so that delivery is inevitable. Each case should be individually assessed, as delivery should not be prevented if the intrauterine environment proves harmful to the fetus (196).

Treatment provided in the acute phase may be beneficial for improving the perinatal outcome but it may be harmful for the fetus both in the short and long term if delivery does not actually occur preterm (196, 197). Access to good prediction models and preventive strategies for women at high risk, and increased knowledge about effective interventions and treatments in the acute phase of threatening PTD, are therefore of major importance (7).

Antenatal corticosteroids are administered on two occasions, with a 24-hour interval, at gestational weeks 23+0 to 33+6 to women with threatening PTD who are expected to deliver within a few days. This treatment increases the perinatal survival rate and reduces the risk of respiratory disorders, enterocolitis and intraventricular hemorrhage. A positive effect on both neonatal mortality and morbidity has been observed even when the interval between administration and delivery is short (198). The long-term outcome in children after antenatal corticosteroid therapy is, however, not known and there is a debate on the risks versus benefits of an additional single dose if more than two weeks have passed since the double dose was given and the woman is still undelivered (199).

Tocolysis is a traditional treatment for women with threatening PTD. It blocks the oxytocin receptors in uterine muscle, leading to reduced tone and contractility (7). It has not been shown to improve neonatal outcome (200); its major function is rather to allow adequate treatment with antenatal corticosteroids for fetal lung maturity. There is no evidence that long-term repeated tocolytic treatment after the completion of corticosteroid therapy has any effect (7).

Antenatal magnesium sulfate ($MgSO_4$) treatment is recommended in most parts of the world for women with imminent PTD at <32+0 gestational weeks. Metaanalyses have shown that this treatment reduces the risk of neonatal brain injury, cerebral hemorrhage and CP (201). Further studies are however needed in order to ascertain optimal dosage and the risk of side effects. A large

Swedish study is ongoing, which has led to a national controlled introduction of $MgSO_4$ to all women expected to delivery <32+0 gestational weeks.

There is an association between vaginal infection or colonization with certain bacteria, e.g. bacterial vaginosis, and spontaneous PTD (202). Metaanalyses concerning screening for and treating bacterial vaginosis with antibiotics have, however, not shown a decreased incidence of PTD (203). Some studies on antibiotic treatment for threatening PTD have even suggested that it could be harmful for the fetus in women with PTL (204-207). In the ORACLE trial, an increased risk of CP was observed in children aged seven whose mothers had been given prenatal antibiotics for PTL (204). Some improvements in short-term outcome, but not at the age of seven years, were found in children born to women with PPROM without contractions who had received prenatal antibiotics (205, 207). Antibiotics treatment in women with PPROM and clinical chorioamnionitis is routine, however, as it has not proven harmful to the child (7).

Finally, studies have suggested acetylsalicylic acid as a potential treatment. It reduced the risk of spontaneous PTD in one study but further research is needed (208).

2.7 Amniocentesis

2.7.1 Background

Mid-trimester genetic amniocentesis is an invasive prenatal procedure, in which amniotic fluid is obtained from the amniotic cavity and used for fetal genetic diagnosis (10). The clinical procedure was introduced in the late 1960s (209).

2.7.2 Indications for mid-trimester genetic amniocentesis

Advanced maternal age is associated with an increased risk of chromosomal abnormalities (210-212) and is the most common indication for mid-trimester genetic amniocentesis (213). The number of Swedish women giving birth to their first child at ≥35 years of age has increased significantly during the last decades. In 1973, 1.7% of primiparas and 8.5% of multiparas were aged >35

years, compared to 12.4% and 28.5%, respectively, in 2015 (214). Other common indications for mid-trimester amniocentesis, related to elevated risk of fetal chromosomal abnormalities, are a) high risk on a first- trimester combined screening test; b) a previous pregnancy or child with chromosomal abnormality; c) family history of chromosomal abnormality or genetic disease; and d) ultrasound finding consistent with fetal malformations. Finally, a few women undergo mid-trimester genetic amniocentesis due to severe anxiety.

2.7.3 Risks associated with mid-trimester amniocentesis

Amniocentesis performed before 14+0 gestational weeks has been reported to be associated with an increased risk of fetal loss (215-217), leakage of amniotic fluid (215, 217) and talipes equinovarus (clubfoot) in the infant (215). It is therefore not recommended to perform an amniocentesis before gestational week 15+0 (218). The reported overall risk of procedure-related fetal loss is inconsistent. Some studies report up to a 1.0% risk (219-221), while more recently reported risks are only 0.06% to 0.13% (10-12). This decrease may possibly be associated with the development of the technique or simply a question of the definition of fetal loss. Several studies report rates of fetal loss at <28+0 gestational weeks (11), while more recent studies have evaluated fetal loss at <24+0 gestational weeks (12, 222). Other studies have a cut-off at four weeks post-procedure (223, 224). One study reported that mid-trimester amniocentesis increases the risk of PTD (225), but this association has not been found by others (222, 226). It has, however, been suggested that some procedure-related miscarriages might be caused by preexisting subclinical IAI (63, 227-229). The same applies for second- and third-trimester hemorrhage following genetic amniocentesis; an old study found an association between amniocentesis and placental abruption (230), which was not supported by more recent data (231). Moreover, a possible association between early amniocentesis and hypertensive disorders of pregnancy has been suggested (232), but this was not confirmed in a more recent study (233). Finally, a few case reports (234-236) suggest that mid-trimester amniocentesis could be followed by clinical chorioamnionitis, defined as maternal temperature >37.8°C and >2 of the following criteria: uterine tenderness, malodorous vaginal discharge, fetal tachycardia (>160 beats/min), maternal tachycardia (>100 beats/min) or leukocytosis (WBC >15,000/ $mm^{3)}$ (237).

2.7.4 Amniocentesis - other applications

The amniotic fluid composition mirrors changes occurring in the uterus, and the protein composition of amniotic fluid obtained at amniocentesis can therefore also reveal other causes of adverse pregnancy outcomes (63). Mid-trimester amniocentesis thus enables the examination of the intrauterine environment in asymptomatic women.

Furthermore, amniocentesis can be performed, and repeated, throughout the entire pregnancy. After mid-trimester, the indications are evaluation of fetal lung maturity in women with PTL or PPROM (238), diagnosis of MIAC or IAI (239), and intrauterine growth restriction (240).

2.7.5 The diagnostic landscape

The number of amniocenteses performed in Sweden has decreased during the last decade, from 4,541 in 2005 to 2,340 in 2014 (241). This reduction is mainly a consequence of developments in prenatal testing and diagnostic techniques, as well as of the risks related to mid-trimester amniocentesis.

The first-trimester combined screening test (10) combines maternal age and nuchal translucency (ultrasound measurement of the fluid-filled area under the skin behind the fetal neck) (242) and maternal plasma biomarkers (protein A (PAPP-A) and free beta human chorionic gonadotropin (free β-hCG)) to estimates likelihoods of trisomy 21, 18 and 13 (243), of which trisomy 21 is the most common (244). Women with results indicating high risk are offered a genetic amniocentesis. The first-trimester combined screening test has a 90% detection rate for trisomy 21, with a false positive rate of 5% (245), resulting in a group of women who still need invasive testing if they choose to ascertain the fetal karyotype.

Another non-invasive method for prenatal testing that further reduced the demand for invasive procedures was introduced in 2011. This method is based on the presence of cell-free fetal DNA in maternal plasma and is known as non-invasive prenatal testing (NIPT). NIPT has higher sensitivity and specificity and lower false positive rates, compared to the first-trimester combined screening test (246). This applies particularly to trisomy 21, for

which the sensitivity is over 99%, with a false positive rate of less than 0.1% (247). NIPT thus minimizes the demand for invasive procedures (Table 5). However, despite recent advances in prenatal screening techniques, NIPT is though not yet a diagnostic procedure.

Table 5. Detection rate of Trisomy 21 and rate of women requirering invasive test (equal to screen positive rate) based on different screening methods. Data from Nicolaides et al. (245) and Gil et al. (247).

Diagnostic method	**Detection rate of Trisomy 21 (%)**	**Screen positive rate (%)**
Maternal age ≥35 years	30	5 – 20
Maternal age ≥35 years and second trimester biochemical screening	50 – 70	5
First-trimester combined screening test	90	5
First-trimester combined screening test and ultrasound examination of the fetal nasal bone, heart and ductus venosus	>95	3
Cell-free fetal DNA	>99.9	<0.1

The introduction of the first-trimester combined screening test has thus resulted in fewer invasive tests. However, the most substantial difference emerged in 2017 when NIPT was introduced in clinical practice at our department at Sahlgrenska University Hospital/Östra. This resulted in a significant decrease in the number of amniocenteses, as well as in fewer children being born with genetic anomalies. Table 6 presents the decrease at our department during the study. Obviously, it was urgent to perform this study when we did, since these samples will not be available for research in the future due to the decline in invasive procedures.

Table 6. Number of amniocenteses performed at Sahlgrenska University Hospital/Östra during the study period

Year	Number of amniocenteses
2008	791
2009	564
2010	450
2011	395
2012	385
2013	334
2014	251
2015	222
2016	173
2017	132

3 Aims

The long-term overall aim with this project is to identify early biomarkers, gain insight into underlying mechanisms and to generate hypotheses that can lead to studies of new preventive strategies, interventions and treatments for asymptomatic women at early gestations with a subsequent spontaneous PTD. Another long-term aim was to contribute to the identification of strong biomarkers or networks of biomarkers detectable in more easily accessible biological compartments than amniotic fluid.

The initial aim of the research presented in this thesis was to identify specific biomarkers related to spontaneous PTD, before the onset of clinical symptoms, and to provide insight into the biological mechanisms underlying the condition by investigating the composition of amniotic fluid from asymptomatic women undergoing mid-trimester amniocentesis for genetic testing. This aim is reflected in the case-cohort study design and the focus on spontaneous PTD (cases) versus term delivery (controls) as a dichotomous trait. Together with systems biology approaches, high-capacity techniques using DNA, RNA, proteins and metabolites may be the key to increased understanding of spontaneous PTD and to obtain a complete picture of the process. Well-designed proteomic studies (172) and multiplex technology have shown promise when it comes to detecting novel predictive biomarkers (248). To fulfil the aim, the constituent papers of this thesis are thus based on advanced omics and multiplex techniques in meticulously designed studies.

During the last couple of years, new data from our group and others have emerged (20, 21), indicating that analyzing gestational duration, instead of comparing spontaneous PTD and term delivery, might increase the likelihood of finding associations contributing to the prediction of spontaneous PTD. Our focus was thereby expanded to include investigating associations between mid-trimester amniotic fluid and gestational duration, aiming at greater understanding of the inflammatory and other pathways leading to the spontaneous onset of both term and preterm labor.

4 Material and methods

4.1 Study design

The constituent papers in this thesis are all based on subsets of a single cohort of women who underwent mid-trimester genetic amniocentesis at Sahlgrenska University Hospital/Östra. The first participant was enrolled in September 2008 and, due to the rapidly developing technology that have led to fewer invasive tests, the last subject was enrolled in December 2017. During that decade, a total of 1,223 women were recruited. The amniocentesis was performed on clinical indication, as described in Section 2.5.2. Women were followed up through their prenatal and obstetric medical records that were retrieved from other medical facilities if necessary. Newborns were followed up until age 28 days. Data concerning maternal characteristics, pregnancy, delivery, neonatal outcome and sampling were recorded in a comprehensive database created specifically for this study with the software FileMaker Pro (Filemaker Inc., California, USA). Sub-groups of women with spontaneous PTD, iatrogenic PTD, other adverse pregnancy outcomes and term deliveries were analyzed separately or compared to one another (Papers I-III and V), as well as analyzed as a cohort (Paper IV). Amniotic fluid samples were divided into aliquots and coupled with clinical data and outcome variables (Fig.7).

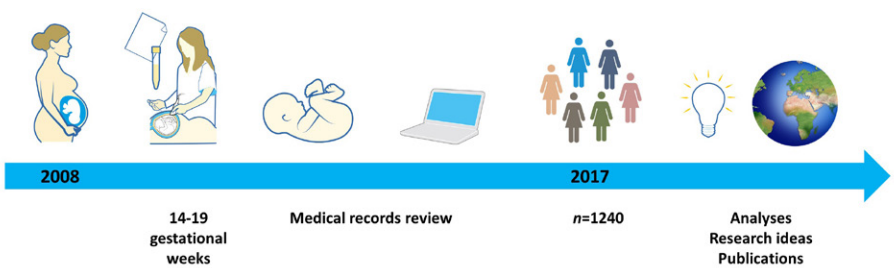

Figure 7. Timeline of study-related procedures. Illustration by Jan Funke.

4.2 Inclusion and exclusion criteria

Women aged over 18 with singleton pregnancies and intact membranes, without PTL or signs of infection and who could understand the Swedish oral and written information were invited to participate. Women were considered ineligible if they were under 18 years of age; had a multiple pregnancy; tested positive for human immune deficiency virus (HIV), hepatitis B or other infectious diseases; had severe mental illness or retardation; carried fetuses with known or suspected malformations; or if samples could not be collected due to logistic problems. Exclusion criteria were declined participation; language barriers; insufficient sample at amniocentesis (a few cases) and enrollment deemed inappropriate for medical or psychological reasons.

4.3 Enrollment strategy

Women were referred to mid-trimester genetic amniocentesis by their prenatal midwives, based on the clinical criteria described in section 2.7.2. They received information about their scheduled appointments, the procedure and this research study by mail prior to their visit. They thus had the opportunity to read the study information at home and to decide whether to participate. On arrival at the department, women (and their partners) were seen by a research staff member. They were given oral information about the study and the opportunity to ask questions. The enrollment strategy evolved over the years including an information screen and a leaflet rack about ongoing research. These were installed in the ultrasound unit waiting room.

4.4 Ethical considerations

Mid-trimester amniotic fluid offers major potential for detection of pathological conditions early in pregnancy in asymptomatic women. Participants in this study are, however, a highly selected population due to their advanced maternal age, homogenous ethnicity and high risk of chromosomal abnormalities. However, due to the risks, albeit limited, to mid-trimester amniocentesis, samples could only be collected in conjunction with clinically indicated procedures. Mid-trimester amniocentesis performed exclusively for research purposes would not have been ethically justifiable since research results do not benefit the participants.

Amniotic fluid is produced continuously. Collection of such a small additional amount as 3 mL did not extend sampling duration or increase the risk of procedure-related complications. Study results did not affect clinical management since a) the analyses were performed after the women were delivered; b) no individual results were reported back to either participants or healthcare staff; and c) data are presented and published on the group level. All clinical data are stored in a password-protected database and kept apart from names and personal identification numbers, which are stored under a separate code key. Source documents are stored in a secure filing cabinet to which access is limited to members of the research group. All material (biological specimens and protocols) was encoded after sampling using consecutive serial numbers so that a single individual could not be identified during the analytical phases. Data and amniotic fluid samples were thereafter connected via these individual serial numbers. Only the study investigators have access to the identification list.

The study was approved by the Regional Ethical Board at the University of Gothenburg, Sweden, initially as early as in 2003. It was, however, not initiated until 2008, after renewed ethical approval. A short description of the ethical approvals are found at the end of this section. Participation was voluntary and women were given the same care regardless of their decision to participate. Written consent to participate was given just prior to the amniocentesis, and both the physician and the woman signed the consent form. Participants were informed that they could discontinue their participation, request that their samples be destroyed or be made anonymous at any time without explanation.

Permit number Dnr. Ö 639-03 was the first ethical approval for the project covering enrollment, sampling, some analyses, biobanking of samples and collection of clinical data through medical records and the Swedish National Board of Health and Welfare's Medical Birth Register.

Permit number T 318-08 derived from a requirement to obtain renewed ethical approval to initiate the project, as a new law had been passed since the first approval. We simultaneously applied to update the written information and to modify the title of the project.

Permit number T 694-11 gave us permission to relate protein findings to a broad spectrum of pregnancy and delivery outcome variables rather than just spontaneous PTD.

Permit number 2019-06022 allowed us to perform a broader variety of laboratory analyses on the biobanked samples, such as oxidative stress, bacteria, metabolites, lipids and carbohydrates.

4.5 Characteristics of the cohort

In total, 3176 women underwent genetic mid-trimester amniocentesis during the study period, of which 24.2% (770/3176) were ineligible. Approximately 51.5% (1240/2406) of the eligible women were enrolled, while the remaining 48.5% (1166/2406) were excluded. Of the 1240 women enrolled, 22 women were excluded retrospectively due to being incorrectly enrolled; suspected fetal malformations (*n*=10), infectious diseases (*n*=5), initial twin gestation (*n*=4), processing deviation (*n*=2) and identity issues (*n*=1), and 40 women had missing data on gestational duration due to missed abortion (*n*=3), termination of pregnancy (*n*=27), lost to follow-up (*n*=7) and had incomplete data (*n*=3). Of the remaining 1178 women, 93.5% (1102/1178) delivered at term while 6.5% (76/1178) of the women had a PTD. As expected, this is slightly higher than the national PTD rate. The rate of spontaneous PTD was 4.3% (51/1178), of which 49.0% (25/51) had PTL and 51.0% (26/51) had PPROM. The rate of iatrogenic PTD was 2.1% (25/1178), of which 48% (12/25) had preeclampsia. A summarizing description of the cohort is presented in Fig.8.

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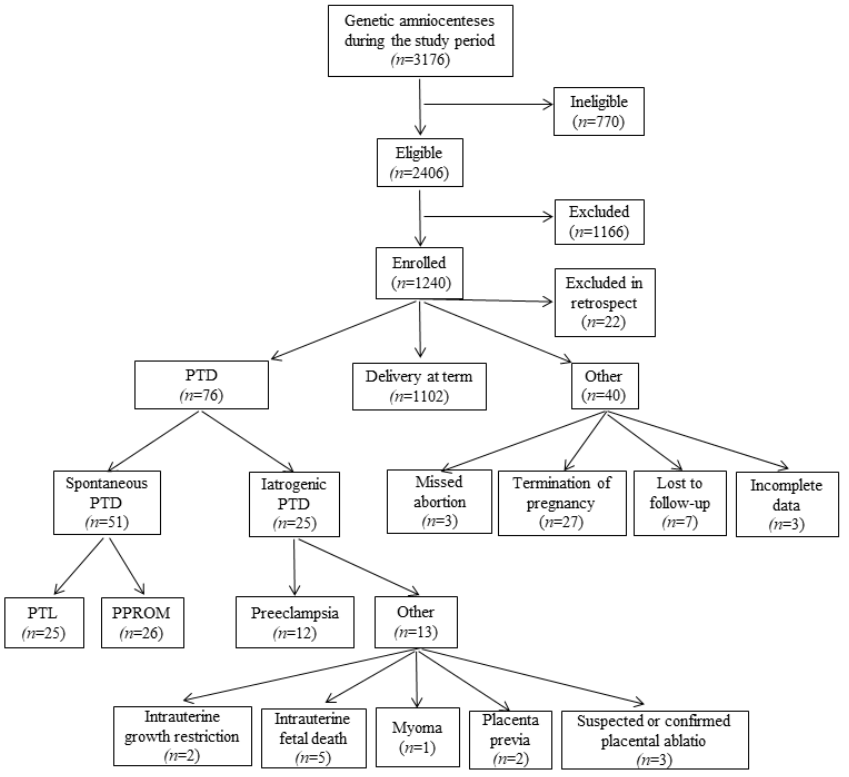

Figure 8. Flowchart of the cohort

Maternal and neonatal characteristics of the study cohort (*n*=1218), from which women with an intrauterine fetal death (*n*=7) and women with "other" outcomes, as shown in Fig.8 (*n*=40), have been excluded, are presented in Table 7 (*n*=1171). This table also presents the maternal and neonatal characteristics of the group of women with a spontaneous PTD (*n*=51).

Table 7. Maternal and neonatal characteristics of the cohort and the subgroup of women with a spontaneous preterm delivery. Continuous variables are presented as the median (interquartile range; IQR) while categorical variables are presented as n (%)

Variable	**Study cohort (*n*=1171)**	**Spontaneous PTD (*n*=51)**
Maternal age at sampling (years)	37 (34 – 39)	37 (35 – 39)
Parity		
0	311 (26.6%)	19 (37.3%)
1	435 (37.1%)	16 (31.4.%)
2	312 (26.6%)	11 (21.6.%)
≥ 3	113 (9.6%)	5 (9.8%)
Maternal BMI at first prenatal visit	23.5 (21.5 – 26.1)	24.9 (22.6 – 27.0)
Smoking at first prenatal visit	59 (5.0%)	5 (9.8%)
IVF	38 (3.2%)	6 (11.8%)
Previous preterm delivery	90 (7.7%)	8 (15.7%)
Gestational age at sampling (weeks+days)	15+5 (15+2 – 16+1)	15+5 (15+1 – 16+1)
Gestational duration (weeks+days)	39+5 (38+5 – 40+6)	35+6 (33+5 – 36+4)
Neonatal sex		
Male	598 (51.1%)	23 (45.1%)
Female	573 (48.9%)	28 (54.9%)
Apgar score <7 at 5 min	11 (0.9%)	1 (2.0%)

Gestational duration of the cohort according to previously described sub-group classifications in setion 2.5.4 and 2.4.1, respectively, are presented in Table 8 (spontaneous PTD) and Table 9 (term delivery). The rate of extremely, severely and moderately PTD is slightly below the average while the group of women with a late or near-term PTD is more than 10 percentage points above average. As expected, the majority of women delivering at term were at full term.

Table 8. Subgroups of PTD (n=76) with the rate presented being within the PTD interval. The overall rate represents the distribution of the study cohort (n=1178)

Definition	**Rate; *n* (%)**	**Overall rate (%)**
Extremely PTD	3 (3.9)	0.3
Severely PTD	9 (11.8)	0.8
Moderately PTD	10 (13.2)	0.9
Late or near-term PTD	54 (71.1)	4.6

Table 9. Subgroups of term deliveries in the study cohort (n=1178) with the rate presented being within the term interval. The overall rate represents the distribution of the study cohort

Definition	**Rate; *n* (%)**	**Overall rate (%)**
Early term	288 (26.1)	24.4
Full term	557 (50.5)	47.3
Late term	187 (17.0)	15.9
Post-term	70 (6.4)	5.9

Fig.9 presents the indication for the amniocenteses in the whole cohort (n=1218). As expected, the most common indication was advanced maternal age.

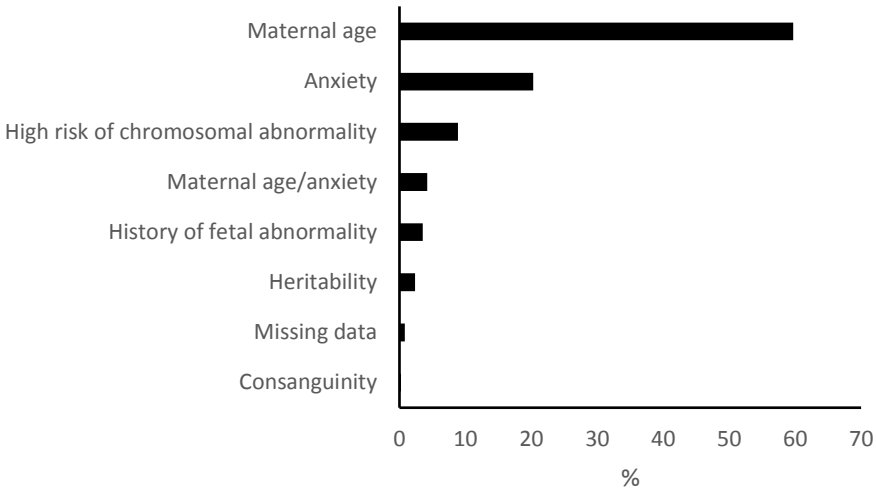

Figure 9. Indication for amniocenteses in the study cohort (n=1218).

4.6 Selection of study participants

The five papers constituting this thesis were performed during different years, and all of them were performed before the whole cohort was collected. As a standard, we included all women with spontaneous PTD on whom we had complete follow-up data at the time of when the respective study was performed. The number of women with a spontaneous PTD in each of the papers thus varies and reflects the number of women who had delivered by the time of analysis.

Initially, we used a nested case-cohort design, to focus on spontaneous PTD versus term delivery, in Papers I-III and Paper V. As time progressed, we also considered gestational duration as an outcome variable in Papers III-V. Among these, Paper IV was the only paper in which gestational duration was analyzed as a continuous variable. Section 4.6.1 to 4.6.5 briefly summarizes the selection of study participants in the respective constituent studies.

4.6.1 Paper I

In Paper I, 19 women with spontaneous PTD (cases) were compared to 118 randomly selected women with delivery at gestational weeks 38+0 to 41+6 (controls). Women with severe chronic diseases were excluded from analysis.

4.6.2 Paper II

The selection process of women for Paper II was similar to that of Paper I. It reports on 24 cases and 40 controls, who were randomly selected among women with a delivery at gestational weeks 38+0 to 41+6. As in Paper I, women with severe chronic diseases were excluded from analysis.

4.6.3 Paper III

Paper III is the one that covers most of the spontaneous PTD cases. It was performed as a three-stage experiment, consisting of an exploratory phase followed by a validation phase and a replication phase. The two first phases reported on essentially the same cohort of women as in Paper II, but with the exclusion of four women due to analytical issues, leaving 22 cases and 37 controls. The replication phase was based on an independent selection of samples from the cohort, consisting of 20 cases and 40 matched controls. The preceding and subsequent control of each case was chosen based on date of sampling and the following criteria; a) maternal age at sampling (± 2 years), b) parity (primiparous/multiparous), c) gestational age at sampling (± 3 days), and d) whether the pregnancy was conceived using IVF (yes/no). Controls were limited to women delivering between 38+0 and 41+6 gestational weeks. Women with certain maternal diseases and fetal complications were excluded from analyses, as well as samples with sampling, processing or analytical deviations.

4.6.4 Paper IV

Paper IV is the only paper with a cohort study design, analyzing gestational duration as a continuous variable. The final cohort that consisted of 784 women, constituting the largest cohort among the constituent papers. To distinguish the phenotype, the study focused on women with spontaneous onset of labor, both at term and at preterm.

4.6.5 Paper V

In Paper V, matched case-control pairs were used. Only women with a delivery at gestational weeks 38+0 to 41+6 were considered eligible controls, as of Papers I-III. Women with sampling or analytical aberrations were excluded, as were women who had chronic diseases that could potentially affect results. The preceding and subsequent control of each case was chosen based on date of sampling and the following criteria; 1) gestational age at sampling (± 3 days), 2) parity (nulliparous/multiparous), 3) IVF (yes/no), 4) maternal age at sampling (± 2 years), 5) BMI group and 6) pregnancy complications or maternal chronic diseases. The final analysis included 36 cases and 36 controls.

4.7 Sample collection and processing

Amniocentesis was performed by an obstetrician under real-time sonography guidance. A 22-gauge (0.7x120 mm) lumbar cannula was inserted transabdominally into the amniotic sac and 10-20 mL of amniotic fluid was extracted. An additional 3 mL was collected from consenting participants. Transplacental amniocentesis was avoided if possible and women were recommended to avoid physical activity for the rest of the day.

Shortly after sample collection, the amniotic fluid was placed in a refrigerator at 4-8 ºC until it was processed later that day. Samples were then centrifuged for 20 minutes at 12 000 *g* at 4ºC (Fig.10). The supernatant was separated from the pellet and divided into aliquots of 200 µL. The pellet was suspended in

phosphate-buffered saline, a water-based salt solution commonly used in biological research, and divided into aliquots of 500 µL. The aliquots were frozen and stored at -80ºC in the local biobank at Sahlgrenska University Hospital/Östra. A standard operating procedure, consisting of detailed step-by-step methodological descriptions, quality manuals and protocols for documentation, was developed. This was carefully followed by all members of the research group in order to achieve uniformity of performance and to ensure a high-quality output without origins, causes and influences that could be affected.

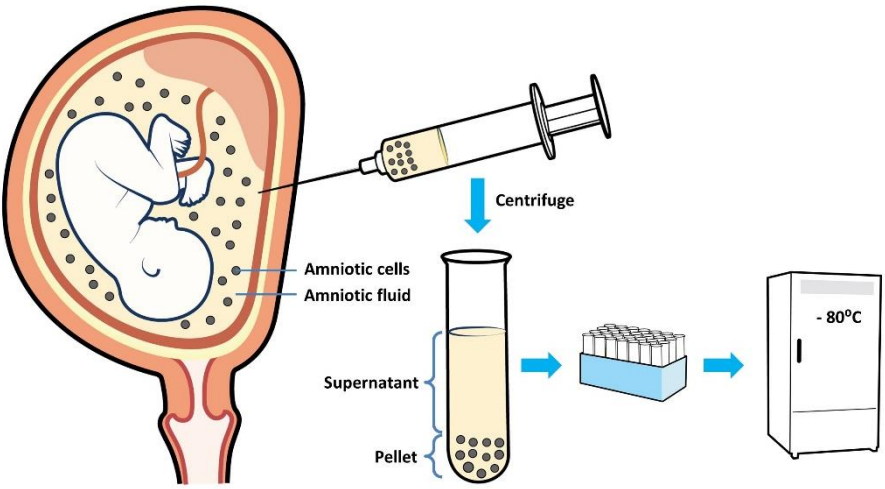

Figure 10. Amniotic fluid sample collection and processing. Illustration: Jan Funke.

4.8 Amniotic fluid analyses

Clinical genetic analysis of the amniotic fluid samples were generally performed with quantitative fluorescent-polymerase chain reaction (QF-PCR), which analyzes chromosomes 13, 18, 21 and the sex chromosomes, accounting for the majority of chromosomal abnormality cases. In some cases, especially with a family history of chromosomal abnormality or genetic disease, a full karyotyping was performed to obtain complete diagnostic information on all chromosomes.

The amniotic fluid collected for research was successively analyzed in batches. Proteins, inflammatory markers and metabolites were analyzed using targeted hypothesis-driven approaches (Luminex, Meso-Scale and enzyme-linked immunosorbent assay (ELISA)), as well as broad untargeted hypothesis-generating approaches (proteomics and metabolomics), to optimize the chances to detect biomarkers associated with spontaneous PTD (Fig.11) and gestational duration (Fig.12).

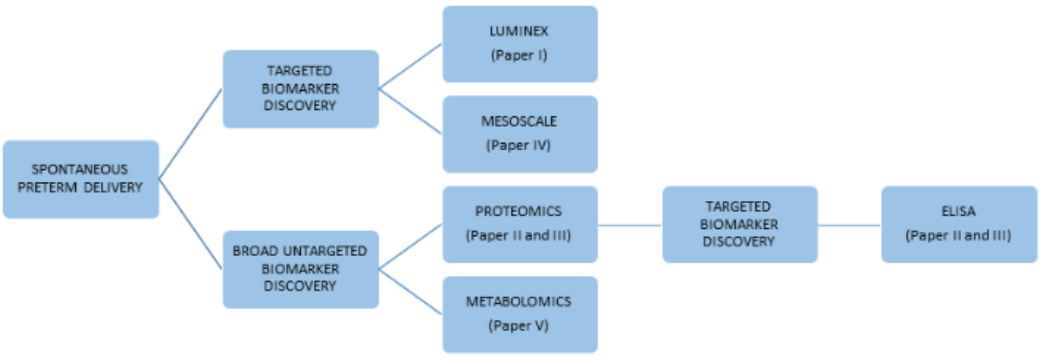

Figure 11. Analyses of mid-trimester amniotic fluid in relation to spontaneous preterm delivery.

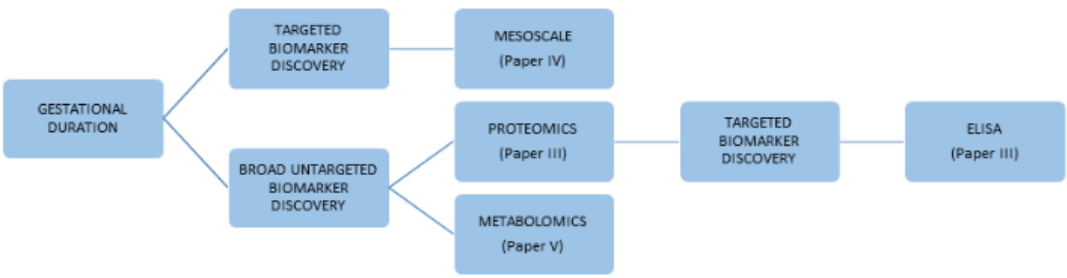

Figure 12. Analyses of mid-trimester amniotic fluid in relation to gestational duration.

Luminex and MesoScale analyses were performed at Statens Serum Institut (SSI) in Copenhagen, Denmark. Proteomics analyses were performed at Charles University, Hradec Kralove, Czech Republic, and metabolomics analyses were performed at Chalmers Mass Spectometry Infrastructure at Chalmers University of Technology, Gothenburg, Sweden. ELISA analyses were performed at the Perinatal Laboratory, Sahlgrenska University Hospital/ Östra, Gothenburg, Sweden.

4.8.1 Multiplex analyses

The hypothesis-driven analyses were developed to solely focus on inflammatory and immunological biomarkers. Due to the complexity of the immune system and the possible network of biomarkers contributing to spontaneous PTD, we performed multiplex analyses, which allow simultaneous measurement of multiple analytes (249). Paper I is based on the first analysis ever of the cohort. When these samples were to be analyzed in 2011, Luminex xMAP technology, previously described in several publications (249-253), was the primary choice for multiplex analyses at SSI. Briefly described, it is a technology based on flowmetric analysis of microbeads encoded with two different fluorescent colors of diverse intensities. Each bead is coated with specific capture antibodies against one of the analytes of interest. The beads are incubated with the samples and the antigens bind to the capture antibodies. The samples are washed to remove excess material and a solution containing antibodies and detector molecules that determines the intensity of the fluorescence is added. The new antibodies bind to the antigens on the beads' antibodies. Samples are incubated again, and the beads pass a Photo Multiplier Tube detector with lasers that read the fluorescence, proportional to the concentration of antigens in the sample. Data are subsequently processed. Due to their unique fluorescence characteristics, assays for several analytes can be performed simultaneously on different sets of beads.

In 2017, we planned for a larger multiplex study with gestational duration as the main outcome (Paper IV). We used a newly developed technology, Meso-Scale Discovery, as it had certain advantages over the Luminex technology. The Meso-Scale technology has been presented in publications (249, 253). Briefly, Meso-Scale combines electrochemiluminescence and MULTI-ARRAY technology. The detection system uses plates fitted with up to ten high-binding carbon electrodes per well, and each electrode is coated with a

different capture antibody. The assay procedure is similar to that of ELISA but quicker; the electrode captures the analytes of interest with an analyte-specific secondary antibody. Labels, called SULFO-TAG, are conjugated to the detection antibodies. On electrochemical stimulation of the plate electrodes' surface, the SULFO-TAG labels emit light that allows determination of the analyte concentration relative to the particular electrode. The combination of electrochemiluminescence and MULTI-ARRAY technology bring speed and high density of information to biological assays.

The analytes selected for the Luminex assay panel were proteins previously identified as associated with term delivery and spontaneous PTD in the late second or third trimester by our group, or linked to neurological developmental abnormality (134, 254, 255). The panel used for Meso-Scale analyses was based on the Luminex panel but further developed to include a few DAMPs (93). These were partly selected, based on the classification of Garg A et al. (256), to explore sterile IAI (96, 97) to some extent. The panel was finally adjusted and developed based on what was technically, financially and realistically feasible, as well as on the availability of commercial kits. Table 10 presents the selected proteins in the respective multiplex analyses with their main function roughly categorized into the following subgroups; cytokines, chemokines or other inflammatory proteins.

Table 10. The selected proteins in the respective panels; [a] part of the Luminex analyses, [b] part of the Meso-Scale Discovery analyses. Data from the UniProt Con-sortium (257) and Meso-Scale Diagnostics (258)

Short protein name	Protein name [a/b]	Main function
Adiponectin [a,b]	Adiponectin	Cytokine
BDNF [a,b]	Brain-derived neurotrophic factor	Other inflammatory protein
CRP [a,b]	C-reactive protein	Other inflammatory protein
GM-CSF [a,b]	Granulocyte-macrophage colony stimulating factor	Cytokine
HMG-1 [b]	High mobility group protein B1	Other inflammatory protein

HSP70 [b]	Heat shock protein 70	Other inflammatory protein
IFN- y' [a]	Interferon gamma	Cytokine
IGFBP-1 [a,b]	Insulin-like growth factor-binding protein 1	Other inflammatory protein
IGFBP-3 [a]	Insulin-like growth factor-binding protein 3	Other inflammatory protein
IL-1β [a,b]	Interleukin-1 beta	Cytokine
IL-6 [a,b]	Interleukin-6	Cytokine
IL-6RA [a]	Interleukin-6 receptor subunit soluble α	Cytokine
IL-8 [a,b]	Interleukin-8	Chemokine
IL-10 [a,b]	Interleukin-10	Cytokine
IL-12 [b]	Interleukin-12	Cytokine
IL12A [a]	Interleukin-12A	Cytokine
IL-17 [a,b]	Interleukin-17	Cytokine
IL-18 [a,b]	Interleukin-18	Cytokine
Leptin [a]	Leptin	Cytokine
MCP-1 (CCL2) [a,b]	Monocyte chemotactic protein 1 (C-C motif chemokine 2)	Chemokine
MIF [a]	Macrophage migration inhibitory factor	Cytokine
MIP-1α (CCL3) [a,b]	Macrophage inflammatory protein 1α (C-C motif chemokine 3)	Chemokine
MIP-1β (CCL4) [b]	Macrophage inflammatory protein-1 beta (C-C motif chemokine 4)	Chemokine

MMP-8 [b]	Matrix metalloproteinase-8 [Neutrophil collagenase]	Other inflammatory protein
MMP-9 [a,b]	Matrix metalloproteinase-9	Other inflammatory protein
NT-3 [a]	Neurotrophin-3	Other inflammatory protein
RANTES [a,b]	T-cell-specific protein RANTES [C-C motif chemokine 5]	Cytokine/chemokine
S100A8 (Protein S100-A8) [b]	S100 calcium-binding protein A8	Other inflammatory protein
TGF-β1 [b]	Transforming growth factor beta-1	Cytokine
TNF-α [a,b]	Tumor necrosis factor α	Cytokine
TNF-β (LT-α) [a,b]	Tumor necrosis factor β (Lymphotoxin-alpha)	Cytokine
TNF-RI [a]	Tumor necrosis factor receptor 1	Cytokine
TREM-1 [a]	Triggering receptor expressed on myeloid cells 1	Other inflammatory protein
Thrombo-spondin-1 [b]	Thrombospondin-1	Other inflammatory protein

4.8.2 "Omics" analyses

High throughput techniques, collectively referred to as "omics" techniques (259) and "high-dimensional biology", allow data mining of complex datasets by simultaneous examination in a biological sample. Changes in the DNA are studied by genomics, in the microRNA by transcriptomics, in proteins by proteomics and in metabolites using metabolomics. These techniques, individually or in combination, aims to increase the understanding of the physiology and mechanisms of pathological conditions or diseases (260-262). Genomics study the expression of genes while proteomics study the protein expression. Metabolomics, however, is used to investigate the consequences of the activity of these genes and proteins. Due to the multi-layered responses to infection and damage detectable by metabolomics, the method may be particularly interesting in the investigation of pathways and processes involved in inflammatory responses (263).

These "omics" techniques are broad and untargeted hypothesis-generating methods. Two of the techniques, proteomics and metabolomics, were applied in the constituent papers of this thesis (Papers II, III and V).

Proteomics provide insight into the proteins' quantity and exploration of the proteomes, covering proteins' overall composition, structure and activity, differing between cells and over time. The mass spectrometry-based method applied in Papers II and III labeled proteins by stable isotope tags followed by liquid chromatography–mass spectrometry (LC-MS). Proteins were digested and multidimensional LC was used to physically separate the multiple components (264, 265) of the amniotic fluid. Finally, samples were analyzed with tandem MS (266), using its mass analysis capabilities which reveals the structural identity of the individual components with high molecular specificity and detection sensitivity. The individual capabilities of each method enhance one another synergistically (264, 265). The peptide identification and quantitation and recorded fragmentation spectra were evaluated in Proteome Discoverer software and Max Quant software, respectively, integrating a false discovery rate analysis, and thereafter compared against the human UniProt database. The sample ratio to global internal standard were calculated based on the intensities of iTRAQ reporter ions, which were corrected using isotope factors supplied with the iTRAQ kit. The identified proteins were further filtered in order to obtain the final dataset. Both a pooled samples strategy (Paper II) and analysis of individual samples (Paper III) were applied. Since the delivery is inflammatory conditioned, many of the proteins that were revealed in the proteomics analyses were naturally linked to inflammation in

one way or another. However, due to the untargeted approach, proteins may also be related to inflammation or immunology by other pathways such as apoptosis or necrosis.

The rapid rise and application of proteomics has resulted in the identification of many potential biomarkers for a number of specific conditions. This abundance highlights the importance of validation in order to confirm or disprove the proteins' potential. This was applied to both Papers II and III, where the results from the exploratory proteomics phase were validated (Papers II and III) and replicated (Paper III).

As the inflammation can alter the local and systemic metabolic profile (267), metabolomics technique was also applied to the cohort (Paper V), with the aim to investigate processes involved in inflammatory pathways of particularly spontaneous PTD but also gestational duration. Metabolomics is used to study small-molecule metabolite profiles (268), end products of cellular processes (269) such as metabolic intermediates, hormones and other signaling molecules (270, 271). Several metabolomics techniques are available, of which LC-MS was used to identify and quantify metabolites in the amniotic fluid samples included in Paper V. Identification is based on the distinct patterns into which analytes fragment, which can be regarded as a mass spectral fingerprint. There are libraries that facilitate identification of a metabolite according to this fragmentation pattern. Metabolomic examination of interactions between inflammation and metabolic changes during gestation may increase our understanding of the etiology of spontaneous PTD (267). It is also a promising method for biomarker discovery (272, 273), for which amniotic fluid, rich in metabolites, becomes an especially interesting biological matrix (267).

4.8.3 Singleplex analysis

The final targeted analysis was singleplex, using ELISA to validate potential biomarkers identified in the exploratory phases of Papers II and III, as well as to replicate proteins from the validation phase of Paper III. In Paper II, only CRP was subjected for validation (protein name and main function is presented in Table 10). Table 11 presents the proteins that were validated in Paper III with their protein accession number and main function roughly categorized into the subgroups, as presented in section 4.8.1.

Table 11. Validated proteins in Papers III. Data from the UniProt Consortium (257)

Short protein name	Protein name
EC-SOD	Extracellular superoxide dismutase (Cu-Zn)
LCN15	Lipocalin-15
MFAP4	Microfibril-associated glycoprotein 4
NGAL	Neutrophil gelatinase-associated lipocalin
PAI-1	Plasminogen activator inhibitor 1
U-II	Urotensin-2

ELISA is the most commonly applied biochemical assay to determine quantitative protein changes in a sample (274) and it is also used for analysis of a single key target protein (249). To detect the presence of a protein in a liquid sample with ELISA, solid-phase enzyme immunoassay is applied, in our case in plates with 96 wells. Antibodies in the wells are specifically targeted for a particular antigen, the protein of interest. Antigens in the sample attach to the surface, where they bind to matching antibodies linked to enzymes, applied over the surface. A substance containing the enzyme's substrate is then added, leading to a color change in the well. Light is thereafter sent through the well by spectrophotometer, and the amount of absorbed light is measured. The darker the color, the higher the antigen concentration (275, 276).

Samples undergoing ELISA were thawed, briefly centrifuged, mixed in a vortex and diluted to different concentrations. The dilution was tested thoroughly, based on the lowest dilution at which the protein can still be detected. Dilution also aimed at preventing protein-protein interactions. When performing analyses such as ELISA, there are factors and challenges that must be taken into consideration and addressed, in order to obtain the most credible results.

4.9 Statistical analyses

The statistical analyses were performed in close collaboration with an experienced statistician. The following software programs were used: SPSS 20.0, 24.0 and 25.0 for Windows XP OS (SPSS Inc, USA), MedCalc 17.7.0 (MedCalc software, Belgium), R version i386 3.0.2 (R version 3.0.2 (2013-09-25), The R Foundation for Statistical Computing, Platform i386-w64-mingw32/i386 (32-bit), R version 3.3.1, and MUVR R package v 0.0.973. Graphical presentation of data in Paper III was designed with the Software Perseus version 1.5.1.6.

In all constituent papers, Mann–Whitney U test or the Student T-test were used to compare continuous variables. These variables were presented as median and interquartile range (IQR: 25th; 75th percentiles). Categorical data were analyzed with the Chi-square test, and in cases of < five individuals at any level, Fisher's exact test was used. Variables were presented as total count (numbers) and frequency distribution (%).

Before analyzing the data, a logarithmic transformation was performed to decrease the effect of outliers and to approach normal distribution, which makes analysis easier. In Papers I and IV, in which multiplex technologies were applied, results were adjusted for plate effects as a potential confounder. Bonferroni correction for multiple comparisons was also performed. In all of the constituent papers, differences were considered statistically significant at $p < 0.05$ using a two-sided alternative hypothesis.

In Papers I and II, the control group was selected by randomization. In Paper I, differences between cases and controls were analyzed using analysis of covariance (ANCOVA). This was also the case in Paper III, in which ANCOVA or Student's t-test was used to compare mean protein concentrations in cases and controls in the exploratory and validation phases. Furthermore, the Pearson correlation was used to analyze correlations between protein concentrations or intensity ratio and gestational age at sampling and gestational duration, respectively, for both cohorts in Paper III. The possible influence of gestational age at sampling was also investigated in Papers IV and V. We adjusted for this variable in all three cases, as there was a strong association between gestational age at sampling and protein concentrations.

A linear mixed model was applied for the replication phase with matched samples in Paper III. The matched samples strategy was also applied in Paper

V, in which a multivariate analysis was undertaken with unbiased variable selection within repeated double cross-validation. Analysis of spontaneous PTD compared with controls was performed as case/control, pair-dependent multilevel analyses with log-fold change between cases and controls as independent variables. Random forest regression models were performed for association between amniotic fluid metabolome and gestational age at sampling and gestational duration at delivery. Statistical significance of multivariate models was assessed using permutation.

Paper IV differed from the others as this was the only study in which gestational duration was analyzed as a continuous variable. It was also the only study in which we selected covariates for which to adjust based on previously reported associations with spontaneous PTD or gestational duration. In the other papers, we instead analyzed whether these and other co-variates affected the outcome and, based on the results, we adjusted for the ones that did. The co-variates in Paper IV were maternal age at sampling, parity, smoking at first prenatal visit and IVF. Initially, linear regression was used to adjust protein concentrations for gestational age at sampling, experimental factors (plate effects) and year of sampling. A Cox regression analysis of the entire study cohort was applied on each of the separate amniotic fluid protein concentrations to explore possible associations with gestational duration. As we were interested in ascertaining whether findings were mostly related to spontaneous PTD or term delivery, Cox regression was censored at 259 days (37+0 gestational weeks) or longer. Based on the results, linear regression models were applied again to evaluate associations between protein concentrations and gestational duration in women with spontaneous onset of labor at term. Finally, linear regression was used to obtain an effect estimate of days/standard deviation (SD). The associations were robust independently of the choice of statistical model. The Cox regression model is a natural choice, rather than the linear model, for modeling survival outcome, while detecting markers with time-varying effects.

Power calculations have not been performed in any of the Papers. In a cohort study like this, we rather had to do the analyses based on the actual numbers since we would hardly have been able to affect the actual numbers. Only in Paper I, a power analysis was used to calculate the smallest effect size possible to detect, based on a given set sample size.

5 Results

5.1 Potential biomarkers associated with gestational duration

Potential biomarkers associated with gestational duration were examined in Papers III-IV. In Paper III, lower concentrations of neutrophil gelatinase-associated lipocalin (NGAL) and plasminogen activator inhibitor 1 (PAI-1) were associated with gestational duration, but neither association was replicated in a second independent cohort. In Paper IV, we found that increased concentrations of thrombospondin-1, macrophage inflammatory protein-1 beta [C-C motif chemokine 4] (MIP-1β) and S100A8, respectively, were associated with lower gestational duration in women with spontaneous onset of labor at term, after Holm-Bonferroni correction. However, the effect was minor, with a decrease in gestational duration by 0.9-1.1 days if concentrations increased by 57% to 104%. No biomarkers associated with gestational duration were identified in Paper V.

5.2 Potential biomarkers of spontaneous preterm delivery

In Paper I, nominally significantly lower concentrations of adiponectin, GM-CSF and macrophage migration inhibitory factor (MIF) was found in amniotic fluid from women with spontaneous PTD, compared to women who delivered at term, after adjustment for plate effects. After a conservative Bonferroni correction, no significance remained. In Paper II, CRP emerged as an interesting biomarker with higher concentrations in women with spontaneous PTD than in women with term delivery. However, its potential was not verified with ELISA. In Paper III, we identified nine potential candidates that were related to spontaneous PTD group. Two of these, NGAL and PAI-1, were validated in the same cohort, with lower concentrations in the spontaneous PTD than in the term delivery group. However, neither association was replicated in a second independent cohort. In summary, no biomarkers that

were associated with spontaneous PTD were identified in Papers I-III, and no potential biomarkers associated with spontaneous PTD emerged in Paper IV or V.

5.3 The association with gestational age at sampling

One of the findings, reported especially in Papers IV and V, was that amniotic fluid protein concentrations changed with gestational age at sampling. The greatest effect was seen in Paper IV, in which thrombospondin-1 and HMG-1 were found to have the strongest associations. Protein concentrations increased 3.4 % and decreased 3.2 %, respectively, per day later that sampling occurred.

6 Discussion

6.1 General discussion

The results of this thesis are generally interesting since there is a biological rationale for several of these candidate proteins being linked to spontaneous PTD, gestational duration and inflammation. Interestingly, certain proteins emerged in several studies, regardless of study design. Insulin-like growth factor-binding protein (IGFBP)-7, NGAL and PAI-1, identified as being associated with both spontaneous PTD and gestational duration in the exploratory phase of Paper III, also emerged as three of the 19 downregulated proteins in Paper II. They were, however, not among the top ten most dysregulated proteins. In Paper IV, protein S100-A8 was significantly associated with gestational duration in women with a spontaneous onset of labor at term. This protein was also identified among the 19 downregulated proteins reported in Paper II.

Many of the women, especially cases of spontaneous PTD, were analyzed in several of the papers. Between Papers I and IV, there was, consciously, an overlap of 148 women, in which amniotic fluid was analyzed using both multiplex methods. Two of the three nominally significantly proteins of Paper I, adiponectin and granulocyte-macrophage colony stimulating factor, were also included in the panel of Paper IV. However, none of them were significantly associated with either spontaneous PTD or gestational duration in Paper IV, in line with the findings of Paper I where no significant differences among women with a spontaneous PTD compared to women with a delivery at term remained after Bonferroni correction for multiple comparisons.

Women excluded from the respective analyses, especially in relation to maternal disease, were addressed specifically for each constituent study based on conditions that might affect the analysis (e.g. were women with diabetes excluded from the metabolomics analyses) or that might have caused spontaneous PTD (e.g. uterine malformations). The exclusion of the first week of the early term group as well as the exclusion of the post-term group from our controls were supported by the theory that the subgroups within the term interval have a somewhat different etiology and rate of adverse neonatal outcomes (82, 83). In regard to women who had a history of previous PTD or

who had undergone IVF, we were consistent throughout all papers. These women were not excluded from analyses, although both previous PTD (277, 278) and IVF have been described as independent predictors of PTD in previous studies (279, 280), as well as in a meta-analysis (281). Women with a spontaneous PTD, which consisted of both PTL and PPROM cases, were not stratified. Our opinion was that the groups were too small for such exclusions or stratifications.

Internal or external environmental, nutritional and genetic factors might have varied over time and influenced the results. The technical environment develops fast, and new technologies can thus be applied within the same project. This was indeed the case with the targeted analyses; Luminex technology was eventually supplemented by the introduction of Meso-Scale technology. Due to the long-term nature of the project, the issue of storage duration of the samples, collected over a 10-year period, should be addressed. There is limited research concerning whether such a long storage period affects protein concentrations in amniotic fluid. In a study from 1998, the stability of angiogenin and IL-6 in paired mid-trimester amniotic fluid samples was investigated. Despite optimal freezing conditions, both proteins' concentrations decreased significantly during the one-year storage period (282). This indicates that the proteins may not be stable. However, in Paper IV, only six of the 26 proteins examined were affected by storage time. In a study by our group, investigating the stability of IL-6 based on different pre-processing conditions, results indicate that this is a stable protein (283). Further research is thus needed to determine the effect of storage time and other factors on protein concentrations.

6.2 Gestational duration

Interestingly, NGAL and PAI-1 were associated with both gestational duration and spontaneous PTD in Paper III, although the findings were not replicated in a second independent cohort. The original case-cohort design in Papers III and V complicated the identification of proteins associated with gestational duration, as the conditions were not really optimal for this variable.

Paper IV consisted of the largest cohort among the constituent papers of this thesis, which provided better conditions and power to detect differences. Further, this was the only study using a pure cohort design in which gestational

duration was examined as a continuous variable. In Paper IV, seven biomarkers were significantly associated with gestational duration at a false discovery rate of <0.1; thrombospondin-1, MIP-1β, S100A8, IL-18, MIP-1α, IGFBP-1 and HMG-1. The increases in thrombospondin-1, MIP-1β and S100A8 survived Bonferroni correction. The results may primarily be relevant in cases of late or near-term spontaneous PTD, representing 71.1% of our PTD cohort, with a median gestational duration (IQR) of 35+6 (33+5 – 36+4) weeks among women with spontaneous PTD. Late spontaneous PTD is associated with less significant neonatal morbidity and mortality compared to spontaneous PTD in the earlier gestational intervals (6). The findings strengthens that this group of women share some common pathways with women who have a spontaneous onset of labor at term. It also adds to the concept of a pregnancy clock, which may be reflected in amniotic fluid.

DAMPs are significant components of the inflammatory processes preceding the spontaneous onset of labor at term. Several DAMPs, specifically amniotic fluid concentrations of HMG1, encoded by the high mobility group box 1 (*HMGB1*) gene, have also been shown to be associated with preterm and term delivery (284-286). Relationships have been reported between DAMPs, in several tissues and biological fluids, and both preterm and term delivery. However, DAMPs are also part of the natural biological pregnancy processes, which must be taken into consideration, especially as sampling was performed at a gestational age during which major reorganization and remodeling occur at the feto-maternal interface, resulting in turn in increased DAMP concentrations. The observed changes could therefore potentially just reflect a normal biological process in a fully developed and functional uterine cavity. Gestational duration prediction based on these biomarkers is thus likely an indicator of a functional amniotic cavity. Either way, the values may indicate feto-placental growth and function and may generate hypotheses for further studies. It is self-evident that the sampling window, gestational weeks 14 to 19, was quite narrow and it is thus unclear whether such correlations continue beyond 19 gestational weeks. Several gestational clocks have been described (15-19, 287), but based on a longitudinal study design with repeated sampling throughout gestation (16). Furthermore, the data concerning "percentage change per day" assumes that percentages changes in a linear fashion, a conclusion that cannot be drawn from our data. However, we are not aware of any publication where this association, adding to the concept of a pregnancy clock reflected in the amniotic fluid, has been described to the same extent as in this paper. It therefore represents an important contribution to the research field.

6.3 Spontaneous preterm delivery

Five potential mid-trimester biomarkers of spontaneous PTD emerged in this project: adiponectin, GM-CSF and MIF in Paper I, and NGAL and PAI-1 in Paper III. All concentrations were lower among women with spontaneous PTD, compared to women with term delivery, but the findings did not survive Bonferroni correction or replication. In total, median concentrations of 18 of the 25 proteins analyzed in Paper I were lower, albeit not significantly, in the spontaneous PTD group, compared with controls (Paper I, Table 3). In Paper III, the majority of the potential biomarkers from the exploratory phase using LC-MS/MS proteomics could not be validated, and the findings of NGAL and PAI-1 could not be replicated. The findings are though novel and interesting as none of nine proteins from the exploratory phase have previously been evaluated in mid-trimester amniotic fluid, although they have previously been described as being associated with spontaneous PTD etiology among symptomatic women in later gestation. Moreover, expression of most of the proteins identified in all the constituent papers was decreased among women with spontaneous PTD. Decreased expression of many cytokines linked to host defense and inflammatory immune capacity have previously been described in mid-trimester plasma samples of healthy pregnancies (26). This may further reflect the high rate of late or near-term spontaneous PTD cases in our cohort, with a somewhat similar etiology as women with a spontaneous onset of labor at term. Infectious and/or inflammatory processes mainly correlate with early, particularly extremely, spontaneous PTD (159) and the earlier in gestation that delivery starts, the more likely the existence of other biological processes and mechanisms that cause onset of the normal delivery process. This further explains the somewhat weak associations in our studies for proteins previously reported as associated with spontaneous PTD.

The Bonferroni correction is conservative, as in the case of Paper I. Some would recommend that it not be performed, but rather to leave it to the reader to judge whether the findings are clinically important or not. The risk if we correct is that we miss important differences, while the risk if we do not correct is the emergence of findings that do not exist.

ELISA did not validate or replicate the findings of Paper III and there are several potential reasons for this. First and most importantly, the results of these two analyses are not comparable or even proportional to one another. The results from the exploratory phase yielded intensity ratios related to ionization/ separation, while ELISA yielded pure concentrations. Validation and replica-

tion were performed by ELISA, but there are other ways to validate and replicate potential biomarkers, i.e. using targeted proteomic analysis by selected or multiple reaction monitoring. There may also be technical and processing differences between the phases, as well as biological differences between the groups, that can explain why these findings were not validated/replicated. Furthermore, the cohorts originated from different years, and environmental conditions may have changed. The dynamic proteome may vary among cells. Its structure depends on a wide range of internal and external factors that were not completely controlled for in this study (or in any human study). Another possible reason that the results from the validation phase did not correlate with those from the replication phase is that we used matched samples for the replication cohort. Finally, at least in women with PPROM, there is a huge variance in protein concentrations, also after stratification according to MIAC and HCA, as well as in the absence of significant differences in clinical and demographical characteristics between groups.

The multicollinearity of the Luminex data, in which the log concentrations of the three statistically significant proteins were strongly correlated (Paper I, Fig.2), must also be kept in mind. This is a problem as the effects of the proteins cannot be kept apart. However, it supports the hypothesis that a coordinated network, rather than a single factor, contributes to the occurrence of spontaneous PTD. Furthermore, the targeted multiplex technology analyses in Papers I and IV solely focused on inflammatory pathways, while spontaneous PTD has multifactorial pathophysiology. For instance, a multiplex study of mid-trimester amniotic fluid in women with a short cervix, that examined multiple pathways rather than a single mediator, found that a group of inflammatory mediators predicted spontaneous PTD (288). A key factor distinguishing that study from ours was that those women had a cervical length of ≤25 mm at the time of sampling. As cervical length was not measured in our cohort, the studies are not really comparable.

6.4 The optimal timing for sampling

The dynamic environment *in utero* changes as pregnancy progresses. However, amniotic fluid samples from asymptomatic women in early gestation, for research purposes, cannot be collectable other than in accordance with clear clinical indications. This limits the time interval to be studied, and raises the question of whether this narrow gestational age interval was optimal

for biomarker discovery. The timing of cytokine release and the local environment in which they act as well as the presence of competing or synergistic elements, cytokine receptor density and tissue responsiveness to each cytokine may potentially differ during pregnancy. Further, as spontaneous PTD is caused by heterogeneous pathways, the respective optimal gestational ages may either overlap or diverse.

Most of our samples were collected between the pro-inflammatory phase of the first trimester and early second trimester and onset of the anti-inflammatory phase of the second trimester. Numerous cytokines are present in mid-trimester amniotic fluid, and previously reported concentrations vary widely. This complicates the possibility to distinguish between "normal" values in healthy pregnancies and those in pathological pregnancies, which precludes the accurate interpretation of results. This is supported by the fact that we found lower concentrations of GM-CSF in mid-trimester amniotic fluid in women with spontaneous PTD in Paper I, while previous data report that the pregnancy itself elicits significantly increased concentrations of GM-CSF in all trimesters, though in maternal serum (30).

6.5 Generalizability

This cohort mainly represents a high-risk group of women with an increased risk of fetal chromosomal abnormalities. The proportion of pregnant women aged ≥35 years in the general Swedish population was 12% in 2015, compared to 74.0% (901/1218) in our cohort. It is therefore unclear how generalizable these data are when it comes to analyzing gestational duration in normal maternal age groups and low-risk pregnancies. Furthermore, the cohort represents women who, from a global perspective, are at low risk of PTD. Our data may therefore not be generalizable to a high-risk cohort. The gestational duration in the spontaneous PTD group is shifted far to the right. Although this does reflect the general rate, we recommend that our results mainly be correlated to the late spontaneous PTD group.

6.6 Clinical importance

The research reported in this thesis has contributed new findings, which are highly significant as they have increased insight into early changes in the amniotic fluid profile. These will hopefully serve as a basis for new hypotheses that may improve understanding of the molecular processes and other biological mechanisms contributing to the development of spontaneous PTD. New knowledge is valuable since PTD can cause neonatal mortality and severe morbidity leading to lifelong disability, and a reduction of the rate would have substantial effects on perinatal health and socioeconomic conditions.

The studies presented in the more comprehensive papers (Papers III-V) are novel and unique when it comes to study design and the use of mid-trimester amniotic fluid samples. Finding biomarkers involved in the process in early pregnancy, long before PTD symptoms have arisen, will enable new strategies for early detection and intervention, treatment and prevention.

The clinical translation value is, however, yet limited. The rationale for performing an invasive procedure such as an amniocentesis to predict pregnancy outcome is weak. It is nonetheless a first step towards biomarker discovery, which can eventually be applied in other more easily accessible biological compartments. The minor effects that we report, with an approximate one-day decrease in gestational duration in women with spontaneous onset of labor at term will lack value in a real clinical setting.

6.7 Comparisons of study designs and analytical approaches

6.7.1 Hypothesis-driven versus hypothesis-generating research

Hypothesis-driven research, where a specific hypothesis is developed in a prospectively designed experiment with pre-defined groups, e.g. case/control, and analyzed with a predetermined assay, has been the classical basic

biological research method. This type of experiment has played, and continues to play, an important role in science and medicine. The introduction of hypothesis-generating research has, however, enabled discoveries that would not have been possible with hypothesis-driven research. The two approaches thus complement each other. Hypothesis-generating research can provide key insights into biological and pathophysiological pathways by leading to results that were not initially intended or expected by the researchers. The novel theoretical basis that this type of research provides will have major impact when applied in clinical medicine (289). However, critical steps after the discovery of potential biomarkers are validation (290) and, when deemed appropriate, replication. The advantages of multiplex technology, compared to proteomics, included simultaneous analysis of a broad panel of potential biomarkers specifically selected for the purpose of the study and on known or hypothesized biomolecular pathways underlying PTD. Other advantages are the small sample volume needed, the cost-effectiveness and the minimal technological challenges, compared to omics techniques (248).

6.7.2 Dichotomous versus continuous variables

Categorizing (grouping) continuous variables may be helpful when data is presented, for instance in tables, and in interpreting results, but it is not necessary for statistical analysis (20). By dichotomizing data, i.e. cases/ controls, a substantial part of the data is lost, reducing the statistical power to detect associations between the variable and an outcome. Studies suggest that the diminished effect by dichotomizing a variable at the median reduces power equal to discarding one-third of all data (291, 292). This complicates research studies that already tend to use too small cohort, especially when examining rare outcomes. Dichotomization may also increase the risk of false positive results (293) and there is a substantial risk of underestimating the variation in outcome between groups as well within each group. Individuals close to, but on opposite sides of the cut-off point, may be characterized as being very different when in fact they are similar. Third, the analysis to two groups conceals and restricts all non-linear relationships between the variable and the outcome (20).

6.7.3 Pooled versus individual samples

Pooling samples means that multiple samples are homogenized into one sample for analysis (165). One advantage to this is that variations between patients, within patients or among sampling or processing conditions (such as between-year variation) may be reduced. It is also much less expensive than analysis of individual samples (294) and useful when the number of specimens is limited (295). The advantages of individual samples, on the other hand, are available information about variance and the freedom to choose an appropriate central measure (296, 297).

6.7.4 Luminex versus Meso-Scale technology

Both Luminex and Meso-Scale platforms are suitable for analyzing trends in multiple cytokine profiles. Quantitative differences are found but the relative differences are comparable. Luminex technology offers better precision, while Meso-Scale has higher sensitivity (249), better accuracy, and lower inter- and intra-assay variation. It also offers a broader dynamic range (249, 253), meaning that high and low expression concentrations can be measured without multiple sample dilution. As stated in Paper I, one protein, interferon (IFN)-γ, was only detected in 0.7% of the samples and was therefore excluded from the analysis. This can happen if there is a broad range of proteins concentrations within the same sample. The Luminex assay is the multiplex technology with the lowest range, which entails difficulties in simultaneously quantifying low and high concentrations of compounds, with loss of accuracy especially in the former case (249). The Meso-Scale platform, which has better low concentration accuracy (249, 298), might have detected IFN-γ better.

6.7.5 Multiplex versus singleplex technology

Compared to multiplex technologies, which allow simultaneous wide-range screening of multiple analytes in small sample volumes (298), ELISA enables the analysis of a single key target protein (249). Several analytes can be analyzed, but several ELISAs would need to be run separately. This would be

more time-consuming and costly and require a larger sample volume, which would be challenging if the source of samples is small (298). However, one issue with multiplex analyses is the mass significance and need to adjust for multiple comparisons.

6.8 Strengths

The value of this research project lies in the fact that it was performed on mid-trimester (14-19 gestational weeks) amniotic fluid samples obtained via trans-abdominal amniocentesis from asymptomatic women. The size of the cohort, with a lost to follow-up rate (including missing data) of only 0.8% (10/1218), comprehensive databases with clinical variables, systematic processes and standardized methodology for sample collection and processing and the number of available aliquots, is unique. The major strengths are the well-designed studies, using high-capacity techniques such as proteomics and metabolomics and multiplex approaches, which may be a key to increased understanding of spontaneous PTD, detection of novel biomarkers and to obtaining a complete picture of the process (172, 248). Collection of samples at this gestational age is becoming increasingly rare due to the introduction of NIPT, and new cohorts like this are thus unlikely in future.

The pathways underlying spontaneous PTD and the factors involved in the onset of labor were examined with a variety of different techniques involving both targeted and non-targeted approaches. This should be considered a strength, as it increases the chances of identifying normal and pathological processes.

A major strength of this project is the high level of data accuracy. In all constituent studies, we collaborated with an experienced statistician and the statistical analyses were thus very thorough. Technical analyses were conducted in collaboration with research groups, rather than just using services to undertake the analyses, in order to extract maximal effects of the data and to be able to discuss findings. This had a major impact on our research.

The database work and medical records reviews were undertaken meticulously by experienced midwives or medical students involved in research. The selection of groups was also meticulous and undertaken stepwise, in order to ensure data accuracy. Results of the exploratory proteomics studies were

validated/replicated, which is otherwise frequently neglected. The fact that three proteins were disqualified from validation in Paper III should also be regarded as a sign of seriousness and accuracy, as a factor present within the amniotic fluid samples might have caused matrix interference. This might in turn have confounded interpretation of results causing bias.

Another major strength is that this was a team-oriented multidisciplinary project in which the team members shared responsibility for the project but contributed different skills and responsibilities. These interdisciplinary collaborations led to optimized and efficient use of available resources. I have been involved in this long-term and extensive project since the very beginning and throughout all phases, including conception, acquiring funding, development, applications, planning and execution. This has led to substantial continuity, which must be regarded as a strength.

6.9 Limitations

The most important limitation is that women undergoing mid-trimester genetic amniocenteses constitute a highly selected population, as they are of a more advanced maternal age and have a higher risk of fetal chromosomal abnormalities than the overall population. This causes selection bias. Such mid-trimester amniotic fluid samples can, however, only be collected in line with clear clinical indications, such as genetic testing. By excluding women with known or suspected fetal abnormalities from the study and women with confirmed fetal abnormalities from analysis, bias was addressed to some extent.

Of all the women who underwent a mid-trimester genetic amniocentesis during the study period, 24.2% were ineligible and 48.5% of the eligible women were excluded. An important limitation is the lack of information about these women. In retrospect, it would have been preferable to ask all women presenting for mid-trimester genetic amniocentesis for permission to review their medical records. Questionnaires regarding dietary patterns and physical activity, collection of blood, saliva or urine samples (possibly reflecting the intrauterine environment as well as amniocentesis), or an ultrasound assessment of the cervix are other factors that might have complemented our data. Finally, we made the cardinal error of designing the study based on what

was known to us at that stage. With current knowledge and experience, we might have been bolder and more innovative.

A case-cohort design, with a control group limited to deliveries between 38+0 and 41+6 gestational weeks and the use of matched cases and controls (as in the replication phase of Paper III as well as in Paper V), are not optimal for studying gestational duration. To maximize the chances of finding associations between protein concentrations and gestational duration, the latter should be treated as a continuous variable, as in Paper IV.

The spontaneous PTD cases consisted of both PTL and PPROM cases, which can complicate detection of true differences. As DAMP expression is much more prone to be elevated in PPROM cases, a stratified analysis might have yielded other results.

The risk of PTD in Sweden is generally low, 5.7% of all pregnancies, and approximately 3.5% are spontaneous. Despite the relatively large cohort of 1218 women, the spontaneous PTD cases available for study are limited (n=51), making it difficult to draw conclusions. Studies of amniotic fluid from the general pregnant population at mid-trimester might have yielded other results, but this would have been neither feasible nor ethical. The studies may thus have been underpowered to detect smaller effect estimates of the biomarkers.

6.10 The project's future

The research project is still in several different phases and this thesis does not mark its conclusion. Parts of the research have been presented at national and international congresses and meetings, attracting broad interest in the project and in the unique cohort of samples and associated medical data. As a first step, we will continue to investigate gestational duration in order to gain a deeper understanding of spontaneous PTD etiology. To gain greater understanding of the biological processes, integrating proteomics and metabolomics data into a multivariate model might be considered, as well as to further study the specific proteins that emerged in several of the papers. Further research is also needed to determine the effect of storage time and different processing conditions on protein concentrations.

Even though spontaneous PTD is the focus of our research, the size of the cohort enables studies of a variety of pregnancy, delivery and neonatal outcomes, for which we have ethical approval to study. We have a substantial number of aliquots left in our biobanks and a comprehensive database with key research variables created especially for the overall project. Furthermore, we have organizational resources and well-organized and established collaborations worldwide. Our ethical approvals permit the connection of data from the project to the extensive, comprehensive and world-renowned registers in Sweden. Together, these advantages enable future research, in which components of mid-trimester amniotic fluid can be linked to other adverse pregnancy outcomes, such as preeclampsia.

Due to the coordinated networks that most likely initiate the onset of delivery, inflammatory, immunological and other pathways have been examined in this thesis, as well as cellular metabolism. However, infections, dietary patterns, environmental factors and genetics probably interact with the inflammatory processes in ways as yet unknown to us. The published papers have high scientific value concerning both negative findings and potential biomarkers. We have now come to a point in our research where the right conditions exist for initiating studies of a more revolutionary character. This and other ongoing projects will now move on to an exciting and innovative level and we are hopeful about what this may contribute to research and society.

7 Conclusions

This thesis presents what should be considered as a snapshot of amniotic fluid composition in the mid-trimester with regard to the subsequent development of spontaneous PTD and the spontaneous onset of labor at term. The cohort represents a highly selected population with a low risk, from a global perspective, of spontaneous PTD. In accordance with the general rate, spontaneous PTDs in this cohort were mainly late cases, and the results should be interpreted accordingly. Alarmins and chemokines can assist in the determination of gestational duration in women with spontaneous onset of labor at term, and the inflammatory processes preceding the spontaneous onset of labor at term are present and detectable as early as in the mid-trimester. DAMPs are involved in natural biological processes such as remodeling of the tissues, and the concentrations measured at mid-trimester may be an indicator of functional fetal membranes and a functional placenta.

The early prediction of spontaneous PTD remains a major challenge due to the complexity inherent in its diverse phenotypes, different underlying pathologies and a limited understanding of sequences and events preceding the condition. Potentially, spontaneous PTD may be the result of acute events not detectable in amniotic fluid as early as in the mid-trimester. Another possibility is that biological signals in early mid-trimester amniotic fluid may be of insufficient strength for accurate risk prediction of spontaneous PTD.

A pregnancy clock, partly controlled by a timing mechanism linked to fetal development that regulates the onset of labor has previously been described in maternal blood/serum and in the placenta. The results presented this thesis and its constituent papers suggest that such a clock is also reflected in amniotic fluid, and that deviations from the clock may precede spontaneous PTD.

Gestational age at sampling is strongly associated with the concentration of proteins in mid-trimester amniotic fluid. Not taking this into consideration in amniotic fluid biomarker studies may introduce bias.

8 Other research during the doctoral studies

During this period, I have been involved in several other projects. Parallel studies of women with and without symptoms of threatening spontaneous PTD, and studies focusing on other pathways such as the infectious pathway (299) as well as being involved in follow-up studies of preterm-born neonates (300). Moreover, we have investigated the stability IL-6 (283), of one of the most promising biomarkers for spontaneous PTD prediction. Finally, I have contributed to several other projects, for which I have been acknowledged.

8.1 Co-authored publications

Stinson L*, Hallingström M*, Barman M, Viklund F, Keelan J, Kacerovsky M, Payne M, Jacobsson B. Comparison of bacterial DNA profiles in mid-trimester amniotic fluid samples from preterm and term deliveries. Front Microbiol. 2020 Mar 24;11:415.
(* Equal contribution)

This is the first paper from the cohort focusing on the infectious mechanisms of spontaneous PTD. The aim was to identify predictive bacterial DNA signatures in mid-trimester amniotic fluid. However, the samples proved to contain low-abundance and low-diversity bacterial DNA, and species typically associated with spontaneous PTD were absent. The majority of all samples were sterile but bacterial DNA was present in approximately one-fifth, suggesting that non-pathogenic bacteria may be present in amniotic fluid as early as in the mid-trimester.

Thorell A, Hallingström M, Hagberg H, Fyhr I-M, Tsiartas P, Olsson I, Chaplin J E, Mallard C, Jacobsson B, Sävman K. Microbial invasion of the amniotic cavity is associated with impaired cognitive and motor function at school age in preterm children. Pediatric Research 2019 Nov 11.

This study aimed to evaluate neurodevelopmental outcome, such as cognitive and motor function, in school-age children born at <34 gestational weeks and exposed to MIAC during gestation. Children exposed to MIAC had significantly lower scores for full-scale IQ, verbal IQ and motor function, compared to the non-MIAC group.

Tsiartas P, Kacerovsky M, Hallingström M, Liman V, Cobo T, Jacobsson B. The effect of latency of time, centrifugation conditions, supernate filtration, and addition of protease inhibitors on amniotic fluid interleukin-6 concentrations. Am J Obstet Gynecol. 2015 Aug;213(2):247-8.

The aim of this study was to evaluate preanalytical handling procedures' effect on IL-6 concentrations, measured by immunoassay. Amniotic fluid was collected from women undergoing elective cesarean section at gestational weeks 38+0 to 41+6. The influence of latency from sampling to centrifugation, centrifugal conditions (centrifugal force, time and temperature), supernate filtration and addition of protease inhibitors (serine, cysteine and metalloproteases) were compared to a standard group. ELISA was used for analysis within 8 months after sampling. IL-6 concentrations were not affected by the different preanalytical handling conditions.

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