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CHARACTERIZING RETAINED PLACENTA: EPIDEMIOLOGY AND PATHOPHYSIOLOGY OF A CRITICAL OBSTETRIC DISORDER

Margit Endler

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Characterizing retained placenta: epidemiology and pathophysiology of a critical obstetric disorder

THESIS FOR DOCTORAL DEGREE (Ph.D.)

By

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To Jakob, Noah and Sacha. My boys.

"Women are not dying of diseases we can't treat. They are dying because societies have yet to make the decision that their lives are worth saving."

- Mahmoud Fathalla

ABSTRACT

Background: Retained placenta is associated with severe postpartum hemorrhage but its etiology and pathophysiology are largely unknown. Certain studies have suggested that retained placenta is associated to defective placentation disorders- pregnancy disorders with an initial defective placentation resulting in increased oxidative stress. The aim of this thesis was to investigate risk factors for and consequences of retained placenta, determine whether retained placenta and defective placentation disorders are epidemiologically associated and to assess if this association is supported at the molecular and histological level.

Methods and Main Results: Study I was a case-control study comparing pregnancy and delivery-related variables in women with retained placenta and controls (n=408 in each group) after singleton vaginal birth. The study found that retained placenta was associated with severe postpartum hemorrhage and that a history of abortion or recurrent miscarriage, pre-eclampsia, preterm delivery and prolonged oxytocin use in the current pregnancy were independent risk factors for retained placenta. **Study II** was a population based cohort study investigating the association between retained placenta and defective placentation disorders (pre-eclampsia, preterm birth, small-for-gestational-age birth and stillbirth) in primiparous women giving vaginal birth at 32-41 gestational weeks between 1997 and 2009 in Sweden (n=386 607). The study found that retained placenta was associated to pre-eclampsia, spontaneous preterm birth, small-for-gestational-age birth and stillbirth. The risk was further increased for women with these disorders among preterm deliveries. **Study III** was a cross-sectional pilot study investigating the antioxidative enzyme Glutathione Peroxidase 1 (GPX1) and the transcription factor Nuclear Factor Kappa-light-chain-enhancer of activated β-cells (NFκB), as markers of antioxidative defence capacity and inflammation, in 29 retained and 31 non-retained placentas. The study found that retained placentas showed a tendency of lower median concentrations GPX1 and were significantly more likely to have a low level of GPX1 protein concentration. There were no differences in expression of NFκB. **Study IV** was a case-control study comparing histological signs of maternal underperfusion and inflammation in retained (n=49) and non-retained (n=47) placentas. The study found that retained placentas had a significantly smaller surface area, were more oblong in shape and showed overall more signs of maternal placental underperfusion compared to non-retained placentas.

Conclusions: Retained placenta is epidemiologically associated to defective placentation disorders, a finding which is supported in part by signs of decreased antioxidative capacity in the placenta and increased histological signs of maternal placental underperfusion. Prolonged oxytocin use may exacerbate the risk of retained placenta. Risk awareness of retained placenta should guide preparedness during the third stage of labor given the high risk of severe postpartum hemorrhage that the disorder entails.

LIST OF SCIENTIFIC PAPERS

I. Endler M, Grünewald C, Saltvedt S. Epidemiology of retained placenta: oxytocin an independent risk factor. Obstet Gynecol. 2012 Apr;119(4):801-9.

II. Endler M, Saltvedt S, Cnattingius S, Stephansson O, Wikström AK. Retained placenta is associated with pre-eclampsia, stillbirth, giving birth to a small-for-gestational-age infant, and spontaneous preterm birth: a national register-based study. BJOG. 2014 Nov;121(12):1462-70

III. Endler M, Saltvedt S, Eweida M, Åkerud H. Oxidative stress and inflammation in retained placenta: a pilot study of protein and gene expression of GPX1 and NFκB. *Submitted*

IV. Endler M, Saltvedt S, Papadogiannakis N. Macroscopic and histological characteristics of retained placenta: a prospectively collected case-control study. *Submitted*

CONTENTS

LIST OF ABBREVIATIONS

aOR	Adjusted odds ratio
BMI	Body mass index
BPMF	Basal plate myometrial fibers
CI	Confidence interval
D&C	Dilatation and curettage
ELISA	Enzyme linked-immunosorbent assay
GPX1	Glutathione Peroxidase 1
ICD-10	International Classification of Diseases 10
Iκβα	Nuclear Factor Kappa-light-chain-enhancer of activated β-cells Inhibitor, alpha
IQR	Interquartile range
LGA	Large-for-gestational-age
mRNA	Messenger RNA
NFκB	Nuclear Factor Kappa-light-chain-enhancer of activated β-cells
PROM	Prelabor rupture of membranes
qRT-PCR	Real-time quantitative reverse transcriptase polymerase chain reaction analysis
RNA	Ribonucleic acid
ROC	Receiver Operating Characteristic
ROS	Reactive oxygen species
OR	Odds ratio
SGA	Small-for-gestational-age

1 INTRODUCTION AND RATIONALE

Retained placenta is globally a major cause of severe postpartum hemorrhage and in areas without access to advanced obstetric care it is often fatal[1]. Despite this, its etiology is unknown and there is no preventative or non-invasive treatment[2]. What motivated this research project was the severity of the consequences of retained placenta in a context lacking research. There existed a need to identify clear risk factors and investigate whether there were biochemical and structural properties characteristic to this poorly understood disorder.

A 2-3% incidence potentially means that millions of women suffer severe postpartum hemorrhage as a result of retained placenta every year[1]. The treatment, manual removal of the placenta, is an invasive procedure involving an increased risk of infection, the risks of full anesthesia as well as the immediate separation of mother and child after delivery[3]. There is no pharmacological treatment for retained placenta that has proved to decrease the need of manual removal of the placenta[2].

At the start of this research project twenty years had passed since the risks associated to retained placenta were analyzed in a large study group in a modern obstetric context[4]. Since then the average woman giving birth is older, heavier, more likely to use assisted reproductive technology, to have a history of previous cesarean section and to give birth with epidural anesthesia after prolonged and augmented labor[5]. Few pathological determinants for retained placenta were known and it was unknown whether these determinants were there at placentation or occurred after delivery. There were no studies investigating whether there were properties specific to retained placenta that would suggest that the disorder was related to other placentation disorders.

The rationale for this project was firstly that knowledge of risk factors for retained placenta could help in identifying women at increased risk of serious hemorrhage, allow clinicians to act rapidly when the condition arises and to take preparatory measures if a high degree of risk is suspected; secondly that exploring the pathophysiology of this poorly understood condition, and its relationship to other pregnancy-related diseases, could be instrumental in its eventual prevention or in its less invasive treatment. This would be of importance most of all to the world´s most vulnerable obstetric population, women giving birth in low resource and poorly staffed settings.

2 BACKGROUND

2.1 RETAINED PLACENTA DEFINED

The timing of manual removal of a non-detached placenta differs globally[6]. The World Health Organization recommends that retained placenta be diagnosed if the placenta has not detached 30 minutes after birth but, weighing the chance of spontaneous delivery against the increasing risk of hemorrhage with delay, recommends manual removal after a further 30 minutes if there are no signs of excessive hemorrhage prior to this[7].

Retained placenta can be separated into three subcategories based on clinical presentation: firstly placentas that are adherent to but can be manually separated from the uterine wall; secondly rare cases that display abnormal invasion into the uterine wall (placenta accreta); and thirdly placentas that are trapped behind a cervix that has contracted after delivery. This thesis is concerned with the first category, the adherent placenta, which is probably the most common form of retained placenta, representing approximately 80% of cases[1]. We have consequently, in addition to its diagnostic code, defined retained placenta by the surgical code for manual removal of the placenta (**Study I** and **II**), which although not excluding the entrapped placenta may reduce its proportional contribution; or by the actual verification during surgery of an adherent plane between the placenta and the uterine wall (**Study III** and **IV**). In **Study III** and **IV** all cases of retained placenta that reached the operating theatre were however found to be adherent obviating the need to exclude any that were entrapped. A recent Cochrane report found that continuous traction of the umbilical cord to detach the placenta after birth, as is routinely performed at the hospital where our studies were performed, reduces the need of manual removal of the placenta, possibly most so in cases where the placenta is trapped behind the cervix[8].

2.2 CONSEQUENCES OF RETAINED PLACENTA

The main complication to retained placenta is postpartum hemorrhage (blood loss >500ml) and severe postpartum hemorrhage (blood loss >1000ml)[4,9,10]. Untreated the fatality rate is somewhere between 2 and 10%[1]. A review of cases of retained placenta in Nigeria reported a fatality rate of 1.8%[11]. Many estimates from clinical settings however do not include deaths in areas where women deliver in less equipped facilities or do not reach health care facilities altogether. In a retrospective study of maternal deaths in rural Tanzania, 70% of which delivered at home, retained placenta accounted for the majority of all deaths[12].

Manual removal of the placenta furthermore increases the risk of postpartum endometritis threefold after adjusting for other sources of infection[3,13]. The procedure is often done under general anesthesia which in the immediate postpartum period is complicated by an increased risk of hypoxia and aspiration during intubation[14].

2.3 RETAINED PLACENTA IN A HISTORICAL PERSPECTIVE

2.3.1 Obstetric practice

In the first decades of the 20th century obstetricians might wait several days for a retained placenta to detach in order to avoid the complications believed to result from manual removal[15,16]. This procedure, with a fatality rate of 10%, was considered one of the most deadly obstetric interventions[17]. Puerperal sepsis due to manual removal may have been as high as 60% at the beginning of the 20th century[18]. In hindsight the high death rate was most probably a result of the delay itself, with manual removal performed on women who were hypovolemic and septic due to severe blood loss, uterine atony and bacteremia[19]. Since bleeding in many cases of retained placenta is retroplacental, it may show first upon manual removal of the placenta leading clinicians to conclude that it was safe to wait. Until the 1950s obstetricians were not in agreement as to what the best practice was[20]. Since then the consensus is to remove the placenta if it has not detached within 30-60 minutes of the birth of the infant or if blood loss is excessive[21].

2.3.2 Increasing incidence

There is a fairly strong consensus that the incidence of postpartum hemorrhage is increasing and that this increase cannot fully be accounted for by changes in demographic or delivery-related factors[22-24]. It is a little less clear whether the incidence of retained placenta is increasing. A historical analysis of national medical archives showed an increase in incidence across nine UK hospitals from 0.7% in 1920 to 2.3% in 1980[25]. However, the rate increase between 1950, when the indications for manual removal may have become more standardized, and 1980 was more inconsistent. A study of 17 002 deliveries in a UK hospital 1947-1951 reported an incidence of 2.3%, very similar to today[20]. Another descriptive study from 1952 showed an incidence of manual removal of the placenta of 3.5% among 12 000 vaginal deliveries in a Harlem hospital[26]. Whether the reported rising incidence in the past century reflects a true increase or merely more consistency in diagnosis is uncertain.

2.4 RETAINED PLACENTA IN A GLOBAL PERSPECTIVE

Inconsistencies in the definition of retained placenta and recording of causes of maternal morbidity and mortality complicate the analysis of global disparities and changes in incidence of retained placenta. It has been suggested that retained placenta is less common in developing countries. A trend of rising incidence from low- to middle- to high-income countries would seem to support this[1,27]. The question is if this disparity is due to differences in obstetric practice, to demographic risk factors or to genetic variations. Interestingly a descriptive study of 152 cases of retained placenta in Louisiana, USA, in 1941 noted that although black women made up 61% of the patient population they only made up 36% of cases of retained placenta[28]. A protective effect of non-white race has also been noted in a recent study[29].

Irrespective of incidence variations the consequences of retained placenta and hemorrhage are beyond doubt more severe in low resource settings. Postpartum hemorrhage causes a third of all maternal deaths worldwide and 99.6% of these deaths occur in low and middle income countries[30]. In Sweden only one woman died as a results of postpartum hemorrhage in the time span 1997-2005 which bluntly illustrates the global inequality in access to obstetric emergency care[31]. Uterine atony is the most common cause of postpartum hemorrhage but hemorrhage due to retained placenta may account for a greater proportion of severe hemorrhage and maternal death[9,32,33]. In other words, in a setting without access to skilled obstetric care, retained placenta may be a more dangerous cause of postpartum hemorrhage than uterine atony.

2.5 NORMAL AND ABNORMAL PLACENTATION

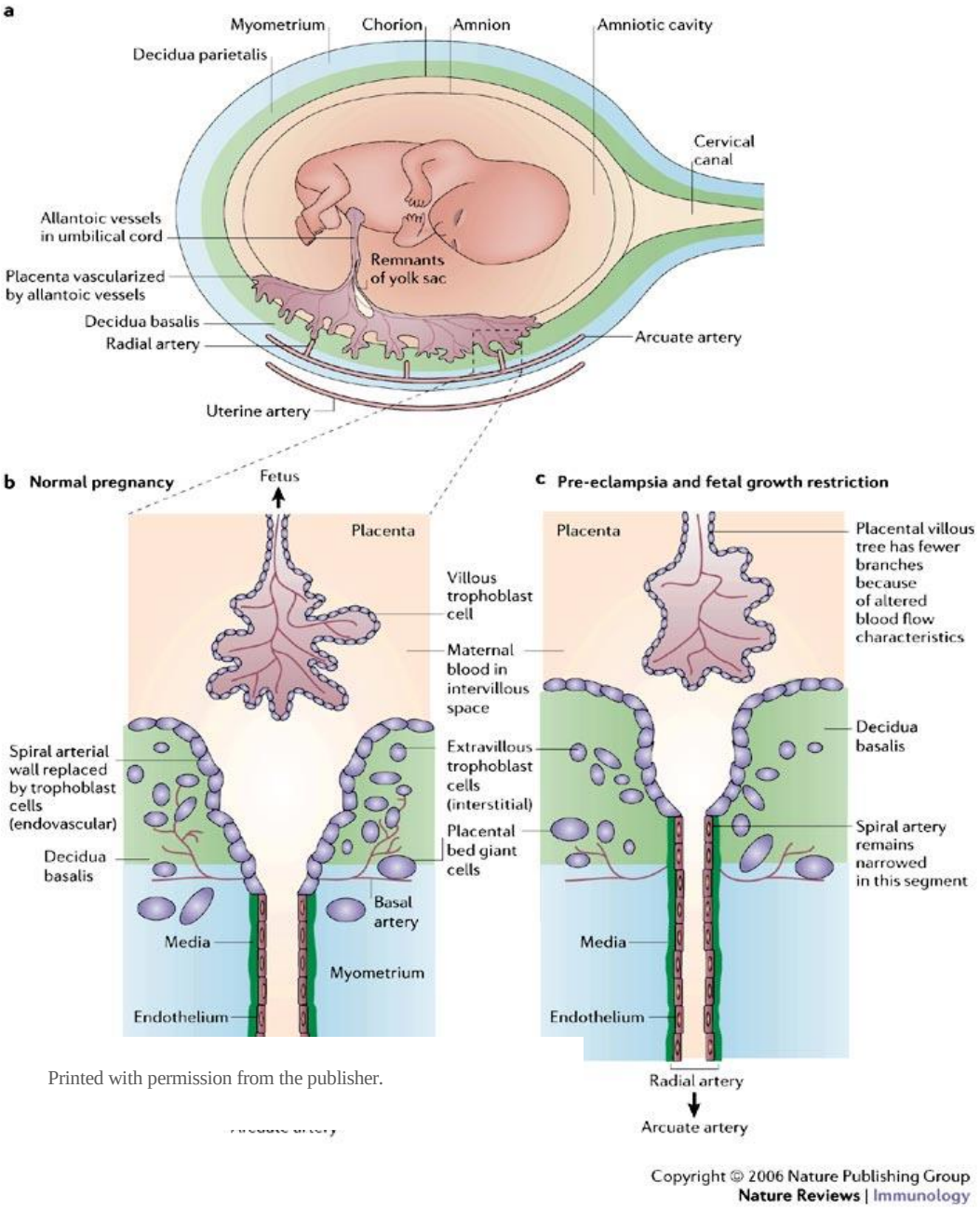
In expectation of the implantation of the embryo, endometrial stromal cells normally transform into decidual cells (decidualization), of which some will become the basal decidua and of which some will surround the embryo, fuse with the smooth chorion and become part of the fetal membranes[34]. Around gestational day 12, extravillous trophoblasts from the developing embryo invade the basal decidua into the superficial myometrium and remodel the normally muscular uterine radial arteries into pliant spiral arteries[34]. This enables the low pressure vascular system needed to ensure blood follow to the developing fetus. Pregnancies that develop pre-eclampsia or end in miscarriage, preterm delivery or small-for-gestational-age (SGA) birth are more likely to show decreased spiral artery remodeling and a more shallow placentation[35] (Figure 1).

In cases of placenta accreta the placental villi are found in direct contact with the myometrium in areas where the decidua is absent. Trophoblast invasion then extends deeper into the

myometrium sometimes penetrating the entire uterine wall (placenta increta and percreta). It is debated whether this is due to increased invasive properties of the extravillous trophoblasts or to defective decidualization resulting in a poor implantation site which then requires deeper attachment[36]. In support of the latter theory the risk of placenta accreta increases if placental attachment occurs over an area with poor decidualization. The risk of accreta is thus increased with implantation in a previously irradiated uterus, implantation in the lower uterine segment (placenta previa), implantation over a previous uterine scar or implantation after previous dilatation and curettage (D&C), an intervention used to evacuate early pregnancy[36].

Figure 1

Normal and abnormal placentation and spiral artery remodeling



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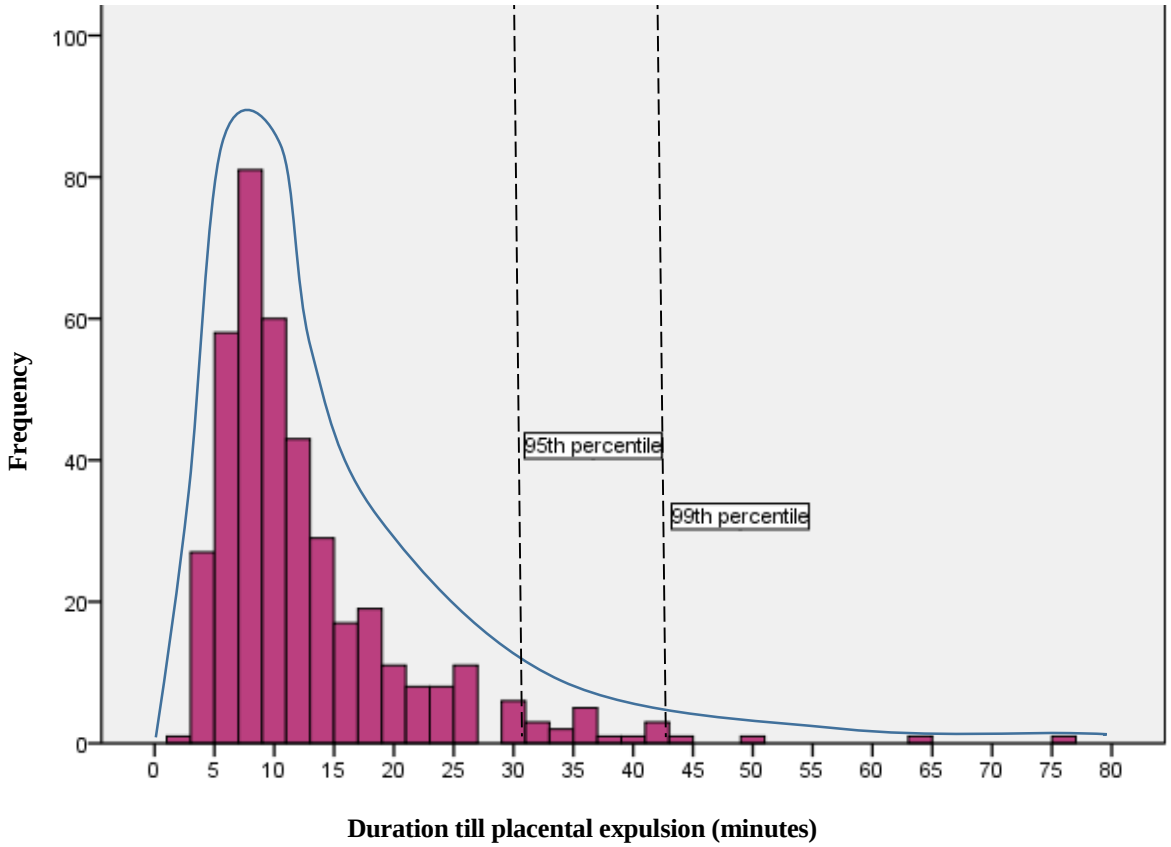
2.6 NORMAL PLACENTAL RELEASE

In clinical practice retained placenta is only diagnosed after vaginal birth. The average time till placental expulsion is between 6 and 10 minutes according to studies spanning over the past 70 years and approximately 80% of placentas will have detached within 15 minutes of the birth of the infant[4,20,37,38]. After 30 minutes only 3.3% remained attached in an analysis of almost 13000 deliveries between 1978 and 1988 but this included those that went on to need manual removal of the placenta[4]. In our analysis of 408 deliveries with spontaneous placental release, of which 398 had data on time till placental expulsion, five percent were retained at 30 minutes, and only one percent at 43 minutes (Figure 2). In 1949 it was demonstrated radiographically that placental detachment requires continued uterine contractions after delivery[20]. Ultrasound studies have since then showed that normal placental separation occurs after a complete cessation of blood flow between the placenta and the myometrium followed by an isolated retroplacental myometrial contraction[39,40]. In a normal third stage there is a gradual thinning of the myometrium in the lower uterine segment and a myometrial thickening of the upper uterine segment which occurs in a linear fashion over time. In a third stage where placental release is relatively delayed (>12 minutes) but ultimately spontaneous, the thickness of the myometrium is reduced overall and the rate of change in each segment slower[38].

Figure 2
Histogram of duration till placental expulsion among spontaneously detached placentas (n=398)



2.7 PATHOPHYSIOLOGY OF RETAINED PLACENTA

2.7.1 Epidemiology

At the start of this research project there was limited knowledge on who was at increased risk of retained placenta. There was some consensus that previous retained placenta, previous abortion, grand multiparity (parity ≥5) and preterm delivery independently predisposed to the disorder[4,13,41,42]. However, there was only one study, dated 1991, with a sample size that allowed a broader risk factor analysis. In this study maternal age >30, non-Asian genetic descent, pre-eclampsia, delivery in a labor bed and augmented labor with oxytocin were also associated to retained placenta[4].

In the years 2014-2015 however, four large epidemiological studies involving retained placenta have been published[27,29,43,44]. Risk factors for retained placenta suggested by these studies are assisted reproductive technology, European genetic descent, stillbirth and delivery-related variables such as induced labor, epidural use, instrumental delivery and prolonged labor. Previous delivery by cesarean section has been a clear risk factor in some of these studies although this is contradicted by earlier studies[4,27,37,44]. Interestingly some of these risk factors were identified in case series as early as 1896[17]. It has been speculated that some of these factors may predispose more to focally accretic placentas (previous uterine surgery) and some more to adherent placenta (preterm labor, dysfunctional labor requiring oxytocin)[1].

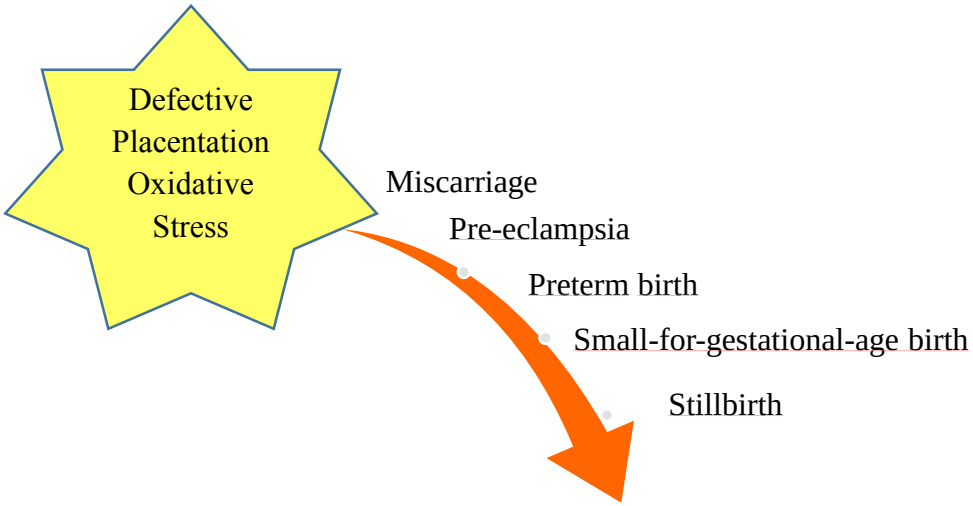
2.7.2 Defective placentation disorders

Pre-eclampsia, preterm birth, fetal growth restriction (as represented by SGA birth), stillbirth and recurrent miscarriage are part of a group of pregnancy complications termed "defective placentation disorders"[45]. These disorders are believed to develop in substantial part as a result of an initial deficient placentation, with an incomplete transformation of spiral arteries into the non-muscularized vascular system that is integral to perfusion of the placenta in normal pregnancy[46]. Perfusion will consequently be diminished or intermittent due to an exaggerated vascular constriction. This intermittent hypoxia results in oxidative stress in the placenta which contributes to a cascade of events resulting in the endothelial cell dysfunction and increased apoptosis characteristic of these disorders[46,47]. It is hypothesized that this will present clinically at different stages of pregnancy as early miscarriage, pre-eclampsia, preterm delivery or SGA birth depending on the severity of the stress[46] (figure 3).

There are some clinical traits to these disorders that are pertinent to this research project:

- They are epidemiologically interrelated and display similar patterns of recurrence and, in some cases, risk asssociations across generations[48-51].
- They display signs of increased oxidative stress or decreased antioxidiative defense capacity in placental tissue[52].
- They are histologically characterized by signs of decidual (maternal) placental underperfusion[53].

Figure 2
Schematic figure of consequences of excessive oxidative stress with progressing duration of pregnancy



2.7.3 Oxidative stress

Oxidative stress occurs when the cell´s antioxidative defenses are overridden by an excess generation of reactive oxygen species (ROS), over time resulting in cell death[54]. Although reactive oxygen species play important roles in cell signaling and immune regulation in the placenta, excessive oxygen is harmful to tissue[55]. Oxidative stress can result both from oxygen deficiency (hypoxia) and from alternating high and low oxygen pressure, such as occurs in pre-eclampsia, with varying intensity in blood perfusion due to the persistent muscularity of spiral arteries[47]. Oxidative stress can be measured directly by quantifying concentrations of ROS, and indirectly by measuring concentrations of antioxidative enzymes or the effects of oxidative stress on proteins and lipids in the form of lipid peroxides or protein carbonyls[54,56]. In pregnancy and labor oxidative stress is heightened so pregnancy and peripartal disorders that

are associated to an increased degree of oxidative stress are so in relation to a state in which oxidative stress is already higher than usual[45].

2.7.4 Histology and relationship to placenta accreta

Placenta accreta is a disorder in which the placenta attaches directly into the myometrium without intervening decidua, creating a plane of attachment that is excessively strong[36]. If and how retained placenta is related to placenta accreta is not clear. In both disorders the placenta does not detach spontaneously following birth of the infant. Basal plate myometrial fibers (BPMF) signifying increased attachment to the myometrium are increased in retained placenta compared to non-retained placentas[57,58]. Risk factors for placenta accreta such as previous cesarean section and previous uterine curettage may also be associated to retained placenta[27,44].

On the other hand some epidemiological and histological factors challenge this association. Placenta accreta is defined by an absence of the basal decidua, something which is not seen in the great majority of cases of retained placenta[57]. The increased incidence of BPMF seen in retained placenta does significantly correlate with hemorrhage or a prolonged third stage[58,59]. Nulliparity, previous miscarriage without surgical treatment and pre-eclampsia are risk factors for retained placenta but not placenta accreta[60].

2.7.5 What animal studies say

Retained placenta in cows increases the risk of endometritis and metritis which delays milk yield and costs the dairy industry in the UK 16 million pounds yearly[61]. Studies in ruminant medicine are therefore numerous. Retained placenta has in these studies been associated to increased oxidative stress, apoptosis, inflammation, a lower ratio of prostaglandin E2 to prostaglandin F2alpha and an elevated steroid hormone receptor status in placental tissue[61]. Antioxidants in the form of Selenium and Vitamin E have proved to decrease the incidence of retained placenta[62,63]. Interestingly both preterm delivery and prolonged labor are risk factors for retained placenta in cows[64].

2.7.6 Limitations of animal studies

In human placentation the fetal chorion is in direct contact with maternal blood (hemochorial placentation) whereas in ruminants discrete areas or cotyledons of the placenta attach into the intact maternal endometrium, creating a multicellular barrier between maternal and fetal blood (epitheliochorial placentation)[65]. In theory hemochorial placentation would facilitate passive

nutrient and gaseous exchange but complicate maternal-fetal immune interactions[66]. What these differences mean to the study of retained placenta in humans however is hard to say. Perhaps of importance, ruminant and human placentation are both villous and the feto-maternal blood flow interaction is similar[65]. The efficiency of this gas exchange is reflected in the similar fetal-to placental weight ratio in these two species. This ratio indicates to what extent the placenta can tolerate stress before is has consequences for the viability of the fetus. A human placenta can compensate for a 50% tissue necrosis before severe consequences for the pregnancy ensue[65]. This compensatory capacity of the placenta is important to keep in mind when studying oxidative stress in relation to clinical outcome.

3 AIMS OF THE THESIS

Study I

- To explore the epidemiology of retained placenta in a context of contemporary obstetric practice and demography in particular in respect to parameters relating to dystocia and the use of oxytocin for labor augmentation.

- To assess the severity of blood loss in retained placenta.

Study II

- To explore the epidemiological association between retained placenta and so-called defective placentation disorders, i.e. pre-eclampsia, spontaneous preterm birth, SGA birth and stillbirth.

Study III

- To assess whether gene and protein markers of oxidative stress and inflammation are increased in retained placentas compared to spontaneously released placentas.

Study IV

- To assess whether histological signs of maternal placental underperfusion and placental inflammation are increased in retained placental tissue compared to spontaneously released placentas.

- To assess whether retained placenta shows histological signs of increased placental attachment.

4 METHODS

4.1 STUDY DESIGNS

Table 1
Overview of study designs and methods

	Study Design	Inclusion Criteria	Number of Participants	Analysis Methods
Study 1	Case-Control	Singleton live births with retained placenta and time-matched controls	816	Logistic regression
Study II	Cohort	Singleton births in primiparous women at 32-41 gestational weeks without fetal malformations or placental abruption	386 607	Logistic regression
Study III	Cross-Sectional	Singleton full-term live births without pre-eclampsia, diabetes or SGA birth	60	Mann-Whitney U test, Student t-test, Spearman´s rank correlation, logistic regression
Study IV	Case-Control	Singleton full-term live births without pre-eclampsia, diabetes or SGA birth	97	Mann-Whitney U test, Student t-test, logistic regression

Study I is a case-control study of all cases of retained placenta (n=408) occurring after vaginal delivery at Södersjukhuset, Stockholm, between January 2007 and March 2010 and the same number of controls giving birth at equivalent dates. Aspects of obstetric history, the current pregnancy, labor and delivery were compared.

Study II is a population based cohort study of all primiparous women in Sweden with singleton vaginal deliveries between January 1997 and December 2009 at 32-41 gestational weeks (n=386 607). The study investigates the association between retained placenta and pre-eclampsia, preterm birth, SGA birth and stillbirth.

Study III is a cross-sectional pilot study with prospectively collected data of 29 retained and 31 non-retained placentas occurring after vaginal delivery between February 2013 and September 2014 at Södersjukhuset, Stockholm, in full-term, singleton, normal-for-gestational-

age live births without pre-eclampsia or diabetes in the mother. The study investigates antioxidative defense capacity and inflammation in retained and non-retained placental tissue.

Study IV is a case-control study of 49 cases of retained placenta and 47 control placentas, part of which were part of **Study III**, and selected based on the same criteria as this study. The study compares histological signs of maternal underperfusion, inflammation and increased placental attachment in retained and non-retained placentas.

4.2 NOTE ON STATISTICS USED

In all studies mean and median values were compared using Students T-test and Mann-Whitney U test respectively depending on whether the data was normally distributed (as assessed by Shapiro-Wilks test of normality). Correlations were assessed using Spearman´s rank correlation coefficient. Risk associations were calculated as odds ratios (OR) with 95 percent confidence intervals (CI) using unconditional logistic regression. The null hypothesis was rejected when $p < 0.05$ or when the 95% CI did not encompass 1.

4.3 THE SWEDISH MATERNAL HEALTH CARE SYSTEM AND STUDY DATA

In **Study I, III and IV** study participants were women with vaginal deliveries at Södersjukhuset, a tertiary level obstetric department in Stockholm with approximately 7500 deliveries per year. Oxytocin is routinely administered after delivery as part of active management of the third stage. Postpartum bleeding is subjectively assessed unless blood loss is estimated to exceed 1000ml in which case the blood is quantified by weighing. In Sweden data from maternal health care centers are linked to the women´s hospital records. At Södersjukhuset specialized nurses go through all patient hospital records and accord the right diagnostic codes at discharge. Complications during pregnancy and delivery are since 1997 classified according to the 10th version of International Classification of Diseases (ICD-10).

In **Study II** the cohort of study participants was assembled from the whole population of women delivering in Sweden, of which 99.7% deliver in hospitals[67]. Data came from the Swedish National Birth Register which includes nationwide demographic data, information on reproductive history and complications that occur during pregnancy, delivery, and the neonatal period on 98% of all live births and stillbirths in Sweden[68]. Data on each pregnancy and delivery is forwarded to the Birth Register through copies of standardized antenatal, obstetric and pediatric records. Individual record linkage between the Birth Register and other registries

are possible through each individual's unique personal registration number, assigned to each Swedish resident.

4.4 STUDY POPULATIONS

Study I included all cases of retained placenta occurring after vaginal delivery at Södersjukhuset between January 2007 and March 2010 (n=427). Stillbirths and multiple births were excluded (n=19). Controls (n=408) were randomly selected by computer generation from women giving birth during the same time period.

In **Study II** a cohort was created based on all vaginal births in nulliparous women in Sweden between January 1997 and December 2009 (Figure 4). Births of infants with fetal malformations and cases of placental abruption were excluded. We thus greatly increased the study size (n=386 607) but narrowed the scope of our analysis in the following manner:

- Focusing on a few interrelated exposures: pre-eclampsia, spontaneous preterm birth, SGA birth and stillbirth.

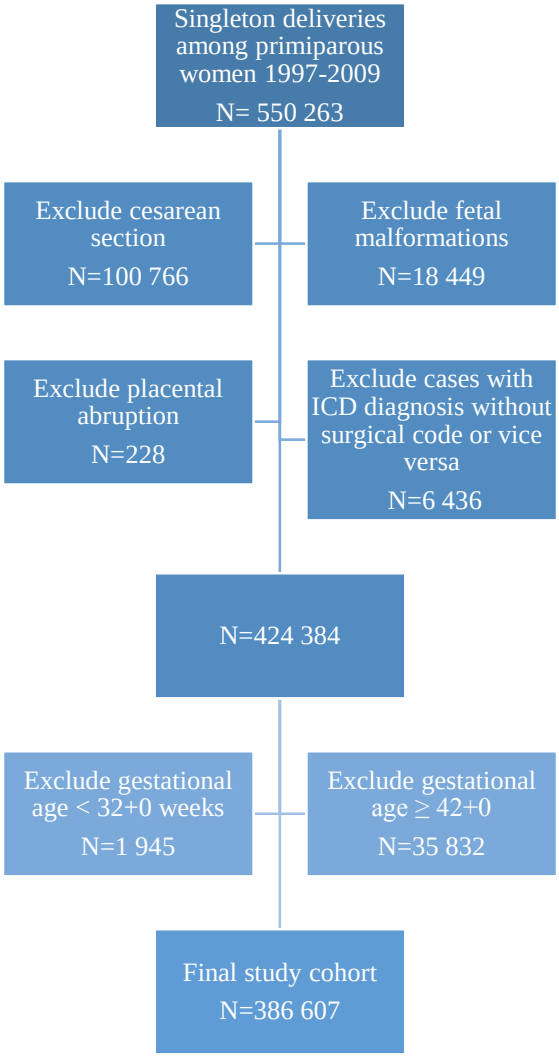
- Subanalyzing a preterm cohort of pre-eclampsia, SGA birth and stillbirth, a group assumed to represent more severe forms of these disorders.

- Selecting only nulliparous women and thereby excluding the effect of previous retained placenta which was the strongest risk factor in our analysis in Study I.

- Excluding births prior to gestational week 32 since before this women with some of our exposures of interest (pre-eclamptic pregnancies and SGA infants) are often delivered by cesarean section which would distort our analysis of association to retained placenta in very early preterm delivery.

Figure 4

Flowchart of inclusion into the study cohort of Study II



Study III and IV are partly based on the same study group. We included all cases of retained placenta occurring at Södersjukhuset between February 2013 and September 2014 that were accessible for collection by the researchers, meaning that they were present at the time of manual removal of the placenta. We excluded the disorders that were shown to be associated to retained placenta in **Study II**: pre-eclampsia, preterm birth, SGA birth and stillbirth. These variables were associated both to our exposure (oxidative stress and inflammation) and to our outcome (retained placenta) and would therefore confound our analysis[52,69]. We also excluded births from mothers with diabetes which is associated to oxidative stress and was overrepresented among cases of retained placenta in **Study I**, although not a significant risk

factor in **Study II**[70]. An equivalent number of controls were randomly selected from women giving birth during the same time period.

After initial inclusion, one case of placenta accreta and one case of non-diagnosed diabetes in the mother were excluded from **Study III** giving a total study group of 29 cases of retained placenta and 31 non-retained placentas. This same diabetic case was excluded after inclusion from **Study IV** as well as three placentas that were accidentally discarded according to routine protocol at the hospital resulting in a study group of 49 cases and 47 controls.

4.5 VARIABLES AND ANALYSIS STUDY I

Outcomes

1. Retained placenta defined by the Swedish National Procedure Code MBA30 ("Manual removal of fetal membranes, placenta or rest thereof"). All cases also has an ICD-10 code for retained placenta recorded (ICD-10 codes O720, O722, O730 or O731).
2. Postpartum hemorrhage as recorded in the patient records (blood loss >500ml), severe postpartum hemorrhage (blood loss >1000ml), very severe postpartum hemorrhage (blood loss >2000ml) and the need of blood transfusion.

Exposures

1. Variables relating to past and current obstetric history: age, parity, body mass index (BMI), smoking status, previous miscarriage, induced abortions, vacuum aspiration, ectopic pregnancies, cesarean section or retained placenta.
2. Variables relating to current pregnancy: hypothyroidism, pregnancy induced or gestational diabetes mellitus, hypertension of pregnancy, pre-eclampsia and SGA birth.
3. Variables relating to labor and delivery in the current pregnancy: gestational age at delivery, prelabor rupture of membranes > 48hrs (PROM), induced labor, epidural use, fever during delivery (T >38ºC), augmented labor by oxytocin (use and duration), labor duration and instrumental delivery.

Statistical analysis

Associations to retained placenta were calculated using unconditional logistic regression. No a priori assumptions were made about associations between retained placenta and each exposure. Duration of labor and duration of augmented labor were stratified into quantile categories. Univariate logistic regression was performed first. The final multivariate model was created

using a forward stepwise regression model in which each exposure variable was entered in order or strength (combined assessment of OR and 95% CI). A sensitivity analysis to test the robustness of results was made using a backward stepwise model. The subanalysis of retained placenta and risk of postpartum hemorrhage was performed using unconditional regression analysis.

4.6 VARIABLES AND ANALYSIS STUDY II

Outcome

Retained placenta was defined by the combined existence in patient records of the ICD-10 code for retained placenta (ICD-10 codes O720, O722, O730 or O731) and the Swedish National Procedure Code for manual removal of the placenta, MBA30 ("Manual removal of fetal membranes, placenta or rest thereof").

Exposures

1. Pre-eclampsia defined as blood pressure ≥ 140/90 and proteinuria ≥ 0.3 g/24 hours; ICD-10 codes O14-15.
2. SGA birth defined as birthweight 2 standard deviations or below the mean weight for gestational age according to the Swedish sex-specific fetal growth curve [71]. Only live births were included. Preeclamptic pregnancies were excluded to isolate SGA birth from the potential causal factor of pre-eclampsia.
3. Stillbirth, encompassing both antepartal and intrapartal death as recorded in the Birth Register.
4. Spontaneous preterm birth defined by: spontaneous onset of labor (induction of labor excluded) of unknown cause (pre-eclampsia, SGA birth and stillbirth excluded) at gestational age below 37 weeks (according to ultrasound assessment at week 18-20).
5. Preterm pre-eclampsia, SGA birth and stillbirth defined as the occurrence of these disorders in the sub-cohort of deliveries prior to 37 gestational weeks.

Statistical analysis

The association between retained placenta and each exposure was calculated in turn according to the models specified above, using unconditional multivariate logistic regression. The analysis was then repeated when the cohort was stratified into preterm (32-36 weeks) and term (37-41 weeks) birth groups.

4.7 MANUAL PLACENTAL REMOVAL AND SAMPLING STUDY III AND IV

Outcome

Retained placenta was defined as the identification during manual removal of an adherent plane between the placenta and the uterine wall. During manual removal one of two researchers was prepared to collect and sample placentas directly upon placental removal.

Sample collection

For **Study III** two periumbilical, two peripheral and one umbilical samples were taken at standardized sites (one cm from the periphery and umbilical insertion respectively) and immediately frozen at -70ºC. The time duration from delivery of the placenta until the samples were frozen was recorded. The remaining placenta was analysed histologically at the division of perinatal pathology as part of **Study IV**. Full-thickness placental biopsies were collected from three standardized macroscopically normal sites as well as sites with focal macroscopic changes. A complete 1 cm thick full-lumen sample of the umbilical cord was collected. The samples were fixed in buffered formalin, paraffin embedded, cut and stained for a detailed standardized microscopic analysis. Within a few days of a retained placenta being recruited to the study a spontaneously delivered placenta was randomly selected and sampled as a control. Variables relating to reproductive history and current delivery, and data relating to the newborn, were recorded for each woman admitted to the two studies.

4.8 VARIABLES AND ANALYSIS STUDY III

Molecular markers

In **Study III** protein and gene expression of Glutathione Peroxidase I (GPX1) and gene expression of NFκB (Nuclear Factor Kappa-light-chain-enhancer of activated β-cells) and its inhibitor IκBα (Nuclear Factor Kappa-light-chain-enhancer of activated β-cells inhibitor, alpha) were analysed and compared between retained and non-retained placentas. These markers of oxidative stress and inflammation were chosen based on the following premises:

1. GPX1 is the main cellular isoform of the antioxidative enzyme GPX, accounts for most enzymatic activity in placental tissue and is vital to cellular antioxidative defense, as illustrated by the fatal effect of severe oxidative stress on GPX1 knock-out mice[72,73]. Several studies have shown that GPX1 enzyme activity and/or mRNA expression is decreased in cases of pre-eclampsia[74-76].

2. NFκB is a transcription factor which upregulates GPX1 in response to oxidative stress, modifies inflammatory response in the placenta and may be upregulated in cases of pre-eclampsia[77-80]. IκBα is the inhibitor of NFκB.

Laboratory analysis

Gene expressions of GPX1, NFκB and IκBα were analyzed by real-time quantitative reverse transcriptase polymerase chain reaction analysis (qrt-PCR). Total RNA was extracted from each placental sample using Bullet Blender Homogenizer (Next advance Inc., USA) and Aurum total RNA mini kit (Bio-Rad Laboratories Inc., USA). Twice the amount of recommended DNase1 was used to ascertain no remaining cDNA. The quantity of total RNA was measured using the Pico Drop spectrophotometer (PicoDrop LTD, UK). Five micro litres of each RNA sample were analyzed using 1% Agarose gel electrophoresis in TAE buffer and visualized using ethidium bromide staining.

Both cDNA synthesis and real-time PCR analysis of GPX1, NFκB and IκBα were performed using iTaq™ Universal SYBR® Green One-Step Kit (Bio-Rad Laboratories Inc., USA. Specific primers were used to measure the gene expression of GPX1, NFκB, IκBα and the housekeeping gene tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ). The real-time PCR products were visualized in 2% agarose gel electrophoresis and ethidium bromide staining. Each sample was analyzed in duplicates and the mean value of each sample was used for quantification. The formula of Livak and Schmittgen was used for quantification of samples and normalization was performed using the housekeeping gene YWHAZ[81].

Sandwich enzyme linked-immunosorbent assay (ELISA) was used for quantitative determination of intracellular GPX1 protein in placental samples using the GPX1 human intracellular ELISA kit (Adipogen International Inc., USA). A standard curve was performed. The absorbance was measured at 450 nanometers using Ascent software v. 2.6. (Thermo Labsystems, Finland). All samples were analysed in duplicates and the researcher was blinded to outcome group.

Statistical analysis

Median values of gene and protein expressions of GPX1and gene expressions of NFκB and IκBα between retained and non-retained placentas were compared. Levels of GPX1 protein were categorized as low or high according to a cut-off based on an ROC curve. The rationale for categorization of GPX1 protein levels was that concentrations were not normally distributed. Associations between a low level of GPX1 protein and retained placenta were calculated using unconditional univariate logistic regression.

4.9 VARIABLES AND ANALYSIS STUDY IV

Histological features

1. Histological features of maternal underperfusion: artheriolopathy, placental infarcts, giant trophoblastic multinucleated giant cells, syncytical knots/villous agglutination, chorangiosis, septal cysts and fibrotic villi.

2. Histological placental inflammation: acute chorionitis, chorioamnionitis, vasculitis of fetal vessels or funisitis.

3. Macroscopic characteristics consistent with placental underperfusion: Weight in relation to gestational age, fetal to placental weight ratio, shape, width and thickness.

Other placental features recorded were umbilical coiling and insertion, distal villous immaturity, thrombosis of the fetal stem vessels, intervillous thrombosis, chronic villitis, placental abruption, and signs of increased placental attachment in the form of placental accreta or basal plate myometrial fibers (BPMF).

Histological analysis

The placentas were weighed after formalin fixation and removal of the fetal membranes and the umbilical cord and defined < the 10^{th}, between the 10^{th} and 90^{th}, or > the 90^{th} percentile for gestational age according to a reference curve[82]. Villous maturation was evaluated in relation to gestational week. The size and configuration of the dominant villous component was assessed, including the layer of trophoblasts and the number and morphology of blood vessels. Fresh and older infarctions were included. Histological sections were taken from at least one of the macroscopically suspected infarcted areas as well as otherwise macroscopically disrupted areas. The proportion of infarcted placental tissue was calculated by dividing the total volume of the

infarcts by the total volume of the placenta. Histological variables were defined and recorded according to pre-specified definitions (Appendix: histological definitions).

All histological analysis was performed at Karolinska University Hospital, Department of perinatal pathology by an experienced perinatal pathologist. The pathologist was blinded in regard to the outcome group for all microscopic analysis.

Statistical analysis

Mean and median values relating to size and shape were compared. Categorical variables relating to maternal placental underperfusion, placental inflammation, placental attachment and fetal vascular trombo-occlusive disease were compared using unconditional univariate logistic regression.

4.10 ETHICAL CONSIDERATIONS

Study I

- All patient data was anonymized and coded. The code for each participant can only be traced back to the participants´ identity through a secure server accessible to only two of the researchers in the study.

Study II

- The data for the patient cohort in study was retrieved from the Swedish Medical Birth Register. The researchers only had access to anonymized data. All data analysis was performed at the physical location of the Unit of Clinical Epidemiology at Karolinska Institutet which means that no patient data ever left the secure server of those premises.

Study III and IV

- Written informed consent was obtained from all study participants. The participants were informed of the purpose of the study and of the fact that she could at any time demand that the biological material applicable to her be destroyed. The sample collection was performed after delivery and therefore involved no physical discomfort to the participant. Placental samples were stored in an approved biobank. The sampled material was marked in code which can only be traced back to the participants´ identity through a secure server accessible to only one of the researchers in the study. No further studies can be performed on this material without further approval by the ethical board.

Ethical permits have been obtained for all four studies from the Local Ethics Committee at Karolinska Institutet, Stockholm, Sweden (2010/438-31/4, 2012/1250-31/4, 2012/15-31/2).

5 RESULTS

5.1 STUDY I: RETAINED PLACENTA IN MODERN OBSTETRICS

5.1.1 Summary of main findings

Nulliparity, previous placental retention, a history of recurrent miscarriage or abortion, pre-eclampsia, preterm birth and augmented labor independently increased the risk of retained placenta in our study, whereas smoking at the start of pregnancy lowered the risk. In regard to augmented labor there was a dose-response relationship between duration of oxytocin exposure and risk of retained placenta. Retained placenta was significantly associated to postpartum hemorrhage, severe and very severe postpartum hemorrhage as well as the need for blood transfusion (Table 2).

5.1.2 Related findings

There were 427 cases of retained placenta among 16 209 vaginal deliveries at Södersjukhuset between January 2007 and March 2010, giving an incidence of retained placenta of 2.6 %.

There was no difference in median age, BMI or gravidity between cases and controls. There was no difference in median gestational age, but preterm and postterm deliveries were more common among retained placentas. In the univariate model women with retained placenta were more likely to have had a previous vacuum aspiration. In the current pregnancy they were more likely to have had prelabor rupture of membranes (PROM), induced labor, received epidural anesthesia, developed fever during delivery and given birth by instrumental delivery. Hypothyroidism and diabetes were numerically more common among women with retained placenta. None of these variables were associated to retained placenta in the multivariate model.

Table 2
Main risk factors for retained placenta and association between retained placenta and postpartum hemorrhage in Study I

	Rate and Risk of Retained Placenta					
	Cases= 408		Controls= 408		Adjusted Odds Ratio	Confidence Interval (95%)
	n	%	n	%		
Model 1[a]						
History, pregnancy and delivery						
Previous Retained Placenta[b]	22	(5.4%)	3	(0.7%)	**12.61**	(3.61-44.08)
Previous Miscarriages						
1 previous miscarriage	93	(23.0%)	75	(18.4%)	**1.52**	(1.04-2.21)
≥2 previous miscarriages	29	(7.2%)	16	(3.9%)	**2.62**	(1.31-5.20)
≥1 previous abortion[b]	98	(24.2%)	74	(18.2%)	**1.58**	(1.09-2.28)
Parity						
1 para	137	(33.6%)	158	(38.7%)	**0.78**	(0.55-1.11)
≥2 para	35	(8.6%)	67	(16.4%)	**0.40**	(0.24-0.70)
Smoking at start of pregnancy	5	(1.4%)	15	(4.3%)	**0.28**	(0.09-0.88)
Pre-eclampsia	27	(6.6%)	8	(2.0%)	**2.85**	(1.20-6.78)
Augmented labor by oxytocin	228	(55.9%)	160	(39.2%)	**1.74**	(1.21-2.48)
Duration of augmented labor						
<50th percentile (10-194 min)	92	(22.5%)	100	(24.5%)	1.24	(0.83-1.84)
50-75th percentile (195-415 min)	59	(14.5%)	43	(10.5%)	**2.00**	(1.20-3.34)
>75th percentile (>415min)	77	(18.9%)	17	(4.2%)	**6.55**	(3.42-12.54)
Preterm delivery	32	(7.8%)	12	(3%)	**3.28**	(1.60-6.70)
Model 2[c]						
Postpartum period						
Blood loss≥500 ml	383	(93.9%)	126	(69.1%)	**33.07**	(20.57-53.16)
Blood loss≥1000ml	305	(74.8%)	27	(6.6%)	**43.44**	(26.57-71.02)
Blood loss≥2000ml	157	(38.5%)	3	(0.7%)	**111.24**	(27.26-454.00)
Blood transfusion	126	(30.9%)	5	(1.2%)	**37.48**	(13.63-103.03)

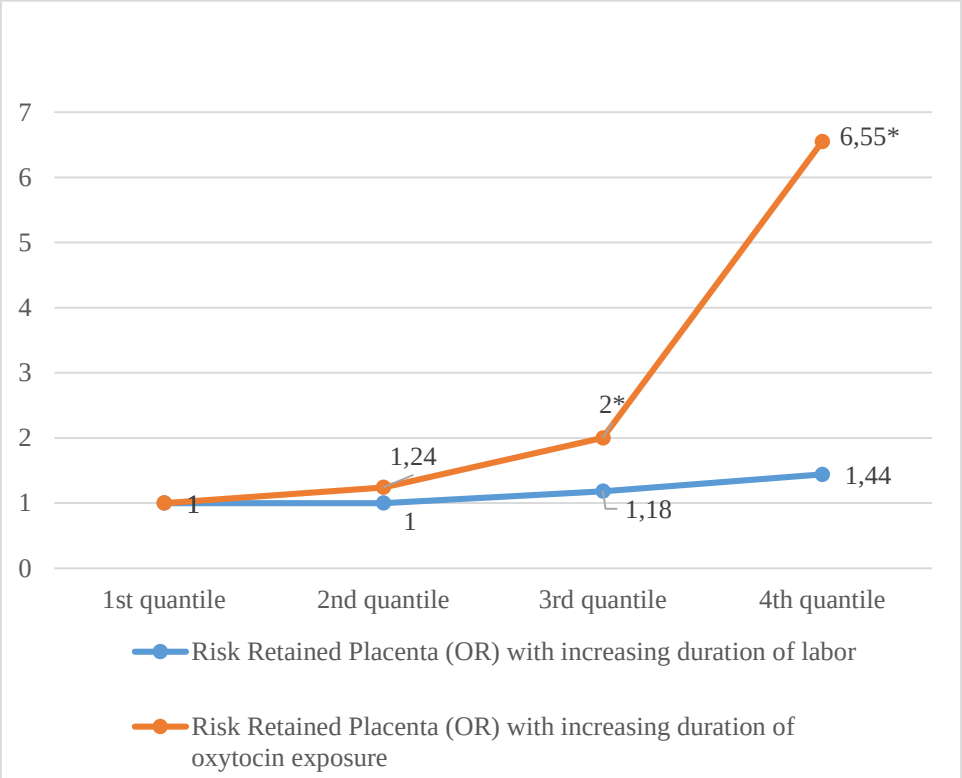
a Model 1 adjusted for previous retained placenta, previous miscarriage, previous abortion, pre-eclampsia and duration of augmented labor.
b There were 4 cases with missing values for data on miscarriage, 3 on previous abortion.
c Model 2 was adjusted for labor duration ≥12 hrs.

5.1.3 Augmented labor and labor duration

Augmented labor by oxytocin was an independent risk factor for retained placenta and the risk of retained placenta increased in an exponential fashion with increasing duration of use (Figure 5). Both women with and without retained placenta had a median latency phase of 5 hours (p= 0.74). In the univariate analysis, women with retained placenta were more likely to have labor duration of more than 12 hours. Both the median duration of the active phase of the first stage

of labor (p<0.04) and the second stage of labor (p<0.001) were longer in cases of retained placenta compared to controls. Neither duration of labor over 12 hours nor increasing duration of the second stage (categorized by quantiles of time) were however associated to retained placenta in the multivariate model (Figure 5).

Figure 5
Risk of retained placenta (odds ratio) in each quantile of duration of augmented labor and duration of the second stage of labor



*p <0.05

5.1.4 Postpartum hemorrhage

Median blood loss was 1600ml among women with retained placenta and 400ml among controls (p>0.001). Retained placenta increased the risk of all measures of postpartum hemorrhage, particularly of severe (>1000 ml) and very severe (>2000 ml) hemorrhage. There was a weak but significant negative correlation (r= -0.2, p<0.001) between total blood loss and time from birth of the infant till manual removal of the placenta among cases of retained placenta.

5.2 STUDY II: RETAINED PLACENTA AND DEFECTIVE PLACENTATION

5.2.1 Main findings

Defective placentation disorders, i.e. pre-eclampsia, spontaneous preterm birth, SGA birth and stillbirth, were all associated to an increased risk of retained placenta. The association was stronger for preterm pre-eclampsia and preterm SGA birth. There was a dose-response relationship in respect to spontaneous preterm birth, with increasing risk of retained placenta for decreasing gestational age at delivery (Table 3 and 4).

Table 3
Risk of retained placenta associated to disorders of defective placentation

	Rate and Risk of Retained Placenta			
			Odds Ratio (95% Confidence Interval)	
	Number	%	Crude	Adjusted [a]
Preeclampsia[b]				
No	7 982	2.13	Ref	Ref
Yes	426	3.55	1.69 (1.53-1.87)	1.37 (1.21-1.54)
Stillbirth[b]				
No	8 340	2.16	Ref	Ref
Yes	68	5.80	2.79 (2.18-3.56)	1.71 (1.28-2.29)
Small for gestational age birth [c]				
No	7 456	2.08	Ref	Ref
Yes	247	3.01	1.46 (1.28-1.66)	1.47 (1.28-1.70)
Spontaneous preterm birth[d]				
No	6 467	1.97	Ref	Ref
35-36 weeks	336	2.90	1.49 (1.33-1.66)	1.55 (1.37-1.75)
32-34 weeks	182	4.60	2.41 (2.07-2.80)	2.35 (1.97-2.81)

a All models were adjusted for maternal age, body mass index, country of birth, previous miscarriages, year of delivery, gestational age, epidural analgesia, labor dystocia, instrumental vaginal delivery, and large for gestational age birth weight. Preeclampsia was additionally adjusted for premature rupture of membranes and induction of labor. Stillbirth was additionally adjusted for induction of labor. Small-for-gestational-age birth was additionally adjusted for induction of labor. Spontaneous preterm labor was additionally adjusted for prelabor rupture of membranes.

b In analyses of preeclampsia and stillbirth, total number of births were 386 607.

c Analysis only includes live births. Pregnancies with preeclampsia were excluded. 1 164 pregnancies with missing data on birth weight were not included in the analysis. Total number of births analyzed was 373 466.

d Analysis only includes live births. Pregnancies with pre-eclampsia, SGA birth or induction of labor were excluded. Total number of births analyzed was 344 307.

5.2.2 Related Findings

The overall rate of retained placenta was 2.17%. Compared to women with spontaneous placental expulsion, those with retained placenta were on average older and had a somewhat higher BMI. In the univariate analysis they were more likely to have been born in a Nordic country, have had previous miscarriage, have used assisted reproductive technology, deliver preterm, have PROM, induced labor, epidural analgesia, labor dystocia, give birth by instrumental delivery and give birth to a large-for-gestational-age (LGA) infant.

In the adjusted analysis only maternal age and previous miscarriage remained as strong independent risk factor for retained placenta. Both these variables showed a dose-response relationship to retained placenta with increasing risk according to increasing age and number of miscarriages respectively. In the analysis of births occurring at term, LGA birth was also an independent risk factor for retained placenta. The remaining variables listed above and adjusted for in the analysis were only marginally associated to retained placenta (adjusted ORs ≤1.30.).

Table 4
Defective placentation disorders and risk of retained placenta in term and preterm deliveries

	Retained Placenta					
			Preterm delivery			**Term delivery**
	n	%	Adjusted [a] OR (95% CI)	n	%	Adjusted [b] OR (95% CI)
Pre-eclampsia						
No	1 064	3.53	Ref	10509	2.06	Ref
Yes	54	4.83	**1.69 (1.25-2.28)**	372	3.42	**1.32 (1.16-1.50)**
Small-for-gestational-age birth[c]						
No	366	3.46	Ref	7605	2.02	Ref
Yes	25	6.39	**2.19 (1.42-3.38)**	222	3.11	**1.43 (1.23-1.66)**
Stillbirth						
No	351	3.59	Ref	753	2.09	Ref
Yes	15	4.10	**1.10 (0.63-1.92)**	53	6.58	**2.36 (1.71-3.27)**

n=number, OR= odds ratio; CI, confidence interval.

a Adjusted for maternal age, country of birth, previous miscarriages, year of delivery, gestational age, PROM and instrumental vaginal delivery. In analyses of pre-eclampsia and stillbirth, total number of births were 18 756. In analysis of SGA birth, total number of births was 17 291.

b Adjusted for maternal age, body mass index, country of birth, previous miscarriages, year of delivery, gestational age, induction of labor, epidural analgesia, labor dystocia, instrumental vaginal delivery, and large-for-gestational-age birth. In analyses of pre-eclampsia and stillbirth, total number of births were 367 851. In analysis of small for gestational age, total number of births was 356 175.

c Defined as a live born infant with a birth weight of >2 standard deviations below the mean birth weight for gestational age according to the sex specific Swedish fetal growth curve. Pregnancies with pre-eclampsia and those missing data on birth weight were excluded.

5.3 STUDY III: RETAINED PLACENTA, OXIDATIVE STRESS, INFLAMMATION

5.3.1 Main findings

Women with retained placenta were more likely to have a low level of GPX1 protein concentration in placental tissue compared to women without retained placenta and this association was strengthened when the analysis was adjusted for duration of labor augmentation. Retained placental tissue showed a tendency of lower median GPX1 protein concentrations compared to non-retained placentas but the differences were not statistically significant (Table 5 and 6).

Table 5
Median protein concentration or gene expression of GPX 1, NFκB and IκBα in retained and non-retained placenta

	Retained Placenta		Non-Retained Placenta		
	Median	Interquartile Range	Median	Interquartile Range	Difference (p-value)
GPX 1 concentration ng/ml					
periumbilical	13.32	(9.81)	17.96	(12.95)	p= 0.22
peripheral	13.27	(16.72)	19.09	(8.85)	p= 0.07
GPX 1 mRNA expression[a]					
periumbilical	1.13	(1.13)	0.88	(0.88)	p= 0.08
peripheral	1.32	(0.91)	1.18	(1.12)	p=0.47
IκBα mRNA expression[a]					
periumbilical	1.14	(1.92)	0.95	(0.95)	p= 0.16
peripheral	1.96	(4.86)	0.99	(2.36)	p= 0.25
NFκB mRNA expression[a]					
periumbilical	1.02	(0.45)	0.99	(0.42)	p= 0.71
peripheral	1.22	(0.61)	1.04	(0.42)	p= 0.17

a Relative expression to YWHAZ housekeeping gene

Table 6
Level of GPX 1 protein concentration and risk of retained placenta

	Risk of retained placenta			
	Unadjusted Odds Ratio	p-value	**Adjusted Odds Ratio[b]**	**p-value**
Low value[a] periumbilical GPX 1	**3.82**	0.02	**3.54**	0.08
Low value[a] peripheral GPX 1	**3.95**	0.02	**7.00**	0.01

a Low-value assessment based on sensitivity-specificity analysis of ROC curve. Periumbilical GPX1 cut-off= 15.62 ng/ml, peripheral GPX 1=19.90 ng/ml.
b Adjusted for duration in minutes of augmented labor.

5.3.2 Related Findings

There were no significant differences between women with and without retained placenta in regard to age, parity, history of previous miscarriage, abortion or cesarean section, use of assisted reproductive therapy, gestational age, epidural use, induction of labor, labor duration over 12hours or instrumental delivery. Median duration of labor augmentation was significantly longer in deliveries with retained placenta. Median blood loss in cases of retained placenta was 1600 ml compared to 400 ml in the group without retained placenta ($p < 0.001$). There was no significant difference in fetal birthweight or placental weight between deliveries with and without retained placenta.

GPX1, NFκB and IκBα mRNA were detected at all placental sampling sites. Median periumbilical and peripheral GPX1 gene expressions were numerically higher in retained placentas (1.13 vs 0.88 for periumbilical samples and 1.32 vs 1.18 for peripheral samples) which approached statistical significance for periumbilical sample ($p=0.08$) but was statistically non-significant in peripheral samples ($p=0.47$). There was no significant difference in median periumbilical or peripheral NFκB and IκBα gene expressions between retained and non-retained placentas.

Periumbilical and peripheral GPX1 protein concentrations did not significantly correlate with duration of labor, duration of augmented labor or total blood loss. Median duration from birth of the infant until placental delivery was 1h 22 min for retained placentas and 8min 13sec for spontaneously released placentas ($p < 0.001$). Duration until placental delivery did however not correlate with peripheral or periumbilical GPX1 protein concentrations in either group.

5.4 STUDY IV RETAINED PLACENTA AND PLACENTAL UNDERPERFUSION

5.4.1 Main findings

All histological markers of placental maternal underperfusion were numerically more common in retained placentas with the exception of chorangiosis. Overall, signs of maternal underperfusion were significantly more common among retained placentas compared to spontaneously released placentas and when small placental size for gestational age was included as a marker of placental insufficiency, this difference was more marked. Retained placentas had a relatively smaller surface area and were more likely to have an oblong shape. Accessory lobes and placentas below the 10^{th} centile for gestational age were numerically more common among retained placentas although the difference was not statistically significant.

5.4.2 Related findings

There were no significant differences between women with and without retained placenta in regard to age, parity, history of previous miscarriage, abortion or cesarean section, use of assisted reproductive therapy, gestational age, epidural use, induction of labor, labor duration ≥ 12 hours or instrumental delivery. Women with retained and without retained placenta received augmented labor at similar rates, however the median duration of augmented labor was significantly longer for those who went on to develop retained placenta (p=0.01).

The differences in mean placental weight and median fetal to placental weight ratio between retained and non-retained placentas were not statistically significant. Signs of fetal vascular thrombo-occlusive disease in the form of intervillous thrombosis, thrombosis of fetal vessels, chorionic villous immaturity, and chronic villitis were similar between retained and non-retained placentas. There was no statistically significant difference in individual or combined markers of acute placental inflammation.

Focal placenta accreta was numerically, but statistically not significantly, more common in retained placentas compared to non-retained placentas (12.3% vs 4.3%; 95% CI 0.60-16.40). BPMF were however significantly more prevalent in retained compared to non-retained placentas (22.4% vs 3.4%; 95% CI 1.36-31.22). Median blood loss in cases of retained placenta with and without focal placenta accreta, and with and without BPMF, was not significantly different and was consistent with the median blood loss for retained placenta overall (1500 ml).

Table 7

Macroscopic and histological features of placental underperfusion in retained and non-retained placentas

	Non-retained placenta (n=47)		Retained placenta (N=49)		Association to retained placenta	Significance
	Number	%	Number	%	Odds Ratio	p-value or 95% CI
Macroscopic Features						
Placental surface area median cm2 (IQR)	396	341-418	342	288-432		**p=0.05**
Oblong shape[a]	3	6.4%	11	26.2%	**5.24**	**1.34-20.21**
Placental weight for gestational age[b]						
<10th percentile	12	25.5%	21	42.9%	2.02	(0.84-4.88)
>90th percentile	5	10.6%	2	4.1%	0.46	(0.08-2.58)
Accessory lobes	1	2.1%	7	14.3%	7.67	(0.91-64.94)
Histological Features						
Artheriolopathy	0	0%	1	2.0%	nc	nc
Villous infarction	9	19.1%	9	18.4%	0.95	(0.34-2.65)
Infarcted volume						
1-5%	1	2.1%	3	6.1%	3.14	(0.31-31.30)
>5%	0	0%	2	4.1%		
Grouped trophoblastic multinucleated giant cells	7	14.9%	11	22.4%	1.65	(0.58-4.71)
Villous syncytial knots	1	2.1%	6	10.2%	5.27	(0.59-46.54)
Villous agglutination	1	2.1%	4	8.2%	4.09	(0.44-38.01)
Chorangiosis	2	4.3%	1	2.0%	0.47	(0.04-5.35)
Placental septal cysts	3	6.4%	5	10.2%	1.67	(0.38-7.40)
Fibrotic villi	0	0%	2	4.0%	nc	nc
Any sign of placental underperfusion[c]	13	27.7%	24	49.0%	**2.51**	**1.07-5.87**
Any sign of placental underperfusion including placental weight[d]	20	42.6%	36	73.5%	**3.74**	**1.59-8.82**

IQR=interquartile range, n=number, nc=not computable. CI =confidence interval, in parenthesis=non-significant.

a Defined as a ratio of larger width to lesser width ≥ 1.3.

b According to a reference curve of placental weight for gestational age [82].

c Presence of one or more of following characteristics: artheriolopathy, infarcted placental volume >1%, grouped trophoblastic multinucleated giant cells, villous syncytial knots or agglutination, chorangiosis, fibrotic villi, placental septal cysts.

d Including also placentas <10th percentile for gestational age.

6 METHODOLOGICAL CONSIDERATIONS

6.1 SYSTEMATIC ERROR

Systematic error in a study arises because of incorrect selection of study groups, incorrect classification of the variables under study or lack of adjustment for factors that interact with these variables to the extent that they affect results.

6.1.1 Selection bias

All studies were designed with attention to the risk of selection bias, the selection of either cases or controls that differ in their exposure-to-outcome distribution from the population from which the study group is recruited. **Study I** thus included all consecutive cases of retained placenta during a three year period and controls were chosen randomly from the same population. As a national register-based study, **Study II** included the whole relevant exposed and unexposed populations. Arguably however, a certain selection bias is introduced by the necessary exclusion of deliveries by cesarean section in both these studies. The question is if pregnancies delivered by cesarean section are different from pregnancies with vaginal deliveries in a way that would affect the associations studied.

Cesarean section is more likely to be performed in a pregnancy with a history of previous cesarean section or pregnancies complicated by pre-eclampsia, SGA birth or prolonged labor. If these factors are associated to retained placenta the relative risks for exposed and unexposed participants in the study would remain unaffected as long as the exposed excluded were not more likely to develop retained placenta than the exposed included. A woman with a history of cesarean section would be more likely to be delivered by cesarean section also in her next pregnancy and thus be excluded from **Study I**. Her risk of retained placenta may also be increased compared to a woman without previous cesarean section, but her relative risk compared to a woman with previous cesarean section who was included in the study would probably not be different[27,44]. However, pregnancies with severe pre-eclampsia and fetal growth restriction may be at increased risk both of exclusion from the study because of delivery by cesarean section, and retained placenta compared to pre-eclamptic and SGA births that were included in the study. If this is the case the strength of the associations found between pre-eclampsia, SGA birth and retained placenta in **Study II** may be underestimated, a so-called type II error. A similar "loss to follow-up" bias may occur in the analysis of prolonged or augmented labor and retained placenta in **Study I**. Increasing duration of labor would be associated both

with exclusion (delivery by cesarean section) and, if our results are correct, an increasing risk of retained placenta.

Study III and IV recruited participants from the same study population as **Study I**. For logistical reasons not all consecutive cases were included, but there is no reason to believe that the ones included, which were primarily from daytime deliveries, differed in their exposures from the ones that were not included. Controls were randomly chosen. No women asked to participate declined to do so.

6.1.2 Information bias and confounding

6.1.2.1 Study I and II

In both **Study I** and **II** outcome and exposures were clearly defined. In the study setting relevant to **Study I**, all medical records are routinely examined by specialist nurses after discharge to assure a correct diagnostic record. In addition to this, augmented labor and labor duration in each participant was assessed by the researcher in individual patient records and partograms. The rates of the variables studied are therefore likely to be reliable for both cases and controls, minimizing information bias. In **Study II** each epidemiological model was built so as to make the relevant exposure as specific as possible. The registration in the Birth Register of gestational age at delivery, birthweight and stillbirth, exposures relevant to **Study** II, are considered highly reliable. The record of the ICD-9 diagnosis for pre-eclampsia in the Register has been validated[83]. However some variables in the Register are incompletely recorded. The incidence of oxytocin use was 8 % among the nulliparous women in **Study II** as compared to 63% among nullipara in **Study I**, which more closely approximates empirical clinical practice. Although associated to retained placenta, oxytocin is not known to be associated to any of the study exposures and its incomplete inclusion in the model should therefore not affect the main associations studied.

During the years 1997-2001, 1.4% of births were diagnosed with the outcome retained placenta in the Register, whereas the corresponding rate during 2006-2009 was 2.7%. The rate increase most likely reflects better registration of the diagnosis during the later years and we adjusted for year of delivery in our statistical models. It is probable that retained placenta was better recorded in **Study I**. However, the studies were in consensus in regard to the associations found. The fact that outcome may be to some extent non-differentially misclassified in **Study II** would tend to push results towards the null (type II error). Error would therefore be on the cautionary side. Consistent with this associations were somewhat weaker in **Study II**.

6.1.2.2 Study III

In **Study III** and **IV** the outcome retained placenta was specifically defined and verified as an adherence to the uterine wall. Strict inclusion criteria limited confounding that would otherwise have been difficult to adjust for in studies of this size. Pre-eclampsia, diabetes, preterm birth, SGA birth and stillbirth, factors that are associated both with retained placenta and the molecular and histological presentation of oxidative stress and/or inflammation, were excluded[52,69]. Other confounding factors that could hypothetically increase oxidative stress levels in placental tissue, and could not be excluded, such as prolonged labor and postpartum hemorrhage did not correlate with GPX1 levels in **Study III**. Duration of labor was not significantly different amongst retained and non-retained placentas. Duration of augmented labor was however significantly longer in cases of retained placenta. For this reason duration of oxytocin exposure was adjusted for in the regression analysis. It is still possible however that augmented labor, prolonged labor and hemorrhage represent residual confounding in the analysis of GPX1 gene and protein levels across groups.

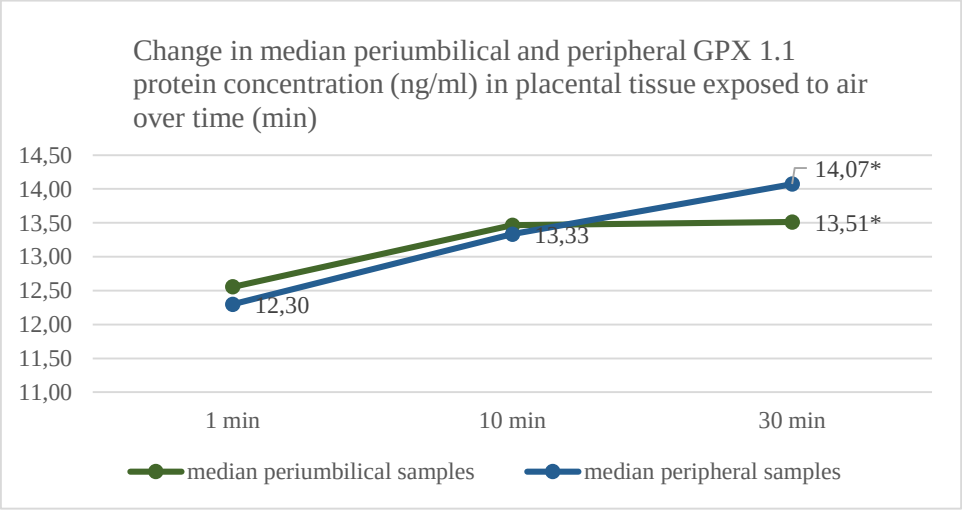
Placental samples from both groups in **Study III** were frozen within approximately 5 minutes of sampling, reducing potential oxidative damage to the placental tissue from exposure to air. All laboratory assessments were done in duplicates. The researcher performing the laboratory analysis was blinded to outcome group.

There is however a black box to gene and protein research on retained placenta inherent to the diagnosis itself. By definition retained placentas have been attached to the uterine wall for at least 30 minutes before samples are taken. Although the time from placental detachment till samples are frozen can be kept short and equal between cases and controls, it is difficult to adjust for this period of attachment or to know in which direction oxidative stress levels go during this phase. In an attempt to answer this question we analyzed GPX1 gene and protein expression in placental samples collected at different time intervals after placental delivery in six placentas exposed to air. The results showed that although both gene and protein levels remained fairly stable up until 30 minutes after placental delivery, there was a small but significant increase in GPX1 protein expression[84] (Figure 6). This increase possibly signifies a compensatory increase of antioxidative enzymes in the placenta after the stress of labor has ceased. It is uncertain however if these results can be extrapolated to retained placentas where blood flow from the fetus is cut-off but where a plane of attachment to the uterine wall remains.

Systematic error is also an assessment of to what degree the study measures what it claims to do. GPX1 and NFκB are adequate overall markers of oxidative stress, inflammation and the overlap between these two processes. As a pilot study, **Study III** was however small in the scope of its analysis which consequently limits to what extent the study can describe the role of oxidative stress and inflammation in retained placenta. To reliably assess oxidative stress would require an analysis of all subtypes of GPX, other antioxidative enzymes as well as by-products of oxidative stress in placental tissue. In a more specific assessment of the association between retained placenta and pre-eclampsia, the analysis of predictive markers of impaired angiogenesis such as serum or placental VEGF (vascular endothelial growth factor) and sFLT-1 (soluble fms-like tyrosine kinase-1) in retained placenta could be considered. These latter factors are also active in inflammatory pathways and could tie into a further analysis of the role of inflammation in retained placenta. An analysis of inflammation might also include products of activated inflammatory pathways in the placenta such as tissue tumor necrosis factor-alpha, placental interleukins, cyclooxygenase-2, and markers of apoptosis.

Figure 6

Change in median periumbilical and peripheral GPX1 protein concentrations in placental tissue exposed to air over time.



*p <0.05

6.1.2.3 Study IV

Outcome was strictly defined in **Study IV** and disorders with histological characteristics that would confound the analysis were excluded. The researcher performing the microscopic analysis was blinded to outcome group. The delivery-related factors discussed above may be relevant to **Study III** in that genes and proteins involved in the regulation of oxidative stress

and inflammation can alter their expression in the time span of labor and delivery. Histological features related to oxidative stress and inflammation will however take longer to develop and do not need to be accounted for in the analysis.

6.2 RANDOM ERROR

Random error refers to the unpredictable variability in measurements due to chance that decide with what degree of statistical confidence results can be stated.

6.2.1 Precision

Study I represents a smaller epidemiological study. The fact that medical records were individually assessed limited the size of the study group which prevented the study rare of rare exposures such as SGA birth. The study was however well-powered to the detailed study of common exposures such as duration of labor and augmented labor, factors that could also be validated by the researcher in individual medical records. In contrast **Study II** greatly increased the sample size at the cost of somewhat lower specificity in recorded variables. The study size enabled the study of associations between retained placenta and several interrelated rare exposures.

Molecular and histological analysis is resource intensive which often limits study group size. Both **Study III** and **IV** would have benefited from a larger study group. Sample size was decided in a power calculation which included an estimate of variance in GPX1 concentrations and baseline incidences of histological features respectively. Placental sampling in both **Study III** and **IV** was performed at standardized locations and layers. Molecular and histological analysis was performed according to standard protocols. Intersample variability in placental tissue in **Study III** was however larger than expected. In **Study IV** the incidence of histological pathology was more common than expected in the control placentas from full-term healthy pregnancies. This variability limited to what extent both studies could distinguish differences with a high degree of confidence.

6.2.2 Variability in levels of oxidative stress

The placenta is a setting of concentrated oxygen metabolism making the study of oxidative stress in pregnancy and labor essentially complex[55]. Oxidative stress levels differ across the placenta depending on proximity to the umbilical insertion and layer of the placental tissue[56,74,85,86]. Prolonged labor induces oxidative stress and apoptosis in the placenta and activates inflammatory pathways and angiogenic regulators[56,84]. Placental sampling after labor

may therefore increase intersample variability which would decrease the precision of the results. Above all, measurements may not reflect the placenta´s normal physiology, something which has given rise to caution against studying placental molecular biology after labor altogether[56]. In the case of retained placenta studying placentas after labor is however the only option available.

6.3 EXTERNAL VALIDITY

External validity is a measure of the extent to which the results of a particular study group can be extrapolated to a larger national, regional or global population.

Study I represents the obstetric context of one large urban hospital. Demographic and delivery-related interventions vary to some extent, primarily on a rural to urban axis in Sweden, but the results are probably applicable to the Swedish population as a whole[87]. **Study II** was a population-based study meaning that sampling bias, that could otherwise limit generalizability on a national level, was largely eliminated. Both these study populations are however Swedish and the delivery-related variables studied reflect a Swedish obstetric context. The associations found may therefore only be applicable to a genetically similar population in a similar obstetric environment.

The extent to which the findings in **Study III** and **IV** can be generalized to a larger population with clinical retained placenta is limited. This is because of the deliberate narrow inclusion of placentas only from full-term otherwise healthy pregnancies. As initial studies testing new hypotheses these studies aimed not so much at generalizability but at creating a platform for future research.

7 DISCUSSION

7.1 THE RESULTS IN CONTEXT

7.1.1 Augmented labor

Study I found a dose-response association between labor augmentation with oxytocin and retained placenta which has not been shown before. Duration of augmented labor was analyzed instead of total dose of oxytocin administered. Given that oxytocin doses needed for optimal myometrial contraction are highly variable among individuals, duration of use may reflect oxytocin receptor saturation as well as does cumulative dose[88]. The study distinguished between augmented labor and prolonged labor and found only augmented labor to be independently

associated to retained placenta. A recent study found prolonged labor to be a risk factor for retained placenta but did not adjust for labor augmentation[29]. However, prolonged labor was suggested as a risk factor for retained placenta in studies prior to the discovery of oxytocin in (1953, Vincent du Vigneaud)[18,28]. Augmented labor and prolonged labor both reflect dysfunctional uterine contractility, it is therefore not unlikely that also prolonged labor is related to the development of retained placenta.

The question remains how oxytocin and prolonged labor are instrumental in the development of retained placenta. Randomized trials have shown that although oxytocin administration after delivery reduces postpartum hemorrhage it does not reduce the incidence of manual removal of the placenta or the length of the third stage[2,89]. There are indications that augmented labor increases postpartum hemorrhage and that prolonged oxytocin exposure during labor gradually desensitizes myometrial cells to its effect[90 91]. The idea that oxytocin itself could cause oxidative stress is counterintuitive, most studies on its physiology indicate that it attenuates oxidative stress and decreases inflammation[92]. Possibly however, both prolonged labor and augmented labor render the uterus less capable of a contractile mechanism needed to detach the placenta postpartum. Oxytocin which is titered up as far as the frequency of contractions allow, would be more likely to saturate contractile capacity, predisposing both to postpartum hemorrhage and retained placenta.

7.1.2 Hemorrhage

In **Study I, III,** and **IV** the estimated median blood loss for cases of retained placenta was consistently between 1500 and 1600 ml. This means that the majority of women with retained placenta suffer severe postpartum hemorrhage. The magnitude of this risk is probably valid despite the fact that blood loss was to some extent subjectively assessed in these studies. Although it is conceivable that blood loss was selectively overdiagnosed in cases of retained placenta, most studies on the contrary indicate that visual assessment underestimates blood loss and increasingly so for larger volumes of blood lost[93,94].

An initial aim of **Study I** was to investigate whether increasing duration till manual removal of the placenta increased total blood loss. What was found was the inverse relationship, suggesting that expedient manual removal increases hemorrhage. Most probably this is a case of confounding by indication: women who bled profusely were taken to surgery without delay. The duration from delivery of the infant till manual removal was then in fact measuring the degree of hemorrhage and not vice versa. One study found gradually rising rates of hemorrhage

and blood transfusion in third stages of labor ≥ 30 minutes with maximum rates at 75 minutes postpartum. However, at 30 minutes 72% of retained placentas had already been manually removed - most likely those that bled profusely[4]. In a working clinical setting the question of optimal timing till manual removal seems difficult to answer and must therefore continue to be guided by individual clinical assessment.

7.1.3 Defective placentation disorders

The hypothesis of defective placentation and increased oxidative stress in retained placenta was derived from the results of **Study I** where recurrent miscarriage, pre-eclampsia and preterm birth were among the strongest associated risk factors, all disorders of placental origin. Recurrent miscarriage is, in this association, likely to represents a tendency of pregnancy loss and not vacuum aspiration sometimes used to treat incomplete miscarriage. Approximately 90% of early miscarriages reach completion with expectant management, the first line of treatment for incomplete miscarriage in Sweden is pharmacological and the study found no association to vacuum aspiration[95]. **Study II** showed that all defective placentation disorders studied were related to retained placenta. This was the first study to show an association between SGA birth and retained placenta as well as stillbirth and retained placenta. The increased risk of retained placenta in preterm pre-eclampsia and preterm SGA birth was also not previously known. Compared with pre-eclampsia and SGA birth at term, early-onset forms are usually clinically more severe, show an increased degree of histological and molecular-level oxidative stress and high resistance uterine artery flow[42,96-101]. The stronger risk associated with preterm disorders therefore supports the hypothesis of a pathophysiological association between retained placenta and defective placentation disorders. The increased risk of retained placenta in preterm birth has been shown in numerous studies but we created a model specific for spontaneous preterm birth that suggested that this association is not a proxy for increased rate of induction of labor or underlying causes of preterm birth such as pre-eclampsia, fetal growth restriction or infection. The dose-response association of increasing risk of retained placenta with decreasing gestational age at birth supports a causal relationship between spontaneous preterm birth and retained placenta[102].

Study I and **II** thus independently indicate pre-eclampsia, preterm delivery and recurrent miscarriage as risk factors for retained placenta. In light of what other studies have shown this strongly suggests that these disorders are associated to retained placenta. Since **Study II** was

performed two other studies have also corroborated an association between retained placenta and stillbirth[29,103].

7.1.4 Epidemiological patterns in retained placenta

In **Study II** maternal age had a dose-response relationship with retained placenta with increasing risk of retained placenta with increasing age. Maternal age >30 or >35 has also been a risk factor in several previous studies on retained placenta which supports this association[27,29,42]. Maternal age is a risk factor for the development of most defective placentation disorders and probably reflects poorer placental perfusion with age[104].

Nulliparity was associated to retained placenta in **Study I** and is consistently a risk factor in other studies of retained placenta. Studies that represent a population with increased parity also suggest grand multiparity as a risk factor for retained placenta[41,42]. The U-formed risk curve in relation to parity, with increased risk for the nullipara as well as the grand multipara, reflects that which has been suggested in the association of pre-eclampsia to parity[105,106].

Like pre-eclampsia, the strongest risk factor for retained placenta is having had the diagnosis in a previous pregnancy[107]. One study found that increased inter-pregnancy interval increased the risk of retained placenta, as it increases the risk of pre-eclampsia[44]. A recent analysis of the genetic component in postpartum hemorrhage found that most family clustering occurred for hemorrhage due to retained placenta[108]. Here again, retained placenta "behaves" epidemiologically like pre-eclampsia.

However, most relevant studies have not shown either increased BMI or diabetes to increase the risk of retained placenta as these factors do for pre-eclampsia[13,29,37,44,69]. Likewise the possibly increased risk of retained placenta in populations of European genetic descent is contrary to the suggested decreased risk of pre-eclampsia in this population[109].

Delivery-related variables such as induction of labor, epidural use and instrumental delivery were either not significantly or only weakly associated to retained placenta in both **Study I** and **II**. These variables have been associated to retained placenta in other studies as well but may, despite statistical adjustments, represent residual confounding and reflect other causal factors such as augmented or prolonged labor and preterm birth.

7.1.5 Placental underperfusion and oxidative stress

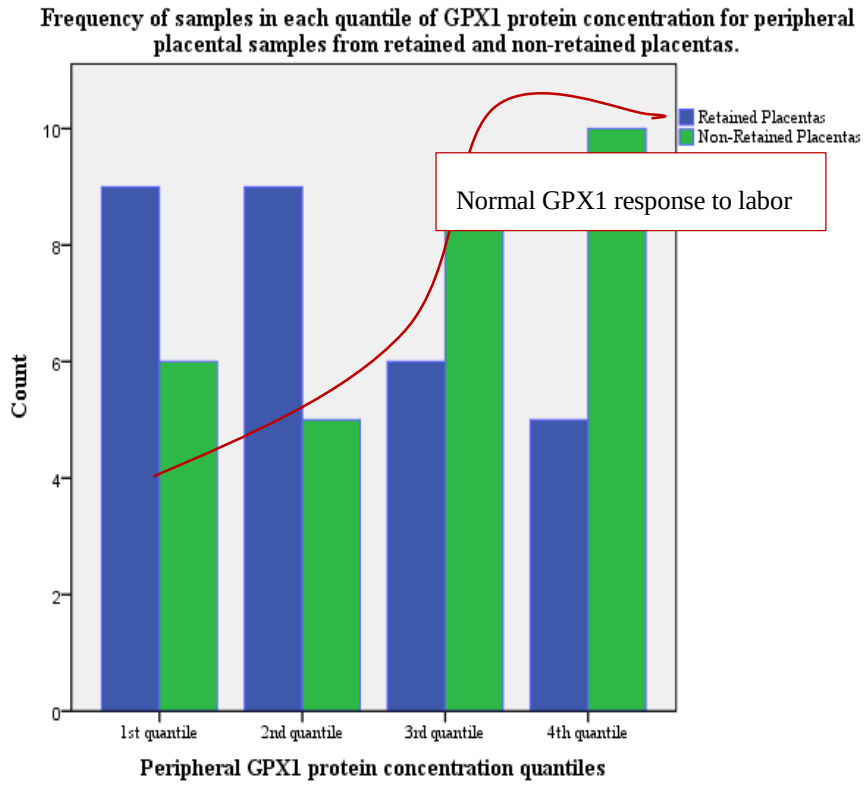
To my knowledge there are no previous studies on any aspect of the molecular biology of retained placenta. **Study III** was therefore the first to investigate the hypothesis of increased oxidative stress and inflammation in retained placenta. The results suggest that antioxidative defense capacity may be decreased, although a larger sample would be needed to state this more conclusively. However, the results were consistent in that the same pattern of decreased expression of GPX1 protein and somewhat increased expression of GPX1 mRNA was seen in both peripheral and periumbilical samples of retained placentas. If these results were to be supported in larger studies, what we might be seeing is either a posttranslational modification that decreases effective antioxidative capacity despite an upregulated gene expression, or the effect of negative feedback, with high mRNA expression in response to low or depleted GPX1 protein. Although decreased GPX1 concentrations cannot confidently be associated to retained placenta in this study it is notable that low GPX protein expression and GPX activity has been associated to pre-eclampsia and fetal growth restriction[74-76].

It is also interesting that retained placenta was significantly associated to low values of GPX1 protein. The categorical analysis of GPX1 in relation to retained placenta is informative since the distributions of GPX1 concentrations were not normal either among retained or non-retained placentas. Biochemical data in humans is often skewed to the left with a preponderance of low or normal values and a tail of higher values signaling disease. It may be that this similar pattern, seen in retained placenta in relation to GPX1, signals an impaired placental capacity to respond to oxidative stress, low values in this case signaling disease (Figure 7). This theory has been put forward for pre-eclampsia in relation to antioxidative defense capacity[75].

Study IV found an association between retained placenta and histological signs of maternal underperfusion which was new as were characteristics of oblong shape and decreased surface area found in retained placenta. These three features have previously been associated to pre-eclampsia and fetal growth restriction[100]. Decreased size, aberrant placental shape as well as the development of accessory lobes, which were also more common among retained placentas in our study, are hypothesized to result from implantation into an area of the uterine cavity with poor perfusion[65,110]. Markers of maternal placental underperfusion likewise reflect decreased perfusion of the placenta (as opposed to low perfusion to the fetus due to abnormalities of the umbilical cord)[110].

Figure 7

Distribution of GPX1 values in peripheral samples of retained and non-retained placentas in Study III with superimposed line showing hypothetical normal GPX1 response to labor which may be absent or depleted in cases of retained placenta



7.1.6 Inflammation

Study III did not support a role for increased inflammation in retained placenta mediated by the NFκB/IκBα pathway. Likewise **Study IV** found no histological signs of increased acute inflammation in the placenta, although chorioamnionitis was a common finding among both retained and non-retained placentas. PROM was not associated to retained placenta in **Study I** and weakly so in **Study II**. Fever during delivery was not associated to retained placenta in **Study I.** Other studies have likewise found PROM to be a weak risk factor for retained placenta[4,27,29,37,69]. However, PROM and fever can at best only be considered proxy variables for infection and NFκB is only an indirect marker of inflammation. Whereas this thesis found no clear support for inflammation as an agent in the development of retained placenta, either at the epidemiological or at the molecular level, further studies are needed to draw any substantial conclusions on the role of inflammation in this disorder.

7.1.7 The relation to placenta accreta

Study IV found that retained placenta had histological similarities to placenta accreta in that the incidence of BPMF was significantly increased and that cases of focal placenta accreta were numerically, although statistically non-significantly, overrepresented among retained placentas. The great majority of retained placentas, however, had no BPMF and a wholly intact decidua in the samples that were analyzed. Since the whole placental surface was not studied, focally accretic sections may have been missed, despite the fact that macroscopically disrupted placental areas were sampled. This leaves the question of the relation of retained placenta to placenta accreta unresolved.

Placenta accreta and retained placenta seem to share risk factors related to previous injury to the endometrium. **Study I** distinguished, to the extent possible, between previous miscarriage and induced abortion, as well as the surgical intervention, which in Sweden is vacuum aspiration, used in a minority of these pregnancies. The study indicated that previous miscarriage and induced abortion were significantly related to retained placenta while vacuum aspiration was not an independent risk factor in itself. Other studies have likewise shown an association primarily to previous induced abortion[4,27]. Previous surgical treatment for miscarriage or abortion is however a variable that is incompletely registered in Swedish obstetric medical records which may affect analysis. Some studies have shown an association to previous dilatation and curettage (D&C)[41,42]. Complicating comparisons across studies is the fact that most countries no longer use D&C, which represents a sharper trauma to the uterine lining and is likely to increase the risk of postoperative intrauterine adhesions[111]. However, it may be telling that an association has been found to this more traumatic form of treatment. Although cesarean section was not a risk factor in our first study it has been shown to be a risk factor in several other studies[27,37,44]. Previous retained placenta was a risk factor in **Study I** as well as in earlier studies[13,42,107]. Postpartum D&C for retained placental products was likely performed in some of these study groups but it is very rarely performed in Sweden. It is possible therefore that this risk factor reflects different etiologies in different studies: either an initial predisposition for retained placenta or previous damage to the uterine wall with subsequent increased placental attachment.

Damage to the uterine lining in a previous pregnancy will probably not alter biological factors that determine the risk of retained placenta, but may affect the implantation site for future pregnancies. This may result, like it does in cases of placenta accreta, in increased placental attachment because of an absent or abnormal decidual transformation of the endometrium. So

although the absence of decidua does not seem to affect the majority of cases of retained placenta it is possible that a portion of cases can be explained by focally damaged endometrium, poor decidual development and compensatory placental attachment as reflected by increased BPMF and focal placenta accreta.

7.2 PATHOPHYSIOLOGICAL MODELS FOR RETAINED PLACENTA

7.2.1 Risk profiles for retained placenta

Placing the results of this research project into the context of what is currently known about retained placenta, I would like to go back to the idea of two different etiological risk profiles for retained placenta: one representing an association to other placental disorders with reduced placental perfusion and one representing previous injury to the uterine lining. The results shown in this project indicate an epidemiological association between retained placenta and defective placentation disorders, supported in part by signs of decreased antioxidative capacity and placental perfusion at the molecular and histological level. This, together with the epidemiological patterns that retained placenta shows in relation to maternal age, parity, risk of repetition and familial clustering suggests that retained placenta has molecular, immunological and perhaps genetic etiological components. These may predispose to the development of retained placenta already at implantation, carry forward into the next pregnancy and perhaps into the next generation. On the other hand, interventions that affect the endometrial lining such as D&C after terminated pregnancy or delivery and cesarean section may increase the risk of retained placenta by disrupting normal decidual development and increasing attachment at implantation in the next pregnancy. Based on the strength of the associations this is probably the less common etiology.

I also suggest that prolonged augmented labor with oxytocin increases the risk of retained placenta, possibly because of the stress that this exposure incurs on the contractile apparatus and oxygenation of the placenta, something which then impedes normal placental release. Suggested increases in incidence of retained placenta, perhaps particularly so in developing countries, and globally differing rates may be due both to increases in D&C and cesarean section rates but also to the increasing use of oxytocin for labor augmentation.

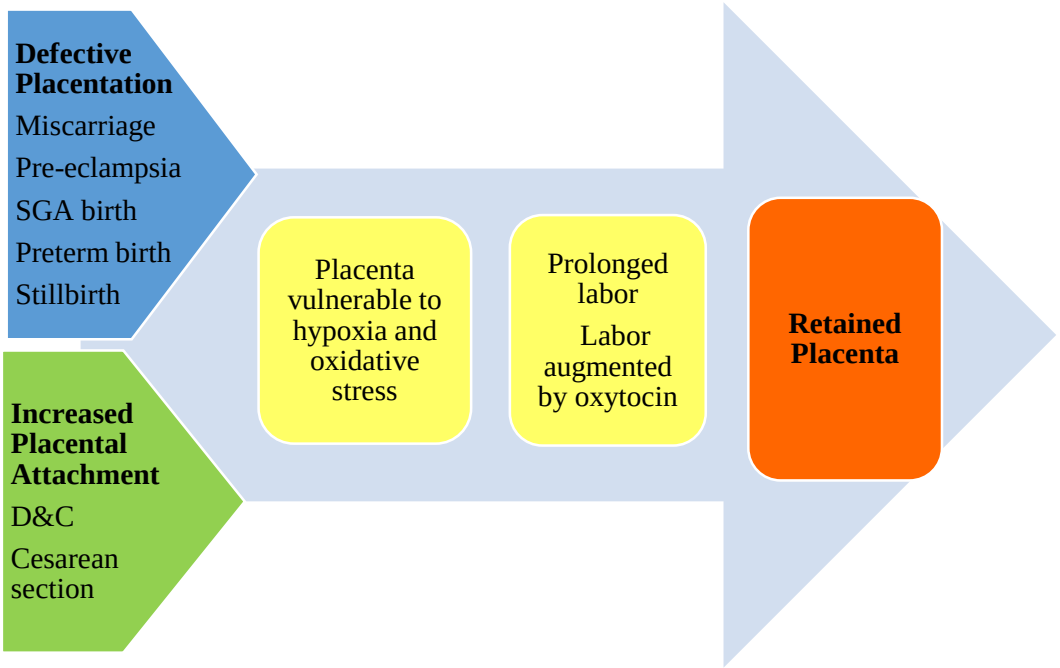
7.2.2 Too deep or too shallow placentation?

If retained placenta originates at placentation it may, as discussed above, occur as a result of two unrelated processes, one resulting in increased placental attachment, the other in deficient,

or too shallow, placentation. A more symbiotic model is that these two, seemingly opposite, placental patterns are themselves related and may as a result take on a similar clinical presentation in the third stage of labor. Some studies have indicated that this may be the case. Placenta accreta has been shown to present with reduced spiral artery remodeling, the pathognomonic pattern of pre-eclampsia[60,112]. Placental weight and spiral artery remodeling has been shown to be reduced in placentas with increased BPMF, a characteristic trait of placenta accreta[59]. A study from as early as 1987 presented the hypothesis that accretic placentation occurred in response to an initial incomplete or shallow placentation[113]. In this combined hypothetical model, placentas predisposed to become retained would be more vulnerable to oxidative stress irrespective of etiology. Prolonged oxytocin exposure which saturates the contractile capacity of the myometrium may then exacerbate an underlying heightened risk for retained placenta (Figure 8).

Figure 8

Schematic model of etiology of retained placenta and association to defective placentation disorders, placenta accreta and augmented labor



8 CONCLUSIONS

- Prolonged augmented labor by oxytocin is associated to an increased risk of retained placenta.

- Retained placenta is associated to postpartum hemorrhage, in particular severe and very severe postpartum hemorrhage.

- Optimal timing till manual delivery of the placenta is difficult to assess in a working clinical setting and must be guided by individual assessment.

- Defective placentation disorders i.e. pre-eclampsia, preterm birth, SGA birth, stillbirth as well as a history of recurrent miscarriage are associated to retained placenta.

- In regard to maternal age, parity and risk of recurrence retained placenta shows epidemiological patterns that are similar to those seen in pre-eclampsia.

- The protein expression of GPX1, an antioxidative enzyme, may be decreased in retained placenta but this needs to be validated in a larger study.

- Histologically, retained placenta shows no signs of increased placental inflammation and retained placental tissue shows no significant increase in NFκB/IκBα mRNA expression that would reflect activation of inflammatory pathways.

- Retained placenta ressembles pre-eclampsia and other defective placentation disorders in regard to placental shape, size and histological signs of maternal placental underperfusion.

- Features of increased placental attachment are overrepresented in retained placentas which may suggest a pathophysiologial association to placenta accreta. The majority of retained placentas however show no signs of increased placental attachment which indicates that retained placenta and placenta accreta are separate disorders.

- In summary, retained placenta is epidemiologically associated to defective placentation disorders something which is supported in part by signs of decreased antioxidative capacity in the placenta and increased histological signs of maternal placental underperfusion.

9 CLINICAL AND SCIENTIFIC IMPLICATIONS

9.1 IMPLICATIONS FOR CLINICAL PRACTICE

Awareness of risk factors for retained placenta can help to identify women at heightened risk and subsequent risk of serious hemorrhage and should guide clinical practice.

- The risk of retained placenta should be assessed in routine obstetric history. In particular a history of retained placenta in a woman giving birth prior to 37 weeks should lead to heightened awareness. This risk level should be increased if the pregnancy is complicated by pre-eclampsia, fetal growth restriction or stillbirth and if there is a history of previous cesarean section. During delivery, prolonged labor augmentation by oxytocin should be compounded to this risk profile.

- The level of predicted risk should guide in what setting a woman delivers, whether a physician should be present during the third stage and whether preparatory measures are taken for manual removal of the placenta.

- The increased risk of retained placenta associated to augmented labor and cesarean section, delivery-related interventions that are to some extent avoidable, should be weighed in the harm-benefit calculation made before they are used in obstetric practice.

9.2 IMPLICATIONS FOR THE RESEARCH FIELD: QUESTIONS THAT REMAIN

There is still a lack of research about retained placenta which is a concern given how serious the disorder is. Further research should be perfomed based on the platform of what is now known. What is most lacking are studies exploring the pathophysiology of retained placenta: studies in molecular biology, immunochemistry and histology that might identify genetic, immunological or molecular features as well as histological characteristics specific to retained placenta.

- In relation to oxidative stress: a broader analysis of antioxidative defense systems and products of oxidative stress in retained placenta at the gene, protein and enzyme activity level.

- In relation to disorders of defective placentation: an analysis of molecular markers associated to pre-eclampsia and fetal growth restriction in retained placentas.

- In relation to histology: a complete analysis of the placental bed in retained placenta compared to non-retained placenta as well as placentas delivered manually at cesarean section; myometrial biopsy sampling at manual removal of the placenta to assess the degree of spiral artery remodeling in retained placenta compared to non-retained placentas.

Knowledge of the pathophysiology of this disorder and its relationship to other pregnancy-related diseases could eventually be instrumental in its prevention or in its less invasive treatment. This is of paramount importance to the women who are most vulnerable to the consequences of postpartum hemorrhage, namely women in poor countries without access to modern obstetric emergency care.

10 POPULÄRVETENSKAPLIG SAMMANFATTNING

Placentaretention (kvarhållen moderkaka) innebär att placentan inte lossnar spontant inom 30 minuter efter barnets födelse och uppstår vid 2-3% av alla förlossningar. Det är en av de främsta orsakerna till allvarlig blödning efter förlossning och i områden utan tillgång till sjukhusvård är tillståndet ofta dödligt. Behandlingen, manuell placentalösning, utsätter den nyförlösta kvinnan för sövningsrisken och en ökad risk för infektion i livmodern. Orsaken bakom placentaretention är inte känd. Detta forskningsprojekt genomfördes för att tydliggöra riskfaktorer för placentaretention, utvärdera sambandet mellan placentaretention och andra graviditetssjukdomar och undersöka om det finns molekylära eller vävnadsstrukturella utmärkande drag för tillståndet.

Studie I, en fall-kontroll studie, bekräftade att placentaretention innebär en kraftigt ökat risk för allvarlig blödning efter förlossning. Studien fann även ett samband mellan placentaretention och långvarig användning av oxytocin för värkstimulering under förlossning. Preeklampsi, förtidsbörd och tidigare missfall var också riskfaktorer för placentaretention i studien. Dessa tillstånd är, tillsammans med dödföddhet och tillväxthämning, tillstånd med så kallad ”defekt placentation”, där den ursprungliga infästningen i livmodern är rubbad. Blodförsörjningen i placentavävnaden är försämrad vid dessa tillstånd vilket kan ses i vävnaden som ökad syreorsakad stress på molekylär nivå och genom strukturella tecken till dålig blodförsörjning på mikroskopisk nivå. Studie II, en stor populationsbaserad studie, fann att alla dessa tillstånd var kopplade till placentaretention.

I Studie III och IV samlades kvarhållna och icke-kvarhållna placentor in efter förlossningen för vävnadsanalys. Studie III fann en tendens till lägre kapacitet att försvara sig mot syreorsakad stress i placentor som varit kvarhållna jämfört med placentor som inte varit kvarhållna. Studie IV fann att kvarhållna placentor var mindre till ytan, mer avlånga i formen och visade fler strukturella tecken till dålig blodförsörjning i vävnaden jämfört med icke-kvarhållna placentor. Både biokemiskt och strukturellt fanns det alltså likheter mellan placentor som varit kvarhållna och placentor där graviditeten komplicerats av tillstånd med defekt placentation.

Sammanfattningsvis är placentaretention epidemiologiskt kopplad till en grupp andra placentära sjukdomar med ursprunglig defekt placentation. De utmärkande molekylära och vävnadsstrukturella dragen i placentan vid dessa tillstånd kan i viss grad också ses vid

placentaretention vilket stärker sambandet. Kunskap om riskfaktorer för placentaretention bör vägas in i hur sjukvårdspersonal behandlar kvinnor efter förlossningen. Att förstå orsaken bakom placentaretention är nästa steg mot att kunna förebygga och behandla ett tillstånd som framför allt för kvinnor i låginkomstländer innebär en livshotande risk.

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12 APPENDIX: HISTOLOGICAL DEFINITIONS

Artheriolopathy: Fibrinoid necrosis of the spiral artery wall, often with dilation of the vessels, presence of acute atherosis and lumen thrombosis.

Basal plate myometrial fibers: Myometrial smooth muscle cells at the maternal floor with intervening decidua.

Chorangiosis : vascular hyperplasia in the terminal chorionic villi and defined according to Altshuler´s criteria [114].

C**horionitis/subchorionitis**: presence of neutrophils in the chorion or subchorion.

Chorioamnionitis: expansion into the chorioamniotic mesoderm and/or amnion.

Chronic villitis: Presence of lymphocytes with or without histiocytes in the stroma of placental villi.

Fetal thrombosis: Thrombosis in the vessels of the umbilical cord, chorionic plate or stem villi.

Funicitis: Inflammation as defined above extending into Wharton´s jelly.

Grouped multinucleated trophoblastic cells: Trophoblastic cells with three or more clustered trophoblasts in the decidua each with three or more nuclei [115].

Placental abruption: Presence of a retroplacental hematoma was recorded as suggestive of the disorder.

Placenta accreta: Absence of intervening decidua between the chorionic plate and the myometrium.

Placental infarction: ischemic necrosis of the villi. Fetal thrombosis was defined as thrombosis in the vessels of the umbilical cord, chorionic plate or stem villi.

Placental septal cysts: Cysts within the septa that separate the cotyledons of the placenta.

Syncytial knots: >10 aggregated syncytical nuclei at the surface of the terminal villi [34].

Umbilical vasculitis: Presence of neutrophils in the walls of the umbilical cord.

Villous agglutination: Syncytial knotting with collapse of the villous space.

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