Recurrent unexplained first-trimester miscarriage

Effects of acetylsalicylic acid, platelet aggregation and thyroid disease

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The most effective therapy for patients with unexplained RPL is often the most simple: antenatal counseling and psychological support.

Holly B Ford, 2009

Abstract

Background/Aims: Recurrent pregnancy loss (RPL) occurs in 1-2% of fertile couples and about 50% of cases are unexplained. Impaired placental circulation, increased platelet aggregation, immunological factors and thyroid autoimmunity have been suggested to be involved. Other placenta-mediated complications have been reduced by inhibition of platelet aggregation with acetylsalicylic acid (ASA). The effect of ASA on RPL has been unclear. These studies aimed at investigating the effect of ASA treatment on RPL and arachidonic acid (AA)-induced platelet aggregation in women with RPL, as well as the effect of thyroid function by analyzing Thyroid Stimulating Hormone (TSH) and thyroid peroxidase antibodies (TPO-ab).

Methods: Women (n=640) with at least three unexplained first-trimester miscarriages were screened for inclusion in a single-center, randomized, placebo-controlled trial (the ASA-RCT, Paper I). Four hundred women were given either 75 mg ASA or placebo daily, beginning at gestational week (gw) 6-7, when fetal heartbeat was detected by vaginal ultrasound. Treatment ended at the latest at gw 36. Treatment compliance was determined by analysis of AA-induced platelet aggregation using multiple electrode impedance aggregometry. All women underwent the same follow-up. Primary outcome was live birth rate (LBR).

In order to define reference values for the multiple electrode impedance aggregometry (the Multiplate analyzer), a longitudinal study was conducted including 79 healthy, non-smoking pregnant women with normal pregnancies (Paper II). Platelet aggregation induced by AA, adenosine diphosphate (ADP), thrombin receptor activating peptide 6 (TRAP) and collagen (COL) were determined four times during pregnancy and three months postpartum.

From the randomized population, 176 and 177 women, respectively, with normal AA-induced platelet aggregation before pregnancy and treated with ASA or placebo, were studied (Paper III). Platelet aggregation was determined before and during pregnancy and results in the randomized groups were compared with one another, as well as with those in the healthy controls from Paper II.

From the screened and eligible population, 495 women with complete thyroid test analyses [thyroid stimulating hormone (TSH), free thyroxine (T4) and thyroid peroxidase antibodies TPO-ab] before pregnancy were included. Risk factors for a new miscarriage were studied, in particular associations with TPO-ab and TSH in the upper normal range.

Results: In the ASA-RCT, all 400 randomized women completed the follow-up. LBR were 83.0% and 85.5% in the ASA and placebo groups, respectively. The mean difference was -2.5% (95% CI to -10.1% to 5.1%). The risk ratio was 0.97 (95% CI 0.89 to 1.06).

In the longitudinal study of platelet aggregation during normal pregnancy, activation of platelets by AA, ADP and TRAP resulted in a minor decrease in platelet aggregation during pregnancy, compared with postpartum. COL-induced platelet aggregation was unchanged. A minor increase in platelet aggregation as pregnancy continued was found related to ADP.

There were no significant differences in AA-induced platelet aggregation when placebo-treated women with RPL were compared with healthy women with normal pregnancies. ASA treatment significantly reduced platelet aggregation during pregnancy, compared with before pregnancy. Gradually increased platelet aggregation was seen in the majority of ASA-treated women as pregnancy progressed. There were only two complete non-responders to ASA.

Miscarriage occurred more often in women with than without TPO-ab (25.7% vs 17.5%). There was no association between TSH in the upper normal range and a new miscarriage. Independent risk factors for a new miscarriage were age, number of previous miscarriages and presence of TPO-ab.

Conclusions: ASA does not prevent a new miscarriage in women with at least three previous first-trimester miscarriages. AA-induced platelet aggregation seems to be similar in women with RPL and in healthy women with normal pregnancies. ASA, 75 mg daily, decreases AA-induced platelet aggregation in most women during pregnancy, but the effect diminishes as pregnancy progresses. TPO-ab, but not TSH in the upper normal range, may be associated with an increased risk of a new miscarriage.

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Populärvetenskaplig sammanfattning

Bakgrund

Upprepade missfall drabbar omkring 1 på 100 av fertila par varav ungefär hälften inte har en påvisbar trolig orsak. Upprepade missfall i tidig graviditet kan definieras på olika sätt utifrån antal tidigare missfall och när i graviditeten de inträffat. I avhandlingens studier, har kvinnorna minst tre oförklarade missfall i följd under graviditetens första tredjedel och dessa tillsammans med samma partner.

Kvinnans ålder och antal tidigare missfall har i tidigare studier visats vara en riskfaktor för ett nytt missfall, men det finns också andra tänkbara förklarande orsaker; kromosomavvikelse hos kvinnan eller mannen, anatomiska avvikelser i livmodern, blodproppssjukdomar, hormonella sjukdomar (sköldkörtelsjukdom) samt livsstilsfaktorer (kraftig övervikt, extensiv träning). Dock, i fler än hälften av fallen visar utredning inte någon tänkbar eller påvisbar orsak.

Försämrad blodcirkulation med ”mikroblodproppar” i moderkakan och därmed sämre blodtillförsel till den nyanlagda graviditeten och fostret, har framförts som en orsak. Men även andra orsaker har föreslagits. Utan någon säkerställd effekt har olika behandlingar prövats såsom blodproppsförebyggande läkemedel (hämning av blodets levringsförmåga), sköldkörtelhormon (tyroxin), progesteron, immunglobulin.

Syftet med denna avhandling är att undersöka;

- om blodproppsförebyggande behandling med acetylsalicylsyra (ASA) kan minska risken för nytt missfall
- om funktionen hos blodplättar (trombocyter), små partiklar i blodet bidragande till blodets levringsförmåga, förändras under normal graviditet jämfört med kvinnors trombocytfunktion utan graviditet
- om trombocytfunktion under normal graviditet är annorlunda jämfört med graviditet föregången av upprepade tidiga missfall
- hur ASA behandling påverkar trombocyterna under graviditet
- om avvikelser i sköldkörtelfunktionen, närvaro av tyroperoxidas (TPO)-antikroppar eller Tyroidea Stimulerande Hormon (TSH) i övre normal-området, kan innebära ökad risk för ett nytt missfall

Arbete I

Denna studie gjordes för att utvärdera om behandling med 75 mg ASA dagligen, efter påvisad graviditet, kunde minska risken för ett nytt missfall hos kvinnor med minst tre tidigare upprepade missfall före tretton fulla graviditetsveckor och där utredning inte visat något avvikande. Paren remitterades till studien från kvinnokliniker på sjukhus samt öppenvård, hänvisades eller tog själva kontakt för att få delta i studien. De flesta kom från Region Västra Götaland och Region Halland.
Fyrahundra kvinnor, 200 kvinnor i varje grupp, lottades till ASA eller piller utan effekt, s.k. placebo, vid påvisad hjärtaktivitet hos fostret (graviditetsvecka 6-7) med ultraljud. Alla följdes med samma kontrollprogram under graviditeten. Trombocytfunktionen mättes hos alla kvinnorna för att mäta effekten av ASA.

Resultatet visade att prognosen är mycket god för dessa kvinnor att föda barn oavsett ASA-behandling eller inte. Omkring 15% av kvinnorna fick ett nytt missfall inom varje grupp och 83% i ASA-gruppen och 85.5% i placebogruppen födde barn. Följsamheten till behandlingen var mycket god.

Arbete II

Trombocyter och dess funktion under graviditet är av stor betydelse för vissa graviditetskomplikationer som preeklampsi (”havandeskapsförgiftning”), dålig fostertillväxt, för tidig födsel och kanske även för upprepade missfall. Det är viktigt att studera trombocytaggregationen (blodplättarnas ”hopklumpning”) under graviditet för att se om behandling med läkemedel som motverkar trombocyt-aggregationen kan minska risken för dessa graviditetskomplikationer.

Sjuttionio av 104 friska gravida kvinnor utan tidigare upprepade missfall och utan komplikationer i aktuell graviditet, studerades avseende trombocytfunktion under graviditet och tre månader efter förlossning då trombocytfunktionen bedöms ha återgått till en normal nivå.

Resultatet visade en något minskad trombocytaggregation under graviditet jämfört med tre månader efter förlossningen.

Arbete III

Arbete III gjordes för att jämföra trombocytaggregationen under graviditet hos kvinnor med upprepade missfall med dem som haft ett eller inget missfall, samt hur trombocytaggregationen påverkades av behandling med 75 mg ASA respektive med placebo. Trombocytaggregation studerades dessutom för att få kunskap om ASA-

effekten vid olika tidpunkter under graviditeten och jämförelse gjordes med trombocytaggregation före behandling.

Resultaten visade att, det inte var någon skillnad i trombocytaggregation som aktiverats av arachidonsyra (AA) under graviditet, för kvinnor med upprepade missfall och de normala kontrollerna. Det finns troligen inget stöd för hypotesen att kvinnor med upprepade missfall skulle ha en ökad AA-aktiverad trombocytaggregation som förklaring till missfallen.

Trombocytaggregationen hos de ASA-behandlade kvinnorna visade mer än 50% reduktion, vilken kvarstod för de allra flesta under hela graviditeten. Reduktionen minskade dock något under graviditetens senare del. Detta är inte visat tidigare och skulle kunna visa på ett behov att under senare del av graviditeten höja dosen av ASA om 75 mg per dygn används som prevention mot de tidigare nämnda graviditets-komplikationerna.

Arbete IV

Underfunktion av sköldkörteln (hypotyreos) är en känd riskfaktor för missfall. Av den anledningen kontrolleras körtelns funktion med ett blodprov, TSH, vid inskrivning på mödravården i tidig graviditet, så att behandling med tyroxin kan sättas in vid behov. Ett annat tecken till påverkan på sköldkörteln är förekomst av TPO-antikroppar i blodet. Det saknas idag kunskap om TSH i sitt övre normalområde ($2.5 < TSH \leq 4.0$ mU/l) med eller utan samtidig förekomst av TPO-antikroppar är riskfaktorer för nytt missfall hos kvinnor med tidigare upprepade missfall i första tredjedelen av graviditeten.

I detta arbete ingick 495 av 640 kvinnor som tidigare utretts för att kunna ingå i studien i arbete I. Alla hade normala prover avseende TSH och fritt T4. Graviditetsutfall (missfall och födsel) jämfördes avseende förekomsten av TPO-antikroppar samt nivån av TSH.

Resultaten visade att TSH i sitt övre normalintervall inte var en riskfaktor för nytt missfall, men att förekomst av TPO-antikroppar skulle kunna vara det.

List of papers

I **Blomqvist L, Hellgren M, Strandell A.**
Acetylsalicylic acid does not prevent first-trimester unexplained recurrent pregnancy loss: A randomized controlled trial. Acta Obstet Gynecol Scand. 2018;97:1365-1372.

II **Blomqvist L, Strandell A, Baghaei F, Hellgren M.**
Platelet aggregation in healthy women during normal pregnancy - a longitudinal study. Platelets. 2019;30:438-444.

III **Blomqvist L, Strandell A, Jeppsson A, Hellgren M.**
Platelet aggregation during pregnancy in women with previous recurrent first-trimester fetal loss, with and without acetylsalicylic acid treatment. Submitted.

IV **Blomqvist L, Filipsson Nyström H, Hellgren M, Strandell A.**
Preconceptual thyroid peroxidase antibody positivity in women with recurrent pregnancy losses may be a risk factor for another miscarriage. In manuscript.

Contents

List of abbreviations

AA	Arachidonic acid
ACOG	American College of Obstetricians and Gynecologists
ADP	Adenosine diphosphate
APL	Apoteket Produktion & Laboratorier
APTT	Activated partial thromboplastin time
APS	Antiphospholipid syndrome
ASA	Acetylsalicylic acid
ASPI-test	Reagent kit used for monitoring of ASPIRIN and other cyclooxygenase inhibitors, after induction by arachidonic acid
ASRM	American Society for Reproductive Medicine
AUC	Area under curve
BMI	Body mass index
BV	Bacterial vaginosis
CI	Confidence interval
COL	Collagen
COX	Cyclooxygenase
DGGG	German Society of Gynecology and Obstetrics (Deutsche Gesellschaft für Gynekologie und Geburtshilfe)
ESHRE	European Society of Human Reproduction and Embryology
Free T4	Free thyroxine
gw	Gestational week
Hb	Hemoglobin
hCG	Human chorionic gonadotropin
ITAKA	Identify of TAblets and KApslar (capsulas)
IVF	In vitro fertilization
IVIG	Intravenous immunoglobulin
LBR	Live birth rate

LC	Leucocyte count
LMWH	Low molecular weight heparin
NK	Natural killer cell
OEGGG	Austrian Society of Gynecology and Obstetrics (Oesterreiche Gesellschaft für Gynekolgie and Geburtshilfe)
OR	Odds ratio
PC	Platelet count
PC(INR)	Prothrombin complex (international normalized ratio)
PCOS	Polycystic ovary syndrome
PFA-100	Platelet Function Analyzer
PI	Principal investigator
PRP	Platelet rich plasma
RCOG	Royal College of Obstetricians and Gynecologists
RCT	Randomized controlled trial
REPL	Recurrent early pregnancy loss
RPL	Recurrent pregnancy loss
RR	Risk ratio
SCH	Subclinical hypothyroidism
SD	Standard deviation
SFOG	Swedish Society of Obstetrics and Gynecology
SGGG	Swiss Society of Gynecology and Obstetrics (Schweizerische Gesellschaft für Gynekologie und Geburtshilfe)
TLC	Tender loving care
TPO-ab	Thyroid peroxidase antibody
TRAP	Thrombin receptor activated peptide 6
TSH	Thyroid Stimulating Hormone
TXA_2	Thromboxane A2
U	Unit
UK	United Kingdom
WHO	World Health Organization

Introduction

Miscarriage

Miscarriage, the spontaneous loss of a pregnancy before the fetus has reached viability (Rai *et al.*, 2006), is the most common complication of pregnancy. Miscarriage, also called spontaneous abortion, can be classified as sporadic or recurrent. A sporadic miscarriage occurs in 30-50% of pregnancies, but only 15% of cases are diagnosed (van Dijk *et al.*, 2016; Rai *et al.*, 2006; Kutteh *et al.*, 2014). Between 12% and 24% of clinically diagnosed pregnancies end in miscarriage, usually in the first trimester (Lee *et al.*, 1996), as illustrated in Figure 1 (Nybo-Andersen, 2000).

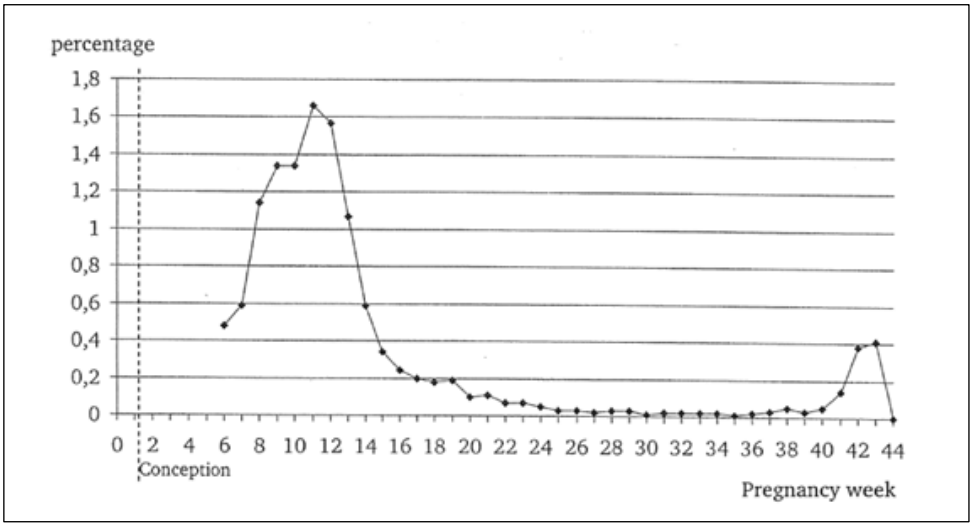

Fig. 1. Weekly risk of fetal loss. Anne-Marie Nybo Andersen. Fetal death, epidemiological studies. PhD thesis. University of Copenhagen. 2000. Published with permission from the author.

Age and risk of miscarriage

Many authors have reported an increased risk of fetal death and spontaneous abortion with increasing maternal age, especially over 30 years, irrespective of previous obstetric history (Nybo-Andersen *et al.*, 2000; Cauchi *et al.*, 1991; Lund *et al.*, 2012; Bhattacharya *et al.*, 2010).

Reproductive behavior in our modern society has changed; many women now choose to delay pregnancy and childbearing. According to UK data, the number of babies born to women aged 35 years and up doubled between 1985 and 2001, from 8% to

16% of all births (Rai *et al.*, 2006). In 1973, women in Sweden had their first child at a mean age of 24 years. In 2016, the corresponding age was 29.2 and in 2018 it was 29.4. In Danderyd, a municipality near Stockholm, the mean age at first childbirth was 31.8 years (highest in the country) in 2018, while it was 31.6 in Stockholm, 30.3 in Gothenburg (the second biggest city in Sweden) and 28.3 in Borås (a middle-sized city, in which the main study was conducted) (Statistics Sweden, 2018). In smaller cities and in rural areas, women had their first child at age 25-26. The level of education and women wanting to establish themselves on the labor market before considering pregnancy and starting a family have been suggested as factors contributing to this variation.

Nybo-Andersen conducted a prospective register study on data from the Civil Registration System in Denmark (Nybo-Andersen *et al.*, 2000) between 1978 and 1992, including 634,272 women and the outcome of 1,221,546 pregnancies. The overall risk of fetal loss, defined as spontaneous abortion, hydatiform mole, ectopic pregnancy or stillbirth, was 13.5%. More than 20% of all pregnancies in 35-year-old women ended in fetal loss, while this occurred in 54.5% of women aged 42 years. About 80% of fetal losses were spontaneous abortions. The risk of spontaneous abortion increased with age: 8.7% in women aged 22 years and 84.1% in women aged 48 and up (Fig. 2) and number of preceding spontaneous abortions (Fig. 3) (Nybo-Andersen *et al.*, 2000).

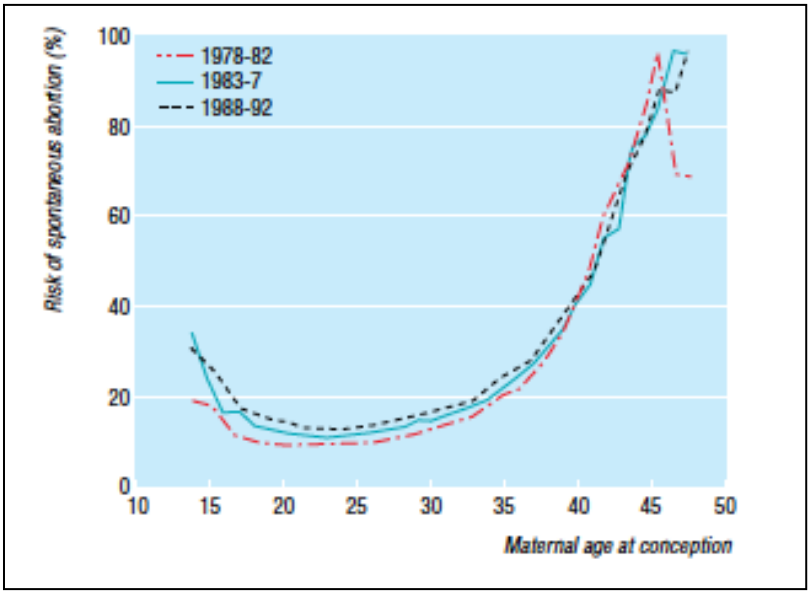

Fig. 2. Risk of spontaneous abortion according to maternal age at conception, stratified according to calendar period (Nybo-Andersen *et al.* Maternal age and fetal loss: population- based register linkage study. BMJ 2000;320:1708). Published with permission from BMJ/Copyright Clearance Center.

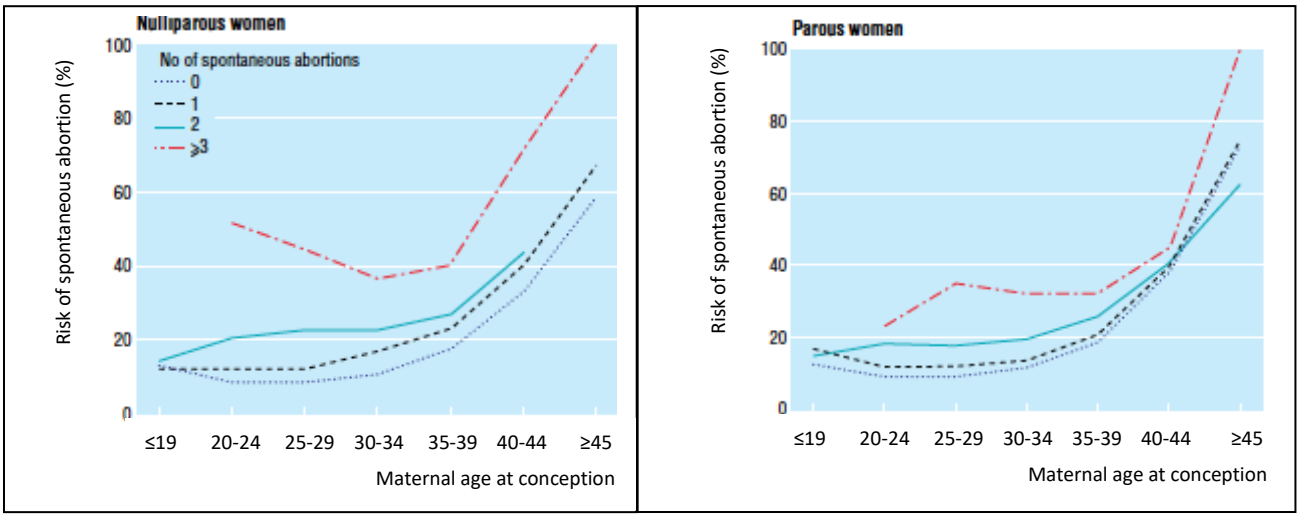

Fig. 3. Risk of spontaneous abortion in nulliparous women (left panel) and parous women (right panel), according to maternal age at conception and number of spontaneous abortions in preceding 10 years (Nybo-Andersen et al. Maternal age and fetal loss: population-based register linkage study BMJ 2000;320:1708). Published with permission from BMJ/Copyright Clearance Center.

The study demonstrated a clear independent effect of maternal age on the risk of spontaneous abortion, in addition to an increased risk of ectopic pregnancy and stillbirth with increasing maternal age (Nybo-Andersen *et al.*, 2000).

Recurrent pregnancy loss

Empirically, recurrent pregnancy loss (RPL) occurs in 1-2% of fertile couples, if defined as at least three consecutive losses, and in 5% if the definition is two or more losses. According to the literature, RPL affects 0.5% to 2.3% of couples if the definition is at least three losses and 5% if it is defined as at least two losses (Stirrat, 1990; Jauniaux *et al.*, 2006; Rasmark Roepke *et al.*, 2017). If only confirmed (by ultrasound and/or histology) pregnancies were included, an incidence of 0.8 to 1.4% has been reported. Adding loss of biochemically diagnosed pregnancies increased the incidence to 2-3% (Larsen *et al.*, 2013).

The definition of RPL is thus debated, ranging from two, not necessarily consecutive, to three consecutive miscarriages. Unfortunately, the terminology in the literature is not consistent. It is difficult to compare results of different studies due to this lack of consensus regarding nomenclature and classification of RPL (Kolte *et al.*, 2015). Most authors seem to have adopted the definitions at least three consecutive pregnancy losses before gestational week (gw) 13 (Clifford *et al.*, 1997; Rai *et al.*, 2000; Wilson *et al.*, 1999) or gw 20 (Jablonowska *et al.*, 1999; Abu-Heija, 2014; Kaur *et al.*, 2016). In recent years, many authors have adopted the definitions at least two losses before gw 13 (D´Uva *et al.*, 2008; Bernardi *et al.*, 2013; Pasquier *et al.*,

2015) or gw 20 (Kaandorp *et al.*, 2010; Vissenberg *et al.*, 2015; van Dijk *et al.*, 2016). Some authors apply gw 24 in their definition instead (Brigham *et al.*, 1999; Clark *et al.*, 2010). Different guidelines are presented in Table 1.

Table 1. Definitions of recurrent pregnancy loss in international guidelines

Guideline	Definition
American College of Obstetricians and Gynecologists (ACOG), 2016	Two or more pregnancy losses before gw 24
American Society for Reproductive Medicine (ASRM), RPL Guidelines 2013	Two or more consecutive miscarriages
European Society of Human Reproduction and Embryology (ESHRE), RPL Guidelines Nov. 2017; Hum Reprod Open 2018	Two or more pregnancy losses before gw 24 (RPL), or before gw 10 (REPL)
Royal College of Obstetricians and Gynecologists (RCOG), Green-top Guideline No.17, 2011	Three or more consecutive pregnancy losses before gw 22
Swedish Society of Obstetrics and Gynecology (SFOG), Guidelines March 2017	Three or more consecutive miscarriages before gw 22
World Health Organization (WHO), 1976	Three or more consecutive miscarriages before gw 20
German (DGGG), Austrian (OEGGG) and Swiss (SGGG) Society of Gynecology and Obstetrics; Geburtshilfe und Frauenheilkunde 2018;78:364-381	Three or more consecutive miscarriages before gw 20

RPL=recurrent pregnancy loss, REPL=recurrent early pregnancy loss, gw=gestational week

RPL is classified as either primary, with no previous live births, or secondary, in which recurrent miscarriages are preceded by live births.

About 50% of RPL are unexplained (Stirrat, 1990; Quenby *et al.*, 1993; Clifford *et al.*, 1997; Yadava *et al.*, 2014; Homer, 2019).

Based on data from the Danish National Patient Register, Knudsen et al. conducted a study on a large unselected Danish population consisting of 300,500 pregnancies, of which about 33,900 were spontaneous abortions (Knudsen *et al.*, 1991). The objective was to investigate the overall risk of a new miscarriage, defined as a spontaneous abortion before gestational week 28, related to the number of previous miscarriages (Table 2). Their results are compared to results reported by other authors during different time periods in Table 3.

To evaluate the live birth rate (LBR) within five years of the RPL investigation, 987 women with at least three consecutive miscarriages were monitored in a prospective register study by Lund. The LBR was 81.3% for women aged 20-24 years, 69.9%

for women aged 30-34 years and 41.7% for women over age 40. Moreover, the number of previous miscarriages had an effect on the LBR, which was 71.9% after three and 50.2% after six miscarriages (Lund *et al.*, 2012) (Fig. 4).

Table 2. Risk of a new miscarriage according to age and number of previous miscarriages

Number of previous abortions	Age <35 years risk (%)	Age ≥35 years risk (%)
0	9.7	24.2
1	14.9	24.0
2	23.8	32.8
3	43.0	54.8
4	53.8	56.3

Modified table from Knudsen et al., 1991 (Eur J Obstet Gynecol Reprod Biol 1991;39:31).

Table 3. Risk of spontaneous abortion expressed in percentage, according to number of previous consecutive spontaneous abortions

Author, Year	Number of previous miscarriages			
	1	2	3	4
Whitehouse, 1930	22	38	73	-
Eastman, 1946	13	37	84	-
Warburton, 1964	24	26	32	-
Knudsen, 1991	16	25	45	54

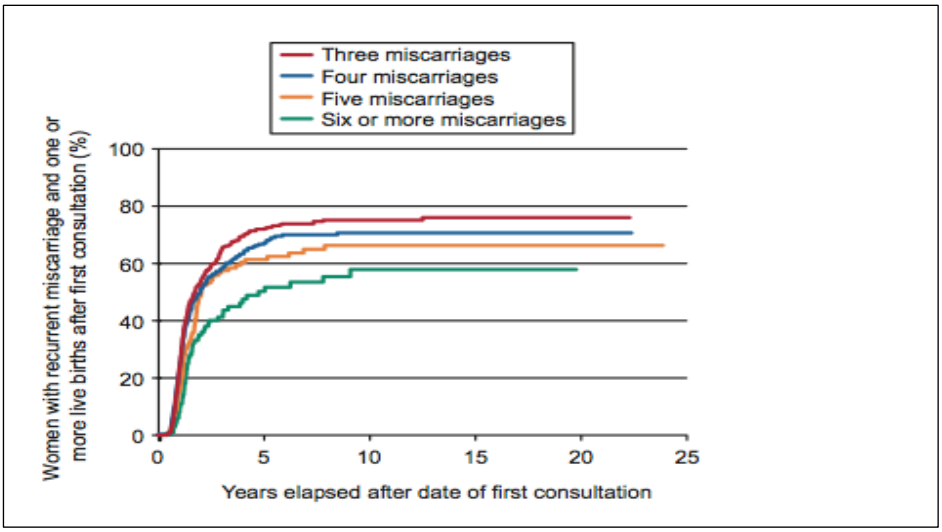

Fig. 4. Chance of a live birth after consultation for recurrent pregnancy loss by number of previous miscarriages (Lund *et al*. Recurrent Miscarriage and Prognosis for Live Birth. Obstet Gynecol 2012;119:37).
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Conceivable and probable causes of recurrent pregnancy loss

Some causes of early RPL are regarded as treatable. There are etiological factors, possible to identify and sometimes to treat, in up to 50% of RPL (Clifford *et al.,* 1997; Bricker *et al.,* 2002; Kutteh *et al.,* 2014). The most important of these are chromosomal aberration, reproductive tract malformation, luteal phase insufficiency, antiphospholipid syndrome, genital infection, hypercoagulable state, iatrogenic factors and lifestyle factors.

Chromosomal aberration

Impaired fertility and increased risk of first-trimester RPL were found when an aberration was carried by either the man or the woman. On investigation of RPL, a chromosomal aberration prevalence of 2-5% was found, mostly consisting of balanced translocations, compared with 0.7% in the general population (Homer 2019; Ford *et al.,* 2009; Rai *et al.,* 2006). Two types of balanced translocation have been reported: reciprocal balanced translocation, in which there is an exchange of two terminal segments from different chromosomes, and Robertsonian translocation, i.e. centric fusion of two acrocentric chromosomes. The most frequent versions are between chromosomes 13 and 14, 14 and 15 and 14 and 21, respectively. In the female, this can present as 45,XX,rob(14;15)(q10;q10). The carrier of a balanced translocation has a normal phenotype and no sign of chromosomal injury but can produce unbalanced gametes and an unbalanced fetus. In general, the translocation prevalence is higher in females than in males (Venkateshwari *et al.,* 2010).

Reproductive tract malformation

Congenital uterine anomalies are found in 1.8% to 37.6% of women with RPL (Rai *et al.,* 2006; Ford *et al.,* 2009; Morley *et al.,* 2013). Intrauterine septum is the most common congenital anomaly, and that which is most closely linked to RPL. Other important uterine anomalies are fibroids and polyps. Depending on the size and location of the anomaly, it can create poorly vascularized endometrium for implantation, and is thus a plausible reason for the miscarriage.

Luteal phase insufficiency with progesterone deficiency

Women with polycystic ovary syndrome (PCOS) are reported to have a RPL prevalence of 8-20% (Bricker *et al.,* 2002; Kutteh *et al.,* 2014; Rai *et al.,* 2000). Insufficient progesterone exposure during early pregnancy results in impaired secretory endometrium, which is important for normal embryo implantation and

growth (Palomba *et al.,* 2015). A positive effect of vaginal progesterone treatment initiated in the luteal phase was demonstrated in a randomized placebo-controlled trial of 700 women with unexplained RPL (Ismail *et al.,* 2018). Highly significant differences in miscarriage rates (12.4% vs. 23.3%) and LBR rates (91.6% vs. 77.4%) were reported. However, a systematic review and meta-analysis, including the above-mentioned randomized controlled trial (RCT), concluded that there were too few studies to draw firm conclusions about the efficacy of progesterone treatment for women with RPL without specifically addressing luteal phase insufficiency (Rasmark Roepke *et al.,* 2018).

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is an autoimmune disease that interferes with coagulation (Yadava *et al.,* 2014). The prevalence of APS in women with RPL is 5-20% (Bricker *et al.,* 2002; Rai *et al.,* 2006; Kutteh *et al.,* 2014; Yadava *et al.,* 2014; Homer, 2019), compared with 2-5% in unselected obstetric patients (Branch *et al.,* 2010). APS is the most important treatable cause of RPL. It is diagnosed by moderate to high blood titers of lupus anticoagulant, anti-cardiolipin antibodies and/or anti-β2-glucoprotein-1 antibodies, at two testing occasions with an interval of at least 12 weeks (Homer *et al.,* 2019). It is a major risk factor underlying adverse pregnancy outcomes, including RPL (Kutteh *et al.,* 2014), and the only thrombophilia with a proven association to RPL (McNamee *et al.,* 2012). Women with the syndrome have a miscarriage rate of 90% in subsequent untreated pregnancies (Rai *et al.,* 2006). The mechanisms by which APS results in RPL are insufficiently understood (Ford *et al.,* 2009) but thrombosis and infarction of the uteroplacental vasculature have been suggested as mediators (Sebire *et al.,* 2002).

Infection

Bacterial vaginosis (BV) in the first trimester is associated with an increased risk of late miscarriage or preterm birth although causality has not been proven (Larsson, *et al.,* 2005). The prevalence of BV in pregnant women is 15.6% to 32.5% (Svare, *et al.,* 2006; Jacobsson, *et al.,* 2002; McGregor, *et al.,* 1995). BV was more frequent in women with a history of late miscarriage (21%) than in women with recurrent pregnancy loss (8%) (Llahi-Camp, *et al.,* 1996), and there was no significant association between BV and a history of RPL (Gözde, *et al.,* 2016). The value of screening and treatment in early pregnancy is under debate.

Inherited thrombophilia
Prospective cohort studies have not shown any significant association between RPL and thrombophilia, including factor V Leiden mutation, prothrombin gene mutation, protein C deficiency, protein S deficiency and antithrombin deficiency.

Iatrogenic and lifestyle factors
Smoking, consumption of alcohol and caffeine intake are not associated with the risk of RPL (Zhang *et al.,* 2010). Couples with RPL should, however, be informed that smoking and alcohol could have a negative impact on their chances of live birth, and cessation is thus recommended nonetheless (ESHRE Nov., 2017). Maternal obesity (BMI $\geq$30 kg/m$^{2)}$ significantly increased the risk of miscarriage in couples with unexplained RPL. There was no increased risk in non-obese women with overweight (Lo *et al.,* 2012). No studies were found regarding the impact of exercise on the chances of live birth in women with RPL. The effect of exercise on the risk of a sporadic first-trimester miscarriage is unclear. In a review by Hegaard (2016) two studies reported that exercise was associated with a lower risk of miscarriage (Xu *et al.,* 2014; Zhang *et al.,* 2011), two others showed the same risk of miscarriage in exercising and non-exercising pregnant women (Maconochie *et al.,* 2007; Clapp, 1989) and one found an increased risk of first-trimester miscarriage in exercising women (Madsen *et al.,* 2007). High-intensity occupational activity has been identified as a risk factor for miscarriage (Schlussel *et al.,* 2008). Treatment for cervical intraepithelial neoplasia (conization) was not associated with early miscarriage and RPL, although it was associated with a significantly increased risk of second-trimester miscarriage (Kyrgiou *et al.,* 2014).

Unexplained recurrent pregnancy loss

As mentioned above, about 50% of RPL are unexplained. This thesis focuses on investigating the respective roles of acetylsalicylic acid (ASA), platelet aggregation and thyroid function.

Platelet aggregation and hemostasis

Impaired platelet aggregation has been suggested to play an important role in placenta-mediated obstetric complications, such as RPL, preeclampsia, intrauterine growth retardation, impaired placental circulation and pregnancy-induced hypertension (Morrison *et al.,* 1985; Dogan Gun *et al.,* 2006; Bujold *et al.,* 2009;

Flood *et al.*, 2010; Sultana *et al.*, 2012; Dempsey *et al.*, 2015; Atallah *et al.*, 2017). The role of platelet aggregation in unexplained recurrent miscarriage is unclear. No longitudinal study of platelet aggregation in women with at least three consecutive unexplained first-trimester RPL has been found. Furthermore, no published trial has demonstrated the effect of low-dose ASA on platelet aggregation during pregnancy in this group.

Platelet aggregation

As platelet reactivity is an important factor in placenta-mediated obstetric complications, it is of great interest to study platelet aggregation during pregnancy, since increased aggregation might be a factor associated with these complications. There are different platelet aggregation receptors on the platelet´s surface (Fig. 5).

Platelet aggregation can be induced by different activators and analyzed with different methods, such as light transmission aggregometry, multiple electrode impedance aggregometry and platelet function analyzer (PFA-100) (Zimmermann *et al.*, 2008; Lordkipanidzé *et al.*, 2007). Born light transmission aggregometry is considered to be the gold standard (Le Quellec *et al.*, 2016).

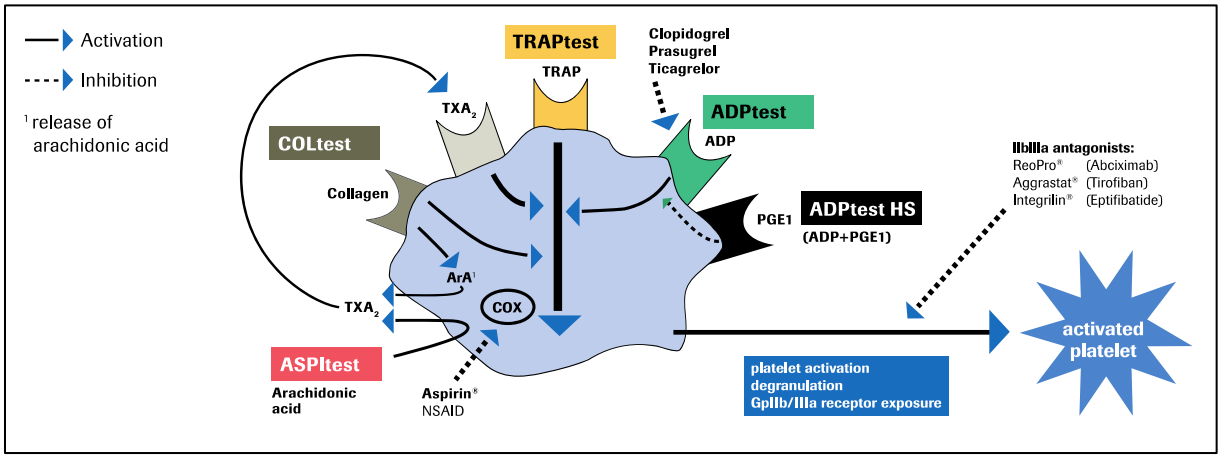

Fig. 5. Receptors on the platelet´s surface through which different activators induce platelet aggregation**.** Published with permission from Roche Diagnostics.

Four activators were used in these studies:

Arachidonic acid (AA) is often used for monitoring ASA and other cyclooxygenase (COX) inhibitors. Platelet aggregation induced by AA is the substrate for COX. COX forms thromboxane A2 (TXA_2) which is a potent platelet agonist and vasoconstrictor.

Collagen (COL) leads to platelet activation via the platelet COL receptor, which generates release of AA, which is converted to TXA_2.
Adenosine diphosphate (ADP) activates platelet aggregation through several receptors on the platelet surface, the most important of which is P2Y12.
Thrombin receptor activating peptide 6 (TRAP) stimulates platelet aggregation via the thrombin receptor. Thrombin is a very potent platelet activator.

Multiple electrode impedance aggregometry (Multiplate analyzing system) was used for platelet aggregation analysis in our studies. This method was chosen because of many advantages: the use of whole blood, no time-consuming centrifugation is needed, results are available within a few minutes, only a very small amount of blood is needed and the method is easy to handle.

The analysis takes place in a single-use test cell containing a dual sensor unit and a Teflon-coated stirring magnet. Upon activation, the platelets adhere to and aggregate on metal sensor wires in the Multiplate test cell. Every Multiplate test cell contains two independent sensor units, each consisting of two silver-coated, highly conductive copper wires with a length of 3.2 mm. When platelets are activated they attach to vascular injuries as well as to artificial surfaces. When they adhere to the sensor wires the electrical resistance between the wires rises (Fig. 6). The increase in impedance is transformed into aggregation units (U) and plotted against time. Two curves are assessed using the two independent sensors in the test cell. The parameters calculated by the software are the mean values of the parameters determined with each curve. The result is shown as the area under the curve (AUC) and presented in units (U) (Fig. 7).

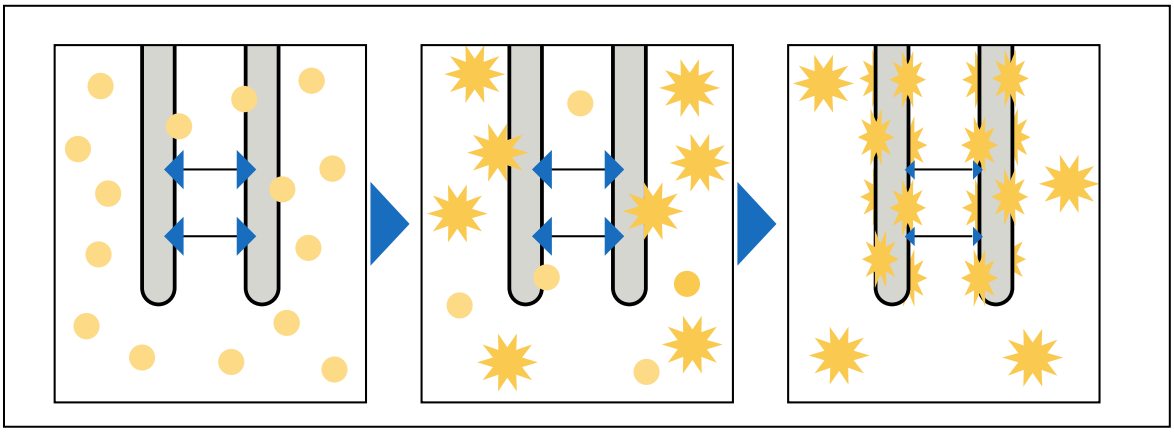

Fig. 6. Activated platelets adhere to the metal sensor wires in the Multiplate test cell and the electrical resistance between the wires rises. The increase of impedance is converted into aggregation units and plotted against time. Published with permission from Roche Diagnostics.

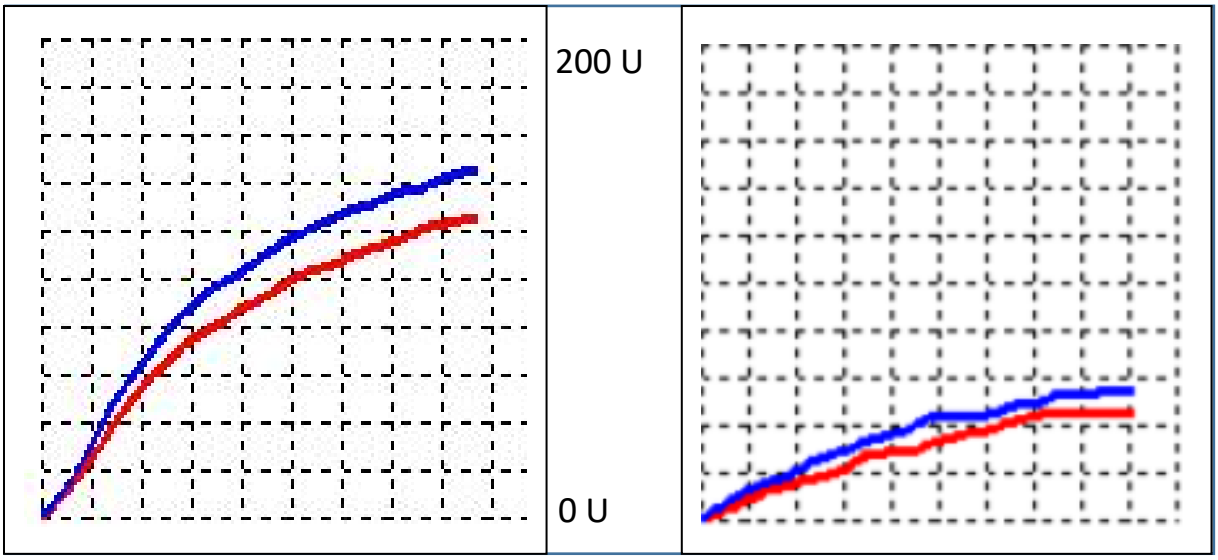

Fig. 7. Area under the curve denotes platelet aggregation after activation by arachidonic acid, without medication (left panel) and under treatment with ASA (right panel). Red and blue denote parallel tests. Published with permission from Roche Diagnostics.
U=aggregation units

Multiplate multiple electrode impedance aggregometry has been used in the evaluation of platelet inhibitors during treatment of non-pregnant individuals (Jambor *et al.*, 2009; Mueller *et al.*, 2009; Paniccia *et al.*, 2009; Le Quelles *et al.*, 2016). When it comes to pregnancy, the method has been used in a small study comparing platelet aggregation during normal pregnancy and preeclampsia, as well as in a larger study concerning non-responsiveness to ASA for prevention of preeclampsia (Can *et al.*, 2010; Navaratnam *et al.*, 2018).

Normal pregnancy

Normal pregnancy leads to changes in the coagulation and fibrinolytic systems, i.e. increased activity of a number of clotting factors, decrease in protein S levels, inhibition of fibrinolysis, and reduced activity of activated protein C, an important anticoagulant (Bremme, 2003).

Platelet reactivity during normal pregnancy remains poorly characterized (Valera *et al.*, 2010). No longitudinal study of platelet aggregation induced by different agonists in healthy women with uncomplicated pregnancy has been found.

Conflicting results have been published regarding platelet aggregation during pregnancy, perhaps due to the use of different agonists and various anticoagulants used in blood sampling.

Norris conducted a small study (n=20) investigating platelet aggregation in healthy primigravidas at gw 12, 20, 28, 32 and 36; during labor; at 1, 24 and 48 hours after delivery; and six weeks postpartum (Norris *et al.,* 1993). Platelet aggregation was induced by AA, COL and ADP and measured by a modification of the method described by Fox (1982). Pregnancy had a significant effect on COL-induced platelet aggregation from gw 20, reaching a maximum during labor but decreasing sharply within 48 hours after delivery. Only a small, non-significant increase remained at six weeks postpartum. Platelet aggregation induced by ADP exhibited a significant increase at gw 36 and a slight increase at six weeks after delivery. Pregnancy had a significant effect on AA-induced platelet aggregation at gw 32 and 36, compared with gw 20, peaking during labor and gradually decreasing until 48 hours after delivery. In summary, the findings of this study were that platelet aggregation induced by AA and COL increased during normal pregnancy. Platelet aggregation was inhibited by ASA (Norris *et al.,* 1993).

Obstetric complications

Platelet aggregation induced by AA and COL has been studied in pregnant women with gestational hypertension, essential hypertension and preeclampsia (Morrison *et al.,* 1985; Norris *et al.,* 1993).

In a study by Whigham, aimed at evaluating platelet aggregation in preeclampsia, two control groups were included, non-pregnant women (n=11) and women with a normal pregnancy (n=9). No significant difference was found between the two control groups in platelet aggregation induced by AA, ADP and COL in a single sample drawn at gw 36. Platelet aggregation was measured photometrically in 0.1 ml of platelet-rich plasma (PRP), using the Bryston aggregometer described by Gordon and Drummond (1974) (Whigham *et al.,* 1978).

Recurrent pregnancy loss

In a study by Flood, platelet aggregation in 30 women with at least three consecutive miscarriages before gw 20, and in 30 healthy women with normal pregnancy and no adverse obstetric history (one or no previous miscarriage), was investigated (Flood *et al.,* 2010). Single samples were drawn in the non-pregnant state at least 12 weeks after the last miscarriage. There was no significant difference between the RPL and control groups in platelet aggregation induced by ADP, COL and TRAP. Women with unexplained RPL had significantly increased platelet aggregation in response to AA (Flood *et al.,* 2010).

A study was conducted by Dempsey in order to evaluate platelet function in very early pregnancy in patients with at least three consecutive unexplained miscarriages. Patients were divided into two cohorts: women with a viable pregnancy at gw 12 and a subsequent live birth (n=30) and women who miscarried before gw 12 (n=9). A single blood test was drawn at gw 4-7. Platelet aggregation was analyzed with a modification of light transmission aggregometry, after induction by AA, ADP, COL and TRAP. In women with unexplained RPL, in whom pregnancy ended in a new miscarriage, there was a significant reduction in platelet aggregation in response to ADP and TRAP, but not to AA or COL (Dempsey *et al.,* 2015).

Thyroid function

General aspects

The effect of thyroid disease and autoimmunity on adverse pregnancy outcome, such as decreased LBR, premature delivery, miscarriage and first-trimester RPL, has been pointed out by many authors (van den Boogaard *et al.*, 2011; Thangaratinam *et al.,* 2011; Mannisto *et al.,* 2013; Dhanwai Kumar *et al.,* 2016), but is also under debate. Hypothyroidism and subclinical hypothyroidism (SCH), defined as elevated serum thyroid stimulating hormone (TSH) with normal serum free thyroxine (free T4) levels (Vissenberg *et al.,* 2012; van Dijk *et al.,* 2016), are regarded as causes of adverse pregnancy outcome (Chan *et al.,* 2015; Faisal *et al.,* 2016). The effect of SCH in women with miscarriage is unclear. Some authors report an increased risk of miscarriage (van den Boogaard *et al.,* 2011; Liu *et al.,* 2014; Maraka *et al.,* 2016; Ma *et al.,* 2016), while some found no association at all (Bernardi *et al.,* 2013; van Dijk *et al.,* 2016; Plowden *et al.,* 2016). Thyroid autoimmunity, detected as thyroid antibodies, mostly thyroglobulin antibodies and thyroid peroxidase antibodies (TPO-ab), has been associated with increased risk of sporadic and recurrent miscarriage (Negro *et al.,* 2006; Vissenberg *et al.,* 2012; Lata *et al.,* 2013; Meena *et al.,* 2016; Wang *et al.,* 2017).

Thyroid peroxidase is an enzyme expressed in the inner membrane of the follicular cell within the thyroid gland (Ruf *et al.,* 2006) and is normally undetectable by the immune system. The production of TPO-ab is regarded as a marker of autoimmune thyroid disease, as the integrity of the follicular cell is disturbed and will put the patient at risk of future thyroid dysfunction (Sadler *et al.,* 2012).

Several pathophysiological theories have been presented to explain the proposed association between thyroid hormone disturbances, with or without TPO-ab, and subfertility and early pregnancy loss, but the exact mechanism is unknown (van den Boogaard *et al.*, 2011). Some theories have focused on endometrial changes, involving T cells and cytotoxic natural killer (NK) cells (Nazarpour *et al.*, 2017), diminished expression of trophoblast endocrine function (Maraka *et al.*, 2017), down-regulation of the thyroid hormone receptors THR-A and THR-B in villous trophoblasts (Ziegelmüller *et al.*, 2015) or endometrial physiology in the implantation process (Aghajanova *et al.*, 2011). More general theories include autoimmunity against the fetal allograft (Prummel *et al.*, 2004) and thyroid hormone deficiency during implantation and in the early stages of embryo development (Coliccia *et al.*, 2014; Vissenberg *et al.*, 2015).

Recurrent pregnancy loss

The prevalence of TPO-ab in euthyroid women of fertile age is 8-14% (Yan *et al.*, 2012; Lin *et al.*, 2014; Vissenberg *et al.*, 2015). In euthyroid women with RPL, defined as at least three unexplained first-trimester miscarriages, positive TPO-ab were found in 10.7% and 23.5%, respectively, in two controlled studies (Ticconi *et al.*, 2011; Yan *et al.*, 2012).

A two- to four-fold increased risk of both sporadic and recurrent miscarriage has been reported by many investigators when TPO-ab are detected in euthyroid women (Negro *et al.*, 2005; Ticconi *et al.*, 2011; Thangaratinam *et al.*, 2011). However, other authors have not found any such association (Esplin *et al.*, 1998; Lata *et al.*, 2013; Plowden *et al.*, 2016).

The ultimate proof of thyroid involvement in RPL would be if thyroxine treatment reduced the risk of a new miscarriage. Again, some studies, both observational and randomized, found an effect of treatment (Negro *et al.*, 2006; Glinoer *et al.*, 2006; Lapoutre *et al.*, 2012; Nazarpour *et al.*, 2017), while other RCTs found no positive effect (Negro *et al.*, 2016; Wang *et al.*, 2017). The TABLET trial was published in 2019, reporting that levothyroxine treatment before conception, in euthyroid women with one or more miscarriages and TPO-ab, neither decreased the miscarriage rate nor increased the LBR. The fact that women with as few as one miscarriage were included, and that no common laboratory threshold for TPO-ab levels was applied to all participating centers, were limitations (Dhillon-Smith *et al.*, 2019).

The European Society of Human Reproduction and Embryology (ESHRE) concluded in their 2017 guideline that the evidence regarding treatment with levothyroxine in women with SCH and RPL is conflicting. Further investigation of this potential treatment effect is thus required. Regarding positive TPO-ab and levothyroxine treatment, the published studies are too small to draw any robust conclusion. There is insufficient evidence to support treatment with levothyroxine in euthyroid women with TPO-ab and RPL outside a clinical trial (ESHRE Guidelines Nov., 2017).

One problem, when comparing studies, is the different definitions of positive TPO-ab, which varies from 10 to 200 kU/l, according to the respective laboratory. Furthermore, the TSH reference values for definition of SCH vary. However, the lower cut-off level is 2.5 mU/l in the majority of the studies.

Suggested treatments for unexplained recurrent pregnancy loss

Different treatments, based on different theories regarding plausible etiologies, have been attempted for unexplained RPL. ASA, low-molecular-weight heparin (LMWH), intravenous immunoglobulin (IVIG), corticosteroids, lipid emulsion and leucocyte immunization have all been tried. There is no conclusive evidence that any currently available specific medical intervention is successful in decreasing the risk of miscarriage in women with unexplained RPL (Dempsey *et al.*, 2015; Rasmark Roepke *et al.*, 2018).

Pharmacological treatment with ASA, LMWH, IVIG and leucocyte immunization have been evaluated in RCTs. For ASA and LMWH summarized in Table 4. The concept of "tender loving care" has also been studied, albeit only in observational studies.

Antiplatelet and anticoagulant therapy

The historical hypothesis behind RPL was that affected women are already in a pro-thrombotic state before pregnancy; indeed, a positive effect of ASA in prevention of placental thromboses has been reported (Jahaninejad, 2014).

Anti-platelet or anticoagulant drugs, or a combination, have been suggested in order to prevent subsequent miscarriage in women with previous early RPL. In addition to

preventing thrombosis, these suggestions have been grounded in different hypotheses concerning disturbed placental circulation, a pro-thrombotic female phenotype or coagulation-cascade activation of the developing trophoblasts (Geer *et al.*, 2010). Pregnancy is a hypercoagulable state with increased levels of pro-coagulant factors (Stirling *et al.*, 1984) and increased number of circulating endothelial microparticles (Carp *et al.*, 2004). It has thus been suggested that miscarriage is a coagulopathy (Rai, 2003). Significantly increased platelet aggregation induced by AA has been reported in women with unexplained RPL (Flood *et al.*, 2010). Furthermore, this theory is supported by the fact that microthrombi and thromboses are common in placental vessels (Rushton, 1988; Dogan-Gun *et al.*, 2006).

Acetylsalicylic acid

A precursor to ASA, found in willow leaves, has been used for health effects for at least 2,400 years. In 1853, the chemist Charles Frédéric Gerhard produced ASA for the first time. During the following fifty years, the chemical structure was established and more efficient production methods were developed. In 1899, Bayer named it aspirin, and its popularity spread around the world. Today, ASA is one of the most widely used medications globally, with about 44,000 tons, or 100 billion pills, consumed each year (Jones *et al.*, 2015). It is included in the World Health Organization´s (WHO) List of Essential Medicines that includes the safest and most effective medicines needed in a health system.

ASA is a non-steroid anti-inflammatory drug with anti-platelet properties that works primarily through inhibition of COX-1 and COX-2. COX-1 regulates the production of prostacyclin and TXA_2. Prostacyclin is a potent vasodilator and inhibitor of platelet aggregation, while TXA_2 is a potent vasoconstrictor and promotes platelet aggregation.

A low dose of ASA has been used during pregnancy mostly to prevent the onset of preeclampsia. Other suggested indications have included prevention of RPL, preterm birth and fetal growth restriction. The majority of RCTs have found no increase in hemorrhagic complications, placental abruption, postpartum hemorrhage or mean blood loss associated with low-dose ASA during pregnancy (ACOG Committee Opinion, 2018).

When it comes to the fetus, no risk of congenital anomalies has been found, although a possible, but very weak, association between ASA use during pregnancy and gastroschisis has been pointed out (Werler *et al.*, 2002; Kozer *et al.*, 2002).

Administration of ASA (60-150 mg) in the third trimester has not been associated with ductal closure (Sibai *et al.*, 1989), persistent pulmonary hypertension, neonatal intracranial hemorrhage or other neonatal hemorrhagic complications (Duley *et al.*, 2007). In a study by Werler, the hypothesis that ASA taken during first-trimester pregnancy would increase the risk of congenital heart defects was tested. No increased risk, compared to that of other structural malformations, was found (Werler *et al.*, 1989). The question of neonatal malformations related to ASA 50-150 mg daily during pregnancy was evaluated, based on Swedish Medical Birth Register data, by the Pharmacological Council in Region Stockholm, Sweden, in 2018. The studied group comprised 6,601 children whose mothers had used ASA during pregnancy to inhibit platelet aggregation. The total rate of malformations was normal, 2.3% compared with the expected 2.1%. An increased rate of cardiac malformations was reported in the ASA group: 63 compared with the expected 46. Twenty-nine had a ventricular septum defect, but 21 had an atrial septum defect, compared with the expected nine. The rate of hydronephrosis was also increased: 19, compared with the expected nine. The authors did not make any recommendation concerning ASA prescription during pregnancy.

When it comes to the effect of anticoagulant treatment in reducing miscarriage rates or improving LBR, studies report diverging results. The interventions in the following briefly presented studies consist of ASA alone, LMWH alone, or ASA and LMWH in combination (Table 4).

Only one RCT was found comparing low-dose ASA with placebo (Tulppala *et al.*, 1997). The other trials compared ASA with other drugs, alone or in combination. There was also a variation in study design.

Based on a meta-analysis (de Jong, 2014) and the results of two subsequent large RCTs (Pasquier *et al.*, 2015; Schleussner *et al.*, 2015), there was no evidence that LMWH alone, ASA alone or the two in combination improved the LBR in women with consecutive unexplained RPL (ESHRE Guideline Nov., 2017).

Table 4. Interventional studies of anti-platelet and anti-coagulant treatments in women with unexplained recurrent pregnancy loss

Author, year	Study design, participants, n	Previous miscarriages	Treatment, participants, n	Outcome	Comments
Pasquier 2015	Multicenter RCT n=256	≥2 consecutive before gw 15	Enoxaparin 40 mg (n=138) / placebo (n=118)	Live birth 66.6% vs. 72.9%	Stratified for no. of previous miscarriages and age
Schleussner 2015	Multicenter RCT n=434	≥2 consecutive before gw 13	Dalteparin 5000 IU (n=220)/ multivitamin pills (n=214)	Ongoing pregnancy at gw 24 86.8% vs. 87.9% Live birth 86.0% vs. 86.7%	
Clark 2010	Multicenter RCT (SPIN) n=294	≥2 consecutive before gw 24	Enoxaparin 40 mg and ASA 75 mg and TLC (n=147)/ TLC alone (n=147)	Miscarriage 22% vs. 20%	
Kaandorp 2010	Multicenter RCT (ALIFE) n=364 (enrolled), n=310 (pregnant)	≥2 before gw 20	ASA 80 mg and fraxiparine 2850 IU (n=103)/ASA 80 mg alone (n=104)/placebo (n=103)	Live birth 54.5%, 50.8% and 57.0%	400 µg folic acid to all participants during pregnancy
Badawy 2008	Single-center RCT n=340	≥3 first-trimester	Enoxaparin 20 mg (n=170)/no treatment (n=170)	Miscarriage 4.1% vs. 8.8% (early) 1.1% vs. 2.3% (late)	500 µg folic acid until gw 13 to all participants
Dolitzky 2006	Multicenter RCT, single-blind n=107	≥3 consecutive first-trimester or ≥2 second-trimester	Enoxaparin 40 mg (n=54)/ASA 100 mg (n=50) Lost to follow-up (n=3)	Live birth 81.5% vs. 84.0%	No placebo group
Tulppala 1997	Single-center RCT n=54		ASA 50 mg (n=27)/placebo (n=27)	Live birth 81.5% vs. 81.5%	
Elmahashi 2014	Single-center RCT, open n=150	≥3 consecutive	Enoxaparin 40 mg and ASA 75 mg (n=75)/ASA 75 mg (n=75)	Miscarriage 29% vs. 47% Live birth 71% vs. 42%	High miscarriage rate, low LBR and preterm delivery rate about 23%
Rai 2000	Single-center observational study n=805	≥3 consecutive before gw 13	ASA 75 mg (n=367)/no treatment (n=438)	Live birth 68.4% vs. 63.5%	

ASA=acetylsalicylic acid, gw=gestational week, LBR=live birth rate, RCT=randomized controlled trial; TLC=tender loving care

Immunoglobulin

Jablonowska conducted a double-blind RCT (n=41) to evaluate the effect of treatment with IVIG (20g/400ml Gammonativ, n=22), compared with placebo (400ml saline, n=19), in order to prevent new miscarriages in a group of women with three or more consecutive unexplained miscarriages before gw 20. All previous pregnancies were documented by ultrasound or histology. The LBR was 77% for IVIG and 79% for placebo (Jablonowska *et al.,* 1999). Another five RCTs were evaluated by Rasmark Roepke in a systematic review. IVIG was compared with placebo in four studies, but the placebo drugs were different (three albumin and one saline). IVIG was compared with ASA+LMWH in the fifth study. No significant effect on LBR was shown (Rasmark Roepke *et al.,* 2018).

Leucocyte immunization

Gatenby published a double-blind RCT comparing immunization with 400 million paternal lymphocytes and immunization with 400 million autologous lymphocytes, in women with at least three consecutive confirmed miscarriages before gw 20. Treatment started before pregnancy and was repeated every 12 weeks until positive pregnancy test. LBRs were 68% and 47% for paternal and autologous lymphocyte immunization, respectively (p=0.1) (Gatenby *et al.,* 1993). In the systematic review by Rasmark Roepke, another four RCTs were evaluated. There were design differences between all trials in terms of start and of testing time-points. The trials were old (published between 1985 and 1994) the results were not trustworthy due to very low certainty of evidence based on a high risk of bias and severe imprecision (Rasmark Roepke *et al.*, 2018).

Psychological support/tender loving care

As early as in in 1954, Javert wrote about the need for psychotherapy for patients with RPL. A therapeutic regimen, including a pre-conceptional consultation for the patient and her partner, and examination for detection of specific medical, gynecological and psychological factors, was offered. Frequent prenatal visits and unlimited phone calls in order to relieve anxiety were also included. “It has been our practice since 1940 to establish a pre-conceptional interview with the patient. The patient was allowed to verbalize her past experiences with the miscarriages, doctors, hospitals and her attitude toward another pregnancy. Then she was referred, with her partner, to the psychosomatic consultant for more detailed review” (Javert, 1954). When these patients had had a successful pregnancy, their subsequent pregnancies were uncomplicated. According to this author, repeat miscarriage is in itself a stress mechanism which may lead to rejection of the next pregnancy (Javert, 1954).

Musters did something very similar: a questionnaire study of 174 women with at least two previous miscarriages. The women preferred a plan for the first trimester in a subsequent pregnancy that involved one single doctor and repeated ultrasounds, as well being shown understanding and being listened to. Furthermore, they wanted healthcare staff to be aware of their obstetric history and show respect for them and their miscarriages (Musters *et al.,* 2013).

Couples with recurrent miscarriage have higher levels of depression, anxiety and feelings of guilt, compared with the general population. Therefore, patients may benefit from regular contact with a dedicated miscarriage clinic (Morley *et al.,* 2013; Fertl *et al.,* 2009; Homer, 2019; Rai *et al.,* 2006). When a follow-up clinic was established, 79% of women with previous recurrent miscarriage attended, and they all found the contact helpful (Lee *et al.,* 1996).

A third of the women seeing psychiatric specialists due to RPL are clinically depressed, and about 20% have anxiety symptoms similar to those in general psychiatric outpatient populations. It is evident that anxiety symptoms may occur in response to miscarriage (Lee *et al.,* 1996).

In 1962, Tupper and Weil suggested that psychotherapy should be included as part of the treatment of RPL. A group of women with three previous consecutive miscarriages, given psychotherapy in the subsequent pregnancy, had a LBR of 84%, compared to 26% in the control group (Tupper *et al.,* 1962). James also concluded that psychotherapy had a positive effect in reducing RPL, as the LBR was 80% in the therapy group, compared with 42% in the group not given therapy (James *et al.,* 1963). However, the small study populations and a major risk of selection bias, constitute limitations in these three old observational studies. The results are thus not applicable.

Supportive care as the sole treatment has been described in several case series, reporting 70-86% LBR in subsequent pregnancies in women with unexplained RPL (Badawy *et al.,* 2008; Fawzy *et al.,* 2008; Brigham *et al.,* 1999; Bricker *et al.,* 2002).

In a study by Stray-Pedersen of couples with three or more unexplained consecutive miscarriages, the LBR was 86% after special psychological support, compared with 33% after routine prenatal care (Stray-Pedersen *et al.,* 1984). The same levels, 86% and 33%, respectively, were found by Liddell, although the fact that the control group included only nine women was a limitation (Liddell *et al.,* 1991). These authors

concluded that psychological support alone may lead to a significant improvement in the outcome of pregnancy.

In summary, the studies on supportive care alone are all observational and suffer from major methodological drawbacks. Rai suggests that patients with RPL should be referred to placebo-controlled trials, in order to elucidate the value, shown in previous studies, of psychological support in improving pregnancy outcome (Rai *et al.*, 2006). This suggestion is wise, since all individuals participating in prospective trials will be thoroughly monitored and taken care of, which will likely be beneficial for women with RPL.

Aims

Aims of the thesis;

- to investigate whether a low dose of ASA, 75 mg daily, during pregnancy can prevent a new miscarriage in women with at least three consecutive unexplained first-trimester miscarriages in the current relationship (Paper I)

- to study platelet aggregation in healthy women during normal pregnancy longitudinally by multiple electrode impedance aggregometry and compare it with platelet aggregation in the non-pregnant state (Paper II)

- to compare platelet aggregation during pregnancy in women with and without RPL for unknown reasons (Paper III)

- to study the effect of ASA on platelet aggregation in women with RPL during pregnancy longitudinally and in comparison with healthy pregnant women (Paper III)

- to investigate the effect of TPO-ab and of TSH levels in the upper normal reference range on the risk of first-trimester miscarriage in women with a history of early RPL (Paper IV)

Study populations

The main study population consisted of women with RPL. One RCT (Paper I) and two post hoc studies (Papers III and IV) were derived from this screened population. A population of healthy women with normal pregnancy constituted the study population in Paper II and contributed a comparison group in Paper III. The formation of the final study populations in the four papers is presented in two flow charts (Figures 8 and 9). The specific methodology related to each paper is presented below.

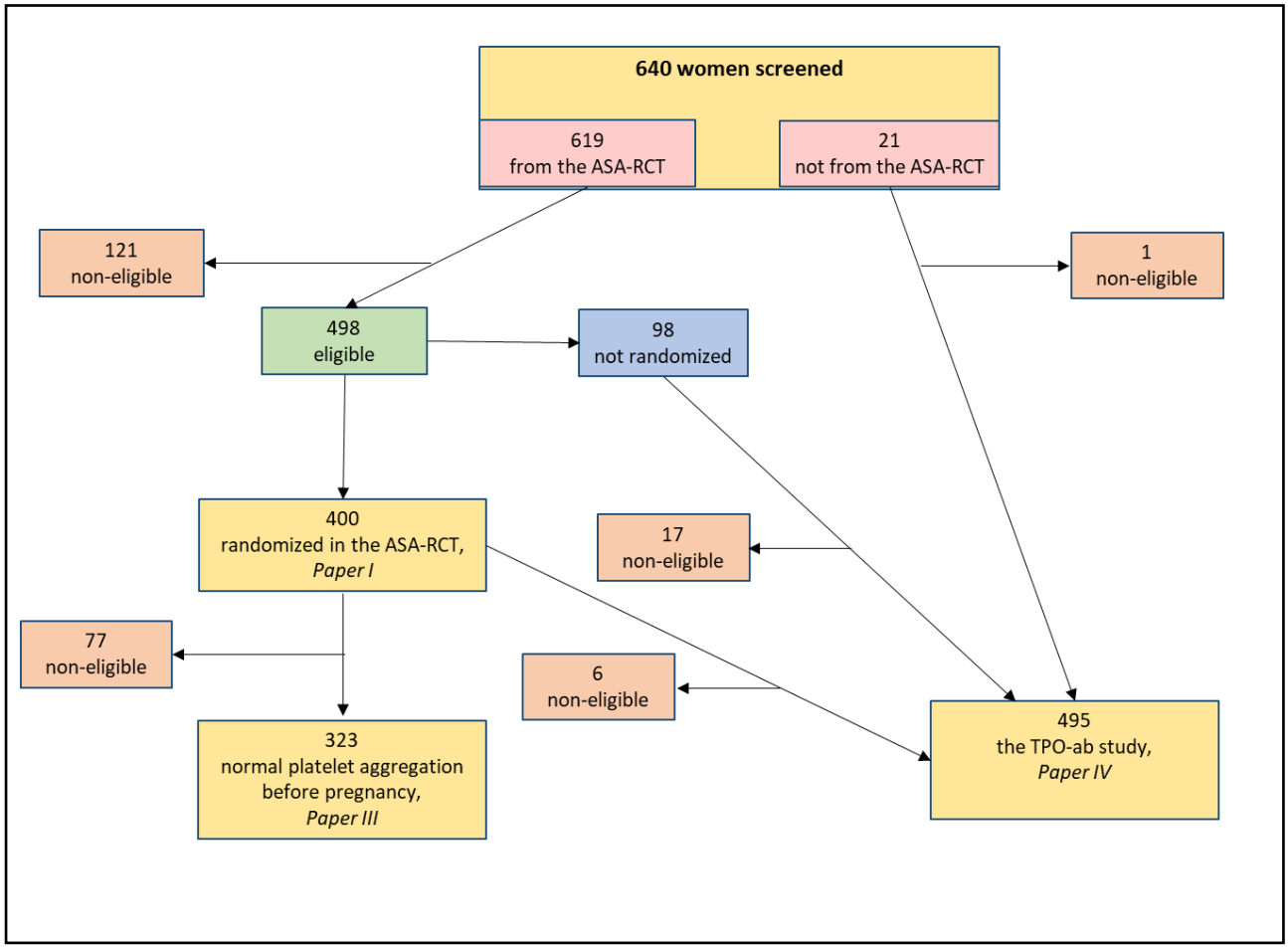

Fig. 8. Flow chart of the study populations of women with recurrent early pregnancy loss. Abbreviation: ASA-RCT=randomized controlled trial on preventive effect of acetylsalicylic acid for recurrent miscarriage; TPO-ab=thyroid peroxidase antibodies

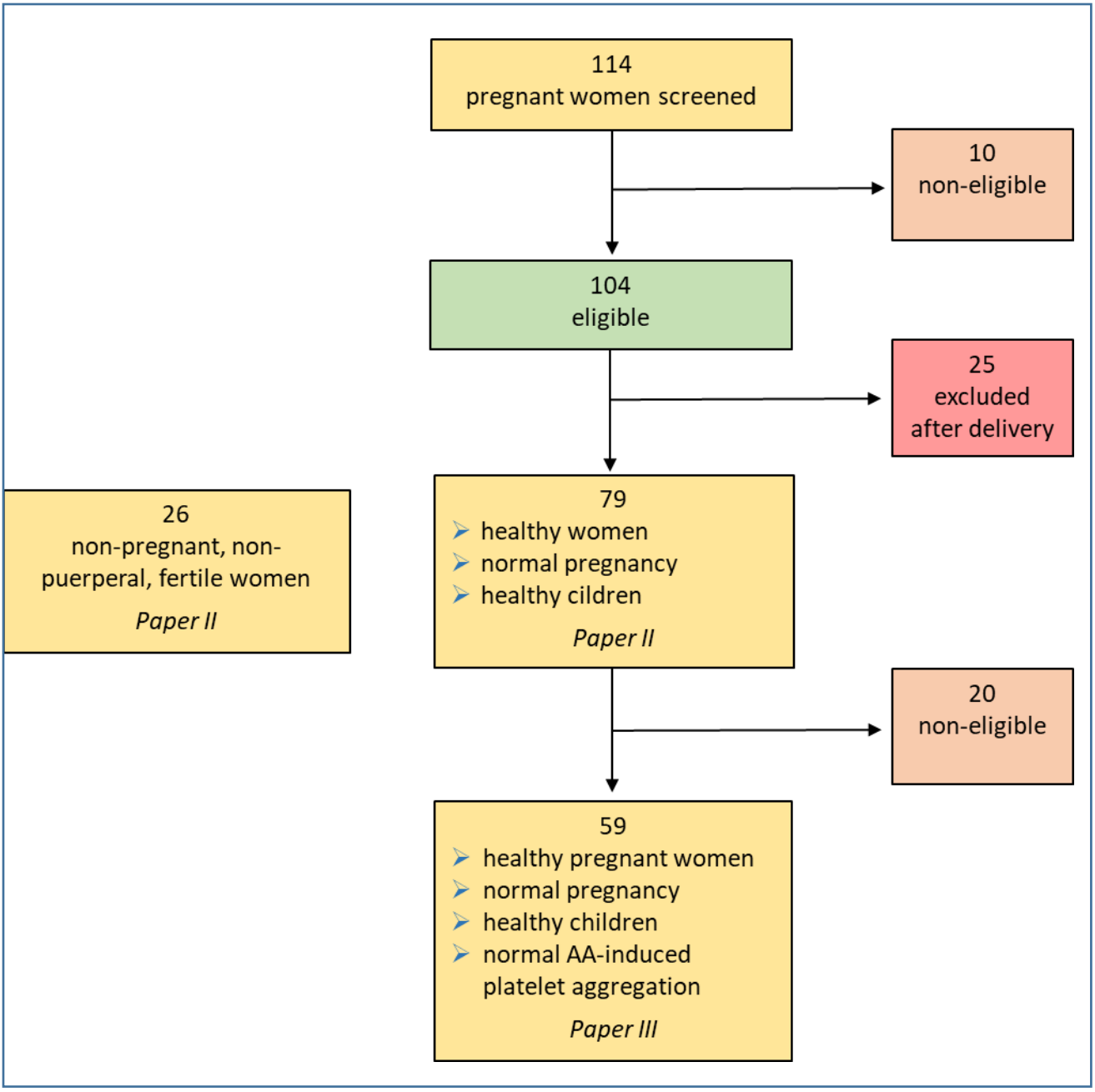

Fig. 9. Flow chart of the study populations of healthy women with normal pregnancy.
Abbreviation: AA=arachidonic acid

Study populations of women with recurrent pregnancy loss

Randomized pregnant women with unexplained first-trimester recurrent pregnancy loss

Paper I: Acetylsalicylic acid does not prevent first-trimester unexplained recurrent pregnancy loss: a randomized controlled trial

After promising results in a pilot case series, the planning of an RCT, hereafter referred to as the ASA-RCT, to test the hypothesis that low-dose ASA would prevent miscarriage, started in the spring of 2007. Later the same year, the principal investigator (PI) visited gynecology departments and clinics in order to inform gynecologists and general practitioners about the trial. Figure 10 describes how women came to screening for the ASA-RCT.

The primary geographical inclusion area was chosen in response to the question "How long would a couple accept to travel for examination, monitoring and taking part in a study of recurrent miscarriage?" Information about the RCT was also spread by newspapers and radio. Many couples, even some living very far away, were referred by their local doctors or contacted the PI themselves, wanting to participate in the study. Most couples came from Region Västra Götaland and Region Halland (n=534) in western Sweden, while some came from other parts of Sweden (n=85).

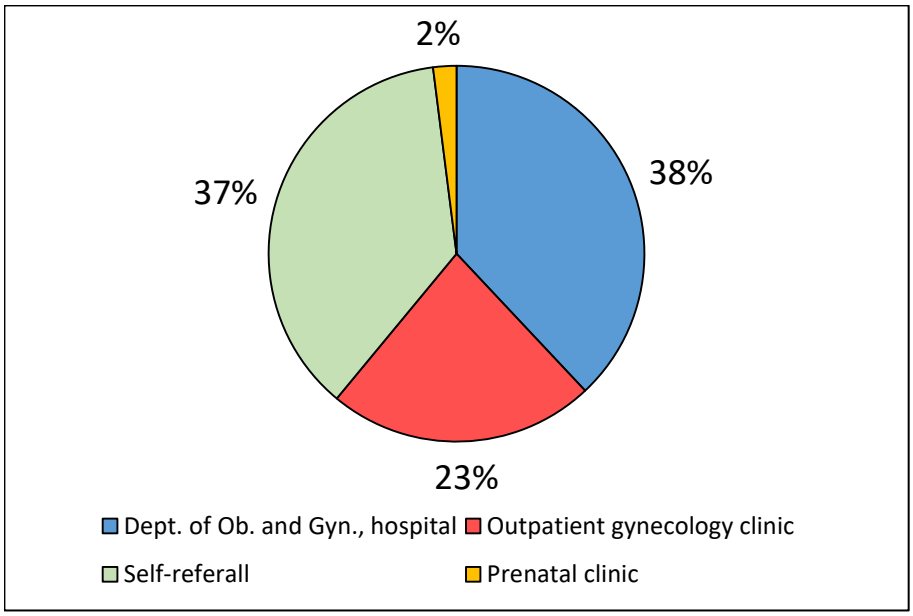

Fig 10. Distribution of the 619 screened women in the ASA-RCT, according to type of referral unit. ASA-RCT=randomized controlled trial on preventive effect of acetylsalicylic acid for recurrent miscarriage

Screening phase
All women had a primary interview by phone with the PI. Medical and obstetrical history and heredity were discussed, and inclusion/exclusion criteria were checked. If nothing abnormal emerged during the interview, the couple was booked for an appointment with the PI for examination and further information about the study.

The workup included general and gynecological examination, transvaginal ultrasound, hydrosonography of the uterine cavity and blood pressure measurement. Laboratory blood tests included hemoglobin (Hb); leucocyte count (LC); platelet count (PC); activated partial thromboplastin time (APTT); prothrombin complex (INR) (PC(INR)); liver enzymes; kidney function; plasma glucose; thyroid function; chromosome analysis; and coagulation tests, including analysis for thrombophilia, antiphospholipid syndrome and lupus anticoagulant.

The workup for the male partner included lifestyle, heredity and chromosome analysis.

Eligiblity
Inclusion criteria were a history of at least three consecutive first-trimester (maximum gestational length 12 weeks and 6 days) miscarriages in the current relationship, no abnormalities in the workup, body mass index (BMI) <35 kg/m², age <40 years at the start of the investigation and no allergy to ASA. Women with ongoing conflicting medical treatment, conception by in vitro fertilization (IVF) on RPL indication or previous participation in the study were excluded. All clinical data were collected at the Department of Obstetrics and Gynecology at Södra Älvsborg Hospital, Borås, Sweden.

Written consent was provided by both partners. All screened couples were seen by the PI, who was responsible for assessing eligibility. The eligible women were provided with the number of the "study cellphone". After the woman had conceived, she reported the positive pregnancy test (human chorionic gonadotropin (hCG) in urine) to the PI. A total of 498 women were eligible in the ASA-RCT (Fig. 8).

Randomization
When a woman reported a positive pregnancy test to the PI, she was booked for a visit to confirm a viable pregnancy. If a fetal heartbeat was detected, a form was filled in and faxed to the pharmacy at Södra Älvsborg Hospital, Borås, where it was completed with a code number in consecutive order.

The randomization was stratified for age (<33/≥33 years), in blocks of 20 and with a 1:1 ratio, for ASA and placebo, in order to ensure similar age-related fertility in both groups. The cut-off age 33 was based on the median in the pilot case series of women with RPL. Both the randomization list and the key code were computer-generated by Apoteket Produktion & Laboratorier (APL), Stockholm, Sweden. The study parcels were produced, labeled and numbered by APL and sent to the pharmacy at Södra Älvsborg Hospital. The parcel was collected by the patient, either at that pharmacy or at another hospital pharmacy. If ordered before 4 AM, the parcel reached the other hospital the next day.

The study drug
In November 2007, APL started production of treatment capsules. The substrate ASA was supplied by Pfizer AB (Sollentuna, Sweden).

The ASA capsules consisted of 75 mg ASA, cellulosum microcristallinum PH 102, Ph Eur 260 mg and capsulae gelatinosae size 0, caramel opaque. The product weight was checked in 5/150 produced capsules, with an accepted difference of ±5%. The capsules were also checked for identity according to the ITAKA-system (Identity of TAblets and KApslar (capsules) and tested positive for ASA, quantitative titration of ASA, decomposition (≤30 minutes according to Ph Eur) and microbiology (according to Ph Eur Category 3). Appearance, quantitative titration of ASA and decomposition were re-checked after 18 and 36 months.

The placebo capsules consisted of cellulosum microcristallinum PH 102, Ph Eur 300 mg and capsulae gelatinosae size 0, caramel opaque. The production process was checked for 10 capsules at every 15 minutes. The accepted difference in capsule weight was ±5%. The placebo capsules were also checked for appearance and identity according to ITAKA and tested for absence of ASA, decomposition and microbiology. Appearance and decomposition were re-checked after 18 and 36 months.

Every study parcel contained three jars, each containing 70 ASA or placebo capsules. The parcels and capsules were identical in appearance. They were produced in two series to ensure drug efficacy over time.

Follow-up during and after pregnancy
Patients were checked at clinical visits at gw 9, 13, 30 and 36 and by telephone call at gw 20. The visits included ordinary prenatal monitoring, assessment of fetal

growth, registration of adverse events including vaginal bleeding and inquiring about side effects of treatment. The study drug was discontinued at gw 36. The PI made a final summary, focusing on the final weeks of pregnancy, delivery and early postpartum period, by phone three weeks after delivery.

Randomized pregnant women with unexplained recurrent early pregnancy loss and normal platelet aggregation before pregnancy

Paper III: Platelet aggregation during pregnancy in women with previous recurrent first trimester fetal loss, with and without acetylsalicylic acid treatment

A post hoc study was performed on a population of women with normal platelet aggregation, induced by AA and analyzed with multiple electrode impedance aggregometry, the ASPI test, in the non-pregnant state. Two groups were derived from the study population of women with RPL. Inclusion criteria were randomization in the ASA-RCT and normal AA-induced platelet aggregation before pregnancy. Among ASA-treated women, 176 had an ASPI test before pregnancy, of which 160 yielded results within reference limits. The corresponding numbers for women treated with placebo were 177 and 163. Table 5 presents the time-points for blood sampling and laboratory analyses.

Table 5. Blood sampling occasions for the laboratory analyses during pregnancy and in the non-pregnant state, Papers I, II and III

Laboratory methods	**Non-pregnant***	**Gestational week**							
		13	8-15	20	20-22	30	30-33	36	37-40
MP AA	I/ II/III	I/III	II/III		II/III	I/III	II/III	I/III	II/III
MP ADP	II/III		II/III		II/III		II/III		II/III
MP collagen	II/III		II/III		II/III		II/III		II/III
MP TRAP	II/III		II/III		II/III		II/III		II/III
Platelet count	I/II/III		II/III	I/III	II/III		II/III		II/III
Hemoglobin	I/III			I/III					
PC(INR)	I/III			I/III					
APTT	I/III			I/III					

Roman numbers refer to the respective papers. AA=arachidonic acid, ADP=adenosine diphosphate, MP=Multiplate, TRAP=thrombin receptor-activating peptide Trap-6, PC(INR)=prothrombin complex (international normalized ratio), APTT (activated partial thromboplastin time)
*Blood samples for Paper II and for controls in Paper III in the non-pregnant state were collected three months post-partum.

A third group was derived from the study population of healthy women with normal pregnancy and is presented below under that heading.

Pregnant women (including biochemical pregnancies) with unexplained recurrent early pregnancy loss, normal TSH and analyzed TPO antibodies

Paper IV: Pre-conceptional thyroid peroxidase antibody positivity in women with recurrent pregnancy losses may be a risk factor for another miscarriage

A cohort was retrieved from the study population of pregnant women with RPL. This cohort included women that were either screened in the ASA-RCT, or outside the trial using the same eligibility criteria (Fig. 8). Conception (at least a positive pregnancy test) and blood sampling for thyroid hormone analyses were additional inclusion criteria. A very early loss of a pregnancy confirmed only with a pregnancy test was defined as a biochemical pregnancy. As part of the screening phase before pregnancy, blood samples were taken for analysis of TSH, free thyroxine (T4) and TPO-ab.

Missing thyroid tests, TSH >4 mU/l and non-retrievable pregnancy outcome were exclusion criteria. In total 495 women were included (Fig 8).

Study populations of healthy women with normal pregnancy

Healthy women with normal pregnancy

Paper II: Platelet aggregation in healthy women during normal pregnancy - a longitudinal study

Women in early pregnancy were invited to take part in a study of platelet aggregation with multiple electrode impedance aggregometry using the Multiplate analyzing system. During the period October 2011 to December 2012, women at gw 6-10 were registered at their first visit to the primary care Prenatal Clinic in Borås, Sweden. They received oral and written information during their first visit to the midwife. If the woman was interested in participation, she was contacted by phone and interviewed by the PI about her medical and obstetric history as well as the inclusion and exclusion criteria. If nothing abnormal emerged, written consent was provided and the woman was included.

The inclusion criteria were: healthy woman, age <40 years, none or one previous miscarriage, willing to come in four times during pregnancy and once three months postpartum for blood sampling.

Exclusion criteria were: previous pregnancy complicated by preeclampsia/ eclampsia, hypertension, thyroid disease, diabetes mellitus or other chronic illness, BMI <35 kg/m², current smoking, family history of thromboembolic or bleeding disease and current medical treatment affecting hemostasis, including platelet function.

Since no blood sampling was possible before pregnancy in this group, the post-partum state was used to assess platelet aggregation in the non-pregnant state.

A total of 114 women accepted participation, of whom 104 were eligible and 79 were finally included in the study (Fig. 9). Blood samples for analysis of PC, and platelet aggregation by the four activators AA, COL, ADP and TRAP were collected at gw 8-15, 20-22, 30-33 and 37-40 and three months postpartum (Table 5).

Healthy women with normal pregnancy and normal platelet aggregation in the non-pregnant state

Paper III: Platelet aggregation during pregnancy in women with previous recurrent first trimester fetal loss, with and without acetylsalicylic acid treatment

This study population derived from Study II served as a control group in Study III, in order to compare platelet aggregation during pregnancy in women with RPL with that in a control group of healthy women with normal pregnancy. Inclusion criteria were AA-induced platelet aggregation within reference limits in the non-pregnant state. This control group comprised 59 women.

Laboratory methods

All participants

Blood sampling from the brachial vein was performed between 7 AM and 3 PM at the Department of Clinical Chemistry, Södra Älvsborg Hospital, Borås. Most blood samples were centrifuged at 20°C for 10 minutes at 2,000 g within two hours and stored at -20°C if not analyzed the same day. Platelet aggregation analyses were

performed on fresh whole blood. All laboratory analyses were analyzed at the same department, except coagulation and immunological samples, which were sent to the Departments of Coagulation and Immunology at Sahlgrenska University Hospital, Gothenburg, Sweden, for analysis. Most biochemical data were collected and stored at the Department of Clinical Chemistry, Södra Älvsborg Hospital, Borås. Results of blood tests analyzed at Sahlgrenska University Hospital were stored with the medical records at the Department of Obstetrics and Gynecology, Södra Älvsborg Hospital, Borås.

Laboratory analyses during the screening phase of the ASA-RCT
The routine tests Hb, LC, PC, liver enzymes, kidney function, thyroid function and glucose were analyzed according to ordinary routine methods.

Coagulation tests, including screening for thrombophilia, antiphospholipid-antibodies, and chromosome analyses were performed with ordinary routine methods.

Platelet aggregation was determined with the Multiplate analyzer method (Roche Diagnostics International Ltd, Rotkreutz, Switzerland). The blood samples were collected in two 3-ml tubes containing hirudin 25 µg/ml and were handled within 30–180 minutes, according to the manufacturer's instructions. The results were not reported by the laboratory until after the study closed. Multiple electrode impedance aggregometry (Multiplate analyzer), using the ASPI test with AA as the initiator, was used as indicator of ASA effect.

An ASPI value <30 aggregation units (U) during ASA treatment was used as the cut-off for normal ASA response, in line with the manufacturer's instructions (Calatzis *et al.,* 2004; Lenk *et al.,* 2013; Toth *et al.,* 2006). Non-responders to ASA were defined as women with ASPI ≥30 U at every test occasion during ASA treatment. Changes in AA-induced aggregation corresponding to a 30%, 40% and 50% reduction from the non-pregnant state were also determined.

Due to a technical delay, the Multiplate analyzing system was not available when the first study patients in the ASA-RCT had their initial visit with the PI. Therefore, 47 women did not have an ASPI test before pregnancy.

TSH, free T4 and TPO-ab were all determined by routine methods. The samples were collected in BD Vacutainer Lithium Heparin PST II, 3 ml. In this study, the upper

normal range for TSH was defined as 2.5 <TSH ≤4.0 mU/l. Positive TPO-ab was defined as ≥10 kU/l, corresponding to the laboratory's cut-off value.

Blood sampling periods and laboratory analyses in the studies
Platelet aggregation with AA as activator, PC, Hb, activated partial thromboplastin time (APTT) and prothrombin complex (international normalized ratio) (PC(INR)) were determined during pregnancy and post-partum. Time-points for blood sampling are shown in Table 5.

In addition, ADP, COL and TRAP were used as activators of platelet aggregation during pregnancy and postpartum in Paper II (see Table 5).

Statistical analyses

Sample size calculations

Paper I
To detect a 15% difference in LBR, given a reference level of 50%, α=0.05, β=0.20 and a two-sided Fisher´s exact test, 170 patients were required in each group. A total of 400 patients was the final target for recruitment, allowing for a 10% loss to follow-up.

Paper II
The number of included women was based on an estimation of how many could be included within a reasonable time period. There were no previously published data on Multiplate analysis in a pregnant population on which to base a formal power calculation.

Paper III
The sample sizes in the different groups were determined by availability of ASPI test results in the non-pregnant state among the eligible subjects in the two original studies (Blomqvist *et al.*, 2018; Blomqvist *et al.*, 2019).

Paper IV
The sample size was determined by the availability of complete thyroid laboratory analysis results and pregnancy outcomes in the study population of pregnant women with RPL.

Statistical methods

A summary of the applied analyses in the four papers is presented in Table 6.

Table 6. Statistical methods applied in the papers

Statistical methods	Paper I	Paper II	Paper III	Paper IV
Descriptive analyses. Mean, SD, median, minimum; maximum or Q1; Q2 for continuous variables. Numbers and % for categorical variables	X	X	X	X
For comparisons between two groups:				
Fisher´s exact test for dichotomous variables	X	X		X
Mann-Whitney U test for continuous variables that are not normally distributed	X	X	X	X
Two-sample t-test for normally distributed continuous variables		X		
Mantel-Haenszel Chi-square test for ordered categorical variables				X
For other analyses:				
Wilcoxon signed rank test for analysis of change within groups	X	X	X	
Linear regression, followed by Wilcoxon Signed Rank Test to analyze whether beta coefficients (slopes) differ from zero over subjects		X		
Spearman´s correlation coefficient for cross-sectional correlation analysis		X		X
Generalized linear model with binomial distribution for the dependent variable, and log-link function to estimate adjusted risk ratio with 95% confidence interval				X
Stepwise logistic regression for selection of risk factors				X

Descriptive statistics

Categorical variables are presented with number and percentages. Continuous variables are presented with mean, standard deviation (SD), median, minimum/ maximum or quartiles (Q1, Q3).

Comparisons between groups

The Fisher´s exact test was used for dichotomous variables, the Mantel-Haenszel Chi-square test for ordered categorical variables, the Mann-Whitney U test was used for non-normally distributed continuous variables and the two-sample t-test for

normally distributed variables. Risk differences and risk ratios with 95% confidence intervals (CI) were calculated. Mean differences with 95% CI for continuous variables were bootstrapped with 10,000 re-samplings.

Comparisons within groups
The Wilcoxon signed rank test was used for analysis of changes over time for continuous variables within-groups.

Correlation and regression analyses
Spearman´s correlation coefficient was used for all cross-sectional correlation analyses. Linear regression analysis was used for calculation of individual beta coefficients (slopes) for trend over time. These slopes was analyzed with the Wilcoxon signed rank test, to assess whether these slopes differed from zero over subjects.

Generalized linear models with binomial distribution for the dependent variable, and log-link function were used to estimate unadjusted and adjusted risk ratio (RR) with 95% confidence interval (CI). RR with exact 95% CI for the estimated proportions were calculated. Stepwise logistic regression was applied to found important risk factors presented with odds ratio (OR) and 95% CI.

All significance tests were two-sided and conducted at the 5% significance level. All analyses were performed using the SAS System version 9 (SAS Institute, Cary, NC, USA).

Regulatory approvals

Permission for drug testing with ASA 75 mg was given by the Swedish Medical Products Agency on Oct. 2, 2007 (Eudra Clinical Trial Application number (Eu-nr) 2007-003839-24, number 151:2007/48666).

Ethical considerations

Ethical considerations must always be evaluated when planning a trial:

- Will randomization, intervention, control treatment and/or follow-up intervene with the patient's integrity or autonomy?
- Will the trial cause displacement effects?
- Is the trial very costly and will any potential benefit be cost-effective?

During the planning of the ASA-RCT, we found that women with RPL had been treated with low-dose ASA despite unproven benefit, which we considered to be unethical and a reason to conduct the trial. No safety issues with low-dose ASA were evident from the literature. After the launch of the trial, patients actively sought participation and they did not consider their integrity or autonomy to be threatened by the study protocol. No displacement effects were anticipated by antenatal clinic staff and the RPL population could be referred to the PI and the department of gynecology where the study was conducted.

Since ASA is very cheap, any treatment regimen would be cost-effective if it were proven to be effective. Now, after the trial has been conducted and ASA proven ineffective when initiated after verification of a viable fetus at gestational week 6-7, it would instead raise ethical concerns to administer ASA treatment under these conditions.

In the other study population, participants were healthy women with normal pregnancies who chose to participate and contribute their free time.

All studies were conducted in accordance with the Declaration of Helsinki. The Regional Ethical Review Board at the University of Gothenburg approved the ASA-RCT (Papers I, III, IV) on July 18, 2007 (number 234-07) and the study of platelet aggregation in healthy pregnant women (Paper II) on July 21, 2010 (number 366-10).

Results

Of the 619 screened women, 51 were excluded after the telephone interview but before the medical investigation, due to age ≥40 years (n=34) or incorrect miscarriage history (n=17). The reasons for exclusion after medical investigation were chromosomal aberration (n=15), thyroid disease (n=11), abnormal laboratory test results (n=8), uterine anomaly (n=2), BMI >35 kg/m^2 (n=3) and dropped out before inclusion (n=31). A total of 121 women were excluded. If the results of the investigation were normal, the woman was included in the study after providing written consent. Randomization occurred after a positive pregnancy test and detection of fetal heartbeat by vaginal ultrasound.

Of the 498 included women, 98 were not randomized due to failure to become pregnant after investigation (n=34), no longer planning pregnancy (n=8), miscarriage before randomization (n=7), adoption (n=8), divorce (n=7), dropped out for personal reasons or other reason (n=34). Four hundred women were finally randomized to treatment with ASA or placebo (Fig. 8).

Paper I

The mean age in the study group was 32.3 (SD 4.3) years and the mean BMI was 23.9 (SD 3.8) kg/m$^{2.}$ The women had had three to seven consecutive miscarriages. Fifty-two percent of the couples had primary RPL, i. e. no children born before the miscarriages. The obstetric and medical history and demographic variables were evenly distributed between the randomization groups except smoking, which was less prevalent in the placebo group. Ninety-eight percent were singleton pregnancies, and the remaining 2% were twin pregnancies, four in each randomization group.

The randomization rate was fairly constant after the first year (Fig. 11). The first woman was randomized at the very beginning of March 2008 and the last at the end of October 2015.

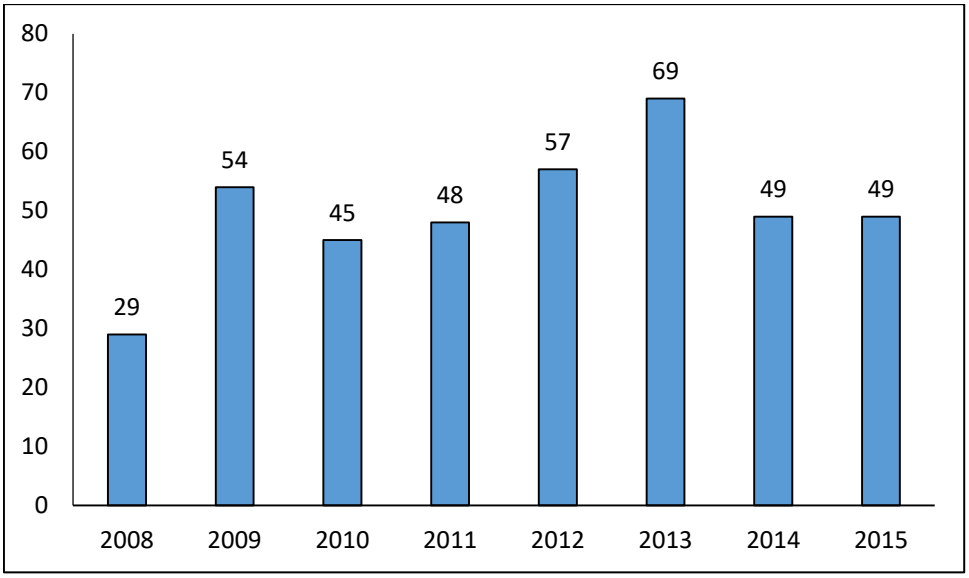

Fig. 11. Number of randomized women per year in the ASA-RCT.

Approximately 90% of the visits at randomization and during follow-up, and all visits at Södra Älvsborg Hospital, were with the PI. The remaining visits were carried out at other hospitals' or gynecologic outpatient clinics, mostly due to long distances from the study site. Not all pregnant women were physically examined toward the end of pregnancy, due to difficulties related to long journeys, especially if they had symptoms that made it difficult to travel. Single physical visits that did not occur for these reasons were replaced with a subsequent check-up by phone. All delivery records were submitted to the PI, followed by a phone call with the woman. All participants reported absolute adherence to treatment.

LBR, the primary outcome of the study, did not differ in the ASA and placebo groups (83.0% vs. 85.5%) in the intention-to-treat analysis. The difference was -2.5% (95% CI -10.1% to 5.1%).

There were similar rates of pregnancy loss in the two treatment groups. In the ASA group, four of five women with late miscarriages (gw 17-21) had a verified uterine infection. The reason for a second-trimester termination of pregnancy was hydrocephalus, detected at routine ultrasound at gw 19+2 days. In the placebo group, one intrauterine fetal death occurred at gw 42. There were few obstetric complications, such as placenta previa, preeclampsia and pregnancy-induced hypertension, and rates were similar in the two study groups. Five (2.5%) and 13 (6.5%) women had a preterm delivery in the ASA and placebo group, respectively. Six (3.6%) and four (2.3%) babies in the ASA and placebo group, respectively, were

small for gestational age. Miscarriage, either early or late, occurred in 33 (16.5%) and 28 (14.0%) in the ASA and placebo group, respectively (RR 1.18 (95%CI 0.74 to 1.87, p=0.58). Pregnancy outcomes for all women in the ASA-RCT are shown in Fig. 12.

Platelet aggregation was analyzed as a test of compliance and showed a marked decrease in ASA-treated women, as shown in Figure 2 in Paper I.

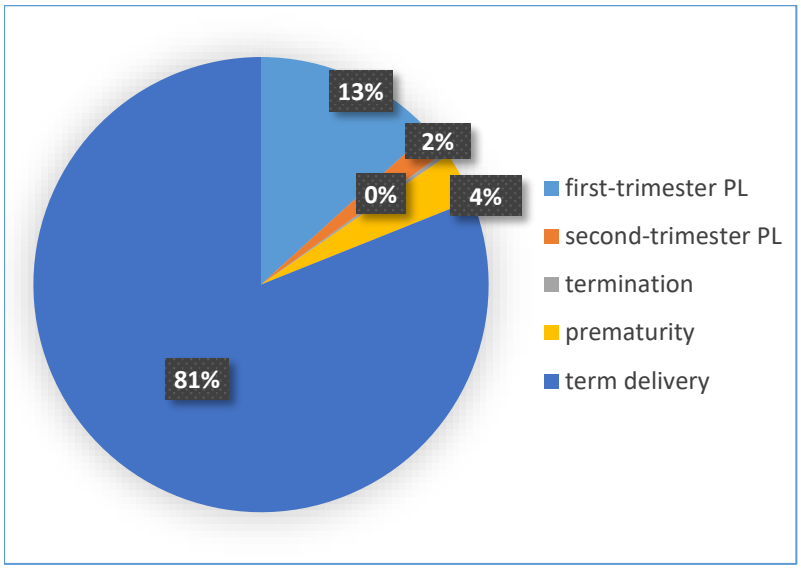

Fig. 12. Pregnancy outcome among 400 randomized women in the ASA-RCT.
PL=pregnancy loss

Paper II

A total of 114 pregnant women accepted participation. Ten women were excluded before start due to chronic illness, BMI ≥35 kg/m^2 or current smoking. Another 25 women were excluded after delivery due to complicated pregnancies. The remaining 79 women had uncomplicated pregnancies and gave birth at term to healthy babies. The mean age was 29.1 (SD 4.8) years and the mean BMI was 23.0 kg/m^2 (SD 3.1). Postpartum (8-12 weeks after delivery) samples from 19 participants were missing.

Lower platelet aggregation at gw 8-15 and 37-40, compared with postpartum, was found after activation with AA, ADP and TRAP. Platelet aggregation after induction with COL was unaltered. A minor increase in ADP-induced aggregation was found as pregnancy proceeded (Table 3 in Paper II). AA-, COL- and TRAP-induced aggregation exhibited no change over time during pregnancy.

PC from all sampling occasions were not available for all participants. Platelet aggregation was therefore compared between women with known and unknown PC. There were no statistically significant differences in platelet aggregation with any activator between women with and without PC determined at the different analysis points during pregnancy. However, significantly lower platelet aggregation was noted postpartum after induction with ADP (Table 7).

No statistically significant difference was found when platelet aggregation after activation with ADP in the two non-pregnant groups was compared. A slightly, but significantly, lower platelet aggregation after activation with AA, COL and TRAP in the postpartum group, compared with the non-pregnant, non-puerperal group, was found.

Table 7. Comparison of platelet aggregation induced by different activators in women with known and unknown platelet count in the non-pregnant state

Activator of platelet aggregation	Platelet aggregation		Comparison between groups
	Platelet count known (n=45)	**Platelet count unknown (n=15)**	
	Mean (SD) Median (Min; Max) (Q1; Q3) n=	**Mean (SD) Median (Min; Max) (Q1; Q3) n=**	**Mean difference (95% CI) p-value**
ADP	62.3 (21.1) 61.0 (19.0; 118.0) (49.0; 76.0) n=45	48.7 (15.2) 46.0 (21.0; 78.0) (36.0; 60.0) n=15	13.7 (1.8; 25.5) p=0.024
AA	82.2 (23.3) 83.0 (15.0; 118.0) (69.0; 100.0) n=45	71.3 (16.4) 68.0 (47.0; 96.0) (63.0; 89.0) n=15	10.8 (-2.2; 23.9) p=0.10
COL	66.2 (18.1) 67.0 (31.0; 98.0) (55.5; 79.0) n=44	57.5 (12.7) 60.0 (33.0; 77.0) (49.0; 67.0) n=15	8.69 (-1.47; 18.86) p=0.092
TRAP	94.2 (19.1) 97.0 (38.0; 147.0) (85.0; 103.0) n=45	84.0 (17.3) 86.0 (55.0; 125.0) (79.0; 91.0) n=15	10.2 (-0.9; 21.4) p=0.072

The t-test was used for comparison between groups.
Calculation of CI for continuous variables is based on the assumption of normality. When variances are not equal ($p<0.05$) the SD is based on Satterthwaite´s approximation; otherwise, the SD is based on the pooled SDs.

Paper III

Medical and obstetric history, medication, platelet aggregation, PC and demographic variables except smoking in the non-pregnant state for ASA- and placebo-treated women from the ASA-RCT were evenly distributed. Fewer placebo-treated women were smokers. The healthy controls had fewer previous complicated pregnancies and less previous illness, medication and smoking. The mean age was 32.3 (4.6), 32.0 (4.2) and 29.3 (4.1) years, respectively, and there was no difference in BMI. Before pregnancy (non-pregnant state), AA-induced platelet aggregation was lower in the ASA group [mean 73.7 (21.3) U], compared with the placebo-treated group [mean 79.9 (23.0) U] and healthy controls [mean 80.6 (20.6) U] 8-12 weeks postpartum (non-pregnant state) ($p<0.001$).

Women in the ASA-RCT had normal values for Hb, PC, PC(INR) and APTT before pregnancy, except one woman in the placebo group, who had PC 76 and 66 $x10^9$/L at the two sampling time-points during pregnancy. As expected, Hb, PC, PC(INR) and APTT were significantly lower at gw 20, compared with the non-pregnant state. Women in the healthy control group had PC above 100 x 10^9/L during pregnancy and postpartum.

No differences were observed in AA-induced platelet aggregation at any time-point during pregnancy and postpartum, when the placebo-treated women with unexplained RPL were compared with healthy women with normal pregnancy (Fig. 13). Significantly decreased AA-induced platelet aggregation was found in ASA-treated women in gw 13, 30 and 36 compared with the pre-pregnant state (Fig. 13). The decrease diminished gradually during pregnancy but was still >50% for 74% of the ASA-treated women at the end of the third trimester. The ASA-treated women had significantly lower AA-induced platelet aggregation at every time-point during pregnancy, compared with both placebo-treated women and healthy women with normal pregnancy.

Full response to ASA was found in 68 of 103 (66%) women, who had ASPI tests (AA-induced platelet aggregation) <30 U at all three time-points during pregnancy. Two women (1.9%) were found to be non-responders (ASPI >30 U at every sampling). The remaining 33 women (32.1%) with varying platelet aggregation (ASPI values more or less than 30 U) at the different time-points during pregnancy were regarded as partial responders. Individual results during ASA treatment in

women with normal AA-induced platelet aggregation and low AA-induced platelet aggregation, respectively, are shown in figures 14 and 15.

The placebo-treated women from the ASA-RCT did not differ in AA-induced platelet aggregation at any time-point during pregnancy, compared with before pregnancy. A small, but statistically significant, decrease in platelet aggregation was observed for the healthy controls with normal pregnancies, when gw 8-15 and 37-40 were compared with the non-pregnant state.

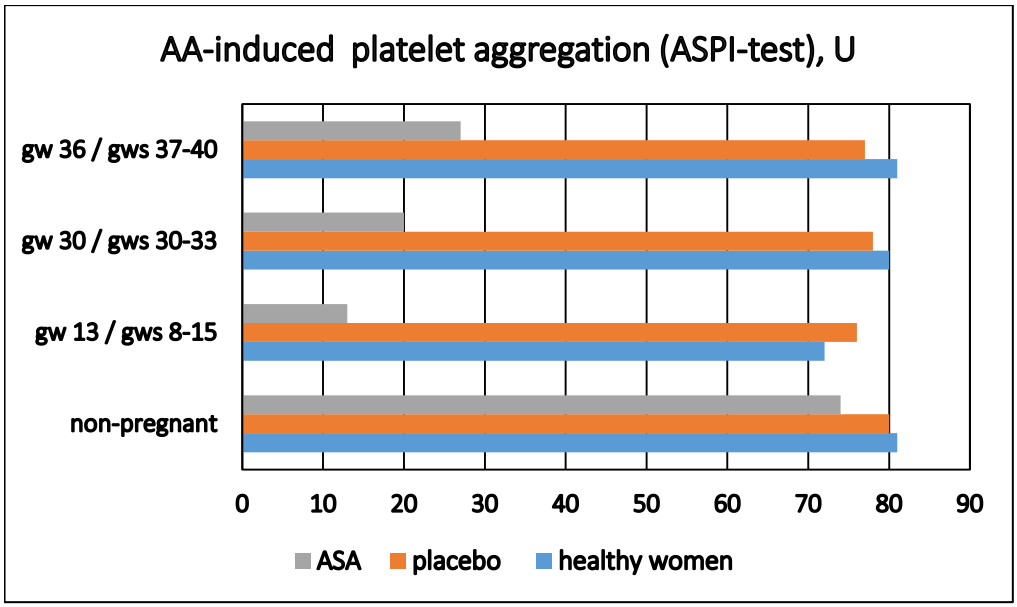

Fig. 13. Comparison of platelet aggregation, measured with the ASPI test (Units), in women with recurrent pregnancy loss treated with ASA or placebo, as well as in healthy women with normal pregnancy.

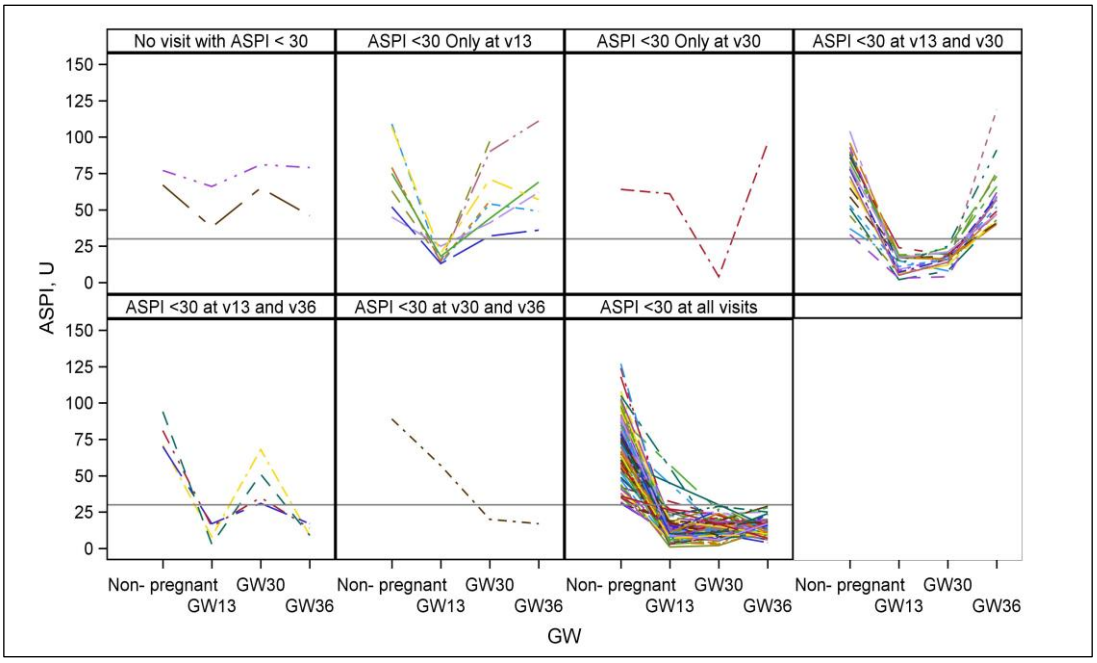

Fig. 14. Arachidonic acid (AA) induced platelet aggregation in pregnant women with unexplained recurrent pregnancy loss and **normal** AA-induced platelet aggregation before pregnancy. Each line indicates an individual woman during treatment with ASA or placebo or no treatment, grouped according to inhibition pattern.

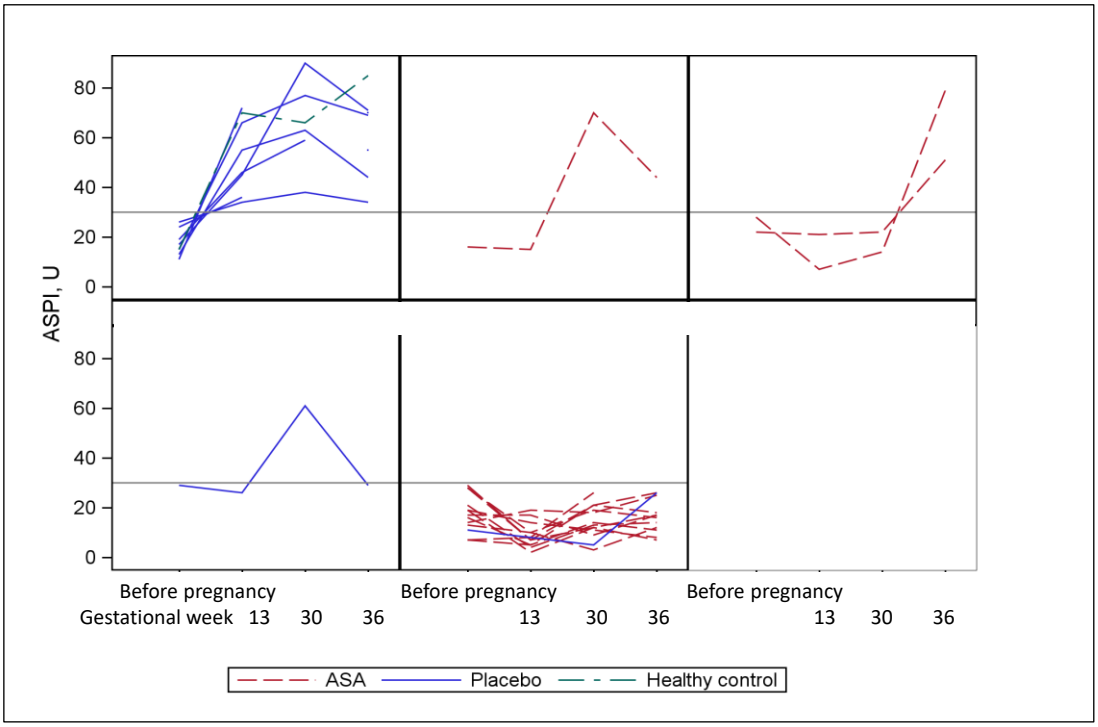

Fig. 15. Arachidonic acid (AA) induced platelet aggregation in pregnant women with unexplained recurrent pregnancy loss and **low** AA-induced platelet aggregation before pregnancy. Each line indicates an individual woman during treatment with ASA or placebo or no treatment, grouped according to inhibition pattern.

At the end of the third trimester, 74% of ASA-treated women demonstrated a 50% decrease in AA-induced platelet aggregation, 80% had a 40% decrease and 81% had a 30% decrease (Fig. 16).

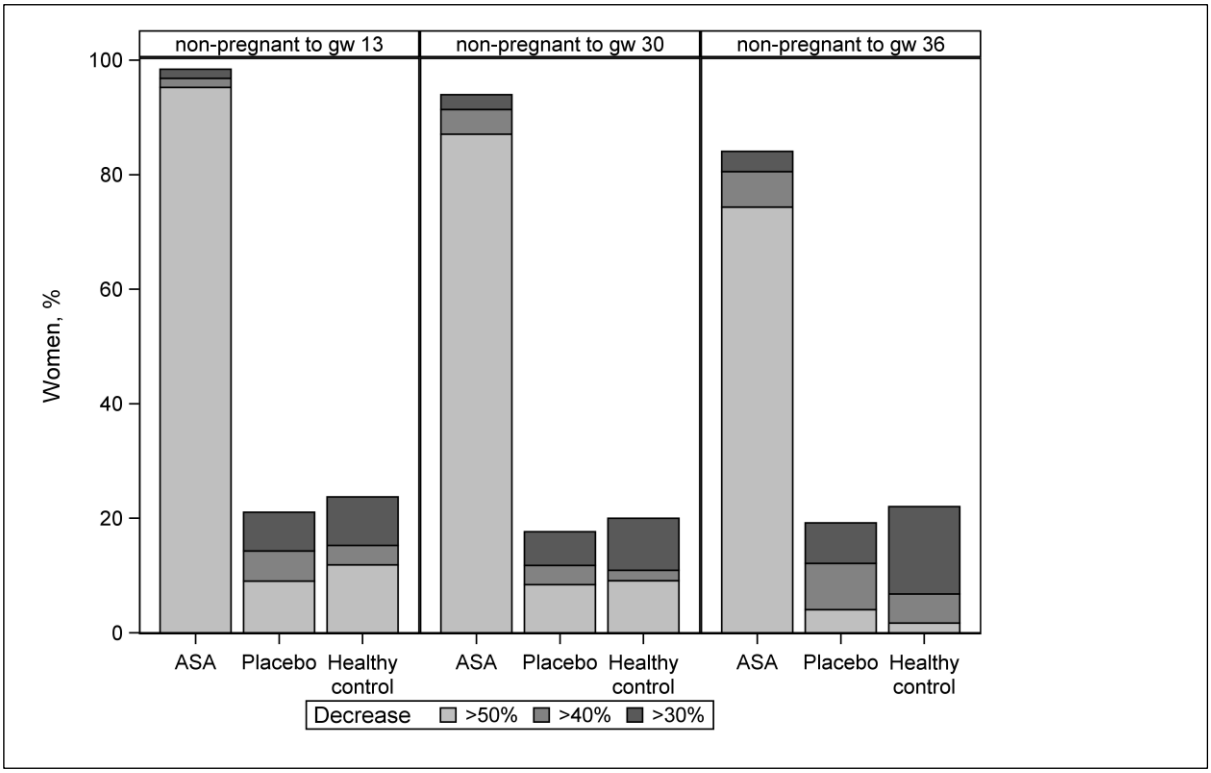

Fig. 16. Comparison of inhibition by ASA, expressed as different levels of decrease in arachidonic acid-induced platelet aggregation, in women with recurrent pregnancy loss treated with ASA or placebo and healthy women with normal pregnancy.

Paper IV

A total of 495 women were included in this study. Of the 619 women assessed for the ASA-RCT, 475 were included. Twenty women who met the inclusion criteria for the ASA-RCT and who had undergone the same tests and examinations as in the RCT, were also included in this study. All participants had complete sets of thyroid blood samples and documented pregnancy outcomes (Fig. 8).

The participants in this study had very similar demographic characteristics as the women in the ASA-RCT: mean age 32.5 years, mean BMI 24.0 kg/m^2 and three to seven consecutive unexplained first-trimester miscarriages in their history. Previous thyroid disease was reported for 32 women (8.6%), but they had all been euthyroid, with ongoing levothyroxine treatment, during the periods in which their miscarriages had occurred.

Among the 495 women, 78% had a live birth and 20% had a first-trimester miscarriage, of which 13 occurred in biochemical pregnancies.

Baseline differences according to TPO status were found regarding the number of previous miscarriages, previous thyroid disease and ongoing treatment with levothyroxine. Baseline differences according to TSH levels were found in the number of previous live births and ongoing treatment with levothyroxine, antihistamines or anti-depressants.

In the univariable regression analyses, three variables were potentially predictive of a new miscarriage: number of previous miscarriages, age and presence of TPO-ab. Women with positive TPO-ab had a higher miscarriage rate than TPO-ab-negative women. The RR for a new first-trimester miscarriage was 1.47 (95%CI 1.02 to 2.11), while the adjusted RR was 1.40 (95%CI 0.99 to 2.00). Miscarriage rates were similar in women with TSH in the upper and lower normal ranges, respectively. The RR was 0.93 (95% CI 0.46 to 1.85) and no confounding factor was applicable. Analysis after exclusion of patients with a biochemical pregnancy as an outcome did not significantly affect the results.

In a stepwise logistic regression analysis, the number of previous miscarriages was the most important risk factor for a new miscarriage, followed by age and presence of TPO-ab.

Discussion

In our studies, we found that treatment with 75 mg ASA daily did not improve the outcome with regard to a new miscarriage or a live birth in women with unexplained RPL (Paper I). A minor decrease was found in platelet aggregation, after induction by AA, ADP and TRAP. No difference was seen regarding to COL. A minor increase in aggregation after ADP activation was found as pregnancy continued. AA-induced platelet aggregation in both the non-pregnant state and during pregnancy is similar in women with and without RPL (Paper III). ASA inhibition of AA-induced platelet aggregation is strong but decreases as pregnancy progresses. TPO-ab may be a risk factor for a new miscarriage, but this issue requires further evaluation (Paper IV). Finally, an important finding is that the LBR was high in all study groups.

Paper I

Variations in RPL populations and interventions make comparisons between studies problematic. Populations may vary depending on the definition of RPL, i.e. at least two or at least three previous miscarriages, gestational age, consecutive or not, or within the same relationship or not. Our decision to include couples with three or more unexplained miscarriages was based on the contemporary definition when the RCT was planned. We found that about 15% had a new miscarriage during the course of the study, with or without active treatment. This rate is similar to the results of other RCTs (Dolitzky *et al.*, 2006; Tulppala *et al.*, 1997), but considerably lower than that found in observational studies (Brigham *et al.*, 1999; Clifford *et al.*, 1997). Thrombosis in decidual vessels is believed to be a possible cause of recurrent miscarriage (Rai, 2003; Carp *et al.*, 2004; Dolitzky *et al.*, 2006; Badawy *et al.*, 2008). Pregnancy itself is a hypercoagulable state (Dolitzky *et al.*, 2006; Rai *et al.*, 2000) and the levels of procoagulant microparticles are higher in women with RPL than in controls (Laude *et al.*, 2001; Stirling *et al.*, 1984).

In the early weeks of pregnancy, intermediate trophoblasts invade the decidual segments of the spiral arteries, causing physiological structural modifications. Insufficiency of the transformed arteries may give rise to early pregnancy loss (Dogan Gun *et al.*, 2006). Intermediate trophoblastic cells are important for implantation and establishment of the utero-placental circulation (Wan *et al.*, 1992;

Gerdsel *et al.,* 2002). Intervillous circulation of maternal blood is likely to be established progressively between gws 8 and 12 (Valentin *et al.,* 1996; Jaffe *et al.,* 1997; Carbillon *et al.,* 2001).

From the histopathological standpoint, recurrent early pregnancy loss is ascribed to lesions in the placental bed, including fibrinoid necrosis and acute atherosis of the spiral arteries (Rushton, 1988; Dogan Gun *et al.,* 2006). These findings are of considerable interest, as they are related to preeclampsia and intrauterine growth retardation later in pregnancy, but they are also interesting in relation to spontaneous abortion in early pregnancy. Examination of freshly aborted fetuses and placentas revealed uteroplacental ischemia and retroplacental hemorrhage in almost one fifth of cases (Rushton, 1988). Comparison of vessels in elective abortion decidua and those in spontaneous abortion decidua revealed many more converted spiral arteries in the former than in the latter (Dogan Gun *et al.,* 2006). This author concluded that spontaneous abortion was associated with a significantly increased incidence of inadequate placentation, particularly reduction or absence of trophoblastic invasion and defect transformation of the spiral arteries (Dogan Gun *et al.,* 2006).

According to our results reported in Papers I and III, we did not find any support for the hypothesis of hypercoagulability, expressed as increased AA-induced platelet aggregation, as a cause of unexplained recurrent first-trimester miscarriage.

In our ASA-RCT (Paper I), treatment with ASA or placebo started after detection of fetal heartbeat by ultrasound, at about seven gestational weeks. Thus we cannot draw any conclusions about pregnancy outcome if treatment had started before ovulation or at positive pregnancy test. The findings of Rushton and Dogan Gun indicate that earlier start of treatment may result in a higher LBR than we found.

Most studies compare ASA with other drugs, alone or in combination. There were, however, no previous robust studies comparing ASA alone with placebo in women with three or more consecutive unexplained first-trimester miscarriages. One small underpowered RCT, in which ASA 50 mg was compared with placebo, showed a similar LBR as our study (Tulppala *et al.,* 1997). In a large observational study with three or more consecutive first-trimester miscarriages, 75 mg ASA was compared with no treatment. LBRs were 68.4% and 63.5%, respectively (Rai R *et al.,* 2000). A comparison of treatment with either 40 mg enoxaparin or 100 mg ASA in women with three or more consecutive first-trimester miscarriages revealed LBRs of 81.5% and 84.0%, respectively (Dolitzky *et al.,* 2006). Although these three studies

included an intervention with ASA treatment, they differed as to the time-point for starting treatment. Treatment started earlier in two of these studies - within five weeks of amenorrhea (Rai *et al.,* 2000) and after positive pregnancy test (Tulppala *et al.,* 1997), respectively - while the third had a similar starting point, i.e. at gw 6-7 (Dolitzky *et al.,* 2006), as in our study.

We chose to include only first-trimester miscarriages, since etiologies for second-trimester miscarriage are different, e.g. uterine infection and cervical incompetence. Four of the seven second-trimester miscarriages in our study were associated with a verified intrauterine infection.

We considered it important to determine blood levels of ASA during treatment in order to verify compliance. In order to find an appropriate method for this, 15 volunteers from the staff at the Department of Obstetrics and Gynecology at Södra Älvsborgs Hospital, healthy and without conflicting medication, had been recruited and given either 75 mg ASA for two weeks or no treatment. However, it was not possible for the hospital laboratory to detect any difference between the groups related to this low ASA dose. We subsequently found that AA-induced platelet aggregation (ASPI test), analyzed with the Multiplate analyzer, was an appropriate method for checking compliance.

In the ASA-RCT, we found a marked decrease in AA-induced platelet aggregation in ASA-treated women, while no corresponding decrease in the placebo-treated group was demonstrated. This finding indicates very good adherence to treatment, but contradicts the hypothesis that platelet aggregation induced by AA is involved in first-trimester RPL after verification of a viable fetus. However, we do not know what would have happened if the ASA treatment would have been started at ovulation or before conception. Furthermore, we cannot rule out that platelet aggregation induced by other activators, such as COL, ADP or TRAP, might have affected the RPL rate.

In our study, as well as in others, there was a high LBR, which indicates a good prognosis for this group. The extensive follow-up program, applied to both study groups, may have been a contributory factor to the high LBR in this study. Many authors emphasize that women with unexplained first-trimester RPL have a very good prognosis (75-86%) for a successful pregnancy if provided with psychological support and high access to caregivers, even in the absence of pharmacological intervention (Clifford *et al*., 1997; Brigham *et al*., 1999; Rai *et al*., 2006; Badawy *et*

al., 2008; Morley *et al*., 2013; Kutteh *et al*., 2014; Musters *et al.,* 2013). This concurs with our perception that our study participants had a high rate of successful pregnancy, and that they also emphasized the importance of the follow-up program and the possibility to easily contact the PI. However, the effect of "tender loving care" has not as yet been properly investigated in a RCT (Rasmark Roepke *et al.,* 2018).

Paper II

There were a few small previous studies describing platelet aggregation during normal pregnancy (Burke *et al.,* 2013; Louden *et al.,* 1990). We sought a suitable method for determining platelet aggregation in order to study differences between healthy women and women with RPL regarding platelet aggregation, ASA effect and treatment compliance. We found that multiple electrode impedance aggregometry was one of the most applied methods in other study populations undergoing ASA treatment (Jambor *et al.*, 2009; Oscarsson *et al.,* 2011). However, we also discovered that there was a lack of robust experience of the method in connection with pregnancy.

Therefore, we undertook this study in order to gain knowledge of platelet aggregation during normal pregnancy in healthy women. Determining platelet aggregation longitudinally during normal pregnancy in healthy women was also a prerequisite to evaluate whether platelet aggregation is increased in women with RPL.

In this study, platelet aggregation was measured longitudinally after induction by AA, ADP, COL and TRAP, respectively. We found a minor decrease in platelet aggregation, after activation by AA, ADP and TRAP, compared with postpartum. No difference was seen regarding COL. A minor increase in aggregation after ADP activation was found as pregnancy continued. We believe that the minor changes in platelet aggregation that we found probably lack clinical significance under normal conditions.

As the women in this study were recruited in early pregnancy, it was impossible to obtain non-pregnant platelet aggregation results before the current pregnancy. Previous studies have shown normalized platelet function within 6 to 12 weeks postpartum (Norris *et al.*, 1994; Louden *et al.*, 1990). Therefore, we chose three months postpartum as the non-pregnant state for analysis. In our study a small but

statistically significant difference was noted when the non-pregnant, puerperal women were compared with non-pregnant, non-puerperal, fertile-age, healthy women. This could be due to a residual effect of pregnancy, different populations or differences in handling the method.

Some older previous studies have shown increased platelet aggregation induced by AA, ADP and COL in normal pregnancy (Morrison *et al.*, 1985; Leuschen *et al.*, 1986). In a small longitudinal study by Louden (1990), eight healthy pregnant women with normal pregnancy and delivery were compared with 12 healthy non-pregnant women regarding platelet aggregation, induced by AA, ADP and COL and analyzed with the Ultra-Flo 100 system. No significant changes after ADP and COL induction were observed, but after AA induction, there was a significant increase in platelet aggregation at gw 32, compared with the first trimester. Platelets seemed to be more reactive to AA stimulation in normal pregnancy, beginning from gw 16 (Louden *et al.*, 1990). In a longitudinal study during pregnancy including 36 non-smoking pregnant women and 30 healthy non-pregnant controls, blood samples for platelet aggregation analysis were drawn (Burke *et al.*, 2013). They were analyzed with light transmission aggregometry after induction by AA, ADP, COL and TRAP. The main findings were significantly reduced platelet reactivity after COL induction in the first trimester and significantly increased platelet reactivity after AA induction in the second and third trimesters.

Paper III

No difference was found in AA-induced platelet aggregation when women with at least three unexplained consecutive first-trimester miscarriages were compared with healthy women with uncomplicated pregnancy and normal delivery at term. Increased platelet aggregation has been suggested by previous authors to be a causal factor of recurrent miscarriage in early pregnancy (Flood *et al.*, 2010). The absence of any difference in AA-induced platelet aggregation between the healthy controls and women with recurrent miscarriages may indicate that AA-induced platelet aggregation is not a cause of recurrent first-trimester miscarriage.

Treatment with 75 mg ASA daily during pregnancy from gw 7 to gw 36 resulted in markedly lower platelet aggregation, but the degree of inhibition decreased as pregnancy progressed. However, AA-induced platelet aggregation remained reduced by more than 50% during the entire pregnancy in the majority of ASA-treated

women. This has not been previously reported. Our study suggests that the ASA dose should be increased during the third trimester in order to yield unaltered effect throughout pregnancy. The ongoing debate about prophylactic doses of ASA for prevention of preeclampsia and other placenta-mediated complications necessitates further studies, including of platelet aggregation. It is important to understand the mechanism through which ASA exerts its effects in these conditions in order to determine the right dose. A low dose of ASA, 75 mg daily, seems to be as effective as a high dose, i.e. 325 mg daily (Patel *et al.,* 2007). The most effective ASA dose in preventing cardiovascular events seems to be 75-150 mg, which has similar efficacy as doses up to 1500 mg ASA daily (Mansour *et al.,* 2009). A systematic review and meta-analysis of the outcomes moderate and severe preeclampsia and intrauterine growth retardation (Roberge *et al.,* 2016) found that treatment with 75-150 mg ASA daily was associated with a reduction of all three studied outcomes. Individualized dosage of ASA after platelet function testing has been found to be effective in preventing preeclampsia in high-risk women (Rey *et al.,* 2011). In our study, we found increased platelet aggregation as pregnancy progressed during treatment with 75 mg ASA. In most RPL studies, 50-100 mg of ASA has been used and no effect on LBR has been demonstrated based on dosage (Kaandorp *et al*., 2009; Tulppala *et al*., 1997; de Jong *et al*., 2014).

Platelet aggregation was analyzed by multiple electrode impedance aggregometry after induction by AA (ASPI test). We found full response to ASA (ASPI <30 U at all testing time-points during pregnancy) in about two-thirds and varying response in one-third of the pregnant women during pregnancy. The Multiplate method is most suitable for detection of changes in platelet aggregation within an individual during drug treatment, as there is wide inter-individual variation.

The definition of non-response is problematic due to different populations, study designs and test methods. A generally accepted definition based on valid diagnostic criteria has not yet been established (Zimmermann *et al.,* 2008). There is a *laboratory definition* of resistance to ASA therapy, i.e. failure of ASA to inhibit TXA_2 production, estimated to affect 24-28% (Zimmermann *et al.,* 2008; Patel *et al.,* 2007) There is also a *clinical definition,* i.e. the occurrence of thrombotic cardiovascular ischemic events despite regular ASA therapy, according to some authors (Patel *et al.,* 2007).

Only two women (1.9%) were non-responders to ASA (all ASPI ≥30 U during pregnancy). This incidence of non-responders is much lower than previously

reported among obstetric populations (Navaratnam *et al.,* 2016; Caron *et al.,* 2009). We defined women with one ASPI value ≥30 U at any testing time-point during pregnancy, in whom the levels at the other time-points were below 30 U, as partial responders; this applied to 32.1%. We have not seen partial responders defined in any other study. Together, our non-responders and partial responders constituted 34%. If this combined group had been redefined as non-responders, the prevalence would be in line with previously reported prevalences of 29-39% non-responsiveness in obstetric populations (Navaratnam *et al.*, 2016). In another cohort of 87 pregnant women, 25 were non-responders to 81 mg ASA daily (Caron *et al.,* 2009). After increasing the dose to 162 mg, only eight women were still non-responders, indicating that the lack of response can be overcome with higher doses in most individuals.

Paper IV

Our cohort was selected to comprise only patients with normal thyroid hormone levels, including those with normal thyroid function as well as those taking levothyroxine, who were euthyroid during their miscarriage history.

We found that women with positive TPO-ab had a higher miscarriage rate than women lacking TPO-ab. This concurs with some previous studies (Negro *et al.,* 2005; Stagnaro-Green *et al.,* 2011), while others have reported no such association in women with RPL (Pratt *et al.,* 1993; Esplin *et al.,* 1998; Rushworth *et al.*, 2000; Yan *et al.,* 2012).

When TSH levels in the upper and lower normal ranges were compared in our study, the miscarriage rates were similar, 21.1% and 21.4%, respectively. TSH in the upper normal range was not thus associated with an increased risk of pregnancy loss. According to the literature, TSH levels in the upper normal range are often referred to as subclinical hypothyroidism, which is formally defined as elevated TSH in combination with normal free T4. Conflicting results regarding the risk for a new miscarriage related to subclinical hypothyroidism in RPL patients have been reported (van den Boogaard *et al.,* 2011; van Dijk *et al.,* 2016).

We identified number of previous miscarriages, age and TPO-ab in a risk factor model. Very few previous studies have investigated these risk factors using a multivariable technique. Two authors report correlations between miscarriage and

higher age and the presence of TPO-ab. Higher age is an independent risk factor for miscarriage and most TPO-ab-positive women are older than women without TPO-ab (Ashrafi *et al.,* 2007; Roberts *et al.,* 2000).

Women with biochemical pregnancy losses were included in the study population; exclusion of these women did not significantly affect the results. In most studies of recurrent (≥three) first-trimester miscarriages focusing on elevated TSH and TPO-ab, biochemical pregnancy has not been discussed. Some studies have mentioned, and included, biochemical pregnancies but the results have not been reported separately (Liu *et al.,* 2014; Kaur *et al.,* 2016; Vissenberg *et al.,* 2016). Only one study explicitly stated that biochemical pregnancies were excluded (van Dijk *et al.,* 2016).

TPO-ab was chosen as a biomarker of thyroid autoimmunity as it is the most common thyroid antibody (Frölich *et al.,* 2017). TPO-ab as a risk factor for a new first-trimester miscarriage in women with RPL is under debate, as illustrated by varying reported results. In our study, positive TPO-ab, but not TSH in the upper normal range, affected the risk of a new miscarriage. It may be the thyroid autoimmunity *per se*, rather than impaired thyroid function, that causes the miscarriage. Normal thyroid function is required for fetal-placental development (Vissenberg *et al.,* 2015), and if thyroid gland reserve capacity is reduced by autoimmune disease, it can manifest in early pregnancy as a miscarriage.

Strengths

The strength of this thesis is that it is based mainly on a large RCT with a complete follow-up and total adherence to the study protocol. By applying the definition of at least three, and not two, consecutive unexplained first-trimester miscarriages, "random miscarriages" were avoided. Thus, the study population was more likely to have been a "true" RPL population. Measurement of platelet aggregation to check compliance added important information. The low prevalence of smokers in the study population was advantageous, since smoking has been associated with non-responsiveness to ASA.

We are not aware of any previous large study that has presented results on platelet aggregation, determined by impedance aggregometry and induced by four different agonists, longitudinally during normal pregnancies in healthy women (Paper II). Nor

were there, to our knowledge, published results of studies undertaking longitudinal comparison during pregnancy of women with and without recurrent unexplained first-trimester miscarriage, or of studies investigating the effect of ASA treatment during pregnancy among women with RPL (Paper III).

The population screened for eligibility for the ASA-RCT was well documented, which enabled conduction of the post hoc studies.

Limitations

Some limitations in the design of the RCT can be noted. The adverse effects were reported in response to an open question and not according to a pre-specified report form at the prenatal visits. Other limitations refer to the planning of laboratory testing. It would have been valuable to have analyzed hCG in early pregnancy for correlation with TSH, as well as to have repeated the thyroid hormone tests during pregnancy. Moreover, the ASPI test was not performed until gw 13, precluding any information on inhibition of platelet aggregation in women who miscarried in the first trimester.

In the cohort of healthy women with normal pregnancy, the loss to follow-up three months postpartum was high but anticipated. Furthermore, the importance of missing PCs in some women at certain time-points during pregnancy had to be investigated, since platelet aggregation is dependent on PC. However, sensitivity analysis revealed that there was no difference in platelet aggregation in women with and without available PC.

Clinical implications

A very strong recommendation, based on high-quality evidence from the ASA-RCT, is that women with a history of RPL **not** be treated with ASA, beginning in early pregnancy when a fetal heartbeat is detected, with the intention of reducing the risk for a new miscarriage. Whether ASA treatment initiated at ovulation or on the date of a missed period would affect the subsequent miscarriage risk is still unknown. Thus, this treatment should only be considered in the context of a RCT.

Our results may have implications on dosage of ASA during pregnancy, when prescribed on other indications such as prevention of preeclampsia. In order to maintain unaltered inhibition of platelet aggregation throughout pregnancy, the dose should be increased at least during the third trimester.

The Multiplate method might have a place in monitoring platelet aggregation during drug treatment in individual pregnant patients, as is already the case in non-pregnant patients. The method is not suitable for determination of minor intra-individual changes.

The presence of TPO-ab as a potential risk factor for a new miscarriage should be recognized when women and their partners seek help for RPL.

Conclusions

- Treatment with 75 mg ASA, initiated after detection of a viable pregnancy, did not prevent a new miscarriage in women with at least three previous first-trimester unexplained miscarriages in the current relationship. This population had a very good prognosis for a subsequent live birth, both with and without ASA treatment.

- Platelet aggregation induced by AA, ADP, and TRAP, and longitudinally measured by multiple electrode impedance aggregometry, underwent minor changes during pregnancy, compared with the non-pregnant state. Platelet aggregation after COL activation was unchanged. These changes probably lack clinical significance under normal conditions.

- During pregnancy and in the non-pregnant state, women with recurrent unexplained miscarriages seem to have the same degree of AA-induced platelet aggregation as healthy women with normal pregnancies. Platelet aggregation induced by AA is not a plausible causal factor for recurrent unexplained first-trimester miscarriage.

- AA-induced platelet aggregation was inhibited, and remained reduced by at least 50% throughout pregnancy, in the majority of ASA-treated women. The reduction was less pronounced later in pregnancy. This may indicate a need to increase prophylactic low dose ASA as pregnancy progresses in order to achieve unaltered effect throughout pregnancy.

- Euthyroid, TPO-ab-positive women with a history of RPL may have an increased risk for a new miscarriage, compared with women without TPO-ab. Age and number of previous miscarriages are strong risk factors for a new miscarriage. The risk of a new miscarriage was not influenced by TSH levels if they were within the normal range.

Future perspectives

Women with unexplained first-trimester RPL are a vulnerable group, for whom no established treatment is available. Women seeking help for RPL are likely to accept experimental treatment, but all suggested treatments should only be given in the context of a RCT at present. For every suggested treatment, there is an underlying mechanism theory that needs to be tested.

This thesis focuses on ASA treatment and the underlying suggested explanation that inhibition of platelet aggregation would reduce the risk of a new miscarriage. A similar LBR, regardless of treatment allocation, was demonstrated. However, it cannot be ruled out that ASA initiated before or at implantation might have a beneficial effect on the risk of a new miscarriage. To test that hypothesis, a placebo-controlled trial evaluating low-dose ASA initiated before ovulation or during the luteal phase is required.

The LBRs were fairly high (85%) in both randomization groups. Both groups attended an extended follow-up program, which raises the question of the impact of the follow-up on the overall results. The concept of 'tender loving care' has been discussed for more than half a century, but has never been tested with a randomized design.

We found major inter-individual variations when healthy women with normal pregnancies were examined with impedance aggregometry for analysis of platelet aggregation, induced by AA, ADP, COL and TRAP. Thus, a larger pregnant population is needed to establish a reference range for the method, if it is to be used clinically in pregnant women treated with platelet aggregation inhibitors.

The inhibition of platelet aggregation by ASA treatment decreased as pregnancy progressed. This finding has not been previously reported and thus requires verification. The clinical implication is to consider increasing the ASA dose as pregnancy progresses, when it is given to prevent obstetric complications such as preeclampsia. The magnitude of the dose increase must be studied in a clinical setting before any recommendation can be made.

The effect of TPO-ab and subclinical hypothyroidism on miscarriage risk among women with RPL must be studied further. The finding that TPO-ab are a potential risk factor must be evaluated in a larger population, preferably with a prospective cohort design, and the protocol should include repeated thyroid tests as well as analysis of hCG. Loss of biochemical pregnancies also merits attention.

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