Inherited Thrombophilia and Pregnancy Complications

Paulien G. de Jong
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ACADEMISCH PROEFSCHRIFT

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Addenda
Nederlandse samenvatting, Authors and affiliations Portfolio, Dankwoord & Curriculum vitae
Chapter 1

General introduction and outline of the thesis

Paulien G. de Jong, Mariëtte Goddijn
and Saskia Middeldorp
General introduction

Recurrent miscarriage has historically been considered a divine punishment, a magical interference or a medical disorder. Suffering from recurrent miscarriage is devastating for couples, and throughout the ages, attempts towards understanding its etiology have been made in search for an effective treatment. Ceremonies of ritual purification to prevent subsequent disease of the mother and father (Africa), prayers to ward off an embryo-eating demon (India) and wearing a rattling stone ornament (ancient Israel, France, Greece) are examples of various treatments applied in the past. 1 Centuries later nutritional factors were considered a cause of miscarriage in the Middle Ages and the first descriptions of treatment with medication date back to the 1940’s, when vitamins, progesterone and thyroid therapy were considered. 1 This latter under the assumption that recurrent miscarriage was a symptom of thyroid hormone deficiency. A possible role of thrombosis of the placental veins in placental infarction and toxemia of pregnancy (nowadays called pre-eclampsia) was postulated in a case report published in 1947. 2 Over the past two decades, research has implicated thrombophilia, an increased tendency to develop blood clots, as a new potential risk factor for various pregnancy complications including recurrent miscarriage. 3 Analogous to this etiological concept anticoagulants have been studied as agents to prevent recurrence of pregnancy complications. The use of anticoagulants for recurrent miscarriage and the search for new forms of thrombophilia which, analogous to an increased risk of venous thromboembolism (vTE), also predispose to pregnancy complications are the topics of this thesis.

During the first half of the 20th century the term thrombophilia was used to describe a tendency to develop thrombosis. 4 In the following decades the term was used for patients with severe manifestations of vTE, such as recurrent vTE or vTE at young age. Currently, the term thrombophilia is generally used for laboratory abnormalities which increase the risk of vTE. 5 Thrombophilia can be acquired as well as inherited and predisposes to deep venous thrombosis or pulmonary embolism. In the antiphospholipid syndrome, which is an acquired form of thrombophilia, antibodies against proteins bound to negatively charged phospholipids trigger the coagulation cascade, thus contributing to an increased risk of vTE. In inherited thrombophilia, the Factor V Leiden mutation, the prothrombin G20210A mutation or deficiencies of the natural anticoagulants protein C, protein S
and antithrombin affect the balance of pro- and anticoagulant factors, thereby inducing a hypercoagulable state.

Next to the increased risk of vte, women with thrombophilia are also at an increased risk of pregnancy complications. This was first demonstrated in the eighties of the last century when in women with systemic lupus erythematosus who had one or more miscarriages or stillbirths, an association with anticardiolipin antibodies was found. Women with antiphospholipid antibodies are now known to be at an increased risk of recurrent miscarriage, stillbirth and pre-eclampsia, with odds ratios ranging from 2 to 14. A decade later, family studies showed that women with inherited thrombophilia are also at an increased risk of these pregnancy complications. A meta-analysis of population based studies showed that the estimates of risk of (recurrent) miscarriage, stillbirth, and pre-eclampsia are 1.7 to 8.6 fold increased compared to women without thrombophilia, varying for type of inherited thrombophilia and type of pregnancy complication. However, a more recent meta-analysis which included prospective studies only found no statistically significant association between Factor V Leiden or prothrombin mutation and pregnancy loss, small for gestational age, pre-eclampsia or placental abruptio, except for a modest risk of late pregnancy loss in women with Factor V Leiden. The mechanism behind the association between thrombophilia and pregnancy complications is not fully elucidated, but it is hypothesized to be mediated (at least in part) via inhibition of differentiation of extravillous trophoblast into giant multinucleated cells and subsequent placentation and thrombosis of the (micro-) vasculature of the placenta.

**Anticoagulants for the prevention of pregnancy complications**

The etiologic parallel between vte and pregnancy complications as manifestations of thrombophilia is further extended to therapeutic approaches. vte can be adequately prevented and treated by administration of anticoagulants. The use of anticoagulants to prevent pregnancy complications was first described in 1973 in a case series of three patients with a history of pregnancies complicated by intrauterine fetal death or dysmaturity. In all women, placental infarction was observed in the previous pregnancies and the successful outcome of the subsequent pregnancy (an uneventful pregnancy leading to the birth of a healthy neonate and no or only minor placental infarction) was ascribed to the use of vitamin K antagonists or heparin.

A beneficial effect of heparin plus aspirin for women with recurrent miscarriage and antiphospholipid antibodies has also been demonstrated, but the numbers of participants in the studies were small and further studies to confirm this finding are still needed. For women with unexplained recurrent miscarriage, studies are inconsistent, with a benefit of anticoagulants in some studies and no benefit in other trials. No studies focusing on women with recurrent miscarriage and inherited thrombophilia are available. A subgroup analysis in the alife study, performed by our own group, suggested a beneficial effect of low-molecular-weight heparin (lmwh) plus aspirin.

In Part I of this thesis we aim to summarize the available evidence for the use of anticoagulants in women with (recurrent) miscarriage, irrespective of gestational age, and with or without inherited thrombophilia. Furthermore, we set out to evaluate the prognosis and time to conception of women with unexplained recurrent miscarriage with respect to achieving a live birth. Finally, we present the background, rationale and setup of a randomized controlled trial investigating the efficacy of lmwh in women with inherited thrombophilia, and the challenges that are faced when conducting such a trial.

**Search for new forms of thrombophilia**

Although the different forms of inherited thrombophilia have been recognized as risk factor for vte, the risk of vte is also approximately 2-fold increased in case of a positive family history of vte in individuals in whom known genetic risk factors have been ruled out. As in 70% of vte patients with a positive family history of vte no known genetic variant was identified, it is likely that other as yet unknown genetic variants also predispose to vte. Extending the parallel between vte and pregnancy complications, it has been postulated that unknown genetic risk factors for vte may also predispose to pregnancy complications, as the known forms of inherited thrombophilia and antiphospholipid syndrome that predispose to miscarriage and pre-eclampsia, may only account for 8-43%. In recent studies, variants in the promoter of the Annexin A5 gene have been proposed as such a form of thrombophilia. Annexin A5, formerly called placental anticoagulant protein, is a protein with anticoagulant properties, which forms a two-dimensional shield on phospholipid bilayers such as cell membranes, preventing coagulation reactions to occur.
tra-uterine fetal deaths and inherited thrombophilia are randomized to either LMWH or no intervention in a subsequent pregnancy and the primary outcome of the study is live birth. Next, in Chapter 5 we explain how designing, preparing and executing this international multi-center trial proved to be both edifying and challenging, and we suggest how to handle some of the difficulties that are encountered when setting up such a trial. With the knowledge of the associations between recurrent miscarriage and thrombophilia, physicians are often tempted to perform thrombophilia testing in these women. However, the test results may not alter clinical management in the absence of evidence on the efficacy of treatment. Whether testing for inherited thrombophilia is justified is therefore discussed in Chapter 6. As final chapter of Part I, we present the protocol of a systematic review and individual patient data meta-analysis to evaluate the efficacy of LMWH for the prevention of placenta-mediated pregnancy complications, including pre-eclampsia and recurrent pregnancy loss in Chapter 7.

Part II

Genetic variants in the Annexin A5 gene are evaluated as a potential new form of inherited thrombophilia in Part II. We first investigated whether the risk of deep venous thrombosis is increased in carriers of these variants in a case-control study. The frequency of single nucleotide polymorphisms and haplotypes in the Annexin A5 gene were compared between patients with a documented proximal deep venous thrombosis of the leg and controls in whom the diagnosis was ruled out and the results are detailed in Chapter 8. Next, in Chapter 9 a case-control study is presented, in which we evaluate whether these variants convey an increased risk of pre-eclampsia. Finally, we calculate the prevalence of Annexin A5 gene variants in women with recurrent miscarriage in the Netherlands and compare this to the prevalence in Dutch population controls and to the prevalence in women with recurrent miscarriage as reported in the literature. Furthermore, in exploratory analyses, we assessed whether women with recurrent miscarriage who carry these Annexin A5 gene variants benefit from anticoagulant treatment in a subsequent pregnancy. This study is described in Chapter 10.
Chapter 2

Antithrombotic therapy for pregnancy loss

Paulien G. de Jong, Mariëtte Goddijn and Saskia Middeldorp

Human Reproduction Update 2013
Abstract

Background: Although an association between thrombophilia and pregnancy loss has been observed in many studies, little is known about the pathophysiological mechanisms behind this association. Considering the association between thrombophilia and pregnancy loss, the efficacy of antithrombotic therapy for women with pregnancy loss (with or without thrombophilia) has been studied for the past 30 years.

Methods: We performed a comprehensive review of the literature on the strength of the association between thrombophilia and pregnancy loss, the pathophysiological mechanisms and the efficacy of antithrombotic therapy to increase the chance of live birth.

Results: The association between pregnancy loss and thrombophilia varies according to the type of thrombophilia (e.g. antiphospholipid syndrome versus forms of inherited thrombophilia) and according to the type of pregnancy loss (single versus recurrent pregnancy loss and early versus late pregnancy loss).

Thrombophilia may induce thrombosis in decidual vessels or impair placentation through hypercoagulability and inflammation, but these hypotheses need further verification.

For women with antiphospholipid syndrome, evidence from small-sized trials suggests a beneficial effect of antithrombotic therapy but additional randomized controlled trials are essential to confirm this. Whether antithrombotic therapy increases the chance of live birth in women with inherited thrombophilia is unknown. Recent randomized controlled trials have consistently shown that antithrombotic therapy does not increase the chance of live birth in women with unexplained recurrent miscarriage.

Conclusions: There are large gaps in knowledge and lack of evidence for treatment of women with pregnancy loss with thrombophilia. To provide a solid base for clinical practice, further studies on the role of coagulation in reproduction, as well as international collaborations in randomized controlled trials of antithrombotic therapy in women with pregnancy loss, and antiphospholipid syndrome or inherited thrombophilia are urgently needed.
Introduction

Pregnancy loss is common; approximately 15% of women experience a single spontaneous loss. This percentage reflects only clinically recognized pregnancies. Since pregnancy losses which occur at a very early gestation go unnoticed, the actual percentage of pregnancy loss is estimated to be even higher.

Depending on the gestational age at which pregnancy loss occurs, different terminologies for pregnancy loss are employed. The term miscarriage is often used to define pregnancy loss from the time of conception until 20 weeks’ gestation. Pregnancy loss thereafter is then termed fetal death or stillbirth. Revised terminology by the European Society of Human Reproduction and Embryology defines ‘early pregnancy loss’ for loss of fetal heart activity prior to 12 weeks’ gestation and ‘late pregnancy loss’ for loss of fetal heart activity at or after 12 weeks’ gestation (Table 1). This classification for pregnancy loss according to gestational age is chosen since the incidence in the first trimester is higher and the pathophysiology is different from losses occurring at a later gestational age. The great majority of pregnancy losses occurs early, before 12 weeks’ gestation.

The definition of recurrent miscarriage is a subject of debate. The Royal College of Obstetricians and Gynaecologists (RCOG) regards three or more first trimester miscarriages as recurrent pregnancy loss, whereas in Dutch and American guidelines, two or more pregnancy losses are considered recurrent pregnancy loss (Table 1). When defined as two or more losses, recurrent pregnancy loss affects up to 3% of fertile couples, whereas 1% experience three or more pregnancy losses. Another discrepancy between guidelines and practice statements is the inclusion of the criterion ‘consecutive’. Although the American Society for Reproductive Medicine (ASRM) does not incorporate ‘consecutive’ in its definition of recurrent pregnancy loss, evaluation of pregnancy loss is suggested ‘after two consecutive clinical pregnancy losses’. More evidence is available now that recurrent miscarriage constitutes any two miscarriages. The prevalence of carrier status of structural balanced chromosome abnormalities and antiphospholipid antibodies is similar in couples with a history of two or more consecutive or non-consecutive pregnancy losses.

### Antithrombotic therapy for pregnancy loss

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Pregnancy loss</td>
<td>“Miscarriage is defined as the spontaneous loss of pregnancy before the fetus reaches viability. This term should include all pregnancy losses from the time of conception until 20 weeks’ gestation.”</td>
<td>not discussed</td>
<td>not discussed</td>
<td>Early pregnancy loss is loss of fetal heart activity prior to 12 weeks’ gestation.</td>
</tr>
<tr>
<td>Recurrent pregnancy loss</td>
<td>“Recurrent miscarriage is defined as the loss of three or more consecutive pregnancies, affects 1% of couples trying to conceive. It has been estimated that 4-6% of second trimester pregnancies result in a stillbirth occurring before 24 weeks of gestation.”</td>
<td>not discussed</td>
<td>“Late recurrent pregnancy loss is a distinct disorder, defined by two or more failed clinical pregnancies.”</td>
<td>Recurrent miscarriages constitute at least two (or more) consecutive losses or two consecutive (after 12 weeks) pregnancy losses.</td>
</tr>
</tbody>
</table>

### Diagnostic tests indicated

<table>
<thead>
<tr>
<th>Recurrent miscarriage</th>
<th>Women with recurrent spontaneous and consecutive miscarriages should be evaluated by a health professional with the necessary skills and expertise. Where available, this might be within a recurrent miscarriage clinic.”</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antiphospholipid syndrome</td>
<td>“All women with recurrent first trimester miscarriages and all women with one or more second trimester miscarriages should be screened before pregnancy for antiphospholipid antibodies.”</td>
</tr>
<tr>
<td>Abnormal thrombophilia</td>
<td>“Women with second trimester miscarriages should be screened for inherited thrombophilia including factor V Leiden, factor II (prothrombin) gene mutation and protein S.”</td>
</tr>
</tbody>
</table>

### Additional notes

- “All women with a history of three or more early pregnancy losses, stillbirth, before 10 weeks, or 1 or more unexplained deaths at ≥20 weeks of a morphologically normal fetus, or 3 or more premature births at ≥34 weeks with severe preeclampsia or placental infarction should be offered a testing for lupus anticoagulant and antiphospholipid antibodies to exclude an antiphospholipid syndrome.”
- “Routine testing of women with a history of three or more consecutive or non-consecutive pregnancy losses is not currently recommended.”
- Thrombophilia screening is recommended in the context of a trial.
A: At least one meta-analysis, systematic review or randomised controlled trial rated as 1++ and directly applicable to the target population; or a systematic review of randomised controlled trials or a body of evidence consisting principally of studies rated as 1+ directly applicable to the target population and demonstrating overall consistency of results.

B: A body of evidence including studies rated as 2++ directly applicable to the target population, and demonstrating overall consistency of results; or extrapolated evidence from studies rated as 1++ or 1+.

C: A body of evidence including studies rated as 2+ directly applicable to the target population and demonstrating overall consistency of results; or extrapolated evidence from studies rated as 2++.

D: Evidence level 3 or 4; or extrapolated evidence from studies rated as 2+.

Good practice point: Recommended best practice based on the clinical experience of the guideline development group.

The ASRM practice committee does not grade the available evidence but refers to original studies only. The ACCP gives recommendations from 1A through 2C. The number (1 or 2) refers to the strength of the recommendation (1: strong recommendation, 2: weak recommendation), and the letters (A, B or C) indicate the quality of the evidence on which the recommendation is based (A: high quality evidence, B: moderate quality evidence, C: low quality evidence).

These differences in nomenclature are relevant, as a number of diagnostic tests are performed once the diagnosis of recurrent pregnancy loss is confirmed. Where possible, we describe the type of pregnancy loss in more detail, e.g. single miscarriage, recurrent miscarriage, biochemical pregnancy together with the definition as mentioned in the study, but this is not always possible.

A majority of pregnancy losses that occur before 10 weeks’ gestation are due to chromosomal errors arising from non-inherited, non-disjunctional events. Late pregnancy losses (e.g. >24 weeks’ gestation) occur sporadically and are more often due to maternal factors, such as preeclampsia. Despite a diagnostic work-up, in approximately 50% of the cases no cause can be identified. Uterine, hormonal and chromosomal abnormalities, endocrine and immune disorders have all been associated with (recurrent) pregnancy loss.

Maternal age is the most important risk factor for pregnancy loss and likely reflects the prevalence of underlying random numeric chromosome errors; the probability of pregnancy loss is around 9% in women aged 20-24 and increases to over 50% in women who are older than 42 years. Although after a first pregnancy loss the chance of live birth in a subsequent pregnancy is similar to the chance of live birth for primigravidae, it decreases with an increasing number of previous pregnancy losses. The psychological distress induced by experiencing recurrent pregnancy loss is relevant, as a number of diagnostic tests are performed once the diagnosis of recurrent pregnancy loss is confirmed.
loss is high. Thrombophilia can be acquired or inherited, and can be identified in approximately half of all patients with venous thromboembolism.

Acquired thrombophilia comprises the antiphospholipid syndrome that consists of clinical criteria combined with persistent presence of laboratory abnormalities, e.g. lupus anticoagulant, antibodies against cardiolipin, or antibodies against beta 2 glycoprotein 1, tested at least 12 weeks apart. Clinical criteria include either venous or arterial thrombosis, or pregnancy morbidity, defined as three or more unexplained pregnancy losses before 10 weeks’ gestation, one or more unexplained intrauterine fetal death beyond 20 weeks’ gestation or one or more premature birth before 34 weeks' gestation, due to eclampsia, severe preeclampsia or recognized features of placental insufficiency.

### Methods

For this comprehensive review, we searched Pubmed to identify relevant articles. We used (combinations of) the following search terms: ‘anticoagulants’, ‘heparin’, ‘low-molecular-weight heparin’, ‘aspirin’, ‘association’, ‘pregnancy complications’ ‘fetal death’, ‘pregnancy loss’, ‘pregnancy outcome’, ‘prognosis’, ‘thrombophilia’ and ‘antiphospholipid syndrome’. Reference lists of identified articles were scanned for relevant citations. For studies describing prognosis of pregnancy after pregnancy loss only contemporary studies were considered (i.e. published from 1995 onward).

### Results

#### Pregnancy loss and thrombophilia

##### Association

Pregnancy loss occurs more frequently in women with thrombophilia, and the strengths of the associations are summarized in **Table II**. The term thrombophilia is used to describe ‘endogenous’ risk factors for venous thromboembolism. Thrombophilia can be acquired or inherited, and can be identified in approximately half of all patients with venous thromboembolism.

Acquired thrombophilia comprises the antiphospholipid syndrome that consists of clinical criteria combined with persistent presence of laboratory abnormalities, e.g. lupus anticoagulant, antibodies against cardiolipin, or antibodies against beta 2 glycoprotein 1, tested at least 12 weeks apart. Clinical criteria include either venous or arterial thrombosis, or pregnancy morbidity, defined as three or more unexplained pregnancy losses before 10 weeks’ gestation, one or more unexplained intrauterine fetal death beyond 20 weeks’ gestation or one or more premature birth before 34 weeks’ gestation, due to eclampsia, severe preeclampsia or recognized features of placental insufficiency.

**Table II - Association between Pregnancy Complications and Thrombophilia.**

<table>
<thead>
<tr>
<th>Type of Thrombophilia</th>
<th>Miscarriage (1st or 2nd trimester OR [95% CI])</th>
<th>Recurrent 1st trimester miscarriage OR [95% CI]</th>
<th>Non-recurrent 2nd trimester miscarriage OR [95% CI]</th>
<th>Stillbirth (1st trimester loss) OR [95% CI]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V Leiden mutation (homozygous)</td>
<td>2.7 (1.2–5.6)</td>
<td>*</td>
<td>*</td>
<td>2.0 (0.4–9.7)</td>
</tr>
<tr>
<td>Factor V Leiden mutation (heterozygous)</td>
<td>1.7 (1.2–2.6)</td>
<td>1.9* (1.2–3.8)</td>
<td>4.1* (1.8–9.9)</td>
<td>2.1 (1.1–4.2)</td>
</tr>
<tr>
<td>Prothrombin G20210A mutation (heterozygous)</td>
<td>2.5 (1.2–5.5)</td>
<td>2.7 (1.4–5.3)</td>
<td>8.6 (2.2–34.0)</td>
<td>2.7 (1.3–5.5)</td>
</tr>
<tr>
<td>Antithrombin deficiency</td>
<td>0.9 (0.2–4.5)</td>
<td>NA</td>
<td>NA</td>
<td>7.6 (0.3–196.4)</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>2.3 (0.2–26.4)</td>
<td>NA</td>
<td>NA</td>
<td>3.1 (0.2–31.5)</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>3.6 (0.4–35.7)</td>
<td>NA</td>
<td>NA</td>
<td>20.1 (2.7–192.2)</td>
</tr>
<tr>
<td>Anticardiolipin antibodies</td>
<td>8.6 (1.2–61.7)</td>
<td>5.1 (1.3–16.0)</td>
<td>NA</td>
<td>3.3 (1.4–8.7)</td>
</tr>
<tr>
<td>Anti-beta 2 glycoprotein 1 antibodies</td>
<td>NA</td>
<td>2.12 (0.63–6.55)</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Lupus anticoagulants (Non-specific inhibitor)</td>
<td>3.0 (1.0–9.8)</td>
<td>NA</td>
<td>14.3 (4.7–42.2)</td>
<td>2.4 (0.8–7.0)</td>
</tr>
</tbody>
</table>

Statistically significant odds ratios are indicated in bold. Note: data are derived from systematic reviews. Terminology of pregnancy loss at various gestational ages may vary among included studies. *Homozygous and heterozygous carriers were grouped together; it is not possible to extract data for zygosity. (NA = not available).
Antiphospholipid syndrome is an example of a syndrome that has been defined without understanding the etiology of the disease, based solely on the observation that young individuals with systemic lupus erythematosus (SLE) often suffer from unprovoked thrombosis or unexplained pregnancy losses and show the presence of antiphospholipid antibodies in their plasma.21

Inherited thrombophilia comprises deficiencies of the natural anticoagulant proteins, antithrombin, protein C, or protein S, that down regulate the formation of thrombin, or the gain of function mutations in coagulation factors Va (leading to activated protein C resistance, Factor V Leiden) and prothrombin (prothrombin G20210A mutation).22 In the 1990’s, inherited thrombophilia was also found to be associated with recurrent pregnancy loss, late pregnancy loss and preeclampsia.27/28

**Pathophysiology**

The association between thrombophilia and pregnancy loss has been identified, but the underlying mechanism remains unclear. One presumption was that pregnancy loss might be caused by thrombosis in decidual vessels.29 However, the concept that recurrent pregnancy loss and preeclampsia can be attributed to thrombosis is likely to represent an oversimplification.

Coagulation and inflammation are closely related pathways30 and several observations have implicated a role for both procoagulant and inflammatory pathways in pregnancy failure.30/31 Inflammatory changes are essential for the various processes of successful embryonic implantation, such as trophoblast invasion, angiogenesis, and placental growth, but how the initial inflammatory response during the implantation period is controlled to protect the semi-allogenic fetus is poorly understood. In procoagulant thrombomodulin-deficient mice, activated coagulation factors induce cell death and inhibit the growth of trophoblast cells.32 In-vitro experiments have shown that antiphospholipid antibodies prevent extravillous trophoblast differentiation.33 In addition, antiphospholipid antibodies are thought to disrupt crystallization of Annexin A5, thereby limiting its anticoagulant properties.34/35 Furthermore, in mice, antiphospholipid antibodies induce complement activation, the degree of which is closely correlated to fetal death.36 In an observational study, levels of circulating procoagulant microparticles were higher in women with recurrent early miscarriage as compared to controls.37 These observations clearly indicate a role for both coagulation and inflammation, but a better understanding of the underlying mechanisms and, most notably, translation to the human situation of implantation and pregnancy failure is urgently needed.

**Plausible biology of beneficial effect of antithrombotic agents**

In light of the presumed pathophysiology of the association between pregnancy loss and thrombophilia, the hypothesis of a beneficial effect of antithrombotic agents has arisen. The mechanism of action of heparin is mostly attributed to its anticoagulant activity, a result of binding to and potentiating the action of antithrombin.38 This anticoagulant acitivity may reduce thrombosis in the (micro) vasculature of the placenta. In addition, blockage of adhesion proteins (selectins) is an anti-inflammatory property of heparin on tumor cell lines in vitro.39 The clinical relevance of this in pregnancy is uncertain, as proper expression and interaction of adhesive molecules is essential for successful implantation. Furthermore, heparin promotes extravillous trophoblast differentiation in placental tissue in vitro,40 although the effect on trophoblast differentiation varies between Ufh and LMWH. LMWH reduces antiphospholipid antibody binding to trophoblast cells in vitro.41 The mechanism of effect of aspirin to increase the chance of live birth in women with pregnancy loss is less clear. As a platelet inhibitor, aspirin may reduce coagulation activity in the placenta. Protease activated receptors (PARs) play a role in coagulation and thrombin mediates activation of platelets via PAR-4. As mentioned previously, in thrombomodulin deficient mice, trophoblast development is inhibited. PAR-4 deficiency of the mother or the absence of maternal platelets restores normal development in one-third of thrombomodulin deficient embryos.42 This indicates that PAR-4 mediated activation of maternal platelets is a likely mechanism responsible for fetal loss in this mouse model. Inhibition of platelets by aspirin is therefore hypothesized to reduce placental coagulation and may contribute to successful placental development.

It was demonstrated in murine models that appropriate complement regulation is necessary to control placental inflammation and that a local increase in complement activation fragments is highly deleterious to the
developing fetus. It is proposed that antiphospholipid antibodies, in addition to their direct effects on platelet and endothelial cell targets, generate complement split products, which ultimately lead to fetal loss in the obstetric antiphospholipid syndrome. As coagulation is intertwined with the immune system, deleterious effects of coagulation, which in turn may be activated by the complement system, may be inhibited by platelet aggregation inhibitors such as aspirin.

Prognosis of live birth without pharmacological treatment after pregnancy loss

**TABLE III** summarizes the results from contemporary observational studies investigating the prognosis of live birth without pharmacological treatment in women with previous pregnancy loss. We have categorized studies for women with antiphospholipid antibodies (not always fulfilling all criteria for antiphospholipid syndrome), and those with and without inherited thrombophilia. Furthermore, if possible, we categorized the type of pregnancy loss: early versus late and single versus recurrent. Live birth rates vary between 0% and 99%, indicating the difficulty in drawing conclusions. Study populations vary and, perhaps more importantly, the onset of follow-up differs per study. It appears that live birth rates in women recruited in very early pregnancy, e.g. from 5 weeks amenorrhea, are substantially lower than in women who are recruited from 12 weeks’ gestation. This is likely explained by the fact that women with early miscarriages are not included in the latter study population.

![Table III - Prognosis of live birth after pregnancy loss without pharmacological treatment; results from contemporary observational studies.](image-url)
The studies by Rai (1995), Rai (2000), Rai (2002), Coppens (2007), Lindqvist (2006), Sugira-ogasawara (2008), Chauleur (2010) and Lund (2010) investigated multiple cohorts; in this table discussed separately. Data for the study by Lindqvist and Merlo (2006) was not available from the paper; provided by Dr. Lindqvist, personal communication. Study population was a subgroup of a cohort study\(^*\) including 2480 gravidae; recruited at a mean of 12 weeks of gestation.

a: Age is presented as mean (+/− standard deviation) or median (range) as reported in original study reports. If age was not specified for subgroups, age for the original total cohort is given.
b: Mean age at time of first pregnancy.
c: Description of onset of follow-up was not often clearly described in original study reports; quoted from the original articles.
d: Inclusion criterion of a minimum of three consecutive losses was irrespective of gestational age, but the median number of late miscarriages (14 – 21+6 weeks’ GA) and stillbirths (after 22 weeks’ GA) was 0.
e: Median number of late miscarriages 1, (range 1–3).
f: Personal communication with study authors. A majority of patients had recurrent early miscarriage.

\(\text{ab2gp1}\), antibeta2glycoprotein 1 antibodies; \(\text{aca}\), anticardiolipin antibodies; \(\text{aps}\), antiphospholipid syndrome; \(\text{fvl}\), Factor V Leiden; \(\text{ga}\), gestational age; \(\text{la}\), lupus anticoagulant; \(\text{ptm}\), prothrombin \(\text{g}20210\text{a}\) mutation; \(\text{na}\), not available

Clinical studies investigating the efficacy of antithrombotic agents for pregnancy loss

Despite the uncertainty of prognosis after pregnancy loss and the mechanisms of action, physicians frequently prescribe antithrombotic agents in pregnancy. Of note, beneficial effects of antithrombotic agents are frequently suggested by results from observational studies that have intrinsic methodological issues undermining their validity to assess efficacy of an intervention. Although clinical trials have been performed, these are generally limited by small sample sizes and often lack a control arm without active intervention.

As described above, pregnancy loss is not a homogeneous disorder. Recurrence of pregnancy loss as well as the presence of thrombophilia may identify women with pregnancy loss who are more likely to benefit from antithrombotic agents than others. In the next paragraphs, we distinguish four groups: 1) women with antiphospholipid syndrome; 2) women with one previous pregnancy loss and inherited thrombophilia; 3) women with recurrent pregnancy loss and inherited thrombophilia; and 4) women with unexplained recurrent pregnancy loss. **TABLE IV** summarizes the trials.
### TABLE IV - Available evidence from randomized controlled trials investigating the efficacy of antithrombotics in women with a history of recurrent pregnancy loss; effect on live birth.

<table>
<thead>
<tr>
<th>Study</th>
<th>Antithrombotic Therapy</th>
<th>Comparator</th>
<th>Follow-up (months)</th>
<th>Live Birth Rate (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study A</td>
<td>Low-dose aspirin</td>
<td>Placebo</td>
<td>12</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td>Study B</td>
<td>Low-dose aspirin</td>
<td>Placebo</td>
<td>24</td>
<td>60</td>
<td>0.03</td>
</tr>
<tr>
<td>Study C</td>
<td>Low-dose aspirin</td>
<td>Placebo</td>
<td>36</td>
<td>70</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Note: Further details and specific antithrombotic regimens can be found in the respective studies.
The study by Tulppala (1997) investigated both women with and without antiphospholipid antibodies; these groups are discussed separately in this Table. The study by Brenner (2005) included women with inherited thrombophilia, antiphospholipid antibodies. The study by Laskin (2009) included women with recurrent pregnancy loss and antiphospholipid antibodies, inherited thrombophilia, or antinuclear antibodies; only the subgroup of women with antiphospholipid antibodies was included in the Table. The study by Giancotti (2012) included women with unexplained recurrent miscarriage, as well as women with recurrent miscarriage associated with antiphospholipid antibodies or inherited thrombophilia, the subgroup of women with antiphospholipid antibodies was excluded from this Table.


Data for this table on live birth were provided by study authors for the studies by Badawy 2008 and Giancotti 2012 (personal communication).

The criteria for antiphospholipid syndrome vary per study.

The risk of bias in individual studies was determined according to the Cochrane Collaboration’s tool for assessing risk of bias antiphospholipid antibodies.

c-The rate of successful pregnancy in those with secondary abortions in thromboprophylaxis group (81.8%) was higher than the control group (9.25%) (p=0.02).

ufh, unfractionated heparin; LMWH, low-molecular-weight heparin; ptt, prothrombin time; ga, gestational age.
Obstetric antiphospholipid syndrome

Single pregnancy loss

Clinical trials on antithrombotic agents that exclusively included women with antiphospholipid syndrome based on the clinical criterion of one late previous pregnancy loss have not been performed.

Recurrent pregnancy loss

Clinical trials that have investigated the efficacy of antithrombotic therapy in women with recurrent pregnancy loss and antiphospholipid syndrome are few and have been summarized in a Cochrane systematic review.\(^{46}\) Two more recent systematic reviews have addressed the efficacy of heparin combined with aspirin versus aspirin only.\(^{47}\)\(^{48}\)

Aspirin only – The pooled results of three very small trials (total number of 71 participants) showed no effect of aspirin only as compared to no treatment (RR of pregnancy loss of 1.05, 95% CI 0.66 – 1.68).\(^{46}\)

Heparin only – A recent trial compared the efficacy of the LMWH (bemiparin 2500 U once daily, n = 80) with aspirin 100mg (n = 61) in women with at least two consecutive miscarriages <20 weeks’ gestation.\(^{46}\) Results suggest a beneficial effect of bemiparin over aspirin (RR for live birth for women treated with bemiparin 1.20, 95% CI 1.00 – 1.43). The allocation was not concealed, as the study was quasi-randomized (sequential assignment of treatment) and for unclear reasons more women were allocated to bemiparin compared to aspirin.

Heparin combined with aspirin – The evidence for a beneficial effect is strongest for treatment with UFH combined with aspirin as compared to aspirin only, in women with recurrent pregnancy loss and antiphospholipid syndrome. Results of one meta-analysis showed that in women with two or more pregnancy losses, treatment with UFH combined with aspirin (n = 103) reduced the chance of first trimester miscarriage when compared to aspirin only (n = 109) (RR 0.26, 95% CI 0.14 – 0.48).\(^{46}\) In two trials in which treatment with LMWH combined with aspirin (n = 96) was compared to aspirin only (n = 90), the pooled relative risk for pregnancy loss was 0.70, without reaching statistical significance (95% CI 0.34 – 1.45).\(^{48}\) When comparing any heparin (UFH or LMWH) combined with aspirin (n = 199) with aspirin only (n = 199), the beneficial effect of heparin of reducing the risk of first trimes-

ter miscarriage was maintained (RR 0.39, 95% CI 0.24 – 0.65), with little statistical heterogeneity (I² 10%).\(^{47}\) Interestingly, in the studies that observed a profound effect of UFH added to aspirin, the chances of a live birth in the aspirin only arms were only 44% and 42%\(^{50}\)\(^{51}\). These are markedly lower than in the comparator arms of the other studies comparing LMWH and aspirin to aspirin only\(^{52}\) or aspirin to placebo,\(^{52}\)\(^{53}\)\(^{54}\) in which the chances of a live birth varied between 68% and 80%, which indicates clinical heterogeneity between the trials. In a pilot randomized trial (n = 56), a prospective cohort study (n = 50) and a recent randomized trial (n = 60), the use of LMWH and UFH (both combined with aspirin) were directly compared and the results did not suggest a difference in effects.\(^{54}\)\(^{55}\) In one trial, two doses of LMWH (enoxaparin 40mg and enoxaparin 20mg, both combined with aspirin) were compared (n = 60); no difference in live birth was observed (RR 1.10, 95% CI 0.81 – 1.49).\(^{53}\) To our knowledge, there are no trials that compared heparin (UFH or LMWH) combined with aspirin with no treatment or placebo.

The evidence for treatment with heparin combined with aspirin was obtained in two studies including women with three or more pregnancy losses,\(^{54}\)\(^{55}\) and one study including women with two or more first or second trimester losses.\(^{47}\) As a result, guidelines of the American College of Chest Physicians (ACCP) recommend UFH or LMWH combined with aspirin for women with antiphospholipid syndrome based on three or more pregnancy losses, but refrain from recommendations for women with antiphospholipid syndrome based on clinical criteria of a single late pregnancy loss or placental insufficiency.\(^{44}\) Guidelines of the Royal College of Obstetricians and Gynecologists state that ‘pregnant women with antiphospholipid syndrome should be considered for treatment with low-dose aspirin combined with heparin to prevent further miscarriage’ without further reference toward clinical criteria of antiphospholipid syndrome in the recommendation.\(^{4}\)

Although evidence for a beneficial effect of heparin combined with aspirin is at hand, this is based on studies with very low numbers of women and further studies reaffirming this efficacy are warranted. Furthermore, whether the efficacy of heparin combined with aspirin is truly similar when UFH is replaced by LMWH needs to be determined, as well as the effect of antithrombotic agents in different subgroups of women with antiphospholipid syndrome based on laboratory or clinical criteria (e.g. women with one late pregnancy loss). This is in agreement with a statement from the ESHRE Special Interest Group for Early Pregnancy (SIGEP) that there is a need
Recurrent pregnancy loss

Aspirin only – To our knowledge, no randomized controlled trials evaluating the efficacy of aspirin in women with inherited thrombophilia and recurrent pregnancy loss have been performed.

Heparin only – Only observational studies that have evaluated the outcomes of women with recurrent pregnancy loss treated with heparin compared to no treatment are available. These studies do not provide evidence for the efficacy of LMWH and should be regarded as hypothesis generating with a need for confirmation in randomized clinical trials. In a retrospective cohort study of women with three or more consecutive pregnancy losses and inherited thrombophilia, live birth was 70.2% in women treated with enoxaparin compared to 43.8% in historical control women who received no intervention. It should be noted that approximately 45% of the included women did not have an established form of thrombophilia, but were classified as such based on presence of C677T mutation in the MTHFR gene for which the association with thrombosis and pregnancy loss is unclear.

Heparin combined with aspirin – To our knowledge, a randomized controlled trial evaluating the efficacy of heparin combined with aspirin limited to women with inherited thrombophilia and recurrent pregnancy loss has not been performed.

Inherited thrombophilia

Little evidence is available for the effect of antithrombotic agents in women with a single pregnancy loss and inherited thrombophilia. Results from several small retrospective and prospective cohort studies in women with inherited thrombophilia, with or without previous pregnancy complications, suggest a beneficial effect of antithrombotic therapy to reduce pregnancy complications. These studies are heterogeneous with regard to study design and study population.

Single pregnancy loss

In a clinical trial, women with one previous pregnancy loss after 10 weeks’ gestation and heterozygous Factor V Leiden mutation, prothrombin G20210A mutation, or protein S deficiency, were allocated to enoxaparin 40mg once daily (n = 80) or to aspirin 100 mg (n = 80). Women who were treated with enoxaparin had a much higher chance of a live birth than those allocated to aspirin (86% and 29% respectively, 57% absolute risk reduction, odds ratio 15.5, 95% CI 7 to 34). However, methodological issues regarding concealment of allocation, lack of generalizability due to very stringent inclusion criteria, and the fact that women who experienced an early miscarriage after randomization were not taken into account were raised. The results of this single study have not been implemented in recent evidence based guidelines.

With the apparent lack of evidence for the efficacy of antithrombotic therapy in women with inherited thrombophilia and a single pregnancy loss, antithrombotic agents are not recommended for this indication.

For an international collaborative RCT to evaluate the type and duration of thromboprophylaxis in antiphospholipid syndrome, before this treatment is used systematically in routine clinical practice. However, based on the currently available evidence of studies with small numbers of participants, clinicians worldwide have adopted practice to prescribe antithrombotic agents to all women with obstetric antiphospholipid syndrome. Acquiring ethical approval for such studies and finding patients who are willing to participate in such randomized trials will, therefore, be a major challenge.

Antithrombotic therapy for pregnancy loss

Recurrent pregnancy loss

Aspirin only – To our knowledge, no randomized controlled trials evaluating the efficacy of aspirin in women with inherited thrombophilia and recurrent pregnancy loss have been performed.

Heparin only – Only observational studies that have evaluated the outcomes of women with recurrent pregnancy loss treated with heparin compared to no treatment are available. These studies do not provide evidence for the efficacy of LMWH and should be regarded as hypothesis generating with a need for confirmation in randomized clinical trials. In a retrospective cohort study of women with three or more consecutive pregnancy losses and inherited thrombophilia, live birth was 70.2% in women treated with enoxaparin compared to 43.8% in historical control women who received no intervention. It should be noted that approximately 45% of the included women did not have an established form of thrombophilia, but were classified as such based on presence of C677T mutation in the MTHFR gene for which the association with thrombosis and pregnancy loss is unclear.

Heparin combined with aspirin – To our knowledge, a randomized controlled trial evaluating the efficacy of heparin combined with aspirin limited to women with inherited thrombophilia and recurrent pregnancy loss has not been performed. In the SPIN, ALIFE, and HABENOX studies (see paragraph detailing unexplained recurrent pregnancy loss), small proportions of the study populations consisted of women with inherited thrombophilia (3.5%, 15.7% and 24.6% respectively). Unfortunately, subgroup analyses were
Based on the available evidence, various guidelines now unanimously recommend against the use of antithrombotic agents in women with unexplained recurrent pregnancy loss (Table 1).6/18

Adverse effects of antithrombotic therapy

Potential adverse effects of antithrombotic therapy include bleeding, local skin reactions such as itching and swelling and the more rare complications, heparin-induced thrombocytopenia (HIT) and heparin-induced osteopenia. In a systematic review the safety of LMWH for thromboprophylaxis and treatment of venous thromboembolism in pregnancy was evaluated, including data from 2777 pregnancies.80 As incidences of significant bleeding and allergic skin reactions were low (1.98%, 95% CI 1.50 – 2.57 and 1.80%, 95% CI 1.34 – 2.37 respectively) and there was only one case of osteoporotic fracture and no case of HIT, the authors concluded that LMWH is safe in pregnancy.

Although the incidence of bleeding and significant bleeding is low, avoiding any such bleeding by withholding ineffective therapy is preferred. Furthermore, local skin reactions due to administration of LMWH were reported in 40% of women treated with nadroparin in the Alife study, and in 29% of women in a prospective observational study.72/81

Testing for thrombophilia

Given the association between recurrent pregnancy loss and thrombophilia, a relevant question is whether testing for thrombophilia should be performed on a routine basis in this clinical setting. Understanding the possible cause of pregnancy loss provides an explanation for patients, and their doctors. However, if a test result only provides insight but does not alter clinical management its yield is limited and testing should not be performed. If, however, effective treatment is available, the results of testing may identify those women in whom antithrombotic therapy increases the chance of live birth in a subsequent pregnancy.

In women with three or more pregnancy losses and antiphospholipid antibodies, antithrombotic therapy increases the chance of live birth and hence testing is indicated. Whether testing for antiphospholipid antibodies is also relevant for women with a single late pregnancy loss (>10 weeks’ gestation) is arguable given the absence of evidence that these

Unexplained recurrent pregnancy loss

Several studies have investigated the efficacy of antithrombotic agents in women with unexplained recurrent pregnancy loss. Although some have reported beneficial effects of antithrombotic agents, concerns regarding study designs and external validity have been raised.74-77 High-quality evidence was obtained by means of five randomized clinical trials,54/71-75/76 of which three compared various antithrombotic agents with no treatment or placebo.54/71/75

In the SPIN study,71 294 women with two or more unexplained pregnancy losses were randomized to enoxaparin 40 mg combined with aspirin 75 mg plus standard surveillance or standard surveillance only. No effect of the medical intervention was observed (odds ratio for successful pregnancy 0.91, 95% confidence interval CI 0.52 – 1.59). In the Alife study, we randomized 364 women with two or more unexplained pregnancy losses to nadroparin 2850 IU combined with aspirin 80 mg, aspirin 80 mg only, or placebo (for aspirin) before conception or at a maximum gestational age of 6 weeks.72 Of these women, 299 became pregnant. The chance of live birth did not differ between the treatment groups (88 of live birth for women who became pregnant were 1.03 (95% CI 0.85 – 1.25) for nadroparin combined with aspirin, and 0.92 (95% CI 0.75 – 1.13) for aspirin only, compared to placebo). In an older trial, 66 women with recurrent pregnancy loss were randomized to aspirin or no treatment. The subgroup analysis of women with unexplained recurrent pregnancy loss (27 women randomized to aspirin and 27 to no treatment) showed no difference in live birth between both groups (i.e. LMWH with aspirin, LMWH only or aspirin only), the results showed no differences in live birth rates between these treatments.73/81

Testing for thrombophilia

Given the association between recurrent pregnancy loss and thrombophilia, a relevant question is whether testing for thrombophilia should be performed on a routine basis in this clinical setting. Understanding the possible cause of pregnancy loss provides an explanation for patients, and their doctors. However, if a test result only provides insight but does not alter clinical management its yield is limited and testing should not be performed. If, however, effective treatment is available, the results of testing may identify those women in whom antithrombotic therapy increases the chance of live birth in a subsequent pregnancy.

In women with three or more pregnancy losses and antiphospholipid antibodies, antithrombotic therapy increases the chance of live birth and hence testing is indicated. Whether testing for antiphospholipid antibodies is also relevant for women with a single late pregnancy loss (>10 weeks’ gestation) is arguable given the absence of evidence that these
women benefit from antithrombotic agents. Likewise, testing women with a single or recurrent pregnancy loss for inherited thrombophilia in routine patient care is not indicated since it is unknown whether women with inherited thrombophilia benefit from antithrombotic agents in a subsequent pregnancy. Obtaining evidence of the efficacy of antithrombotic therapy in these women is essential, and until this evidence is available, we argue that testing for inherited thrombophilia for this indication should only be performed in the context of clinical trials.

As a result of the lack of evidence, recommendations regarding investigations in cases of (recurrent) pregnancy loss differ per guideline and per country and are listed in Table I. For example, RCOG guidelines recommend testing for antiphospholipid syndrome and inherited thrombophilia in women with previous second trimester losses; ACCP does not recommend for or against testing for antiphospholipid syndrome in women with late pregnancy loss; and ACCP and British Society for Haematology guidelines suggest not to screen for inherited thrombophilia for women with a history of pregnancy complications.

**Future perspectives**

Although some insight into the pathophysiology of (recurrent) pregnancy loss and its association with thrombophilia has been gained, much has yet to be unravelled. The discussed mechanisms by which both acquired and inherited thrombophilia may play a causal role in the pathology of pregnancy loss are plausible as supported by the in-vitro and animal studies. However, further studies investigating these mechanisms as well as verification of generated hypotheses in humans, are required. Ultimately, we need to obtain evidence from high quality randomized clinical trials regarding benefits and harms of antithrombotic agents to increase live birth in women with both acquired and inherited thrombophilia. Recently, the ALIFE2 study (www.trialregister.nl, NTR3361) has started recruiting; this is a trial in which women with inherited thrombophilia and recurrent pregnancy loss will be randomized to either treatment with LMWH plus standard pregnancy surveillance or standard pregnancy surveillance only. Randomized trials investigating the effect of antithrombotic therapy in women with antiphospholipid syndrome are needed.

**Conclusions**

Over the past few years, a great body of evidence has been obtained in the field of (recurrent) pregnancy loss and its association with thrombophilia, but further compelling evidence is awaited. Guidelines for the management of women with recurrent pregnancy loss with or without thrombophilia are conflicting in terms of terminology and investigations, but unanimous regarding the need for further studies. In order to understand the association between thrombophilia and recurrent pregnancy loss, the mechanistic role of coagulation in reproduction has to be elucidated. For clinical practice, antithrombotic agents should not be given to women with unexplained recurrent pregnancy loss. Although antithrombotic agents for women with antiphospholipid syndrome are widely prescribed, the evidence underpinning this strategy is very limited. Randomized controlled trials investigating which women, particularly those with thrombophilia, benefit from antithrombotic therapy to increase their chance of live birth are essential. To overcome the challenges posed by conducting these clinical trials, (international) collaboration and guideline adherence are required, and antithrombotic therapy should be prescribed only if recommended or in the context of such a clinical trial.


35. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two or more recurrent spontaneous abortions. RCOG 2011.


Chapter 3

Long-term prognosis of women with recurrent miscarriage with or without inherited thrombophilia

Paulien G. de Jong, Stef P. Kaandorp, Remi O. Kool, Barbara A. Hutten, Saskia Middeldorp and Mariëtte Goddijn

Submitted for publication
Abstract

Several studies evaluated the outcome of the first pregnancy after recurrent miscarriage. We aimed to determine the chance of a live birth over an extended follow-up period and to investigate the relevance of prognostic variables for time to live birth, including inherited thrombophilia. We collected data on pregnancies occurring in women who previously participated in the ALIFE study (ISRCTN 58496168), a randomized controlled trial investigating the efficacy of antithrombotic therapy on live birth in women with unexplained recurrent miscarriage. Follow-up data were available for 271 of 364 (74%) women, with a median follow-up of 84 months (15 to 169 months). The median time to a live birth was 19 months [9 to 105 months], and the cumulative probability of live birth, taking competing risks into account, was 15%, 55%, 77% and 81% after 1, 2, 5 and 10 years respectively. The cumulative probability of live birth was similar in women with and without inherited thrombophilia (hazard ratio 1.15, 95% confidence interval 0.77 – 1.72). The 81% cumulative incidence of live birth after 10 years is reassuring information for women with unexplained recurrent miscarriage with or without inherited thrombophilia. This is essential information when counselling couples with recurrent miscarriage.

Introduction

Recurrent miscarriage, as defined by the loss of two or more pregnancies before the fetus reaches a viable age, affects approximately 3% of women attempting to conceive. Hematologists are often involved in the evaluation and management of these patients, especially when thrombophilia is present. Low-molecular-weight heparin and acetylsalicylic acid are prescribed to women diagnosed with recurrent miscarriage in antiphospholipid syndrome to increase the chance of live birth in a subsequent pregnancy. Whether anticoagulants increase the chance of live birth in women with recurrent miscarriage and inherited thrombophilia is currently being evaluated (NTR3361, www.trialregister.nl).

When counselling couples with recurrent miscarriage, not only information regarding their chance of a live birth in a subsequent pregnancy is important, but also their chance of a live birth if more pregnancy attempts are made. Women with recurrent miscarriage are prone to undergo many diagnostic tests and ineffective therapeutic interventions. If the prognosis
Materials and methods

Study population and design

The study population consisted of women who had participated in the ALIFE study (ISRCTN 58496168), as reported previously. In short, from 2004 through 2008, 364 women with two or more unexplained miscarriages before 20 weeks’ gestation were randomized to receive either low-molecular-weight heparin (LMWH) plus acetylsalicylic acid (ASA), ASA only or placebo (for ASA). At time of randomization, they were either attempting to conceive or less than six weeks pregnant. Unexplained recurrent miscarriage was diagnosed in case of absence of abnormal parental karyotype, significant intrauterine abnormalities, lupus anticoagulant or anti-cardiolipin IgG and IgM antibodies, and abnormal fasting level of homocysteine.

Between November 2012 and September 2014, we sent information on the study and a request for participation by postal mail, and subsequently contacted women by phone to collect follow-up data. Data on all pregnancies after the ALIFE study including dates and outcomes were collected using a predefined case record form. Only for women who were not reached after multiple attempts but for whom medical charts were available in the Academic Medical Center (the central study centre), medical charts were reviewed.

We defined the date of the last pregnancy before randomization in ALIFE as baseline (start of follow-up).

Outcome

The primary outcome was time to birth of a living neonate in the period from the last pregnancy prior to randomization in the ALIFE study until the end of follow-up.

Statistical analyses

The probability of a live birth was estimated using a competing risks analysis, where reaching the age of 46 years was considered as a competing risk for live birth. Data were censored when a live birth had not occurred or the age of 46 had not been reached before the end of follow-up.
We used data of women who participated in the follow-up study (primary analysis). We also performed a secondary analysis, including all women who participated in the ALIFE study (n=364). For women without available follow-up data, only data available from their participation in the ALIFE study were included. In the secondary analysis, data of women of whom no follow-up data were available were censored when a miscarriage, extra-uterine pregnancy or termination of pregnancy occurred in the ALIFE study period, when pregnancy was not reached within 24 months after randomization in the ALIFE study or at July 2009 (end of the ALIFE study), whichever came first. For women who dropped out, were lost to follow-up in the ALIFE study or who were not reached in follow-up, data were censored at the timing of drop out (if known) or at 1 year post-randomization.

To investigate predictors of time to live birth, data of women of whom follow-up data were available were used. We first explored the relative prognostic significance of maternal age (age at the time of the last pregnancy before ALIFE, as categorical covariate (categorized as age younger than 26, 26 to 30, 31 to 35, 36 to 40, and 41 years or older), the number of preceding miscarriages (two versus three or more miscarriages as dichotomous covariate), previous live birth (yes or no) and inherited thrombophilia (presence of any form of inherited thrombophilia, yes or no; and Factor V Leiden separately, yes or no) for time to live birth in univariate analyses. Next, a multivariable proportional hazards model corrected for competing risks was performed, to evaluate the individual contributions of the predictors. Inclusion in the final model was determined by backward stepwise elimination, with a probability of \( P \)-to-remove \( \geq 0.2 \).

Statistical analyses were performed using SPSS version 20.0 software and using the R statistical package version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). Two-sided probability values of \(<0.05\) were considered statistically significant.

**Ethics**

The ALIFE study was approved by the local ethics committee (ethics registration no. 02/173) and all women provided written informed consent. For this follow-up study, the local ethics committee of the Academic Medical Center did not deem formal assessment necessary due to the nature of the questionnaires (waiver registration no. 2011_336#C20121219).

**Results**

A flow chart of the study population is depicted in **FIGURE 1**. Of 364 participants in the ALIFE study, 3 (0.8%) women had died after participation in the study, and follow-up data were available for 271 (74%) women, including 16 women of whom additional follow-up data were retrieved from medical charts. Of the 167 women who did not have a live birth during ALIFE, follow-up data were available for 113 (68%) women. Baseline characteristics for the full cohort and for women of whom follow-up data were available are summarized separately (**TABLE 1**). The mean age of the full cohort at baseline was 32.7 (range 20-42) years and the median time of follow-up since last pregnancy prior to randomization was 7 (range 1 to 14) years for women of whom follow-up data were obtained.

**FIGURE 1 - Flow chart of study population.**

The flow chart indicates which women were included in the primary and secondary analysis.
Live birth and time to live birth

In the primary analysis of 271 women with complete follow-up data, 213 (79%) achieved a live birth (Table II). Of these, 158 (58%) had a live birth during their participation in the ALIFE study, whereas 55 women (20%) had at least one live birth thereafter. The number of pregnancies calculated from the start of follow-up ranged from 0 to 12, with 10 women who had 6 or more pregnancies. The median time to live birth was 19 months, ranging from 9 months to 105 months.

Figure II shows the cumulative probability of live birth, taking into account competing risks. The probabilities of live birth were 15%, 55%, 77% and 81% after 1, 2, 5 and 10 years respectively, with only limited increase of the probability after 4 years.

In the secondary analysis, also including data of women of whom only data during their participation in the ALIFE study were available, the cumulative probability of live birth did not differ materially from the primary analyses (increasing from 15%, 54%, 77%, to 81% after 1, 2, 5 and 10 years respectively, with only limited increase of the probability after 4 years.

In the secondary analysis, also including data of women of whom only data during their participation in the ALIFE study were available, the cumulative probability of live birth did not differ materially from the primary analyses (increasing from 15%, 54%, 77%, to 81% after 1, 2, 5 and 10 years respectively, with only limited increase of the probability after 4 years.

Determinants of prognosis

Results of the univariate and multivariable analyses of the association between patient characteristics and time to live birth are shown in Table III. Although age was not significantly associated with time to live birth in the univariate analysis, a trend for an inverse association was observed when the age categories (31-35, 36-40, ≥41 years) were compared to the reference category (26-30 years). A history of three or more previous miscarriages was significantly associated with a longer time to live birth in univariate analysis when compared to a history of two miscarriages.

In the multivariable analysis with all potential prognostic variables in the model (full model), only the number of previous miscarriages (HR 0.75, 95% CI 0.57 – 0.97) was an independent predictor of time to live birth, while a previous live birth and presence of any form of inherited thrombophilia were not significantly associated. After stepwise backward elimination only the number of previous miscarriages remained in the model, corresponding to live birth probabilities of 15%, 63%, 86% and 88% after 1, 2, 5 and 10 years in women with two miscarriages versus 15%, 50%, 71% and
73% in women with three or more miscarriages (FIGURE II-B).

Factor V Leiden, when tested independently of the other forms of inherited thrombophilia, was not associated with time to live birth in both the univariate analysis (HR 0.90, 95% CI 0.52 – 1.57) and multivariable analysis.

TABLE II - Number of pregnancies and live births during follow-up in women with recurrent miscarriage.

<table>
<thead>
<tr>
<th>Number of subsequent pregnancies</th>
<th>Complete follow-up available n=271</th>
<th>Full cohort ALIFE study n=384</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>20 (7.4)</td>
<td>46 (12.6)</td>
</tr>
<tr>
<td>1</td>
<td>77 (28.4)</td>
<td>144 (39.6)</td>
</tr>
<tr>
<td>2</td>
<td>88 (32.5)</td>
<td>88 (24.2)</td>
</tr>
<tr>
<td>3</td>
<td>38 (14.0)</td>
<td>38 (10.4)</td>
</tr>
<tr>
<td>4</td>
<td>26 (9.6)</td>
<td>26 (7.1)</td>
</tr>
<tr>
<td>5</td>
<td>12 (4.4)</td>
<td>12 (3.3)</td>
</tr>
<tr>
<td>6</td>
<td>4 (1.5)</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td>7</td>
<td>4 (1.5)</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td>8</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
</tr>
<tr>
<td>12</td>
<td>1 (0.4)</td>
<td>1 (0.3)</td>
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Frequency of live births

<table>
<thead>
<tr>
<th>Number of live births</th>
<th>Complete follow-up available n=271</th>
<th>Full cohort ALIFE study n=384</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>58 (21)</td>
<td>112 (31)</td>
</tr>
<tr>
<td>1</td>
<td>114 (42)</td>
<td>153 (42)</td>
</tr>
<tr>
<td>2</td>
<td>88 (32)</td>
<td>87 (24)</td>
</tr>
<tr>
<td>3</td>
<td>10 (4)</td>
<td>10 (3)</td>
</tr>
<tr>
<td>4</td>
<td>2 (0.7)</td>
<td>2 (0.5)</td>
</tr>
</tbody>
</table>

Pregnancy in which first live birth was achieved n (%) (cumulative %)

<table>
<thead>
<tr>
<th>Pregnancy in which first live birth was achieved</th>
<th>Complete follow-up available n=271</th>
<th>Full cohort ALIFE study n=384</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st</td>
<td>167 (62) (62)</td>
<td>206 (57) (57)</td>
</tr>
<tr>
<td>2nd</td>
<td>30 (11) (73)</td>
<td>30 (8) (65)</td>
</tr>
<tr>
<td>3rd</td>
<td>8 (3) (76)</td>
<td>8 (2) (67)</td>
</tr>
<tr>
<td>4th</td>
<td>5 (2) (78)</td>
<td>5 (1) (68)</td>
</tr>
<tr>
<td>5th</td>
<td>2 (0.7) (78)</td>
<td>2 (0.5) (69)</td>
</tr>
<tr>
<td>6th</td>
<td>1 (0.4) (78)</td>
<td>1 (0.3) (69)</td>
</tr>
</tbody>
</table>

Percentage totals do not always meet 100% due to rounding.
C

(A) Cumulative probability of a first live birth or competing risk for live birth (i.e. reaching the age of 46) in 271 women with unexplained recurrent miscarriage. (B) Cumulative probability of a first live birth or competing risk for live birth (i.e. reaching the age of 46) after unexplained recurrent miscarriage, compared between women with 2 vs 3 or more previous miscarriages. (C) Cumulative probability of a first live birth or competing risk for live birth (i.e. reaching the age of 46) after unexplained recurrent miscarriage; compared between women with inherited thrombophilia vs no inherited thrombophilia. t = 0 indicates the start of follow-up, defined as the date of the last pregnancy before randomization in the ALIFE study.

<table>
<thead>
<tr>
<th>HR (95% CI)</th>
<th>HR (95% CI)</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Younger than 26</td>
<td>0.78 (0.47 – 1.30)</td>
<td>0.74 (0.45 – 1.21)</td>
</tr>
<tr>
<td>26 to 30</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>31 to 35</td>
<td>0.79 (0.57 – 1.10)</td>
<td>0.80 (0.58 – 1.11)</td>
</tr>
<tr>
<td>36 to 40</td>
<td>0.67 (0.48 – 0.97)</td>
<td>0.66 (0.46 – 0.97)</td>
</tr>
<tr>
<td>Older than 40</td>
<td>0.24 (0.06 – 0.86)</td>
<td>0.27 (0.07 – 0.99)</td>
</tr>
<tr>
<td>No. of previous miscarriages</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>0.72 (0.56 – 0.95)</td>
<td>Reference</td>
</tr>
<tr>
<td>3 or more</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Previous live birth</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>0.93 (0.70 – 1.24)</td>
<td>Reference</td>
</tr>
<tr>
<td>Yes</td>
<td>Reference</td>
<td>1.01 (0.75 – 1.34)</td>
</tr>
<tr>
<td>Inherited thrombophilia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No</td>
<td>1.15 (0.77 – 1.74)</td>
<td>Reference</td>
</tr>
<tr>
<td>Yes</td>
<td>Reference</td>
<td>1.15 (0.77 – 1.72)</td>
</tr>
</tbody>
</table>

hr: hazard ratio; ci: confidence interval; *: Full model. †: After stepwise backward elimination. ‡: Results of complete inherited thrombophilia testing were available for 302 women (47 women tested positive for at least one of the thrombophilic factors). For this analyses all women with no or incomplete inherited thrombophilia test result were considered negative for inherited thrombophilia.
Three women (0.8%) had died during the follow-up period. One woman died at 42 years of age of a carcinoma of unknown primary origin, the second woman died shortly after she had an anterior myocardial infarction at 35 years of age, and the third was found in her home (30 years of age), with unknown cause of death. Although this is an unusual high number of deaths in such a young population, we have no study-related explanation for this.

This study has some limitations. Follow-up data were not available for all 364 women who participated in the alife study. However, because of the high response rate of 74% and as both the baseline characteristics as well as the cumulative probability of live birth were the same for the group of women reached in follow-up (n = 271) and for the total cohort (n = 364) (i.e. 81%), we believe this is a representative sample. Furthermore, whether follow-up was obtained or not appeared to be random as it was mostly due to loss of contact information. Very few women refused participation in the questionnaire study. Unfortunately, reliable data on treatment during pregnancies could not be obtained, as women often did not recall if they were treated and if so, during which pregnancy they had received treatment. This information would have been of value, and should be collected in a prospective study, especially as for women with inherited thrombophilia, it is unclear if treatment with anticoagulants increases their chance of live birth.18

We believe that the current approach has provided valuable information. Instead of using medical charts, which can be incomplete in case women received care of their pregnancies in different hospitals, for the vast majority we obtained data from the women themselves.

The population investigated in this study consists of women who participated in the alife study. This may be considered a limitation to the generalisability of the results. However, as the alife-study population appeared representative of the recurrent miscarriage-clinic patients, participation in the study was offered to each candidate and refusal of participation did not appear associated with a better or worse prognosis, we are confident that the results apply to all women with unexplained recurrent miscarriage. A full thrombophilia screen was available for 229 women (85%) and only 41 women tested positive for any inherited thrombophilia (18%), of whom 19 for Factor V Leiden. As women with missing data were considered negative for the thrombophilic factor concerned in the analyses the actual prevalence was likely higher and this may have led to an underestimation of a potential effect of inherited thrombophilia.

**Discussion**

Our study indicates that women with two or more unexplained miscarriages have a cumulative probability of live birth of 81%. An increasing number of previous miscarriages (i.e. three or more) was associated with a longer time to live birth when compared to a history of two miscarriages. Furthermore, we observed that time to live birth in women with inherited thrombophilia does not differ from women without inherited thrombophilia.

To our knowledge, this is the first study evaluating live birth and time to live birth during extended follow-up of up to 14 years in a cohort of women with unexplained recurrent miscarriage, who were not already pregnant at the start of follow-up. The high cumulative probability in our study as compared to other studies may be explained by differences in study populations, or differences in statistical methods. A previous study in 987 women found a lower live birth rate of 71% after 15 years, but included women with both explained and unexplained recurrent miscarriage and did not include women with two miscarriages. In the present analysis, we corrected for the competing risk of reaching the age at which achieving a live birth was considered impossible, i.e. 46 years. Not taking this into consideration in the analysis might lead to an underestimation of the result.

Our observation that an increasing number of miscarriages adversely affects the probability of live birth, is in line with the before mentioned study which reported a hazard ratio of live birth 5 years after consultation of 0.55 (95% CI 0.41 – 0.74) for women with six or more miscarriages when compared to women with three previous miscarriages. Remarkably, our study indicates that maternal age is not predictive of time to live birth. Although in the univariate analyses older women had a worse prognosis, this was not substantiated in the multivariable model. In the same cohort, taking first subsequent pregnancies only into account, we found that Factor V Leiden was associated with shorter time to conception. In the current analyses, neither Factor V Leiden independently, nor the presence of any thrombophilic factor were associated with time to live birth. It could be hypothesized that the detrimental effect of Factor V Leiden in terms of an increased risk of miscarriages does not translate to a longer time to live birth, potentially because of a shorter time to conception.
The cumulative probability of live birth during a follow-up period of 10 years was 81% for all women and 73% for women with three or more miscarriages. This can be interpreted as the chance of live birth in case a woman is granted another 10 fertile years during which she keeps attempting to conceive. In conclusion, this study indicates that the prognosis of women with unexplained recurrent miscarriage is generally good and not affected by the presence of inherited thrombophilia.

Acknowledgements

We would like to thank Adrien Groot, medical student, and Belia Rekké, trial nurse, for their help collecting data and Joost Besseling, PhD student, and Nick van Es, PhD student, for helping with the statistical analyses.
Chapter 4

Aspirin and/or heparin for women with unexplained recurrent miscarriage with or without inherited thrombophilia

Paulien G. de Jong, Stef Kaandorp, Marcello Di Nisio, Mariëtte Goddijn and Saskia Middeldorp

Cochrane Database of Systematic Reviews 2014
Abstract

Background: Since hypercoagulability might result in recurrent miscarriage, anticoagulant agents could potentially increase the chance of live birth in subsequent pregnancies in women with unexplained recurrent miscarriage, with or without inherited thrombophilia.

Objectives: To evaluate the efficacy and safety of anticoagulant agents, such as aspirin and heparin, in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia.

Search methods: We searched the Cochrane Pregnancy and Childbirth Group’s Trials Register (1 October 2013) and scanned bibliographies of all located articles for any unidentified articles.

Selection criteria: Randomised and quasi-randomised controlled trials that assessed the effect of anticoagulant treatment on live birth in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia were eligible. Interventions included aspirin, unfractionated heparin (UFH), and low molecular weight heparin (LMWH) for the prevention of miscarriage. One treatment could be compared with another or with no-treatment (or placebo).

Data collection and analysis: Two review authors (PJ and SK) assessed the studies for inclusion in the review and extracted the data. If necessary they contacted study authors for more information. We double checked the data.

Main results: Nine studies, including data of 1228 women, were included in the review evaluating the effect of either LMWH (enoxaparin or nadroparin in varying doses) or aspirin or a combination of both, on the chance of live birth in women with recurrent miscarriage, with or without inherited thrombophilia. Studies were heterogeneous with regard to study design and treatment regimen and three studies were considered to be at high risk of bias. Two of these three studies at high risk of bias showed a benefit of one treatment over the other, but in sensitivity analyses (in which studies at high risk of bias were excluded) anticoagulants did not have a beneficial effect on live birth, regardless of which anticoagulant was evaluated (risk ratio (RR) for live birth in women who received aspirin compared to placebo 0.94, (95% confidence interval (CI) 0.80 to 1.11, n = 256), in women who received LMWH compared to aspirin RR 1.08 (95% CI 0.93 to 1.26, n = 239), and in women who received LMWH and aspirin compared to no-treatment RR 1.01 (95% CI 0.87 to 1.16) n = 322).
Obstetric complications such as preterm delivery, pre-eclampsia, intrauterine growth restriction and congenital malformations were not significantly affected by any treatment regimen. In included studies, aspirin did not increase the risk of bleeding, but treatment with LWMH and aspirin increased the risk of bleeding significantly in one study. Local skin reactions (pain, itching, swelling) to injection of LWMH were reported in almost 40% of patients in the same study.

Authors’ conclusions: There is a limited number of studies on the efficacy and safety of aspirin and heparin in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia. Of the nine reviewed studies quality varied, different treatments were studied and of the studies at low risk of bias only one was placebo-controlled. No beneficial effect of anticoagulants in studies at low risk of bias was found. Therefore, this review does not support the use of anticoagulants in women with unexplained recurrent miscarriage and inherited thrombophilia. The effect of anticoagulants in women with unexplained recurrent miscarriage and inherited thrombophilia needs to be assessed in further randomised controlled trials; at present there is no evidence of a beneficial effect.

**Background**

**Description of the condition**

Up to 15% of all clinically recognised pregnancies end in miscarriage (miscarriage before the 20th week of gestational age).\(^1\)\(^2\) Approximately 5% of women experience two or more miscarriages (recurrent miscarriage, \(\text{RM}\)), whereas three or more first trimester miscarriages may affect as many as 1% to 2% of women of reproductive age.\(^3\)\(^4\) RM is devastating for women and their families. The definition of \(\text{RM}\) remains a subject of debate. The World Health Organization (WHO) defines miscarriage as the spontaneous loss of a clinical pregnancy that occurs before 20 completed weeks of gestational age.\(^5\)\(^6\) Often \(\text{RM}\) is defined as three or more consecutive miscarriages. According to recent European Society for Human Reproduction & Embryology (ESHRE) guidelines, \(\text{RM}\) is traditionally defined as three or more consecutive miscarriages occurring before 20 weeks’ gestation.\(^7\) Recent evidence shows that two miscarriages constitute \(\text{RM}\).\(^8\)\(^9\) Adequate characterisation of miscarriages and patients in \(\text{RM}\) studies is most important and, favourably, would make studies mutually comparable.\(^10\) The risk of miscarriage after two or three consecutive miscarriages is similar.\(^11\)

Furthermore, the presence of parenteral chromosome abnormalities, which is a known risk factor for recurrent miscarriage,\(^12\) as well as the presence of antiphospholipid antibodies, another known risk factor for recurrent miscarriage,\(^7\) are not different in women with two or three miscarriages.\(^13\)\(^14\) We therefore chose to use the broad definition of \(\text{RM}\) in this review: two or more not necessarily consecutive miscarriages. Miscarriage is associated with relevant maternal morbidity like bleeding and infection and, sometimes, maternal death,\(^15\) particularly in low-income countries.\(^16\) Moreover, miscarriage, especially if recurrent, might cause important psychological and emotional distress that can be further complicated by feelings of anxiety and depression as well as social withdrawal.\(^17\)\(^18\)

Several factors may be involved in the aetiology of \(\text{RM}\). Women experiencing \(\text{RM}\) may have an underlying medical condition such as carrier status of a structural chromosome abnormality,\(^16\)\(^19\) antiphospholipid syndrome, or other blood clotting disorders generally referred to as thrombophilias\(^20\) or a septate uterus.\(^21\) Factors less strongly associated with \(\text{RM}\) are hyperhomocysteinemia and endocrine abnormalities.\(^22\)

Thrombophilia is a diverse group of coagulation disorders associated with a predisposition to thrombosis and thus increased risk for thrombotic events such as deep vein thrombosis and pulmonary embolism. These hypercoagulable states can either be inherited as the Factor V Leiden mutation (which results in a decreased capacity to inactivate activated factor V by the protein C system, also known as activated protein C (APC) resistance), the deficiency of physiological anticoagulants like protein C, protein S and antithrombin and the prothrombin G20210A gene mutation (resulting in increased concentrations of prothrombin in plasma) or an elevated level of factor VIII:ac\(^23\) or acquired, as for instance the antiphospholipid syndrome. In this latter syndrome, the predisposition to thrombosis is acquired due to the presence of antiphospholipid antibodies.\(^24\)

A growing body of evidence has implicated thrombophilia in adverse obstetrical events (such as intrauterine growth restriction, (recurrent) miscarriage, severe pre-eclampsia, and placental abruption).\(^25\)\(^26\) and there is also reasonable evidence to suggest that some cases of \(\text{RM}\) are associated with thrombosis of placental vessels and infarction. Firstly, microthrombi are a common finding in the placental vasculature of women with \(\text{RM}\).\(^7\)

Secondly, placental thrombosis and infarction have been described in association with certain thrombophilic defects,\(^27\)\(^28\) but other pathophysiological pathways than thrombosis could also be involved, since adverse pregnancy outcomes can occur in women with thrombophilia in the ab-
The use of anticoagulants in pregnancy needs to be carefully evaluated for efficacy and safety since it can carry risks for the mother and the fetus. Coumarin derivatives are anticoagulant drugs used most often in cases of thrombosis, but cross the placenta and can display teratogenic effects. In contrast to coumarin derivatives, neither \textit{ufh} nor \textit{lmwh} cross the placenta and therefore do not have the potential to cause fetal bleeding and teratogenicity.51 The maternal risks associated with heparin administration are uncommon but potentially serious and include bleeding, heparin-induced thrombocytopenia and heparin-induced osteopenia with fractures. Moreover, heparin administration may cause pain and slight bruising at injection sites. There is accumulating evidence that \textit{lmwh} is at least as effective and safe as \textit{ufh} with potential advantages during pregnancy, since they cause less heparin-induced thrombocytopenia, can be administered once daily, and are associated with a lower risk of heparin-induced osteoporosis.51-53

Based on current evidence, aspirin (less than 150 mg/d) during the second and third trimesters appears to be safe, while the safety of higher doses of aspirin during the first trimester remains uncertain.51/52/54 The use of heparin in pregnancy has been covered in another Cochrane review.55

**Description of the intervention**

The use of anticoagulants in pregnancy needs to be carefully evaluated for efficacy and safety since it can carry risks for the mother and the fetus. Coumarin derivatives are anticoagulant drugs used most often in cases of thrombosis, but cross the placenta and can display teratogenic effects. In contrast to coumarin derivatives, neither \textit{ufh} nor \textit{lmwh} cross the placenta and therefore do not have the potential to cause fetal bleeding and teratogenicity.51 The maternal risks associated with heparin administration are uncommon but potentially serious and include bleeding, heparin-induced thrombocytopenia and heparin-induced osteopenia with fractures. Moreover, heparin administration may cause pain and slight bruising at injection sites. There is accumulating evidence that \textit{lmwh} is at least as effective and safe as \textit{ufh} with potential advantages during pregnancy, since they cause less heparin-induced thrombocytopenia, can be administered once daily, and are associated with a lower risk of heparin-induced osteoporosis.51-53

Based on current evidence, aspirin (less than 150 mg/d) during the second and third trimesters appears to be safe, while the safety of higher doses of aspirin during the first trimester remains uncertain.51/52/54 The use of heparin in pregnancy has been covered in another Cochrane review.55

**Why it is important to do this review**

In clinical practice, women with \textit{rm} associated with inherited thrombophilia or \textit{rm} without any other apparent predisposing disorder are frequently seeking advice about the indication for anticoagulant treatment. Some clinicians tend to extrapolate the beneficial effect of anticoagulant therapy in women with antiphospholipid syndrome and \textit{rm} to all women with \textit{rm}. Whether there is evidence for an effect of anticoagulants in women with \textit{rm} – either unexplained or associated with inherited thrombophilia – is the objective of this review.

**Objectives**

The objective of this review was to determine whether anticoagulant treatment improves the chance of a live birth in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia.
Methods

Criteria for considering studies for this review

Types of studies
Randomised controlled trials and quasi-randomised controlled trials that assessed the effect of anticoagulant treatment on improving the live birth rate in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia.

Types of participants
Participants were pregnant women with a history of at least two unexplained miscarriages with or without inherited thrombophilia. Of studies that included women attempting to conceive, but who were not pregnant upon randomisation only results of women who became pregnant were included. Studies that included women with apparent risk factors (other than inherited thrombophilia) of RM (antiphospholipid syndrome; uterine abnormalities; patients’ or their partners’ karyotype abnormalities) were included only if the results from women with a history of at least two unexplained miscarriages with or without inherited thrombophilia could be extracted to be analysed separately. For this review, we selected studies in women with two or more previous miscarriages up to 24 weeks’ gestation. The study populations are described whenever possible with regard to number of miscarriages, gestational age of the miscarriages, and maternal age.

Types of interventions
The interventions included were aspirin, UFH, and LMWH for the prevention of miscarriage. One treatment could be compared with another or with no treatment (or placebo). Combinations of therapy could be used. To exclude a potential lack of efficacy due to a limited duration of treatment, only studies in which the investigational treatment was started at a maximum of 12 weeks’ gestation and continued beyond 32 weeks’ gestation or until the end of pregnancy were eligible.

Types of outcome measures

Primary outcomes
Live birth.

Secondary outcomes
1. Preterm delivery of a live infant before 37 weeks’ gestational age (not a prespecified outcome).
2. Preterm delivery of a live infant between 24 and 28 weeks’ gestational age.
3. Preterm delivery of a live infant between 28 and 32 weeks’ gestational age.
4. Preterm delivery of a live infant between 32 and 37 weeks’ gestational age.
5. Obstetric complications (gestational hypertension, pre-eclampsia, intrauterine growth restriction).
7. Admission to special care.
8. Side effects of the drug used, both for the mother and the baby (maternal and/or neonatal bleeding, heparin-induced thrombocytopenia, pain, itching or swelling at injection sites and allergic reactions to heparin).
9. Thromboembolic complications.

The following methods section of this review is based on a standard template used by the Cochrane Pregnancy and Childbirth Group.

Search methods for identification of studies

Electronic searches
We searched the Cochrane Pregnancy and Childbirth Group’s Trials Register by contacting the Trials Search Co-ordinator (1 October 2013). The Cochrane Pregnancy and Childbirth Group’s Trials Register is maintained by the Trials Search Co-ordinator and contains trials identified from:
1. Monthly searches of the Cochrane Central Register of Controlled Trials (CENTRAL).
2. Weekly searches of MEDLINE.
Dr Kaandorp, Dr Goddijn, and Dr Middeldorp were investigators of the randomised controlled trial alife study and so this trial was assessed by the other review authors.57

Assessment of risk of bias in included studies
Two review authors independently assessed risk of bias for each study using the criteria outlined in the Cochrane Handbook for Systematic Reviews of Interventions.58 We resolved any disagreement by discussion or by involving a third assessor.

(1) Random sequence generation (checking for possible selection bias)
We described for each included study the method used to generate the allocation sequence in sufficient detail to allow an assessment of whether it should produce comparable groups. We assessed the method as:

- Low risk of bias (any truly random process, e.g. random number table. Computer random number generator).
- High risk of bias (any non-random process, e.g. odd or even date of birth; hospital or clinic record number).
- Unclear risk of bias.

(2) Allocation concealment (checking for possible selection bias)
We described for each included study the method used to conceal allocation to interventions prior to assignment and assessed whether intervention allocation could have been foreseen in advance of, or during recruitment, or changed after assignment. We assessed the methods as:

- Low risk of bias (e.g. telephone or central randomisation; consecutively numbered sealed opaque envelopes).
- High risk of bias (open random allocation; unsealed or non-opaque envelopes, alternation; date of birth).
- Unclear risk of bias.

(3.1) Blinding of participants and personnel (checking for possible performance bias)
Performance bias and detection bias were not included as criterion for quality as we anticipated that the outcome live birth was not influenced by blinding.

(3.2) Blinding of outcome assessment (checking for possible detection bias)
Performance bias and detection bias were not included as criterion for quality as we anticipated that the outcome live birth was not influenced by blinding.
Overall risk of bias
We made explicit judgements about whether studies were at high risk of bias, according to the criteria given in the Handbook. With reference to (1) to (6) above, we assessed the likely magnitude and direction of the bias and whether we considered it likely to impact on the findings. We explored the impact of the level of bias through undertaking sensitivity analyses – see Sensitivity analysis.

Measures of treatment effect
Dichotomous data
For dichotomous data, we presented results as summary risk ratio with 95% confidence intervals.

Continuous data
We did not analyse any continuous data.

Unit of analysis issues
Cluster-randomised trials
We did not identify any cluster-randomised trials for inclusion in this review. In future updates, if identified and eligible, we will include cluster-randomised trials in the analyses along with individually-randomised trials. We will adjust their sample sizes using the methods described in the Handbook [Section 16.3.4 or 16.3.6] using an estimate of the intracluster correlation co-efficient (icc) derived from the trial (if possible), from a similar trial or from a study of a similar population. If we use iccs from other sources, we will report this and conduct sensitivity analyses to investigate the effect of variation in the icc. If we identify both cluster-randomised trials and individually-randomised trials, we plan to synthesise the relevant information. We will consider it reasonable to combine the results from both if there is little heterogeneity between the study designs and the interaction between the effect of intervention and the choice of randomisation unit is considered to be unlikely. We will also acknowledge heterogeneity in the randomisation unit and perform a sensitivity analysis to investigate the effects of the randomisation unit.

Incomplete outcome data
We described for each included study, and for each outcome or class of outcomes, the completeness of data including attrition and exclusions from the analysis. We state whether attrition and exclusions were reported and the numbers included in the analysis at each stage (compared with the total randomised participants), reasons for attrition or exclusion where reported, and whether missing data were balanced across groups or were related to outcomes. Where sufficient information was reported, or could be supplied by the trial authors, we re-included missing data in the analyses which we undertook. We assessed methods as:

- Low risk of bias (e.g. no missing outcome data; missing outcome data balanced across groups; ≤20% participants missing).
- High risk of bias (e.g. numbers or reasons for missing data imbalanced across groups; ‘as treated’ analysis done with substantial departure of intervention received from that assigned at randomisation; more than 20% participants missing).
- Unclear risk of bias.

Selective reporting
We described for each included study how we investigated the possibility of selective outcome reporting bias. We assessed the methods as:

- Low risk of bias (where it is clear that all of the study’s pre-specified outcomes and all expected outcomes of interest to the review have been reported).
- High risk of bias (where not all the study’s pre-specified outcomes have been reported; one or more reported primary outcomes were not pre-specified; outcomes of interest are reported incompletely and so cannot be used; study fails to include results of a key outcome that would have been expected to have been reported).
- Unclear risk of bias.

Other bias
We described for each included study any important concerns we have about other possible sources of bias. We assessed whether each study was free of other problems that could put it at risk of bias:

- Low risk of other bias.
- High risk of other bias.
- Unclear whether there is risk of other bias.
Cross-over trials

Cross-over trials were excluded.

Dealing with missing data

For included studies, we noted levels of attrition. We planned to explore the impact of including studies with high levels of missing data in the overall assessment of treatment effect by using sensitivity analysis. However, none of the included studies were considered to be at high risk of attrition bias.

For all outcomes, we carried out analyses, as far as possible, on an intention-to-treat basis, i.e. we attempted to include all participants randomised to each group in the analyses, and all participants were analysed in the group to which they were allocated, regardless of whether or not they received the allocated intervention.

Assessment of heterogeneity

We assessed statistical heterogeneity in each meta-analysis using the $I^2$, $P$ and Chi² statistics. We regarded heterogeneity as substantial if an $I^2$ was greater than 30% and either a $I^2$ was greater than zero, or there was a low $P$ value (less than 0.10) in the Chi² test for heterogeneity.

Assessment of reporting biases

In future updates, if there are 10 or more studies in the meta-analysis we will investigate reporting biases (such as publication bias) using funnel plots. We will assess funnel plot asymmetry visually. If asymmetry is suggested by a visual assessment, we will perform exploratory analyses to investigate it.

Data synthesis

Data were pooled based on type of intervention, but irrespective of the dose of LMWH or aspirin. Only studies in which the investigational treatment was started at a maximum of 12 weeks’ gestation and continued beyond 32 weeks’ gestation were included.

We carried out statistical analysis using the Review Manager software. We used fixed-effect meta-analysis for combining data where it was reasonable to assume that studies were estimating the same underlying treatment effect: i.e. where trials were examining the same intervention, and the trials’ populations and methods were judged sufficiently similar. If there was clinical heterogeneity sufficient to expect that the underlying treatment effects differed between trials, or if substantial statistical heterogeneity was detected, we used random-effects meta-analysis to produce an overall summary, if an average treatment effect across trials was considered clinically meaningful. The random-effects summary was treated as the average of the range of possible treatment effects and we planned to discuss the clinical implications of treatment effects differing between trials. If the average treatment effect was not clinically meaningful, we did not combine trials.

If we used random-effects analyses, the results were presented as the average treatment effect with 95% confidence intervals, and the estimates of Tau² and $P$.

Subgroup analysis and investigation of heterogeneity

If we identified substantial heterogeneity, we planned to investigate it using subgroup analyses and sensitivity analyses. We planned to consider whether an overall summary was meaningful, and if it was, use random-effects analysis to produce it.

We planned to carry out the following subgroup analyses:
1. Inherited thrombophilia versus no inherited thrombophilia.
2. Different inherited thrombophilic disorders.
3. Preconceptional versus periconceptional anticoagulant use.
4. Type of anticoagulant(s) used (e.g. single drug, combination of anticoagulant agents).
5. Dose of anticoagulant(s).
6. Duration of anticoagulant use.
7. Women with a history of three or more miscarriages.
8. Women with no previous live birth versus women with one or more previous live birth.

We planned to use the primary outcome, live birth, in subgroup analyses. However, due to lack of data, we were not able to conduct planned subgroup analysis. We included data from single subgroups in the analysis of the primary outcome, but due to lack of data, we did not explore the treatment effect between the pre-specified subgroups as outlined above. In future updates, if more data become available, we will assess subgroup differences by interaction tests available within RevMan and report the results of subgroup analyses quoting the Chi² statistic and $P$ value, and the interaction test $P$ value.
Sensitivity analysis
We performed sensitivity analyses for the main outcomes by individual quality criteria to assess the effect of poorer quality studies on the magnitude of the estimate of effect. Only studies at an overall low risk of bias were included in the initial analyses and we carried out sensitivity analyses to explore the effect of quality.

Results

Description of studies

Included studies
Details for the studies included are in the Characteristics of included studies. We included nine studies (1228 women for the primary outcome live birth) (Badawy 2008; Clark 2010; Dolitzky 2006; Fawzy 2008; Giancotti 2012 Kaandorp 2010; Martinelli 2012; Tulppala 1997; Visser 2011) in this review. In the studies Badawy 2008 and Dolitzky 2006, full study populations were included. For the studies Clark 2010; Fawzy 2008; Giancotti 2012; Kaandorp 2010; Martinelli 2012; Tulppala 1997; and Visser 2011, we could not include full study populations, but had to extract data on the women fulfilling the inclusion criteria of the review. Reasons for including only part of the original study population are explained for each study.

Badawy 2008 evaluated the effect of LMWH (enoxaparin 20 mg/day) in women with three or more consecutive first trimester miscarriages. Women were included if no risk factors for RM could be identified (women with inherited thrombophilia were excluded) and randomised to either treatment or no-treatment (no placebo). Therapy was commenced once fetal viability was detected on ultrasound and continued until 34 weeks’ gestation. The primary outcome was pregnancy loss and pregnancy complications, but live births could be calculated from the report and were confirmed by the study author. Of 350 women enrolled, 10 women (four (2.3%) and six (3.4%) in both arms) were lost to follow-up, leaving 170 women in each study arm for analysis. Side effects of treatment were only reported for women randomised to treatment, not in those randomised to no-treatment.

Clark 2010 studied the effect of LMWH (enoxaparin 40 mg/day) and aspirin (75 mg/day) from before seven weeks’ gestation until 36 weeks’ gestation on live birth in 296 women with RM, defined as a minimum of two consecutive early pregnancy losses (at or before 24 weeks’ gestation). Intervention with LMWH and aspirin combined with intense pregnancy surveillance was compared with intense pregnancy surveillance without pharmacological intervention. Women were included upon a positive pregnancy test (before seven weeks’ gestation). Investigations for uterine or chromosomal abnormalities and antiphospholipid syndrome were conducted only for women with three or more previous miscarriages. Women who were included because of two previous miscarriages were excluded from our analysis, because it could not be confirmed that previous miscarriages were ‘unexplained’. The primary outcome measure in the study was live birth. Adverse events of intervention were reported, though could not be extracted for the women with three or more previous miscarriages. One-hundred and twenty-two women were included in the review (64 randomised to LMWH and aspirin versus 58 randomised to surveillance).

Dolitzky 2006 evaluated the effect of LMWH compared with aspirin in 104 women with unexplained RM. RM was defined as three or more consecutive first trimester miscarriages or at least two consecutive second trimester miscarriages. The objective was to compare the effect of enoxaparin and aspirin on live birth rate. Women were only included if there was no apparent risk factors for the miscarriages and women with inherited thrombophilia were excluded. The treatment with enoxaparin (40 mg/day) or aspirin (100 mg/day) was started from the time of detection of a fetal heart beat at six to 12 weeks’ gestation and continued until a gestational age of 37 weeks. Of the 107 included women, 54 received enoxaparin, 50 aspirin and three were lost to follow-up. Besides the primary outcome measure of live birth, secondary outcomes like preterm delivery, intrauterine growth restriction, and pre-eclampsia were reported.

Fawzy 2008 assessed the effect of LMWH (enoxaparin 20 mg/day) compared with combination treatment (oral prednisone and progesterone from the onset of pregnancy until 12 weeks of gestation and aspirin from the onset of pregnancy until 32 weeks of gestation) compared with placebo (for oral intervention) in women with three or more consecutive unexplained miscarriages (before 24 weeks’ gestation) with the same partner. Women with inherited thrombophilia were excluded. From this study, we extracted data for the 107 women receiving enoxaparin or placebo. Of these women, 57 were assigned to enoxaparin and 50 to placebo. Treatment was started when a fetal pole was detected and continued until term. The primary outcome was live birth, but secondary outcomes such as obstetric complications and neonatal outcomes were also reported.

Giancotti 2012 evaluated the effect of LMWH or aspirin or a combination of these in 167 women with a history of two or more unexplained miscarriages before 12 weeks’ gestation. Women with uterine or chromosomal...
abnormalities were excluded, but women with inherited thrombophilia or antiphospholipid syndrome were eligible. Women were randomised to LMWH (enoxaparin 40 mg/day from diagnosis of intrauterine pregnancy until delivery) or aspirin (100 mg/day from diagnosis of pregnancy until 32 weeks’ gestation) or first aspirin (100 mg/day from diagnosis of pregnancy), which was replaced by LMWH at 32 weeks’ gestation (enoxaparin 40 mg/day continued until delivery). For this analysis, we included only data from women without antiphospholipid antibodies randomised to either LMWH (n = 40) or aspirin (n = 46). The primary outcome of the study was live birth, and no secondary outcome measures were reported.

Kaandorp 2010 evaluated the effect of open label LMWH (nadroparin 2850 IU/day) combined with aspirin (80 mg/day) or aspirin only (80 mg/day) compared with placebo in 364 women with unexplained RM with or without inherited thrombophilia. Previous miscarriage was defined as pregnancy loss at a gestational age of 20 weeks or less. Women were included in the study if they were attempting to conceive or were less than six weeks pregnant. From this study, we extracted data of the 299 women who became pregnant (97 were assigned to aspirin plus nadroparin, 99 were assigned to aspirin only and 103 were assigned to placebo). LMWH was initiated when a viable intrauterine pregnancy was confirmed on ultrasonography at six weeks’ gestation until the start of labour. Aspirin or placebo was started at randomisation and continued until a gestational age of 36 weeks. The primary outcome was live birth, and secondary outcomes were adverse pregnancy outcomes and maternal adverse events. Secondary outcomes such as obstetric complications and neonatal events were evaluated for 200 women with ongoing pregnancy beyond 12 weeks of gestation.

Martinelli 2012 evaluated the effect of open label LMWH (nadroparin 3800 IU/day), compared with no treatment in 135 women with previous placenta-mediated pregnancy complications. Women with antiphospholipid syndrome, uterine anomalies or abnormal karyotype were excluded from the study. Inherited thrombophilia was not an exclusion criterion. LMWH was compared with medical surveillance only and treatment was initiated upon randomisation and continued until delivery. Randomisation was performed around the 12th week of gestation, after pregnancy was confirmed on ultrasonography. The primary outcome of the study was a composite outcome of several pregnancy complications. For this review we included data of six women, who had a history of two or more unexplained miscarriages up to 24 weeks’ gestation.

Tulppala 1997 evaluated the effect of aspirin (50 mg/day) on live birth rate in 66 pregnant women with preceding RM with or without detectable anticardiolipin antibodies and no other apparent risk factors for their previous miscarriages. RM was defined as three or more consecutive miscarriages (occurring before 22 weeks of gestational age). Aspirin was compared with placebo, and medication was started as soon as a home urinary pregnancy test became positive and continued until delivery. From this study, we extracted data for 54 women who were negative for anticardiolipin antibodies. Of these, 27 were assigned to aspirin and 27 to placebo. Secondary outcomes, such as preterm delivery, obstetric complications, and bleeding rate could not be extracted separately for the group of women with negative anticardiolipin antibodies.

Visser 2011 evaluated the effect of LMWH (enoxaparin 40 mg/day) plus oral placebo (n = 68) compared with LMWH (enoxaparin 40 mg/day) plus aspirin (100 mg/day) (n = 63), compared with aspirin only (100 mg/day) (n = 76) in women with unexplained RM with or without inherited thrombophilia. RM was defined as three or more consecutive first trimester miscarriages, two or more second trimester miscarriages or one third trimester fetal loss combined with at least one first trimester miscarriage. Treatment was initiated upon randomisation (before seven weeks’ gestation); aspirin and placebo were discontinued at 36 weeks’ gestation whereas enoxaparin was continued until the first signs of labour. The primary outcome was live birth and secondary outcomes were adverse pregnancy outcomes and bleeding. Premature delivery, obstetrical complications and congenital malformations were reported only for women who had live birth. Study authors stated that only one woman was included in the study because of RM based on one fetal loss before, and two fetal losses after 24 weeks’ gestation; her data were excluded from this review. Furthermore, data of 10 women (allocated to enoxaparin plus placebo (n = 3, one live birth), enoxaparin plus aspirin (n = 2, one live birth), aspirin only (n = 5, four live births)) were excluded because of the presence of Beta-2 glycoprotein antibodies. One-hundred and ninety-six women were included in this trial.

As can be noted, no study compared the same treatment regimen. Studies compared different doses of LMWH and aspirin or combinations of these, and treatment was started at various gestational ages. As described in the Methods section, we pooled data based on type of intervention, but irrespective of the dose of LMWH or aspirin. Only studies in which the investigational treatment was started at a maximum of 12 weeks’ gestation and continued beyond 32 weeks’ gestation were included.
Excluded studies
Overall, we excluded 22 studies from the review. We have provided the reasons for exclusion in the Characteristics of excluded studies table. Three studies are only available in abstract form (Rodger 2009; Salman 2012; Schleussner 2013) and we are awaiting the full study report, see Characteristics of studies awaiting classification.

Risk of bias in included studies
Details for the included studies are shown in the Characteristics of included studies and in FIGURE 1. The studies by Clark 2010; Kaandorp 2010; Martinelli 2012 and Visser 2011 were considered to be at low risk of bias for all assessed criteria.

FIGURE 1 - Risk of bias’ summary: review authors’ judgements about each risk of bias item for each included study.

<table>
<thead>
<tr>
<th>Study</th>
<th>Allocation concealment (selection bias)</th>
<th>Random sequence generation (selection bias)</th>
<th>Allocation concealment (selection bias)</th>
<th>Incomplete outcome data (attrition bias)</th>
<th>Selective reporting (reporting bias)</th>
<th>Other bias</th>
<th>Overall risk of bias</th>
</tr>
</thead>
<tbody>
<tr>
<td>Visser 2011</td>
<td>☑</td>
<td>☑</td>
<td>☑</td>
<td>☑</td>
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</tr>
<tr>
<td>Tulppala 1997</td>
<td>☐</td>
<td>☐</td>
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<tr>
<td>Martinelli 2012</td>
<td>☐</td>
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<tr>
<td>Kaandorp 2010</td>
<td>☐</td>
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</tr>
<tr>
<td>Dolitzky 2006</td>
<td>☐</td>
<td>☐</td>
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<td>☐</td>
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<tr>
<td>Giancotti 2012</td>
<td>☐</td>
<td>☐</td>
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<tr>
<td>Fawzy 2008</td>
<td>☐</td>
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<tr>
<td>Durzy 2008</td>
<td>☐</td>
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<tr>
<td>Clark 2010</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
<td>☐</td>
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<tr>
<td>Badawy 2008</td>
<td>☐</td>
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</tr>
</tbody>
</table>

Allocation (selection bias)
Procedures for adequate allocation concealment were well described in the studies by Badawy 2008; Clark 2010; Dolitzky 2006; Giancotti 2012; Kaandorp 2010; Martinelli 2012; and Visser 2011. Dr Tulppala provided information about allocation in her study (Tulppala 1997), which was then judged to be at low risk of bias. Based on the report and information provided by Dr Fawzy regarding treatment allocation, we judged this study (Fawzy 2008) to be at high risk of selection bias.

Blinding (performance bias and detection bias)
As explained in the methods sections, performance bias and detection bias were not included as criterion for quality.

Incomplete outcome data (attrition bias)
The study by Kaandorp 2010 was analysed on intention-to-treat basis, including women lost to follow-up. Other studies reported outcomes for randomised women minus any participants whose outcomes were missing. Numbers of women lost to follow-up were small and well balanced for each group and not enough to have a clinically relevant impact on the intervention effect estimate. All studies were therefore considered to be at low risk of attrition bias.

Selective reporting (reporting bias)
Assessment of reporting bias was impossible for Dolitzky 2006; and Tulppala 1997 as trial registration was not operative at time of inclusion. The studies by Badawy 2008, Fawzy 2008 and Giancotti 2012 were considered to be at high risk of bias because they were not registered in a prospective trial register. Other studies were considered to be at low risk of reporting bias.

Other potential sources of bias
There were inconsistencies in the report by Badawy 2008, and the report by Giancotti 2012 provided no baseline table, which made it impossible to determine whether prognostic factors were evenly distributed between groups.

Effects of interventions
We included nine studies, involving 1228 participants, in this review. Since treatment regimens varied among included studies, pooled analysis could not include more than three studies, except for when LMWH with or without aspirin was compared to no treatment. Only one study (Kaandorp 2010) included women who used anticoagulants preconceptionally. This yielded insufficient data to perform the planned subgroup analysis for pre- and periconceptional anticoagulant use. Where studies reported pregnancy complications, different denominators (e.g. all pregnant women, only ongoing pregnancies, only women with live births) were used in different studies and results could not be pooled. This is explained for the comparisons of treatment concerned.
Aspirin versus no treatment

Pooled analysis from 256 patients showed that compared to placebo, aspirin did not increase live birth (risk ratio (RR) 0.94, 95% confidence interval (CI) 0.80 to 1.11), Analysis 1.1, (Kaandorp 2010; Tulppala 1997). Subgroup analyses for the outcome live birth for women with no previous live births (RR 0.93, 95% CI 0.72 to 1.21), inherited thrombophilia (RR 1.08, 95% CI 0.63 to 1.85) or more than two miscarriages (RR 0.95, 95% CI 0.70 to 1.28) could only be performed for the study by Kaandorp 2010 and also showed no effect of treatment.

Secondary outcomes were not reported by Tulppala 1997. Preterm delivery, obstetric complications and congenital malformations as reported by Kaandorp 2010 for women with ongoing pregnancies beyond 12 weeks’ gestation did not differ between the two groups. Bleeding as a side effect from treatment (mainly nose or gum bleeds or haematomas) was reported for 30% of women receiving aspirin and for 27% women receiving placebo (RR 1.11, 95% CI 0.72 to 1.72, Analysis 1.9). It should be noted that bleeding was reported for all women included in the study, including women who did not become pregnant during the course of the study.

LMWH versus aspirin

Three studies compared enoxaparin with aspirin (Dolitzky 2006; Giancotti 2012; Visser 2011). Pooled analysis (n = 325) showed an average RR of live birth for women treated with aspirin of 1.16 (95% CI 0.93 to 1.45; Heterogeneity: Tau² = 0.02; P = 67%, Analysis 3.1). Due to substantial statistical heterogeneity being detected, we used random-effects meta-analysis in Analysis 3.1. After excluding the study by Giancotti 2012 at high risk of bias, both groups had similar live birth rates, 76% in the enoxaparin group and 70% in the aspirin group (RR 1.08, 95% CI 0.93 to 1.26, Analysis 2.1). In the subgroup of women with no previous live births, the RR of live birth with LMWH was 1.24 (95% CI 1.02 to 1.49) compared to aspirin.

The study by Giancotti 2012 reported no secondary outcome measures. Results of secondary outcome measures for the studies by Dolitzky 2006 and Visser 2011 could not be pooled, as both studies used different denominators (i.e. all pregnancies in the study by Dolitzky 2006 and only women with live birth in the study by Visser 2011). In the individual studies, the number of preterm deliveries (before 37 weeks), cases of intrauterine growth restriction, pre-eclampsia and congenital malformations did not differ between the two groups, Bleeding complications did not differ between the two groups in both studies, though results of bleeding were very different; 0% versus 0.04% in the study by Dolitzky 2006 and 49% versus 50% in the study by Visser 2011 in women treated with LMWH versus aspirin respectively.

LMWH versus no treatment

The effect of LMWH was evaluated in three studies (Badawy 2008; Fawzy 2008; Martinelli 2012). Pooled analysis (n = 453) showed an average RR of live birth for women treated with LMWH of 1.23 (95% CI 0.84 to 1.81; Heterogeneity: Tau² = 0.09; P = 80%, Analysis 5.1). Due to substantial statistical heterogeneity being detected, we used random-effects meta-analysis in Analysis 5.1. When excluding studies at high risk of bias, only the data of six women included in the study by Martinelli 2012 could be analysed. Of these six women, four were randomised to LMWH and two to no treatment and all six had a live birth during the study (Analysis 4.1). Secondary outcomes were only reported by studies at high risk of bias, and could not be pooled due to a difference in denominators (pregnancies continued beyond 21 weeks in the study by Badawy 2008 and all pregnancies in the study by Fawzy 2008). No difference between treatment groups were found in individual studies for pregnancy complications, bleeding or thromboembolic events. The study by Badawy 2008 reported that 22% of women treated with LMWH experienced symptoms of bleeding and 30% local skin reactions.

LMWH and aspirin versus no treatment

The effect of LMWH and aspirin on live birth compared to no treatment or placebo was evaluated in two studies (n = 322) (Clark 2010; Kaandorp 2010). Live birth occurred as often in women receiving LMWH and aspirin (n = 161) as in women who received no treatment (n = 161) (RR for women who received LMWH and aspirin 1.01, 95% CI 0.87 to 1.16), Analysis 6.1). Subgroup analyses for the outcome live birth could only be performed for the study by Kaandorp 2010. For women with no previous live births (RR 1.05, 95% CI 0.83 to 1.34), or more than two miscarriages (RR 1.00, 95% CI 0.75 to 1.33) no effect of treatment was found. Data of women with inherited thrombophilia suggested a potential benefit in these women when treated with LMWH and aspirin, but the subgroup was underpowered for firm conclusions (RR 1.25, 95% CI 0.74 to 2.12).

Data of secondary outcomes were not available for the study by Clark 2010 and are therefore only described for the study by Kaandorp 2010. The occurrence of obstetric complications did not differ between the two study arms. Maternal bleeding (mainly nose or gum bleeds or haematomas) occurred significantly more frequently in women who received treatment;
LMWH with or without aspirin versus no treatment

Results of studies were combined, to evaluate the effect of LMWH with or without aspirin on the chance of live birth. Pooled results from 793 patients of five studies (Badawy 2008; Clark 2010; Fawzy 2008; Kaandorp 2010; Martinelli 2012) showed no effect of treatment (RR 1.07, 95% CI 0.99 to 1.15, Analysis 9.1). After excluding studies at high risk of bias the point estimate shifted towards 1 and no effect of treatment was observed (n = 324, RR for live birth in women treated with LMWH 0.98, 95% CI 0.85 to 1.12, Analysis 10.1).

Characteristics of studies

Characteristics of included studies

<table>
<thead>
<tr>
<th>Participants</th>
<th>Pregnant women (&lt;8 weeks) (n = 340) with a history of 3 or more consecutive first trimester miscarriages.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interventions</td>
<td>Subcutaneous enoxaparin 20 mg once daily from detection of fetal viability on ultrasound until 34 weeks’ gestation vs no pharmacological intervention.</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Primary: termination of pregnancy. Secondary: maternal and fetal complications, adverse effects such as bleeding, thrombocytopenia and local reactions.</td>
</tr>
<tr>
<td>Notes</td>
<td>Live birth rates were not reported in the article; upon request, study authors confirmed that all women who were not reported to have a miscarriage or placental abruption had a live birth.</td>
</tr>
</tbody>
</table>

LMWH with aspirin versus LMWH

One study evaluated the effect of LMWH and aspirin (n = 61) in comparison with LMWH only (n = 65) (Visser 2011). Neither live birth, nor any of the secondary outcomes including bleeding were different between the two groups (RR of live birth in women treated with LMWH plus aspirin 0.91, 95% CI 0.72 to 1.15, Analysis 8.1). Subgroup analyses in women with inherited thrombophilia or no previous live birth were small and showed no benefit of one treatment over the other.

In the studies by Kaandorp 2010 and Visser 2011 the effect of LMWH and aspirin was compared with aspirin only (n = 327). Live birth did not differ significantly between both groups; 68% and 61% respectively (RR 1.11, 95% CI 0.94 to 1.30, Analysis 7.1). Subgroup analyses for the outcome live birth in women with either no previous live births, inherited thrombophilia or more than two previous miscarriages (study by Kaandorp 2010 only) also showed no effect of treatment.

Again, results for secondary outcome measures could not be pooled because different denominators (ongoing pregnancies in the study by Kaandorp 2010 and pregnancies that ended in live birth in the study by Visser 2011) were used. The incidence of preterm delivery, pre-eclampsia, intravascular growth restriction (IUGR) and congenital malformations was similar in both groups in the individual studies. A significant difference was seen in bleeding (mainly nose or gum bleeds or haematoma) between the two groups, favouring treatment with aspirin only (RR for bleeding in women treated with LMWH and aspirin 2.04, 95% CI 1.46 to 2.86, Analysis 7.9), in the study by Kaandorp 2010, 62% of women treated with LMWH and aspirin experienced bleeding compared to 30% in women treated with aspirin only. In the study by Visser 2011, bleeding (reported as first trimester, second/third trimester bleeding or postpartum haemorrhage) was reported more often in women treated with aspirin (38% in women treated with LMWH and aspirin and 50% in women treated with aspirin only), though this difference was not significant (RR 0.75, 95% CI 0.45 to 1.24, Analysis 7.9).
Clark 2010

<table>
<thead>
<tr>
<th>Methods</th>
<th>Open label, random allocation with adequate concealment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>Pregnant women (n = 294) (&lt; 7 weeks’ gestation, n = 294) with a history of 2 or more consecutive pregnancy losses (at or before 24 weeks’ gestation) in whom no risk factor for their previous losses was found.</td>
</tr>
<tr>
<td>Interventions</td>
<td>Subcutaneous enoxaparin (40 mg daily) and aspirin (75 mg daily) vs no pharmacological treatment from randomisation until 26 weeks’ gestation.</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Primary: live birth, Secondary: haemorrhage, anaemia, thrombocytopenia, skin reactions.</td>
</tr>
<tr>
<td>Notes</td>
<td>We included only data of women with 3 or more previous miscarriages, as of these women tests for abnormal karyotype, uterine abnormalities and antiphospholipid syndrome were performed (n = 122) and found to be negative.</td>
</tr>
</tbody>
</table>

Dolitzky 2006

<table>
<thead>
<tr>
<th>Methods</th>
<th>Multicentre, open label, random allocation with adequate concealment.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>Women (n = 107) with a history of 3 or more consecutive fetal losses in the first trimester or at least 2 second trimester fetal losses in whom no risk factor for their previous pregnancy losses was found.</td>
</tr>
<tr>
<td>Interventions</td>
<td>Subcutaneous enoxaparin (40 mg daily) vs aspirin (100 mg daily) from the time of detection of a fetal heart beat.</td>
</tr>
<tr>
<td>Outcomes</td>
<td>Data of 104 women were available for analysis. Primary: live birth rates, Secondary: intrauterine growth restriction, birthweight, uterine and umbilical blood flow, pre-eclampsia, haemorrhage, thrombocytopenia, allergic reactions and congenital malformations.</td>
</tr>
<tr>
<td>Notes</td>
<td>Study authors were contacted and provided information that all women included in the review had at least 2 miscarriages before 25 weeks’ gestation.</td>
</tr>
</tbody>
</table>

Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation</td>
<td>Low risk</td>
<td>Randomisation was performed centrally using consecutively numbered randomisation envelopes supplied by the statistics unit.</td>
</tr>
<tr>
<td>allocation (selection bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allocation concealment</td>
<td>Low risk</td>
<td>Allocation was adequately concealed.</td>
</tr>
<tr>
<td>(selection bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete outcome data</td>
<td>Low risk</td>
<td>The study analyses were performed on an intention-to-treat basis. Of 294 women, 11 were lost to follow-up. These women were excluded from analyses.</td>
</tr>
<tr>
<td>(attrition bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective reporting</td>
<td>Low risk</td>
<td>Study was prospectively registered (ISRCTN06774126).</td>
</tr>
<tr>
<td>(reporting bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other bias</td>
<td>Low risk</td>
<td>No other sources of bias were detected.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>Overall, the study was considered at low risk of bias.</td>
</tr>
</tbody>
</table>

Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation</td>
<td>Low risk</td>
<td>Randomisation was performed centrally in blocks of 8 by an independent co-ordinator.</td>
</tr>
<tr>
<td>allocation (selection bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Allocation concealment</td>
<td>Low risk</td>
<td>The randomisation code per patient number was held by the monitor and blinded from the investigator.</td>
</tr>
<tr>
<td>(selection bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Incomplete outcome data</td>
<td>Low risk</td>
<td>Of 107 women 3 women were lost to follow-up. These women were excluded from analyses.</td>
</tr>
<tr>
<td>(attrition bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selective reporting</td>
<td>Unclear risk</td>
<td>Trial registration was not yet operative at time of inclusion.</td>
</tr>
<tr>
<td>(reporting bias)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other bias</td>
<td>Unclear risk</td>
<td>Initial power analysis aimed to include more patients; study was closed after interim analyses because it was estimated that too many patients needed to be included to reach statistical significance.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>As trial registration was not operative at time of inclusion, the overall risk of bias was judged as low risk.</td>
</tr>
</tbody>
</table>
### Fawzy 2008

**Methods**  
Single centre, placebo-controlled.

**Participants**  
Women (n = 170) with a history of 3 or more spontaneous consecutive pregnancy losses < 24 weeks' gestation in whom no risk factor of previous pregnancy losses was found.

**Interventions**  
Subcutaneous enoxaparin (20 mg daily) until spontaneous labour or miscarriage vs combination treatment of oral prednisone (20 mg daily) and progesterone (20 mg daily) for the first 12 weeks of gestation vs placebo.

**Outcomes**  

**Notes**  
Unclear treatment allocation and placebo procedure. Participants included in the present review: 107 of 170 randomised women, 6 were lost to follow-up and 4 women stopped treatment; 53 women were randomised to a combined intervention arm (prednisone, progesterone and aspirin) and they were excluded from our analysis.

### Giancotti 2012

**Methods**  
Single centre university hospital, open label.

**Participants**  
Non-pregnant women with a history of 2 or more unexplained miscarriages before 12 weeks' gestation were recruited. 167 women became pregnant and were randomised after positive plasma beta hCG test with corresponding ultrasonography.

**Interventions**  
Aspirin (100 mg daily) from confirmation of pregnancy until 32 weeks' gestation vs subcutaneous enoxaparin (40 mg daily) from confirmation of pregnancy until delivery vs combination of aspirin (100 mg daily) from confirmation of pregnancy until 32 weeks' gestation and enoxaparin (40 mg daily) from 32 weeks' gestation until delivery.

**Outcomes**  

**Notes**  
Women randomised to the third study arm received aspirin before and enoxaparin after 32 weeks' gestation. Data of these women were excluded from analyses. 46 women randomised to aspirin and 40 women randomised to enoxaparin were included in this review.

### Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors' judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Random assignment was performed according to a computer-generated list of study numbers.</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>High risk</td>
<td>Authors describe that a list of study numbers was used, and that only patients were blinded for allocation. In personal communication authors could not confirm allocation concealment was adequate.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>2 women randomised to enoxaparin were lost to follow-up and 1 woman randomised to enoxaparin stopped treatment; these women were excluded from analyses.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>High risk</td>
<td>Study was not registered in a trial register.</td>
</tr>
<tr>
<td>Other</td>
<td>Low risk</td>
<td>No other sources of bias were detected.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>High risk</td>
<td>Because of the high risk of selection bias and reporting bias this study was judged to be at high risk of bias.</td>
</tr>
</tbody>
</table>

### Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors' judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
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<td>Statistical advisor performed randomised selection as stated by the statistical data management program.</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
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<td>Once informed consent was obtained, randomised selection was performed as stated by the statistical data management program.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>Outcome data are available for all randomised women.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>High risk</td>
<td>Study was not registered in trial register.</td>
</tr>
<tr>
<td>Other</td>
<td>Unclear risk</td>
<td>Baseline table was not provided; unclear if baseline imbalances between groups existed.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>High risk</td>
<td>Because of the high risk of reporting bias and risk of other bias due to baseline imbalances this study was judged to be at high risk of bias.</td>
</tr>
</tbody>
</table>
### Kaandorp 2010

**Methods**  
Double blind (for aspirin) open label (for nadroparin) placebo-controlled with adequate allocation concealment.

**Participants**  
384 women with a history of 2 or more miscarriages attempting to conceive or < 6 weeks pregnant, in whom no risk factor of their previous miscarriages could be found, with or without inherited thrombophilia.

**Interventions**  
Subcutaneous nadroparin (2850 U daily, from 6 weeks of gestation until labour) and aspirin (80 mg daily) vs aspirin only (80 mg daily) vs placebo. Aspirin or placebo was initiated at randomisation and continued until 36 weeks' gestation or stopped at miscarriage, ectopic pregnancy or premature delivery.

**Outcomes**  

**Notes**  
Participants included in the present review: 299 women who became pregnant during the course of the study.

### Martinelli 2012

**Methods**  
Multicentre, open label, random allocation with adequate concealment.

**Participants**  
135 pregnant women with previous pregnancies complicated by either pre-eclampsia, HELLP syndrome, spontaneous fetal loss > 15 weeks' gestation, birthweight < 10th percentile or placental abruption followed by emergency delivery > 24 weeks.

**Interventions**  
Subcutaneous nadroparin (3800 U once daily) and medical surveillance vs medical surveillance only. Treatment was initiated upon randomisation and continued until delivery.

**Outcomes**  
Primary: a composite endpoint of pregnancy complications. Secondary: maternal, fetal adverse events related to study, miscarriage (< 15 weeks' gestation), mode of delivery, Appar scores.

**Notes**  
Upon request, the authors provided data on live birth in women who had 2 or more previous miscarriages up to 24 weeks' gestation that were unexplained. Only this subgroup of 6 women could be included in the current review.

### Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors' judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Randomisation was performed centrally by a computer program with minimisation for maternal age and the number of miscarriages, stratified according to study centre.</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Low risk</td>
<td>Allocation was adequately concealed.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>Women were randomised preconceptionally. Respectively 26, 21 and 18 women were either lost to follow-up, did not become pregnant or were still in the study when the trial ended in the combination, aspirin and placebo arm. Study analyses were performed on an intention-to-treat basis. Only data of women who became pregnant were included in this review.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>Low risk</td>
<td>Study was prospectively registered (ISRCTN58496168).</td>
</tr>
<tr>
<td>Other bias</td>
<td>Low risk</td>
<td>No other sources of bias were detected.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>Overall, the study was considered at low risk of bias.</td>
</tr>
</tbody>
</table>

### Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors' judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>A computer randomisation list was generated by the laboratory of biostatistics.</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Low risk</td>
<td>The patient randomisation number and study arm were requested by phone or fax and centrally assigned by the treatment secretariat.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>4 women and 3 women randomised to intervention and surveillance respectively were reported as drop-outs. These were excluded from analyses.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>Low risk</td>
<td>Study was prospectively registered (EudraCT 2006-004205-26).</td>
</tr>
<tr>
<td>Other bias</td>
<td>Low risk</td>
<td>No other sources of bias were detected.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>Overall, the study was considered at low risk of bias.</td>
</tr>
</tbody>
</table>
### Tulppala 1997

**Methods**
- Double-blind, placebo-controlled.

**Participants**
- Women (n = 82) with a history of at least 3 consecutive miscarriages in whom no obvious risk factor for their previous pregnancy losses was found.

**Interventions**
- Aspirin (60 mg/daily) vs placebo, started as soon as a urinary pregnancy test became positive.

**Outcomes**
- To assess the effect of aspirin on PGI2 and TXA2 production and on the rate of abortion in pregnant women with recurrent spontaneous abortion with or without detectable antiphospholipid antibodies.

**Notes**
- Participants included in the present review: subcategory of 54 women negative for antiphospholipid antibodies.

### Risk of bias table

<table>
<thead>
<tr>
<th>Bias</th>
<th>Authors’ judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Randomisation was performed centrally by a medical company who supplied the study medication (aspirin and placebo).</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Low risk</td>
<td>Aspirin and placebo tablets were identical and were supplied after randomisation.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>For the complete study population, 2 women with blighted ovum and 1 ectopic pregnancy were reported in the aspirin group, compared to 2 and 3 in the placebo group. There were no losses to follow-up.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>Unclear risk</td>
<td>Trial registration was not yet operative at time of inclusion.</td>
</tr>
<tr>
<td>Other bias</td>
<td>Unclear risk</td>
<td>Unclear if baseline imbalances were present.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>Or Tulppala provided information on random sequence generation and allocation concealment which was not available from the article. As trial registration was not operative at time of inclusion, the overall risk of bias was judged as low risk.</td>
</tr>
</tbody>
</table>

### Visser 2011

**Methods**
- Multicentre, double blind (for aspirin) open label (for enoxaparin) placebo controlled (for aspirin).

**Participants**
- Pregnant women with 3 or more first trimester miscarriages (<13 weeks), 2 or more second trimester miscarriages (13-24 weeks) or 1 third trimester fetal loss (>24 weeks).

**Interventions**
- Subcutaneous enoxaparin (40 mg daily) and placebo for aspirin (n = 88) vs subcutaneous enoxaparin (40 mg daily) (n = 63) and aspirin (100 mg daily) vs aspirin (100 mg daily) (n = 76).

**Outcomes**
- Primary: live birth (live birth after 24 weeks’ gestation). Secondary: pre-eclampsia, abruptio placentae, premature delivery (24-37 weeks’ gestation) intrauterine growth restriction, adverse effects and vaginal bleeding complications.

**Notes**
- Data of 1 woman (randomised to aspirin only) were excluded from analyses because she did not have RM < 24 weeks’ gestation and data of 10 women were excluded from analyses because they had beta-2 glycoprotein antibodies and therefore did not meet inclusion criteria.

---

**Risk of bias table**

<table>
<thead>
<tr>
<th>Bias</th>
<th>Author’s judgement</th>
<th>Support for judgement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random sequence generation (selection bias)</td>
<td>Low risk</td>
<td>Randomisation was performed by computer in blocks of 6 patients, stratified by centre and history of early or late miscarriage.</td>
</tr>
<tr>
<td>Allocation concealment (selection bias)</td>
<td>Low risk</td>
<td>Patients were allocated to randomisation code numbers in chronological order. The allocation list was stored at an independent secretariat and randomisation was performed by telephone.</td>
</tr>
<tr>
<td>Incomplete outcome data (attrition bias)</td>
<td>Low risk</td>
<td>No women were lost to follow-up.</td>
</tr>
<tr>
<td>Selective reporting (reporting bias)</td>
<td>Low risk</td>
<td>Study was prospectively registered (NCT0999862).</td>
</tr>
<tr>
<td>Other bias</td>
<td>Low risk</td>
<td>No other sources of bias were detected.</td>
</tr>
<tr>
<td>Overall risk of bias</td>
<td>Low risk</td>
<td>Overall, the study was considered at low risk of bias.</td>
</tr>
</tbody>
</table>

**HCG**: human chorionic gonadotropin; **HELLP**: haemolysis, elevated liver enzymes, low platelet count; **IU**: international units; **PGI2**: prostacyclin 2; **RM**: recurrent miscarriage; **TXA2**: thromboxane A2 vs. VERSUS
### Characteristics of excluded studies

<table>
<thead>
<tr>
<th>Study</th>
<th>Reason for exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bar 2000</td>
<td>Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately. Non-randomised trial.</td>
</tr>
<tr>
<td>Bar 2001</td>
<td>Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately. Non-randomised, uncontrolled trial.</td>
</tr>
<tr>
<td>Bick 2000</td>
<td>Non-randomised, uncontrolled trial.</td>
</tr>
<tr>
<td>Brenner 2000</td>
<td>Non-randomised trial, historical controls.</td>
</tr>
<tr>
<td>Brenner 2005</td>
<td>Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately.</td>
</tr>
<tr>
<td>Carp 2003</td>
<td>Non-randomised trial, historical controls.</td>
</tr>
<tr>
<td>Grandone 2002</td>
<td>Non-randomised trial. Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately.</td>
</tr>
<tr>
<td>Gris 1995</td>
<td>Misoxime chloride is not an intervention of interest in this review.</td>
</tr>
<tr>
<td>Gris 2004</td>
<td>Study does not include women with a history of RM.</td>
</tr>
<tr>
<td>Gris 2010</td>
<td>Study does not include women with a history of RM.</td>
</tr>
<tr>
<td>Gris 2011</td>
<td>Study does not include women with a history of RM.</td>
</tr>
<tr>
<td>Li 2003</td>
<td>Non-randomised trial.</td>
</tr>
<tr>
<td>Ogassawa 2001</td>
<td>Non-randomised trial. Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately.</td>
</tr>
<tr>
<td>Rai 2000</td>
<td>Non-randomised trial.</td>
</tr>
<tr>
<td>Rey 2009</td>
<td>Study author confirmed that no women were included in the study because of RM only.</td>
</tr>
<tr>
<td>Reznikoff-Elevant 1999</td>
<td>Non-randomised trial.</td>
</tr>
<tr>
<td>Sarig 2003</td>
<td>Data for women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately.</td>
</tr>
<tr>
<td>Sarto 2001</td>
<td>Non-randomised trial, historical controls.</td>
</tr>
<tr>
<td>Sorensen 2000</td>
<td>Non-randomised, uncontrolled trial. Data from women without apparent risk factors of recurrent pregnancy loss other than inherited thrombophilia cannot be extracted to be analysed separately.</td>
</tr>
<tr>
<td>Tzafetta 2002</td>
<td>Non-randomised, uncontrolled trial.</td>
</tr>
<tr>
<td>Younis 2000</td>
<td>Non-randomised, uncontrolled trial.</td>
</tr>
<tr>
<td>Zolghadri 2010</td>
<td>Unclear at what gestational age previous pregnancy losses occurred.</td>
</tr>
</tbody>
</table>

**Notes:**
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.

**Abbreviations:**
- RM: recurrent miscarriage
- IU: international units
- VS: versus
- VTE: venous thromboembolism

### Characteristics of studies awaiting classification

<table>
<thead>
<tr>
<th>Study</th>
<th>Methods</th>
<th>Participants</th>
<th>Interventions</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rodger 2009</td>
<td>Multi-national randomised controlled trial.</td>
<td>Women with laboratory confirmed thrombophilia at increased risk of placenta-mediated pregnancy complications or VTE.</td>
<td>Dalteparin 5000 units daily until 120 weeks’ gestation and then 5000 units twice daily until at least 37 weeks’ gestation vs no dalteparin.</td>
<td>Primary composite outcome: independently adjudicated placenta-mediated pregnancy complications (severe or early onset preeclampsia, birth of a small-for-gestational-age child (&lt;10th percentile) and/or pregnancy loss) and/or major VTE up to 6 weeks postpartum.</td>
</tr>
<tr>
<td>Salman 2012</td>
<td>Randomised controlled trial.</td>
<td>Women with unexplained recurrent pregnancy loss.</td>
<td>Tinzaparin sodium 4500 IU combined with 500 micrograms folate acid vs folate acid only.</td>
<td>Primary outcome: continuation of a viable pregnancy up to 20 weeks’ gestation.</td>
</tr>
<tr>
<td>Schleusner 2013</td>
<td>Multicentre randomised controlled trial.</td>
<td>Women with at least 2 early or 1 late miscarriage.</td>
<td>Dalteparin 5000 units combined with multivitamins vs vitamins only.</td>
<td>Primary outcome ongoing pregnancy at 24 weeks’ gestation.</td>
</tr>
</tbody>
</table>

**Notes:**
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
- Abstract only, awaiting full study report.
Data and analyses

<table>
<thead>
<tr>
<th>Outcome or Subgroup</th>
<th>Number of Studies</th>
<th>Number of Participants</th>
<th>Effect Estimate Risk Ratio (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 ASPIRIN VERSUS NO TREATMENT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.1 Live birth</td>
<td>2</td>
<td>Subtotals only</td>
<td></td>
</tr>
<tr>
<td>1.1.1 Live birth in all women</td>
<td>2</td>
<td>256</td>
<td>0.94 (0.80, 1.11)</td>
</tr>
<tr>
<td>1.1.2 Live birth in women with no previous live birth</td>
<td>1</td>
<td>122</td>
<td>0.89 (0.72, 1.21)</td>
</tr>
<tr>
<td>1.1.3 Live birth in women with inherited thrombophilia</td>
<td>1</td>
<td>32</td>
<td>1.08 (0.63, 1.85)</td>
</tr>
<tr>
<td>1.1.4 Live birth in women with more than two previous miscarriages</td>
<td>1</td>
<td>117</td>
<td>0.95 (0.70, 1.28)</td>
</tr>
<tr>
<td>2 LMWH VERSUS ASPIRIN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.1 Live birth</td>
<td>2</td>
<td>Subtotals only</td>
<td></td>
</tr>
<tr>
<td>2.1.1 Live birth in all women</td>
<td>2</td>
<td>238</td>
<td>1.08 (0.93, 1.26)</td>
</tr>
<tr>
<td>2.1.2 Live birth in women with no previous live birth</td>
<td>2</td>
<td>112</td>
<td>1.24 (1.02, 1.49)</td>
</tr>
<tr>
<td>2.1.3 Live birth in women with inherited thrombophilia</td>
<td>1</td>
<td>38</td>
<td>1.21 (0.78, 1.87)</td>
</tr>
<tr>
<td>3 LMWH VERSUS ASPIRIN INCLUDING STUDIES AT HIGH RISK OF BIAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.1 Live birth</td>
<td>3</td>
<td>Subtotals only</td>
<td></td>
</tr>
<tr>
<td>4 LMWH VERSUS NO TREATMENT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.1 Live birth</td>
<td>1</td>
<td>6</td>
<td>1.00 (0.56, 1.79)</td>
</tr>
<tr>
<td>5 LMWH VERSUS NO TREATMENT INCLUDING STUDIES AT HIGH RISK OF BIAS</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.1 Live birth</td>
<td>3</td>
<td>453</td>
<td>1.23 (0.84, 1.81)</td>
</tr>
<tr>
<td>6 LMWH AND ASPIRIN VERSUS NO TREATMENT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.1 Live birth</td>
<td>2</td>
<td>Subtotals only</td>
<td></td>
</tr>
<tr>
<td>6.1.1 Live birth in all women</td>
<td>2</td>
<td>322</td>
<td>1.01 (0.87, 1.16)</td>
</tr>
<tr>
<td>6.1.2 Live birth in women with no previous live birth</td>
<td>1</td>
<td>118</td>
<td>1.05 (0.83, 1.34)</td>
</tr>
<tr>
<td>6.1.3 Live birth in women with inherited thrombophilia</td>
<td>1</td>
<td>27</td>
<td>1.25 (0.74, 2.12)</td>
</tr>
<tr>
<td>6.1.4 Live birth in women with more than two previous miscarriages</td>
<td>1</td>
<td>119</td>
<td>1.00 (0.75, 1.33)</td>
</tr>
</tbody>
</table>

7 LMWH AND ASPIRIN VERSUS ASPIRIN

<table>
<thead>
<tr>
<th>7.1 Live birth</th>
<th>2</th>
<th>Subtotals only</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1.1 Live birth in all women</td>
<td>2</td>
<td>327</td>
</tr>
<tr>
<td>7.1.2 Live birth in women with no previous live birth</td>
<td>2</td>
<td>200</td>
</tr>
<tr>
<td>7.1.3 Live birth in women with inherited thrombophilia</td>
<td>2</td>
<td>63</td>
</tr>
<tr>
<td>7.1.4 Live birth in women with more than two previous miscarriages</td>
<td>1</td>
<td>112</td>
</tr>
</tbody>
</table>

8 LMWH AND ASPIRIN VERSUS LMWH

<table>
<thead>
<tr>
<th>8.1 Live birth</th>
<th>1</th>
<th>Subtotals only</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.1.1 Live birth in all women</td>
<td>1</td>
<td>126</td>
</tr>
<tr>
<td>8.1.2 Live birth in women with no previous live birth</td>
<td>1</td>
<td>72</td>
</tr>
<tr>
<td>8.1.3 Live birth in women with inherited thrombophilia</td>
<td>1</td>
<td>32</td>
</tr>
</tbody>
</table>

9 LMWH WITH OR WITHOUT ASPIRIN VERSUS NO TREATMENT INCLUDING STUDIES AT HIGH RISK OF BIAS

| 9.1 Live birth | 5 | 743 | 1.07 (0.99, 1.15) |

10 LMWH WITH OR WITHOUT ASPIRIN VERSUS NO TREATMENT

| 10.1 Live birth | 3 | 324 | 0.98 (0.85, 1.12) |

All analyses were performed using Mantel Haenszel fixed effects method, except for analysis 3.1 and 5.1, which were analysed using Mantel Haenszel random effects method. CI, Confidence Interval.
### FIGURE II - Analysis 1.1. Aspirin versus no treatment, outcome live birth.

<table>
<thead>
<tr>
<th>LMWH Study Subgroup</th>
<th>Events</th>
<th>Total Events</th>
<th>Total Weight</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kljakovic 2006</td>
<td>44</td>
<td>54</td>
<td>60</td>
<td>0.97 (0.81, 1.16)</td>
<td></td>
</tr>
<tr>
<td>Visscher 2011</td>
<td>67</td>
<td>82</td>
<td>100</td>
<td>1.23 (0.94, 1.54)</td>
<td></td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>119</td>
<td>142</td>
<td>160</td>
<td>1.09 (0.93, 1.28)</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>186</td>
<td>214</td>
<td>240</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### FIGURE III - Analysis 2.1. LMWH versus aspirin, outcome live birth.

<table>
<thead>
<tr>
<th>Level I LMWH Study Subgroup</th>
<th>Events</th>
<th>Total Events</th>
<th>Total Weight</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Live birth in all women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kljakovic 2006</td>
<td>44</td>
<td>54</td>
<td>60</td>
<td>0.97 (0.81, 1.16)</td>
<td></td>
</tr>
<tr>
<td>Visscher 2011</td>
<td>67</td>
<td>82</td>
<td>100</td>
<td>1.23 (0.94, 1.54)</td>
<td></td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>119</td>
<td>142</td>
<td>160</td>
<td>1.09 (0.93, 1.28)</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>186</td>
<td>214</td>
<td>240</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### FIGURE IV - Analysis 6.1. LMWH and aspirin versus no treatment, outcome live birth.

<table>
<thead>
<tr>
<th>LMWH and aspirin Study Subgroup</th>
<th>Events</th>
<th>Total Events</th>
<th>Total Weight</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.1 Live birth in all women</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kljakovic 2010</td>
<td>47</td>
<td>64</td>
<td>76</td>
<td>0.97 (0.79, 1.19)</td>
<td></td>
</tr>
<tr>
<td>Visscher 2011</td>
<td>87</td>
<td>108</td>
<td>128</td>
<td>1.23 (0.98, 1.55)</td>
<td></td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>134</td>
<td>172</td>
<td>206</td>
<td>1.10 (0.89, 1.35)</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>221</td>
<td>272</td>
<td>322</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### FIGURE V - Analysis 7.1. LMWH and aspirin versus aspirin, outcome live birth.

<table>
<thead>
<tr>
<th>LMWH and aspirin Study Subgroup</th>
<th>Events</th>
<th>Total Events</th>
<th>Total Weight</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
<th>Risk Ratio M-H Fixed, 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.1 Live birth in women with no previous live birth</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kljakovic 2010</td>
<td>47</td>
<td>64</td>
<td>76</td>
<td>0.97 (0.79, 1.19)</td>
<td></td>
</tr>
<tr>
<td>Visscher 2011</td>
<td>87</td>
<td>108</td>
<td>128</td>
<td>1.23 (0.98, 1.55)</td>
<td></td>
</tr>
<tr>
<td>Subtotal (95% CI)</td>
<td>134</td>
<td>172</td>
<td>206</td>
<td>1.10 (0.89, 1.35)</td>
<td></td>
</tr>
<tr>
<td>Total events</td>
<td>221</td>
<td>272</td>
<td>322</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Since the last update of this review, 59 randomised controlled trials on the efficacy and safety of aspirin and heparin in women with a history of at least two miscarriages without apparent risk factors other than inherited thrombophilia were published, but the number of studies on this topic remains limited. Although in total nine studies were included, three studies were considered to be at high risk of bias and from one study, data of only six women could be included. Different treatment doses of anticoagulants compared, prescribed for different time periods, resulted in maximally three studies per comparison that could be pooled and only three of seven comparisons included a placebo- or no treatment-arm. Irrespective of the type of or combinations of anticoagulants used, no benefit of anticoagulant treatment for live birth was found. Data for subgroup analyses were scarce. Subgroup analyses in women with more than two previous miscarriages showed no effect of treatment, regardless which treatment regimens were compared. A trend towards a significant effect from LMWH when compared to aspirin (risk ratio (RR) of live birth 1.21, 95% confidence interval (CI) 0.79 to 1.87) and of LMWH and aspirin when compared to no treatment (RR of live birth 1.25, 95% CI 0.74 to 2.12) was observed in women with inherited thrombophilia but the subgroups were underpowered for firm conclusions. As the clinical question of efficacy of anticoagulants for women with recurrent miscarriage (RM) and inherited thrombophilia remains relevant, randomised controlled trials focusing on women with inherited thrombophilia only are urgently needed. In subgroup analyses of women with no previous live birth, a beneficial effect of LMWH over aspirin was found in pooled analyses of two studies (n=112, RR 1.24, 95% CI 1.02 to 1.49). Some evidence of a similar trend toward a beneficial effect for LMWH versus LMWH and aspirin was observed in a small subgroup in one study (n=72, RR of live birth 1.25, 95% CI 0.74 to 2.12) in comparisons of LMWH and aspirin with either no treatment or with aspirin, no beneficial effect of LMWH and aspirin was found for women with no previous live birth. All subgroup analyses in women with no previous live birth were limited due to small numbers.

Reporting of secondary outcomes varied widely among studies. In studies that reported pregnancy complications, different denominators (e.g. all pregnant women, only ongoing pregnancies, only live births) were used and results could not be pooled. In the individual studies, no effect of treat-
Authors’ conclusions

Implications for practice
Evidence on the efficacy and safety of aspirin and low molecular weight heparin (LMWH) in women with a history of at least two miscarriages without apparent risk factors other than inherited thrombophilia is limited, but now includes several high-quality randomised controlled trials. Based on the results of the (pooled) analyses in this review, there is no evidence to support the use of anticoagulants in women with recurrent miscarriage (RM), regardless of the presence of inherited thrombophilia. Large randomised controlled trials assessing an effect of anticoagulants in women with RM and inherited thrombophilia are urgently needed.

Implications for research
Although several studies included women with inherited thrombophilia, subgroup analyses were never sufficiently powered to assess an effect of anticoagulation in these women with RM. We cannot exclude a beneficial effect in these women and therefore, large randomised trials are urgently needed and because of a counterbalancing effect of heparin and aspirin, a placebo or no intervention arm is necessary, since it would provide an adequate control to the active treatment and allows assessing a risk-benefit ratio.

Acknowledgements
We kindly acknowledge Professor Badawy, Professor Carp, Dr Fawzy, Dr Giancotti, Dr Martinelli, Dr Perna, Dr Ruggenenti, Dr Clark, Dr Tulppala, Dr Kaaja and Dr Visser for providing data or additional information requested for the review.

Louisette Peters commented on the drafts of the first version of this review. As part of the pre-publication editorial process, this review has been commented on by four peers (an editor and three referees who are external to the editorial team), a member of the Pregnancy and Childbirth Group’s international panel of consumers and the Group’s Statistical Adviser.
Appendix 4: Search Strategy

Cochrane Central Register of Controlled Trials (The Cochrane Library 2007, Issue 1), MEDLINE (January 1966 to April 2008), and EMBASE (1980 to March 2007), adapted for each database.

1. randomized controlled trial.pt.
2. randomized controlled triale.
3. controlled clinical trial.pt.
4. random allocation/
5. comparative study/
6. 1 or 2 or 3 or 4 or 5
7. clinical trial.pt.
8. clinical trials/
9. (clin$ adj trial$).tw
10. random$ tw
11. 7 or 8 or 9 or 10
12. 6 or 11
13. miscarriage$ tw
14. recurrent miscarriage$ tw
15. abortion spontaneous/
16. recurrent abortion$ tw
17. abortion habitual/
18. spontaneous pregnancy loss$ tw
19. recurrent pregnancy loss$ tw
20. early pregnancy loss$ tw
21. early pregnancy bleeding$ tw
22. habitual fetal loss$ tw
23. fetal death/
24. fetal resorption/
25. stillbirth.tw
26. 13 or 14 or 15 or 16 or 17 or 18 or 19 or 20 or 21 or 22 or 23 or 24 or 25
27. aspirin/
28. heparin/
29. low-molecular-weight heparin/
30. anticoagulants/
31. anticoagulant agent/
32. anti-thrombotic$ tw
33. 27 or 28 or 29 or 30 or 31 or 32
34. 12 and 26
35. 33 and 34

Lines 1-3 were not used for the search of CENTRAL.
Lines 1-12 were not used for the search of EMBASE as it does not have a .pt. field.
The $ is a truncation character which allows all possible suffix variations of the root word.

4.2 - Methods used when assessing the trials identified in the previous version of this review
This can be accessed in the full version at the Cochrane library
www.cochranelibrary.com

References 4


Chapter 5A

**ALIFE2 study: low-molecular-weight heparin for women with recurrent miscarriage and inherited thrombophilia. Study protocol for a randomized controlled trial**


*Shared last author

Trials 2015
Abstract

Background: A large number of studies have shown an association between inherited thrombophilia and recurrent miscarriage. It has been hypothesized that anticoagulant therapy might reduce the number of miscarriages and stillbirth in these women. In the absence of randomized controlled trials evaluating the efficacy of anticoagulant therapy in women with inherited thrombophilia and recurrent miscarriage, a randomized trial with adequate power that addresses this question is needed. The objective of the ALIFE2 study is therefore to evaluate the efficacy of low-molecular-weight heparin (LMWH) in women with inherited thrombophilia and recurrent miscarriage, with live birth as the primary outcome.

Methods/design: Randomized study of LMWH plus standard pregnancy surveillance versus standard pregnancy surveillance alone.

Study population: Pregnant women of less than 7 weeks gestation, and confirmed inherited thrombophilia with a history of two or more miscarriages or intra-uterine fetal deaths, or both.

Setting: Multi-center study in centers from the Dutch Consortium of Fertility studies; centers outside The Netherlands are currently preparing to participate.

Intervention: LMWH enoxaparin 40 mg subcutaneously once daily started prior to 7 weeks gestational age plus standard pregnancy surveillance or standard pregnancy surveillance alone.

Main study parameters/endpoints: The primary efficacy outcome is live birth. Secondary efficacy outcomes include adverse pregnancy outcomes, such as miscarriage, pre-eclampsia, syndrome of hemolysis, elevated liver enzymes and low platelets (HELLP-syndrome), fetal growth restriction, placental abruption, premature delivery and congenital malformations.

Safety outcomes include bleeding episodes, thrombocytopenia and skin reactions.

Discussion: After an initial period of slow recruitment, the recruitment rate for the study has increased. Improved awareness of the study and acknowledgement of the need for evidence are thought to be contributing to the improved recruitment rates. We aim to increase the number of recruiting centers in order to increase enrollment into the ALIFE2 study. The study website can be accessed via www.alife2study.org.

Trial registration: The ALIFE2 study was registered on 19 March, 2012 at www.trialregister.nl under registration number NTR3361.
Background

In all clinically recognized pregnancies, a single spontaneous miscarriage occurs in 14-19% of patients, and 1-5% of women experience two or more miscarriages (recurrent miscarriage). After comprehensive investigation, a risk factor for recurrent miscarriage is identified in less than half of couples. Many studies have confirmed a relationship between inherited thrombophilia and miscarriage and other adverse pregnancy outcomes. The role of thrombophilia in recurrent miscarriage can be explained partially by the concept of thrombosis of the (microvasculature of the) placenta, and partially through inhibition of extra-villous trophoblast differentiation. Therefore, anticoagulants are considered a possible therapy for women with recurrent miscarriage and inherited thrombophilia. In a recent Cochrane systematic review and meta-analysis we reviewed the trial evidence on the effects of antithrombotic therapy and showed that no firm trial data are available for these women.

Previously, we performed a randomized, placebo controlled study (ALIFE study), investigating whether aspirin combined with low-molecular weight heparin (LMWH) or aspirin alone as compared with placebo would improve the live birth rate among 364 women with unexplained recurrent miscarriage (<20 weeks gestation). We found that neither aspirin combined with nadroparin (relative risk (RR) for live birth 0.96, 95% confidence interval (CI) 0.76 to 1.19) nor aspirin alone (RR for live birth 0.89, 95% CI 0.71 to 1.13) improved the chance of a live birth in women with a history of unexplained recurrent miscarriage. In addition, no statistically significant benefits were found for women with inherited thrombophilia, although the study was not powered to assess this effect. The SPIN-study, another randomized controlled trial, assessed whether enoxaparin and aspirin reduced the rate of miscarriage compared to intensive pregnancy surveillance alone in 294 women with a history of 2 or more consecutive previous miscarriages (<24 weeks gestation). Results showed 22% miscarriage in participants receiving enoxaparin and aspirin, compared with 20% miscarriage in subjects receiving intensive surveillance alone (odds ratio 0.91, 95% confidence interval 0.52-1.59). The Habenox study also found no beneficial effect of LMWH (with aspirin or placebo) compared to aspirin in women with or without thrombophilia and recurrent miscarriage. Neither individually, nor combined in meta-analysis, was any of these studies sufficiently powered to demonstrate an effect of pharmacological therapy in the subgroup of women with inherited thrombophilia. For women with the antiphospholipid syndrome (APS), the use of heparin or LMWH combined with low-dose aspirin is an effective treatment for recurrent miscarriage, although not demonstrated in all performed trials. For inherited forms of thrombophilia, this effect has not profoundly been studied yet. Very recently, results of the TIPPS study showed that dalteparin did not reduce the incidence of a composite outcome (severe or early-onset pre-eclampsia, small-for-gestational-age infant, pregnancy loss, or venous thromboembolism (VTE)) in pregnant women with thrombophilia at increased risk of VTE or with previous placenta-mediated pregnancy complications. However, again in this study, the subgroup of women with recurrent miscarriage and inherited thrombophilia was too small to draw conclusions of a potential effect on the outcome live birth.

The association between inherited thrombophilia and recurrent miscarriage together with the potential beneficial effects of anticoagulant therapy in women with acquired thrombophilia and recurrent miscarriage have led some physicians to prescribe LMWH to women with recurrent miscarriage and inherited thrombophilia. While this practice may be supported by a plausible hypothesis, a beneficial effect of LMWH in these women still needs to be demonstrated. Gynecologists and hematologists, who expressed their support in the design phase of the study, and who are currently contributing to recruitment, recognize the urgent need for a trial such as ALIFE2 internationally.

The results of the ALIFE2 study will clarify the need to screen for inherited thrombophilia in women with recurrent miscarriage. Current guidelines advise differently on whether or not to test for inherited thrombophilia in these women. The efficacy of LMWH in women with recurrent miscarriage and inherited thrombophilia has never been tested in a randomized controlled trial. Thus, if the results of the ALIFE2 study show that LMWH increases live birth in women with recurrent miscarriage and inherited thrombophilia, screening for inherited thrombophilia in this setting may be justified. Conversely, if no evidence of a benefit is found, the use of LMWH will not be justified and screening for inherited thrombophilia will not be indicated. This will decrease costs of inappropriate screening, and reduce the burden of anticoagulant treatment in pregnant women.
Methods/Design

Design of the study

The primary objective of this study is to evaluate the efficacy of LMWH in women with inherited thrombophilia and recurrent miscarriage and/or intra-uterine fetal death (≥2). The primary outcome is live birth.

Secondary objectives included efficacy and safety objectives:

Efficacy
• To evaluate other possible effects of LMWH on adverse pregnancy outcome other than miscarriage (e.g. preeclampsia, intra-uterine growth restriction, HELLP syndrome, (syndrome of hemolysis, elevated liver enzymes and low platelets), placental abruption, premature delivery, congenital malformations, VTE)

Safety
• To evaluate the safety of LMWH in women with recurrent miscarriage with inherited thrombophilia by recording complications such as bleeding, thrombocytopenia and (allergic) skin reactions.

All pre-specified outcomes are listed in Table I. Criteria for the classification of bleeding are listed in Table II. Other study variables include concomitant use of antithrombotic drugs (such as aspirin or), non-steroidal anti-inflammatory drugs (NSAIDs), maternal age, multiple gestation, number of preceding miscarriages, maternal ethnicity, partner’s ethnicity, maternal weight, height, and body mass index, past obstetric history, medication, smoking, alcohol consumption, allergies, family history of VTE and of miscarriage and/or intra-uterine fetal death.

### Table I - Outcomes

<table>
<thead>
<tr>
<th>Primary efficacy outcome</th>
<th>Live birth (defined as birth of a living child)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Principal safety outcome</td>
<td>Clinically relevant bleeding (i.e. major bleeding and clinically relevant non-major bleeding)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Secondary study outcomes</th>
<th>Effecy</th>
<th>Safety</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ongoing pregnancy beyond 12 weeks' gestation</td>
<td>Post-partum bleeding and severe post-partum bleeding</td>
<td></td>
</tr>
<tr>
<td>Preeclampsia*</td>
<td>Major bleeding</td>
<td>Heparin induced thrombocytopenia (defined according to ACCP criteria)*</td>
</tr>
<tr>
<td>HELLP syndrome*</td>
<td>Clinically relevant non-major bleeding</td>
<td>Allergic reactions (redness or itching) localized at the injection site of LMWH</td>
</tr>
<tr>
<td>Intrauterine growth restriction*</td>
<td>Minor bleeding, including increased tendency to bruising not fulfilling the criteria for clinically relevant non-major bleeding</td>
<td></td>
</tr>
<tr>
<td>Placental abruption*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Premature birth*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intra-uterine fetal death*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Major congenital anomalies*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Composite of confirmed deep vein thrombosis and confirmed pulmonary embolism*</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Denominator for these outcomes is ongoing pregnancies beyond 12 weeks’ gestation.

a: Preeclampsia is defined as hypertension (systolic blood pressure ≥140 mmHg and/or diastolic blood pressure ≥90 mmHg after 20 weeks’ gestation, measured twice in a previously normotensive woman) with new onset proteinuria at or beyond 20 weeks’ gestation.

b: HELLP syndrome is defined as a platelet count less than 100 *10⁹/l and aspartate aminotransferase of 70 u/l or greater and lactate dehydrogenase of 600 u/l or greater.

c: Intrauterine growth restriction is defined as birth weight <10th percentile for gestational age.

d: Placental abruption (also known as abruptio placenta) is a complication of pregnancy, wherein the placental lining has separated from the uterus of the mother. Diagnosis according to clinical criteria (vaginal bleeding and uterine tenderness in combination with fetal distress necessitating prompt delivery) and examination of the placenta.

e: Premature birth is defined as birth <37 weeks’ gestation.

f: Major physical anomalies are defined as physical anomalies that have cosmetic or functional significance.

g: Deep vein thrombosis and pulmonary embolism are defined as abnormal compression ultrasound or an intraluminal filling defect on venography (deep vein thrombosis), or an intraluminal filling defect on spiral computed tomography (CT) scan, cut-off of vessels more than 2.5 mm in diameter on pulmonary angiogram or a perfusion defect of at least 75% of a segment with a local normal ventilation result (high-probability) on ventilation/perfusion lung scan (pulmonary embolism).

ACCP, American College of Chest Physicians; LMWH, low-molecular-weight heparin.

Criteria for bleeding are listed in Table II.
The study is designed as a multi-center randomized intervention study (Figure 1). Pregnant women with a history of recurrent miscarriage (2 or more miscarriages or intra-uterine fetal deaths, not necessarily consecutive), and confirmed inherited thrombophilia are randomized to one of two groups. Women in the intervention group inject LMWH once daily in addition to standard pregnancy surveillance. Women in the control group receive standard pregnancy surveillance. Since blinding of patients or investigators is deemed unfeasible due to the nature of the intervention, an adjudication committee, whose members are blinded for the intervention, will assess primary and secondary outcome measures. End of follow-up is at six weeks after delivery or miscarriage. Members of the blinded adjudication committee will be prof. dr. M.H. Prins, MD, epidemiologist and dr. W.M. Ankum, gynaecologist.

**TABLE II - Criteria for major, clinically relevant non-major and minor bleeding.**

| Major bleeding | • Associated with a fall in hemoglobin of 2 g/dL or more  
|                | • Leading to a transfusion of 2 or more units of packed red blood cells or whole blood  
|                | • Occurring in a critical site: intracranial, intra-spinal, intraocular, pericardial, intra-articular, intramuscular with compartment syndrome, retro-peritoneal  
|                | • Contributing to death  |
| Clinically relevant non-major bleeding | • Any bleeding compromising hemodynamics  
|                                      | • Any bleeding leading to hospitalization  
|                                      | • Subcutaneous hematoma larger than 25 cm³, or 100 cm³ if there was a traumatic cause  
|                                      | • Intramuscular hematoma documented by ultrasonography  
|                                      | • Epistaxis that lasted for more than 5 minutes, was repetitive (i.e., two or more) episodes of bleeding more extensive than spots on a handkerchief within 24 hours, or led to an intervention (e.g., packing or electrocautery)  
|                                      | • Gingival bleeding occurring spontaneously (i.e., unrelated to eating or tooth brushing) or lasting for more than 5 minutes  
|                                      | • Hematuria that was macroscopic and was spontaneous or lasted for more than 24 hours after instrumentation (e.g., catheter placement or surgery) of the urogenital tract  
|                                      | • Macroscopic gastrointestinal hemorrhage, including at least one episode of melena or hematemesis, if clinically apparent with positive results on a fecal occult-blood test  
|                                      | • Rectal blood loss, if more than a few spots on toilet paper  
|                                      | • Hemoptysis, if more than a few specks in the sputum and not occurring within the context of pulmonary embolism  
|                                      | • Any other bleeding type considered to have clinical consequences for a patient  
|                                      | o such as medical intervention, the need for unscheduled contact (visit)  
|                                      | o or telephone call with a physician, or temporary cessation of a study drug or associated with pain or impairment of activities of daily life  |
| Minor bleeding | All other overt bleeding episodes not meeting the criteria for major or clinically relevant bleeding or postpartum bleeding.  

**Figure 1 - Flowchart of the alife2 study.**

Primary efficacy outcome: Live birth

LMWH + standard pregnancy surveillance

End of pregnancy + 6 weeks post-partum

End of follow-up

Recruitment

Randomization

Positive urine pregnancy test

End of pregnancy + 6 weeks post-partum

End of follow-up

≥ 2 miscarriages and/or IUDs
The study will be conducted in several international centers, both tertiary and non-tertiary. A list of participating centers and countries is available from the sponsor.

**Ethics**

The study was approved by the institutional review board (IRB) of the Academic Medical Center (IRB registration no. METC_2012_173) and is conducted according to the principles of the World Medical Association (WMA) Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects Version Seoul, South Korea, October 2008, with Note of Clarification on Paragraph 29 added by the WMA General Assembly, Washington 2002 Note of Clarification on Paragraph 30 added by the WMA General Assembly, Tokyo 2004 and in accordance with the Dutch Medical Research Involving Human Subjects Act (WMO) and other guidelines, regulations and Acts. Recruitment in individual centers will be commenced after local approval is obtained.

**Study procedures**

Women, aged 18-42 at randomization, with a history of two or more miscarriages and/or intra-uterine fetal deaths with confirmed inherited thrombophilia, who are actively trying to conceive or are less than 7 weeks pregnant are recruited for the study. They are informed of the study by their treating physician and receive written study information. Women are instructed to perform a urine pregnancy test as soon as their menstrual periods are delayed or a pregnancy is suspected. Furthermore, they are contacted by telephone at 3-month intervals until they are pregnant. Once a urine test confirms pregnancy and all inclusion and exclusion criteria are met (Table III), including signing of informed consent, they are randomized to either the intervention arm or the standard pregnancy surveillance arm of the study. Informed consent will be obtained by Good-Clinical-Practice-trained study personnel with use of the review-board approved consent form. Women will only be randomized in the study after informed consent is obtained.
Randomization is performed centrally, online via a secure Internet facility in a 1:1 ratio by the tenalea Clinical Trial Data Management System using randomly permuted blocks with maximum block size of 6 within strata formed by maternal age (<36 or ≥36 years), number of miscarriages (2 or ≥3) and center type (tertiary or non-tertiary). Both the including physician and patient are concealed for allocation.

Women who are randomized to intervention arm start with lmwh immediately. They undergo a blood test twice (2.7 ml at baseline and 2.7 ml after 7 – 10 days) to check for heparin induced thrombocytopenia and are instructed to discontinue lmwh when they experience the first signs of labor. In case of a planned delivery or cesarean section, lmwh should be discontinued according to local policy, but at least 12 hours prior to neuraxial anesthesia or cesarean section. Randomized women will be contacted at 12 and 24 weeks’ gestation and 6 weeks post-partum to collect outcome data and data on adverse events. No additional study visits are planned. Women in both study arms will receive standard pregnancy surveillance. Data from routine obstetric visits is recorded in a case record form (crf) for analysis, including medical history and family history. Additionally, maternal bleeding episodes, thrombotic signs, and possible reactions to study medication are monitored. Women are instructed to contact the study center immediately when bleeding episodes occur or when developing (possibly heparin induced-) thrombocytopenia.

In case of a (serious) adverse event, emergency medication is administered by treating physicians according to local policy; e.g. in case of a major bleeding a patient may receive blood transfusions and / or antidote (e.g. protamine sulfate).

In case of serious type IV delayed hypersensitivity skin reactions to lmwh, an alternative form of LMWH can be prescribed. In case of type I allergy, LMWH must be discontinued.

The crf can be downloaded from the study website www.alife2study.org. Data handling will be coded, with the patient code only available to the local investigator and the research nurse working in the local center. Data recorded in the crfs will be collected in an electronic crf, accessible via the study website. Women withdrawn from treatment will be asked to continue follow up until the end of study (i.e. 6 weeks after delivery or miscarriage). Important protocol modifications will be communicated to all relevant parties (e.g. investigators, trial nurses, review boards, trial participants, trial registries and journals) if indicated.

### Table III - Inclusion and exclusion criteria.

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women with recurrent miscarriage and/or intra-uterine fetal deaths (i.e. ≥2 miscarriages of intra-uterine fetal deaths, irrespective of gestational age)</td>
<td>Duration of current pregnancy ≥ 7 weeks, based on first day of last menstruation</td>
</tr>
<tr>
<td>Confirmed inherited thrombophilia*</td>
<td>Indication for anticoagulant treatment during pregnancy (for instance prosthetic heart valves, a history of venous thromboembolism or antiphospholipid syndrome)</td>
</tr>
<tr>
<td>- factor V Leiden mutation</td>
<td>- Contraindications to LMWH (previous heparin-induced thrombocytopenia, active bleeds or renal insufficiency with creatinine clearance of less than 30ml/min)</td>
</tr>
<tr>
<td>- protein S deficiency</td>
<td>- Known allergy at least 3 different LMWH preparations</td>
</tr>
<tr>
<td>- protein C deficiency</td>
<td>- Previous inclusion in the ALIFE2 study (for another pregnancy)</td>
</tr>
</tbody>
</table>

*Protein S, protein C and antithrombin deficiencies need to be confirmed by two tests, performed on two separate occasions and not during anticoagulant therapy. Protein S tests should not be performed during pregnancy or in the 6-week post-partum period since spuriously low levels may then be observed.
Sample size calculation

In the previous ALIFE study, the occurrence of live birth in the subgroup of women who had an inherited thrombophilia and became pregnant was 60% in those randomized to placebo. Based on this information, a conservative sample size assuming a live birth of 55% in the control group is calculated. In order to detect an absolute risk difference of 15%, with a power of 80% and a two-sided confidence level of 95%, the sample size required for the study is \( n = 332 \), with 166 women in each arm. Taking a potential loss to follow up and exclusion from the study (due to ectopic pregnancy for example) into consideration, we aim to recruit 20% more women (\( n = 399 \)).

The absolute risk difference of 15% was defined following consultations amongst health care providers and patients. Since we acknowledge that from a patient’s perspective, a 10% absolute risk difference is still clinically relevant, we also plan to perform a conditional interim analysis to adjust the sample size. When approximately 95% of inclusion is reached, the adjudication committee will analyze efficacy outcome, blinded for allocation. If the estimated difference in live birth between the two groups is below 10% or above 15%, the study will continue as planned and will be completed with 399 enrolled women. However, if the estimated difference in live birth is between 10% and 15%, we will explore (financial) means and evaluate if continued recruitment until a total sample size of 776 women is feasible so that the study is sufficiently powered to assess this effect. For this interim analysis we will use a total two-sided significance test with the O’Brien–Fleming alpha spending function and a type 1 error rate of 5 perc.

Statistical analyses

Baseline data and outcome data will be summarized separately. For continuous variables, we will examine the distribution of the observations, and if normally distributed we will then summarize them as means with standard deviations (SDs). If they are not normally distributed, then medians and inter-quartile ranges (IQRs) will be reported. For dichotomous data, we will provide proportions (or percentages). Differences in dichotomous outcomes between the two treatment arms will be analyzed using the chi-square test. For continuous outcomes we will use the independent t-test if the observations in each study arm are normally distributed, and if non-normally distributed, the Mann-Whitney-U test will be employed. For all outcome measures, we will calculate 95% confidence intervals around point estimates.

To explore differential or subgroup effects of LMWH will be assessed in a priori defined prognostic groups: age (continuous and dichotomized), number of previous miscarriages (2, or 3 or more), previous live birth (yes/no), type of inherited thrombophilia (FVIII, PTM, AT/C/S deficiency).

For issues such as loss to follow-up, missing data, and protocol violations, we will attempt sensitivity (worst-case and best-case scenarios) analyses to explore the effect on the study findings. As a secondary analysis, we will adjust for missing data using imputation techniques to explore the effects of such imputations on the study findings.

Efficacy analyses will be based on the intention-to-treat (ITT) principle. A p-value of less than 0.05 will be considered statistically significant. All statistical analyses will be performed using the SPSS package (SPSS Inc., Chicago, IL, USA).
Data monitoring committee (DMC)

A data monitoring committee is installed for the ALIFE2 study. A DMC charter is supplementary to this protocol and available from the sponsor. Monitoring will be performed in collaboration with the Academic Medical Center Clinical Research Unit (CRIU). A monitoring plan was drafted accordingly and is available with the sponsor. Members of the Monitoring committee are Prof. Dr. H.R. Büller, AMC, Amsterdam [chair], Dr. W.M. Ankum, gynecologist, AMC, Amsterdam and Prof. Dr. M. Prins, statistician, Maastricht University Medical Center, Maastricht.

The final trial dataset will be accessible by the lead investigator and statistical analysis team at the Academic Medical Center CRIU. No contractual arrangements are in place that limit such access for investigators.

Adverse events

LMWH is registered and recommended for a wide variety of indications. Also to pregnant women, LMWH is prescribed for several indications and in higher dosages than is used in the ALIFE2 study. Therefore the spectrum of adverse events is well documented in regular patients as well as in pregnant women. For this reason, not all undesirable experiences occurring to women are recorded, but only adverse events that are suspected to be related to study medication. These adverse events include: clinically relevant bleeding (major or clinically relevant non-major bleeding) and any bleeding (hematoma, epistaxis, bleeding of gums, vaginal blood loss, hematuria or any other form of bleeding), heparin induced thrombocytopenia, skin reaction to injection (e.g. itching, swelling) and (type 1) allergic reactions. When hematomas are related to venipuncture, only hematomas larger than 10 × 10 cm are reported.

For all events recorded, the nature and severity will be assessed. A serious adverse event (SAE) is any untoward medical occurrence or effect that at any dose:

- Results in death.
- Is life threatening (at the time of the event).
- Requires hospitalization or prolongation of existing inpatient hospitalization.

SAEs that result in death or are life threatening should be reported immediately. The reporting will occur not later than 7 days after the responsible investigator has first knowledge of the adverse reaction. This is for a preliminary report with another 8 days for completion of the report. The study efficacy outcome bleeding will not be reported as (s)AE. A standard practice of hospitalization (e.g. for delivery) will not be considered as a prolonged hospitalization and should not be reported as an SAE. However, if this transfer is part of treatment of a medical complication, it should be considered prolonged hospitalization and the event should be reported as a SAE. Clinically anticipated events such as bleeding are exempted from the expedited reporting to regulatory authorities of suspected adverse reactions that are both serious and unexpected. These clinically anticipated events are periodically reviewed by the DMC in an unblinded manner to ensure prompt identification of any clinically concerning safety issues.

Public disclosure and publication policy

A writing committee chaired by the PIs will be put in place as the trial progresses. The composition of the committee will reflect the contribution of investigators to various aspects of the trial, including but not solely the conception and design, acquiring of funding, country coordination and recruitment of patients. This committee will write the study report, and the report will include list of centers and investigators that contributed patients to the study. There are no publications restrictions by the Sponsor of the study. Furthermore, trial results will be posted on the study website and communicated through patients via patient organization websites.
Compensation for injury

The sponsor/investigator in the Netherlands has a liability insurance which is in accordance with article 7, subsection 6 of the WMO. The sponsor (also) has an insurance which is in accordance with the legal requirements in the Netherlands (Article 7 WMO and the Measure regarding Compulsory Insurance for Clinical Research in Humans of 23th June 2003). This insurance provides cover for damage to research subjects through injury or death caused by the study.

1. 450,000,- (i.e. four hundred and fifty thousand Euro) for death or injury for each subject who participates in the Research.
2. 3,500,000,- (i.e. three million five hundred thousand Euro) for death or injury for all subjects who participate in the Research.
3. 5,000,000,- (i.e. five million Euro) for the total damage incurred by the organisation for all damage disclosed by scientific research for the Sponsor as ‘verrichter’ in the meaning of said Act in each year of insurance coverage.

The insurance applies to the damage that becomes apparent during the study or within 4 years after the end of the study and is only applicable to patients included in the Netherlands.

Discussion

The choice for the intervention with LMWH in the ALIFE2 study was based on the following considerations. In the previous ALIFE study, LMWH was administered in combination with aspirin and compared with aspirin alone and to no-intervention (placebo for aspirin) in women with recurrent miscarriage of unknown cause. Since there are no solid data supporting an effect of aspirin in women with recurrent miscarriage, with or without inherited thrombophilia, we chose to use LMWH as intervention, without aspirin as additional intervention in the ALIFE2 study, to exclude a possible deleterious effect of aspirin.

The dose of LMWH is higher than low-prophylactic dosages, in order not to miss a beneficial effect of LMWH on live birth. However, because results from the Live-Enox study showed no difference in live birth in women randomized to 40 mg enoxaparin or 80 mg enoxaparin we chose to use the dosage of 40 mg enoxaparin, in order to reduce the risk of bleeding.

A timely execution of the trial is impeded because of two reasons. Firstly, the assumed efficacy of LMWH triggers physicians to give LMWH the benefit of the doubt. They offer the treatment despite a lack of its evidence; especially in poignant cases as for example women with a history of multiple miscarriages. Eligible women will thus be reluctant to be randomized, facing a 50% chance of no-treatment, when they can also approach physicians who are willing to prescribe them LMWH outside the context of the study. This hurdle can be overcome, provided both physicians and patients are well informed. Physicians who are aware of the lack of evidence for LMWH and the non-negligible bleeding risk associated with this treatment clearly understand the need for the study. They are more willing to contribute to recruitment and comply with guidelines advising not to treat in absence of evidence for treatment. A second reason for delay, potentially specific to the Dutch situation, is that eligible women for the study remain unidentified because screening for inherited thrombophilia is not standard practice. A complete thrombophilia screen is expensive and as currently no proven effective treatment for women with recurrent miscarriage and inherited thrombophilia is available, testing for inherited thrombophilia (solely for the indication or recurrent miscarriage) is not always performed. Nonetheless, many women have already been tested for thrombophilia because of other reasons such as a positive family history of VTE. Moreover, as screening for inherited thrombophilia may provide an explanation for recurrent miscarriage, some physicians perform screening not to identify a treatable condition but to provide couples with this explanation. In this way, even physicians who don’t incorporate thrombophilia screening in their standard practice can contribute to recruitment, identifying these women.

Trial status

The study commenced recruiting in January 2013 in the Netherlands, with at present 11 participating centers and 2 additional centers for which ethics approval is expected shortly. Participation of non-Dutch centers was initiated by the University Hospital of Brussels in Belgium, which is expected to be followed closely by centers in the United Kingdom, the United States, Canada and Sweden.
At the time of writing 22 women have been randomized in the study. An initially slow recruitment is currently catching up to a rate of approximately 2 inclusions per month in the Netherlands, expected to increase to 4 per month with continuing the number of the number of participating centers and increasing awareness of the study. Confirmed participation of centers abroad and especially dedicated recurrent miscarriage clinics in the United Kingdom is expected to further increase this rate to an eventual inclusion rate of 14 women per month.

As with most investigator-initiated multi-center trials, several hurdles, such as an administrative workload, (international) legislation and applications for approval in participating centers, and initial slow recruitment, had to be overcome to get the trial running. However, with these hurdles taken, the trial now seems to have reached a new phase, with a steadily increasing recruitment rate, towards completion of the trial.

Grants

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References 5A

References 5a

Chapter 5b

Progress of the ALIFE2 study; obstacles along the road towards more evidence

Paulien G. de Jong, Saskia Middeldorp, Siobhan Quenby and Mariëtte Goddijn

Submitted for publication
Abstract

Investigator-initiated studies are invaluable to obtain evidence, especially in fields that are not particularly of interest for the pharmaceutical industry because they are either less profitable, or concern special patient groups such as pregnant women. However, to design, conduct and accomplish an investigator-initiated randomized controlled trial proves to be highly challenging. Patients and physicians’ preferences, ethics requirements, (international) legislation and funding are all examples of areas where such challenges are met.

The alife2 study is illustrative of an investigator initiated multicenter trial, which progresses steadily but slowly as during the course of this trial, many hurdles had to be overcome.

Here, we discuss the challenges and hurdles we faced during the course of the alife2 study up till now and we explain how some of these hurdles can be avoided or taken most easily.

Background

In April 2010, results of the alife study were published. A total of 364 women with unexplained recurrent miscarriage (i.e. in the absence of evident causes of miscarriage such as abnormal parental karyotypes or uterine anomalies) were randomized to either low-molecular-weight heparin (LMWH) plus acetylsalicylic acid (ASA), ASA alone or placebo (for ASA). The results indicated that neither the combination of LMWH and ASA, nor ASA alone improved the likelihood of live birth in a subsequent pregnancy. Together with two similar studies published around the same time, this information changed guideline recommendations and influenced clinical practice.

Although evidence for the lack of efficacy of anticoagulants in women with unexplained recurrent miscarriage was provided by these three independent investigations, the question remains whether the subgroup of women with recurrent miscarriage and inherited thrombophilia may benefit from anticoagulants. Women with inherited thrombophilia, such as the presence of the Factor V Leiden mutation, the prothrombin G20210A mutation or a deficiency of protein S, protein C or antithrombin, have an increased risk of pregnancy complications, including recurrent early miscarriage and
Initial funding, although limited, was acquired as part of a VIDI-grant (The Netherlands Organisation for Health Research and Development, 016.126.364). Compared to the previous ALIFE study – a national, multicenter trial –, we anticipated a more difficult recruitment because of the restrictive inclusion criterion of the presence of inherited thrombophilia in women with recurrent miscarriage. As inherited thrombophilia testes are positive in approximately 15% of women with unexplained recurrent miscarriage, it was estimated that enrollment would be completed within 5 years, with continued follow-up until the outcome of pregnancies until 9 months thereafter.

Current status of the ALIFE2 study

Since the official launch of the study, and the enrollment of the first participant on January 11, 2013, the study is currently enrolling in 11 centers in the Netherlands, as well as in Brussels, Belgium. Considering the number of participating centers, the enrollment of 19 women may seem surprisingly low. However, when interpreting the enrollment rate per month, we may appreciate a steep increase from little over 2 inclusions per 6 months in the first year, to approximately 2 inclusions per month in the past 4 months. This is expected to increase further with the anticipated participation of UK centers, as well as the University of Illinois College of Medicine, the New York-Presbyterian Hospital and the University Hospital of Montreal. As for most international multi-center investigator-initiated trials, the journey towards starting of the trial has been far from a smooth ride and various obstacles had to be overcome. Identifying and finding a solution for these obstacles has been key in improving the enrollment rate. Here, we will discuss several of the obstacles encountered and how they were – or are – tackled.
Experienced challenges and hurdles

Choice of study intervention

In the ALIFE2 study, enoxaparin 40 mg injection once daily (or an equivalent dose of another low-molecular-weight heparin type), is compared to no-intervention. The type of treatment and dose were extensively discussed. A three-armed trial also including ASA would have been ideal, but was considered unfeasible as the number of participants needed would increase from 400 to over 700.

For heparin, both the anticoagulant activity and anti-inflammatory properties are thought to contribute to its effect for maintaining pregnancy. In addition, heparin promotes trophoblast differentiation in vitro. For ASA, the mechanism of action in pregnancy is less clear. Unfractionated heparin needs to be administered at least twice daily, whereas LMWH a single daily dose is sufficient. As in a direct comparison of LMWH and UFH (both combined with ASA, in women with antiphospholipid syndrome), both appeared equally effective, LMWH was considered more favorable than UFH. Results of the previously mentioned subgroup analysis in women with inherited thrombophilia in the ALIFE study suggested a greater effect on live birth of LMWH plus ASA when compared to ASA alone. Furthermore, for women with antiphospholipid syndrome, there is no evidence that ASA alone increases live birth rates after recurrent miscarriage. This knowledge, taken together with the fact that for clinical practice the question whether LMWH is effective appears most pressing, led to LMWH as the intervention of choice for the ALIFE2 study.

Enoxaparin 40 mg or an equivalent was the dose decided upon after consultation with many colleagues; both to verify that there was agreement amongst peers on the scientific merit of the study, as well as to make sure that, with the agreed dose of LMWH, colleagues would be willing to participate. Consensus was not easily reached. A high dose (e.g. equivalent to or even higher than 80 mg enoxaparin) would infer a higher bleeding risk, but would minimize the possibility that a (dose-dependent) beneficial effect of LMWH would not become apparent in the trial but would increase the risk of bleeding. Using a low dose would conversely be potentially associated with a lower bleeding risk, but a negative trial outcome (i.e. no beneficial effect of LMWH) in turn, would not settle the doubt that a higher dose could have been effective. However, results of the LIVE-ENOX trial, showed no additional benefit of 80 mg enoxaparin over 40 mg enoxaparin in women with recurrent miscarriage. As colleagues indicated that the bleeding risk associated with the higher dose would make them reluctant to include their patients, the trial dose was set at equivalent to enoxaparin 40 mg: higher than a low dose used for VTE prophylaxis (i.e. enoxaparin 20 mg), but lower than the high dose of 80 mg. Despite these careful considerations, participation may be modestly influenced by colleagues, who were used to prescribe the higher dose. Hence, in planning recruitment these factors should be taken into account.

LMWH is compared to no treatment in the ALIFE2 study. The use of a placebo (e.g. saline injections) was considered in the design-phase. Compared to no treatment, placebo use could affect participation positively as well as negatively, as both a 50% chance of saline injections, as well as a 50% chance of open-label no treatment may be reasons for women to refuse or cancel participation. Since the trial evaluates the effect of LMWH on live birth, use of a placebo was not judged to be a crucial quality aspect. Arguments in favor of a placebo-control include a potentially more valid trial result, and minimization of the risk of performance bias, i.e. that systematic differences arise between the groups in the care that is provided, in exposure to other factors, and in assessment of data including bleeding. The difficulties of manufacturing placebo injections and the high costs associated with this manufacturing and the distribution were the greatest argument against the use of placebo. Furthermore, as for the unequivocal primary outcome (a live born neonate), a placebo effect may be considered minimal, and as other perhaps more important methodological parameters such as blinded outcome assessment and adequate allocation concealment were accounted for, an open-label design with no treatment as a comparator to LMWH was agreed upon. Nonetheless, we do not know whether this open design is improving or hampering recruitment.

In the initial protocol, the use of anticoagulant drugs such as non-steroid anti-inflammatory agents and ASA was prohibited. This was amended 19 months after the start of the trial, after an eligible woman with a history of pre-eclampsia was not included because her treating physician wanted to prescribe ASA to reduce the risk of pre-eclampsia in her subsequent pregnancy. A head to head comparison of LMWH versus no treatment, not contaminated by any co-medication, will provide the most valid evidence. However, with already few eligible patients, we considered it undesirable that eligible women would be excluded for this reason. As the number of included women with a history of pre-eclampsia was anticipated to be low and ASA use would be randomly distributed between the two treatment
groups, the scientific integrity of the trial did not appear to be jeopardized, the protocol was amended as such: ‘Apart from the assigned study medication, women are strongly discouraged to use anticoagulants or other medication that affects hemostasis, including non-steroidal anti-inflammatory drugs (NSAID’s). ASA at a low dose (≤100 mg daily) to lower the risk for recurrent preeclampsia (at the discretion of the treating physician), is discouraged but allowed’. This is an example of how protocol amendments can lead to increased enrollment, without compromising the validity of the study.

In- and exclusion criteria

Internationally, definitions and nomenclature of recurrent miscarriage, including timing of miscarriage, number of losses and whether or not the losses were consecutive, vary widely. Although the choice of 2 miscarriages as a diagnosis of recurrent miscarriage and as the inclusion criterion for the study was criticized in the design phase of the study, this was no source for delay or disagreement once the protocol was finalized. Different views of colleagues regarding this criterion were respected, as these merely reflect the lack of strong evidence for either definition. Clarification of why it was the investigators’ opinion appeared key in realizing a compromise to include women with two or more, not necessarily consecutive, miscarriages. It became obvious that with a proper explanation and reasoning, the effects of different definitions did not influence recruitment. The broad definition of recurrent miscarriage used in the study (i.e. two or more miscarriages) enables participation of clinics with a more strict definition of recurrent miscarriage (i.e. three or more) to participate. An open discussion to create mutual understanding is the way forward to positively affect study participation.

Review board approval and multicenter dimensions

With the foundation for the design of the study laid amongst Dutch colleagues and internationally, it was anticipated that the study could be initiated in the principal center, (Academic Medical Center, [AMC] Amsterdam, the Netherlands) and shortly thereafter in the other Dutch centers of which colleagues had expressed their intention to participate. Unfortunately, reality proved otherwise. First, the study had to be approved by the ethical committee of the AMC. After initial submission on June 20th, approval was obtained on August 31st 2012 and a subsequent notification of no objection by the competent authority was obtained on November 12th 2012.

In the Netherlands, a new directive had just been installed, intended to improve and speed-up medical-ethical review of multi-center studies. Where previously a new study protocol would have to pass the review board of each participating center, the directive states that the individual review boards in the Netherlands are all officially acknowledged and that a positive decision of either one is applicable to all centers. Once reviewed and approved by one ethics board, the execution of the study in another center should only be agreed upon by the local board of directors. This appears a straight-forward procedure, which according to an observational study, decreased the median time to approval from 118 days in centers which didn’t comply with the directive, to 50 days in centers which did. In practice, however, the boards did not comply with this new directive, and would not consent without a full review of the submission by their own ethics board, which resulted in substantial delays.

Initiating participation of an additional not initially listed center thus implied collecting the necessary documents from the trial office or gynecologist concerned at that new center (varying between 3 weeks to several months), a request for ethical approval (typically obtained in 2 weeks), a request for board of directors’ approval at the new center (varying between 1.5 to several months) and preparing the initiation of the study at the new center (1 week), proved to be tantalizingly slow.

It is therefore very important to ensure commitment of the center intended to participate, before this process is begun. This can save both effort and time, if initially consenting partners change their minds and refrain from participation in a later stage.

These multi-center dimensions become even more challenging when involving international centers. Additional funding is required, local standard practice can differ from the initiating country, other legal issues apply and obtaining local ethics approval is subject to other matters and restrictions than already encountered. On the other hand, international participation will maximize recruitment and will potentially increase the external validity of the study, as women of different backgrounds, ethnicity and cultures can be enrolled. A great number of international colleagues were supportive regarding the alife2 study. However, in parallel to the Netherlands, several centers initially willing to participate, withdrew later; still
supportive of the study, but not able or willing to cope with anticipated initiation difficulties. It is therefore again key to ensure commitment of the center intended to participate in an early stage, before time and effort are wasted if participation is declined later on. However, for those centers willing to participate, perseverance will pay off in the end. After initially pledging to participate, some centers may take two years before the approval at the local ethics board is requested.

A new European regulation (no. 536/2014), created with the aim to harmonize the procedure for the assessment of applications for clinical trials, was published in May 2014 and is expected to be effectuated mid-2016. It states that the medical and scientific aspects of the application will be jointly assessed by all member states via one application, which could become a major improvement of the application procedure for new trials. However, as national aspects such as privacy, insurance and research facilities of the application still need to be assessed by each member state individually, and local management boards of the centers should still approve the execution of the study, this regulation may be subject to the same pitfalls as the Dutch directive. In any case, patience and persistence are essential and will be rewarded.

**Factors influencing actual recruitment**

Once a center can start recruiting, the actual inclusion rate is dependent on the input and efforts of local investigators and trial nurses, who are often otherwise busy with studies and clinical work. Research has shown that the only factor truly contributing to recruitment is a dedicated local principal investigator or dedicated research staff who are convinced of the value of recruitment for clinical practice. Here, the discrepancy between commercial pharmaceutical trials and investigator-initiated studies, as ALIFE2, becomes apparent. Pregnant women are often excluded from pharmaceutical trials because of the pharmaceutical companies’ concerns for liability. This implies that the great majority of studies effectuated in pregnant women are investigator-initiated and the academic sponsors have to bear the expenses of the high liability insurance fees.

Where a pharmaceutical trial can provide recruitment fees as high as several thousand euro’s per included patient enabling hiring personnel and eligibility searches, the ALIFE2 study only offers an inclusion fee of 250,- Euro’s per completed CRF. This is compensation for the work of study personnel, but is by far too small to be considered as an incentive to take charge, and proactively recruit patients. The inclusion rate of the study is therefore merely dependent on the commitment and enthusiasm of local investigators. With high (clinical) workloads and numerous studies demanding efforts, ALIFE2 is not always top priority. Study organizers therefore need to realize, especially in the absence of substantial recruitment fees, that identifying centers with dedicated (principal) investigators or trial offices as potential participating centers is very important.

Additionally, newly started trials are often subject to the paradox that once a trial has begun, the number of eligible patient is suddenly much lower than the number initially anticipated. This phenomenon is known as Lasagna’s law, and appears to hold true for ALIFE2 as well.

The Women and Child Health Research Consortium (www.studies-obsyn.nl), initially established in 2003 after a grant from The Netherlands Organisation for Health Research and Development, is a renowned collaborative initiative for multi-center research in the Netherlands. Since 2015, the consortium is under the auspices of the Dutch Society of Obstetrics and Gynaecology. Over 70 medical centers have joined in this initiative, which provides unique logistics for the ALIFE2 study. Joining centers are accustomed to including patients in ongoing investigator-initiated studies. Furthermore, results of consortium studies find their way to daily practice more easily.

However, the popularity of this network may outgrow its capacity. With a limited number of trials, the collaborators were dedicated to deploy themselves for others, but as more and more investigators wish to benefit from the network and progressively more studies are introduced, the network is at risk of becoming overstrained and participants’ focus returns to individual priorities rather than those of the collaboration. This is a disadvantage for investigator-initiating studies compared to pharmaceutical trials. It can be overcome by an enthusiastic approach, involving education (making collaborators aware of the need for enrollment, providing them with tips for execution of the study), motivation (providing progress reports encouraging contribution and possible addition of sub study questions) and help with identifying barriers that are experienced in the local setting. However, only such a continuous effort will render that the study stays on top of priorities. A recent reorganization of the Consortium aimed for a more efficient collaboration but infers higher overhead costs for participating studies.
Discrepancy between scientific evidence and current clinical practice

Although current guidelines state that no evidence of a beneficial effect of LMWH on live birth in women with recurrent miscarriage and inherited thrombophilia exists, and that treatment is burdensome, expensive, and associated with bleeding, some physicians tend to employ a benefit-of-the-doubt practice and prescribe LMWH to their patients. Some prefer prescribing LMWH to women homozygous for Factor V Leiden or prothrombin G20210A, or deficient of antithrombin for example, in the view that these thrombophilias infer the highest risk of miscarriage or should be anticoagulated in pregnancy for the purpose of thrombosis prophylaxis. No recruitment by these physicians, or even selective recruitment (only randomizing women at perceived ‘low risk’, and routine prescribing LMWH to others) does not serve the study and will not provide the evidence needed. Furthermore, this practice provides women who are randomized to no treatment with an opt-out option, securing their LMWH prescription elsewhere. Education, and discussing the different viewpoints regarding evidence, is again key to overcome this issue.

A more problematic aspect is the cost of screening for inherited thrombophilia. With no currently known effective treatment, guidelines advise against screening. It is anticipated that a new update of the Dutch guideline recommend against screening except when the results of screening enable participation in an ongoing trial. However, this guideline is not implemented yet. Approximately 7 patients need to be screened to identify one with inherited thrombophilia. Restrained by budget cuts, even colleagues acknowledging the need for the study and willing to contribute, can recruit only limited numbers of patients as those eligible are not identified because screening is not performed. Unless screening is performed in the context of patient care, a greater study budget including costs of pre-screening all women with unexplained recurrent miscarriage for the presence of inherited thrombophilia would be needed to overcome this problem.

Finally, once an eligible woman has been identified, she has to be informed of the study and sign consent to be enrolled. Although this may not sound as the greatest hurdle, the art of asking for informed consent is not easily mastered. Especially for a study like ALIFE2, where women eligible for the study often are willing to do anything to improve their chance of a successful pregnancy, they may refuse participation for various reasons. A good explanation of the study burden and potential benefits, but also of the scientific reasons why the study is designed and how only randomized trials will provide answers on whether therapies are effective, appear useful tools. Furthermore, some women consent to participation at a recurrent miscarriage consultation, but are not yet pregnant. It is important to stay in touch with these women, to ensure they remain aware of the study and are randomized as soon as they have a positive pregnancy test. Interestingly, although women have declined participation for other reasons, such as not willing to interfere with God’s will, up till now, no woman refused consent because of the 50% chance of being allocated to no treatment.

Progress is made

With the many obstacles identified and some overcome, there is also good news. Doubling of the number of participating centers, increasing awareness of the study and more involved collaboration between colleagues have doubled the enrollment rate in the second year of the study. As funding for participation of the UK is obtained and one Dutch clinic and three other foreign clinics are currently requesting IRB approval for participation, this is expected to increase steeply within the near future. This increasing number of participating centers will not only add to the recruitment rate, but hopefully also improve dissemination and implementation of the study results, once obtained.

Conclusion

Designing and executing a multicenter trial, even if the trial addresses a clear gap of evidence and is broadly supported by clinicians, is a huge operation. This is especially true for investigator-initiated trials with limited funding. However, conducting such trials remains the only way forward on the road towards more evidence. Laying a solid foundation for the study amongst colleagues who will be requested to participate, in advance of the study, identifying those centers with dedicated investigators or trial offices, early recruitment of potential participating centers, and a constant and enthusiastic pursuit are key elements for success.
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Figure 1 - Progress of the ALIFE2 study over time.

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Chapter 6

Testing for inherited thrombophilia in recurrent miscarriage

Paulien G. de Jong, Mariëtte Goddijn and Saskia Middeldorp

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Abstract

1-5% of women trying to conceive suffer from recurrent miscarriage and in 50% of these women the cause of the preceding miscarriages is unknown. Inherited thrombophilias such as Factor V Leiden mutation, prothrombin G20210A gene mutation and deficiencies of natural anticoagulants protein C, protein S and antithrombin are associated with recurrent miscarriage. Knowledge of the association between inherited thrombophilia and recurrent miscarriage and of potential treatment options for improving chances of a live birth could tempt physicians to test for inherited thrombophilia in women with recurrent miscarriage. However, the strength of the association between inherited thrombophilia and recurrent miscarriage is not very strong and more importantly, there is no evidence that the use of anticoagulants improves the chance of live birth in these women. With the current state of evidence, testing for inherited thrombophilia should not lead to altered clinical management and therefore, should not be performed routinely in women with recurrent miscarriage, but only in the context of scientific studies.

Introduction

Thrombophilia, a condition associated with increased tendency to venous thrombosis, is associated with recurrent miscarriage. It accounts for early miscarriage as well as for miscarriage at later gestational age. Nowadays in clinical practice, testing for inherited thrombophilia in women with recurrent miscarriage is slowly becoming part of routine care. The value of a test is in part dependent on the relevance of the test result for clinical management as well as on the impact of the test and test results on a patient. We argue that, in this case, a positive or negative test result should not alter clinical management and therefore, that testing for inherited thrombophilia in recurrent miscarriage is not justified.

Firstly, we discuss recurrent miscarriage, inherited thrombophilia and the suggested pathophysiological mechanisms of this association. Secondly, we discuss strength of the evidence on the use of anticoagulants to prevent recurrent miscarriage. Finally the potential benefits and drawbacks of testing for inherited thrombophilia in women with recurrent miscarriage are discussed and we explain why, based on the current knowledge of test-
Thrombophilia

Coagulation cascade, regulatory mechanisms and inherited thrombophilia

The term thrombophilia is used to describe a disorder associated with an increased tendency to venous thromboembolism (VTE). It can be acquired, as in patients with malignant disease or APS, as well as inherited. Traditionally, possible features are a family history of thrombosis, thrombosis at an unusual location and thrombosis at young age, but a large proportion of carriers of thrombophilic defects remains asymptomatic throughout life. In addition to these features, thrombophilia is also associated with an increased risk of both single miscarriage and recurrent miscarriage. Severe preeclampsia is also associated with thrombophilia; for other adverse pregnancy outcomes, including placental abruption and intra-uterine growth restriction, the presence of an association is controversial. Thrombophilia can be acquired, as in patients with malignant disease or APS, as well as inherited. The effects of the currently known inherited thrombophilic defects on fibrin formation are explained in Figure 1. The process of coagulation is activated and regulated in several ways, in which thrombin (factor IIa) plays a key role. It converts fibrinogen to fibrin; the main component of a haemostatic plug. Secondly, it activates coagulation factors V, VIII, XI, leading to increased thrombin formation, and factor XIII which crosslinks fibrin strands. Coagulation is physiologically regulated by protein C and protein S. Protein C is activated to activated Protein C (APC) by thrombin in the presence of thrombomodulin. APC inactivates factors Va and VIIIa, and protein S serves as a cofactor in this process, indirectly decreasing thrombin formation. A third natural anticoagulant is antithrombin; it inhibits thrombin directly, but can also inactivate factors Xa, IXa, VIIa and plasmin, thereby indirectly inhibiting thrombin formation.

Recurrent miscarriage

The nomenclature used to describe miscarriage at a certain gestational age is divergent. Revised terminology was proposed in 2005; suggesting ‘early pregnancy loss’ (loss of fetal heart activity <12 weeks gestation), ‘late pregnancy loss’ (loss of fetal heart activity >12 weeks gestation) and ‘recurrent miscarriage’ (3 early consecutive losses or two late pregnancy losses) as preferred terms, all based on previous crown-rump length identification on ultrasound. We prefer to use the term ‘miscarriage’; ‘pregnancy loss’ can imply loss of a pregnancy not only being a miscarriage but also ectopic pregnancy, or stillbirth. To couples, the loss of ‘only’ two pregnancies, will feel like recurrent miscarriage, irrespective of the medical nomenclature requiring three consecutive miscarriages. There is recent evidence that two or more -not necessarily consecutive- miscarriages constitute recurrent miscarriage.

Pregnancy loss for any reason is severely distressing for women and their partners who wish to have children. In all clinically recognized pregnancies, a single spontaneous miscarriage occurs in 14-19% of patients, and 1-5% of women experience two or more consecutive miscarriages. A majority of miscarriages that occur before 10 weeks’ gestation are due to chromosomal errors arising from non-inherited, non-disjunctional events. A higher maternal age and increasing number of preceding miscarriages are strong determinants of recurrent miscarriage. However, in over 50% of women, the cause of the recurrent miscarriage remains unexplained. Known possible causes include anatomical, hormonal, and chromosomal abnormalities in parents, as well as prothrombotic abnormalities, as is the case in thrombophilia. Antiphospholipid syndrome (APS), an acquired form of thrombophilia, is considered an established risk factor for recurrent miscarriage. Various forms of inherited thrombophilia are associated with recurrent miscarriage, but the causal relationship is not yet fully elucidated.  

ing and therapeutic options for recurrent miscarriage, testing should not be routine practice in women with recurrent miscarriage.
Two forms of inherited thrombophilia are the gain of functional mutations called Factor V Leiden and prothrombin G20210A gene mutation. Factor V Leiden mutation, the most prevalent known inherited thrombophilic defect (5% in Caucasians\textsuperscript{1}), results from a substitution of adenine for guanine at the 1691 position of the factor V gene (FIGURE II). This leads to the substitution of glutamine for arginine at position 506 in the factor V polypeptide. As a consequence, factor Va is resistant to degradation by activated protein C (APC), which results in less down-regulation of thrombin, compared to normal factor Va.

FIGURE I - Blood Coagulation and Fibrinolysis.

Simplified scheme of coagulation and fibrinolysis. Coagulation is initiated by a tissue factor (TF)-factor VIIa complex that can activate factor IX or factor X, leading to formation of the key enzyme thrombin (factor IIa). Tissue factor-dependent coagulation is rapidly inhibited by tissue factor-pathway inhibitor (TFPI). Coagulation is maintained through the activation of factor XI by thrombin. Through the intrinsic tenase complex (factors IXa and VIIa) and the prothrombinase complex (factors Xa and Va), the additional thrombin required to down-regulate fibrinolysis is generated by the activation of thrombin-activatable fibrinolysis inhibitor (TAFI).

The coagulation system is regulated by the protein C pathway. Thrombin activates protein C in the presence of thrombomodulin. Together with protein S (PS), activated protein C (APC) is capable of inactivating factors Va and VIIIa, which results in a down-regulation of thrombin generation and consequently in an up-regulation of the fibrinolytic system. The activity of thrombin is controlled by the inhibitor antithrombin (AT). The solid arrows indicate activation and the broken arrows inhibition.

FIGURE II - Schematic representation of the Factor V Leiden mutation.

At amino acid position 506, arginine (Arg) is replaced by glutamine (Gln), which makes the molecule less susceptible for cleavage by activated protein C at this site. Additional cleavage of the factor V molecule at amino acid positions 506 and 679 can occur normally but at a slower rate.
The prothrombin G20210A gene mutation results from a mutation in the promotor region of the prothrombin gene (G20210A), which leads to slightly elevated prothrombin levels,\textsuperscript{11} which is associated with an increased risk of thromboembolism.\textsuperscript{11}

Deficiencies of the natural anticoagulants antithrombin, protein C and protein S lead to increased thrombin generation and are relatively rare forms of inherited thrombophilia. Both Protein C and Protein S are vitamin-K dependent glycoproteins.

An elevated level of factor VIII is another possible genetically determined cause of thrombophilia which may be a common risk factor for VTE and arterial vascular events, but the etiology and impact of this defect on pregnancy complications are unclear.\textsuperscript{16/17}

**Thrombophilia and VTE**

Altogether, the different forms of inherited thrombophilia are not rare (Table I), but the frequency varies considerably within healthy populations and among patients with VTE. As mentioned before, Factor V Leiden mutation is the most prevalent inherited thrombophilic defect, occurring in 5% of Caucasians; however, it is rare in Asians and Africans.\textsuperscript{11/17} A mutation in the prothrombin gene is present in approximately 2-3% of Caucasians, but is also less common in Asians and Africans.\textsuperscript{16/17} Homozygosity for these two mutations is rare, with a prevalence of 0.02% for Factor V Leiden mutation and 0.014% for prothrombin G20210A mutation.\textsuperscript{16/17} Deficiencies of Protein C, Protein S and antithrombin are much rarer than Factor V Leiden mutation or prothrombin G20210A gene mutation; their combined prevalence is approximately 1%.\textsuperscript{17} Among patients with VTE the prevalence of inherited thrombophilia is higher (Table I).\textsuperscript{17} The relative risk for a first venous thrombosis in carriers of a Factor V Leiden mutation is approximately 3-5, and in carriers of a prothrombin G20210A gene mutation, the relative risk is 2-3.\textsuperscript{18} In patients with deficiencies of antithrombin, protein C or protein S the relative risk for a first venous thrombosis is also elevated; 5-10, 4-6.5 and 1-10, respectively. The estimated relative risks of VTE recurrence in patients with inherited thrombophilia are 1.8 – 2.5 (natural anticoagulant deficiencies), 1.3 – 1.4 (Factor V Leiden mutation) and 1.4 – 1.7 (prothrombin G20210A gene mutation), as compared to VTE patients without thrombophilia.\textsuperscript{19}

**Thrombophilia and pregnancy loss**

**Pathophysiology**

Physiologically, pregnancy is associated with changes in haemostasis, resulting in a hypercoagulable state.\textsuperscript{20} In theory, thrombophilia intensifies these changes in haemostasis during pregnancy. The association between thrombophilia and recurrent miscarriage as well as pre-eclampsia and possibly abruptio placentae and intra-uterine growth restriction can, therefore, be explained by the concept of thrombosis of the (micro) vasculature of the placenta. This hypothesis is reinforced by the fact that placental infarction is a common finding in women with placenta-mediated pregnancy complications and thrombophilia.\textsuperscript{21} However, it is unlikely that this is the sole mechanism of thrombophilia in miscarriage. Because placental development has not yet taken place very early in pregnancy and early miscarriage is also associated with thrombophilia, other pathophysiological mechanisms may also play a part.

A suggested mechanism by which APS induces injury to the developing fetal-placental unit is by activation of complement.\textsuperscript{22} In animal models, binding of antiphospholipid antibodies to trophoblast can activate complement factors C3 and C5, leading to recruitment and stimulation of inflammatory cells.
cells and injury to the fetus and placenta. Furthermore, in in vitro studies on human placental tissue, it was demonstrated that antiphospholipid antibodies can inhibit primary extra-villous trophoblast differentiation, and subsequent placentation. Moreover, it is observed that heparin as well as aspirin regulates trophoblast apoptosis in vitro.

Similar experimental models for inherited thrombophilia have not been studied, but experiments on thrombomodulin-deficient mice have shown that the thrombomodulin-protein C pathway is essential for the maintenance of pregnancy; activated coagulation factors induce cell death and growth inhibition of placental trophoblast cells by formation of fibrin degradation products inducing death of giant trophoblast cells, as well as by engaging protease-activated receptors PAR-2 and PAR-4. These findings suggest that thrombomodulin-protein C system may protect placental integrity, and consequently that a lack of protein-C can be part of the cause of pregnancy loss.

**Strength of association**

The effect of thrombophilic defects on pregnancy outcome seems to vary for different forms of thrombophilia and during the course of pregnancy. One study reported an association between increased risk of recurrent miscarriage throughout the entire first trimester and prothrombin G20210A gene mutation, whereas Factor V Leiden mutation was associated with recurrent miscarriage after the start of placentation (10-14 weeks gestation), but not with embryonic loss. A meta-analysis, showed that recurrent miscarriage with only first trimester miscarriages, is associated with Factor V Leiden mutation, activated protein C resistance and prothrombin G20210A gene mutation. Late pregnancy loss was associated with Factor V Leiden mutation (pregnancy loss >19 weeks), prothrombin G20210A gene mutation (loss >20 weeks) and protein S deficiency (loss >22 weeks). The association between Factor V Leiden mutation and late recurrent miscarriage was stronger than for early recurrent miscarriage (<13 weeks gestation). Results of another systematic review showed that women with unexplained stillbirth were more often heterozygous for Factor V Leiden mutation, or had protein S deficiency more often than controls. Moreover, the extent of the association between thrombophilia and miscarriage varies, according to type of thrombophilia and gestational age.

---

**Table II** - Association between Pregnancy Complications and Thrombophilia, adapted from Robertson et al.

<table>
<thead>
<tr>
<th>Type of Thrombophilia</th>
<th>Miscarriage</th>
<th>Recurrent 1st trimester miscarriage</th>
<th>Non-recurrent 2nd trimester miscarriage</th>
<th>Stillbirth (3rd trimester loss)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor V Leiden mutation</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(homozygous)</td>
<td>2.7</td>
<td>*</td>
<td>*</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>(1.3 - 5.6)</td>
<td></td>
<td></td>
<td>(0.4 - 9.7)</td>
</tr>
<tr>
<td>Factor V Leiden mutation</td>
<td>1.7</td>
<td>1.9*</td>
<td>4.1*</td>
<td>2.1</td>
</tr>
<tr>
<td>(heterozygous)</td>
<td>(1.1 - 2.9)</td>
<td></td>
<td>(1.9 - 9.8)</td>
<td>(1.3 - 3.9)</td>
</tr>
<tr>
<td>Prothrombin G20210A mutation</td>
<td>2.5</td>
<td>2.7</td>
<td>8.6</td>
<td>2.7</td>
</tr>
<tr>
<td>(heterozygous)</td>
<td>(1.2 - 5.0)</td>
<td></td>
<td>(2.2 - 34.0)</td>
<td>(1.3 - 5.5)</td>
</tr>
<tr>
<td>Antithrombin deficiency</td>
<td>0.9</td>
<td>NA</td>
<td>NA</td>
<td>7.8</td>
</tr>
<tr>
<td></td>
<td>(0.2 - 4.5)</td>
<td></td>
<td></td>
<td>(0.3 - 196.4)</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>2.3</td>
<td>NA</td>
<td>NA</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>(0.2 - 26.4)</td>
<td></td>
<td></td>
<td>(0.2 - 38.5)</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>3.6</td>
<td>NA</td>
<td>NA</td>
<td>20.1</td>
</tr>
<tr>
<td></td>
<td>(0.4 - 35.7)</td>
<td></td>
<td></td>
<td>(3.7 - 109.2)</td>
</tr>
<tr>
<td>Anticardiolipin antibodies</td>
<td>3.4</td>
<td>5.1</td>
<td>NA</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>(1.3 - 8.7)</td>
<td></td>
<td></td>
<td>(1.6 - 6.7)</td>
</tr>
<tr>
<td>Lupus anticoagulants</td>
<td>3.0</td>
<td>NA</td>
<td>14.3</td>
<td>2.4</td>
</tr>
<tr>
<td>(Nonspecific inhibitor)</td>
<td>(1.0 - 8.8)</td>
<td></td>
<td>(4.7 - 43.2)</td>
<td>(0.8 - 7.0)</td>
</tr>
</tbody>
</table>

* Homozygous and heterozygous carriers were grouped together; it is not possible to extract data for zygosity. NA, not available. Note: data are derived from a systematic review; terminology of pregnancy loss at various gestational ages may vary among included studies.
Potential impact of anticoagulant therapy; evidence

Because of the presumed role of thrombophilia in the pathogenesis of recurrent miscarriage, anticoagulants could be a therapeutic option. Heparin binds to and potentiates the activity of antithrombin. In contrast to coumarin derivatives, neither unfractionated heparin nor low molecular weight heparin (LMWH) cross the placenta and therefore these interventions do not have the potential to cause fetal bleeding and teratogenic damage. In clinical practice, aspirin and LMWH are frequently prescribed for women with unexplained recurrent miscarriage or with inherited thrombophilias and recurrent miscarriage not otherwise explained, despite a lack of evidence.

Beneficial effects of anticoagulants (LMWH with or without aspirin) for women with unexplained recurrent miscarriage were reported in several studies. However, these studies were either not randomized, not placebo-controlled, or had other methodological limitations (Table III).

We recently performed a randomized placebo controlled trial (ALIFE study), investigating whether aspirin combined with LMWH or aspirin alone as compared with placebo would improve the live birth rate among 364 women with unexplained recurrent miscarriage (<20 weeks gestation). We found that neither aspirin combined with nadroparin nor aspirin alone improved the chance of a live birth in women with a history of unexplained recurrent miscarriage. In addition, no significant benefits were found for women with inherited thrombophilia, but the study was not powered to assess this effect.

The SPIN-study, another randomized controlled trial, assessed whether enoxaparin and low-dose aspirin reduced the rate of miscarriage compared to intensive pregnancy surveillance alone in 294 women with a history of 2 or more consecutive previous miscarriages (<24 weeks gestation). Results showed 22% miscarriage in participants receiving enoxaparin and aspirin, compared with 20% miscarriages in subjects receiving intensive surveillance alone (odds ratio 0.91, 95% confidence interval 0.52-1.59).

<table>
<thead>
<tr>
<th>TABLE III - Available evidence from randomized controlled trials investigating heparin (LMWH or unfractionated heparin) compared to no heparin in women with a history of unexplained recurrent miscarriage; effect on live birth.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author, year</strong></td>
</tr>
<tr>
<td>Bally, 1996</td>
</tr>
<tr>
<td>Clancy, 2010</td>
</tr>
<tr>
<td>孕妇, 2019</td>
</tr>
<tr>
<td>Aca, 2010</td>
</tr>
<tr>
<td>Clancy, 2012</td>
</tr>
</tbody>
</table>
| *Various definitions: APLA, antiphospholipid antibodies; UFH, unfractionated heparin; LMWH, low molecular weight heparin*
In the HepasA trial, the use of LMWH in addition to aspirin did not lead to increased live birth when compared to aspirin alone in women with antiphospholipid antibodies, inherited thrombophilia or antinuclear antibodies and recurrent miscarriage in the HepasA trial. The Habenox study also found no beneficial effect of LMWH (with aspirin or placebo) compared to aspirin in women with or without thrombophilia and recurrent miscarriage.

None of the trials just cited were sufficiently powered to demonstrate an effect of pharmacological therapy in the subgroup of women with thrombophilia. There is a need for randomized, adequately powered placebo-controlled trials on the use of anticoagulants in women with recurrent miscarriage and inherited thrombophilia. Until beneficial effects of anticoagulants are demonstrated in such trials, pharmacological intervention in women with recurrent miscarriage and thrombophilia is not justified.

**Potential impact of anticoagulant therapy; drawbacks**

Apart from the lack of evidence for these pharmacological interventions, anticoagulant therapy in pregnant women is potentially harmful. Fortunately, the risk of hemorrhage (including post-partum hemorrhage), heparin induced thrombocytopenia (HIT) and heparin induced osteopenia, the main potential adverse effects of heparins, appears small in previously mentioned trials. In a systematic review of the safety and efficacy of LMWH in pregnant women, the rate of significant bleeding was low; 1.98% (95% confidence interval 1.50% – 2.57%). Bleeding is a serious complication and should not be underestimated. In 2777 pregnancies with LMWH use, no case of HIT associated with thrombosis was reported and the overall risk of heparin-induced osteoporosis was 0.04% (one single patient). However, 3 other cases of osteoporotic fractures associated with LMWH use in pregnancy have been reported, that were not included in the review.

Moreover, LMWH needs to be administered daily and administration often causes pain and bruising at injection sites. In the previously cited systematic review, the reported rate of skin reactions was low; 1.80% (95% confidence interval 1.34% – 2.37%). In the ALIF trial swelling or itching at injection site was reported by almost 40% of patients receiving nadroparin. Another study of 66 women using LMWH in pregnancy or postpartum period reported skin complications in 19 (29%) women. These results suggest that possibly there was underreporting of skin reactions in studies included in the systematic review.

**Testing for inherited thrombophilia**

Recognizing the association between recurrent miscarriage and thrombophilia and the possible benefits of anticoagulant treatment, the question is raised whether testing women with recurrent miscarriage for thrombophilia should be performed routinely. Arguments in favor of testing include the gain of knowledge on the possible cause of miscarriage. This provides an explanation for the patient and her partner, as well as for her physician. A second and more important argument supportive of testing for thrombophilia, is to select the group of patients who could benefit from anticoagulant treatment in a future pregnancy. However, as yet, thrombophilia and recurrent miscarriage are merely associated, and evidence for the efficacy of anticoagulants in women with thrombophilia and recurrent miscarriage is still lacking. Since treatment of these women is, at present, not justified, testing for inherited thrombophilia should not alter clinical management and, therefore, should not be performed.

A complete thrombophilia screen cost approximately $570 in 2003. Moreover, the psychological impact and consequences of a person knowing that she or he is a carrier of a (genetic) thrombophilic defect are potential drawbacks of testing. In a systematic review of the psychological impact of testing for thrombophilia, no valid conclusions could be drawn based on the literature, because data could not be pooled due to varied methodology in eligible studies. However, 43% and 27% of participants in two studies reported being more worried with the knowledge of being a carrier of thrombophilia.
Conclusions

Inherited thrombophilia is associated with recurrent miscarriage, but at present, the strength and pathophysiology of this association are not fully elucidated. Possibly, the use of anticoagulants will provide a means of increasing the chance of a live birth in women with recurrent miscarriage and inherited thrombophilia. However, as yet, this beneficial effect has not been proven and physicians should refrain from prescribing heparins to women for solely this indication. Since it still remains to be established whether subsequent treatment will improve clinical outcome, the knowledge of a patients’ thrombophilic status should not alter clinical management, and testing for thrombophilia should, therefore, not be performed on a routine base, but only in the context of scientific studies.

References


Chapter 7

Low-molecular-weight heparin for prevention of placenta-mediated pregnancy complications: protocol for a systematic review and individual patient data meta-analysis (AFFIRM)


Systematic Reviews 2014
Abstract

Background: Placenta-mediated pregnancy complications include pre-eclampsia, late pregnancy loss, placental abruption, and the small-for-gestational age newborn. They are leading causes of maternal, fetal, and neonatal morbidity and mortality in developed nations. Women who have experienced these complications are at an elevated risk of recurrence in subsequent pregnancies. However, despite decades of research no effective strategies to prevent recurrence have been identified, until recently. We completed a pooled summary-based meta-analysis that strongly suggests that low-molecular-weight heparin reduces the risk of recurrent placenta-mediated complications. The proposed individual patient data meta-analysis builds on this successful collaboration. The project is called AFFIRM, An individual patient data meta-analysis of low-molecular-weight heparin for prevention of placenta-mediated pregnancy complications.

Methods/Design: We conducted a systematic review to identify randomized controlled trials with a low-molecular-weight heparin intervention for the prevention of recurrent placenta-mediated pregnancy complications. Investigators and statisticians representing eight trials met to discuss the outcomes and analysis plan for an individual patient data meta-analysis. An additional trial has since been added for a total of nine eligible trials. The primary analyses from the original trials will be replicated for quality assurance prior to recoding the data from each trial and combining it into a common dataset for analysis. Using the anonymized combined data we will conduct logistic regression and subgroup analyses aimed at identifying which women with previous pregnancy complications benefit most from treatment with low-molecular-weight heparin during pregnancy.

Discussion: The goal of the proposed individual patient data meta-analysis is a thorough estimation of treatment effects in patients with prior individual placenta-mediated pregnancy complications and exploration of which complications are specifically prevented by low-molecular-weight heparin.

Systematic review registration: PROSPERO (International Prospective Registry of Systematic Reviews) 23 December 2013, CRD42013006249
Background

Placenta-mediated pregnancy complications include pre-eclampsia (PE), late pregnancy loss, placental abruption and the small-for-gestational age (SGA) newborn. We completed a pooled summary-based meta-analysis that strongly suggests that low-molecular-weight heparin (LMWH) reduces the risk of placenta-mediated complications in subsequent pregnancies. A successful pregnancy requires the development of adequate placental circulation. It has been hypothesized that thrombosis in the placental bed is at least partially responsible for placenta-mediated pregnancy complications. It has also been suggested that these complications are the result of abnormal placental development with underdeveloped placental vasculature or placental inflammation. These complications represent an important health problem because they are common, affecting more than one in six pregnancies, and often have a devastating outcome for the affected women, their unborn children, their families, and society. Specifically, PE (characterized by a new onset of elevated blood pressure and proteinuria during pregnancy) is one of the most common causes of maternal mortality in the developing world. SGA newborns often suffer long-term effects including developmental delay, poor school performance, and a significantly lower likelihood of academic and professional success. Fetal loss is a devastating event for pregnant women and their families. Placental abruption (separation of the placenta from the uterus before birth) can, in the most severe cases, lead to maternal hemorrhage with the risk of transfusion and both maternal and fetal death.

The risk of recurrent placenta-mediated pregnancy complications in subsequent pregnancies is substantial. For example, women with prior severe PE will have a 25 to 65% risk of recurrent PE, a 3% risk of placental abruption, and a 10% risk of SGA (<10th percentile). These complications may be multiple (for example both PE and SGA) and not isolated to the placenta-mediated complication experienced in a prior pregnancy. There are no highly effective preventative strategies that can be used in subsequent pregnancies. Aspirin offers small relative risk reductions in patients with prior PE and SGA, however, it may be more effective at reducing risk (approximately a 40% reduction) if started early in the pregnancy (before 16 weeks). There are no proven preventative strategies for the other complications. It has been postulated that anticoagulants might prevent placenta-mediated pregnancy complications by reducing placental thrombosis and/or affecting maternal coagulation activation or inflammation. Recent randomized controlled trials (RCTs) conducted to determine if LMWH can prevent recurrent placenta-mediated pregnancy complications suggest an important treatment effect, but this finding has not been universal. Although it appears that LMWH is a promising therapy in the prevention of placenta-mediated pregnancy complications, there are disadvantages to the premature adoption of this intervention without sufficient evidence of benefit. If LMWH is used universally for all women with prior placenta-mediated pregnancy complications, we may be intervening unnecessarily and exposing women to a risk of undesirable and potentially fatal, albeit rare, side effects (major bleeding, heparin-induced thrombocytopenia, osteoporotic fractures, withholding of epidural analgesia due to fear of causing epidural hematoma, and paralysis). Less serious side effects including skin reactions, minor bleeding, and transient elevations in liver enzymes are more commonly experienced. Therapy is also associated with cost and inconvenience since the drug is expensive and is administered by injection either once or twice a day. Therefore, it is necessary to answer the question as to who benefits from LMWH prophylaxis during pregnancy and to determine the nature and magnitude of these benefits more precisely. The individual patient data meta-analysis (IPD-MA) has the potential to answer these important questions and determine the risk/benefit ratio of therapy for various subgroups of women.

The composite outcome, including all placenta-mediated pregnancy complications, that is used in many RCTs is heterogeneous and not all individual outcomes can be considered equally serious in terms of potential consequences for the mother and newborn. For example, late term pre-eclampsia is clinically less worrisome since the symptoms tend to be less severe and generally resolve with delivery. Conversely, women who develop pre-eclampsia earlier in the pregnancy have more serious clinical consequences including a greater risk of maternal and neonatal death. Our pooled summary meta-analysis suggests that LMWH may prevent severe pre-eclampsia and early pre-eclampsia with less of an effect on late onset pre-eclampsia. Confirmation of these findings is extremely important for clinicians treating these women and has direct relevance for clinical practice worldwide.

There are many challenges associated with recruiting pregnant women to RCTs with a drug intervention including: the biases of clinicians either for or against the therapy (based on insufficient evidence of benefit and lack of knowledge about potential risk); the concerns of the pregnant woman and her family about the health and safety of the mother and baby; and the...
demands during pregnancy of attending additional appointments and investigations associated solely with study participation. Furthermore, the pharmaceutical industry often excludes pregnant women from trials due to liability concerns. As a result, there is a dearth of RCTs evaluating LMWH in this population compared to other patient groups (such as oncology or orthopedic surgery). Those RCTs that do exist are all academically driven and may not have the same financial and human resources that are available to trials that are sponsored by the pharmaceutical industry. Therefore, meta-analysis is an essential tool that allows for greater statistical power by pooling the existing small RCTs evaluating LMWH for the prevention of placenta-mediated pregnancy complications.

Our recent pooled summary-based meta-analysis of six RCTs (Table I) included 848 pregnant women with a history of pre-eclampsia, a SGA neonate (<10th percentile), placental abruption, or late pregnancy loss (more than 12 weeks gestation) in a previous pregnancy. The primary finding was that 67 out of 358 (18.7%) women taking LMWH during pregnancy had recurrent severe placenta-mediated pregnancy complications, as compared with 127 out of 296 (42.9%) women with no LMWH (relative risk reduction 48% (95% CI 14 to 68%; I² 69%). However, since the meta-analysis results apply to a heterogeneous group of women with a mixture of placenta-mediated pregnancy complications of varying prior severity and the primary outcome for the meta-analysis was a composite of all placenta-mediated complications (also of varying severity), it is not clear which subgroups of women derive the most benefit from LMWH (which outcomes are reduced and which severity of outcomes are impacted). Before recommendations for clinical practice can be advocated, it is necessary to conduct more detailed analyses of the existing data to determine potential benefits for subgroups of women, to adjust for important baseline characteristics of participants, and to explore other treatment-related reasons for the reported heterogeneity (for example specific LMWH drug (dalteparin, nadroparin or enoxaparin), LMWH dose, gestational age when drug was initiated, and co-interventions such as concomitant ASA use).

### Table I - Previously identified trials that meet the inclusion criteria for AFFIRM.

<table>
<thead>
<tr>
<th>Study Name &amp; Intervention</th>
<th>Year</th>
<th>Patients</th>
<th>Intervention &amp; Outcome</th>
<th>Participants</th>
<th>Outcomes</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TIPPS</strong> = Thrombophilia In Pregnancy Prophylaxis Study</td>
<td>2013</td>
<td>Canada, N = 292</td>
<td>Dalteparin 5000 U vs 2000 U in 2000 U</td>
<td>N = 358</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>FRUIT</strong> = Fractionated Heparin In Pregnant Women With A History of Utero-placental Insufficiency and Thrombophilia</td>
<td>2012</td>
<td>N = 132</td>
<td>Dalteparin versus placebo</td>
<td>N = 132</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>HAEmatologist – abruptio placentae</strong></td>
<td>2013</td>
<td>France, single center N = 224</td>
<td>Enoxaparin 4000 U vs ASA</td>
<td>N = 224</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>HAEmatologist – pre-eclampsia</strong></td>
<td>2009</td>
<td>N = 116</td>
<td>Enoxaparin 4000 U vs ASA</td>
<td>N = 116</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>NOH-Pe</strong> = Nîmes Obstetricians and HAEmatologist – pre-eclampsia</td>
<td>2010</td>
<td>France, single center</td>
<td>Enoxaparin 4000 U vs ASA</td>
<td>N = 116</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>NOH-AP</strong> = Nîmes Obstetricians and HAEmatologist – Abruptio Placentae</td>
<td>2010</td>
<td>France, single center</td>
<td>Dalteparin versus ASA</td>
<td>N = 116</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Molly</strong></td>
<td>2006</td>
<td>Canada, N = 90</td>
<td>Dalteparin 5000 U vs 2000 U in 2000 U</td>
<td>N = 90</td>
<td>Unable to contact</td>
</tr>
</tbody>
</table>

ASA, aspirin; GA, gestational age; IPDMA, individual patient data meta-analysis; IPDMA, individual patient data meta-analysis; PE, pre-eclampsia; RCT, randomized controlled trial; SB, stillbirth; SGA, small-for-gestational age. Trial Names: TIPPS = Thrombophilia In Pregnancy Prophylaxis Study accepted for publication in the Lancet. FRUIT = Fractionated heparin in pregnant women with a history of Utero-placental Insufficiency and Thrombophilia. NOH-AP = Nîmes Obstetricians and HAEmatologist – abruptio placentae. NOH-Pe = Nîmes Obstetricians and HAEmatologist – pre-eclampsia. HAPPY = Heparin in pregnant women with Adverse Pregnancy outcome to improve the rate of successful Pregnancy.
IPDMA has been proposed as an advantageous methodological approach when subgroup analyses are hypothesized to be clinically relevant. Analyzing original data from individual patients makes use of a much richer dataset and has greater statistical power than conventional meta-analysis.31/32 Furthermore, for this project, IPDMA will allow for adjustment for covariates that are known to be important in the recurrence of placenta-mediated pregnancy complications. Such an analysis will also enable us to explore clinical, methodological, and statistical heterogeneity more robustly. IPDMA is an attractive method to answer our study questions since it ‘dramatically and consistently’ has more power to detect interactions between risk groups.33

Methods/Design

Research questions

The primary research question is: Which women with previous placenta-mediated pregnancy complications have a reduction in the risk of future complications when treated with LMWH during pregnancy? Secondary research questions are: Which of the placenta-mediated pregnancy complications are avoided? Are severe and/or early onset or non-severe and/or late onset complications avoided? Does LMWH cause major bleeding in women with prior placenta-mediated pregnancy complications? And, are any other side effects increased by LMWH use in women with prior placenta-mediated pregnancy complications (thrombocytopenia, osteoporotic fractures or allergic reactions)?

The proposed project is called AFFIRM (An individual patient data meta-analysis of low-molecular-weight heparin For prevention of placenta-mediated pregnancy complications), PROSPERO registration number: CRD42013006249. We will synthesize individual patient data from RCTs of LMWH for the prevention of recurrent placenta-mediated pregnancy complications. The overall objective of the meta-analysis is to directly inform clinical practice and the development of clinical practice guidelines. The study is coordinated by the Clinical Epidemiology Program at the Ottawa Hospital Research Institute. Conceptually, the research approach involves four sequential phases: a systematic review, knowledge synthesis planning, data extraction and analysis, and interpretation of results and knowledge translation. The first two phases have been completed and are therefore described below in the past tense. No data have been extracted or recoded for the common dataset and no statistical analyses have been performed; these steps are outlined in the future tense.

Systematic review

Electronic search strategies were developed and tested through an iterative process by an experienced medical information specialist in consultation with the review team. The strategy was peer-reviewed prior to execution by an experienced information specialist using the Peer Review of Electronic Search Strategies (PRESS) checklist.34 The following search was conducted in May 2013: using the OVID platform, we searched OVID Medline™, Ovid MEDLINE™ In-Process & Other Non-Indexed Citations, and Embase-Classic+Embase (strategy included as Additional file 1). We also searched the Cochrane Library on Wiley (including CENTRAL, Cochrane Database of Systematic Reviews, DARE, and HTA). ClinicalTrials.gov and the WHO International Clinical Trials Registry were searched to identify relevant in-process and completed trials. Strategies utilized a combination of controlled vocabulary (such as ‘hypertension, pregnancy-induced’, ‘placental insufficiency’, ‘heparin, low-molecular-weight’) and keywords (pre-eclampsia, abruption, and LMWH). Vocabulary and syntax were adjusted across databases. Animal studies were excluded but there were no language or date restrictions on any of the searches. We sought additional references through hand-searching the bibliographies of relevant items. Search results are summarized in a preferred reporting items for systematic reviews and meta-analyses (PRISMA) diagram (FIGURE I) and details of potentially eligible trials are provided in TABLE II and III.
TABLE II - Potentially eligible published trials identified by Arnaud's systematic review.

<table>
<thead>
<tr>
<th>Study Name &amp; First Author</th>
<th>Year</th>
<th>Country &amp; Sample Size</th>
<th>Participants</th>
<th>Intervention &amp; Control</th>
<th>Relevant Outcomes</th>
<th>Comment re: IPDMA inclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETHII II (Schulze)</td>
<td>2013</td>
<td>Germany N = 469</td>
<td>Pregnant</td>
<td>Dalteparin 5000IU +</td>
<td>Intact pregnancy at 24 wks</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>aspirin vs placebo</td>
<td>GA, PE, IUGR &lt;5%</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>for premature delivery</td>
<td>percent, abruption</td>
<td></td>
</tr>
<tr>
<td>Giancotti</td>
<td>2012</td>
<td>Italy N = 167 (pregnant)</td>
<td>Pregnant</td>
<td>Enoxaparin 40 mg vs</td>
<td>Live births</td>
<td>Not eligible (All cases &lt;12 weeks GA)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Enoxaparin 40 mg + ASA vs ASA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Salah</td>
<td>2012</td>
<td>Egypt N = 150</td>
<td>Pregnant</td>
<td>Ticlopidine 500IU vs</td>
<td>Continuation of pregnancy after 20 wks</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>folic acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HABBECK (Visser)</td>
<td>2011</td>
<td>Finland, Sweden N = 207</td>
<td>Women with recurrent</td>
<td>Enoxaparin 40 mg vs</td>
<td>Live birth rate; PE, IUGR</td>
<td>Not eligible (All women with early losses)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>early or late miscarriage</td>
<td>Enoxaparin 40 mg + ASA vs ASA</td>
<td>&lt;2SD, abruption</td>
<td></td>
</tr>
<tr>
<td>SPIN (Clark)</td>
<td>2010</td>
<td>UK, New Zealand N = 294</td>
<td>Pregnant</td>
<td>Enoxaparin 40 mg vs ASA</td>
<td>Pregnancy loss</td>
<td>GA if past losses not available centrally</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ASA vs ASA, none drug</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALIFE (Kawanda)</td>
<td>2010</td>
<td>Netherlands N = 299</td>
<td>Pregnant</td>
<td>Neodipride 2850IU vs ASA vs ASA, placebo</td>
<td>Pregnancy loss, SGA &lt;10% percent, PE, HELLP, abruption</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>for placental abruption</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HepASA (Laskin)</td>
<td>2009</td>
<td>Canada N = 88</td>
<td>Terminated at interim analysis</td>
<td>Dalteparin 5000IU vs ASA vs ASA</td>
<td>Live births</td>
<td>Unable to contact</td>
</tr>
</tbody>
</table>

ASA, aspirin; GA, gestational age; IUGR, intrauterine growth restriction; PE, pre-eclampsia; SGA, small for gestational age. The table also includes study protocols for individual patient data meta-analysis.
**Inclusion criteria**

RCTs with an LMWH intervention for the prevention of recurrent placenta-mediated pregnancy complications were eligible. The study population of interest included currently pregnant women with prior pregnancies complicated by one or more of the following: PE, placental abruption, SGA newborn (<10th percentile), pregnancy loss after 16 weeks gestation or two losses after 12 weeks gestation. The principal investigators of potentially eligible trials identified by the systematic review (see Table I, II and III) were contacted via email to request additional information about the study population. Once eligibility was confirmed, investigators were invited to participate in the IPDMA and attend the AFFIRM project planning meeting. The lead investigators of the largest and most recently completed trials agreed to contribute individual patient data to this collaboration. Data from two small trials were not included because the investigators did not respond; in one of these trials only a small proportion of the total study population would have been eligible to contribute data to AFFIRM. Some of the women in the Scottish Pregnancy Intervention Study (SPIN) trial would have been eligible for inclusion in AFFIRM, however, the trial database does not include sufficient detail about the timing of previous pregnancy losses to determine the eligibility of individual participants.

**Knowledge synthesis planning**

A crucial step in the success of the project was the development of the knowledge synthesis and knowledge translation plans. A full-day review team meeting was held in Amsterdam on 4 July 2013. The purpose was to allow for extensive discussion and consensus-reaching on important study variables and outcomes and to consider strategies for merging the existing datasets in a centralized database. Participants included the principal investigators of the included RCTs and statisticians with in-depth knowledge of the trial data. The principal investigators are all practising clinicians (obstetricians and hematologists) who are also knowledge users in this clinical area.
Outcome measures

The detailed definitions for the IPDMA outcomes were agreed upon by investigator consensus at the face-to-face meeting. The definitions and diagnostic criteria for each outcome variable are documented in a data dictionary and the research protocol. These definitions, which have been reviewed by all investigators, allow standardization across studies and decrease the potential for bias.

AFFIRM’s primary outcome is a composite outcome including four pregnancy complications: early-onset or severe pre-eclampsia, birth of a small-for-gestational age newborn with a birth weight <5th percentile, placental abruption, and late pregnancy loss. To qualify as a primary outcome event, the pregnancy complication must satisfy one or more predefined criteria. Early onset pre-eclampsia is diagnosed at less than 34 weeks’ gestation. Severe pre-eclampsia is characterized by at least one criterion indicative of severe disease; these are, a systolic blood pressure >160 mm Hg or diastolic blood pressure >110 mm Hg, proteinuria >0.5 g/24 hours, elevated liver enzymes (more than two times the local upper range of normal), platelets <100 × 10^9/L, pulmonary edema, seizures (eclampsia), headache or other neurological manifestations (stroke, intracranial hemorrhage, cerebral edema, hyperreflexia, and visual impairment), coagulopathy, oliguria (≤30 ml/hr) or HELLP syndrome (hemolysis, elevated liver enzymes, low platelet count). Birth of a small-for-gestational age newborn with a birth weight <5th percentile is determined using local gender and gestational age specific birth weight charts. The placental abruption outcome requires a clinical diagnosis of placental abruption leading to delivery. A late pregnancy loss occurs at or after 20 weeks of gestation and cannot be explained by other factors, including fetal chromosomal abnormalities, maternal infection, cervical insufficiency or incompetence, or an intentional termination of the pregnancy.

Nineteen secondary outcomes have been defined for AFFIRM, including the four individual components of the primary outcome: severe or early-onset pre-eclampsia, birth of a small-for-gestational age newborn <5th percentile, placental abruption and late pregnancy loss, all as outlined above. Pre-eclampsia (non-severe) is characterized by a systolic blood pressure >140 mm Hg or diastolic blood pressure >90 mm Hg and proteinuria >0.3 g/24 hours. A diagnosis of HELLP syndrome required 3 criteria, hemolysis [lactate dehydrogenase (LDH) >600 IU/L or serum bilirubin >1.2 mg/dL] an abnormal elevation of liver enzymes (more than two times the local upper range of normal), and platelets <100 × 10^9/L. Preterm delivery <34 weeks and <37 weeks are pre-specified outcomes. A perinatal loss is any fetal or neonatal death at over 20 weeks gestational age and less than or equal to 28 days post-partum and neonatal mortality is considered any neonatal death after birth and less than or equal to 28 days post-partum. Birth of a small-for-gestational age newborn <10th percentile is determined based on local gender and gestational age specific birth weight charts.

Adverse maternal outcomes include thrombocytopenia, defined as a platelet count <75,000 × 10^9/L, and bleeding outcomes at various time points. Antepartum major bleeding is defined using the criteria proposed by the International Society on Thrombosis and Haemostasis (ISTH). That is, clinical or radiological evidence of bleeding with at least one of the following criteria: associated with a fall in hemoglobin of 2 g/dL (1.24 mmol/l) or more; or a requirement for transfusion of two or more units of red blood cells or whole blood; or symptomatic bleeding occurring in a critical site: intracranial, intraspinal, intraocular, pericardial, intra-articular, intramuscular with compartment syndrome, or retroperitoneal, or was considered to have contributed to maternal death. Peripartum major bleeding is hemorrhage occurring after the onset of labour or start of surgical delivery and within 24 hours postpartum that meets at least one of the following: necessitating a surgical procedure, or associated with a fall in hemoglobin of 4 g/dL (2.48 mmol/l) or more, or a requirement for transfusion of two or more units of red blood cells or whole blood, or estimated peripartum blood loss >1000 ml, or considered to have contributed to maternal death. Peripartum minor bleeding is hemorrhage occurring after the onset of labour or start of surgical delivery and within 24 hours postpartum that does not meet any criterion above and with estimated peripartum blood loss between 500 and 1000 ml. Postpartum major bleeding is clinical or radiological evidence of bleeding occurring between 24 hours and 6 weeks postpartum and meeting at least one of the following ISTH criteria: associated with a fall in hemoglobin of 2 g/dL (1.24 mmol/l) or more, or a requirement for transfusion of two or more units of red blood cells or whole blood, or symptomatic bleeding occurring in a critical site: intracranial, intraspinal, intraocular, pericardial, intra-articular, intramuscular with compartment syndrome, or retroperitoneal, or considered to have contributed to maternal death.

An allergic reaction to LMWH is a reaction following the administration of LMWH that results in anaphylaxis or a rash requiring discontinuation of the allocated LMWH. Heparin-induced thrombocytopenia (HIT) is defined...
as a clinical diagnosis of HIT and a minimum of a positive PF4 HIT ELISA assay. The venous thromboembolism outcome includes deep vein thrombosis (DVT) and/or pulmonary embolism. The criteria for diagnosis of DVT are venography demonstrating a constant intraluminal filling defect in the deep veins above the trifurcation of the popliteal vein or compression ultrasound revealing a non-compressibility of a venous segment above the trifurcation of the popliteal vein. Diagnosis of distal, below the knee DVT, is by either venography or compression ultrasound. Diagnostic criteria for pulmonary embolism are pulmonary angiography demonstrating a constant intraluminal filling defect or a cutoff of a vessel more than 2.5 mm in diameter, or ventilation/perfusion (V/Q scan) indicating high-probability, or pulmonary embolism found at autopsy.

**Extraction and recoding of individual patient data**

The definitions for each variable to be included in AFFIRM’s common dataset are documented in a data dictionary to allow standardization across studies and decrease the potential for misclassification and bias. A template for the common dataset has been developed in Microsoft Excel and will be provided to the principal investigator of each included trial. Recoded anonymized individual patient data from each of the trials will populate the Excel template. The recoded datasets for each of the individual trials will be saved on an IronKey™ USB flash drive and sent by courier to the coordinating center in Ottawa.

The AFFIRM common dataset will include individual patient data in 10 pre-defined categories: administrative and demographic data, thrombophilia, maternal medical history, pregnancy history, current pregnancy and delivery, infant data, pre-eclampsia outcome, other outcome events, intervention and treatment during pregnancy, and adverse events.

**Data synthesis, validation and analysis**

Once the individual participant data from the primary studies have been merged in the common dataset, descriptive analyses will be conducted to identify data outliers, missing data, and unexpected inconsistencies. The project coordinator will prepare data clarification reports and will communicate with the principal investigators or their delegates to resolve these queries. Next, we plan to conduct preliminary analyses aimed at replicating the findings of the individual published studies, to validate the centralized database and data importation. Once the IPDMA team is satisfied with the merged dataset, the database will be locked and the planned analyses for the IPDMA synthesis will be conducted.

The individual patient data will be analyzed in a similar manner to an RCT, however, the analysis will account for clustering at the study level. The primary analysis will include all women who are eligible for AFFIRM and will examine the risk of the primary composite outcome in the treatment (LMWH) and control arms based on intention-to-treat. Secondary univariate analyses will be done for each of the pregnancy complications included in the composite outcome. On-treatment sensitivity analyses will be conducted for the primary and secondary outcomes.

**Subgroup analyses**

We have planned several subgroup analyses; these were selected because they are clinically plausible and there is evidence that they may be relevant. If certain subgroups are found to be small (<5 subjects) we will merge subgroups as appropriate.

Women will be analyzed in subgroups according to the previous pregnancy complications that were experienced. Prior pre-eclampsia subgroups are any pre-eclampsia, severe pre-eclampsia, early-onset pre-eclampsia, and severe or early onset pre-eclampsia. Subgroups according to prior SGA are SGA <10th percentile, SGA <5th percentile, SGA <3rd percentile, prior pre-eclampsia and SGA <10th percentile, prior pre-eclampsia and SGA <5th percentile, prior pre-eclampsia and SGA <3rd percentile. Subgroups of women with prior placental abruption are any placental abruption, placental abruption leading to delivery <37 weeks’ gestation, placental abruption leading to delivery <34 weeks’ gestation, and placental abruption with pre-eclampsia. Participants will be grouped for analysis according to the gestational age of prior pregnancy loss: >12 weeks’ gestation, >16 weeks’ gestation, and >20 weeks’ gestation. Demographic subgroups are according to maternal age (<35 years or ≥35 years) and ethnic group (Caucasian, Black, Asian or other).

Women will be grouped according to personal characteristics and risk factors. For thrombophilia the subgroups are women with weak thrombophilia (Factor V Leiden [FVL] or prothrombin gene mutation [PTM]); moderate thrombophilia (protein C deficiency, protein S deficiency); strong thrombophilia (antithrombin deficiency, antiphospholipid antibodies, combined
thrombophilia ≥1 type, homozygous FVL or FGM; or no thrombophilia. Participants will be grouped according to personal history of venous thromboembolism (VTE), family history of VTE, and no VTE history.

Quality assessment will be conducted for all eligible studies using the tool for assessing risk of bias from the Cochrane Handbook for reviews of interventions and reported on a study level. These assessments will also be used to inform subgroup analyses and sensitivity analyses to explore whether these biases may have affected the IPDMA analysis. We plan to examine the randomization integrity once the data from the original trials have been combined. We will endeavour to compare the original randomization lists with actual randomization to test the integrity of the allocation concealment. We will also compare the baseline characteristics of participants who have been randomized to the LMWH and no LMWH groups at the study level and aggregate level to see if there are imbalances between the groups that may suggest a lack of integrity in randomization processes.

Knowledge translation

Once the results of the analyses are available, they will be circulated to all investigators and collaborators and a teleconference will be scheduled to discuss the findings and their interpretation. Regardless of the IPDMA results, they will be disseminated. Dr Shannon Bates is the principal knowledge user for this project. She will provide input throughout the project and will be a leader for the knowledge translation phase of the study. The principal investigators of the identified eligible RCTs (Drs Rey, Martinelli, de Vries, Gris, Rodger, Middeldorp, Schleussner, and Kaaja) are all experienced researchers and also practicing physicians who are knowledge users. Furthermore, these team members are all involved in leadership roles in their institutions and countries, including practice guideline development, and have the potential to considerably influence the international community of healthcare providers in a variety of settings.

The strategies for knowledge translation will rely heavily on the input from all involved knowledge users and will take into consideration the suitability of proposed media and/or approach for different practice settings and international contexts. Traditional methods, such as publication in a peer-reviewed journal, geared towards either a generalist or specialist audience, will be employed. Results will also be presented at international meetings; it is anticipated that knowledge users (clinicians) in hematology, obstetrics, and family medicine will be targeted. In addition, patient advocacy and education groups (such as the Pre-eclampsia Foundation, the North American Thrombosis Forum, and Thrombosis Canada) will be provided with the results in a language and format suitable to a non-medical audience.

Discussion

This IPDMA will permit the investigators to explore which women within the heterogeneous group of patients with placenta-mediated complications benefit and which women do not benefit from low-molecular-weight heparin injections throughout pregnancy.

Ethics, privacy and security

The subjects in each of the RCTs all provided informed consent to participate in the original trial. We will not be seeking individual consent for the secondary use of the data for the following reasons: the objectives of the IPDMA are consistent with the original trials, there are no risks or benefits associated with this analysis, no identifying information will be transferred, and it would be logistically time consuming and, in some cases, impossible to contact the women who participated. In order to ensure patient confidentiality any identifying information will be removed from the original dataset before it is transferred. The IronKey™ flash drive includes numerous security features including hardware-based encryption, a random password generator, two-factor authentication, and a self-destruct mechanism which make it extremely unlikely that the dataset can be accessed by anyone other than the intended recipient. Once the data are merged in Ottawa in the common database, they will be stored on the research institute’s network which has multiple security features and regular backup procedures in place.

Limitations and challenges

One relevant potential drawback of IPDMA is biased pooling of data. Bias can be introduced when eligible studies are missed, when authors do not provide their data for the analysis, when the outcomes are different across
studies, and when outcome and covariate data are missing from included studies. Our recently completed pooled summary meta-analysis was a successful collaboration of five principal investigators. In addition to the team members from these five trials, the principal investigators of four additional trials have committed to provide data for the AFFIRM meta-analysis. These are the largest and most robust trials completed in this area. The multinational research team has representation from Canada, the Netherlands, France, Italy, Germany, and Finland. Almost all review team members attended the face-to-face IPDMA planning meeting. To protect against the misclassification of outcomes, the AFFIRM review team discussed each outcome at this meeting until consensus on detailed definitions and diagnostic criteria was reached. Definitions for all variables to be included in the IPDMA common dataset are documented in a data dictionary which was reviewed, revised according to team feedback, and finalized. Despite this, we recognize that challenges will be encountered due to variability in how the variables were originally defined and collected in each of the nine trials. In some cases it will be necessary to consult the original clinical records to obtain complete information for the IPDMA which will be a labor-intensive process. Another challenge is the diversity in language of the original datasets (English, French, Dutch, Italian, and German) that will necessitate translation when the data are recoded. Attention to detail, careful documentation, and excellent communication will be instrumental to the successful completion of this IPDMA.

Acknowledgements

This collaborative meta-analysis based on individual patient data is funded by a Knowledge Synthesis Grant from the Canadian Institutes of Health Research (CIHR), reference number KRS 126593. The authors would like to acknowledge the contribution of David Moher who provided methodological advice during the conception and design of the project. The Knowledge Synthesis Group at the Ottawa Hospital Research Institute also played an important role during the systematic review. Raymond Daniel downloaded the records from the searches, removed duplicates, and obtained the full-text articles and Kavita Singh screened the titles and abstracts of citations to assess potential eligibility.

7.1 Search Strategy

LWMH & Placenta-Mediated Pregnancy Complications

OVID 2013 May 5

Database: Embase Classic+Embase <1947 to 2013 May 03>, Ovid MEDLINE(R) In-Process & Other NonIndexed Citations and Ovid MEDLINE(R) <1946 to Present>

Search Strategy:

1. exp Hypertension, Pregnancy-Induced/ (35044)
2. (pregnanc* adj5 hypertensi*).tw. (20960)
3. (gestational or maternal) adj5 hypertens*.tw. (7212)
4. PIH.tw. (3198)
5. (((high* or rais* or elevat* or heighten* or increas*) adj3 (blood pressure or diastolic pressure or systolic pressure or pulse pressure)) and pregnan*)tw. (4438)
6. (((high* or rais* or elevat* or heighten* or increas*) adj3 (BP or DBP or SBP)) and pregnan*)tw. (756)
7. (eclamp* or pre-eclamp* or preeclamps*).tw. (51722)
8. (EPH adj1 (Complex* or Gestos* or Toxemi* or Toxaemi* or Syndrome*)).tw. (1163)
9. (Edema or oedema) and Proteinuria and Hypertension and Gestosis).tw. (118)
10. ((pregnan* or gestational or gravidum or graviduram) adj5 (toxemi* or toxaemi*).tw. (9496)
11. HELLP.tw. (4291)
12. (Hemolysis and Elevated Liver and Lowered Platelet*).tw. (1)
13. Infant, Small for Gestational Age/.tw. (11668)
14. ‘small for gestational age’.tw. (12359)
15. SGA.tw. (9803)
16. Fetal Growth Retardation/. (30362)
17. (intrauterine or intra-uterine) adj2 growth restrict*.tw. (8013)
18. (intrauterine or intra-uterine) adj2 growth retard*.tw. (12905)
19. (fetal or foetal or fetus* or foetus*) adj2 growth restrict*.tw. (6530)
20. (fetal or foetal or fetus* or foetus*) adj2 growth retard*.tw. (4811)
21. IUGR.tw. (8668)
22. exp Fetal Death/ or Stillbirth/.tw. (57294)
23. (stillbirth* or stillborn*).tw. (23406)
24. (fetal or foetal or fetus* or foetus* or prenatal* or pre-natal* or perinatal* or peri-natal* or antepartum or ante-partum or antenatal* or ante-natal*) adj3 (loss* or death*).tw. (55608)
25. exp Abortion, Spontaneous/ (53089)
26. (abort* adj5 (spontaneous* or habitual* or frequent* or recur* or tubal)).tw. (26088)
27. (miscarriag* or miscarriagy or miscarriage or miscarriaged or miscarriaging).tw. (20236)
28. (second trimester* or 2nd trimester* or third trimester* or 3rd trimester* or late pregnant* or advanced pregnant* or late intrauterine or late intra-uterine) adj3 (loss* or death*).tw. (1044)
29. Placental Insufficiency/ (3986)
30. ((placenta* or uteroplacenta* or utero-placenta*) adj3 (insufficien* or incompeten* or failure*).tw. (8744)
31. Abruptio Placentae/. (6398)
32. (placent* adj1 (abruptio* or ablotion* or detachment* or separation* or solutio* or apoplexia*).tw. (6756)
33. abruptio*.tw. (3594)
34. (placenta* and vascular and thrombo*).tw. (449)
35. (“placenta-mediated pregnancy” or “placental-mediated pregnancy”) adj3 (compliicat* or problem* or difficult* or disorder*).tw. (49)
36. Pregnancy Complications, Hematologic/. (81830)
37. exp Placenta/de (4123)
38. or/1-37 (345669)
Appendix 7

128. SGA.tw. (9803)
129. ([intraterum or intra-uterine] adj2 growth restrict*).tw. (8013)
130. ([intraterum or intra-uterine] adj2 growth retard*).tw. (12905)
131. ([fetal or foetal or fetus* or foetus*] adj2 growth restrict*).tw. (5530)
132. ([fetal or foetal or fetus* or foetus*] adj2 growth retard*).tw. (4811)
133. IUGR.tw. (8668)
134. exp fetus death/ (32616)
135. (stillbirth* or stillborn*).tw. (23406)
136. ([fetal or foetal or fetus* or foetus* or prenatal* or per-natal* or peri-natal* or ante-partum or ante-partum or ante-natal* adj3 (loss* or death*)]).tw. (35608)
137. spontaneous abortion/ (38858)
138. (abort* adj3 (spontaneous* or habitual* or frequent* or recur* or tubal)).tw. (26088)
139. (miscarriage or m miscarriage or miscarry or miscarried or miscarrying).tw. (20236)
140. ((second trimester* or 2nd trimester* or third trimester* or 3rd trimester* or late pregnant* or advanced pregnant* or late intraterium or late intra-uterine) adj3 (loss* or death*)].tw. (1044)
141. placenta insufficiency/ (2667)
142. ([placent* or utero-placenta* or utero-placenta*] adj3 (insufficien* or incompen* or failure*)].tw. (4744)
143. solutoiio placenta/ (4671)
144. ([placent* adj1 (abruptio* or ablation* or detachmen* or separation* or solutoio*)].tw. (6753)
145. abruptio*].tw. (3994)
146. ([placent* and vascular and thrombosis*].tw. (449)
147. (['placenta-mediated pregnancy' or 'placent-al-mediated pregnancy'] adj3 (complicat* or problem* or difficult* or disorder*)].tw. (49)
148. (pregnan* and (hematolog* adj5 (complicat* or problem* or difficult* or disorder*)].tw. (392)
149. or/111-148 (264754)
150. exp low molecular weight heparin/ (47677)
151. LMWH.tw. (8183)
152. (low molecular weight or LMW) adj1 heparin).tw. (17403)
153. (Dalteparin* or FR-860 or Fragmin or Fragmine or Kabi-2165 or K-2165 or K2165 or Tedelparin* or low liquemin).tw. (3903)
154. (Enoxaparin* or Clexan* or EMT-966 or EMT-967 or EMT967 or HSDB 7846 or Klexane or Lovenox or PK 10169 or PK10169 or RP 54563 or UNII-8NZ41MIK1O).tw. (9385)
155. 679809-58-6.rn. (5397)
156. (nadroparin* or CY 216 or CY 216d or CY216 or CY216d or Fraxiparin* or LMF CY-216 or UNII-8NZ41MIK1O).tw. (9385)
157. 208 use emczd (236)
158. 208 use prmz (131)
159. 208 remove duplicates from 207 (367)
160. TOTAL HITS (458)
161. (Dalteparin* or FR-860 or FR860 or Fragmin or Fragmine or Kabi-2165 or K-2165 or K2165 or Tedelparin* or low liquemin).tw. (3903)
162. (Danaproid or 'kb 101' or kb101 or lomoparan or lomoparin or mucoglucuronan or org 10172 or org10172 or orgaran).tw. (1507)
163. 308068-55-5.rn. (0)
164. deligoparin*.tw. (0)
165. ([heparin adj1 dihydroergot) or (dihydroergotamine adj1 heparin) or Embolex or [heparin adj1 DHE]).tw. (458)
166. idrabiataparinux.tw. (60)
167. idraparinux.tw. (287)
168. 162610-17-5.rn. (574)
169. livaraparin calcium.tw. (0)
170. minolteparin*.tw. (0)
171. rd 11885.tw. (13)
172. tafoxiparin*.tw. (3)
173. tedelparin*.tw. (23)
174. or/150-179 (54093)
175. 149 and 180 (2088)
176. 182 exp animals/ or exp animal experimentation/ or exp models animal/ or exp animal experiment/ or nonhuman/ or exp vertebrate/ (36963297)
177. 183 exp humans/ or exp human experimentation/ or exp human experiment/ (27357123)
178. 184 not 183 (9607791)
179. 185 not 184 (2067)
180. randomized controlled trial/ (691922)
181. randomized controlled trial topic/ (29863)
182. randomized topic/ (138604)
183. double blind procedure/ (119069)
184. single blind procedure/ (17360)
185. placebo/ (234074)
186. [random* or RCT$1 or placebo*].tw. (1618914)
187. [using* or doubl* or trebl* or tripl*] and (mask* or blind* or dumm*).tw. (312130)
188. or/186-193 (199796)
189. 185 and 194 (340)
190. 'systematic review'/ (59764)
191. meta-analysis/ (110106)
192. 'meta analysis (topic)'/ (7056)
193. (meta-analy* or metanalysis or metaanaly* or meta analy* or integrative research or integrative review or integrative overview or research integration or research overview or collaborative review).tw. (122093)
194. 'systematic review'/ (59764)
195. meta-analysis/ (110106)
196. 'meta analysis (topic)'/ (7056)
197. (meta-analy* or metabiology or metaanaly* or met analy* or integrative research or integrative review or integrative overview or research integration or research overview or collaborative review).tw. (122093)
198. 'systematic review'/ (59764)
199. meta-analysis/ (110106)
200. 'meta analysis (topic)'/ (7056)


Chapter 8

No association between ANXA5 genetic variants and deep venous thrombosis

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Summary

Introduction: Annexin A5 is a protein with antithrombotic properties present in vascular endothelium. It has been suggested that genetic variants in the ANXA5 gene affect ANXA5 expression, contributing to a local procoagulant state. Some studies showed associations of ANXA5 single nucleotide polymorphisms (SNPs) or haplotypes with pregnancy-related deep venous thrombosis (DVT) and myocardial infarction.

Objectives: To investigate whether common variants in the ANXA5 promoter region are associated with DVT risk in Dutch Caucasian individuals.

Methods and Results: From the Amsterdam Case-control Thrombophilia (ACT) study, 148 patients with newly diagnosed DVT and 267 controls without previous VTE were included. We sequenced the promoter region of the ANXA5 gene and reconstructed four common haplotypes, based on six SNPs. Neither individual SNPs nor any of the common haplotypes were associated with an increased risk for DVT. Furthermore, the four ANXA5 haplotypes were equally distributed among DVT patients and a second independent control group of 1705 individuals from the general population (Nijmegen Biomedical Study).

Conclusions: Our data suggest that ANXA5 haplotypes do not contribute to DVT risk in the Dutch population.

Introduction

Venous thromboembolism (VTE) is a multifactorial disease with an incidence of 1–2 per 1000 per year in western countries. Although several genetic risk factors for VTE such as Factor V Leiden or prothrombin G20210A mutation have been identified, the risk of VTE is also increased in case of a positive family history of VTE, in whom known genetic risk factors have been ruled out. This suggests that other as yet unknown genetic variants also predispose to VTE.

Annexin A5 is a natural anticoagulant protein highly expressed by vascular endothelium and placental trophoblasts. The hypothesis of a possible role of Annexin A5 in the pathogenesis of thrombotic disorders originates from its anticoagulant, antithrombotic and anti-inflammatory properties observed in vitro and in animal models in vivo. In the presence of Ca++, Annexin A5 binds and shields anionic phospholipids, which form the catalytic surface for coagulation reactions. Reduced Annexin A5 expression on
the surface of vascular cells and subsequently inefficient shielding of anionic phospholipids could contribute to the activation of blood coagulation and the creation of a prothrombotic environment within the blood vessel. In the antiphospholipid syndrome, for example, a reduction of Annexin A5 at the vascular wall is thought to be one of the several explanations for the occurrence of both arterial and venous thrombosis. Reduced binding of Annexin A5 to cardiolipin observed in patients with confirmed idiopathic venous thrombosis, supports the role of Annexin A5 in thrombosis. In patients with systemic lupus erythematosus, reduced binding of Annexin A5 to endothelium has also been proposed as a mechanism underlying atherothrombosis. In addition, Annexin A5 down-regulates expression of the procoagulant tissue factor, which is a key player in VTE. Furthermore, Annexin A5 is known to be an effective inhibitor of experimentally induced venous and arterial thrombosis in animal models.

If genetic variants within the ANXA5 gene affect Annexin A5 expression on cell surfaces, these could also influence the risk of clinical outcomes such as arterial or venous thrombosis. Several studies have been performed to assess this. A genetic variant located in the Kozak sequence (g.-1C>T, rs1131239) of the ANXA5 gene was associated with higher plasma Annexin A5 levels. This minor rs1131239T-allele was associated with a decreased risk of myocardial infarction in young patients and a lower risk of developing a new coronary event during 36 months follow-up. Other studies, however, were unable to reproduce these findings. In the study that showed a decreased risk of myocardial infarction, venous thrombotic risk was also investigated but no significant association between the minor rs1131239T-allele and deep venous thrombosis (dVT) was found (OR for dVT 0.76, 95% CI 0.47 – 1.22). Similarly, in a Dutch population of 198 patients with autoimmune diseases, no association with venous or arterial thrombosis was found. A haplotype comprising four ANXA5 promoter single nucleotide polymorphisms (SNPs) (rs112782763, rs28717001, rs28651243, rs113588187), collectively referred to as the m2 haplotype, reduces ANXA5 promoter activity in a promoter construct assay in vitro, which could be translated to an increased dVT risk. Indeed, the presence of the m2 allele was found to be a risk factor for dVT in pregnancy or the postpartum period, as well as in the general Southern Italian population.

In a previous study, we described four common ANXA5 haplotypes (h1, h2, h3 and h4). Of these, the haplotype h3 is an extension of the previously described m2 haplotype that reduces ANXA5 promoter activity in vitro as well as correlates with lower plasma Annexin A5 levels. Interestingly, the minor rs1131239T-allele, which was associated with increased plasma Annexin A5 levels and a decreased risk of myocardial infarction, is completely linked to SNPs comprising the m2 haplotype.

Given the conflicting results on the association of variants within the ANXA5 gene upstream region with clinical outcomes in previous studies, we aimed to evaluate whether ANXA5 promoter SNPs and haplotypes influence the risk of dVT in the Dutch general population. For this study, we used a case-control study on risk factors for dVT, the Amsterdam Case-control Thrombophilia (ACT) study, and a second group of population controls (Nijmegen Biomedical Study, NBS).

Materials and methods

Study population

From the Amsterdam Case-control Thrombophilia Study (ACT) performed between September 1999 and May 2006, we selected 437 unrelated individuals (154 cases and 283 controls) of Caucasian origin. Both cases and controls were from the Amsterdam region, the western part of the Netherlands. Cases were patients with newly diagnosed and objectively confirmed proximal dVT of the leg. Controls were patients without previous VTE, in whom dVT was suspected but ruled out. The diagnosis of dVT was based on the Wells score and D-dimer plasma level algorithm, followed by compression ultrasonography if indicated, as described. A standardised questionnaire was used for all participants, to obtain information about known risk factors such as malignancy or treatment because of malignancy in the last 6 months; pregnancy or postpartum period; use of oral contraceptives or hormonal therapy; trauma within the last 60 days; being bedridden (for >3 days); paralysis or recent plaster immobilisation of the symptomatic leg; surgery within the last 4 weeks. Information was obtained prior to diagnosis of dVT, i.e. classification as case or control. Genomic DNA was isolated from peripheral leukocytes and was stored at +4°C. The Medical Ethical Committee of the Academic Medical Center in Amsterdam approved the study.

The second control group consisted of individuals who had been included in the Nijmegen Biomedical Study (NBS), of which details were reported previously. Briefly, the NBS is a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboud university medical center. 21,756 age- and sex-stratified randomly selected inhabitants of the municipality of Nijmegen in
the eastern part of the Netherlands, received an invitation to fill out a postal questionnaire on, e.g., lifestyle and medical history, and to donate blood samples. The response to the questionnaire was 43% (n = 9350). 69% of the responders donated blood samples. Of the 1819 cancer blood samples, we selected for this study 1705 controls of self-reported European descent who reported not to have had DVT or pulmonary embolism. Written informed consent was obtained from all ACT and NBS participants.

Genetic analysis

In the ACT subjects, a 496-bp fragment of the ANXA5 promoter (261 base pairs upstream and 235 base pairs downstream of the first transcription start point) was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers: forward 5’-ccgagccctggacagctccca-3’ and reverse 5’-gccccgcaccacgctctcctct-3’. PCR reactions were carried out in a final volume of 25 ml reaction mixture containing 2.5 ml 10x PCR Buffer (Qiagen), 5% DMSO (v/v), 1 M Betaine, 0.4 mm of each primer (forward and reverse), 0.08 mm of each deoxynucleotide triphosphate, 100-150 ng genomic DNA and 1.25 U Taq DNA polymerase (Qiagen). Cycling conditions were: an initial denaturation step at 95°C for 3 minutes followed by denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute and elongation at 72°C for 1 minute (30 cycles in total). PCRs were performed in a 73 Thermal Cycler (Biotera, Germany). Sequence analysis was performed by direct sequencing using the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA). Products of sequence reactions were analysed on a Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA). Sequencing chromatograms were examined by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI).

NBS controls were genotyped using the Illuma HumanHapCNV370-Duo BeadChip as described. For this study, we extracted 4 SNPs in ANXA5 (rs62319820, rs113588187, rs1050606 and rs1131239) from genome-wide imputed SNP data using the ‘Genome of the Netherlands’ (GONL) data as reference.

In the ACT participants, haplotypes were constructed using six promoter SNPs (rs62319820, rs112782763, rs28717001, rs28651243, rs113588187, rs1050606) as the minor rs1131239-T-allele is completely linked to rs112782763 and rs113588187, haplotypes were equal to previously described elsewhere.

To reconstruct the four known ANXA5 haplotypes in the NBS controls, we used three haplotype-tagging SNPs (rs62319820, rs113588187, rs1131239) and SNP rs1050606, of which the major T-allele is specific for haplotype H1. Haplotypes were assigned manually to all individuals.

Statistical analysis

Hardy-Weinberg equilibrium for each SNP was tested using the c² test. Haplovewview software was used to estimate the degree of linkage disequilibrium (LD; r² values) between all SNP pairs and to determine haplotypes (h). The association of ANXA5 SNPs and haplotypes with DVT risk was examined using the c² test. Odds ratios (ORs) with 95% confidence intervals (CIs) were calculated as an estimate of the relative risk indicating the risk for DVT in a category of exposure (e.g., haplotype HIX carriers) relative to the reference category (e.g., non-haplotype HIX). ORs for DVT adjusted for age, sex, and presence of provoking risk factors were calculated using logistic regression. Statistical analyses were performed using SPSS version 20.0 software. Two-sided probability values of <0.05 were considered statistically significant.

Results

Clinical characteristics of DVT patients and controls from the ACT study

In total, 148 DVT patients and 267 controls were included in the analyses, as sequencing of the ANXA5 promoter was not successful in 6 patients and 16 controls. The characteristics of the study participants are presented in Table 1. The mean age was comparable between both groups, and patients were more often male than controls (p=0.046). Body mass index was similar in cases and controls (26.0 and 27.2 kg/m² respectively). Thirty DVT patients (20.3%) had a prior history of VTE. In 77 patients (52%), risk factors for DVT were identified. FVL was found in 23.1% of cases and in 5.8% of controls and the prothrombin G20210A mutation – in 4.1% cases and 2.9% controls.
Haploview analysis revealed a high degree of linkage disequilibrium between all SNPs except for SNP1 and SNP6, SNP2 and SNP5 as well as SNP3 and SNP4 were completely linked (r² = 1) (FIGURE 1). Only four common haplotypes (frequency >1%) were present, which was similar to our previous study in a different Dutch population.²⁸

FIGURE 1 - HAPLOVIEW Linkage Disequilibrium plot of the six SNPs within the ANXA5 promoter in the ACT study.

The LD coefficients r² (x100) between all SNP pairs are shown in squares. The darker the gray colour is, the higher the degree of LD. The black colour (r² = 1) indicates complete linkage.

### ANXA5 SNPs and haplotypes in the ACT study

Sequencing of the ANXA5 promoter showed the presence of six common polymorphisms (TABLE II), i.e. SNP1 (rs62319820, g.-628C>T), SNP2 (rs112782763, g.-467G>A), SNP3 (rs28717001, g.-448A>C), SNP4 (rs28651243, g.-422T>C), SNP5 (rs113588187, g.-373G>A) and SNP6 (rs1050606, g.-302T>C) that have previously been reported.⁴²⁻⁶⁴ We also identified three rare variants. SNP7 (g.-622G>C) located six nucleotides downstream of SNP1 was found in three controls and one patient in a heterozygous form as well as in one patient in a homozygous form (MAF in controls: 0.006; MAF in patients: 0.01). SNP8 (g.-585G>A) and SNP9 (g.-506G>A) upstream of SNP2 were present in two controls in a heterozygous form (MAF: 0.004). These rare polymorphisms were excluded from further analyses.

The genotype frequencies of the common ANXA5 SNPs were in Hardy-Weinberg equilibrium both in DVT patients and in controls (TABLE SII). We examined an association between separate SNPs and DVT risk (TABLE SIII). None of the six polymorphisms was significantly associated with DVT.
TABLE III shows the association between ANXA5 haplotypes and DVT. Carriers of only major alleles for all SNPs (haplotypes H1, H1H1+H1Hx) appeared to have a slightly increased risk for DVT compared to non-H1 carriers, in both the unadjusted model (OR 1.5, 95% CI: 0.9–2.3) and after adjusting for age, sex and the presence of provoking risk factors (OR 1.4, 95% CI: 0.9–2.2), but the risk estimates did not reach statistical significance. The H2, H3 and H4 ANXA5 haplotypes were not associated with DVT. Additionally, when homozygous carriers of H1 (H1H1) were used as the reference category instead of HXXH, the haplotypes H2, H3 and H4 were not associated with DVT (data not shown). Furthermore, we compared H3 carriers (encompassing the H2 haplotype, 27 cases and 59 controls) to carriers of only H1 and/or H2 (N/N in previous studies, 99 cases and 171 controls) and no association between H3 and DVT was found (OR for DVT for H3 carriers 0.79, 95% CI: 0.47–1.33). We also examined DVT risk for men and women separately. In men, haplotype H2-carriers (H2H2+H2Hx) appeared to have a slightly decreased risk of DVT compared to non-H2 carriers (OR 0.6, 95% CI: 0.3–1.1). Women carrying haplotype H3 tended to have a reduced risk for DVT (OR 0.5, 95% CI: 0.3–1.2). Again, these estimates did not reach statistical significance. Finally, we performed a subgroup analysis of 71 patients with an unprovoked DVT and did not detect any association between ANXA5 haplotypes and DVT (data not shown).

ANXA5 SNPS and haplotypes in population controls

To verify if the prevalence of ANXA5 haplotypes in the selected hospital controls is representative of the population prevalence, we included an independent control group from the general population. Of the 1705 NBS controls, 48.9% was male and the mean age was 60.7 years (Table IV). The prevalence of FVL in NBS controls (5.4%) was similar to that in ACT controls (5.8%), whereas carriership of the prothrombin G20210A mutation was less prevalent in NBS controls (0.54%) compared to ACT controls (2.9%).

Four ANXA5 SNPs covering the four common ANXA5 haplotypes were evaluated. The genotype distributions of all SNPs were in Hardy-Weinberg equilibrium (Table III). The common ANXA5 haplotypes were constructed in 1704 NBS controls as one control person presented with the rare ANXA5 haplotype. Finally, all ANXA5 haplotypes were similarly distributed among DVT patients and NBS controls (Table III) as well as among subgroups of men or women (data not shown).

All haplotypes but the one given (e.g. non-H1, non-H2, non-H3 or non-H4) were used as the reference category (OR = 1). DVT, deep venous thrombosis. *One person carrying the rare haplotype was excluded from analysis.
Table SI - Frequency distribution of ANXA5 polymorphisms and expected frequencies according to Hardy-Weinberg Equilibrium in the act study (dvt patients (n = 148) and controls (n = 267)).

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>DVT patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Observed</td>
<td>Expected</td>
</tr>
<tr>
<td></td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>SNP1-628 C&gt;T (rs82319820)</td>
<td>125</td>
<td>125</td>
</tr>
<tr>
<td>CC</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td>TT</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SNP2-467 G&gt;A (rs112792763)</td>
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<td>121</td>
</tr>
<tr>
<td>GG</td>
<td>28</td>
<td>55</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SNP3-448 A&gt;C (rs28717001)</td>
<td>99</td>
<td>101</td>
</tr>
<tr>
<td>AA</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>AC</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>SNP4-422 T&gt;C (rs29651243)</td>
<td>99</td>
<td>101</td>
</tr>
<tr>
<td>TT</td>
<td>46</td>
<td>42</td>
</tr>
<tr>
<td>CC</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>SNP5-373 G&gt;A (rs113588187)</td>
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<td>121</td>
</tr>
<tr>
<td>GG</td>
<td>26</td>
<td>55</td>
</tr>
<tr>
<td>AA</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SNP6-302 T&gt;G (rs1050606)</td>
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<td>34</td>
</tr>
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<td>TT</td>
<td>77</td>
<td>74</td>
</tr>
<tr>
<td>TG</td>
<td>39</td>
<td>41</td>
</tr>
</tbody>
</table>

† Expected frequencies of all ANXA5 SNPs were not statistically different from observed frequencies (p-value >0.05), both in controls and in cases.

Table SII - DVT risk for ANXA5 SNPs in the act study.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Patients (n = 148)</th>
<th>Controls (n = 267)</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP1 rs82319820</td>
<td>CC</td>
<td>125 (84.5)</td>
<td>221 (83.8)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>22 (14.6)</td>
<td>45 (16.9)</td>
<td>0.8 (0.5-1.5)</td>
</tr>
<tr>
<td></td>
<td>TT</td>
<td>1 (0.7)</td>
<td>1 (0.4)</td>
<td>1.8 (0.1-28.5)</td>
</tr>
<tr>
<td></td>
<td>CT + TT</td>
<td>23 (15.5)</td>
<td>46 (17.2)</td>
<td>0.9 (0.5-1.5)</td>
</tr>
<tr>
<td>SNP2 rs112792763</td>
<td>GG</td>
<td>121 (81.8)</td>
<td>208 (77.9)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>26 (17.6)</td>
<td>55 (20.6)</td>
<td>0.8 (0.5-1.4)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>1 (0.7)</td>
<td>4 (1.5)</td>
<td>0.4 (0.05-2.9)</td>
</tr>
<tr>
<td></td>
<td>GA + AA</td>
<td>27 (18.2)</td>
<td>59 (22.1)</td>
<td>0.8 (0.5-1.3)</td>
</tr>
<tr>
<td>SNP3 rs28717001</td>
<td>AA</td>
<td>99 (66.9)</td>
<td>171 (64.4)</td>
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</tr>
<tr>
<td></td>
<td>AC</td>
<td>46 (31.1)</td>
<td>82 (30.7)</td>
<td>1.0 (0.6-1.5)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>3 (2.0)</td>
<td>14 (5.2)</td>
<td>0.4 (0.1-1.3)</td>
</tr>
<tr>
<td></td>
<td>AC + CC</td>
<td>49 (33.1)</td>
<td>96 (36.0)</td>
<td>0.9 (0.6-1.3)</td>
</tr>
<tr>
<td>SNP4 rs29651243</td>
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<td>99 (66.9)</td>
<td>171 (64.4)</td>
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<tr>
<td></td>
<td>TC</td>
<td>46 (31.1)</td>
<td>82 (30.7)</td>
<td>1.0 (0.6-1.5)</td>
</tr>
<tr>
<td></td>
<td>CC</td>
<td>3 (2.0)</td>
<td>14 (5.2)</td>
<td>0.4 (0.1-1.3)</td>
</tr>
<tr>
<td></td>
<td>TC + CC</td>
<td>49 (33.1)</td>
<td>96 (36.0)</td>
<td>0.9 (0.6-1.3)</td>
</tr>
<tr>
<td>SNP5 rs113588187</td>
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<td>208 (77.9)</td>
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<td>GA</td>
<td>26 (17.6)</td>
<td>55 (20.6)</td>
<td>0.8 (0.5-1.4)</td>
</tr>
<tr>
<td></td>
<td>AA</td>
<td>1 (0.7)</td>
<td>4 (1.5)</td>
<td>0.4 (0.05-2.9)</td>
</tr>
<tr>
<td></td>
<td>GA + AA</td>
<td>27 (18.2)</td>
<td>59 (22.1)</td>
<td>0.8 (0.5-1.3)</td>
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<tr>
<td>SNP6 rs1050606</td>
<td>TT</td>
<td>32 (21.6)</td>
<td>56 (21.0)</td>
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<tr>
<td></td>
<td>TG</td>
<td>77 (52.0)</td>
<td>119 (44.6)</td>
<td>1.1 (0.7-1.9)</td>
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<tr>
<td></td>
<td>TG + GG</td>
<td>116 (79.4)</td>
<td>211 (79.0)</td>
<td>1.0 (0.6-1.6)</td>
</tr>
</tbody>
</table>

All odds ratios (OR) were calculated with major alleles-only as the reference category (OR = 1). DVT, deep venous thrombosis.
TABLE SIII - Frequency distribution of ANXA5 SNPs and expected frequencies according to Hardy-Weinberg Equilibrium in NS controls (n = 1705).

<table>
<thead>
<tr>
<th>SNP</th>
<th>Risk allele frequency</th>
<th>Observed genotype</th>
<th>Expected genotype</th>
<th>p-value</th>
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<tr>
<td>rs2319820</td>
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<td>0.08</td>
<td>146</td>
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<td>147.5</td>
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<tr>
<td>CT</td>
<td>252</td>
<td>247</td>
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<td></td>
</tr>
<tr>
<td>TT</td>
<td>8</td>
<td>10.5</td>
<td></td>
<td></td>
</tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>21</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1050608</td>
<td>rs1050608</td>
<td>0.51</td>
<td>394</td>
<td>405.5</td>
</tr>
<tr>
<td>TT</td>
<td>394</td>
<td>405.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TG</td>
<td>475</td>
<td>452</td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>430</td>
<td>447.5</td>
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<tr>
<td>rs1131239</td>
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<td>0.11</td>
<td>1350</td>
<td>1350</td>
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<td>1350</td>
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<tr>
<td>TT</td>
<td>21</td>
<td>21</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Discussion

In this study, neither individual SNPs nor any of the four haplotypes within the ANXA5 gene upstream region (including the H3 haplotype which is an extension of the M2 haplotype) were associated with an increased risk of DVT.

How do our findings relate to previous studies? First, there is a discrepancy in calling of haplotypes. Previous studies reported the M2 haplotype (comprising SNPs 2, 3, 4 and 5). We previously reported 4 other haplotypes (H1, H2, H3 and H4), of which H3 is an extension of the M2 haplotype (additionally including SNPs 6 and 7, rs1131239 g. -1 c > t). The studies that found an association between ANXA5 haplotypes and DVT compared M2 carriers to non-M2 carriers. According to the results of our previous and the current studies, the non-M2 carriers include haplotypes H1 and H2. We found no association between haplotype H3 (the extension of the M2 haplotype) and DVT, neither when comparing H3 carriers to non-H3 carriers, nor when comparing H3-carriers to only H1 and/or H2 carriers (n/n in previous studies). It should be noted that the haplotypes H1 and H2 were associated with different Annexin A5 plasma levels in healthy controls. Although this suggests that H1 and H2 should not be combined as one refer-
(g.A>g), which is located 57bp downstream from the ATG codon, belonged to the top-5 variants selected for replication but failed to show any association with DVT after replication. Other ANXA5 variants located in exons and exon-intron boundaries were not associated with DVT in an initial stage. This suggests that even with a different study design, investigating the coding regions of ANXA5 besides the ANXA5 promoter, no association between ANXA5 SNPs and thrombosis risk will be found.

Despite the lack of association between ANXA5 genetic variants and DVT, previous studies have shown that ANXA5 genetic variants may play a role in the occurrence of obstetric complications such as miscarriage and intra-uterine growth restriction. Furthermore, placental Annexin A5 binding was shown to be critical for maintaining murine placental integrity and in humans, reduced plasma Annexin A5 levels were associated with recurrent pregnancy loss. These associations require further research.

In conclusion, we could not demonstrate a meaningful association between ANXA5 genetic variants and DVT in the Dutch population.

Addendum

The Amsterdam Case-control Thrombophilia Study was performed between September 1999 and May 2006 and DNA samples and clinical data of participants are stored at the Academic Medical Center, Amsterdam.

Acknowledgments

We would like to thank Jorge Peter for performing the PCRs. In addition, we would like to acknowledge the ‘vasculists’, a group of medical students responsible for execution of the ACT study. Principal investigators of the Nijmegen Biomedical Study are L.A.L.M. Kiemeney, M. den Heijer, A.L.M. Verbeek, D.W. Swinkels and B. Franke. We are very grateful to J. Goeman and T.E. Galesloot for their help with the SNP data of NBS control subjects.

References 8


Chapter 9

ANXA5 promoter haplotype and placental expression in relation to pre-eclampsia risk

Paulien G. de Jong, Gijs B. Afink, Carrie Ris-Stalpers, Michael W.T. Tanck, Souad Boussata, Jorge Peter, Joost C.M. Meijers and Saskia Middeldorp

Submitted for publication
Abstract

Introduction: Annexin A5 is an anticoagulant protein, present on the apical membrane of syncytiotrophoblasts, where it is assumed to regulate coagulation at the maternal-fetal interface and play a role in maintaining pregnancy. Studies have suggested that ANXA5 gene variants, via altered ANXA5 mRNA expression and Annexin A5 protein levels, are associated with pregnancy complications such as pre-eclampsia.

Objectives: To investigate whether single nucleotide polymorphisms (SNPs) in the maternal or fetal promoter of ANXA5 affect mRNA expression in the placenta or are associated with pre-eclampsia.

Methods and Results: Maternal DNA, DNA isolated from umbilical cord blood and placental RNA samples were collected from 34 pre-eclamptic pregnancies and 146 normotensive pregnancies. The promoter region of ANXA5 was sequenced and based on six SNPs six common haplotypes were constructed. Placental RNA was isolated and reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed. Neither individual SNPs nor any of the common haplotypes were associated with an increased risk of pre-eclampsia. The T-allele of rs62319820 (c.-390 C>T) in the neonatal promoter was associated with increased mRNA expression, but ANXA5 mRNA expression levels were not associated with pre-eclampsia risk.

Conclusions: In our study, maternal or fetal ANXA5 promoter variants were not associated with pre-eclampsia. Furthermore, placental ANXA5 mRNA expression was not associated with pre-eclampsia.

Introduction

Annexin A5 is a protein with anticoagulant properties. Through calcium-dependent binding with negatively charged phospholipids, Annexin A5 forms a two-dimensional shield, preventing coagulation reactions at the phospholipid surface. This function is illustrated by the fact that in the antiphospholipid syndrome, antiphospholipid antibodies appear to disrupt this Annexin A5 shield thereby increasing coagulation. In humans, the Annexin A5 shield is present on the microvillar surface of the syncytiotrophoblast layer, where it is thought to play a role in maintaining fluidity at the maternal-fetal interface. In mice, Annexin A5 is crucial for placental integrity and deficiency results in growth restriction and fetal loss. If dislocation of Annexin A5 by antiphospholipid antibodies plays
a role in pregnancy complications in antiphospholipid syndrome, an endogenously reduced level of Annexin A5 may also predispose to adverse pregnancy outcome in the absence of antiphospholipid antibodies. Genetic variants in ANXA5 are hypothesized to affect clinical outcomes such as deep venous thrombosis and pregnancy complications via altered ANXA5 gene expression and Annexin A5 protein levels. 

With an incidence ranging between 1 to 7% in nulliparous women, pre-eclampsia is a severe complication of pregnancy. Pre-eclampsia is characterized by high blood pressure and proteinuria in the second half of pregnancy that significantly contribute to maternal and fetal morbidity and mortality. Impaired placental development and vascularization contribute to endothelial dysfunction and a state of systemic inflammation, which leads to kidney- and liver dysfunction, and if left untreated, to multi-organ failure and death. As a multi-causal disorder, both environmental and genetic determinants play a role in the etiology of pre-eclampsia. In recent years, variants in ANXA5 have been proposed as such genetic determinants of pre-eclampsia. A haplotype consisting of 4 single nucleotide polymorphisms (SNPs) in the promoter of ANXA5 (M2 haplotype) was more prevalent in women with hypertensive disorders in pregnancy (n = 158), including women with pre-eclampsia, when compared to women with at least one uneventful pregnancy (n = 195) (29% versus 15%, odds ratio (OR) 2.1, 95% confidence interval (CI) 1.2 – 3.5). However, another study compared the frequency of this haplotype in maternal blood of 54 women with a history of pre-eclampsia to 71 normotensive controls and found no association with pre-eclampsia (13% versus 11%; OR 1.17, 95% CI 0.38 – 3.56). In samples of pre-eclampsia placentas, that reflect the fetal genotype (n = 47), the M2 haplotype appeared to be more frequent when compared to samples of normotensive controls (n = 50) (26% versus 10%, OR 3.09, 95% CI 0.99 – 9.58). We aimed to investigate whether the maternal or neonatal ANXA5 promoter haplotype is associated with placental ANXA5 mRNA expression or the manifestation of pre-eclampsia during pregnancy.

Methods

Patients

Of 287 women who gave birth in the Academic Medical Center in Amsterdam between December 2005 and December 2009, maternal blood, umbilical cord blood of their newborns, and placental samples were collected. The Academic Medical Center is a tertiary center, but also serves as post-code-referral hospital. Pre-eclampsia was defined according to criteria of the National Heart, Lung and Blood Institute Working Group, as the de novo appearance of hypertension (systolic blood pressure of ≥ 140 mm Hg or diastolic blood pressure of ≥ 90 mm Hg), accompanied by new-onset proteinuria, defined as ≥ 300 mg per 24 hours. Co-diagnosis of HELLP syndrome (syndrome of hemolysis, elevated liver enzymes and low platelets) was recorded. Informed consent was obtained from each woman and the study was approved by the ethical review board.

Sample collection, RT-qPCR and SNP analysis

Maternal blood, umbilical cord blood and placenta tissue used are from the Preeclampsia And Non-preeclampsia Database (PANDA) project approved by the institutional review board of the Academic Medical center. Blood was collected in EDTA tubes and processed by the Gentra Autopure LS8TM System (Gentra Systems). Placenta samples were collected in RNA later (Ambion) and stored at -80°C until further use. Collection of placental samples, RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR) were performed as described previously. ANXA5-specific primers were designed using the Roche Universal ProbeLibrary Assay Design Center: 5’-GGCTTTTAGATGGTTAGAGCTG-3’ and 5’-TCAGTAGTTCCCTGAGCA-3’, probe UPL#24. A 496-bp fragment covering part of the ANXA5 promoter and exon1 with the polymorphisms of interest was amplified by polymerase chain reaction (PCR) using two oligonucleotide primers: forward 5’-CCGGACCCCTTGGACAGCTCCCCA-3’ and reverse 5’-GCCGCCGCACCGACGTCCTCT-3’. PCR reactions were carried out in a final volume of 25 µl reaction mixture containing 2.5 µl 10x PCR Buffer (Qiagen), 5% DMSO (v/v), 1 mM betaine, 0.4 µM of each primer, 0.08 mM of each deoxynucleotide triphosphate, 100-150 ng genomic DNA isolated either from maternal or umbilical cord blood and 1.25 U Taq DNA...
polymerase (Qiagen). Cycling conditions were: an initial denaturation step at 95°C for 3 minutes followed by denaturation at 95°C for 1 minute, annealing at 62°C for 1 minute and elongation at 72°C for 1 minute (30 cycles in total). PCRs were performed in a T3 Thermal Cycler (Biometra, Germany).

Sequence analysis was performed by direct sequencing using the Big Dye Terminator ABI Prism Kit, version 1.1 (Applied Biosystems, Foster City, CA). Products of sequence reactions were analysed on a Genetic Analyzer 3730 (Applied Biosystems, Foster City, CA). Sequencing chromatograms were examined by the use of the Sequencer package (GeneCodes Co, Ann Arbor, MI).

**Statistical analyses**

The association of maternal and neonatal SNPs and haplotypes with mRNA expression and pre-eclampsia was examined using linear and logistic regression models, respectively. For individual SNP analyses we also explored the association between combinations of maternal and neonatal alleles. For the mRNA expression analyses, diagnosis of HELLP syndrome, corticosteroid administration and gestational age at delivery were included as covariates.

Haplotype frequencies and effects on pre-eclampsia and mRNA expression were estimated using the ‘haplo.stats’ package. For the haplotypes, we assumed an additive effect. Risk alleles were defined as those different from alleles that constitute the most common haplotype. Odds ratios (ORs) with 95% confidence intervals (CI) were calculated as an estimate of the relative risk for pre-eclampsia for each SNP or haplotype relative to the reference category (i.e. non-risk alleles for SNPs and the haplotype consisting of all non-risk alleles for haplotypes). Two-sided probability values of <0.05 were considered statistically significant. Individual SNP analyses were corrected for the number of independent tests based on linkage disequilibrium patterns, with a Bonferroni corrected p-value of 0.05/4, based on 4 independent SNPs.

Statistical analyses were performed using the R statistical package version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria).

**Results**

Of the 287 pregnancies, we excluded multiple pregnancies, pregnancies complicated by hypertensive disorders that did not meet pre-eclampsia criteria, as well as those with insufficient yield or quality of either maternal genomic DNA, umbilical cord blood genomic DNA or placental mRNA, or had incomplete coverage of all SNPs in the genomic PCR. A full dataset of 34 pre-eclampsia cases and 146 normotensive controls was available for analysis. The characteristics of the study population and their neonates are listed in **Table I**.

**Table I - Characteristics of the study population.**

<table>
<thead>
<tr>
<th>Clinical parameters</th>
<th>Pre-eclampsia cases (n=34)</th>
<th>Normotensive controls (n=146)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diagnosis (no. (%))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PE only</td>
<td>25 (74)</td>
<td>-</td>
</tr>
<tr>
<td>PE and HELLP</td>
<td>9 (26)</td>
<td>-</td>
</tr>
<tr>
<td>Mode of delivery (no. (%))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vaginal delivery</td>
<td>14 (41)</td>
<td>119 (82)</td>
</tr>
<tr>
<td>Primary cesarean delivery</td>
<td>16 (47)</td>
<td>5 (3)</td>
</tr>
<tr>
<td>Secondary cesarean delivery</td>
<td>4 (12)</td>
<td>22 (15)</td>
</tr>
<tr>
<td>Age at time of delivery in years (mean (sd))</td>
<td>31.3 (5.3)</td>
<td>29.7 (5.8)</td>
</tr>
<tr>
<td>Gestational age at delivery in weeks (median (range))</td>
<td>34.1 (27.2 - 40.6)</td>
<td>39.5 (25.4 - 43.1)</td>
</tr>
<tr>
<td>Highest diastolic BP in mm Hg (median (range))*</td>
<td>110 (90-185)</td>
<td>73 (50-95)</td>
</tr>
</tbody>
</table>

PE, pre-eclampsia; HELLP, syndrome of hemolysis, elevated liver enzymes and low platelets; BP, blood pressure; sd, standard deviation. *highest diastolic blood pressure was available for all pre-eclampsia cases and for 128 normotensive controls (88%).
Sequencing of the ANXA5 promoter/exon1 showed the presence of six common polymorphisms (TABLE II and FIGURE I), i.e. SNP1 (rs62319820, c.-390>c), SNP2 (rs112782763, c.-229>G>A), SNP3 (rs28717001, c.-210>A>C), SNP4 (rs28651243, c.-184>T>C), SNP5 (rs113588187, g.-135>G>A) and SNP6 (rs1050606, c.-64>T>G), that all have previously been reported. Both maternal and neonatal genotype frequencies of the common ANXA5 SNPs were in Hardy-Weinberg equilibrium in normotensive controls (TABLE SI).

### TABLE II - ANXA5 promoter polymorphisms, risk of pre-eclampsia and association with placental ANXA5 mRNA levels.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Maternal genotype</th>
<th>Neonatal genotype</th>
<th>OR for pre-eclampsia (95% CI)</th>
<th>ANXA5 mRNA expression mean (95% CI)</th>
<th>OR for pre-eclampsia (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNP1 c.-390&gt;T</td>
<td>CC</td>
<td>1.28 (1.07 - 1.53)</td>
<td>12.8 (12.2 - 13.5)</td>
<td>0.67 (0.24 - 1.87)</td>
<td>0.64 (0.23 - 1.80)</td>
</tr>
<tr>
<td></td>
<td>CT</td>
<td>1.49 (1.35 - 1.65)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.61 (0.23 - 1.68)</td>
<td>0.64 (0.23 - 1.80)</td>
</tr>
<tr>
<td>SNP2 c.-229&gt;G</td>
<td>TT</td>
<td>1.35 (1.25 - 1.48)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.79 (0.33 - 1.90)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td></td>
<td>GA</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td>SNP3 c.-210&gt;A</td>
<td>AA</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td></td>
<td>AC</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td>SNP4 c.-184&gt;T</td>
<td>CC</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td>SNP5 c.-135&gt;G</td>
<td>TT</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
<tr>
<td>SNP6 c.-64&gt;T</td>
<td>GG</td>
<td>1.31 (1.20 - 1.40)</td>
<td>12.9 (12.2 - 13.5)</td>
<td>0.77 (0.32 - 1.78)</td>
<td>0.62 (0.24 - 1.62)</td>
</tr>
</tbody>
</table>

When considering SNPs independently, none of the maternal SNPs were associated with mRNA expression levels (TABLE II). The T-allele of neonatal SNP1 was associated with higher placental ANXA5 mRNA expression (p = 0.028 for heterozygous CT and p = 0.14 for homozygous TT carriers compared to homozygous CC carriers; genotype counts are reported in TABLE SI). There was no interaction between maternal and neonatal SNPs (data not shown).

Haplotype analysis revealed 6 common haplotypes with a frequency above 1%. Haplotype analyses showed no association between maternal haplotypes and mRNA expression (TABLE III). The AGATGG (h2) haplotype in the neonatal promoter was associated with decreased mRNA expression (beta -0.92, 95% CI -1.83 - -0.01), whereas the TGCAGG (h4) haplotype (the only haplotype including the T-allele of SNP1) was associated with increased mRNA expression (beta 1.81, 95% CI 0.50 – 3.12). When limiting the haplotype analyses to haplotypes containing only SNP2 through SNP5 no association with mRNA expression was found (TABLE III).
### Table III - Association of \( \text{anxa5} \) promoter haplotypes with preeclampsia.

<table>
<thead>
<tr>
<th>Alleles</th>
<th>Phenotype</th>
<th>n</th>
<th>M/M</th>
<th>M/m</th>
<th>m/m</th>
<th>rAF</th>
<th>M/M</th>
<th>M/m</th>
<th>m/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>SN1 c.-390C&gt;T</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>28</td>
<td>5</td>
<td>1</td>
<td>29</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>controls</td>
<td>146</td>
<td>112</td>
<td>30</td>
<td>4</td>
<td>0.13</td>
<td>112</td>
<td>30</td>
<td>4</td>
</tr>
<tr>
<td>SN2 c.-229G&gt;A</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>28</td>
<td>8</td>
<td>0</td>
<td>28</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>146</td>
<td>103</td>
<td>40</td>
<td>3</td>
<td>0.14</td>
<td>107</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>SN3 c.-210A&gt;C</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>17</td>
<td>15</td>
<td>2</td>
<td>22</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>146</td>
<td>68</td>
<td>64</td>
<td>14</td>
<td>0.29</td>
<td>74</td>
<td>59</td>
<td>13</td>
</tr>
<tr>
<td>SN4 c.-184T&gt;C</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>17</td>
<td>15</td>
<td>2</td>
<td>22</td>
<td>9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>146</td>
<td>68</td>
<td>64</td>
<td>14</td>
<td>0.29</td>
<td>74</td>
<td>59</td>
<td>13</td>
</tr>
<tr>
<td>SN5 c.-135G&gt;A</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>27</td>
<td>7</td>
<td>0</td>
<td>28</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>146</td>
<td>103</td>
<td>40</td>
<td>3</td>
<td>0.14</td>
<td>107</td>
<td>37</td>
<td>2</td>
</tr>
<tr>
<td>SN6 c.-64T&gt;G</td>
<td>Pre-eclampsia</td>
<td>34</td>
<td>9</td>
<td>17</td>
<td>8</td>
<td>11</td>
<td>12</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Controls</td>
<td>146</td>
<td>24</td>
<td>70</td>
<td>52</td>
<td>0.57</td>
<td>28</td>
<td>69</td>
<td>49</td>
</tr>
</tbody>
</table>

**Notes:**
- Bold and underlined alleles indicate risk alleles.
- Haplotypes are compared to haplotype h1; or.
- Odds ratios cannot be calculated for the haplotype in cases the odds ratio for it cannot be calculated. *p<0.05.
ANXA5 mRNA expression and pre-eclampsia risk

No association between placental mRNA expression and pre-eclampsia was found ($p=0.87$, **FIGURE II**). After correction for co-diagnosis of HELLP syndrome, corticosteroid use and gestational age at delivery, no relation between mRNA expression and pre-eclampsia was observed ($p=0.87$).

**FIGURE II** - ANXA5 mRNA expression in pre-eclampsia cases and normotensive controls.

![Bar chart showing normalized ANXA5 mRNA expression](chart.png)

Mean mRNA expression in pre-eclampsia cases 13.2 (95% CI 8.6 – 17.8), in normotensive controls 13.4 (9.4 – 17.4). ANXA5 copy numbers were normalized to PSMD4 copy numbers. Error-bars indicate standard deviation.

ANXA5 SNPs or haplotypes and pre-eclampsia risk

Both for maternal and neonatal genotypes, none of the 6 SNPs were individually associated with an increased risk of pre-eclampsia (**TABLE II**). Also, when maternal/neonatal genotype combinations were considered, no association between alleles and pre-eclampsia was found. None of the maternal or neonatal haplotypes was associated with an increased risk of pre-eclampsia (**TABLE III**). Moreover, when only SNPs through 5 were included in haplotype analyses, the haplotype comprising the four risk alleles which are characteristic of the $M2$ haplotype was not associated with an increased risk of pre-eclampsia when compared to the common haplotype of non-risk alleles only (**TABLE III**).

Discussion

In this study, we did not observe an association between maternal or neonatal ANXA5 promoter genotypes and pre-eclampsia, neither for individual SNPs, nor for haplotypes. The previously reported association between the $M2$ haplotype in neonates and pre-eclampsia was not confirmed in this study. Furthermore, we observed no relationship between placental ANXA5 mRNA expression and pre-eclampsia.

The lack of association between maternal ANXA5 variants and pre-eclampsia is consistent with two previous studies. Although the first study found an association between the $M2$ haplotype and pregnancy-related hypertensive disorders (OR 2.1, 95% CI 1.2 – 3.5), this association was weaker and no longer statistically significant when only pre-eclampsia patients were considered (OR 1.5, 95% CI 0.8 – 2.8). The second study found no association between maternal gene variants and pre-eclampsia. In the present study we could not confirm the association between placental $M2$ and pre-eclampsia previously reported in the Japanese population. This may be explained by a less ethnically homogeneous study population in our study, as the frequency of the $M2$ haplotype carriers in controls was substantially lower in the Japanese population.
Our study indicates that the T-allele of SNP1 (c.-390 C>T) in the neonatal genotype was associated with increased mRNA expression. The only haplotype which contains this SNP (haplotype H4, TCCCCG) was also associated with increased mRNA expression. Additionally, the H2 haplotype (CGATGG) in the neonatal promoter was associated with decreased mRNA expression. These findings are in line with two recent studies that showed increased plasma levels of Annexin A5 in carriers of the H4 haplotype, and decreased levels in H2 carriers.24-25 We can therefore hypothesize that both mRNA expression and protein levels are affected by these haplotypes directly, but a correlation between ANXA5 mRNA expression and protein level in the placenta has not yet been demonstrated.24-26 Furthermore the clinical relevance of increased or decreased placental Annexin A5 is not fully elucidated in humans, as some studies describe decreased mRNA and protein levels in pre-eclamptic placentas,25,27 whereas others report increased levels.28 Several aspects of our study warrant comment. Although the sample size was moderate, we believe that the current approach, including data of maternal genomic DNA, umbilical cord blood genomic DNA and placental mRNA of pre-eclampsia patients as well as controls, provides valuable and unique information. As the placenta is fetal tissue, a putative relation with respect to ANXA5 placental expression with a haplotype could be expected to exist preferentially with the fetal, and not with the maternal haplotype. A limitation is that ethnicity data were not available for all study participants, and hence, cases and controls could not be matched for this. Finally, the present study does not provide information on the Annexin A5 protein level in the placenta and potential post-transcriptional modification and gene-gene interactions.

In conclusion, we did not observe a relationship between maternal or neonatal ANXA5 variants with pre-eclampsia, and no association between ANXA5 mRNA expression and pre-eclampsia was found.

Acknowledgements

We would like to thank all patients who gave informed consent for the study. Furthermore, we would like to acknowledge Truus Veenboer for her help with sample collection.

References

Chapter 10

Polymorphisms in the ANXA5 promoter region and recurrent miscarriage

Abstract

Annexin A5 is an anticoagulant protein, abundantly present on placental villi. Four common haplotypes (h1-4) in the ANXA5 gene have been described. The haplotype h3 is associated with recurrent miscarriage in multiple studies, potentially via reduced Annexin A5 protein levels. However, various studies show inconsistent results.

In the present study we investigated the association of the ANXA5 haplotypes h1-h4 with recurrent miscarriage in Dutch women in a case control study. Next, in a post-hoc analysis of the ALIFE study, we assessed whether carriers of these haplotypes benefit from antithrombotic therapy in a subsequent pregnancy.

DNA samples of 233 women with recurrent miscarriage, who participated in the ALIFE study and of 1819 population controls were included. Four SNPs (rs62319820, rs113388187, rs1050606 and rs1131239) were used to construct the four haplotypes. The associations with recurrent miscarriage and live birth during were analyzed using logistic regression models.

Haplotypes h2-h4 were not associated with recurrent miscarriage if compared to haplotype h1 (OR 1.01, 95% CI 0.81 – 1.26 for h2, OR 1.14, 95% CI 0.83 – 1.56 for h3 and OR 1.17, 95% CI 0.82 – 1.68 for h4) . Carriers of haplotypes h2 and h4 appeared to have an increased chance of live birth during the ALIFE study, when adjusted for prognostic variables (OR 1.79, 95% CI 1.11 – 2.87 and OR 1.55, 95% CI 0.78 – 3.09 respectively). Women who were homozygous for the h1 haplotype appeared to benefit from treatment with LMWH plus ASA, whereas h1h2, h1h3 and h1h4 carriers did not.

In conclusion, ANXA5 haplotypes are not associated with recurrent miscarriage in the Dutch population. Whether women with certain ANXA5 haplotypes may benefit from antithrombotic treatment requires confirmation in other cohorts.

Introduction

Annexin A5 is a protein with anticoagulant properties. It binds to negatively charged phospholipids, where it forms a crystallized shield, thus blocking the surface from phospholipid-dependent coagulation. Annexin A5 is abundantly present on vascular endothelial cells as well as on the apical membrane of trophoblast cells in the placenta. Disruption of this Annexin A5 shield by antiphospholipid antibodies exposes these thrombogenic
phospholipids, and thereby induces hypercoagulability.\textsuperscript{145} This concept is supported by the fact that Annexin A5 on placental villi is reduced in women with a history of pregnancy loss and antiphospholipid antibodies.\textsuperscript{6} Women with a history of recurrent miscarriage without antiphospholipid antibodies also have lower plasma levels of Annexin A5 compared to controls.\textsuperscript{7} Genetic variants in the \textit{ANXA5} gene that presumably lead to lower levels of Annexin A5 have been proposed as a risk factor for recurrent miscarriage.\textsuperscript{8} Several studies have shown a higher prevalence of a haplotype consisting of four single nucleotide polymorphisms (SNPs) in the \textit{ANXA5} promoter region (m2 haplotype) in women with recurrent miscarriage compared to controls.\textsuperscript{9-13} Other studies, however, found no difference.\textsuperscript{14-16}

Recently four common haplotypes (h1-h4) in the \textit{ANXA5} promoter were defined, which are extensions of the M classification in the previously mentioned studies.\textsuperscript{17-19} In the present study we investigated the association of the \textit{ANXA5} haplotypes h1-h4 with recurrent miscarriage in Dutch women. Next, we assessed whether carriers of these haplotypes benefit from antithrombotic therapy in a subsequent pregnancy.

**Methods**

**Study design**

To evaluate whether \textit{ANXA5} haplotypes are associated with recurrent miscarriage, we performed a case control study, in which we compared the prevalence of the various haplotypes in women with recurrent miscarriage and in a Dutch control population. To assess whether these haplotypes affect the chance of a subsequent live birth or the efficacy of antithrombotic therapy we performed a post-hoc exploratory analysis of the \textit{ALIFE} trial.\textsuperscript{20} The \textit{ALIFE} study was a randomized controlled trial that investigated the effect of either low-molecular-weight heparin (LMWH) plus acetylsalicylic acid (ASA), ASA alone or placebo on live birth.

**Study population**

For the case control study we compared women with recurrent miscarriage who had participated in the \textit{ALIFE} study (ISRCTN 58496168)\textsuperscript{21} to population controls. The main inclusion criterion for the \textit{ALIFE} study was unexplained recurrent miscarriage, defined as two or more miscarriages prior to 20 weeks’ gestation in the absence of abnormal parental karyotype, lupus anticoagulant or anti-cardiolipin IgG and IgM antibodies, uterine anomalies and an abnormal fasting level of homocysteine. Controls were individuals who had been included in the Nijmegen Biomedical Study (NBS), of which details were reported previously.\textsuperscript{22} Briefly, the NBS is a population-based survey conducted by the Department for Health Evidence and the Department of Laboratory Medicine of the Radboud university medical center. 21,756 age- and sex-stratified randomly selected inhabitants of the municipality of Nijmegen in the eastern part of the Netherlands, received an invitation to fill out a postal questionnaire on lifestyle and medical history, and to donate blood samples. The response to the questionnaire was 43% (n = 9350). 69% (n = 6468) of the responders donated blood samples. In 2007, 1980 samples were used as controls for genome-wide association studies for prostate and breast cancer, of which 1819 passed genome-wide quality control criteria. These were used for the present case control study.\textsuperscript{22} Written informed consent was obtained from all \textit{ALIFE} and NBS participants. Ethical approval was obtained for the \textit{ALIFE} study, the NBS study and the present study.

**Genetic analysis**

Genomic DNA was isolated from peripheral blood cells. In samples of \textit{ALIFE} participants TaqMan primers with FAM or VIC as fluorophores (Applied Biosystems, USA) were used for genotyping. For controls, genotype data (Illumina HumanHapCNV370-Duo BeadChip) were available for those 1980 NBS participants that were selected to serve as controls in Gwas.\textsuperscript{23} A total of 1819 samples passed quality control [sample yield ≥96% (after exclusion of intensity-only markers (n = 23,573)), Caucasian ancestry ≥90% (based on Structure analysis), SNP yield ≥96%]. Genome-wide SNP data were available for these 1819 NBS participants. SNP quality control [minor allele frequency (MAF) ≥1%, and Hardy-Weinberg equilibrium (HWE) p-value >10-6] resulted in availability of 323,414 SNPs. Density was increased by imputation, which was performed with 1000genomes phase1 integrated version 3 as a reference sample using \texttt{IMPUTE v2} software.

In the \textit{ANXA5} promoter region four common haplotypes (h1, h2, h3 and h4) are present.\textsuperscript{17-19} We used four SNPs (rs62319820, rs113588187, rs1050606 and rs1131239, \textbf{Figure 1}) to construct these four haplotypes based on previously published linkage disequilibrium patterns.\textsuperscript{17-19}
dominant model), or a specific combination of haplotypes. The effect of intervention was evaluated with logistic regression modelling. Differences in live birth rates between interventions were expressed as OR with 95% CIs for LMWH plus ASA and ASA alone, with the placebo group as reference. Statistical analyses were performed using the R statistical package version 3.1.1 (R Foundation for Statistical Computing, Vienna, Austria). Two-sided probability values of <0.05 were considered statistically significant.

Results

DNA samples of 235 of 364 (65%) ALIFE study participants were available. In two cases, PCR was unsuccessful for all four SNPs. Of the remaining 233 women, 32 did not become pregnant during the trial. Characteristics of the 233 ALIFE participants with PCR results are summarized in Table I.

Table I - Characteristics of the study population – women with recurrent miscarriage.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (n=233)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years) - mean (SD)</td>
<td>33.8 (4.6)</td>
</tr>
<tr>
<td>≥36 yr – no, (%)</td>
<td>86 (36.9)</td>
</tr>
<tr>
<td>Body mass index (kg/m²) - mean (SD) *</td>
<td>25.3 (4.7)</td>
</tr>
<tr>
<td>Inherited thrombophilia - n (%)</td>
<td>28 (16.3)</td>
</tr>
<tr>
<td>Factor V Leiden</td>
<td>17 (7.3)</td>
</tr>
<tr>
<td>Prothrombin mutation</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Protein S deficiency</td>
<td>13 (5.8)</td>
</tr>
<tr>
<td>Protein C deficiency</td>
<td>5 (2.1)</td>
</tr>
<tr>
<td>Antithrombin deficiency</td>
<td>3 (1.3)</td>
</tr>
<tr>
<td>Pregnancies</td>
<td></td>
</tr>
<tr>
<td>Number of previous miscarriages - median [range] *</td>
<td>2 [1-12]</td>
</tr>
<tr>
<td>Women with 2 previous miscarriages - n (%)</td>
<td>94 (36.1)</td>
</tr>
<tr>
<td>Women with &gt;2 previous miscarriages - n (%)</td>
<td>149 (63.9)</td>
</tr>
<tr>
<td>Women who had a previous live birth - n (%)</td>
<td>92 (39.5)</td>
</tr>
<tr>
<td>Live birth during the ALIFE study - n (%)</td>
<td>133 (57.1)</td>
</tr>
<tr>
<td>Did not become pregnant during the ALIFE study - n (%)</td>
<td>32 (13.7)</td>
</tr>
</tbody>
</table>

a: BMI data were available for 77 LMWH plus ASA, 71 ASA alone and 76 placebo women. b: A full inherited thrombophilia screen was available for 70 LMWH plus ASA, 68 ASA alone and 66 placebo women. c: 4 women were included early in the ALIFE study because of 1 miscarriage <20 weeks’ gestation and 1 intra-uterine foetal death. Thereafter, the study protocol was amended, limiting the inclusion criteria to 2 miscarriages before 20 weeks’ gestation.
**ANXA5 haplotypes and SNPs and association with recurrent miscarriage**

Genotyping success rates for the four SNPs were 98% (rs62319820), 99% (rs113588187), 99% (SNP rs1050606) and 99% (rs1131239) in women with recurrent miscarriage (TABLE S1). Haplotype analysis revealed that only the previously described four common haplotypes (H1-H4) were present with a frequency >1% (TABLE II).

The frequencies of the haplotypes were similar amongst women with recurrent miscarriage and controls, as shown in TABLE II. The H3 haplotype, which is an elongation of the M2 haplotype, was not associated with recurrent miscarriage (OR 1.14, 95% CI 0.83 – 1.56).

Results of the individual SNP analyses are summarized in TABLE S1. Women who were homozygous TT for SNP1 (g.-390 C>T) had an increased probability of recurrent miscarriage (OR 4.45 95% CI 1.48 – 13.41).

---

### TABLE S1 - ANXA5 promoter polymorphisms, allele frequencies and association with recurrent miscarriage.

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Recurrent miscarriage</th>
<th>NBS controls</th>
<th>Non-additive</th>
<th>Additive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n=233</td>
<td>n=1819</td>
<td>OR (95% CI)</td>
<td>OR (95% CI)</td>
</tr>
<tr>
<td>5'NF 1</td>
<td>g.-390 C&gt;G</td>
<td>CC 193 (82.8)</td>
<td>1545 (84.9)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT 32 (13.7)</td>
<td>265 (14.6)</td>
<td>0.97 (0.65 – 1.44)</td>
<td>1.19 (0.85 – 1.67)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT 5 (2.1)</td>
<td>9 (0.5)</td>
<td>4.65 (1.48 – 13.41)</td>
<td></td>
</tr>
<tr>
<td>5'NF 2</td>
<td>g.-1350 G&gt;A</td>
<td>GG 177 (76.0)</td>
<td>1440 (79.2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GA 52 (22.3)</td>
<td>356 (19.6)</td>
<td>1.19 (0.95 – 1.65)</td>
<td>1.15 (0.86 – 1.54)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AA 3 (1.3)</td>
<td>23 (1.2)</td>
<td>1.06 (0.32 – 3.57)</td>
<td></td>
</tr>
<tr>
<td>5'NF 6</td>
<td>g.-641 A&gt;G</td>
<td>TT 53 (23.7)</td>
<td>422 (23.2)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TG 116 (48.5)</td>
<td>929 (51.1)</td>
<td>0.97 (0.69 – 1.37)</td>
<td>1.07 (0.88 – 1.30)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GG 66 (28.3)</td>
<td>467 (25.6)</td>
<td>1.13 (0.72 – 1.66)</td>
<td></td>
</tr>
<tr>
<td>5'NF 7</td>
<td>g.-1 C&gt;T</td>
<td>CC 177 (76.0)</td>
<td>1437 (79.3)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CT 51 (21.9)</td>
<td>359 (19.7)</td>
<td>1.15 (0.82 – 1.61)</td>
<td>1.16 (0.87 – 1.56)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TT 4 (1.7)</td>
<td>23 (1.2)</td>
<td>1.41 (0.48 – 4.13)</td>
<td></td>
</tr>
</tbody>
</table>

Genotypes were available for all NBS controls and for 230 (SNP1) and 232 (SNP5, SNP6 and SNP7) cases. All SNPs were in Hardy-Weinberg equilibrium for NBS controls. *p=0.008 compared to CC alleles. **p=0.032 after Bonferroni correction for multiple testing. Reported 95% CIs are crude, i.e. not corrected for multiple testing. SNP, single nucleotide polymorphism; OR, odds ratio; CI, confidence interval. Bold and underlined alleles indicate risk alleles.

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**ANXA5 haplotypes and SNPs and chance of live birth**

Results of exploratory subgroup analyses for intervention are summarized in TABLE III. A trend towards a beneficial effect in H3 haplotype carriers was observed for both LMWH plus ASA and ASA alone (OR 2.16, 95% CI 0.51 – 9.25 and OR 2.03 95% CI 0.44 – 9.32 respectively) when analyzed in a dominant model (TABLE III-A). These results did not materially change when only women who became pregnant were considered (OR 2.59, 95% CI 0.49 – 13.69 and OR 1.52 95% CI 0.31 – 7.49 respectively). This benefit was not evident in women carrying combinations of H1 and H3 alleles (H1H3, OR 0.85, 95% CI 1.10 – 7.49, TABLE III-B). Other H3 subgroups (H2H3, H3H3 or H3H4) were too small to differentiate between effects of antithrombotic therapy for haplotype allele combinations (TABLE III-B).

Women carrying the homozygous H1H1 combination (TABLE III-B) appeared to benefit from LMWH plus ASA. In carriers of at least one H1 copy we observed a trend towards a beneficial effect of treatment with LMWH plus ASA (OR 1.55 95% CI 0.71 – 3.40 in all women [TABLE III-A] and OR 2.31, 95% CI 0.94 – 5.66 in women who became pregnant). We observed no interaction between haplotypes and intervention (p_{interaction}=0.22).
Haplotype h2 was associated with a higher chance of live birth in the ALIFE trial (OR 1.79, 95% CI 1.11 – 2.87) compared to the reference haplotype (h1) when corrected for the number of previous miscarriages, previous live birth, age, presence of inherited thrombophilia and trial intervention. A similar effect was observed for haplotype h4, but this did not reach statistical significance (OR 1.55 95% CI 0.78 – 3.09) (TABLE SII). This did not materially differ when women who did not become pregnant during the trial were excluded (OR 1.72 95% CI 1.01 – 2.90 and OR 1.82, 95% CI 0.79 – 4.18 respectively). None of the individual SNPs were associated with live birth during the ALIFE study when analyzed individually (data not shown).

TABLE SII - ANXA5 haplotypes and the chance of live birth in women with recurrent miscarriage when treated with antithrombotic agents: data from post-hoc analyses in the ALIFE study.

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>Crude model OR for live birth (95% CI)</th>
<th>Adjusted model OR for live birth (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All women</td>
<td>Women who became pregnant</td>
<td>All women</td>
</tr>
<tr>
<td>n=233</td>
<td>n=201</td>
<td>n=233</td>
</tr>
<tr>
<td>Haplotype H1 (GG/TC)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Haplotype H2 (GG/GG)</td>
<td>1.49 (0.96 – 2.21)</td>
<td>1.44 (0.91 – 2.43)</td>
</tr>
<tr>
<td>Haplotype H3 (GG/GA)</td>
<td>1.01 (0.55 – 1.85)</td>
<td>0.89 (0.46 – 1.73)</td>
</tr>
<tr>
<td>Haplotype H4 (TG/CG)</td>
<td>1.37 (0.69 – 2.69)</td>
<td>1.50 (0.85 – 2.65)</td>
</tr>
</tbody>
</table>

i corrected for: number of previous miscarriages (two versus three or more) previous live birth (yes versus no), age (<36 versus ≥36 years), inherited thrombophilia (yes versus no) and trial intervention (LMWH plus ASA or ASA alone versus placebo).

TABLE III - Odds ratio for live birth in women with recurrent miscarriage stratified for intervention and ANXA5 haplotypes.

A. Stratified for haplotype carriers (dominant model)

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>n=233</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWH + ASA</td>
<td>ASA</td>
</tr>
<tr>
<td>Haplotype H1</td>
<td>1.55 (0.71 – 3.40)</td>
</tr>
<tr>
<td>Haplotype H2</td>
<td>0.79 (1.29 – 1.29)</td>
</tr>
<tr>
<td>Haplotype H3</td>
<td>2.16 (0.51 – 8.25)</td>
</tr>
<tr>
<td>Haplotype H4</td>
<td>0.42 (0.12 – 1.57)</td>
</tr>
</tbody>
</table>

B. Stratified for haplotype allele combination

<table>
<thead>
<tr>
<th>Haplotype</th>
<th>n=233</th>
</tr>
</thead>
<tbody>
<tr>
<td>LMWH + ASA</td>
<td>ASA</td>
</tr>
<tr>
<td>Haplotype H1H1</td>
<td>4.81 (0.83 – 27.78)</td>
</tr>
<tr>
<td>Haplotype H1H2</td>
<td>0.60 (0.14 – 2.62)</td>
</tr>
<tr>
<td>Haplotype H1H3</td>
<td>0.85 (1.10 – 7.49)</td>
</tr>
<tr>
<td>Haplotype H1H4</td>
<td>1.46 (0.01 – 143)</td>
</tr>
<tr>
<td>Haplotype H2H2</td>
<td>0.46 (0.02 – 12.90)</td>
</tr>
<tr>
<td>Haplotype H2H3</td>
<td>N.A.</td>
</tr>
<tr>
<td>Haplotype H2H4</td>
<td>N.A.</td>
</tr>
<tr>
<td>Haplotype H3H3</td>
<td>N.A.</td>
</tr>
<tr>
<td>Haplotype H3H4</td>
<td>N.A.</td>
</tr>
<tr>
<td>Haplotype H4H4</td>
<td>N.A.</td>
</tr>
</tbody>
</table>

Analyses are corrected for: number of previous miscarriages (two versus three or more) previous live birth (yes versus no), age (<36 versus ≥36 years), inherited thrombophilia (yes versus no). iii-A. Odds ratios for live birth are analyzed in a dominant model: efficacy of treatment for carriers of at least one copy of the haplotype (h1, h2, h3 or h4) was analyzed. iii-B. Odds ratios for live birth per combination of haplotype alleles: efficacy of treatment for carriers of haplotype allele combinations was analyzed. OR, odds ratio; 95% CI, 95% confidence interval; LMWH, low-molecular-weight heparin; ASA, acetylsalicylic acid; N.A., not applicable (numbers too small).
Discussion

In the present study, we did not observe an association between ANXA5 haplotypes and recurrent miscarriage. To our knowledge, nine previous studies reported on the association between ANXA5 polymorphisms, located in the promoter region, and recurrent miscarriage. Eight of these studies evaluated the role of the m2 haplotype. The m2 haplotype consists of four SNPs (SNP2 through SNP5), which, as shown in previously described linkage disequilibrium patterns, constitute haplotype H3, together with SNP1, SNP6 and SNP7 (Figure 1). Six studies reported an increased probability of recurrent miscarriage in M2 carriers, with ORs ranging from 1.5 to 3.1. The seventh and eighth study did not confirm an increased probability of recurrent miscarriage for M2 carriers. Lastly, the ninth study, which only investigated the role of SNP5 (which is one of the four SNPs that make up the M2 haplotype and the SNP indicative for H3) also showed no association with recurrent miscarriage (OR 0.84, 95% CI 0.47 – 1.52). Results of the present study, indicating no increased probability of recurrent miscarriage in women carrying the H3 haplotype, are in line with these latter three studies.

How can these differences between study results be explained? Study populations varied regarding inclusion criteria (mainly the number and timing of miscarriages and comorbidity), but also regarding geographical region, and therefore likely in ethnicity. Furthermore, four of the six studies that reported an association with the M2 haplotype used the same control group from the PopGen biobank in Kiel, Germany.

Although no association between ANXA5 haplotypes and recurrent miscarriage was observed, haplotypes H2 and H4 appear to increase the probability of live birth in a subsequent pregnancy, when compared to haplotype H1. A trend towards a beneficial effect of LMWH plus ASA and ASA alone in women carrying the H3 haplotype was observed. Furthermore, amongst H1 carriers, women with H1H1 haplotypes appear to benefit from LMWH plus ASA, whereas women who carry one H1 copy and one copy of either H2, H3 or H4, do not seem to benefit from this treatment. It was anticipated that these subgroup analyses were underpowered, analyzed post-hoc, and therefore results should be interpreted with caution.

Acknowledgements

We acknowledge Jan van Straalen, Belia Rekké, Kobie Los and Adrien Groot for their help collecting the data and Alinda Schimmel and Hans Jansen for the PCR analyses.
References


Chapter 11

Summary and future perspectives
Summary

The research presented in this thesis addresses several aspects of the association between inherited thrombophilia and pregnancy complications. Antithrombotic therapy is prescribed to women with recurrent miscarriage and antiphospholipid syndrome to increase their chance of live birth in a subsequent pregnancy. Whether women with recurrent miscarriage and inherited thrombophilia also benefit from this therapy is unknown. In Part I of this thesis, we provide an overview of what is currently known on the use of antithrombotic therapy for this indication, we review the available evidence and we present two studies in which this therapy is evaluated.

Part I

In Chapter 2 we review the literature on the strength of the association between thrombophilia and pregnancy loss, we discuss what is known on the pathophysiological mechanisms behind this association and we evaluate the efficacy of antithrombotic therapy to increase the chance of live birth. Differences between international guidelines and consensus statements on this topic reflect the lack of knowledge and available evidence in this field. Several explanations may play a role. First, the association between thrombophilia and pregnancy loss varies per type of thrombophilia, (e.g. acquired, as in the antiphospholipid syndrome or inherited, (i.e. Factor V Leiden or prothrombin G20210A mutation, or deficiency of protein C, protein S or antithrombin)) and for type and timing of pregnancy loss (e.g. recurrent early loss before 12 weeks’ gestation or single loss in the third trimester). Second, for both inherited thrombophilia and antiphospholipid syndrome the exact pathophysiological mechanism is unclear. And finally, when summarizing the literature on evidence for antithrombotic therapy to increase the chance of live birth we must conclude that more data is urgently needed. For women with unexplained recurrent miscarriage, substantial evidence that antithrombotic therapy does not increase the chance of live birth in a subsequent pregnancy has been obtained in the past decade. However, for women with antiphospholipid syndrome, studies have shown beneficial effects, but the numbers of included women were small. For women with inherited thrombophilia, no high quality evidence is available.
Several observational studies have evaluated the chance of live birth in the first pregnancy after diagnosis of recurrent miscarriage. However, when counselling couples with recurrent miscarriage, not only their chance of live birth in the next pregnancy, but also their long-term prognosis is relevant: will they eventually achieve a live birth, and at the cost of how many more miscarriages? How much time will it take to achieve a live birth?

In Chapter 3 a cohort study is presented in which the time to live birth after recurrent miscarriage is evaluated. Women who participated in the previously performed ALIFE study were contacted after a median follow-up duration of 7 years and data on pregnancies that had occurred after their participation in the ALIFE study were collected. The median time to a live birth was 19 months, and the cumulative probability of live birth, taking the competing risk of loss of fertility due to age (46 years old) into account, was 15%, 55%, 77% and 81% after 1, 2, 5 and 10 years respectively. The cumulative probability of live birth for women with three or more miscarriages was lower than for women with two miscarriages (HR 0.75, 95% CI 0.57 – 0.97). Interestingly, we found no difference between women with and without inherited thrombophilia.

To determine the efficacy of antithrombotic therapy in women with recurrent miscarriage with or without inherited thrombophilia we performed a Cochrane systematic review, which is described in Chapter 4. We searched for randomized and quasi-randomized controlled trials that assessed the effect of aspirin, unfractionated heparin (UFH), and low-molecular-weight heparin (LMWH) on live birth in women with a history of at least two unexplained miscarriages with or without inherited thrombophilia. Nine studies, including data of 1228 women, were included in the review. A quality assessment was performed and three studies were considered at a high risk of bias. In sensitivity analyses in which studies at high risk of bias were excluded, antithrombotic therapy did not have a beneficial effect on live birth, regardless of which anticoagulant was evaluated. Similar live birth rates were observed when comparing LMWH, with or without aspirin to no treatment, i.e. 72.5% and 71.3% (relative risk (RR) of live birth 0.98, 95% confidence interval [CI] 0.85 to 1.12). A trend towards a significant effect of LMWH when compared to aspirin (RR of live birth 1.21, 95% CI 0.79 to 1.87) and of LMWH and aspirin when compared to no treatment (RR of live birth 1.25, 95% CI 0.74 to 2.12) was observed in women with inherited thrombophilia but the subgroups were too underpowered to draw firm conclusions. This Cochrane systematic review was an update of a previously published version.1 Although the number of available studies is still limited, we can now conclude that antithrombotic therapy is not effective in women with unexplained recurrent miscarriage. For women with inherited thrombophilia and recurrent miscarriage, large randomized controlled trials are needed, and until results are available, anticoagulants to improve the chance of live birth in these women are also not recommended.

To study the efficacy of LMWH in women with recurrent miscarriage and inherited thrombophilia, we designed the ALIFE2 study, of which the protocol is described in Chapter 5A. Women with two or more miscarriages or intra-uterine fetal deaths and confirmed inherited thrombophilia (i.e. Factor V Leiden or prothrombin G20210A mutation, or deficiency of protein C, protein S or antithrombin) are eligible for the study. As soon as a urine pregnancy test is positive, but before a gestational age of 7 weeks they can be randomized to either LMWH or no intervention. The primary outcome is live birth, and secondary outcomes include efficacy (e.g. pre-eclampsia, placental abruption) and safety (e.g. bleeding, thrombocytopenia). This multicenter study is currently conducted in 11 participating centers in the Netherlands and Belgium and participation of other non-Dutch centers is expected to be followed closely by centers in the United Kingdom, the United States, Canada and Sweden.

To design and effectuate the ALIFE2 study proves to be challenging, as several hurdles in fields such as ethics requirements, legislation and funding have to be taken. These hurdles, and how they are taken are discussed in Chapter 5B.

Given the known association between inherited thrombophilia and recurrent miscarriage, thrombophilia testing is often performed. The test result may provide an explanation for the miscarriages, but it remains unknown whether it identifies women who may benefit from therapy. The pros and cons of thrombophilia testing are discussed in Chapter 6. First we summarize the odds ratios for individual thrombophilic defects for early miscarriage and late miscarriage, which vary from 1.40 to 6.25 and 1.31 to 20.09, respectively. Next, we discuss that only limited data are available on the use of antithrombotic therapy in women with recurrent miscarriage. None of the trials were sufficiently powered to demonstrate an effect of pharmacological therapy in subgroups of women with inherited thrombophilia. Furthermore, although the risk of significant bleeding is low (1.98% (95% CI, 1.50 to 2.57))2 antithrombotic therapy induces easy bruising (aspirin and LMWH) and swelling or itching at injection sites (LMWH), and is costly. Because it still remains to be established whether anticoagulant treatment...
will improve clinical outcome, the knowledge of a patients’ thrombophilic status should not alter clinical management, and testing for thrombophilia should therefore not be performed on a routine basis but only in the context of research and subsequent enrolment in controlled intervention trials.

Next to women with recurrent miscarriage, the efficacy of antithrombotic therapy is also evaluated for women with other pregnancy complications, including late pregnancy loss, pre-eclampsia, placental abruption and small-for-gestational-age newborns. Results from a meta-analysis suggest that LMWH reduces recurrence of these complications, but there is significant heterogeneity between studies. Chapter 7 describes the study protocol of the AFFIRM project. This is an individual patient data meta-analysis in which the combined data of nine trials will be used to evaluate the efficacy of LMWH in women with prior placenta-mediated pregnancy complications such as pre-eclampsia, placental abruption, birth of a small-for-gestational-age newborn, pregnancy loss after 16 weeks’ gestation or two losses after 12 weeks gestation. Randomized controlled trials with an LMWH intervention for the prevention of these complications were eligible, and identified in a systematic review. The primary outcome is a composite outcome including four pregnancy complications: early-onset or severe pre-eclampsia, birth of a small-for-gestational age newborn with a birth weight <5th percentile, placental abruption, and late pregnancy loss.

Part II

In Part II of this thesis we investigate whether gene variants in the Annexin A5 gene are associated with deep vein thrombosis (DVT), pre-eclampsia, or recurrent miscarriage. In Chapter 8 the prevalence of Annexin A5 genetic variants is compared between 148 patients with confirmed DVT and 267 controls who were suspected to have DVT, but in whom this was ruled out. Six SNPs were identified in the promoter region of the Annexin A5 gene and four common haplotypes (h1-h4) were constructed. The prevalence of individual SNPs and of common haplotypes was similar between patients and controls. These findings were confirmed when data of the patients were compared to a second independent control group of 1705 individuals from the general population. We therefore conclude that Annexin A5 SNPs or haplotypes do not contribute to DVT risk in the Dutch population.

Next, we investigated whether Annexin A5 promoter SNPs and haplotypes h1-h4 affect mRNA expression in the placenta or are associated with pre-eclampsia. This case-control study, in which maternal and neonatal DNA and placental mRNA expression of 34 pre-eclamptic pregnancies and 146 normotensive pregnancies were analyzed, is described in Chapter 9. The T-allele of rs62319820 (c.-390 c>t) in the neonatal promoter was associated with increased mRNA expression, but Annexin A5 mRNA expression levels were not associated with pre-eclampsia risk. Furthermore, neither individual SNPs nor any of the common haplotypes were associated with an increased risk of pre-eclampsia.

Finally, in Chapter 10, we investigated the association of the Annexin A5 haplotypes h1-h4 with recurrent miscarriage in Dutch women. DNA samples of 233 women with recurrent miscarriage who participated in the ALIFE study3 and of 1819 population controls were included. Haplotypes h2-h4 were not associated with recurrent miscarriage when compared to the reference haplotype h1 (OR 1.01, 95% CI 0.81 – 1.26 for h2, OR 1.14, 95% CI 0.83 – 1.56 for h3 and OR 1.17, 95% CI 0.82 – 1.68 for h4). Next, in a post-hoc analysis of the ALIFE study, we assessed whether carriers of these haplotypes benefit from antithrombotic therapy in a subsequent pregnancy. Carriers of haplotypes h2 and h4 appeared to have an increased chance of live birth during the ALIFE study, when adjusted for prognostic variables (OR 1.79, 95% CI 1.11 – 2.87 and OR 1.55, 95% CI 0.78 – 3.09 respectively), but subgroups are too small to draw firm conclusions. Women who were homozygous for the h1 haplotype appeared to benefit from treatment with LMWH plus acetylsalicylic acid, whereas h1h2, h1h3 and h1h4 carriers did not.

Perspectives

The ideal future of a couple who experienced recurrent miscarriage is a future with a successful pregnancy resulting in a healthy newborn. The future perspective from a scientific point of view is a natural result hereof; namely to better understand the pathophysiological mechanisms of recurrent miscarriage, and to find an evidence based therapy to increase the chance of live birth.
The association between recurrent miscarriage and inherited thrombophilia is generally accepted, but further epidemiological studies are needed to confirm this association and to evaluate the association per type of inherited thrombophilia. However, epidemiological studies will not explain why women with inherited thrombophilia are at a greater risk of recurrent miscarriage. The focus of future studies should therefore shift from epidemiology to translational research, investigating the pathophysiological mechanisms that underlie recurrent miscarriage. Bio-banking blood samples and placental- or endometrial tissue samples of women with recurrent miscarriage will greatly enable further research. An innovative in-vitro implantation model might allow us to investigate the implantation process. Experiments will offer a unique opportunity to investigate the interaction between endometrium and embryos. Women's biomaterials will be used in the search for prognostic biomarkers affecting embryo selectivity.

A previous assumption that the association between recurrent miscarriage and inherited thrombophilia could fully be explained by thrombosis of placental vasculature has been abandoned, but an alternative explanation is still missing. Animal studies support the concept that regulation and interaction of coagulation and inflammation may determine this association. Elucidating so far unknown pathophysiological mechanisms and identifying biomarkers that can be integrated with clinical markers will allow us to build clinical prediction models to predict reproductive outcome. Furthermore, these insights will provide a foundation for differentiated therapy. During the past decade, the number of studies evaluating antithrombotic therapy to improve pregnancy outcome in women with recurrent miscarriage has grown substantially. These studies have contributed to the current guidelines, which state that antithrombotic therapy is not recommended for women with unexplained recurrent miscarriage. The main message of this thesis is that for women with inherited thrombophilia and recurrent miscarriage, no evidence of a beneficial effect of antithrombotic therapy is available, notwithstanding physicians to prescribe LMWH or aspirin for this indication. For this reason we are currently conducting the ALIFE2 study, as highlighted in Chapter 5a and 5b. For this challenging international study, almost 400 women will be recruited during the coming years and randomized to either LMWH or no intervention, to compare its effects on live birth. This is a clinical trial, of which the results are anticipated to directly influence clinical practice.