The association between hereditary thrombophilias and pregnancy loss

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The correlation between hereditary thrombophilia and fetal loss is supported by several observations. In murine models, the protein C system has been shown to be essential for the maintenance of pregnancy, as it indirectly acts as a growth factor for trophoblast cells and protects them from apoptosis. In humans, it has been shown that the placenta replaces the yolk sac as an essential source of blood supply to the embryo during the 8th and 9th weeks of gestation. Furthermore, meta-analysis of epidemiological data demonstrates a correlation between thrombophilic polymorphisms such as factor V Leiden and prothrombin 20210G→A and isolated or recurrent fetal losses. Finally, therapeutic non-controlled trials indicate the benefits and safety of low-molecular weight heparins as secondary prophylaxis. However, it is still necessary to further clarify the association between thrombophilia and fetal loss, especially during a first pregnancy, with regard to the type of pregnancy loss and with respect to other related factors.

Key words: thrombophilia, pregnancy, miscarriages.

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Pregnancy loss is one of the leading problems in women's health issues - 9-13% of women in the reproductive age group experience one clinically recognized loss, 5% experience two or more losses, and 1 to 2% suffer three or more losses.[1-6] Although several medical causes have been established, up to 50% of cases of recurrent pregnancy loss, defined as two or more spontaneous abortions, still remain unexplained after standard gynecological, hormonal, and karyotypic investigations.

Pregnancy loss is a well-established complication of several acquired thrombophilic disorders, including the antiphospholipid/cofactor syndrome[7] and essential thrombocythemia.[8] In these cases, administration of antithrombotic therapies has been shown to facilitate successful pregnancies.[9,10] More recently, a number of case-control, cohort, and cross-sectional studies performed in women with poor pregnancy outcomes have shown that inherited hypercoagulable disorders that promote thrombosis, collectively termed inherited thrombophilia, may increase susceptibility to fetal loss. During the initial descriptions of thrombophilia-related clinical manifestations, which focused on thrombotic patients, or on families with constitutional thrombophilias, this association was not noted. However, in 1996, the first retrospective case-control study, based on a cohort of women from inherited thrombophilia-related thrombotic families, identified a significant risk for stillbirth in women with the most severe thrombophilias, i.e. combined defects or antithrombin deficiency.[11] The risk of miscarriage or stillbirth was not significant in women with the factor V Leiden mutation. Discrepancies between studies focused on women with pregnancy loss and those focused on thrombotic women carrying inherited thrombophilia explain in part why the available results have been considered to be conflicting with respect to the presence and magnitude of the association. An additional point added to the debate was the factor V Leiden mutation which, associated with reduced intrapartum blood loss in a published work[12] (although the method of loss assessment used was mainly subjective), had been claimed to induce an evolutionary advantage. It was difficult to imagine a protecting predisposition in mothers also favoring the interruption of their lineage. Evaluations of the epidemiological association, by meta-analysis of existing studies, have reinforced the link.[13-15] In addition, these studies have examined specific factors, including the timing and definition of

pregnancy loss. Here we would like to clarify the relationship between inherited thrombophilia and pregnancy loss, based on data from animal models, understanding of the evolution of human pregnancy, analysis of epidemiological studies, and the use of antithrombotic agents for treatment of at-risk pregnancies.

Evidence

What have we learnt from animal models?

Two primitive blood-related steps are necessary for the normal evolution of gestation. The initial formation of the yolk sac vascular system and its connection to the embryonic circulation is crucial for embryo survival in both mammals and birds. Later on, the establishment of a hemochorial placenta and the onset of the umbilico-placental circulation is essential for fetal survival in mammals when the yolk sac circulation disappears.

The formation of the yolk sac vascular system

The yolk sac consists of an outer endoderm and inner mesoderm layer and during development, blood islands (lined with endothelium) form between them. A fusing process termed vasculogenesis occurs, denoting the *in situ* differentiation of endothelial cells from the mesoderm and their coalescence into primary vessels. Vasculogenesis leads to the formation of the first major intra-embryonic blood vessel, the aorta, and to the formation of the primary vascular plexus in the yolk sac. These primary vessels are remodeled into arteries and veins with the onset of embryonic circulation, in order to develop a functional vascular loop and to accommodate cardiac output. The remodeling of the primary vascular plexus into a more mature vascular system is thought to occur by a process termed angiogenesis. Angiogenesis in the yolk sac involves capillary sprouting, splitting and remodeling, which leads to the reorganization of the primary vessels to produce an extensive vascular network. Recent data obtained in the chick embryo indicate that arterio-venous differentiation and patterning are controlled by hemodynamic forces.[16] The yolk sac vasculature fuses with the embryonic vasculature, and functions as a primitive placenta. The formation of a mature vasculature depends on the precisely co-ordinated and sequential action of a variety of angiogenic growth factors, including members of the vascular endothelial growth factor, angiopoietin and ephrin families, on their corresponding receptors. As most elegantly demonstrated by genetic deletion studies in mice, vascular endothelial growth factor-A is required at the earliest stages of vascular development in an accurately dose-dependent manner so that even haploid insufficiency results in early embryonic lethality, whereas angiopoietin and ephrin are involved during later vascular remodeling. Vascular endothelial growth factor-A activities are mediated by two high affinity receptors with tyrosine kinase activity, Flt-1 (vascular endothelial growth factor receptor-1) and KDR/Flk1 (vascular endothelial growth factor receptor-2). Vascular endothelial growth factor receptor-2 knockouts result in impairment at early stages of vasculogenesis and angiogenesis, with embryonic lethality, absence of blood islands and of embryonic and yolk sac blood vessels. Neuropilins are co-receptors, binding two structurally disparate ligands (members of both the vascular endothelial growth factor family and the semaphorin family). They may act as adaptor proteins for receptor tyrosine kinases, vascular endothelial growth factor receptor-2 in the case of vascular endothelial growth factor. Double neuropilin 1 and neuropilin 2 knockout mice totally lack blood vessel formation in the yolk sac and die very early *in utero*, this abnormal vascular phenotype resembling that of vascular endothelial growth factor receptor-2 deficient mice.

The formation of the hemochorial placenta

As the placenta develops, the embryo-derived trophoblast cells breach maternal blood vessels, and the embryonic trophoblast comes directly into contact with the maternal blood supply. A successful pregnancy outcome is highly dependent on the establishment and maintenance of an adequate placental circulation. Given that thrombophilia results in a hypercoagulable state, uncontrolled and enhanced coagulation in the intervillous spaces of the placenta may induce fibrin deposition, ultimately leading to placental infarction in the most severe cases. Thus, in this simple scheme, maternal-inherited thrombophilia may induce thrombotic events in the placenta, leading to its functional failure and to pregnancy loss.

Interestingly, placental cells not only express the anticoagulant thrombomodulin – protein C – endothelial protein C receptor system, a known regulator of blood coagulation, but also express the cell-surface receptor tissue factor which triggers blood coagulation. In this unique situation, the same cells constitutively express both the principal inhibitory pathway of blood coagulation and the receptor for the initiation of coagulation. Furthermore, these cells are in direct contact with the maternal blood supply. Mouse knock-out models have increased our understanding of the effect of inherited thrombophilia on the pathophysiology of fetal loss. Thrombomodulin and endothelial protein C receptor play a crucial role during development - mice lacking either of these molecules die during mid-gestation.[17,18] Embryos

become growth-arrested around E8.5. Restoration of thrombomodulin expression specifically in the placenta leads thrombomodulin-null embryos to develop normally during mid-gestation.[19] Conversely, specific loss of thrombomodulin function in endothelial cells does not affect embryonic development.[20] Whereas placental expression of thrombomodulin is only a prerequisite for continued embryonic development, the crucial site where endothelial protein C receptor expression is required during development is the placenta.

The growth defects of both thrombomodulin and endothelial protein C receptor knock-out embryos suggest that the protein C system plays a role in regulating cell proliferation during development. Proliferation is decreased not only in the thrombomodulin knock-out embryo, but also in the corresponding placenta, with stunted proliferation of the diploid ectoplacental trophoblast cells.[21] This effect is due to altered activation of protease-activated receptors. Normally, endothelial protein C receptor-bound protein C, which has been activated by thrombin-bound thrombomodulin, partially proteolyses and engages cell-surface protease-activated receptor 1, ultimately leading to enhanced trophoblast cell proliferation. In case of failure of the thrombomodulin-protein C-endothelial protein C receptor system, the unopposed coagulation enzymes thrombin and factor Xa can directly activate protease activated receptors 2 and 4, which inhibit trophoblast cell proliferation (Figure 1).[21]

There is also a reduced number of polyploid giant trophoblast cells in thrombomodulin-null mice, as a consequence of their selective death,[21] suggesting that thrombomodulin mediates an indirect anti-apoptotic effect. This effect of thrombomodulin in the placenta depends on the inhibition of fibrinolysis through thrombin-activated fibrinolysis inhibitor. Cellular death observed in thrombomodulin-null mice is related to fibrin deposited in the intervillous space, and is inhibited by antifibrinolytic agents. This effect is not mediated *in vitro* by fibrin itself, but by fibrin split products (Figure 1).[21]

Thus, thrombomodulin exerts dual effects - it regulates proliferation of trophoblast cells through activation of protein C and subsequent protease-activated receptor 1 activation, and it protects giant trophoblast cells from apoptosis induced by fibrin degradation products (through inhibition of fibrinolysis and the formation of fibrin degradation products). The latter effect of thrombomodulin provides a rationale for the phenotypic differences observed in embryos lacking thrombomodulin versus endothelial protein C receptor. Endothelial protein C receptor is necessary for protease activated receptor 1 engagement by activated protein C, but is not necessary for

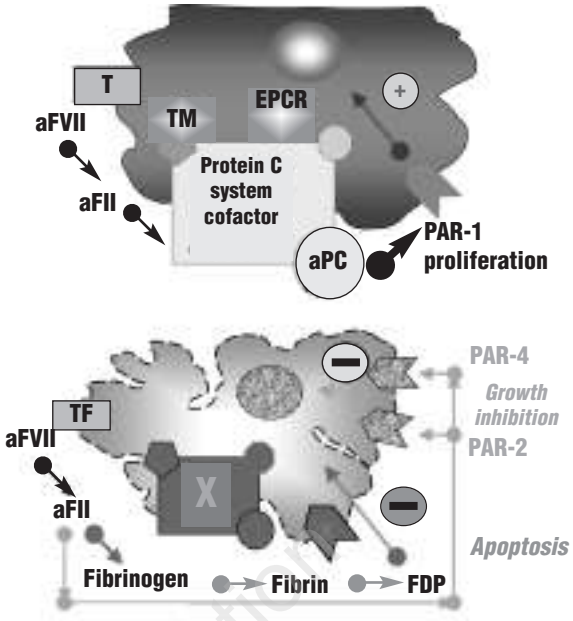


Figure 1. The role of the thrombomodulin-protein C-endothelial protion C factor system, at the surface of trophoblast cells, in the maintenance of murine pregnancy, according to Issermann.[20] TF: tissue factor; TM: thrombomodulin; EPCR: endothelial cell protein C receptor; aPC: activated protein C; aFVII: activated factor VII; aFII: thrombin; PAR: proteolysis-activated receptor; FDP: fibrin degradation products. Upper: normal pregnancy, proliferation of trophoblasts; lower: abnormal pregnancy in the case of failure of the TM-aPC-EPCR system, inhibition of the proliferation and apoptosis of trophoblasts.

the activation of thrombin activated fibrinolysis inhibitor by thrombomodulin-bound thrombin. Endothelial protein C receptor-null mice are not distinguished by a rapid resorption of the embryos. The persistent activation of thrombin activated fibrinolysis inhibitor allows for continuous inhibition of fibrinolysis, which is consistent with the detectable fibrin deposition in the placenta of endothelial protein C receptor-null embryos.

Thrombin generation itself, at the trophoblast cell surface in the placenta intervillous space, has dual effects. In the presence of a functional thrombomodulin-protein C-endothelial protein C receptor system, thrombin favors the evolution of pregnancy through its positive effects on placentation. In the absence of a functional system, thrombin impairs the evolution of pregnancy through its negative effects on placentation (direct activation of inhibitory protease activated receptors, and generation of fibrin degradation products).

The recent use of a novel transgenic strategy to generate mouse models expressing 1% to 18% of normal protein C levels has evidenced that maternal protein C is vital for sustaining pregnancy after the initial stages of embryogenesis.[22] Blastocyst develop-

ment prior to implantation was not protein C dependent. In mothers with low protein C levels, at 7.5 days postcoitum, multiple hemorrhages and extensive fibrin deposition were observed at the ecctoplacental cone region and extensive trophoblast giant cell death was revealed in all embryos. The ability of trophoblast giant cells to generate activated protein C from maternal protein C is central to embryonic survival. It can be predicted from these mouse knock-out models that acquired and constitutional impairments of the thrombomodulin-protein C-endothelial protein C receptor system may have deleterious effects on pregnancy outcome. Soluble factors in the maternal blood including protein C, protein C co-factors (protein S, factor V Leiden), antiphospholipid/anticofactor antibodies interfering with the function of the protein C system, and specific autoantibodies interfering with the various components of the system are likely candidates. The related trophoblast cell surface receptors, thrombomodulin and the endothelial protein C receptor, are also candidate targets for pregnancy loss. As the genome of the embryo results from contributions of both the maternal and paternal genomes, it opens the door to an as yet unconsidered potential paternal role in pregnancy loss. We may need to expand our thinking from women at risk of pregnancy loss to couples at risk of pregnancy loss. In this regard, a recent communication by H. Weiller (*International Symposium on Women's Health Issues in Thrombosis and Haemostasis, Budapest, February 2005*) described a new mouse model of enhanced pregnancy loss, wherein factor V Leiden-positive mice (which do not develop pregnancy loss) are mated with mice expressing low-functional thrombomodulin (by itself not associated with pregnancy loss). The resulting model of enhanced pregnancy loss would seem to have potentially major implications for understanding the basis of fetal loss in humans.[23]

What can we learn from the natural evolution of human pregnancy?

Human pregnancy is far from being a homogeneous developmental process. It is not until the beginning of the 8th week that contact of maternal uterine spiral arteries with the placental intervillous space can be recognized.[24] Ultrasonography has shown that the arterial signal in the yolk sac circulation disappears, and that the umbilico-placental circulation increases between the beginning of the 8th and 10th weeks of gestation. The placenta replaces the yolk sac as an essential source of blood supply to the embryo at that time.[25] Maternal hemostatic defects are thus unlikely to play a deleterious role before this crucial period. The first trimester of pregnancy is a heterogeneous period in terms of the relationship between embryo placental cells and components of the maternal blood. It is thus important to re-classify pregnancy loss during the first trimester: fetal loss from the beginning of the 10th week of gestation (umbilico-placental circulation), embryo loss up to the beginning of the 8th week (yolk sac circulation), and embryo loss from the beginning of the 8th week to the end of the 9th week (various relative intensities of the two types of circulation). For the purposes of epidemiological analysis, we believe that hemostasis-related risk factors for pregnancy loss should be limited to those cases at 10 weeks of gestation or later. The clinical definitions used in the available studies are the following: *embryonic loss*: between 5 and 10 weeks of gestation; *fetal loss*: after 10 weeks; *miscarriages*: before 20 weeks; *intra-uterine fetal death* (*still birth*): after 20 weeks; *first trimester loss*: between 5 and 12 weeks; *second trimester loss*: between 13 and 24 weeks; *third trimester loss*: after 24 weeks.

What can we learn from the analysis of epidemiological studies?

Results from the initial retrospective EPCOT case-control study,[11] based on a cohort of women from families with inherited thrombophilia-related thrombosis, demonstrated that the risk of fetal loss was increased by a mean relative value of 35% in women with thrombophilia. The mean risk was higher mainly for stillbirth (3.6-fold), for women with combined defects (14.3-fold), those with antithrombin deficiency (5.2-fold), and those with protein C deficiency (3.3-fold). The ensuing prospective EPCOT study,[26] which included all pregnancy losses from the 4th to the 18th week of gestation, found that, in women with a previous pregnancy loss, there was a mean increase of 40% for the risk of loss in subsequent untreated pregnancies. This risk was similar among women with different types of thrombophilia - 40% increase for factor V Leiden-positive women and 60% increase for antithrombin-deficient women. When women were treated with antithrombotic treatment throughout pregnancy, the risk of loss was lowered by only 30%.

However, these results are open to criticism, based on methodology. In this type of study, prevalence of miscarriages is not sufficient (roughly 30%) to obtain an informative evaluation. Miscarriages cannot, due to the low number, be analyzed according to the timing or definition of fetal loss, resulting in the pooling of relevant data (pregnancy losses from the 10th week) with irrelevant manifestations (losses before the 10th week). By analogy, if the factor V Leiden mutation had initially been described only as a protecting factor against intrapartum blood loss, studying 500 patients would have been sufficient.[12] To ignore the fact that mutation, in rare cases, also pre-

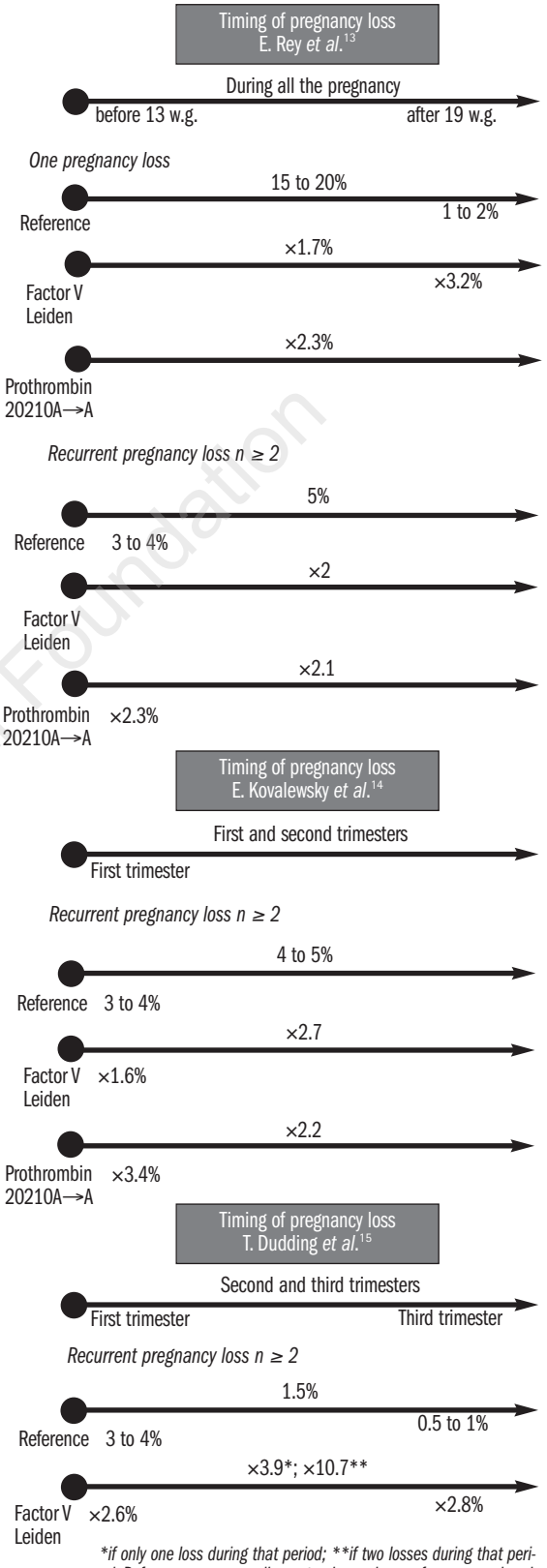
disposes to venous thromboembolic disease during pregnancy, then necessitates the study of roughly 2000 patients to achieve statistical significance. Finally, co-factors which enhance the thrombotic risk in women carrying the factor V Leiden mutation are not necessarily those which enhance the risk of fetal loss, a pathophysiology which, in animals, is not directly thrombosis-related.

The second approach for analysis of the correlation between thrombophilia and fetal loss focused on all patients with pregnancy loss. However, these analyses are complicated because data have been pooled from women with *gestational vascular complications*, including pre-eclampsia, fetal growth retardation and placental abruption, the pathophysiologies of which may be different from that of unexplained pregnancy loss.[27] Another confounding point is the frequent absence of separation in studies between primary pregnancy loss (childless patients) and secondary loss, between recurrent and non-recurrent pregnancy loss, and the variable degree of selection of the patients (all patients with pregnancy loss versus only unexplained cases).

The two most common polymorphisms in hereditary thrombophilia, the factor V Leiden mutation and the prothrombin 20210A allele, occur with sufficient prevalence to allow an in-depth analysis. There is a demonstrated dose-effect relationship: homozygous factor V Leiden mutation carriers have a greater risk of fetal loss (×2) and of stillbirth (×4.85) than heterozygous carriers.[28] In addition, the risk of loss is greater in mutated female siblings of thrombophilic probands with the factor V Leiden mutation (2.3-fold after the first trimester).[2] A single prospective study has analyzed the outcome of untreated pregnancies in women who had at least three pregnancy losses, each occurring before 13 weeks. In women positive for the factor V Leiden mutation, the recurrence rate of pregnancy loss was increased four-fold. Analysis of cases in which factor V Leiden mutation-positive women experienced fetal loss after 12 weeks indicates that the prospective risk is increased eight-fold.[30] Results of three meta-analyses[13-15] (Table 1) indicate that women with heterozygous factor V Leiden mutation exhibit a two- to three-fold increase in the risk of pregnancy loss. Fewer data are available concerning the prothrombin 20210A allele (Table 1), but the limited results suggest that women carrying a heterozygous prothrombin 20210A allele also exhibit a two- to thee-fold increased risk of pregnancy loss.

We have performed a large case-control study on 33,000 first pregnancies. Preliminary results demonstrate that the factor V Leiden and the prothrombin 20210A polymorphisms are associated with a greater risk of pregnancy loss from the 10th week on in Caucasian women.[31] However, most carriers of these

Table 1. Mean variations of the relative risk of pregnancy loss, according to their previous number and to their timing during intended pregnancies, given by the three meta-analyses[13-15] performed on the factor V Leiden polymorphism and on the prothrombin 20210G→A allele.



**if only one loss during that period; **if two losses during that period. Reference: corresponding natural prevalence of pregnancy loss in the normal population; w.g.: weeks of gestation.*

polymorphisms will not develop any clinical signs and remain undiagnosed, because these conditions present a small clinical risk. The question is how to predict which women with the factor V Leiden or the prothrombin 20210A polymorphism will be at risk of pregnancy loss. Maternal co-factors, including antiphospholipid/co-factor antibodies, homocysteine, protein Z (a vitamin K protein thought to modulate, through its associated protein Z-dependent protease inhibitor, the first amounts of activated coagulation factor X), anti-protein Z antibodies, anti-endothelial protein C receptor antibodies, and soluble endothelial protein C receptor plasma concentrations are currently under investigation.[32-36] Data concerning potential paternal co-factors are currently unavailable.

What are the available therapeutic data?

The main experimental antithrombotic treatments which have been tested during at-risk pregnancies are low-molecular weight heparins. Trials are open, non-placebo controlled studies in which the clinical outcomes are compared with either the spontaneous previous pregnancies,[37-40] or with pregnancy outcomes of a parallel non-treated group,[41] or with the outcomes of a parallel group treated by another antithrombotic drug.[42]

The safety of low-molecular weight heparin treatments during pregnancy has, of course, been questioned. An analysis of 2777 pregnancies, in which women were treated with fractionated heparins, demonstrated the general safety of these agents. Significant bleeding, generally associated with primary obstetric causes, occurred in only 1.98% of the cases. There were minimal allergic skin reactions (1.8%), no incidents of heparin-induced thrombocytopenia, few cases of thrombocytopenia unrelated to fractionated heparins (0.11%), and rare instances of osteoporotic fracture (0.04%).[43]

Brenner and his group studied the effect of enoxaparin (40 mg daily; 80 mg in complex thrombophilia), in women with thrombophila (either inherited or acquired, n = 50), and with a history of pregnancy loss (three miscarriages in the first trimester, or two in the second, or one in the last trimester): 75% of the treated pregnancies were successful in terms of live births, compared with 20% of the previous untreated pregnancies.[38] In a second trial (Live-Enox study), the same group compared two doses of enoxaparin (40 versus 80 mg) in 180 women. This study confirmed the positive therapeutic effect of low-molecular weight heparins, with no significant difference in effect between the two dosages (84% vs. 78%).[39]

In another study, Carp and his group followed the pregnancies of 85 women with thrombophilia, and a history of at least three miscarriages before 20 weeks of gestation. Seventy percent of the treated women (40 mg enoxaparin daily) had a live birth, compared with 44% of the non-treated women, the difference being mainly in patients with primary miscarriages.[41]

We compared the effect of enoxaparin (40 mg daily from the beginning of the 8th week) with the effect of low-dose aspirin (100 mg daily)[42] on pregnancy outcome in 160 women who had had an unexplained pregnancy loss after the 10th week during a first or second pregnancy and who carried the factor V Leiden mutation or the prothrombin 200210A polymorphism or were deficient in protein S, but who were negative for antiphospholipid/co-factor antibodies and for high homocysteine plasma levels. All these women also took folic acid (5 mg daily) before pregnancy. Eighty-six percent of the 80 pregnancies treated with low-molecular weight heparin resulted in normal births, but only 29% of the 80 pregnancies treated with low-dose aspirin resulted in normal births. The superiority of enoxaparin over low-dose aspirin was apparent for each of the three inherited thrombophilias. An associated low level of protein Z (< 1 mg/L) or positive anti-protein Z antibodies were pejorative risk factors. The neonates whose mothers were treated with enoxaparin had a higher birth weight than those whose mothers where treated with low-dose aspirin.

Practical recommendations

The proposed recommendations are based on the available meta-analyses,[13-15] and therapeutic studies,[37-42] on a systematic review of the safety of the low-molecular weight heparins during pregnancy,[43] and on published recommendations.[44-46]

Because most carriers of a hereditary thrombophilia will not develop pregnancy loss, routine screening of all pregnant women for thrombophilia is currently not recommended. Screening for congenital thrombophilia has been suggested for women with recurrent pregnancy loss (three or more miscarriages),[45] and for women with fetal loss, including three or more first trimester losses, two or more second trimester losses, or any stillbirth.[46]

From our point of view, we recommend that earlier screening should now be considered, at least in (childless) women with a primary first unexplained fetal loss from the 10th week of gestation. This concurs with the inference from animal models, and with our preliminary results.[31] We currently do not know whether this recommendation should be expanded to women with a secondary first unexplained fetal loss from the 10th week. Recent recommendations have suggested the use of low-dose

aspirin therapy plus either mini-dose heparin or prophylactic low-molecular weight heparin for women with congenital thrombophilia and recurrent miscarriages, a second trimester or later loss.[45] However, no study has shown the benefits of the use of low-dose aspirin in such an indication, and minidose unfractionated heparin has some practical limitations and induces more side-effects than low-molecular weight heparin. Low-molecular weight heparin is thus the more tempting option and carries a minimal risk in pregnancy. We have no other potentially effective treatments, and prospective randomized trials are lacking. It can thus be recommended that women with pregnancy loss and thrombophilia in a subsequent gestation should be enrolled in clinical trials or treated on an individual basis, with a pragmatic choice[47] of low-molecular weight heparins. However, in women with a first pregnancy loss from the 10th week and protein S deficiency, or heterozygous factor V Leiden or prothrombin 20210G→A polymorphism, low-molecular weight heparin is more efficient than low-dose aspirin[42] and should probably be the first-line treatment, if any, for this indication.

Conclusions

The association between maternal-inherited thrombophilias and pregnancy loss is based on mouse knock-out studies and is concordant with physiological and epidemiological human data. The two most common inherited polymorphisms, the factor V Leiden mutation and the prothrombin 20210A allele, confer a modest risk of venous thrombosis, and also induce a modest but significant risk of pregnancy loss after the 10th week of gestation. Additional co-factors which probably enhance this risk have yet to be identified. Fundamental results obtained from knock-out mice suggest that maternal soluble co-factors which impair the thrombomodulin-protein C-endothelial protein C receptor system, but also trophoblast cell-surface and intracellular co-factors, play a role in embryonic development. Polymorphisms in these genes are inheritable from both the mother and the father. Moving from the identification of women at risk to the identification of couples at risk will be the new challenge, ultimately resulting in more accurate treatment of at-risk pregnancies. Low-molecular-weight heparins, which are safe during pregnancy, are the first-line treatment, particularly for women with a first fetal loss after the 10th week and who carry the factor V Leiden mutation, the prothrombin 20210A allele, or who are deficient in protein S.

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