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Thrombophilia in Assisted Reproductive Technology – Place and Needs of Thromboprophylaxis

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1. Introduction

Assisted reproductive techniques (ART) have become part of the routine care, with a prevalence ranging from 0.1 to 3.9% of all live born children in Europe and an average of 2% in some parts of the USA [1]. Despite the initial dramatic improvements in success rates and significant increments in ART uptake, the live birth rate resulting from these techniques has recently plateaued. Unfortunately a common non successful termination of ART procedures are due to failure of implantation of high quality graded embryo(s). Implantation failure may recur and three or more in vitro fertilization (IVF) cycles without pregnancy are usually regarded as repeated implantation failures (RIF).

Recently after improving embryo culture media and optimizing controlled ovarian hyperstimulation (COH) protocols, the predominant part of IVF cycles results in embryo transfer (ET) but only about one third [2] of all cycles reach clinically achieved pregnancy. This is evidence that most embryos failed in an early stage of pregnancy establishment. RIF after IVF procedures emphasize the clinical importance of this crucial step in ART and forces efforts to investigate the firm mechanism of implantation and to find approach to increase pregnancy outcome success.

The implantation of the blastocyst into the endometrium in human pregnancy is a very complex corresponding signalling process including specific receptors being expressed on cells surface both on embryo and maternal cells. The specific signalling processes throughout implantation passed in the similar, although not the same algorithm in the spontaneous cycle and after IVF embryo transfer. Plenty of factors have been recognized to affect either success,

or failure rate of IVF embryo transfer. Mother side factors include age, parity, hormonal levels before stimulation, antral follicles count, endometrial thickness and quality of transformed endometrium [3, 4]. Embryo grading [5] and place of ET in uterus are other implantation limiting factors. It turns out that not only endometrium, but also extracellular matrix molecules, endothelium and blood circulation factors were involved in remodelling of the endometrium, which is associated with the embryo acceptance process.

A couple of factors [6], having functions in coagulation and fibrinolysis cascades, were found to be connected with the transformation processes in the endometrium during the implantation. In relation with that, the alteration of the function and activity of blood coagulation factors could influence blastocyst acceptance in the endometrium. One proposed cause for implantation failure could be maternal thrombophilias. Thrombophilia was represented as a condition of hypercoagulable state with variety of causes [7] – inherited defects in coagulation factors, anticoagulation and fibrinolysis processes. All these coagulation factors changes result in an increased capacity of blood to form thrombin. The formation of increased thrombin amount, main factor triggering formation of thrombus, is associated with an increased risk of thrombosis development [8]. The study of inherited thrombophilia impact on implantation failure is conducive to IVF procedure because of in vitro embryo selection before transfer. Thus a part of implantation failures due to chromosomal abnormalities [9] of the conceptus have been eliminated as a cause of negative pregnancy outcome.

In the recent twenty years, a number of heritable disorders predisposing to thrombosis have been identified. Except the well-known deficiencies in anticoagulation factors – protein C, S and antithrombin III [10], nowadays, the defects in factor V, factor XIII and factor II of coagulation and polymorphism in plasminogen activator inhibitor (PAI-1) and platelet adhesion proteins [11], have been widely discussed. The prevalence of inherited thrombophilic factors in the different populations varies widely from being absent to being found in up to 15% of healthy individuals [12, 13]. In this relation, a discussion of the impact of inherited thrombophilia should be performed only in the range of corresponding population with well-known distribution of factors in health individuals.

Thrombophilic defects have been shown to be associated with an increased risk not only of venous thrombosis but also with fetal loss and gestational complications. Multiple studies [14, 15, 16, 17] have shown that thrombophilias increase the risk of recurrent first- and second-trimester pregnancy losses through placental bed thrombosis. To date, meta-analyses of relatively small case-control studies have demonstrated a small but significant increase (OR 1.5–4.0) in embryonic and fetal loss, abruption, intrauterine growth restriction and preeclampsia in association with inherited thrombophilias [18, 19]. Prospective cohort studies have similarly supported a minor contribution of inherited thrombophilias on perinatal outcomes [20, 21].

Maternal risk was also increased with increments in the rates of preeclampsia, gestational diabetes, placenta praevia and the consequent need for Caesarean section. All of these conditions are being increasingly recognized as having their origins in the first trimester with abnormal implantation and trophoblast development being the key pathophysiological

processes. Furthermore, several investigators have also studied the relationship between thrombophilias and implantation with conflicting results [22, 23, 24]. An additional confusing fact is that the development of the intervillous space occurs after 10 week of gestation, making it somewhat difficult to explain implantation failure solely with microthrombosis in decidual vessels. Current evidence concerning thrombophilias and recurrent IVF failure remains limited and inconclusive. Therefore, there is need to evaluate the present prominence of the association of inherited thrombophilia factors with IVF failure not only with thrombotic changes during the implantation process, but also to examine the possible influence of the factors on maternal-embryo receptor interaction and the influence on embryo development.

The notion that coagulation disorders may lead to implantation failure has led to the use of anticoagulants, mainly heparin, during assisted reproduction. The role of heparin in assisted conception in women with inherited and acquired thrombophilia has been thought classically to be prevention of thrombosis in relation to implantation and placental development. It is postulated, potentially, a much wider role for heparin in assisted conception due to its ability to interact with a wide variety of proteins, which can alter the physiological processes of implantation and trophoblast development, a process that may be adversely influenced by assisted conception per se. Although the process of implantation is enigmatic, anticoagulant therapy is now being examined as a preventative measure for women with a history of placental-mediated pregnancy complications and many clinics are embarking on the use of low molecular weight heparins (LMWH), again based on biological plausibility rather than evidence of efficacy. Despite the heterogeneity of studies, the results suggest a potential improvement in pregnancy outcomes with anticoagulant therapy.

The approval of the outlined potential of LMWH to alter the molecular processes underpinning successful implantation is urgently required giving the potential for clinical translation to increased pregnancy and live birth rate and a reduction in adverse perinatal outcomes for all women undergoing ART. Important notes discuss the present chapter connected with appropriate time, type and dose of anticoagulant prophylaxis according to patients' type and severity of thrombophilia. This was given after elucidation of the place of inherited thrombophilic factors in implantation process and type and action of some off-label drugs used in improving ART success.

2. Overview of human embryo implantation stages. Adhesion glycoproteins and coagulation factors in implantation process

In the middle of the eighties it was found that the implantation is not one-stage process in primates [25] and is subdivided in a few separate different steps in which many factors both from the mother and embryo have been involved. Prior to the real implantation and after the shedding of zona pellucida, the embryo is oriented toward the endometrium. This stage of implantation was named apposition. During this process, there is no contact between embryo and endometrial cells. The apposition was followed by an adhesion of the embryo to the endometrial cells surface. This step was time limited and was performed through cell surface

receptor communication. In the window of implantation [26] – a short period where it is only possible to realize the implantation process, both endometrial and embryonic cells express adhesion molecules and materialize the adhesion. The adhesion molecules are mainly receptors from the integrin family [27]. Generally, integrins are a group of cell surface adhesion molecules playing role in allowing cell to cell interaction. Within the endometrium, the expression of certain integrins changes during the menstrual cycle [28, 29]. Three integrins, in particular $\alpha v\beta 3$, $\alpha 4\beta 1$ and $\alpha 1\beta 1$, are thought to play a vital role in the implantation. $\alpha v\beta 3$ is the integrin which amount changes most prominently during the implantation window. Heterodimer $\alpha v\beta 3$ consists of two subunits αv and $\beta 3$, which execute different functions. $\beta 3$ subunit is connected with receptor-to-receptor interaction: the subunit recognizes the extracellular matrix ligand osteopontin, which is a protein ligand expressed by the endometrium during the window of implantation. The interaction between integrin $\alpha v\beta 3$ on endometrial and embryonic cell surface with osteopontin actually realize first cell-to-cell contact – embryo adhesion [30]. The expression of the intact heterodimer $\alpha v\beta 3$ is rate-limited by the production of the $\beta 3$ subunit, which is regulated directly by the transcription factors. $\beta 3$ subunit also is a part of other integrins. One of them is $\alpha IIb\beta 3$ integrin, involved in the process of platelet aggregation [31]. One common polymorphism in the gene sequence of $\beta 3$ subunit (1567 T>C) was found to increase the subunit affinity to ligands and could influence the platelet interaction, as well as the adhesion process during implantation.

The last and the most extended and complex process during implantation is the trophoblast invasion within endometrium. The trophoblast invasion requires plenty of up- and down-regulation of many factors both from mother and embryo, which results in degradation of decidua extracellular matrix (ECM) and subsequently endometrium and myometrium vessels invasion. The initial invasion of the trophoblast into the decidua requires the up-regulation of proteases (especially matrix metalloproteinases - MMP) to degrade the ECM. The enzyme activity of migration-behaviour part of cytotrophoblast – extravillous cytotrophoblast (EVT), is controlled by urokinase plasminogen activator (uPA) [32]. uPA is able to activate matrix metalloproteinases (MMPs), produced by EVTs. In vitro studies have shown that after activation by uPA the migrating trophoblast up-regulates MMP2 [33], MMP3, MMP9 [34] and cathepsins [35]. Plasminogen activator inhibitor-1 (PAI-1) is a primary uPA regulator that inhibits uPA by forming a covalent complex, thus controlling the thrombotic/fibrinolytic process [36]. In addition, through its binding to the ECM, PAI-1 regulates cell adhesion and migration by interfering with the binding between cellular integrins or uPA receptor (uPAR) and vitronectin. A 2675 4G/5G sequence polymorphism in the PAI-1 gene promoter has been correlated with increased levels of plasma PAI-1 [37]. The carrier status for 4G4G phenotype results in higher activity than the 5G allele, because in addition to the binding site for the transcriptional activator, the latter also contains a binding site for a transcriptional repressor. In the presence of 4G allele and the absence of bound repressor, the basal level of PAI-1 transcription is increased [38]. The 4G allele of PAI-1 has been recently linked to venous thromboembolism [39] and coronary disease [37], however relation with recurrent pregnancy loss and implantation failure is under discussion.

After the initial invasion into decidua, EVT gains endometrial vessels and breaches their wall allowing first contact of embryo cells with maternal blood [40]. During this deep invasion step, EVT opened and remodelled spiral arteries and arterioles to produce high-conductance vessels, necessary for the further developing fetus. The haemostasis into decidua during vessel invasion was ensured by up-regulation of tissue factor (TF) and activation of extrinsic coagulation cascade, and simultaneously increased PAI-1 activity [41]. The result of increased TF activity is generation of thrombin. In cases of increased thrombin production, such as in inherited thrombophilias conditions, the decidual cells produced anti-angiogenic soluble fms-like tyrosine kinase-1 factor (sFlt-1), which inhibits enzymes related with EVT invasion [41]. Insufficient shallow invasion of EVT into decidua results in incomplete vascular transformation and underperfused embryonic cells, which could lead to early pregnancy loss. Inherited changes in coagulation factors increased the amount of plasma and local thrombin. The most discussed are Factor V Leiden and 20210 G>A substitution in prothrombin gene. The prothrombin (FII) mutation involves guanine-to-adenine transition at nucleotide position in the 3'-untranslated region of the prothrombin gene. The mutation 20210 G>A is associated with both increased plasma concentration of prothrombin, and an increased risk of thrombosis [42]. Factor V Leiden (FVL) represents an altered Factor V (FV) of coagulation proteins, due to substitution of adenine for guanine at nucleotide position 1691 (1691 G>A) in exon 10 of the factor's gene. As a result of the mutation, circulating half time of life of FV is increased dramatically. This has been represented with permanently increased risk for blood clotting formation. This two inherited thrombophilic factors have had still debatable role in increasing the risk for late, as well as early recurrent pregnancy loss.

In the preimplantation period and during the decidual invasion of EVT, simultaneous cell division processes have passed in the embryo. Cell division and differentiation have been connected with continuous changes (activation and inactivation) in gene activity. One fundamental process to regulation of mammalian gene activity is methylation status of the genome [43]. The gene methylation is critical precisely to early embryonic development [44]. Methylation of DNA parts, responsible for gene activity, was realized by donor chemical compounds, such as S-adenosylmethionine (SAM). Methyl groups, passed from folic acid through a series of enzymes, contribute to the production of SAM. As such, folic acid is indispensable for embryonic development [45]. An enzyme critical to the folic acid pathway is methylenetetrahydrofolate reductase (MTHFR). Deficiencies of folic acid or defects in MTHFR have demonstrated DNA hypomethylation and abnormal biochemical and phenotypic changes in cell development and interaction [46, 47].

Most common MTHFR inherited gene changes are 677 C>T 1298 A>C single-nucleotide polymorphisms (SNPs) [48]. Homozygous variant for 677 C>T (genotype TT) creates a thermolabile enzyme with only 30% of wild-type activity. The decreasing of MTHFR activity reduces the amount of SAM and thus methylation processes. Low MTHFR activity causes enzyme block in methionine metabolism and leads to increased homocystein (Hcy) in blood plasma. Concentration of Hcy more than 15 $\mu\text{mol/l}$ was found to be related with increased thrombosis development, due to endothelial injury and coagulation cascade activation. The increased plasma Hcy and 677 C>T polymorphism was discussed as a risk factor for arterial

and venous thrombosis development. The impact on early pregnancy loss and implantation failure is still disputed point although its role in fertility has not been extensively studied [49, 50, 51]. Another SNP 1298 A>C in MTHFR affects the SAM regulatory domain and has ~60% of wild-type activity [52, 53]. The effect of 1298 A>C on MTHFR and thus on SAM metabolism is not so pronounced as the effect of 677 C>T, and have been discussed as an additional risk factor in presence of both polymorphisms simultaneously.

3. Diverse influence of thrombophilic factors on implantation and risk for RIF

3.1. MTHFR polymorphisms and embryo development

Recent studies try to establish a connection between folic acid metabolism, preimplantation and implantation embryo development. It was shown that folic acid is present in the follicular fluid. Its supplementation decreases serum and follicular fluid homocysteine levels and is associated with better quality and more mature oocytes used in IVF procedures [54]. During maturation oocytes express receptors for folic acid transport protein [55]. This finding enforces the idea for the crucial role of folate and folate metabolism in the regulation of gene expression through methylation and demethylation of regulatory parts of the genes using SAM. By hystopathological investigation some authors have found defective chorionic villous vascularization [56], as well as significantly smaller median area, perimeter and diameter per chorionic vascular element [57] in women with elevated total homocystein levels. Fluctuating data for the prevalence of MTHFR polymorphism in women with repeated IVF failure was presented supporting these hypotheses. Dobson et al. [50] investigated maternal and paternal carrier status for 677 C>T and 1298 A>C in MTHFR in 197 couples, who underwent IVF procedure. They do not found significant impact of both SNPs, although in women with TT genotype decreased pregnancy rate compared with CT and CC genotype was established (33.3% compared with 47.7% and 47.9%). The authors do not exclude any other additional confounding factors concerning IVF failure, such as hyperhomocysteinemia due to alimentary factors or immunological and/or additional thrombophilic factors. Many other authors establish fluctuating prevalence of TT genotype among women failed to conceive after IVF/ICSI: Qublan et al. [24] found 22.2% prevalence of TT genotype. They established this polymorphism as the most common SNP among women with IVF implantation failure. Before that Martinelli et al. [58] found 19% prevalence of TT genotype evaluating 162 women with failed IVF/ICSI treatment. Moreover, Azem et al. [59] who investigated 45 women with a history of four or more failed IVF cycles reported an incidence of 17.8%.

The discrepancy between the reported prevalence could be related with the number of failed IVF cycles [60] in patient groups, as well as with the selection criteria for the control group of women. The isolated impact assessment of MTHFR SNPs depends on a variable number of other investigated thrombophilic factors [22, 23], included in any other study. The ethnic variability of polymorphisms should be also considered. An important obscured factor for difficulties in proper evaluation of MTHFR polymorphism impact is the wide spread prophyl-

lactic prenatal vitamins taking containing high dose folic acid. Alimentary intake of folic acid masks the suggested roles of MTHFR genotypes on IVF failure. In relation with that a ten-time increased dose of folic acid supplementation [61, 62,63] was recommended for women undergoing IVF.

3.2. FII 20210 G>A: Thrombin generation influence on cytotrophoblast invasion

Normal implantation is associated with thrombin-induced fibrin deposition in the absence of overt bleeding [64]. The excess of fibrin deposition in forming intervillous spaces could have negative effect on the implantation process. The serine protease thrombin is a key element in fibrin accumulation [23]. In presence of increased amount of thrombin the decidual cells produce anti-angiogenic soluble fms-like tyrosine kinase-1 factor, which inhibits enzymes related with EVT proliferation [41]. Thus in early pregnancy, thrombin may act as an autocrine/paracrine enhancer of sFlt-1 expression on the decidual cells to promote implantation failure by interfering with local vascular transformation [64]. The prothrombin role on implantation was supported by experimental data for reduced infertility due to increased topical concentration of thrombin in both fallopian tubes, although the mechanism of action of locally administrated thrombin is undefined [65]. The possible impact of FII 20210 G>A on the implantation failure is also supported by the found distinct prevalence of FII 20210 G>A and FVL on early pregnancy loss [66]. In recurrent pregnancy loss before 10 week of gestation was established a more pronounced prevalence of 20210 G>A mutation compared with FVL. The slight increase of APC (natural inhibitor of FV) at early pregnancy could explain normal level of FV activity during the embryonic period (5 to 10 week of gestation) [67]. Indirectly, this finding could be referred to a high prevalence of 20210 G>A mutation in IVF failure. Some studies fail to find relation between 20210 G>A mutation and implantation failure. Others establish weak [23] or strong [24] correlation between 20210 G>A and unsuccessful IVF attempts. The prevalence of FII 20210 G>A in Caucasian is between 1 and 3% and thus the low occurrence enforces larger study arrangement to evaluate the factor's impact on implantation.

3.3. Advantages and disadvantages of FVL during implantation

One of the first and frequently discussed studies concerning IVF failure and presence of inherited thrombophilia is published by Grandone at al. [68]. The study includes a small number of women with more than 3 failed IVF attempts (n=18) compared with 216 women with at least one successful terminated pregnancy. The authors reported significantly higher occurrence of FVL and FII 20210 G>A in patients comparing with controls, although the frequency of FVL in health subjects was found relatively low (1.9%) (common occurrence in Caucasians between 7 and 10% [13]). These authors included in the patients' group women with pregnancy loss, which moreover embarrassed the evaluation of the impact of thrombophilic factors on implantation process. After this report, series of consequent papers discussed the influence of thrombophilia on implantation process, especially after IVF procedure. Some studies found [23, 24] but others not [58, 69] a relation of FVL and FII 20210 G>A with implantation failure. The conflicting results show the necessity of large and specific inclusion criteria fulfilling studies to detect the real connection between the presence of increased thrombin

generating factors and implantation outcomes. Interesting diverse outcomes for the prevalence were found by some authors: a part of them established a relatively lower prevalence of FVL in women with IVF implantation failure compared with controls [60]; others reported an increased implantation success after the first IVF attempt in FVL carriers. On this basis the hypothesis for positive effect of thrombin deposition during trophoblastic invasion was established. This selective advantage of FVL carriers on implantation was described for the first time by Gopel et al. [70], who found 90% successful implantation rate after first IVF attempt in FVL carriers comparing to 49% rate in non-carriers. It should be known that the authors included both mother and fetus positive FVL genotype. This stand referred to another discussion concerning the significance of paternal [71] and fetal thrombophilia for early pregnancy development [72]. Similar results show Martinelli et al. [58] reporting 86% pregnancy rate after first IVF attempt in FVL and FII 20210 G>A carriers compared to 68% pregnancy rate in non-carriers (non-significant difference). Although not all authors [72] share the proposition, FVL could improve implantation rate in IVF and spontaneous cycles. The increased implantation rate in FVL carriers could have been balanced by abortions or miscarriages later in pregnancy. The discussions for evolutionary advantages and disadvantages of FVL still remain, probably because there should be some unknown benefits, or the mutation would have been eradicated from population.

3.4. PAI-1 4G/5G: Hypofibrinolysis and impaired deep cytotrophoblast invasion

During implantation, an accurate balance of coagulation, fibrin deposition and fibrinolysis is mandatory for trophoblastic invasion. Accumulation of fibrin forces conversion of plasminogen to plasmin, thus stimulating the process of fibrinolysis. Fibrinolysis is important for modulation of the extracellular matrix mediated by the plasminogen activation system. Plasminogen activation facilitates cell migration through targeted proteolysis and local dissolution of the basement membrane [73]. So extended fibrinolysis is crucial for implantation process. Inhibition of fibrinolysis after increased activity of plasminogen activator inhibitors such as PAI-1 could impair proper deep trophoblastic invasion. High PAI-1 activity, particularly due to inherited changes in PAI-1 gene expression, is associated with inhibition of the conversion of plasminogen to plasmin and subsequent hypofibrinolysis. Hypofibrinolysis as a result of the 4G allele (especially genotype 4G/4G) of the PAI-1 gene appears to be a possible risk factor for implantation failure by limiting trophoblastic invasion [74].

A limited number of papers report for a possible negative impact of 4G/4G genotype on impaired implantation process. Coulam et al. [23], who investigated 42 women with implantation failure, found a significantly higher prevalence of 4G/4G genotype in patients compared with controls (respectively 38% and 10%). They also found distinct higher occurrence of 4G allele in patients vs. controls (74% and 20%, $p=0.007$). Goodman et al. [22] also found higher but not significant prevalence of 4G/4G genotype in 73 controls and 70 women experiencing implantation failure (26% vs. 36%). Some others [75] found similar high prevalence of polymorphisms but they also included women with early pregnancy loss in the study group. The impact of 4G/5G polymorphism frequently was evaluated in the context of multigenetic carrier status [23].

3.5. PL A1/A2 in integrin beta 3 platelet activity and adhesion properties of endometrium

To the best of recently knowledge, a few observations have been published on the possible association between the platelet integrin polymorphisms and recurrent pregnancy loss development. The hypothesis for the connection between PL A1/A2 of GP IIb/IIIa and increased risk of pregnancy loss has been based on the supposition that impairment of platelet function is related with disturbance in uteroplacental vascular system. Increased platelet aggregation, as a result of the presence of allele A2 could establish prothrombotic conditions and increase thrombus formation in intervillous space, leading to poor fetal outcome.

Some recent studies find association between beta 3 integrin and recurrent pregnancy loss [76] In our pilot study [60] of 67 women with primary sterility and 96 healthy control subjects, we found a significantly higher prevalence of PL A1/A2 in women with implantation failure after assisted reproduction technology (ART) in comparison with controls (OR: 2.6, 95% CI: 1.1-6.3, $p=0.033$). These data suggest that the carriers of PL A1/A2 are at higher risk of implantation failure and do not have successful ART outcome. In a separate study [77] we have found more pronounce prevalence of PL A1/A2 in women with RPL before 10 week of gestation compared with carriers status in women with late pregnancy loss (after 10 week of gestation) (41.8% vs 29.3%, OR 1.73; 95% CI 0.93 - 3.21, $p=0.084$). Compared patients' groups were not FVL and/or FII 20210 G>A carriers. This finding emphasizes the more probable PL A1/A2 influence on early than late pregnancy loss. The impact of increased platelet activity on implantation process is particularly embarrassed, because investigated polymorphism A1/A2 concerns beta3 subunits of platelet integrin α IIb/beta3, which is also part of integrin α v β 3, related with embryo adhesion to endometrium [78]. An in vitro investigation has found that changes in the peptide structure of beta3 subunit have had influence on the receptor activity to its ligands - osteopontin and vitronectin [31]. Sajid et al. [31] established that cell lines which expressed α v/beta3 receptor with PL A1/A2 had an enhanced haptotactic migratory response to vitronectin and osteopontin. As the initiation of fetus adhesion to the endometrium has been connected mainly with the activity of integrin α v/beta3, polymorphism A1/A2 could play a significant role not only in platelet aggregation, but also in adhesion and endometrium to embryo interaction during implantation.

The influence of thrombophilic genetic factors in pregnancy outcome after assisted reproduction is connected with modification of endometrium adhesion properties and effect on trophoblastic invasion ability. Independent significance of thrombophilia is hard to evaluate because of assembled information for many other recently discussed IVF failure risk factors which are outside the thrombophilic group factors. Apoptosis and cell arrest tumor suppressor factor p53 [79], inducing angiogenesis vascular endothelial growth factor (VEGF) [80] or Leukemia inhibitory factor (LIF) [81] are a part of these agents. These factors should be considered in the impact of thrombophilic factors discussion because the recent approach in consultation after IVF failure investigations is to identify the combination of defects [80] instead of one factor that will lead to implantation failure. Moreover, the fetal role in thrombophilia should also be discussed because of established positive polymorphism carrier status in placental tissue [82]. This finding necessarily includes a role of the father's genetic pattern in pregnancy destiny. Nevertheless the beginning of adequate trophoblastic invasion is tightly

regulated by thrombophilic genetic factors expression. Hence, increased activity of one or more thrombogenic agents could modify implantation process. To improve pregnancy outcome after assisted reproduction the type, dose and beginning of effective treatment for imbalances in expression of haemostatic proteins at the time of implantation needs to be established.

4. LMWH action in implantation beyond anticoagulant effects

The role of heparin in assisted conception in improving outcomes in women with inherited or acquired thrombophilia has been thought classically to be prevention of thrombosis in relation to implantation and placental development. But there is, potentially, a much wider role for heparin in assisted conception due to its ability to interact with a wide variety of proteins which have been involved in implantation process [83,84]. Heparin's influence on the physiological processes of implantation and trophoblast development, due to interaction with this growth factors and adhesion molecules responsible for embryo adhesion and invasion could influence the assisted conception entirely. Several lines of evidence from *in vivo* and *in vitro* studies suggest a beneficial effect of heparin on embryo implantation through interactions with several adhesion molecules, growth factors, cytokines and enzymes such as matrix metalloproteinases [83]. This finding clarified non-related with coagulation beneficial effect of heparins upon implantation processes.

L-Selectin. Interaction between adhesion molecules on trophoblast and endometrial cells is mediated by lectins, termed selectins. Three different selectins have been identified: P-, E- and L-selectin - which recognize and bind to crucial carbohydrate determinants on selectin ligands. On the blastocyst side, strong L-selectin staining has been observed over the entire embryo surface corresponding to oligosaccharide-based ligands on the maternal side which are up-regulated during the window of implantation [85]. The selectin adhesion system may therefore constitute an initial step in the implantation process. Heparin may have negative effect on selectin-mediated cell adhesion. The inhibitory effect is molecular weight dependent: Tinzaparin, which is known to have about 22–36% fragments greater than 8 kDa, also significantly impaired L-selectin binding, while, enoxaparin with 0–18% fragments > 8 kDa did not impact on L-selectin expression [86]. Therefore, care with the choice of LMWH depending from molecular weight would be required, as the use of UFH or a LMWH with a high contribution of large fragments could impair selectin expression and implantation.

Glycoproteins. E-cadherin, a glycoprotein connected with cell-to-cell adhesion is expressed by a variety of tissues including endometrium. The suppression of E-cadherin expression is associated with disruption of cell-to-cell adhesion and acquisition of invasive growth [87]. E-cadherin is up-regulated by estradiol via the estrogen receptor beta and down-regulated by progesterone [88]. Down regulation by increased progesterone levels during luteal phase of cycle facilitated trophoblast invasion. Heparins, particularly enoxaparin have been shown to down-regulate decidual E-cadherin expression [89], thereby potentially explaining the observations that LMWH can promote extravillous trophoblast differentiation

4.1. Local growth factors

Heparin-binding (HB) epidermal growth factor (EGF)-like growth factor functions as a mitogen and is potent survival factor during stress [90]. It has been shown that cells expressing the transmembrane form of HB-EGF adhere to human blastocysts and HB-EGF acts like a potent growth factor for enhancing embryo development to blastocyst and mediation of embryo hatching [91]. Separately, in cell culture was found enhanced differentiation and invasive activity of first trimester trophoblast in presence of HB-EGF. HB-EGF activation is heparins- dependable and therefore, LMWH may potentiate HB-EGF function [92].

Insulin-like growth factors I (IGF-I) and II (IGF-II) are potent mitogenic and differentiation-promoting growth factors and are also implicated in implantation and fetal development [93]. Importantly, they have their activity modulated by glucoseaminoglycans, in particular heparins [94]. Heparin and LMWH increase free IGF-I in a dose-dependent manner and so facilitated implantation [95].

4.2. Cytokines

Transforming growth factors (TGF) β 1 to 3 are expressed both in endometrial and trophoblast cells, and have been shown to inhibit trophoblast proliferation and invasion [96]. LMWH inhibits TGF- β 1 expression, attenuates collagen and fibronectin deposition and assists trophoblast invasion [97].

Leukaemia inhibitory factor (LIF), is known to regulate differentiation, proliferation and survival of various cells in the embryo as well as in the adult. It also has been found to enhance the blastocyst formation and hatching [98]. To date, no studies have examined the interaction of LIF and heparin, although the given interactions of HB-EGF and TGF- β 1 with heparin, potential up-regulation of LIF expression is feasible with experimental analysis urgently required [85].

The presence of **Interleukin-1 (IL-1)**, a pro-inflammatory cytokine in the site of implantation increases endometrial epithelial cell β 3 integrin expression with an improved blastocyst adhesion [99]. The effect of LMWH on trophoblast or blastocyst IL-1 expression has not been examined but the found increase of IL-1 expression in activated leukocytes again raising the possibility that modulation of integrin expression may be possible [100].

Interleukin-11 (IL-11) is another pleiotropic cytokine which functions as a hematopoietic growth factor and immunoregulator exhibiting anti-inflammatory effects by regulating immune effector cell function but has additional positive roles in decidualization [101]. The augmentation of IL-11 signalling and induction by heparin may prove to be beneficial both in terms of implantation and placental development [102].

IL-6 in addition to its roles as adipokine, which regulates the acute phase response and haematopoiesis, is also implicated in reproduction [103]. The effect of heparin on endometrial IL-6 production is not known but LMWH stimulates IL-6 production by peripheral blood and also enoxaparin had identical effects to recombinant IL-6 in reducing embryonic absorption in a pro-abortive murine model [104].

Granulocyte-macrophage colony-stimulating factor (GM-CSF) stimulates proliferation and differentiation of myeloid precursors into several cell types, but it is also an important determinant of pregnancy outcome: the factor actions as an immune-regulatory agent contributing to maternal immune tolerance of the fetal-placental tissues, and as a trophic growth and viability factor in preimplantation embryo development and regulation of placental morphogenesis [105]. LMWH is also capable of binding to GM-CSF [106]. Again although direct evidence for heparin is lacking, a positive effect on this pathway is possible.

Matrix metalloproteinases (MMPs). MMPs are a family of 22 endopeptidases capable of degrading all components of the ECM and are important mediators of cell behaviour including cell-matrix and cell-cell interactions. In vitro studies suggest that successful implantation and placentation result from the balance between secretion of MMPs from the trophoblast and their inhibition by their natural antagonist tissue inhibitors [107]. The gene knockout studies in mice suggest that MMP-9 is critical for implantation [108]. MMP-9 is known to degrade collagen IV, the main component of the basement membrane, and in conjunction with MMP-2 may enable the invasion of trophoblast cells through the decidua and into the maternal vasculature [109]. Although divergent effects of heparin on MMPs have been observed, LMWH in therapeutic doses has been shown to induce trophoblast MMP-2 and MMP-9 transcription and protein expression. Therefore, LMWH appears capable of improving the invasive capacity of trophoblast cells by regulating MMP degradative capacity [110].

5. Dose and initiation of LMWH therapy in ART according recognized thrombophilic mutations

5.1. Needs of anticoagulant therapy

No guideline suggests a type of pharmacological non-hormonal support for women who have experienced several ART failures with or without thrombophilic factors presence. The possibility that implantation failure depends on hypercoagulability state cannot be ruled out because of a relatively rare investigation of embryo and male partner thrombophilic status. But in IVF/ICSI procedures where embryo aneuploidy, thyroid autoimmunity [111] and other immunological factors [112] are eliminated, and thus high grade embryos are transferred in highly receptive endometrium, therefore inherited thrombophilia could constitute as a main etiologic reason in implantation failure. Also insufficient number randomized studies explore the needs of anticoagulant therapy in RIF and thrombophilia.

The first and last till July 2012 placebo-controlled, randomized trial to evaluate the efficacy of thromboprophylaxis using enoxaparin 40 mg/day in a cohort of 83 women with a history of three or more previous IVF failures, who had at least one thrombophilic defect, was published [113]. Patients who received LMWH for thromboprophylaxis had a significant increase implantation and pregnancy rates compared with the placebo controls (20.9 vs 6.1% and 31 vs 9.6%, respectively; $p < 0.001$ and $p < 0.05$, respectively). A significant increase in the live birth rate was observed in the heparin-treated group compared with placebo (23.8 vs 2.4%, respec-

tively; $p < 0.01$). This study was criticized for its “methodological weakness” and for heparin being used prior to demonstration of its efficacy in carefully designed randomized controlled clinical trials [114]. Disadvantages of this study are the availability in the study group of patients with acquired thrombophilic disorders such as lupus anticoagulant and anticardiolipin antibodies (ACL) which impedes inherited thrombophilia impact evaluation. A weakness is connected also with the insufficient patients’ number and not symmetrical appearance of inherited thrombophilic factors among LMWH – treated and non-treated groups.

Some other investigators found a positive impact of LMWH application in women with RIF: Urman et al. [115] found in a cohort of 150 women with two or more failed assisted reproduction treatment cycles, who were randomly assigned to receive 1 mg/kg/day of LMWH or no treatment from oocyte retrieval to 12th week, implantation rates 24.5 and 19.8% in the LMWH and control groups, respectively ($p = 0.33$), and live births 34.7 versus 26.7%, respectively ($p = 0.29$). These authors tendentiously excluded from investigation groups women with inherited thrombophilia with intention to avoid possible thrombotic events during implantation process as a result of thrombophilic factors presence.

An interesting study present Lodigiani et al. [116] who introduce LMWH therapy during the time of controlled ovarian hyperstimulation and discontinued on the day of β -human chorionic gonadotrophin application. The authors found an improved outcome of ART in thrombophilic women used 40 mg enoxaparin daily compared with a control group without LMWH (25% vs 13.5% pregnancy rate, p -ns). This study indirectly proved the needs for anticoagulant prophylaxis in thrombophilia presence.

In spite the lack of large randomized trials, an increasing number of clinics embark the use of LMWH prophylaxis in RIF, particular in inherited thrombophilia cases. American College of Chest Physicians (ACCP) Evidence-Based Clinical Practice Guidelines (9th edition, 2012) [117] do not recommend the routine use of LMWH in women with pregnancy complications and inherited thrombophilia, except for the cases of pregnancy after IVF with severe ovarian hyperstimulation syndrome (OHSS) where guidelines recommend a 3 months prophylaxis. A suggestion for the use of prophylactic or intermediate dose LMWH was done only for women carriers of FVL or FII 20120 G>A in homozygous variant who have a family history for venous thromboembolism (VTE). For pregnant women with all other thrombophilias, a close follows up of pregnancy without routine LWMH application has been recommended. The next large number investigation will display the proper place of heparins in implantation failure escape. Until then LMWH prophylaxis should be undertaken with caution, knowing the possible side effects of therapy according to long-term daily treatment. Recommendations for close follow-up of women with LMWH application was given to avoid bleeding, skin reactions, and heparin-induced thrombocytopenia [118]. Osteoporosis and osteoporotic fractures development was reported only in women with therapeutic dose of LMWH, who have taken more than 30 weeks during pregnancy [119]. All advantages and disadvantages of LMWH prophylaxis should be explicated to women treated with heparins.

5.2. Beginning of LMWH prophylaxis

No consensus concerning initiation of anticoagulant therapy in IVF cycle performance was established. Some authors start the intervention at 6 weeks of gestation following the confirmation of a viable pregnancy and using postulates for early stage of placenta development [120]. As was mentioned, another guideline [116] introduces LMWH therapy during the time of controlled ovarian hyperstimulation, which found improved pregnancy outcome after ART in women with thrombophilic mutations. If it estimates, according founded LMWH action during implantation period, the prophylaxis recommended beginning should be later than embryo transfer day [83, 84].

5.3. Dose regiment of LMWH

When it was referred to VTE events in non-pregnant populations, weight-based LMWH dosage regimens provide effective treatment of acute VTE without the need to monitor anti-Xa levels. In pregnancy, the volume of distribution and renal clearance increases, leading to a lower peak and shorter duration of anti-Xa activity, but with continued use the anti-Xa activity can become prolonged [121]. Therapeutic doses after ART may be indicated during pregnancy in women who present with acute VTE, those at high risk of recurrent thromboembolism and women with major cardiac disease at risk of arterial embolism [122]. The therapeutic dose is 1 mg/kg enoxaparin twice daily. The dose was based on their weight at presentation, rounded to the nearest 10 kg, and was not adjusted as the woman's weight increased during pregnancy. In other cases a prophylactic dose of enoxaparin, 40 mg once daily was recommended: in women with recurrent pregnancy loss with no history of VTE Brenner et al. [123] did not found advantage to increase enoxaparin from 40 mg/day to 80 mg/day (40 mg twice daily). They found similarly effective and safe the both dose regiments with a live birth of 84% and 78% in the enoxaparin 40 mg/day and 80 mg/day groups, respectively.

5.4. Duration and discontinuation of LMWH therapy

A part of authors discontinued LMWH therapy after the end of 3rd lunar month or in the end of controlled ovarian hyperstimulation [116]. ACCP Guideline recommends discontinuation of LMWH at least 24 h prior to induction of labor or cesarean section (or expected time of neuraxial anesthesia) rather than continuing LMWH until the time of delivery.

5.5. Monitoring of LMWH

Platelet blood count measure was recommended after the start of LMWH prophylaxis to avoid possible complication connected with heparin induced thrombocytopenia (HIT). A poor correlation was found with LMWH use and anti-Xa levels monitoring. Although that in prophylactic dose of enoxaparin range of 0.2 to 0.4 U/mL of anti-Xa activity was recommended. When therapeutic dose LMWH was used range of 0.77 to 0.86 U/mL was suggested [124].

Recently a new marker was introduced in monitoring of thrombosis risk patients. Platelet-leukocyte aggregates (PLA) are heterotypic cell complexes. The interaction between platelets

and leukocytes manipulates their function in the processes of hemostasis and inflammation [125]. The circulating PLA are a more sensitive marker of *in vivo* platelet activation than platelet surface P-selectin. PLA could be use as indirect gauge for LMWH therapy in high risk pregnancy complicated in inherited thrombophilia factors [126] because both women with combination of prothrombotic factors and women with single thrombophilic factor showed increased levels of whole blood PLA compared with control group [126].

6. Place of aspirin in RIF – Combined therapy with LMWH

As have been supposed, one of the causes of RIF may be found in impaired uterine perfusion [127]. It has been suggested that Acetylsalicylic acid (low dose aspirin <100 mg/d) may increase the uterine blood flow by inhibiting platelet aggregation and reducing vasoconstriction, possibly leading to a more favourable endometrium for embryo implantation [128]. Aspirin may also suppress the negative effects of prostaglandins on the implantation, such as the induction of uterine contractions or an inflammatory response [129]. Furthermore, aspirin improves the chance of a live birth in women with antiphospholipid syndrome with a history of recurrent miscarriage [130], although newer studies show that it is not effective in women with unexplained recurrent miscarriage [120]. Whereas some studies could not demonstrate any benefit in IVF outcome [131, 132], others reported an increase in pregnancy rate, sometimes with even statistically significant impact [133, 134, 135]. The aspirin influence on IVF outcome and in particular, the action connected with inherited thrombophilia, again is not elucidated, although Zhao et al. [81] found increased alphaV/beta3 integrin expression in the uterine endometrium in aspirin presence. Speculating from the above finding the polymorphism A1/A2 in beta3 subunits of integrin alphaV/beta3 could be related with impaired embryo adhesion but this should be confirmed in future studies. If genotypes A1/A2 and A2/A2 of PL A1/A2 are associated with enhanced platelet thrombogenicity and so with increased risk of implantation failure, an inclusion of prophylactic antiaggregant therapy [136] to prevent poor pregnancy outcome should be considered. Later on, the detecting of the genotype for PL A1/A2 could be important, because of the raised data for acetylsalicylic acid and other antiplatelet agents' resistance [137, 138] in the presence of A2 allele.

Another important gap in the literature was the question when to start and when to stop aspirin and how long should aspirin be given. Usually authors start aspirin just after pregnancy establishment, continue throughout pregnancy and cease 3 weeks before the delivery [139]. Others begin therapy along with the initiation of controlled ovarian hyperstimulation.

In conclusion, in a recent systematic review [140] the authors' conclude that the use of low-dose aspirin for women undergoing IVF could not be recommended due to lack of adequate trial data. Only for women who fulfill the laboratory criteria for antiphospholipid antibody (APLA) syndrome and meet the clinical APLA criteria based on a history of three or more pregnancy losses, ACCP recommends [117] antepartum administration of low-dose aspirin, 75 to 100 mg/d combined with prophylactic dose LMWH.

7. Metformin, thrombophilia and pregnancy outcome after IVF

Metformin, an oral biguanide insulin-sensitizing agent, acts by inhibiting hepatic glucose production without hypoglycaemia because it does not increase the insulin secretion [141]. This drug enhances the effects of insulin on glucose uptake in skeletal muscles and adipocytes, decreases intestinal absorption of glucose and leads to lowering androgen production in PCO patients [142]. Widely it is known that decreased androgen production after using metformin improves ovulation as well as pregnancy outcome in women with polycystic ovaries [143]. Insulin has a direct effect on ovarian steroidogenesis by the stimulation of androgen production by the thecal cells. Hyperinsulinaemia can inhibit the insulin-like growth factor-1 (IGF-1)-binding protein production by the liver with a subsequent increase in IGF-1. This IGF-1 in conjunction with LH could stimulate ovarian thecal cell androgen production. So, decreasing insulin resistance by metformin recovers menstrual cycle, ovulation and increased pregnancy rate in PCOS. Independently, metformin has a direct inhibitory effect on androstenedione production in human ovarian thecal-like androgen-producing tumour cells [144]. These findings could explain the mechanism for the decrease in androgen levels with the use of metformin. Along with the androgen-decreasing action, metformin was found to reduce plasma plasminogen activator inhibitor-1 (PAI-1) concentration in both diabetic and non-diabetic obese subjects. In vitro studies found dose-dependently decreased PAI-1 production under both basal and interleukin-1 beta-stimulated conditions after metformin application [145]. A large cohort study of PCOS patients has found a relation between the increased prevalence of 4G allele of PAI-1 4G/5G polymorphism and elevated PAI-1 levels [46]. The correlation between the reduction in the plasma insulin and PAI-1 levels after treatment with metformin in early pregnancy, suggests an early initiation of this therapy in COH-IVF cycles. This approach should be considered especially in PCO patients with insulin resistance as well as in patients carriers of 4G allele (homo- and heterozygous state).

If further studies support the reduction of miscarriage rate by metformin, the latter will become the only known treatment that appears to decrease the poor pregnancy outcome in PCOS women to date. The impact of using metformin as an adjunct to controlled ovarian stimulation and IVF also is promising and requires additional investigation particularly in women with of increased PAI-1 levels due to 4G/5G polymorphism.

Correlation between the increased PAI-1 levels and the suggestive findings of improved pregnancy outcome with metformin use supports the proposed pathogenic role of PAI-1 in early pregnancy complications. That forces extended metformin application in connection with increasing amount of data support for the safe use of metformin throughout pregnancy [147, 148], and because of the absence of reports for any drug's related teratogenicity effects [148], and also very low side effect incidences.

8. Other anticoagulant drugs instead LMWH

Fondaparinux is a synthetic pentasaccharide, a direct Factor Xa inhibitor. Fondaparinux forms a high affinity binding site for the anti-coagulant factor antithrombin III (ATIII). Binding to ATIII has been shown to increase the anti-coagulant activity of antithrombin III 1000 fold. In contrast to heparin, fondaparinux does not inhibit thrombin. Limited case-reports studies have shown effective treatment of inherited thrombophilia (Protein S deficiency) using Fondaparinux in case of heparin intolerance [149]. In situations where pregnant women cannot receive LMWH, fondaparinux may be a valuable alternative during pregnancy. Fetal safety is always an issue when considering maternal pharmacologic treatment. In a recently published report, a minor transplacental passage of fondaparinux was found in vivo [150] but until larger scale studies are available, the use of fondaparinux in pregnant women should be limited to those patients with either severe allergic reactions to heparin, or eventually to those with HIT.

Lepirudin and bivalirudin are anticoagulants, with parenteral action that functions as direct thrombin inhibitors. Direct Thrombin Inhibitors are recombinant derivats of hirudin. Lepirudin may be used as an anticoagulant when heparins (unfractionated or LMWH) are contraindicated because of heparin-induced thrombocytopenia. For pregnant women, ACCP [117] suggest limiting the use of fondaparinux and parenteral direct thrombin inhibitors to those with severe allergic reactions to heparin who cannot receive danaparoid.

Dabigatran, an oral anticoagulant from the class of the direct thrombin inhibitors does not require frequent blood tests for international normalized ratio (INR) monitoring while offering similar results in terms of efficacy like indirect anticoagulants. Clinical experience is lacking in populations for whom anticoagulants are routinely used, such as patients with pregnancy-associated thrombosis.

Rivaroxaban and apixaban are oral anticoagulants, the first available orally as active direct factor Xa inhibitors. Rivaroxaban is well absorbed from the gut and maximum inhibition of factor Xa occurs four hours after a dose. Rivaroxaban is a highly selective direct Factor Xa inhibitor with oral bioavailability and rapid onset of action. ACCP [117] recommends avoiding the use of oral direct thrombin (dabigatran) and anti-Xa (rivaroxaban, apixaban) inhibitors during pregnancy needing thromboprophylaxis.

9. Closing remarks

The maternal risk during pregnancy was increased with increments in the rates of recurrent pregnancy loss leading to placenta praevia, cervical incompetence, and consequently need for Caesarean section. All of these conditions are being increasingly recognized as having their origins in the first trimester. The abnormal implantation and trophoblast development have had the key role in these severe obstetrics complications. So endorsement of complicated due

to inherited thrombophilic factor presence IVF achieved pregnancy in its very early stage of development could improve maternal as well as fetal outcome.

A couple of studies have shown a significant increase in the implantation and pregnancy rates after IVF procedures when use LMWH compared with those who did not. Although the process of implantation is not well understood, anticoagulant therapy is now being examined as a preventative measure for women undergoing IVF. Given the increased risk of adverse outcome associated with ART pregnancies, clinics commence to use LMWH, again based on biological plausibility rather than evidence of efficacy. Presence of thrombophilic factors such as FII 20210 G>A or polymorphisms 4G/5G in PAI-1 is desirable condition for LMWH prophylaxis even immediately after embryo transfer. Distinctly, presence of 4G allele in PAI-1 gene is suggestion to metformin complement also not only in PCO patients with insulin resistance but in all 4G allele carriers. In FVL carriers, the postpone of LMWH could be considered till positive pregnancy test, because of suspicious positive effect of the mutation on the implantation process. A ten-time increased dose of folic acid supplementation should be applied even before the start of COH in MTHFR 677 TT genotype carriers to ensure not only better implantation chances but also improved follicular development during ovarian stimulation. Despite the found increased alphaV/beta3 integrin expression in the uterine endometrium in aspirin presence and separately raised acetylsalicylic acid resistance in beta3 polymorphism A1/A2 presence, the need for application and dose adjustment in the polymorphism appearance is still not clarified. An upcoming prospective randomized trial on LMWH and other supplement therapy would support scientific evidence for future clinical applications of medicines possibly related with after-IVF implantation outcome in inherited thrombophilia presence.

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