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Evidence-based Update: Immunological Evaluation of Recurrent Implantation Failure

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Abstract

Recurrent implantation failure (RIF) is generally defined as the failure to achieve clinical pregnancy after the transfer of at least 6 good quality embryos in fresh or frozen IVF cycles or of at least 4 embryos in two egg donations. Patients with RIF represents an heterogeneous group in which diverse immunological or inflammatory factors have been described, representing globally the more frequent known causes, including antiphospholipid syndrome or autoimmune thyroid disease, among others. However, there is a lack of evidence-based reviews focusing on inflammatory RIF (iRIF). We performed a systematic review of the published literature on iRIF from 1988 to July 2016, in order to build an updated evidence-based classification of immunological diagnostic tests and assigned recommendations accordingly. This review intends to offer a useful immunological workout of women suffering RIF from a multidisciplinary approach.

Keywords: Recurrent implantation failure; *In-vitro* fertilization; Embryo transfer; Anti-phospholipid syndrome; Thyroid autoimmunity; Celiac disease, Mother/foetal allorecognition

Recurrent Implantation Failure Associated to Immunological or Inflammatory Factors

Definition and prevalence of recurrent implantation failure

To date there is a lack of a clear-cut consensus on the definition of recurrent implantation failure (RIF), which impacts on the absence of reliable data about its incidence and prevalence [1]. In the clinical practice, we can only be certain that a woman is suffering implantation failure in the setting of *in-vitro* fertilization (IVF), given that natural implantation failure may occur unnoticed.

There is a wide variability in the definition of RIF. Three generic definitions can be considered according to: the number of unsuccessful assisted reproduction treatment cycles; the number of embryos transferred; or a combination of both factors [2]. RIF is generally defined as the failure to achieve clinical pregnancy after the transfer of two good quality embryos, in at least three fresh or frozen IVF cycles/embryo transfers (6 embryos in total) or in at least two egg donations (EG, i.e. 4 embryos in total) [3]. Some studies define RIF as the failure to achieve pregnancy following repeated IVF cycles (2 to 6 IVF cycles, in which at least 10 high-grade embryos were transferred to the uterus [4]. Considering that embryo quality is closely related to maternal age, some definitions will only consider failed IVF transfer cycle in women younger than 40 years [5].

The incidence of pregnancy loss after natural implantation is high, estimated from 25% to 40% [6]. Of the total number of pregnancies that are lost, 75% represent a failure of implantation and are therefore not clinically recognized as pregnancies [7]. Failed implantation represents the major limiting factor in assisted reproduction. Besides, the supporting evidence of immunological or inflammatory causes of RIF (from here referred as iRIF) has not been re-appraised by the current guideline consensus [4], despite growing new evidence of the involvement of immunological alterations on pregnancy outcome. There are no existing evidence-based guidelines focusing on immunological factors of iRIF. This review intends to provide an updated picture of the causes and factors contributing to iRIF and to classify them according to the current evidence for the diagnosis of couples with RIF.

Causes of RIF

The most common causes of RIF are generally classified as maternal and/or embryological factors [4]. Maternal factors include: uterine anatomical anomalies; endometrium pathology resulting in altered endometrial receptivity; states of hypercoagulability; and immunological factors (iRIF). Considering embryological factors, it is important to note how good quality embryos are defined. Routine assessment is usually done based

on morphological factors but not on genetic embryo study. Indeed, studies on pre-implantation genetic diagnosis (PGD) refer up to 67% of aneuploidy embryos in patients with RIF [8].

The immune system plays a key role in maternal-placental-foetal cross-talk, embryo development and tropism, normal implantation and placentation and at all phases contribution for pregnancy success [9]. The innate immunity has a major representation at materno-foetal interface, most immune cells (70%) being CD56+ natural killer (NK) cells followed by 20% of monocytes [10]. Between 10% to 15% of all cells found in the decidua are lymphocytes, mainly regulatory T cells (Treg). Clonal expansions of allospecific uterine and peripheral Treg together with the proliferation of uNKs and DCs are involved in the maintenance of immune tolerance to the foetus [11,12].

Extravillous cytotrophoblast cells are derived from the villous core of the placental cotyledon. During early placental maturation, these cells take on invasive qualities, which may be regulated by a number of factors, including MHC expression patterns, integrin expression patterns, in situ oxygen tension and NK cell populations at this site. Foetal derived extravillous cytotrophoblast cells intimately contact maternal immune effector cells in the lacunae, thereby exposing the foetus to potential MHC-restricted recognition. Human placenta is hemochorial, and at least four different points of direct contact between maternal blood and embryo-derived cells have been reported.

In this review, we will focus on immunological factors that are related with iRIF and the current available evidence.

Methodology

Methods used to select the evidence

1. Hand-searchers of published literature
2. Searches of electronic databases

Description of methods

The workout panel of immunological tests that should be performed face to a couple with RIF has not been clearly established. In the last years, there is growing evidence on the role of several immunological factors that may be involved in the pathophysiology of RIF. We sought to determine the degree of evidence based on the following points:

1. Framing the question: Has this specific immunological test been associated with iRIF?
2. Identifying relevant publications through wide search of medical/scientific databases (Medline, Cochrane Reviews, Ovid).
3. Assessing study quality
4. Summarizing evidence and interpreting finding. Each immunological parameter was categorized according to the quality of the supporting evidence and assigns strength of recommendation (Table 1 and Table 2).

Table 1: Categorization of evidence and basis of recommendation and strength of recommendation.

Ia	From meta-analysis of randomized controlled studies
Ib	From at least one randomized controlled study
IIa	From at least control trial without randomization
IIb	From at least one other type of quasi-experimental study
III	From non-experimental descriptive studies, such as comparative, correlation, or case-control studies
IV	From expert committees reports or opinion or clinical experience of respected authorities or both
A	Based on Category I evidence
B	Based on Category II evidence or extrapolated from Category I evidence
C	Based on Category III evidence or extrapolated from Category I or II evidence
D	Based on Category IV evidence or extrapolated from Category I, II or III evidence

Table 2: Evidence-based categorization and strength of recommendation assigned for the immunological tests proposed for the evaluation of recurrent implantation failure.

Immunological factor	Evidence Grade	Strength of Recommendation	Number of patients	Ref.
Antiphospholipid syndrome	IIb	B	7064	20, 22, 25, 28, 29
Thyroid autoimmunity	IIb	B	3016	63, 64, 66, 67, 68
Celiac disease	III	C	2078	71

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pNK cell expansion	III	C	530	37, 38, 39, 40, 42, 44
uNK cells expansion	III	C	30	44, 53
Maternal KIR and HLA-C haplotype of the couple	III	C	420	54, 60
Blood pro-inflammatory dysbalance (ratio IFN-g/ IL-10; TNF-alpha/IL-10; Th1/Th2/Th17)	III	C	102	78, 79
Endometrial pro-inflammatory cytokine profile	III	C	394	80

iRIF: Are there Inflammatory or Immunological Contributing Factors?

According to the consensus evaluation of couples with recurrent miscarriages (RM), the main cause of RM is indeed inflammatory or immune-based and the search for antiphospholipid antibodies is mandatory [13,14]. Other potential inflammatory or immunological causes of RM, such as uncontrolled autoimmune thyroid disease and diabetes mellitus, are also recognized. The potential contribution of these factors to the identification of iRIF remains to be established. RM, as well as RIF, are multifactorial disorders with complex pathophysiology [15].

Among humoral factors, it has been reported that recurrent miscarriage could be related with anti-idiotipic activity [16]. An adequate idiotipyc-anti-idiotipyc network is necessary to maintain blastocysts implantation and avoid the rejection against trophoblast and this activity play a role in ensuring a correct uterine contractility [17]. This ability to bind idiotypes is related to certain flexible molecules, such as IgG1 and IgG3. It is worthy to note, that IgG1 and IgG3 deficiencies has been related with recurrent spontaneous abortion patients. So this situation could turn out in an idiotipyc-anti-idiotipyc network unbalance and lead to impair in the maternal-fetal tolerance indeed. These patients were treated with low dose immunoglobulin (IVIg) with a high successful pregnancy rate [18].

Here we present current evidences supporting the pathophysiological role of immunological factors in RIF occurrence:

Antiphospholipid syndrome

is an autoimmune and multisystemic disorder characterized by vascular thrombosis and/or pregnancy morbidity in association with the presence of circulating antiphospholipid antibodies (aPL) [19]. Antiphospholipid antibodies (aPL) are a heterogeneous group of antibodies binding to several phospholipids (phosphatidylserine, cardiolipin, phosphatidylinositol) and phospholipid-protein complexes. The proteins, also called cofactors involved are: 2GPI, prothrombin and activated protein C. In clinical practice, anti-cardiolipin (aCL), anti-2 glycoprotein I (2GPI) both IgG and IgM antibodies, and lupus anticoagulant (LA) are evaluated, although other aPL may play a relevant role in the setting of OAPS, such as anti-annexin A5 [20], plasminogen-activator inhibitor-1, plasmin, annexin A2 and thrombin [21]. In particular, 2GPI and prothrombin account for more than 90% of all the antibody binding activity [19]. A recent review of twenty-nine cohort and case-control studies of aPL antibodies in RIF women (encompassing 5,270 patients) disclosed a prevalence of antibodies in infertile patients from 0%–45% and 3-fold higher risk of implantation failure in aPL positive patients [22].

In the last years, the β2-GPI role has become more significant in clinical practice since it has been identified as the most important antigen in APS. It is a highly glycosylated protein present in the plasma, and belongs to the complement control protein (CCP) superfamily. Normally, β2-GPI in plasma circulates in a circular conformation with a low affinity for anionic surfaces and the epitope for the antibodies is shielded from plasma [23]. When it encounters cells that expose anionic phospholipids on their surface like endothelial cells, monocytes, platelets and trophoblast cells, β2-GPI will bind to these phospholipids, undergoing a conformational change. This change exposes the epitope for the antibodies, and stabilizes β2-GPI in its hockey stick-like conformation. The binding of the autoantibodies results in the generation of bivalent complexes that have much stronger affinity for anionic phospholipids expressed on these cells. There are many different receptors that bind β2-GPI: toll-like receptor (TLR)-2, TLR4, annexin 2, apolipoprotein E receptor 2 (ApoER2) and glycoprotein Ib alpha (GPIbα) [19].

Several mechanisms have been proposed to explain the pathogenic involvement of aPL in OAPS. One of them states that they can bind to receptors (ApoER2 and GPIbα) present in the platelet surface and promote platelet activation and aggregation, inducing thrombosis and thrombocytopenia that is frequent feature of APS [24]. Another mechanism is the interaction with endothelial cells by binding to several receptors as annexin A2, and thus promoting expression of adhesion molecules (ICAM-1 and VCAM-1) and monocyte adhesion. In addition, there is a tissue factor (TF) up-regulation that could contribute to the prothrombotic effects of aPL. Moreover, vascular endothelial growth factor (VEGF) may stimulate TF expression in monocytes [25].

From a clinical point of view, aPL are reported as the most frequent acquired risk factors for recurrent pregnancy loss (RPL). Different pathogenic mechanisms have been suggested to play a role in OAPS manifestations: first, the occurrence of thrombotic events. Intraplacental thrombosis with maternal–foetal blood exchange impairment was suggested to be the main pathogenic mechanism in RPL. aPL may induce a procoagulant state at the placenta through several mechanisms. One of them is the breakage of the anticoagulant annexin A5 shield on trophoblast by aPL, mainly by anti-β2-GPI antibodies. Rand et al. reported that women with aPL have significantly lower distribution of annexin A5 covering the intervillous surfaces of their placentas

in comparison with normal controls [26]. Second, defective placentation is a relevant pathogenic mechanism in APS. In addition to thrombosis, aPL binding to trophoblast leads to cellular injury, apoptosis, inhibition of proliferation and syncytia formation, decreased human chorionic gonadotropin (hCG) production and defective invasiveness. The high expression of β2-GPI on the trophoblast cell membranes that binds to phosphatidylserine, may explain the aPL/anti-β2-GPI antibody placental tropism. And third, inflammatory local events also take place that induce changes in the maternal immune response from a Th2 and regulatory (Treg) responses towards a Th1 and Th17 inflammatory responses [27].

The APS may occur alone (primary APS), or in association with an underlying autoimmune connective tissue disorder (secondary APS) [28]. Although the prevalence of aPL in RM is clearly established, and numerous studies demonstrating their role in iRIF [29], there is still a lack of consensus [30]. Moreover, we and others have proposed the inclusion of RIF as clinical criteria for APS [28,31], since aPL are pathogenic antibodies and the implantation failure could have analogous pathophysiology to that of an early miscarriage.

Expansion of peripheral natural killer cells

Peripheral blood NK (pbNK) cells consist of different subsets, of which the CD16+ / CD56dim cytotoxic NK cells are the most abundant (60% to 95%) [32]. Multiple studies have associated an altered proportions or function of pNK cells to RPL, although their role in this condition remains to be elucidated. Blood NK cells and NK cells subsets may represent a surrogate marker of an underlying systemic pro-inflammatory status in a subgroup of patients with RM or RIF, as first described for OAPS [33] and more recently, dysfunction of these cells subsets has been suggested to be associated to juvenile rheumatoid arthritis, type I diabetes and autoimmune thyroid diseases [34,35] or even NK cells expansion in the neonates from mothers with autoimmune thyroiditis [36].

Peripheral natural killer (pNK) cells have been associated with RPL, infertility and preeclampsia. In a large series study of patients and controls, we showed that pNK cells' subsets did not vary significantly during the menstrual cycle and that the cut-off level of 13% of cytotoxic pNK cells and age 35 was significantly associated with RM and RIF and defined a subgroup of patients of immunological alterations [37-40]. Other authors have reported that elevated numbers of NKT cells in peripheral blood also correlate with recurrent pregnancy loss or implantation failure [41-43].

Deregulation of uterine natural killer cells:

The uNK cells are the most abundant immune cells infiltrating the implantation site and remain in high numbers during early gestation [44]. These cells make up 70-90% of uterine lymphocytes [45]. The main population of uterine NK (uNK) cells is CD56bright/CD16- cells with low cytotoxic capacity while high producers of cytokines, chemokines and growth factors, with a quite different phenotype of pNK cells [46]. The primary role of uNK cells is to promote the uterine vascular changes for maximizing maternal blood flow through the placenta and endometrial invasion. Several studies have shown that NK cells play important roles during pregnancy in embryo implantation, immunosurveillance, angiogenesis, remodelling of the spiral arteries to utero-placental arteries, supporting proper trophoblast and placental growth and by producing immunomodulatory molecules [47,48]. This primarily occurs via the secretion of cytokines and chemokines including IL-8, VEGF, SDF-1, and IP-10 that are involved in tissue remodelling, trophoblast migration, and/or neo-angiogenesis and placentation. The cytokine profile in decidual NK cells is quite different from that in peripheral blood. In early pregnancy, decidual CD56bright NK cells produce mainly TGF-beta (NK3 cells), IL-10 (NKr1 cells) and in smaller quantities IFN-gamma (NK1 cells), IL-4, IL-5 or IL-13 (NK2). Conversely, in peripheral blood in early pregnancy women, the main population is IFNϒ-producing NK1 cells and secondary IL-10. And finally, in non-pregnant women, peripheral blood NK cell produce mainly TNF alpha and IFN gamma [49]. In the contrary, alterations in uNK cells' profile may induce increased cytolytic activity, inhibition of placental hCG secretion, complement activation, cytokine imbalance, failure in the generation of Th2-type responses favouring Th1/Th17 responses. There is controversy whether uNK proportions can be used as a biomarker of recurrent pregnancy failure [50,51].

There may be functional similarities among HLA-C, HLA-E, and HLA-G that promote recognition by immune cells at the maternal–foetal interface. Interactions of these class I molecules with the abundant NK-like immune cells at the maternal–foetal interface may avert NK cell receptor-mediated killing of extravillous cytotrophoblast. Interactions of trophoblast HLA-C, HLA-E, and/or HLA-G with surface receptors on either NK cells (KIR ligands) or other maternal immune effector cells may modulate immune cell cytokine expression profiles [52] The expression by foetus-derived cells of any or all of the trophoblast MHC class I products could also promote essential decidual and vascular invasion.

MThese receptors regulate the activation and function of the NK cell and they may be grouped in inhibitory receptors (some killer immunoglobulin-like receptors, KIRs like KIR2DL1 or NKG2A between others and activating receptors (NKp46, NKp30, NKp44, NKG2D, DNAM-1, NKp80, 2B4 and NTBA) [48]. Fukui et al. [46] reported that the percentage of CD56 bright/IFNγ+/ TNFα+ (NK1) cells was significantly higher in women with RPL compared with healthy controls. The expression of NKp44 between CD56dim cells and CD56bright cells in women with RIF was significantly different. The expression of NKp44 on CD56 bright cells was up regulated compared with controls. This suggests that RIF may be associated with high NK cell cytotoxicity. In women with reproductive failure such as RM, RIF and preeclampsia, a lower expression of NKp46 on pNK cells as well as on uNK cells has been reported. This leads to higher production of TNFα and lower production of IFNγ, inducing abnormal vessel remodelling [46].

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Allorecognition: MHC molecules and NK cells receptors

Although associations between HLA typing and RPL have been suggested, very few investigations have specifically addressed the role of trophoblast MHC class I molecules (HLA-C, HLA-E, and HLA-G) in RIF. Hiby et al. proposed that placentation is regulated as a result of interactions between maternal killer Ig-like receptors (KIRs) expressed by uNK cells and their ligands, HLA-C molecules, displayed by invading foetal trophoblast cells [54,55].

There are two main trophoblast populations: the villous trophoblast, which is in contact with maternal blood, and the extravillous trophoblast (EVT), which invades the decidua, spiral arteries, and myometrium. Villous trophoblast does not express HLA class I or class II molecules, while EVT is characterized by the expression of HLA-C and of the non-classical HLA-class I molecules, HLA-E and HLA-G [10,48]. All HLA-C allotypes are ligands for the highly variable KIRs expressed by uNK cells. HLA-E, is also a ligand for most uNK cells because it binds the CD94/NKG2A receptor expressed by >90% of uNK cells [56]. HLA-G binds to members of the LILR family (LILRB1 and LILRB2) that are found on decidual macrophages and a minority of uNK cells [57]. Trophoblastic cells are the prime source of HLA-G and play a key role in implantation by modulating cytokine secretion to control trophoblastic cell invasion and to maintain a local immunosuppressive state. Soluble HLA-G (sHLA-G) circulates in maternal blood during pregnancy. It has been shown that women with pre-ovulatory low sHLA-G levels appear to be on risk for early abortion after IVF [58].

As trophoblast cells do not spontaneously express neither classical HLA-A and HLA-B nor MHC class II products, it has been suggested that an aberrant expression of these molecules might result in an adverse maternal immune response to the implanting foetus. Data supporting this hypothesis remain limited. Alloimmune interaction of KIRs on the maternal uNK cells and paternal HLA-C on trophoblast may determine alteration in the implantation process [55]. Certain combinations of maternal KIR ligands and paternal HLA-C can be unfavourable for placentation. Indeed, mothers lacking most or all activating KIR (AA genotype were at a greatly increased risk of preeclampsia and worse pregnancy success) when the foetus possessed HLA-C belonging to the HLA-C2 group [59].

It seems that successful implantation depends on a fine balance of NK cell activation and inhibition, which might be influenced by the KIR gene frequencies of the patients and their partners [45,60].

Untreated hypothyroidism

reproductive disorders ranging from abnormal sexual development to menstrual irregularities and infertility. In women of fertile age, thyroid autoimmunity is undoubtedly the most common cause of hypothyroidism and in most patients thyroid peroxidase antibodies are found [61]. At the cellular level, thyroid hormones synergize with FSH to exert direct stimulatory effects on granulose cell functions, including morphological differentiation. Thyroid hormones facilitate FSH-mediated LH/hCG receptor induction and progesterone secretion. Hence, the occurrence of gonadal dysfunction may further result from inadequate thyroid hormone availability at the level of the ovary [61]. Moreover, both gonadotropins and thyroxin appear to be necessary to achieve maximum fertilization rates and blastocyst development. Recently, Cramer et al. showed that TSH is a significant predictor of fertilization failure in women undergoing IVF [63]. These data support the important role of thyroid hormones in oocyte physiology.

A cut-off level of TSH 2.5 mIU/L was set after demonstrating lower implantation rates in women undergoing IVF with TSH above these levels compared with with TSH <2.5 mIU/L [64]. A recent study has highlighted the importance of positive anti-thyroid antibodies (ATA, anti-peroxidase and anti-thyroglobulin), with women with ATA showing lower fertilization rate, implantation rate and pregnancy rate and higher risk for abortion following IVF-ET when compared with those without ATA [65-67] compatible with previous study of this association. Treatment with levothyroxine can reverse such dysfunction and thus should improve fertility. Despite levothyroxine treatment, women with hypothyroidism have a significantly decreased chance of achieving a pregnancy following IVF compared to euthyroid patients [68]. This is also relevant in therapy decision making, even with normal hormone levels [69].

Undiagnosed coeliac disease

Coeliac disease (CD) is a common autoimmune condition characterized by a heightened immunological response to ingested gluten, with estimated prevalence rates in adults of 0.2-1% in the United States and Europe [70]. CD is a chronic immune-mediated gluten-dependent enteropathy induced by ingestion of gluten-containing products, characterized by intestinal malabsorption and subtotal or total atrophy of intestinal villi, which improves after exclusion of gluten from the diet. Current data illuminating the association between CD and RM are marginal [71]. Yet recent studies showed that there might be a correlation between the CD and RM and iRIF [37,71]. It seems that RM patients with CD might benefit from a gluten-free diet, which could be similarly recommended for iRIF [72].

Cytokine imbalance

Cytokines released at the feto-maternal interface play a central role in the survival of the foetus, not only by influencing the maternal immune system, but also by regulating angiogenesis and vascular development. For instance, a shift towards Th2 and regulatory T cell (Treg) in normal pregnancy, the expression of Fas ligand by trophoblast cells and the inhibition of complement activation, are crucial to ensure the tolerance at the maternal-foetal interface. In physiological pregnancies, CD4+CD25+FoxP3+Tregare increased, playing a critical role in maternal immune tolerance confronting to a semi-allogeneic foetal antigens and in embryo implantation. The mechanisms by which Treg may prevent allorejection are through the secretion of IL-10, TGF-, heme oxygenase isoform 1 (HO-1), indoleamine 2,3- dioxygenase (IDO) and leukaemia inhibitory factor (LIF) rather than decrease the Th1 cytokines [73]. In addition, there is also an appropriate balance between inhibitory signals (PD1/PDL1 co-stimulatory pathway, Stat3 and

TGF-1) and co-stimulatory signals (CD80 and CD86), involved in this tolerance [73].

The key role of Treg in preparing endometrium for implantation is supported by several studies in mice showing that Treg cell deficiency in the peri-implantation period, causes either implantation failure or embryo resorption son after implantation [74]. Recent data show that uterine DCs play a central role for successful implantation. The number uterine DCs increase at the implantation period and depletion of DCs results in severe implantation failure. DC depletion impairs uterine NK cell maturation, tissue remodelling and angiogenesis [75-77].

The strict model of the classical Th1/Th2 imbalance as a prerequisite for RM has been challenged by the finding that T cell cytokines are released not only by T cells, but also by NK cells and DCs, as well as a more accurate Th1/Th2/Th17/Treg model, and growing understanding that cytokines, chemokines and growth factors can develop different functions in the context of the environment in which they are released. Serum ratios of Th1/Th2-Treg cytokines or TNF-alpha/IL-10 and IFN-gamma/IL-10 producing T CD4 cells have been developed showing significantly higher levels in RPL patients with respect to controls [78,79]. More recently, an endometrial immune profile has been proposed to better understand dysregulations (mainly overactivation of pro-inflammatory cytokines IL-15, IL-18 and the TNF Weak inducer of Apoptosis (TWEAK) pathways and associated with the activation of the uNK cytotoxic receptor NKp46 and Th1 predominance [80].

Conclusions and Future Directions

Defining the evidence-based immunological studies is essential for the appropriate evaluation and management of couples with RIF. Although there are diverse factors converging in the outcome of IVF (causes of sterility, genetic alterations of the embryo, hormonal treatment combinations, technical procedures, other maternal factors, etc.), immunological as well as haematological or hormonal factors play a relevant role in successful gestation, as has been highlighted for the case of women with RM. There is an urgent need of further validating these data with clinical trials and of standardizing both the evaluation and interpretation of immunological tests. Now is the time for everyone, gynecologists, embryologists, hematologists, endocrinologists and immunologists, to work together more closely and to fully utilize one another's specific knowledge and experience, so as to come up with better answers to improve the outcome of pregnancy and the life-quality of couples undergoing this process. This will impact also on the reduction of IVF-related costs.

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