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What has to be pointed out in unexplained recurrent pregnancy loss research in the unsolved fields: lessons from clinic. An Italian RPL Unit experience.

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ABSTRACT
Recurrent pregnancy loss (RPL) is a controversial field both in research and clinical approaches. Despite the most recent guidelines (ESHRE 2017), an agreement in diagnostic work-up to apply in these patients, as well as in management and treatment, has not been reached, especially in unexplained RPL (uRPL). This is due to the lack of a strong evidence-based level in this field, since the discrepancies among the different RPL research groups, in terms of definition, etiological factors and management cannot lead to organize all the results in systematic reviews. Therefore, common shared cornerstones are required to homogenize research parameters, since the right interplay between clinical management and experimental approaches could lead to contribute in the development of stronger evidences. In this review, we highlight what has to be pointed out in RPL debated subfields and how the experimental approach is necessary to overhaul discrepancies in clinical management. The experience of our RPL Unit has been reported to show how research experience could contribute in modifying the clinical approach.

Keywords: Recurrent Pregnancy Loss; Diagnostic Work-Up; Clinical Management; Treatment

SOMMARIO
L' abortività spontanea ricorrente è un campo dibattuto sia dal punto di vista clinico che di ricerca. Non vi è infatti, un accordo sul tipo di work-up diagnostico clinico da applicare, né sul management o sulle modalità terapeutiche da adottare, in particolar modo nella poliabortività inspiegata. Le discrepanze, in termini di definizione, fattori eziologici riconducibili a tale patologia e di approccio clinico, riscontrate tra i diversi gruppi di ricerca, non permettono di raggiungere un livello di "evidence-based medicine", in quanto non è possibile organizzare i dati non omogenei in review sistematiche. Per tale motivo, risulta essenziale omogeneizzare i parametri di ricerca nel campo della poliabortività, per raggiungere livelli di evidenza maggiori. In questa review vengono evidenziate e discusse le principali discordanze evidenziabili nella letteratura scientifica internazionale sull' approccio alla poliabortività, confrontando tali problematiche con le evidenze raggiunte dai progetti di ricerca messi in atto nel nostro centro di poliabortività e, come tali risultati, possano contribuire a modificare l'approccio clinico nelle pazienti poliabortive.

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INTRODUCTION

Recurrent pregnancy loss (RPL) is a very debated and controversial field for researchers and clinicians. There is no agreement between different groups about clinical diagnostic work-up and management, as well as for personalized potential treatment. The lack of evidence for several aspects, such as etiological factors to investigate or therapy to apply, is due to the inability to perform systematic reviews or metanalysis: if we think that also RPL definition (2 or 3 pregnancy loss? Should they be consecutive? Should non-visualized pregnancy losses, biochemical and/or PULs, and not only intrauterine clinical miscarriages be included? What about molar or ectopic pregnancy? Should we talk about recurrent pregnancy loss or recurrent miscarriage?) is not shared by the several research groups in this field, it becomes clear that it will be difficult to reach an evidence-based medicine level in this disease, by comparing and putting together all different group' s studies.

BACKGROUND

1. Definition of RPL

a. Lessons from clinic

The most recent specific guidelines (ESHRE 2017, European Society of Human Reproduction and Embryology)[1]) defined RPL as the loss of two or more pregnancies before the 24th week of gestation, recommending a clinical evaluation on couple starting from the second pregnancy loss, since abnormal test results for factors defined as proven or probable causes of RPL occur with equal frequency in women with two or more pregnancy losses versus three or more: this is an example how suggestions that came from the clinic, which the gynecologist observes in the patients could be an "inspiration" for research and can lead to a modifications in the guidelines, by reaching an evidence-based level (the previous most accepted RPL definition referred to three or more consecutive losses).

Previous ESHRE guidelines and the RCOG Green Top Guideline, 2011 defined recurrent miscarriage (RM) as ***three or more consecutive*** pregnancy losses before ***20 and 24 weeks of gestation***, respectively[2-3], even if there was already an accepted trend in scientific literature to talk about RM from *two or more* spontaneous miscarriages, , since common etiological factors were found in women with two or three or more miscarriages[4]. This trend agreed with the ASRM (American Society for Reproductive Medicine) 2013 guidelines definition[5-6], in which RM is defined as two or more *failed clinical pregnancies, not necessarily consecutive*, before 20th week of gestation.

b. What to point out

ASRM refers to *failed clinical pregnancies* in its definition: in this case pregnancy has to be documented by ultrasonography or histopathological examination.

This concept is in contrast with ESHRE opinion, which considers non-visualized pregnancy losses (a broad spectrum condition including biochemical pregnancy losses and failed PULs – pregnancy of unknown locations) relevant for their negative prognostic value on subsequent live birth chances in unexplained early recurrent miscarriages (defined as two or more pregnancy demises before 10 week of gestational age)[7]. Therefore, according to ESHRE guidelines the right terminology to use is RPL, since RM referred only to intrauterine clinical miscarriages[1].

Furthermore, ESHRE guidelines 2017, ASRM 2013 Practice Committee and other authors have recently claimed that in defining RPL or RM, respectively, the pregnancy losses could be non consecutive, departing from the traditional definition.[8,5,9]. Conversely, a recent study has shown how only pregnancy losses following a live birth could have a negative prognostic value in subsequent pregnancies. A live birth could remove the negative impact on secondary RM patients' prognosis related to previous pregnancy losses. This could be explained by the immunological memory derived from a previous successful pregnancy of suppressing regulatory T cells in peri-uterine lymph nodes: these cells can suppress the immune response against fetal antigens[10, 11] and this mechanism is lacking in RM patients. RM patient, in fact, have an altered endometrial peri-implantation memory T cell phenotype[12]. This concept shows clearly that RM patients are a heterogeneous population that needs to be stratified[13].

This last point has also a great impact on another important distinguish which has to be done in RM definition, thus to have a better stratification of these patients and individualized treatment: primary or secondary RM. Primary RM is described as RPL without a previous ongoing pregnancy (viable pregnancy) beyond 24 weeks of gestation. RM is defined as secondary, if there was an episode of RPL after one or more previous pregnancies progressing beyond 24 weeks of gestation. As said before, secondary RM patients have different immunological background that can influence the future prognosis[10,11]. In addition

to memory Treg cells, TNF-α levels are higher in early pregnancy in secondary than primary RM, due also to a genetic background[14]. Furthermore, a higher rate of aneuploidy has been shown in secondary RPL: thus, these patients might benefit from preimplantation genetic screening (PGS)[15]. RM patients show an increased risk for obstetrical complications such as preterm delivery, fetal growth restriction, and gestational diabetes mellitus. With particular regard, primary RM patients are more prone to adverse pregnancy outcomes than women affected by secondary RM. A more specific surveillance in primary RM should be recommended for obstetrical complication detection at the earlier stage of their occurrences[16]. It is also important to take into account that the same study showed no difference in the proportion of positive diagnostic tests performed to evaluate RM patients, when primary and secondary RM groups were compared (Chromosomal abnormalities; Uterine anatomic defects, such as septum, unicornuate and bicornuate uteri, fibroids and polyps >1.0 cm in the uterine cavity, and Asherman's syndrome adhesions; Autoimmune disorders, Lupus anticoagulant (LAC); Anticardiolipin antibodies (ACA); Factor V Leiden mutation ; Mutations for the G20210A prothrombin (factor II) gene; protein C, protein S, antithrombin III; Thyroid function (TSH), Antithyroid peroxidase (TPO) and antithyroid antibody; Blood glucose level; Antinuclear antibody (ANA); Rheumatoid factor(RF)) (16). Another difference to consider is that secondary RM is likely associated to a particular immune response towards male-specific minor antigens, due to its association with specific HLA alleles (HY-restricting HLA class II alleles)[15, 17-19].

2. Risk factors

There is strong evidence that age is a risk factor for RPL: the risk of pregnancy loss increases after 40 years old. Also stress has been shown to be associated with RPL, because of psyco-neuro-endocrine-immunological connection, but there is no evidence that it can directly cause the pregnancy losses. Conversely, further studies are needed to reach the evidence-based to recommend the screening for endometritis or abnormal decidualization, that seems to be linked to RPL[1].

a. Lessons from clinic

Based on our observation in patient's care, we noticed that there was a higher incidence of ectopic pregnancy (EP) in RPL. A case-control retrospective study has been conducted by our group to investigate the potential association between RPL and EP: RPL patients are at increased risk of EP (with a higher incidence in primary RPL), which seems to be related to the higher number of pregnancies in this group compared to physiological pregnancies[20].

3. Etiology

Beyond its well-known etiological factors, such as uterine abnormalities, hormonal causes, parental chromosomal abnormalities, autoimmune diseases, antiphospholipid antibody syndrome and major thrombophilia, about 40-50% of the cases remains unexplained[1].

a. Lessons from clinic

The most common clinical condition detected in these patients is the presence of several slight aberrations in different aspects fields involved in RPL, by designing a threshold model as occurs in multifactorial diseases: in most of the cases there is not a major etiological factor that can directly explains and causes the pregnancy losses. Threshold model instead of a pie one, seems to be the most appropriate approach to RPL etiology,as already discussed in a paper by Christiansen O.B. et al. 2008[21].

UNEXPLAINED RPL

In the unexplained cases an aberrant immune response and oxidative stress at fetal-maternal interface have been hypothesized to be involved, as well as genetic/epigenetic mechanisms.

1. Aberrant immune response

During pregnancy the inflammation and immune tolerance processes are slightly tuned: the earliest phases of pregnancy are characterized by an inflammation process responsible for a proper implantation. This inflammatory stage should switch to a modulation of the immune response to guarantee a "non-rejection" of the semi-allogenic fetus. The decidualized stromal cells are the "gatekeepers" of this key immune switching mechanism at the fetal maternal interface, involving different immune cells, such as regulatory T cells, NK cells, M2 macrophages[22-25]. In the last stage of pregnancy, and especially in activating labour, an inflammation process is required again, therefore a new switching process is needed[26-32]. All aberrations in placentation process, and in turn in the modulation and tuning of the immune system, can lead to pregnancy complications, such as RPL[33-35]. RPL is an immune pathological condition characterized by an increased pro-inflammatory response (enhanced Th1, Th17 response with a higher secretion of TNF and IL-6; reduced TH2 response with suppressed production and release of IL-10, G-CSF, and less levels in Treg and uNK), predisposition to break autotolerance

(autoantibodies involved: ACA, ANA, anti ds-DNA, anti-TPO) and dysregulation of the maternal immune response to fetal or trophoblast antigens (molecules involved: KIR, HLA, MBL, HY)[36]. The immunotolerance processes at the maternal-fetal interface pass through also epigenetic modifications that involve, as emerged from recent studies, not only the fetal components and the trophoblastic tissue, but also and especially the maternal side, the decidua. In fact, it has been demonstrated, in animal models, how the lack of T cells effector accumulation at the decidua level is partly attributable to epigenetic silencing, at the level of the decidualized stromal cells, of the genes coding for the pro-inflammatory chemokines for T cell recruitment. Such epigenetic gene silencing mechanisms may be potential markers for targeted therapeutic applications in pregnancy complications, such as uRPL[37].

a. Lessons from clinic

According to the observation that RPL patients have a more pro-inflammatory prone immune system, we notice, looking at the anamnesis in human papilloma virus (HPV) infections, that RPL patients have a lower prevalence of HPV+ DNA tests, suggesting that this increased tendency in pro-inflammatory activation of the immune system could be protective for HPV infections[38].

2. Role of ANAs in uRPL

ANA are one of the most studied and discussed immunological factors in uRPL. The most recent guidelines of ESHRE on RPL recommend ANA assay only for explanation purposes, when no others of the RPL known causes are identified, since there is not yet a level of evidence-based medicine to be considered as a direct etiological factor and to be included in the diagnostic work-up of such patients: the prognosis linked to this factor remains unclear, and at present there are no specific therapies in this regard. The ANA assay could therefore be considered, according to these guidelines, since most of the case-control studies documents an association with uRPL, and there is some evidence from small prospective studies that the ANA status negatively affects the prognosis. Furthermore, it is not yet clear if the positivity of ANA can identify a subset of patients affected by RPL which could respond beneficially to different approaches of immunotherapy[1].

a. Lessons from clinic

ANA Human IgG induce abortion in mouse models in vivo by deposition of immunocomplexes in placental tissue.

The results from our study group in this regard, show that ANA are able to compromise the outcome of pregnancy and suggest a possible mechanism involved in the ANA-mediated damage. During pregnancy, the deposition of immune complexes by ANA on placental tissues may involve the activation of the complement, which is in turn associated with negative obstetric outcome. Histopathological placental staining of mice treated with ANA + IgG suggests that inflammation may contribute to placental lesions. The identification, through the coloration of hematoxylin-eosin of the inflammatory infiltrate in the placental tissue, collected from mice treated with ANA + IgG by RPL patients, suggests that the deposition of immune complexes leads to the activation of the complement and the chemotaxis of the inflammatory cells, probably involved in adverse outcomes of pregnancy[39].

Uterine flow indices, antinuclear autoantibodies and unexplained recurrent miscarriage.

We have also investigated ANA role in the modifications of uterine flow indices. To sum up, our main findings were: 1) ANA + women with uRM have a significantly increased PI (pulsatility index) throughout the menstrual cycle and a reduced FI (flow index) compared to all other categories of studied women; 2) ANA women with uRM have increased values of VI (vascularization index); 3) no differences were found in terms of VFI (vascularization flow index) and RI (resistivity index), regardless of the status of the uRM and/or ANA.

A potential explanation for these results could be that ANA in women with uRM are associated with a lower uterine blood flow intensity compared to physiology, as evidenced by the reduction of FI (blood flow intensity index). To counteract the impairment of uterine blood flow, these women may experience an increase in the pulsatility index of the uterine arteries, as evidenced by the increase in PI. This compensation mechanism could, in turn, explain why no differences were found in terms of VFI (index of both vascolarization and organ perfusion). These hypothesized mechanisms may not be found in uRM women who are ANA-, in which an increase in terms of VI has been detected. It is possible that in these women, in the absence of ANA, a greater vascular density (measured in terms of VI) in the uterus develops in response to other causative factors for RM, other than ANA. Alternatively, since there were no differences between VI in ANA- and ANA

+ uRM women and ANA- controls, it is also possible that an increased VI may represent an independent risk factor for RM[40].

Uterine and placental flow indices, antinuclear autoantibodies and uRPL: should they be investigated during pregnancy as potential related risk factors?

According to our recent study, higher values of VI and VFI were found in pregnant RPL ANA- women compared with RPL ANA + women who do not take LMWH therapy during pregnancy, reflecting a possible placental flow compromise correlated with the ANA status, characterized by an alteration of vascularization (VI) and organ perfusion (in terms of VFI). This reduced blood flow supply disappears in the same two groups treated with LMWH; therefore, it could be hypothesized that LMWH may improve vascularization and placental perfusion in women with uRPL ANA +, leading this subgroup of patients in the same clinical conditions as RPL ANA- women. The beneficial effects of treatment with LMWH, in addition to its well-known anticoagulant action, could be related to the innovative hypothesis according to which ANA can alter the blood supply at the placenta level for the developing embryo, without any thrombotic manifestation in placental vessels.

This is the first study showing an effect of LMWH on placental VI and VFI indices in relation to RPL and ANA status. According to our data, LMWH also seems to change the intensity of blood flow, measured in term of FI, since the RPL group treated with LMWH shows higher FI values than the untreated RPL group and the control group. The same differences were found among the three groups mentioned above, if only the ANA + patients are considered. Such data could strengthen the hypothesis that LMWH can potentially balance the insufficient placental blood supply in the RPL condition, especially in ANA + patients. This effect of LMWH could have an important clinical application, since it has been shown that the state of ANA +, especially in the first trimester, could have a negative influence on the outcome of pregnancy (41 data not yet published; Abstract from AGUI 2018 Congress, Rome).

Antinuclear autoantibodies and pregnancy outcomes in women with unexplained recurrent miscarriage.

Data from our studies showed that ANA status in non-pregnant uRM women was not associated with the number of previous abortions or with the gestational age (GA) in which abortions occurred. However, the proportion of women with primary uRM was higher in the ANA + than in the ANA-: this suggests that ANA can influence the mechanisms involved in the implantation and in the early development of pregnancy, since in women with secondary uRM, these mechanisms have occurred successfully at least once. Our results suggest a possible relationship between pregnancy outcome and ANA status and its modifications during pregnancy. In fact, all prospectively observed abortions occurred in women that were ANA + before pregnancy, which remained ANA + in the first trimester, while no abortions were observed in women that were ANA + before pregnancy, which were then found to be ANA- in the first trimester. The disappearance of ANA positivity at the beginning of pregnancy has been associated with a favorable outcome of pregnancy and this could indicate an improvement in systemic autoimmunity[42].

3. Role of Antiphospholipid antibodies (aPLs) in uRPL

Another interesting topic in the etiological factors of RPL is related to one of its certain causes: the antiphospholipid syndrome (APS). While there is a strong recommendation to look for ACA and LAC[1], such strong evidence has not been demonstrated for anti-ß2 glycoprotein I (aβ2GPI) antibodies: their association with RPL is possible, but it has not been demonstrated and no data are available on prognosis and possible therapy[1]. For this reason, this antibody could have a relevant scientific interest in the cases of uRPL. Indeed, screening for aβ2GPI antibodies could be considered in women with RPL to improve future knowledge. The results of a recent prospective study, although they need to be confirmed, suggest that a decrease in aβ2GPI (IgM) antibodies by anticoagulant treatments was related to a better outcome in pregnancy[43].

Furthermore, the role of aPLs in the pathogenesis of RPL is not yet clear: it has been suggested that they can act through complement activation[44] and, as a consequence, they can potentially cause pregnancy complications associated with APS (preeclampsia, placental dysfunctions, neonatal mortality)[45]. It is evident that the direct pathogenetic mechanism of the aPLs, and in particular of the aß2GPI antibodies, at the level of the maternal-fetal interface, is not yet known and its identification could represent a marker for potential targeted therapies.

a. Lessons from clinic

Antiphospholipid antibodies in amniotic fluid: potential role in aberrant implantation processes not related to antiphospholipid antibody syndrome.

The main finding highlighted by our survey in this regard, is that all the aPL antibodies (Abs) analyzed in the amniotic fluid have been found as measurable in all patients: this is the first study in which aCL and anti-β2GPI Abs were detected in the amniotic fluid of negative peripheral blood patients for aPL Abs.

These data could have a potential clinical implication that suggests a physiological role of local production of these antibodies in pregnancy and a possible role, if their levels are modified, in certain pregnancy complications.

Another important finding of our study is that women with RPL, autoimmune hypothyroidism (ahT) or smokers have a statistically significant difference in the levels of anti-beta2GPI Abs (IgM) compared to the control group. The highest levels in these pathological conditions were detected only for the IgM class: these data supported the idea of a local production of the investigated antibodies, since all patients were negative to the APL Abs test on peripheral blood and since the IgM cannot cross the placental barrier (data not yet published). The scientific literature reinforces this hypothesis, since the presence of β2GPI as a phospholipid antigen in the cell membrane of trophoblastic cells and placental endothelial cells, has been demonstrated[46-48]. The pathological conditions taken into account are characterized by an aberrant implantation process, caused by immunological anomalies (RPL and ahT) and by aberrant oxidative stress processes (RPL and smoke), which lead to placental endothelial dysfunction, dysregulation in neoangiogenesis pathways and accelerated apoptotic processes in the trophoblastic cell line. The presence of anti-β2GPI Abs in the amniotic fluid could represents a direct marker of placental dysfunction and abnormal implantation, since an accelerated apoptotic process could expose the β2GPI antigen located on the trophoblast cell membrane, if damaged. A possible explanation for the detection of these antibodies during physiological pregnancy could be based on the continuous remodeling of the maternal-fetal interface, in order to ensure adequate placental modifications, necessary for the developing fetus during the different stages of pregnancy. However, further studies are needed to understand the local pathophysiological mechanism at the maternal-fetal interface related to the presence of aPL Abs in the amniotic fluid.

4. Oxidative stress (OS)

OS at the placental-fetal interface has been involved in the pathogenesis of pregnancy complications such as RPL: an increase in total pro-oxidant levels and OS indexes and a decrease in total antioxidant mechanisms have been shown in RPL pregnant women, together with higher rates of endothelial dysfunction compared to physiological pregnancies. In particular elevated levels of ox-LDLs, one of the most studied OS biomarker, and lower antioxidant defenses have been shown in RPL women, leading to endothelial dysfunctions and placental injuries-associated complications[49].

a. Lessons from clinic

There is an evidence suggesting that women with a history of recurrent miscarriage are at a higher risk of coronary heart disease (CHD) and cardiovascular diseases (CVD). CVD and RPL a shared common etiopathogenetic pathway: aberrant oxidative stress processes, involving some recently studied biomarkers such as oxidized LDLs (ox-LDLs) and their scavenger receptor LOX-1. Recent studies focus the attention on LOX-1 full length isoform, codified by OLR1 gene, and its splicing isoform Loxin, lacking of exon 5, and thus leading to a non-functional receptor. An aberrant ratio between the two isoforms is strictly associated with an increased CV risk. Likewise, RPL seems to be associated to an unbalanced oxidative stress, involving OLR1 and Loxin peripheral blood expression, especially in the first trimester when the implantation process takes place. In fact, LOX-1 and its ligands, ox-LDLs, are responsible for placental injury by promoting an uncontrolled pro-inflammatory response, an aberrant angiogenesis process, trophoblast invasion inhibition and enhanced apoptosis, increased endothelial dysfunction (as have been studied in preeclampsia field)[49].

GENERAL PROGNOSIS

It has been estimated that approximately two thirds of women with RPL had at least one live birth after the first consultation at the RPL clinic, with the majority of the children being born within 5 years. The proportion of women with a subsequent live birth was negatively affected by increasing number of previous miscarriages and increasing age at first consultation[50].

a. Lessons from clinic

The gestational week in which the miscarriages occur could be taken into account as a prognostic

factor, since we have demonstrated how an unexplained RPL patient tends to have a recurrence in the same gestational age, in terms of miscarriage, that becomes specific for each patient. These data lead to the concept that unexplained causes for RPL could act in the same way on recurrence pattern for each specific patient condition, affecting the mechanism that controls each specific gestational age pregnancy development: to investigate the gestational week of recurrence could help the clinicians to draw the appropriate and personalized diagnostic work-up for a specific patient[51].

TREATMENT: OUR RESEARCH FOCUS ON LOW MOLECULAR WEIGHT HEPARIN.

Regarding the treatment of uRPL, there are no therapies for which scientific evidence of their effectiveness has been demonstrated[1]. However, in the field of clinical practice, low molecular weight heparin (LMWH) has been widely used as an empirical therapy. The rationale behind its use in uRPL is that, in addition to well-documented anticoagulant effects and inhibitory action on the complement system, LMWH potentially exerts immunomodulatory and anti-inflammatory actions, which could counteract the pro-inflammatory response implicated in the pathogenesis of RPL. In addition, LMWH may also be involved in the regulation of key pregnancy processes at the maternal-fetal interface, such as the prevention of trophoblast apoptosis, the increase in trophoblast invasion, the improvement of endothelial and vascular microenvironments, and the regulation of implantation of the embryo[52]. LMWH could, moreover, offer an interesting research aspect, not yet clarified, linked to possible immuno-epigenetic modifications induced at the maternal-fetal interface and / or at the maternal peripheral level. In fact, it has been widely demonstrated in the literature that microRNAs circulating in maternal peripheral venous blood may be specific and sensitive immunological markers of prediction for adverse pregnancy outcomes such as RPL[53].

At present, however, there is no consensus on its use in uRPL because different studies have found discrepant results, due to the differences found in the research design (in terms of definition, randomization and starting-week of gestation- treatment, diagnostic screening applied for uRPL). In fact, even in the most recent ESHRE guidelines, treatment with LMWH is not recommended in the uRPL, as a level of evidence has not been reached to indicate its use: LMWH does not seem to increase the "live birth rate" in women suffering from uRPL[1].

a. Lessons from clinic

Effects of low molecular weight heparin on the polarization and cytokinin profile of macrophages and T helper (Th) cells in vitro

In a recent published- study, we examined the *in vitro* effects of LMWH on macrophages and Th cells, two key actors in promoting immune tolerance during pregnancy.

From our data regarding macrophages, LMWH induces a predominantly pro-inflammatory, M1-like phenotype, reflecting a general activation of macrophages. In addition, the decrease in secretion of CCL2 (expressed and secreted by decidual macrophages, associated with the recruitment of Th cells, as well as a reduced production of proinflammatory cytokines by macrophages) and CCL22 (Th2 associated), the increase of chemokine CCL20 (Th17 associated), following exposure to LMWH is in line with the induction of an inflammatory profile of macrophages. Although the role of the Th17 response in pregnancy is not fully clarified, it is known to be linked to the production of mainly proinflammatory cytokines, while the Th2 response is known to favor the continuation of pregnancy. Therefore, an increase in CCL20 and a decrease in CCL2 and CCL22, together with changes in the phenotype, indicate that macrophages exposed to LMWH show a predominantly activated pro-inflammatory profile in vitro.

Regarding the effect of LMWH on T lymphocytes, the decrease in the percentage of Treg cells and an increase in the secretion of IFN-γ (Th1 associated), as well as the tendency to an increased proportion of lymphoblasts, results in line with an effect of general immunological activation and mainly pro-inflammatory response induced by LMWH, as in the case of macrophages.

To our knowledge, our data constitute the first documentation of the direct effects of LMWH on Th cells *in vitro* and potentially represent a crucial point for RM treatment, since immune modulation is particularly necessary at the maternal-fetal interface and involves Treg cells and M2 regulatory macrophages as key actors in immune tolerance processes, that have been shown to be inadequate in RM.

In addition to the effects of LMWH on macrophages and Th cells, we also evaluated the effects of LMWH on placenta explants, since the placenta is able to induce effects promoting tolerance on macrophages and Th cells. In this regard, a reduction in the placenta explants of

TRAIL secretion, which is mainly localized at the syncytiotrophoblast and has beneficial role in fetal immune tolerance, has shown to be induced by LMWH and could have in turn negative effects for fetal tolerance.

A mainly pro-inflammatory effect on macrophages and Th cells, two central cells that promote tolerance at the maternal-fetal interface, may suggest the possibility of a non-optimal use of LMWH in RM, when administered for its immune-modulatory role. RM is characterized by a proinflammatory profile, with an increase in the Th17 and Th1 responses and a reduced number of M2 macrophages, IL-10 secreting. Therefore, the possible pro-inflammatory effects of LMWH, may rather suggest a role in early pregnancy, especially during implantation, when a primarily pro-inflammatory process is required. In fact, it has been hypothesized that an inadequate implantation process, with a weak pro-inflammatory response, could be a contributing factor to the etiopathogenesis of RM . An early treatment in pregnancy with LMWH is also supported by its well-documented effect on the prevention of trophoblast apoptosis, on the strengthening of trophoblast invasiveness, on the improvement of endothelial and vascular environments and on the regulation of the embryo implantation. Furthermore, it has been shown that LMWH modulates the decidualization of endometrial stromal cells (ESC) in vitro, improving endometrial receptivity and supporting the early stages of the implantation process. These processes have been shown to be abnormal in women with RM, with a consequent extension of the implantation window and a greater capacity of the decidualized endometrium in allowing the implantation of poor quality embryos (super-endometrial receptivity). Therefore, although speculatively, LMWH could also be administered during the luteal phase and be useful for a proper implantation, reducing the risk of RM. It has been shown that the administration of LMWH from the luteal phase could improve the implant and the live birth rate in women with repeated implantation failure (RIF) during assisted reproduction techniques.

The contradictory results in LMWH clinical trials could be partly explained by the attempt to put together non-homogeneous data, since, hypothetically, an early start of treatment with LMWH could be useful, while its use later in pregnancy could have negative immunological effects. Since most of the studies were not designed to test the hypothesis of beneficial effects of LMWH when given at the time of initial implantation, further studies are clearly needed to confirm this hypothesis[52].

Next step will be to further investigate *in vivo* LMWH immuno-epigenetic effects on uRPL patients' pregnancies.

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This is not an exhaustive essay about RPL, but it can be an example how to work and reasoning in this field, integrating clinical and research information in favour of patient's care. Authors declare no conflict of interest.

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