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BRUNA DE OLIVEIRA GONÇALVES

Aetiopathogenesis of Recurrent implantation failure after assisted reproductive techniques

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Trabalho realizado sob a orientação de:

DOUTORA MARIA JOÃO DA SILVA FERNANDES LEAL CARVALHO

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Aetiopathogenesis of Recurrent implantation failure after assisted reproductive techniques

Autores:

Bruna de Oliveira Gonçalves

Estudante do 6º ano do Mestrado Integrado em Medicina da Faculdade de Medicina, Universidade de Coimbra, Portugal

Doutora Maria João Da Silva Fernandes Leal Carvalho

Assistente Convidada, Faculdade de Medicina, Universidade de Coimbra, Portugal

Azinhaga de Santa Comba, Celas, 3000-548 Coimbra

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LIST OF ABBREVIATIONS

Ago – Argonaute

AKT – PTEN/protein kinase B

ANGPTL4 – Angiopoietin-like protein

ANXA5 – Annexin A5

APS – Antiphospholipid Syndrome

ART – Assisted Reproductive Techniques

ASRM – American Society for Reproductive Medicine

ATF3 – Activating transcription factor 3

BMI – Body mass index

CAM – Cell adhesion molecule

CE – Chronic Endometritis

CI – Confidence interval

cMP – Circulating cell-derived microparticle

ECM – Extracellular matrix

ERA – Endometrial receptivity array

ESC – Endometrial stromal cells

ESHRE – European Society of Human Reproduction and Embryology

FET – Frozen-thawed embryo transfer

FISH – Fluorescence in situ hybridization

FSH – Follicle-stimulating hormone

GAPDH – Glyceraldehyde-3-phosphate dehydrogenase

GnRH – Gonadotrophin-releasing hormone

hCG – Human chorionic gonadotrophin

HHV-6a – Human herpes virus 6a

HLA – Human leucocyte antigen

ICSI – Intracytoplasmic Sperm Injection

IGFBP – Insulin-like growth factor binding protein

IL – Interleukin

INF-γ - Interferon-gamma

IVF – In Vitro Fertilisation

KIR – Killer immunoglobulin-like receptor

LH – Luteinizing hormone

LIF – Leukaemia inhibitory factor

LPA – Lysobisphosphatidic acid

MAPK – Mitogen-activated protein kinase

MMP – Metalloproteinase

mtDNA – Mitochondrial DNA

MTHFR – Methylene tetrahydrofolate reductase

ncRNA – Non-coding RNA

NK – Natural killer

PCR – Polymerase chain reaction

PECAMI – Platelet and endothelial cell adhesion molecule I

PG – Prostaglandin

PGT-A – Pre-implantation Genetic Testing for Aneuploidy

PRL – Prolactin

PROK – Prokineticin

RCT – Randomized controlled trials

RIF – Recurrent implantation failure

RISC – RNA-induced silencing complex

RLX – Relaxin

RPL – Recurrent pregnancy loss

TGF – Transforming growth factor

Th – Helper T cells

TIMP – Tissue inhibitor of metalloproteinases

TNF-α - Tumour necrosis factor-alpha

Treg – Regulatory T cells

UFH – Unfractionated heparin

UTR – Untranslated region

VEGF – Vascular endothelial growth factor

WOI – Window of implantation

ZP – Zona pellucida

ABSTRACT

Embryo implantation represents the major obstacle to reproductive process. Recurrent implantation failure (RIF) is defined as the incapacity to conceive after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in women younger than 40 years old. However, there is lack of homogeneity regarding this definition. Despite progresses in assisted reproductive techniques, about 15% of infertile couples undergoing in vitro fertilisation will struggle with RIF.

Pathogenesis of RIF still needs better understanding. The purpose of this review is to systematise the existing data about possible causes and mechanisms behind it.

Pubmed database was systematically searched and relevant articles for our goal were selected. Selection criteria included studies wrote in English or Portuguese published between 2011 and September 2021. A total of 221 articles were obtained. After analysis, 74 articles were included and organized in sections.

Multiple different causes can underlie RIF. There are maternal risk factors that predispose to it, including advanced maternal age, body mass index, smoking, stress and sleep habits. On the other side, male factors can also influence embryo implantation such as smoking, genital infections, chemotherapy or radiotherapy, among other factors. Embryo factors are especially related to aneuploidy, decreased levels of mitochondrial DNA and development stage in the moment of transfer. Uterine factors include anatomical anomalies, chronic endometritis, and endometriosis. Besides, vaginal microbiota might alter endometrial metabolome impairing embryo implantation. Endometrial receptivity might be altered due to immunological factors, genetic factors, and altered molecule expression. Endometrial immune milieu is not fully described but RIF endometrium has been found to have increased Th1/Th2 ratio, altered percentage of natural killer cells and decreased activity of T regulatory cells. Genetic factors refer to polymorphisms of genes crucial for immunotolerance, cell proliferation, vascular remodelling and angiogenesis, and genes that code non-coding RNAs. Lastly, adhesion molecules, metalloproteinases and multiple cytokines are some of the molecules that can be altered in the endometrium of a woman suffering from RIF. Thrombophilia might be a risk factor for RIF, but this is still a controversial issue.

RIF's aetiopathogenesis establishment is fundamental to clinical practice. This allows the identification of potential biomarkers and prognosis factors, and the development of directed complementary diagnostic methods. Furthermore, the development of new therapeutic approaches relies on the comprehension of aetiopathogenesis. Therefore, to better understand this pathology and the specific mechanisms behind it, further, larger and randomized controlled trials are needed.

Keywords Recurrent implantation failure • fertilisation in vitro • aetiology • embryo implantation • endometrium

RESUMO

O principal obstáculo do processo reprodutivo é a implantação embrionária. A falência recorrente da implantação (RIF) é definida como a incapacidade de engravidar após a transferência de pelo menos 4 embriões de boa qualidade transferidos em pelo menos 3 ciclos de fertilização in vitro em mulheres com menos de 40 anos. No entanto, esta definição não é consensual. Apesar dos progressos nas técnicas de reprodução medicamente assistida, cerca de 15% dos casais submetidos a fertilização in vitro são diagnosticados com RIF.

A etiopatogenia da RIF ainda não está totalmente esclarecida. Este artigo tem por objetivo sistematizar os dados existentes sobre as possíveis causas e mecanismos subjacentes sob a forma de revisão bibliográfica.

A pesquisa bibliográfica sistemática foi realizada na base de dados Pubmed e os artigos mais relevantes para o nosso objetivo foram selecionados. Os critérios de seleção incluíam artigos publicados entre 2011 e setembro de 2021, escritos em Inglês ou Português. Um total de 221 resultados foi obtido. Após análise, foram incluídos 74 artigos cuja informação foi organizada em subtemas.

Múltiplas causas podem estar na origem da RIF. Alguns fatores de risco maternos, tais como idade materna avançada, índice de massa corporal aumentado, tabagismo, stress e hábitos de sono, predispõem para a sua ocorrência. Fatores masculinos podem também influenciar a implantação embrionária incluindo tabagismo, infeções genitais, quimioterapia ou radioterapia. Os fatores embrionários dizem respeito sobretudo a aneuploidia, níveis diminuídos de DNA mitocondrial e à fase de desenvolvimento do embrião aquando da transferência. Fatores uterinos incluem anomalias anatómicas, endometrite crónica e endometriose. Além disto, o microbioma vaginal pode alterar o metaboloma do endométrio, prejudicando a implantação embrionária. A recetividade do endométrio pode também ser alterada por fatores imunológicos, genéticos ou por alteração da expressão de moléculas. O meio imunológico do endométrio ainda não está totalmente descrito, mas descobriu-se que o endométrio de mulheres com RIF apresenta elevação da relação Th1/Th2, alteração da quantidade de células natural killer e diminuição da atividade dos linfócitos T reguladores. Os fatores genéticos referem-se a polimorfismos de genes essenciais para a imunotolerância, proliferação celular, remodelling vascular e angiogénese, e de genes codificadores de RNAs não codificantes. Por fim, moléculas de adesão, metaloproteinases e múltiplas citocinas podem estar alteradas no endométrio destas mulheres. Condições trombofílicas podem ser um fator de risco para a RIF, mas este continua a ser um assunto controverso.

O esclarecimento da etiopatogenia da RIF é fundamental para a prática clínica. Isto permitiria a identificação de potenciais biomarcadores e de fatores de prognóstico, bem como o desenvolvimento de métodos complementares de diagnóstico direcionados. Além disto,

permitiria também o desenvolvimento de novas abordagens terapêuticas. Assim sendo, novos estudos randomizados com grandes amostras populacionais são necessários de modo a melhor esclarecer a etiologia da RIF e os mecanismos específicos a ela associados.

Palavras-chave Falência recorrente da implantação • fertilização in vitro • etiologia • implantação do embrião • endométrio

INTRODUCTION

Human reproduction is a quite ineffective even to apparently fertile couples. The chance of becoming pregnant is only 25-30% per menstrual cycle and only around half of these conceptions will surpass 20 weeks of gestation. Embryo implantation represents the major obstacle to reproductive process, with 75% of the lost pregnancies being caused by implantation failure (1).

Implantation is a process wherein the endometrium incorporates the embryo after its apposition, adhesion to the endometrium's luminal surface and, finally, invasion through the epithelium into a deep layer (2, 3). This event occurs during the luteal phase of the menstrual cycle (4), eight to ten days after ovulation, being this period known as the window of implantation (WOI) during which the endometrium becomes receptive and optimal to embed the embryo (5). In assisted reproductive techniques (ART), implantation is considered successful when an intrauterine gestational sac becomes detectable by ultrasound, usually at 5 weeks of gestation. In fact, there is no ultrasonographic evidence of that structure when implantation fails which can occur in two moments: (i) during adhesion or migration, with no production of human chorionic gonadotrophin (hCG) or (ii) after a successful migration, when the embryo is already producing hCG detectable in blood or urine, but its development stops. In clinical practice, the latter situation is labelled as a biochemical pregnancy (2).

Recurrent implantation failure (RIF) is defined as the incapacity to conceive after transfer of at least four good-quality embryos in a minimum of three fresh or frozen cycles in women younger than 40 years old (2). However, there is lack of homogeneity regarding this definition. A recent study demonstrates that the majority of clinicians consider RIF as the failure to implant three or more embryos (6). Actually, from this threshold on, the rate of false positive diagnosis decreases considerably (one out of four cases will be wrongly classified with this condition) (7).

Despite progresses in ART, the pregnancy rate is settled at about 30% per in vitro fertilisation (IVF) cycle and the prevalence of RIF is estimated at 10% (6, 7). Pioneer research on real prevalence of RIF has demonstrated that only 52% IVF cycles result on a clinical pregnancy at first attempt, with decreasing probability cycle after cycle, and that 15% of infertile couples undergoing IVF will struggle with RIF, a higher prevalence than previously expected (7). Due to this low success rate of IVF many couples are likely to fail three attempts randomly. Thereby, we cannot consider RIF as the same as recurrent IVF failure, but as a subgroup of it (2).

Nowadays, even though RIF is associated to poor prognosis, Koot *et al.* observed a cumulative incidence of live birth of 49% after a follow-up of 5.5 years (8).

Pathogenesis of RIF still needs better understanding. Currently, woman's age, obesity, maternal metabolic disorders (e.g., diabetes mellitus or thyroid disorders) embryo quality, sperm quality, uterine and immunological factors, and thrombophilia are known to influence implantation process. Nevertheless, most cases remain unexplained (1, 4, 5). It is of utmost importance to find the underlying cause in each patient through a multidisciplinary approach in order to personalize treatment and distinguish couples who benefit from continuing treatment from those who should considerer other alternatives (2, 8).

This review aims to systematise and stablish the aetiology and pathogenesis of RIF, given its importance towards patients' management and development of new approaches.

MATERIALS AND METHODS

This is a literature review on the aetiology and pathophysiological mechanisms underlying RIF. *Pubmed* database was searched for indexed articles between March and September 2021.

As an initial approach, search was performed adopting the keywords “Recurrent implantation failure AND ("Fertilisation in Vitro"[Mesh] OR ivf) AND ("etiology" [Subheading] OR "Therapeutics"[Mesh]). Selection criteria included studies wrote in English or Portuguese languages and published between 2011 and September 2021. A total of 221 articles were obtained. Thereafter, the respective titles and abstracts were analysed and 122 articles were excluded because their focus was not RIF or their main objective was not relevant to determine RIF aetiology and pathogenesis. In addition, it was given priority to meta-analysis, systematic reviews, literature reviews, randomized controlled trials, case-control studies, and cohort studies. Later, after integral reading of the articles and based on scientific relevance 29 more articles were excluded, remaining 70 articles included (*Fig. 1)*.

In addition, some of the articles included were a source of references because of the citations they provided. Additional search was also conducted combining the keywords “recurrent implantation failure AND obesity”. In this way, 4 more articles were added making up a total of 74 references underpinning this review.

Reference management was conducted with the online manager *EndNote Web* and the citation style used was *Vancouver*.

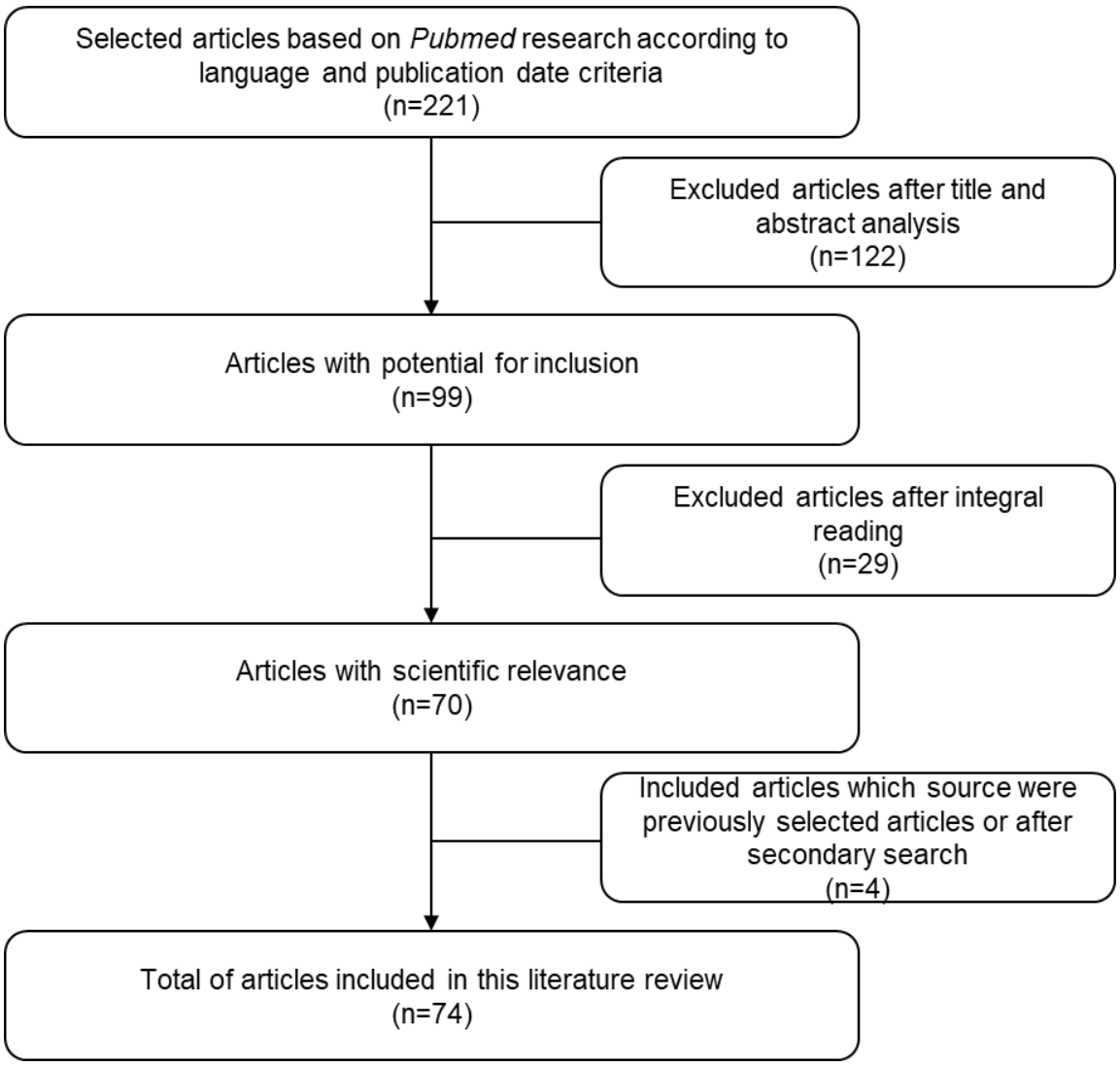

Figure 1 - Flowchart with the literature review phases.

RESULTS

Embryo implantation requires a good quality embryo, a receptive endometrium, and an efficient communication between them. For a long a time, it was thought that embryo and endometrium were the only players behind RIF aetiology, however other aspects such as maternal immune system and thrombophilia are known to preclude implantation.

1 Maternal factors

1.1 Maternal age

Embryo's good quality is one of the requirements for implantation in the endometrium. It is known that aneuploidy rate increases with maternal age (9), although there are other age-related changes that can affect implantation. In 2016, Shapiro *et al.* compared women under 35 years-old (< 35) with women with 35 years or older (≥ 35) and found a significantly reduced oocyte reserve, endometrial thickness, and blastocyst formation in the older group. In this retrospective cohort study were evaluated two risk factors of embryo-endometrium asynchrony: prematurely elevated progesterone levels and delayed (day 6) blastulation. Women ≥ 35 had 1.36 increased risk of having one risk factor (95% Confidence interval (CI) 1.24-1.50) and 1.61 increased risk of having both (95% CI 1.17-2.21), which translates into a proportion of 68.1% asynchronous transfers, comparing with 50% in younger women. In addition, implantation, pregnancy, and live birth rates were significantly higher in women <35 years (10). Furthermore, from the evaluation of the national average implantation rates reported by the United States Centre for Disease Control and Prevention, it was found that implantation rate for frozen-thawed embryo transfers (FET) declines much slower than for fresh transfers, suggesting distinct age-related alterations between the two types of cycle (9, 10).

1.2 Body mass index

Overweight (Body mass index (BMI) ≥ 25 kg/m^2) and obesity (BMI ≥ 30 kg/m^2) are known risk factors for menstrual disfunction, infertility and higher rates of pregnancy complications (11). It was demonstrated that obese women undergoing IVF had lower chance of implantation, clinical pregnancy and live births and that those odds decreased with increasing BMI (11). This might be due to poor oocyte quality, suggested by the need of greater daily doses of gonadotropin (9, 11), and to metabolic changes, namely upregulated glycemia, LDL cholesterol, triglycerides and TSH, that have deleterious effect on endometrial receptivity (12). In fact, Bellver *et al.* reported that obese women experience 1-day WOI displacement when compared to normal weight and overweight women (25.1% vs. 9.7% respectively; p = 0.02). Endometrial receptivity was diagnosed through endometrial receptivity array (ERA) which revealed a pre-receptive endometrium, namely, a delayed displacement (12). These

findings might be at the origin of embryo-endometrium asynchrony leading to the lower implantation rates.

1.3 Smoking

For a long time, smoking is deemed an important detrimental factor towards pregnancy. Cigarette smoke reduces oocyte reserve, impairs embryo implantation and fertilisation during IVF cycles (2). It also has negative effects on male fertility, since it reduces sperm counts and motility, alters sperm morphology and increases DNA damage (2, 9).

In addition, not only smokers are affected but also second-hand tobacco smokers have 52% chance of implantation failure and less 25% chance of achieving a live birth (13).

Therefore, women are strongly advised to avoid tobacco smoke exposure when trying to get pregnant and during pregnancy.

1.4 Stress

Cortisol is known as “the stress hormone” once its production increases with emotional or physical stress rise. Research had shown that elevated cortisol levels were associated to 2.7 times higher risk of miscarriage (95% CI 1.2-6.2) (9).

In 2014, Coughlan *et al.* conducted a cross-sectional study to evaluate stress levels in women with RIF and in women with recurrent pregnancy loss (RPL), which showed that both groups experience similar stress levels and that those were higher than the ones from their fertile counterparts. However, the author cautioned that it was impossible to infer from those results if the stress experienced was a cause or a consequence of poor reproductive outcome. Previous studies had shown that emotional stress during ART does not impair pregnancy rates (14).

Given the mentioned results, it is of utmost importance to give adequate counsellor to infertile couples undergoing IVF, to provide them information and skills to cope with this stressful process.

1.5 Sleep habits and circadian rhythm

Circadian rhythm is a 24-hour endogenous clock that can be disrupted by variations in light-dark and sleep patterns. It is a well-known fact that pre-ovulatory luteinizing hormone (LH) peak is under control of circadian rhythm. Besides ovulation control, circadian rhythm also influences embryo, uterus, and placenta molecular components (15). Furthermore, sleep patterns influence oestradiol and progesterone levels along with hypothalamic hormones during pregnancy (16).

In fact, women with RIF sleep substantially less daily than fertile women (454.63±56.56 min vs 507.35±57.29 min, p=0.03) and there is a negative correlation between total sleep time and light exposure period for the RIF group (r=-0.68; p=0.02) (16).

Female shift workers were proved to have decreased fertility and poor reproductive outcomes (15, 16). In order to investigate light-dark alterations on endometrium receptivity in mice, Goldstein *et al.* performed a study in which embryos retrieved from females exposed to normal light-dark conditions were transferred to dams exposed to circadian disturbance. The variables on study were number of pups, resorptions and implantations and no significant differences were observed comparing those from the study group to the ones from control group. Considering this, embryo implantation impairment is not only due to alterations of endometrium receptivity, but rather due to ovarian and embryo factors. More studies are needed to clarify how circadian disruption impacts ovarian function and embryo development (15).

2 Male factors

Male factors cause 20% of infertility cases and 30-40% of infertility cases are caused by both male and female factors (17). Sperm DNA quality is affected by smoking, genital infections, former chemotherapy or radiotherapy, defects in chromatin remodelling during spermatogenesis, production of free radicals in seminiferous tubules, among other factors (2, 18). Conventionally, men infertility is assessed through semen analysis including volume, sperm concentration, motility, and morphology, even though its utility has been a question of debate (19).

Sperm aneuploidy is associated with higher risk of embryo aneuploidy and higher rates of implantation failure after intracytoplasmic sperm injection (ICSI). In addition, a decrease on sperm concentration increases the probability of abnormal fluorescence in situ hybridization (FISH) results (20). Thus, traditional semen analysis can be useful to predict the risk of aneuploidy and, therefore, to indirectly predict RIF.

Men with poor quality semen are prone to have higher levels of DNA fragmentation which is deemed to impair embryo development and ART outcomes (17, 18). However, there is no sufficient data to include sperm DNA testing in current clinical practice (17). In fact, no correlation was found between sperm DNA fragmentation index and sperm aneuploidy nor embryo aneuploidy rates (18). Furthermore, this index cannot be used as a predictive factor of RIF, once no significant differences were found between couples with RIF and their fertile counterparts (17) and higher percentages of DNA fragmentation do not increase the risk of RIF (19). However, these prospective studies rely on small samples and use different methodologies, so further larger randomized controlled trials (RCT) are needed to determine whether there is a relation between DNA fragmentation index and RIF or not.

3 Embryo factors

3.1 Aneuploidy

Chromosomal aneuploidy is a cause of poor-quality embryos and consequently impairs embryo implantation. Using FISH on blastomeres from day-3 embryos, chromosomal abnormalities rate was found to be significantly higher in women with three or more failed IVF cycles when compared with controls (67% vs. 36%, respectively) (21).

Kort *et al.* also compared embryo aneuploidy rate of infertile couples with fertile controls and concluded that couples with previous failed IVF cycles had significantly higher chance of embryo aneuploidy in pre-implantation genetic testing for aneuploidy (PGT-A) (OR 1.356; 95% CI 1.129-1.629; p=0.0012). These anomalies may occur after fertilisation and have maternal or paternal origin. Maternal aneuploidies are generally due to meiotic errors and paternal aneuploidies are especially related to mitotic errors (22). In addition, aneuploid embryos may also have origin on aneuploid gametes produced by couples whom carry translocations, inversions, deletions or mosaicism (21).

A possible explanation for the higher implantation failure rate of aneuploid embryos is the endometrial biosensor function. Besides endometrial receptive and embryo nourishing functions, decidua also has a selective function. This tissue is capable of controlling maternal response to the embryo precluding the implantation of poor-quality embryos that will become pregnancies destined to fail (23).

3.2 Mitochondrial DNA

Mitochondria is the organelle responsible for energy production and cellular aging, so embryo development relies on it. Mitochondrial genes encode oxidative phosphorylation complex subunits and transcriptional and translational equipment of the organelle (24).

Mitochondrial DNA (mtDNA) was found to be influenced by maternal age (older women had oocytes with less amounts of mtDNA). However, women of the same age were found to have different levels of mtDNA leading to the conclusion that there are other factors influencing them. It is thought that implantation can be impaired by mtDNA levels, since lower amounts of mtDNA in oocytes and blastomeres were related to less energy production and, consequently lower capacity to support preimplantation development. Therefore, it was suggested that mtDNA copy number could be used as a biomarker of oocyte quality (24).

3.3 Zona hardening

Zona pellucida (ZP) is an acellular structure that involves the embryo and has a key role in fertilisation. After fertilisation, it naturally hardens to prevent fertilisation with more spermatozoa, maintain the embryo integrity before implantation and facilitate the transport through fallopian tubes (21). When embryo reaches the blastocyst stage ZP suffers rupture

due to physical expansion and to action of lysins released by the embryo and the uterus. Sometimes ZP fails to hatch, which precludes implantation (21).

3.4 Day of transfer and transfer technique

Despite embryo quality, day 5 or 6 blastocysts have higher implantation potential than day 2 cleavage stage embryos mostly because better quality embryos have higher chance of development (2, 9). Although controversial, FET seem to be superior to fresh embryo transfers in terms of implantation success. This might be due to good-quality embryos better chance of survival to freezing, and to a hormonal treatment that simulates natural cycles since ovarian stimulation protocols and oocyte retrieval were applied in previous cycles (9). In addition, ultrasound guided embryo transfer improves implantation rate (2, 13).

4 Uterine factors

4.1 Anatomical anomalies

Intrauterine pathologies may negatively impact ART results and are estimated to be present in 25% to 50% of infertile couples (2, 25). These anomalies hamper pregnancy through implantation failure or spontaneous miscarriage (25). In fact, Cenksoy *et al.* concluded, after office hysteroscopic screening, that among 157 women suffering from RIF, 44.9% had unrecognized structural uterine anomalies. After hysteroscopy 75 women achieved pregnancy 36 of which had their endometrial pathology corrected. In addition, implantation and pregnancy rates were significantly higher after polypectomy ($p = 0.001$) (25). Therefore, these rates improvement corroborate the hypothesis that uterine anomalies preclude embryo implantation.

Uterine abnormalities can be classified as congenital or acquired. Congenital anomalies include septate uterus and bicornuate uteri, while acquired anomalies include fibroids, endometrial polyps, and intrauterine adhesions (2). The most common hysteroscopic findings are endometrial polyps and intrauterine adhesions (25).

Oppositely to bicornuate uteri, septate uterus has a negative effect on pregnancy rate. Distortion of the uterine cavity and deficient blood supply increases the miscarriage rate, which falls from 80% to 30% after hysteroscopic metroplasty (2).

Submucosal fibroids are proved to hamper implantation once they distort uterine cavity, increase uterine contractility, alter cytokine profile and vascularisation, and promote chronic inflammation of the endometrium. Besides, higher implantation and pregnancy rates were reported after hysteroscopic resection of fibroids (2). Intramural and subserosal fibroids are a controversial matter because they are non-distorting and thus far there is conflicting evidence as to whether they hinder implantation and pregnancy rates or not. However, three meta-analyses concluded that women with intramural myomas have lower implantation rates when

compared to healthy uterine controls and that pregnancy rates did not improve after myomectomy (2). These findings suggest that uterine cavity distortion is not the only cause underlying embryo implantation impairment, but there are other mechanisms involved that justify further investigation.

Endometrial polyps are the most common anomaly found among RIF women (25) and it is proved that polypectomy improves implantation and pregnancy rates (2, 25). At last, intrauterine adhesions were found in 8.5% to 9% of women suffering from RIF (2, 25) and they preclude implantation by blocking embryo's attachment to endometrial surface (2). Similarly to intramural fibroids, there is no consensus about the outcomes of hysteroscopic adhesiolysis since there are studies demonstrating that this procedure improved fertility outcomes (2) while others found no difference in implantation rate ($p > 0.05$) (25).

4.2 Infection

For a long time, endometrium was thought to be sterile, whereas with the evolution of sequencing techniques endometrial microbiome was identified (26). The dominant microorganism of this structure is *Lactobacillus,* which holds a protective function. In fact, when these species are diminished there is a predisposition to gynaecological diseases such as chronic endometritis (CE) (27).

CE consists of a persistent inflammatory state of the endometrial lining. The gold standard for the diagnosis is histological analysis of an endometrial biopsy where plasma cells are present within endometrial stromal cells (ESC) (27-29). Recent research demonstrated a prevalence of CE among women suffering from RIF of approximately 14%, but the author cautioned that this number might be lower than the documented by previous studies (30-37%) due to the small size of the sample (29).

A systematic review from 2020 focused on summarize the existing data about how CE pathophysiology could underlie RIF and established a complex model that includes (27):

i. Cytokine dysregulation – CE alters microRNA expression that in turn modulate the expression of cytokines and growth factors genes. Women with CE have their gene expression altered towards a pro-inflammatory environment, which will be discussed further bellow. In addition, CE increases CXCL13 and CXCL1 expression in endometrium that are responsible for attracting circulating B cells. Interleukin-11 (IL-11 was found downregulated in CE. This cytokine promotes trophoblast invasion by stimulating adhesion molecules endometrial expression and has a key role in decidualisation, angiogenesis and spiral arteries remodelling by improving progesterone action. Furthermore, CE has an anti-apoptotic effect in endometrium. This pathology enhances the expression of BCL-2 that is an inhibitor of CASP-8, a pro-apoptotic gene. The reduction of apoptosis leads to abnormal endometrial proliferation and differentiation and possible formation of polyps, which worsens implantation capacity.

ii. Leucocyte dysregulation – B cells, absent on healthy endometrium, are recruited to endometrial epithelium and stroma triggering a humoral immune response; uterine natural killer (uNK) cells are downregulated impairing vascular remodelling and immunomodulation, and CD4+ T lymphocytes are upregulated which disrupts Th1/Th2 and Th17/Treg balances, essential for the tolerance of the paternal antigens in embryo.

iii. Altered uterine contractility – during normal menstrual cycle endometrium adapts its contractility to the phase of the cycle. Usually during midluteal phase endometrial waves are not propagating to facilitate implantation. However, women with CE have their contractility pattern altered with propagating waves, either retrograde or anterograde, during midluteal phase, which hampers implantation.

iv. Altered vascularisation – blood vessels in CE suffer multiple anatomopathological alterations such as high vascular density and endothelial proliferation, swelling, thickening of the wall, luminal occlusion and fibrinoid degeneration. These findings impair endometrial receptivity in two different ways. Firstly, high vessel density increases oxygenation that can be harmful due to production of reactive oxygen species, and placental development requires a quite hypoxic environment. Oppositely, swelling and luminal occlusion could reduce blood flow to the extent that placental development is precluded.

v. Altered decidualisation – prolactin (PRL) and IGFBP-1, both decidualisation markers, are decreased in CE. Oestrogen and progesterone receptors are overexpressed in CE endometrial tissue, reflecting a resistance towards ovarian hormones. The number of decidualized ESCs are significantly increased in CE but with less differentiation (27, 28). Decidua has a key role in embryo implantation, endometrial immune tolerance and establishment of the placenta, so it can be concluded that CE has a negative impact on endometrial receptivity and pregnancy development.

vi. Altered autophagy – autophagy is an essential mechanism of protein recycling that modulates the inflammatory response and Treg cells. In CE autophagy is enhanced which leads to an environmental modification less propitious for implantation.

4.3 Endometriosis

Endometriosis consists of hormone-sensitive endometrial tissue that develops outside the uterine cavity (28). It is commonly considered a cause of infertility specially resulting from implantation impairment (30). However, this happens to be a controversial theme among scientific community when it comes to explain the implantation failure underlying mechanism. In fact, two different reviews support two different points of view. Miravet-Valenciano *et al.* suggests that only oocyte quality and quantity are affected, but not endometrial receptivity. They support this hypothesis on studies that found no differences in implantation and pregnancy rates between women with endometriosis and healthy women after transfer of

healthy donor oocytes. Furthermore, there are studies that report no differences in endometrial gene expression between endometriosis and control groups in the ERA (30). On the other hand, Lessey *et al.* defends that endometriosis disrupts endometrial receptivity of eutopic endometrium. This review is based on research that correlate the inflammatory nature of the disease with progesterone resistance and extra oestrogen action, both detrimental for implantation and decidua differentiation. In addition, endometriosis leads to altered molecular expression such as reduced leukaemia inhibitory factor (LIF), and overactivation of proliferative pathways such as PTEN/protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) pathways, both known to hamper decidualisation (31).

A recent prospective study aimed to evaluate endometriosis impact on implantation comparing women with recurrent unilateral endometriomas (group I) with women with tubal factor infertility (group II). This paper reported lower oocyte reserve in group I, since anti-müllerian hormone reflects it and was significantly lower (2.1 ± 1.75 vs. 3.2 ± 1.4; p<.005). The number of oocytes recovered was also lower (8.1 ± 3.9 vs. 10.1 ± 6.8; p<.005), but nevertheless the embryo quality was similar between groups. Furthermore, group I had a significantly lower implantation rate when compared to group II (17.1% vs. 24%; p<0.005) (32).. In addition, this study has also related endometriomas size with the chance of implantation. Endometriomas smaller than 3 cm had similar pregnancy rate to the control group, and bigger than 3 cm had 1.5 less chance of embryo implantation than the control group (15.8% vs. 24%, respectively; p<.005) (32). Thus, despite same quality oocytes, endometriosis group's implantation rate was far decreased, which supports the hypothesis of eutopic endometrial receptivity being disrupted by endometriosis

5 Hydrosalpinges

In the time span included in our survey and with our search equation, no original articles about hydrosalpinxes were found. However, some reviews mentioned it as the probable cause of RIF when present (2, 9).

Hydrosalpinx refers to a Fallopian tube filled with fluid. Women with hydrosalpinges have half the chance of achieving a live birth after IVF compared with women with no hydrosalpinges (2). This lower success rate is associated with implantation impairment. Although the exact mechanisms are unclear, it is thought that the fluid impairs embryo development due to its lack of nutrients and toxic effect, endometrial receptivity due to lower LIF expression and may physically push the embryo out of the uterus (2, 9). Furthermore, the reviews refer that salpingectomy significantly improves IVF outcomes. In conclusion, this pathology should be systematically assessed when women are diagnosed with RIF and treated if present (2, 9).

6 Immunological factors

Endometrial immune milieu is not fully described and, thus far, no method to assess cellular populations achieved scientific community consensus about its use. Despite the lack of information about immune cell populations in the uterus, it is known that they change over the course of menstrual cycle (33). Characteristics of the considered studies are detailed in *Table 1*.

6.1 Th1/Th2 ratios

Helper T cells (Th) are a subset of CD4+ T lymphocytes composed by Th1, Th2 and Th17 cells. These cells produce their own cytokines profile: Th1 produce IL-2, tumour necrosis factor-alpha (TNF-α) and interferon-gamma (INF-γ) while Th2 produce IL-4, IL-5, IL-6, IL-10, and IL-13 (4, 34). Normal human pregnancy is characterised by Th2 predominant immunity (34), however some authors refute this hypothesis and defend that inflammatory Th1 cytokines are crucial for preparation of implantation (35).

Liang *et al.* conjectured that controlled ovarian stimulation implemented during IVF, could have impact on blood's lymphocyte subtypes. Therefore, Th1/Th2 ratio, NK cytotoxicity and percentage of peripheral blood lymphocytes were assessed at three moments of controlled ovarian stimulation. The results were compared between three groups that undergone ART: a successfully pregnant, a non-pregnant and a third RIF group (34). Th1/Th2 ratios were deduced from TNF-α/IL-10 and INF-γ/IL-10 ratios. Cytokine measurements were made at the day before the first gonadotrophin-releasing hormone (GnRH) administration (GnRH day), on the day before the first recombinant follicle-stimulating hormone (FSH) administration (Gn day), and on the day before hCG administration (hCG day). No significant differences were found in the percentage of lymphocytes nor in the NK cytotoxicity. On the other hand, TNF-α/IL-10 and INF-γ/IL-10 ratios were significantly higher in non-pregnant women than in successfully pregnant women ($p<0.05$) at the hCG day, but not at GnRH or Gn days. These ratios were also significantly higher in RIF group when compared to successful pregnancy group, but no difference was found between RIF and non-pregnant women. In addition, TNF-α/IL-10 and INF-γ/IL-10 ratios suffer a significant decrease at hCG day in the successful pregnancy group ($p<0.05$) but not in the non-pregnancy group neither at other time points (34). These findings show that similarly to natural cycles during controlled ovarian stimulation the Th1 to Th2 immunity shift prior to the WOI is related to better IVF outcomes (34). Lashley *et al.* found concordant results regarding to the proportion of circulating T cells and Th1/Th2 ratio when comparing women suffering from RIF with fertile control. They further concluded that, although Th1 cytokines increased in a higher proportion, both subsets of cytokines were increased in RIF (35). More recent research described, once more, a

significantly higher expression of Th1 cells and a higher Th1/Th2 ratio among women with poor fertility outcomes (33).

Furthermore, in a subsequent study, Liang *et al.* evaluated the cytokine profile of women with RIF and concluded that they suffer a pro-inflammatory shift. Pro-inflammatory cytokines, from which Th1 cytokines take part, were significantly increased. On the other hand, TGF-β, an anti-inflammatory cytokine, was significantly decreased (36).

Lastly, all the mentioned studies documented a Th1 cell and cytokine dominance among women with history of RIF. This altered ratio impairs embryo implantation because the excess of Th1 cytokines activates an inflammatory response, triggers macrophages that directly harm the trophoblast, inhibits trophoblast's growth and invasion, and impairs placental differentiation and proliferation due to promotion of thrombotic events in the uterus (9, 34, 36)

6.2 Regulatory T cells

Regulatory T cells (Treg) have a key role in preventing an inflammatory overreaction and, thus, are responsible for immune tolerance. In mice, these cells start to accumulate in lymph nodes and in the uterus just before ovulation and after mating, suggesting that their immune suppressive function might be important from an early stage, before conception and WOI. Women with unexplained infertility (both RIF and women who conceived after the first IVF attempt) were found to have Tregs with significant reduced suppressive capacity which could explain the more aggressive alloimmune proliferative response and the intensified production of Th1 and Th2 cytokines with no shift from Th1 dominance to Th2 (35).

Furthermore, Tregs differentiation is stimulated by TGF-β which, as said before, is decreased in RIF women. As a result, Tregs are expected to proliferate less compromising the immune tolerance towards the allogenic paternal material mediated by them (36). In fact, women with history of unexplained RIF have significantly less Tregs ($p=0.034$) than fertile women (33).

6.3 Natural killer cells

NK cells belong to innate immune system and are the most common immune cells in the preimplantation endometrium, therefore come in close contact with the embryo (37-39). NK cells can be classified according to membrane surface markers into $CD16^{+}$ $CD56^{dim}$, the most abundant in the peripheral blood (pNK), and $CD16^{-}$ $CD56^{bright}$, the dominant population in the endometrium (uNK). It is known that the cytotoxic activity of $CD56^{bright}$ cells is weaker than the one performed by $CD56^{dim}$ cells (38). These cells express on their surface killer immunoglobulin-like receptors (KIRs) which recognise trophoblast's human leucocyte antigen (HLA) class I molecules. These receptors modulate NK cells cytotoxicity and production of soluble factors needed to maintain a semi-allograft embryo (37). Embryo implantation is

preceded by a boost in uNK cell proliferation (rise from 5% of ESCs in the early mid-luteal phase to 40% in the late luteal phase), that occurs in response to progesterone and IL-15 production (4, 33). The function of uNK is not fully understood, but it is thought that these cells secrete angiogenic factors like vascular endothelial growth factor (VEGF) taking part in arteries remodelling during decidualisation and placentation (27), and that they also establish a limit that prevents trophoblast from excessively invade the endometrium (40).

Extravillous trophoblast cells contact with both $CD56^{dim}$, at the intervillous space where maternal blood contacts with chorionic villi, and $CD56^{bright}$, present in the placenta (38). Although these two types of NK cells are quite different, the majority of studies are based on pNK cells because some patients do not agree to provide endometrial samples since it is an invasive procedure and uNK levels vary over the menstrual cycle and with the location on the uterus (38, 40).

Implantation success relies on the immune tolerance of the mother towards the embryo. There are studies showing that high percentage and cytotoxicity of pNK cells are associated with RIF and poor IVF outcomes (38, 39). Oppositely to Liang *et al.* that found no difference in pNK cells cytotoxicity between women who successfully conceived after their first IVF attempt and women who failed (34), Karami *et al.* described significantly higher median percentage of pNK cells cytotoxicity in women with IVF failure compared to women with successful IVF (31.3% vs. 10.73%, $p<0.001$) (38). These contradictory findings might be due to the small samples of the studies and to the different methods used to quantify NK cells activity (flow cytometry to calculate the percentage of dead K562 cells vs. colorimetric technique followed by ELISA to quantify the LDH release, respectively). To further clarify this matter, a study with a larger, standardized, and reproducible method evaluated the pNK cells cytotoxicity by flow cytometry based on the presence of the activation marker CD69. It was described that pNK cells were significantly more activated compared to controls ($p=0.005$). In addition, uNK cells were also found to be present in a significantly higher proportion in the endometrium of women with RIF than in controls (53.2% vs. 45.2%, $p<0.001$) (33). Until now, the mechanisms underlying this local immune change are not fully understood but there are some theories. It can be due to KIR haplotypes that can activate uNK cells remaining from previous failed pregnancies or to the higher expression of pro-inflammatory cytokines, such as TNF-α and INF-γ, that enhance NK cells cytotoxicity (33).

A recent retrospective cohort study analysed the relationship between pNK cells and ovarian reserve and function in women who underwent IVF. It was observed that the number of oocytes retrieved had a positive linear relation with NK cell percentage and that the AMH ratio stayed constant until pNK cells reached a percentage of 18%, moment from which both number of oocytes and AMH ratio started to decrease (39). These findings suggest that NK

cells can have a positive impact on ovarian response during IVF treatment, whereas higher levels of NK cells (>18%) are detrimental for embryo implantation and IVF outcomes (39).

Some authors defend that human herpes virus 6a (HHV-6a) increases uNK cell percentages impairing implantation (33). However, Coulam *et al.* found no difference in expression of uNK markers between positive and negative HHV-6a samples, but rather in neutrophil markers that were significantly increased in positive samples (41).

6.4 B cells

B cells might have a key role in the maternal immune tolerance, since Th2 cytokine dominance is thought to promote the production of NK cell blocking antibodies and immunosuppression. The expression of CD19, a marker of B cells, was found to be significantly increased in RIF patients compared to controls ($p<0.001$). When these cells are raised and the Th2 shift does not occur, a viable hypothesis to the impairment of implantation is that B cells have the opposite effect and enhance the immune response against the embryo (33).

***Table1* – Immunological factors: main characteristics of the studies considered**

Authors and years	Country	Study design	Participants and inclusion criteria	Samples, timing and methods	Main outcomes
Karami, 2012	Iran	Case control study	43 patients (23 with RSA and 20 with IVF failure), 43 women with previous pregnancies; <45 years old	Peripheral blood samples. Flow cytometry assay. Cytotoxicity assay with an enzymatic colorimetric assay using LDH quantified in an ELISA reader.	NK cell cytotoxicity and percentage of $CD56^{dim}$ cells in patients with RSA or with IVF failure were significantly higher than in controls.
Liang, 2015	China	Prospective cohort study	51 patients undergoing IVF (24 pregnant after index IVF cycle, 27 non-pregnant); ≤35 years old 18 RIF patients	Peripheral blood samples collected on GnRH, Gn and hCG days of COS. Cytokine's (INF-γ, TNF-α, IL-10) concentration measurement. Flow cytometry assay.	IFN-γ/IL-10 and TNF-α/IL-10 ratios were significantly higher in non-pregnant compared with pregnant patients. IFN-γ/IL-10 and TNF-α/IL-10 ratios were significantly higher in RIF patients compared with pregnant ones. No differences were found between RIF and non-pregnant groups. INF-γ/IL-10 and TNF-α/IL-10 ratios were significantly higher in non-pregnant women than in pregnant women ($p<0.05$) at the hCG day Th1 to Th2 shift during COS prior to the WOI is related to IVF outcome.
Liang, 2015	China	Prospective case control study	34 RIF patients (2 to 6 IVF/ICSI cycles, >10 good-quality embryos), 25 controls (successful pregnancy after index IVF/ICSI cycle), ≤38 years old	Peripheral blood samples collected on mid-luteal phase. Flow cytometry assay. Cytokines (IFN-γ, TNF-α, IL-6, IL-1β, IL-4, IL-10 and TGF-β1) were detected by BD Cytometric Bead Array immunoassay.	RIF patients suffer a shift towards pro-inflammatory status in peripheral blood.

Lashley, 2015	Netherlands	Case control study	22 RIF patients (≥3 IVF failures), 32 infertile patients with successful IVF, 21 fertile women	Peripheral blood samples. Isolation of PBMC. Flow cytometry assay. Cytokine's concentrations measurements.	Higher levels of Th1 and Th2 cytokines and a significantly higher Th1/Th2 ratio were observed in RIF and IVF control compared to fertile control. Diminished suppressive capacity of Tregs in RIF patients and IVF control.
Coulam, 2018	USA	Case control study	30 RIF patients (≥4 embryos transferred), 10 fertile controls	Endometrial samples collected on luteal phase. qRT-PCR to determine endometrial immune profile and HHV-6 detection. Immunohistochemistry.	37% of RIF patients had HHV-6 while no control had it (p=0.038). Significant increase in neutrophil markers' mRNA was found in HHV-6-positive samples. No difference was observed in expression of NK cell-related markers nor T cells.
Marron, 2019	Ireland	Case control study	178 RIF patients (>2 failed embryo transfers), 155 RPL patients (>2 miscarriages), 130 primary infertility, 114 secondary infertility, 35 controls (ART patients due to male factors)	Endometrial samples collected after 5 days of vaginal progesterone of a hormone replacement therapy cycle. Flow cytometry assay.	All adverse outcome populations had increased peripheral NK cells compared to the controls. RIF but not RPL had higher levels of uterine NK cells (median 53.2% vs. 45.2%, p< 0.0001). All adverse outcome populations had higher Th1 expression, Th1/2 and CD4/8 ratios. T regulatory cells were significantly lower in all abnormal groups compared to controls (p = 0.034).
Hur, 2020	Republic of Korea	Retrospective cohort study	936 RIF patients	Data extracted from patients' medical record. Measurement of AMH, FSH, E_2 and NK cell percentage on peripheral blood.	NK cells percentage is positively associated with ovarian response as long as it keeps <18%.

AMH anti-mullerian hormone, *COS* controlled ovarian stimulation, E_2 oestradiol, *ELISA* Enzyme-Linked Immunosorbent Assay, *FSH* follicular stimulating hormone, *Gn* Gonadotrophin, *GnRH* gonadotrophin-releasing hormone, *HCG* Human chorionic gonadotrophin, *HHV*-6 human herpes virus-6, *IL* Interleukin, *INF-*γ Interferon-gamma, *IVF* In Vitro Fertilisation, *LDH* Lactate dehydrogenase, *NK* Natural killer, *qRT-PCR* quantitative real-time polymerase chain reaction, *PBMC* Peripheral blood mononuclear cells, *RIF* Recurrent implantation failure, *RPL* recurrent pregnancy loss, *RSA* Recurrent spontaneous abortion, *TGF-*β*1* Transforming growth factor beta-1, *Th* T helper, *TNF-*α Tumour necrosis factor-alpha, *WOI* Window of implantation.

7 Genetic factors

The studies included in this section are detailed on *Table 2.*

7.1 HLA and KIR polymorphisms

As the need to explain RIF's underlying mechanisms grew, scientific community started to investigate its association with higher prevalence of certain genetic polymorphisms. In fact, RIF has been related to a variety of polymorphisms and HLA class I (HLA-C and HLA-G) genes are two of the most explored ones.

HLA-G belongs to the non-classic human leucocyte antigen class I family and it has been suggested that it has an immunomodulatory function: lower concentrations of HLA-G promote a Th1 cytokine dominant profile and enhances T lymphocytes alloreactivity. The HLA-G gene is composed of eight exons and seven introns translated into four membrane-bound proteins and three soluble proteins, according to the splicing site (37, 42). Soluble isoforms (sHLA-G) can be detected in non-pregnant women and in men's serum, though its concentration is two to four-fold higher in pregnant women's blood. This gene is particularly prone to polymorphisms in the 14-base pair (bp) of the 3' untranslated region (3'UTR) (37). A meta-analysis that aimed to evaluate if the HLA-G 14-bp insertion/deletion polymorphism was related to RIF concluded that the 14-bp insertion phenotype was associated with a significantly lower expression of HLA-G mRNA and sHLA-G compared to the deletion phenotype. In addition, HLA-G 14-bp insertion was shown to be more frequent in women suffering from RIF. All in all, HLA-G 14-bp insertion appears to enhance the risk of RIF in Caucasians (42).

Furthermore, trophoblast cells express both maternal and paternal HLA-C molecules that contact with the KIRs expressed by uNK cells. As said before, KIRs can either inhibit or activate NK cells. In a simplistic way, KIRs function is defined by two main haplotypes: A, that encodes inhibitory KIRs, and B, that encodes activating KIRs (43). The HLA-C molecules are composed by two allotypes – C1 and C2 – that are ligands for different A and B KIR haplotypes. It is known that the C2 binding to KIRs is stronger than the C1 one (44). Alecsandru et al. demonstrated that homozygous women for KIR A haplotype (KIR AA) have significantly higher miscarriage rates and lower live birth rates than KIR AB and KIR BB women after double embryo transfer. These differences were not observed after single embryo transfer, probably because there were less paternal HLA-C molecules presented to uNK cells. In addition, these rates worsen even more when the embryo expresses HLA-C2 molecules and when donated oocytes were used, since these HLA-C molecules are different from the expressed by the mother (these embryos only have "paternal" HLA-C). To summarise, KIR AA carriers have absence of immune NK cells activation when they contact with trophoblast cells, especially if they express HLA-C2, which results in impaired embryo invasion and placentation and consequently in implantation failure. Furthermore, the pregnancy outcomes worsen the more

HLA-C2 genes the embryo has more than the mother (43). These conclusions highlight that embryo implantation is a process that requires a certain degree of maternal immune activation, and that total inhibition of immune response could lead to foetal rejection.

7.2 P53 gene polymorphisms

Several studies referred p53 as one of the genes that could contribute to recurrence of implantation failure (42, 45, 46).

The p53 gene encodes a tumour suppressor protein responsible for inducing apoptosis and angiogenesis. Its function can be altered by a single-nucleotide polymorphism at the codon 72 that consists in either a C or a G allele. C alleles are translated into a proline (P72 variant), G alleles are translated into an arginine (R72 variant). The R72 variant is considerably more effective in leading to apoptosis, increasing LIF and preventing cellular transformation (46).

In fact, it was described that women with RIF had higher prevalence of PP genotype on p53 gene compared with oocyte donors (11.4% vs. 6%, respectively). In addition, it was concluded that the odds of implantation were substantially lower in patients carrying two P72 alleles (33.3% for RR/RP vs. 7.3% for PP; p=0.001) as well as the pregnancy rate (69.4% for RR/RP vs. 33.3% for PP; p=0.011) (46). Turienzo *et al.* also demonstrated that RIF women had higher prevalence of R72P polymorphism than fertile women and the presence of a P allele increases the chance of RIF by 2.78-fold (45). These outcomes probably result from impaired trophoblast growth due to cells arrest at G1 checkpoint of cell cycle and from decreased secretion of LIF, leading to implantation failure. In contrast, the lack of opportune apoptosis results in development of abnormal cells and tissues leading to miscarriage (46).

7.3 Vascular endothelial growth factor gene polymorphisms

Decidualisation is one of the most important steps for a successful pregnancy. Besides stromal oedema, cell proliferation and differentiation and leucocyte invasion, this process includes enhancement of vascular permeability, vascular remodelling, and formation of new vessels (47).

VEGF is a potent angiogenic growth factor involved in vasodilatation and vascular permeability, and in proliferation, differentiation and migration of endothelial cells promoting new vessels formation. VEGF is expressed in the human endometrium during mid-secretory phase, VEGF receptor 1 increases during mid-secretory phase and VEGF receptor 2 reaches its highest expression in late proliferative phase. Their expression is regulated by oestrogen and progesterone. (48). Pregnant women were found to have higher levels of this growth factor, which can be explained by blastocyst's production of VEGF that regulates trophoblast proliferation, invasion, and metabolic activity. Moreover, follicular granulosa and theca lutein

cells express VEGF indicating that it can be involved in follicular vascularisation and, thus, responsible for good-quality oocytes (48).

Several VEGF polymorphisms were studied and two of them were particularly related to infertility conditions: VEGF -1154 G/A and VEGF +405 G/C. Both polymorphisms are associated with lower VEGF expression (45, 48). Turienzo *et al.* explored the prevalence and influence of VEGF -1154 G/A polymorphism and determined that women carrying an A allele had 1.84 more chance of developing RIF (CI 95% 1.002-3.422) (45). Boudjenah *et al.* investigated the association between VEGF +405 G/C polymorphism and RIF and found that the CC genotype had higher frequency among RIF women than among controls (17.5% vs. 5.3%, p=0.01) (48). In both cases, women have lower levels of VEGF and embryos possibly repeat to fail due to impaired decidualisation and early placentation, decreased trophoblast proliferation and invasion, and dysfunctional ovarian angiogenesis that leads to poor-quality oocytes. Additionally, it is important to note that partners genotype can also influence blastocyst VEGF production. In the case of VEGF +405 G/C polymorphism, paternal genotype can either counter maternal C allele by transmission of a G allele or increase the risk of RIF by transmission of a C allele (48).

7.4 Non-coding RNA polymorphisms

Polymorphisms of non-coding RNAs (ncRNAs) such as microRNA (miRNA), circular RNA (circRNA) or long non-coding RNA (lncRNA) have been associated to several pathological alterations leading to cancer, degenerative diseases, and immune and metabolic disorders. These ncRNAs are responsible for regulating post-transcriptional gene expression. MiRNAs bind to mRNA leading to its degradation and suppressing translation. On the other hand, circRNAs and lncRNA act like miRNA sponges preventing them from binding to mRNA and allowing translation. The interactions between these ncRNA form regulatory RNA networks that control mRNA expression (49-53).

As miRNAs regulate processes like cell proliferation, differentiation, and apoptosis, clinicians started to question if their polymorphisms or polymorphisms of ncRNAs that compete with them, could preclude implantation (50). In fact, several studies described that miRNA, circRNA and lncRNA polymorphisms correlate with RIF prevalence either by increasing or decreasing it. However, the specific role of them in RIF remain unclear and additional investigations are needed (49-53).

Good-quality embryos produce extracellular vesicles that contain proteins, lipids, miRNAs, and other molecules to communicate with endometrium. These embryo's miRNAs are thought to alter endometrial gene expression in other to enhance receptivity. Kim *et al.* explored this hypothesis in mice embryos and concluded that competent (outgrowth) embryos had 226 of 1926 miRNAs upregulated compared to non-competent embryos (blastocyst and

non-outgrowth). Ten of the upregulated miRNAs were related to cellular and developmental processes particularly important for implantation (e.g., apoptosis suppression, epithelial cells proliferation) and six of them were found to be in the vesicles secreted by the outgrowth embryo. Although this theory could help to explain how embryo's quality and stage of development influence endometrium receptivity and implantation, as said before the specific role of miRNAs is yet to be discovered (54).

In addition, miRNA binds to Argonaute (Ago) proteins family to form the RNA-induced silencing complex (RISC) whose function is to inhibit mRNA translation. Ago proteins are crucial for RISCs performance, e.g., Ago2 leads the complex towards the target RNA. RISCs regulate gene expression related to growth and development of cells. Ryu *et al.* performed a case control study to explore if AGO1 and AGO2 gene polymorphisms were associated with RIF. They concluded that AGO2 rs4961280C>A polymorphism significantly influenced RIF prevalence since the presence of an A allele increased it. Furthermore, they concluded that different allele and genotype combinations of AGO1 and AGO2 polymorphisms could either be associated with higher or lower prevalence of RIF. In addition, AGO1 and AGO2 polymorphisms were related to clinical repercussions such as white blood cells proportion specifically CD3+ T cells, CD4+ helper T cells and CD8+ suppressor T cells influencing the inflammatory status. FSH, LH and blood urea nitrogen concentration were also found to be influenced by these gene's polymorphisms. Although these alterations could be a possible cause of RIF, the specific role of AGO1 and AGO2 genes in RIF pathogenesis remains unclear (55).

7.5 Mitochondrial DNA

Mitochondrial DNA had been previously related to oocyte and embryo quality (24). Eker *et al.* performed a study to explore if endometrial mtDNA amount was related with RIF. In fact, it was reported that the levels of MT-ND1 and MT-CO2 genes were significantly higher in RIF patients compared to fertile controls ($p=0.010$ and $p=0.019$, respectively). This can possibly be related to increased oxidative stress that modifies endometrial receptivity eventually leading to RIF (24).

***Table 2* – Genetic factors: main characteristics of the studies considered**

Authors and years	Country	Study design	Participants and inclusion criteria	Samples, timing and methods	Main outcomes
Boudjenah, 2012	France	Case control study	171 patients undergoing ICSI for male infertility or IVF failure: 40 women with RIF (≥ 10 embryos transfered), 131 controls (conceived before 10 embryo transfers)	Peripheral blood samples. PCR for VEGF gene.	VEGF +405 C/C polymorphism frequency was significantly higher in RIF women.
Alecsandru, 2014	Spain	Retrospective cohort study	291 women with RM (>3 unexplained miscarriages after ART) or RIF (>3 failed IVF cycles or >2 failed cycles with donor oocytes)	Peripheral blood samples. PCR-SSO for KIR gene.	Cycles with DET have significantly higher miscarriage rates in mothers with KIR AA haplotype compared with those with KIR AB and KIR BB haplotype.
Lledo, 2014	Spain	Prospective case control study	44 RIF patients, 54 RPL patients, 83 controls (oocyte donors)	Peripheral blood samples. RT-PCR for p53 R72P polymorphism.	PP genotype frequency in RIF patients was 11.4% vs 18.5% for those with RPL and 6% in controls ($p < 0.01$). PP genotype had higher risk of lower pregnancy rate [OR = 4.32, 95% CI = 1.39–13.51] Implantation rate was significantly decreased in patients homozygous for P72 (33.3% for RR/RP and 7.3% for PP; $p=0.001$).
Fan, 2017	China	Meta-analysis	5 case control studies		HLA-G 14-bp insertion allele may increase the risk of RIF in Caucasians.

Kim, 2019	Republic of Korea	Case control study	150 blastocysts on 4.5 dpc, 150 non-outgrowth embryos (hatched embryos not attached to fibronectin-coated dishes) and 150 outgrowth embryos (hatched and attached) on 7.5 dpc	Female mice underwent an ovary stimulation protocol and were mated with fertile males. 2-cell embryos were collected 1.5 dpc, cultured for 3 days and transferred to fibronectin-coated dishes. MiRNA microarray hybridisation.	226 of 1926 miRNAs were upregulated in outgrowth compared with blastocyst and non-outgrowth embryos. Ten of those 226 were associated with properties important for implantation. Six of the ten miRNAs were found to be released by the embryo: let-7b-5p, miR-92a-3p, miR-200c-3p, miR-291a-3p, miR-425-5p, and miR-429-3p
Ryu, 2019	Republic of Korea	Case control study	167 RIF patients (≥ 2 failed IVF cycles), 211 controls	Peripheral blood samples. PCR-RFLP for AGO1 rs595961G>A, AGO1 rs636832G>A, AGO2 rs22927779C>G, AGO2 rs4961280C>A and RT-PCR for AGO2 rs11996715C>A	AGO2 rs4961280C>A genotypes were significantly associated with RIF prevalence.
Lee, 2019	Republic of Korea	Case control study	118 RIF patients (≥ 2 failed IVF cycles), 228 healthy controls	Peripheral blood samples. Assessment of the coagulation status. PCR-RFLP for miR-25, miR-32, miR-125 and miR-222 polymorphisms.	MicroRNA polymorphisms frequency did not significantly differ between RIF patients and controls. Specific polymorphism combinations influenced clinical features, increasing the risk of RIF.

Lee, 2019	Republic of Korea	Case control study	119 RIF patients (≥ 10 embryos in ≥ 2 cycles), 212 controls with no history of pregnancy loss	Peripheral blood samples. PCR-RFLP for miR-605A>G (rs2043556), miR-608G>C (rs4919510), miR-631I>D (rs5745925), miR-938C>T (rs12416605), and miR-1302-3C>T (rs7589328).	miR-1302-3C>T polymorphism was associated with decreased incidence of RIF, being a potential biomarker.
Alecsandru, 2020	Spain	Prospective observational cohort study	204 women with RM (≥2 unexplained miscarriages) or RIF (≥2 failed ART cycles with at least 4 good-quality embryos)	Peripheral blood samples of women in study, partners, and sperm donors. Mouth swabs of newborns. Miscarriage tissue. PCR-SSO for HLA-C and KIR genes.	Significant differences were observed in miscarriage rates when DET was performed: 45.2% for KIR AA, 11.4% for AB, and 5.6% for BB (P<.001). No differences were observed after single embryo transfers. In KIR AA patients, LBR decreases and miscarriage rate increases with the increase of the embryo HLA-C2 load.
Turienzo, 2020	Spain	Case control study	89 RPL patients, 77 RIF (≥ 4 good quality embryos in 3 cycles) patients, 89 controls (oocyte donors)	Peripheral blood samples. RT-PCR for rs1042522 p53 R72P; rs1800896 IL-11 -1082AG; rs1570360 VEGF -1154 AG; rs11668344 IL-10; rs429358 APOE R112C.	p53 R72P polymorphism and E4 isoform of APOE gene prevalence were significantly higher in RPL and RIF. p53 R72P and VEGF -1154 AG polymorphisms are risk factors to RIF. E4 isoform of APOE is a risk factor for RPL.
Kim, 2020	Republic of Korea	Case control study	120 RIF patients, 219 control participants (≥ 10 embryos in ≥ 2 cycles)	Peripheral blood samples after 12h of fasting. Homocysteine, folic acid, total cholesterol, uric acid, BUN, Cr, blood coagulation status, TSH, oestradiol, PRL, FSH and LH assessment PCR-RFLP for miR-27aA>G, miR-423C>A, miR-449bA>G, and miR-604A>G.	miR-449bA>G polymorphism has a synergistic effect with coagulation conditions and increases the risk of RIF.

Zhao, 2021	China	Case control study	18 RIF patients (≥3 failed IVF cycles), 16 controls (tubal obstruction, ≤2 IVF cycles until conception)	Endometrial samples collected in mid-secretory phase. A competing endogenous RNAs network was constructed based on data from Gene Expression Omnibus database. qRT-PCR for circRNA, miRNA and mRNA.	hsa_circ_0038383/miR-196b-5p/HOXA9 axis may be a pathway that affects uterine receptivity and embryo implantation.
Lee, 2021	Republic of Korea	Case control study	155 RIF patients (≥2 failed IVF cycles with ≥10 embryos), 330 healthy controls	Peripheral blood samples. PCR-RFLP for HOTAIR gene polymorphisms, including rs4759314, rs920778, rs7958904, and rs1899663.	HOTAIR rs1899663 and rs7958904 polymorphisms were significantly associated with RIF occurrence.
Eker, 2021	Turkey	Case control study	50 women aged 25-42-year-old: 25 RIF patients (≥3 IVF cycles), 25 controls (history of ≥1 live birth)	Endometrial sampling using a pipelle at the 20-24 days of the menstrual cycle. Droplet digital PCR for quantification of MT-ND1, MT-CO2 and GAPDH. Nuclear GAPDH gene copy number was used to normalize mitochondrial DNA amount.	Mitochondrial target gene (MT-ND1 and MT-CO2) copy number was higher in RIF patients than in healthy controls.

APOE Apolipoprotein E, *BUN* Blood urea nitrogen, *CI* Confidence interval, *Cr* Creatinine, *dpc* days post-coitum, *DET* Double embryo transfer, *FSH* Follicle-stimulating hormone, *GAPDH* Glyceraldehyde-3-phosphate dehydrogenase, *HLA* Human leucocyte antigen, *HOTAIR* Homeobox transcript antisense RNA, *ICSI* Intracytoplasmic sperm injection, *IVF* In vitro fertilisation, *LBR* Live birth rate, *LH* Luteinizing hormone, *MT-ND1* mitochondrially encoded NADH dehydrogenase I, *MT-CO2* mitochondrially encoded cytochrome C oxidase II, *PCR* Polymerase chain reaction, *PRL* Prolactin, *RFLP* Restriction fragment length polymorphism, *RIF* Recurrent implantation failure, *RM* Recurrent miscarriage, *RPL* Recurrent pregnancy loss, *RT-PCR* Real-time polymerase chain reaction, *SSO* sequence-specific oligonucleotide, *TSH* Thyroid-stimulating hormone, *VEGF* vascular endothelial growth factor**.**

8 Altered gene and molecule expression

As mentioned before, a successful implantation depends on an effective and synchronized embryo-endometrium cross-talking. This communication involves different molecules, such as adhesion molecules, extracellular matrix proteins, cytokines, and growth factors (56). Consequently, is a process regulated by the endometrial expression of several genes, which is sensitive to hormonal variation along menstrual cycle, ovarian stimulation, and pathologies like endometriosis (57). The following studies were all performed in RIF patients of unidentified cause and are detailed on *Table 3*.

8.1 Constitutive endometrial deregulation

Firstly, failed implantations were thought to be caused only by poor-quality embryos, then it became evident that embryo-endometrium asynchrony had a negative impact on implantation through WOI displacement and, finally, the hypothesis of an endometrial dysfunction as a causative factor of RIF started to be considered (23).

Lédée *et al.* explored this hypothesis through a study in which the expression of selected genes was evaluated during the WOI in preconceptional endometrium of patients with history of RIF and compared with fertile controls (58). Gene expression was quantified by real-time quantitative polymerase chain reaction (PCR) and among the 7706 evaluated genes, 4519 were found to have a different expression in the RIF group. After exploring the biological function these genes perform, they were grouped into (i) molecular and cellular functions and (ii) physiological system development and function. In RIF patients, the first group was mainly composed by genes that regulate cell morphology, cellular development and organization, and cell cycle. Blood cells morphogenesis, morphology and shape changes were particularly affected (58). The second group was composed by differentially expressed genes associated to haematological system since haematopoiesis and differentiation of leucocytes were altered. The abnormal T lymphocytes and dendritic cells, and the anomalous NK cell migration were reflected on an impaired cell-mediated immune response that might reduce the immune tolerance towards the semi-allograft embryo. In addition, nervous system development genes were downregulated, which impairs its function (58). Genes involved in endometrium receptivity were also found to be differentially expressed, but this will be discussed in the following sections.

In 2016, Koot *et al.* designed a case-control study to investigate if a cohort with history of RIF had a different specific transcriptome. Endometrial samples (n=115) were collected 6 or 7 days after LH peak in a natural cycle and then samples were distributed randomly into a signature discovery set (n=81) or an independent validation set (n=34). They concluded that 303 genes were differentially expressed in RIF samples and that they were capable of distinguish RIF patients from the control cohort with a positive predictive value of 100%, a

sensitivity of 58% and a negative predictive value of 81% (57). The function of the genes differentially expressed reinforce the previous findings of Lédée *et al.* Downregulation is predominant especially among genes involving cell cycle regulation, cell division and other proliferative processes, which is translated into an endometrium with poor proliferative capacity. Genes responsible for cilia formation are also downregulated, which can have particular importance in underlying mechanisms of RIF since ciliated cells are present during the WOI of healthy women. Upregulated genes are less frequent and are responsible for extracellular organization, cell motility and expression of transcription factors such as the forkhead transcription factor FOXK2 (important for ovary development and function) (57).

Nowadays, the available test to evaluate endometrial receptivity is the ERA, however this is based on the endometrial gene expression of healthy individuals, so it is not able to differentiate women suffering from RIF from their fertile counterparts. On the other hand, the new gene set established by Koot *et al.* identifies RIF patients and classifies them according to their prognosis, since patients with low (<10%) or high (>90%) misclassification rate have low chance of a successful implantation and patients with an intermediate classification error have higher probability of embryo implantation (57).

Lastly, these biological functions alterations might impair endometrium receptive-selective balance. Low receptivity and high selectivity are related to RIF, whereas high receptivity and low selectivity are associated to recurrent miscarriages (23).

8.2 Oestrogen receptors

Normal endometrial receptivity has been associated with downregulation of oestrogen receptors isoform α (ER-α). The presence of high levels of oestradiol upregulates these receptors expression and progesterone downregulates them. It was shown that for all women (women with RIF and fertile women) oestradiol levels decreased with advancement in endometrial maturation phase determined by ERA (59). However, these receptors were found to have higher mRNA levels in women suffering from RIF compared with women undergoing ART due to male infertility (60). Although, it is assumed that higher levels of ER-α disrupt endometrial receptivity, the mechanisms by which it occurs and its correlation with oestradiol levels are not determined (59).

8.3 Adhesion molecules

Cell adhesion molecules (CAMs) are crucial for apposition and adhesion during embryo implantation and can be divided into four groups: integrins, cadherins, selectins and immunoglobulins (61)

Integrins are glycoproteins localised at the surface of cells that regulate intercellular and cell-matrix interactions. These molecules reach their higher expression on endometrium

during WOI and it had been shown that their abnormal expression was associated to conditions of subfertility, thus scientists started to explore if it was also associated with RIF (62). In 2011, Lédée *et al.* described that integrin patterns were downregulated in a group of repeated unexplained IVF failure compared with the control group, which they argue that could be a risk factor for coagulation conditions (58). One year later, Dos Santos *et al.* described, as well, downregulation of β_3 integrin in the endometrium of women with RIF (60). However, in 2013, Coughlan *et al.* conducted a prospective nonrandomized study in which they compared women with RIF and fertile women and found no differences in integrin's endometrial expression. In addition, they documented that the expression of α_1 and α_4 was higher in glandular epithelium than in luminal epithelium in both groups suggesting that those cells might have different functions thus far unknown. A weakness of this study is the small control cohort (n=6), which makes drawing a conclusion difficult (62).

In 2018, Guo *et al.* investigated the differentially expressed genes between a RIF group and a control group of women who got pregnant after their first treatment cycle. They found 1809 up-regulated genes and 710 downregulated genes in the RIF group, most of them related to molecular functions, especially adhesion, and cellular components. After some research, they concluded that five CAMs with significantly decreased mRNA levels could be associated with RIF which were ICAM2 ($p<0.01$), ITGB2 ($p<0.01$), platelet and endothelial cell adhesion molecule I (PECAMI) ($p<0.001$), SELP ($p<0.05$) and TEK ($p<0.05$), however only PECAMI had a significantly decreased protein expression ($p<0.05$) (61). PECAMI participates in cellular adherence, cell proliferation, apoptosis, leucocyte migration, and in inflammatory and immune responses. These functions are all important for the implantation process from embryo adhesion to cell proliferation and establishment of an inflammatory environment essential for endometrial receptivity (61). In conclusion, reduced expression of PECAMI molecules might be an underlying factor for RIF.

In addition, they concluded that the lack of PECAMI caused a reduction in the expression of TGF-β1 ($p<0.05$) in the RIF group (61).

8.4 Metalloproteinases

Metalloproteinases (MMPs) are a group of proteolytic enzymes whose activity depend on the presence of zinc. They degrade extracellular matrix (ECM) proteins, a crucial action for the third step of embryo implantation – invasion – and for vascular remodelling. Before invasion, MMPs are also involved in the orientation of the blastocyst towards endometrium during apposition. MMPs are divided and named according to their substrate: collagenases, matrilysins, stromelysins, gelatinases, membrane-type MMPs and unnamed MMPs. Their expression is regulated by countless types of cytokines, hormones, and growth factors (IL-1, -

6, -10 and -15, steroids, TNF-α, TGF-β, LIF, and IGFBP-1 and -2) secreted by trophoblast, decidua and uNK cells (63).

The tissue inhibitors of metalloproteinases (TIMPs) have the function of inhibiting MMPs action and, therefore, regulating the volume of ECM necessary for many physiological processes, namely the embryo implantation. TIMPs expression is regulated by several cytokines such as TNF-α, INF-γ, TGF-β and IL, and by the amount of MMPs secreted by the invading trophoblast (decidua produces large quantities of TIMPs in the presence of high amounts of MMPs) (63).

Cytokine alterations previously mentioned in *1. Th1/Th2 ratio* section may also interfere with MMPs activity. Increased levels of TNF-α inhibit MMP-2 precluding trophoblast invasion, and higher levels of IFN-γ promote extravillous trophoblast cells apoptosis, decreasing its growth and production of MMPs. Both scenarios could underlie implantation failure (63).

In conclusion, metalloproteinases are intrinsically involved in successful embryo development and implantation. Therefore, any deregulation of their expression or of their control mechanisms can modify embryo-endometrium cross-talk impairing embryo implantation (63)

8.5 Cytokines

8.5.1 Transforming growth factor β1

TGF-β1 is expressed in epithelium and stroma of the endometrium and in decidual cells. This cytokine stimulates endometrial proliferation, trophoblast adherence and invasiveness during embryo implantation, because contributes for cell survival and apoptosis control (50, 61). Its altered expression might lead to implantation failure. In fact, it was found that RIF group had significantly lower expression of TGF-β1 ($p<0.05$) than the control group and that it was correlated with PECAMI expression. In more detail, like previously mentioned, low PECAMI expression reduces the TGF-β1 expression and both were found to be depleted in the endometrium of women suffering from RIF (61).

8.5.2 Interleukins

Lédée *et al.* found that IL-18 expression was downregulated in women with RIF, which influences Th cells differentiation and impairs vascular remodelling. IL-18 expression is presumed to influence colony-stimulating factor (CSF) signalling that was also altered enhancing the previous alterations (58). In addition, IL-15 expression was found to be significantly increased ($p=0.009$) in ESCs from women with RIF compared with women with proven fertility. IL-15 has a key role in proliferation and cytotoxicity activation of NK cells and is responsible for the recruitment and differentiation of pNK cells into uNK. Therefore, it was documented a positive correlation between uNK cells and IL-15 in the endometrial stroma

(p=0.001, r=0.427). This might be one of the mechanisms underlying RIF due to the increased number and cytotoxicity of uNK cells (64). Conversely, Dos Santos *et al.* documented that IL-15 was lower in the endometrium of women with RIF (60), which could impair implantation due to the lack of uNK cells number and activity. As we can see, this is one more controversial issue to add to the list.

8.5.3 Leukaemia inhibitory factor and activating transcription factor 3

LIF is a cytokine expressed by both blastocyst and endometrium that stimulates the expression of several proteins on the endometrial epithelium promoting uterine receptivity. The endometrial epithelium expresses its receptor (LIFR) and after binding they contribute to the JAK-STAT pathway activation, an essential step in the regulation of epithelial intercellular junctions, polarity, and function during the WOI (56). In more detail, LIF participates in the syncytiotrophoblast's differentiation and regulates the invasion of trophoblast cells, firstly by stimulating the adhesion to the extracellular matrix and secondly by promoting TIMPs expression that limit MMPs activity. In addition, LIF has a key role in the stimulation of hCG production and secretion by the placenta. This hormone is essential for the maintenance of a healthy pregnancy, once it keeps *corpus luteum* production of progesterone, and regulates trophoblast invasion and vascular remodelling (63).

In 2012, Mariee *et al.* documented that LIF expression could be used as an IVF prognosis factor since in immunohistochemistry LIF staining was significantly lower in glandular epithelium of women with RIF compared to women with proven fertility. At that time, despite LIF role in implantation process was not clear, existing data based on LIF knock-out mice indicated that the lack of this cytokine precluded implantation (64).

Nowadays, LIF function is slightly clearer and it was demonstrated *in vitro* that its reduction impaired embryo implantation and its augmentation promoted implantation. It was observed that the activating transcription factor 3 (ATF3) promoted LIF transcription and expression, and that the expression of this transcription factor was reduced in RIF patients compared to controls. ATF3 is a transcription factor inducible by stress and is involved in cell cycle regulation, neutrophil migration, and sexual differentiation (56). Therefore, by promoting ATF3 expression in RIF patients, we could be able to ameliorate endometrial receptivity by upregulating LIF expression.

8.5.4 Leptin and Adiponectin

Leptin is a hormone, produced by adipocytes, responsible for regulating food intake and energy balance, yet it has a part in reproductive function as well. This substance has been also described to be secreted by trophoblast's cells and endometrium's cells. Its functions consist in promoting preimplantation embryo development and integrin expression, and in increasing the levels of IL-1, LIF and their receptors (60).

Adiponectin is another hormone involved in energy balance through regulation of lipid and glucose metabolism and has a crucial role in preventing metabolic events due to its anti-inflammatory, antiangiogenic, and antiatherosclerotic properties. In addition, it was discovered that adiponectin promotes embryo invasion and formation of the syncytiotrophoblast, modulates ovulation, steroid synthesis and secretion, and provides energy and an inflammatory response to the endometrial cells. Its reduction had been described in infertility predisposing conditions, such as endometriosis and polycystic ovary syndrome, and in pre-eclampsia (60).

These adipokines' function depends on their linkage to the respective receptors: Ob-R for leptin, and AdipoR1 and AdipoR2 for adiponectin. The expression of these receptors in the endometrium reaches a peak during the WOI (60).

Dos Santos *et al.* measured the expression of these molecules and their receptors in the endometrium of women with RIF and compared them with fertile controls. They found out that leptin expression at mRNA and protein levels was reduced and that its receptor was upregulated in the RIF women. On the other side, adiponectin expression was not significantly altered, while AdipoR1 and AdipoR2 were both diminished at mRNA and protein levels in the RIF group compared with controls (60). Although the leptin reduction is thought to be a specific condition of RIF among reproductive pathologies and a cause for the upregulation of Ob-R, the mechanisms by which they affect implantation are unknown. Conversely, the reduction of adiponectin receptors on the endometrium prevents this hormone from acting on the endometrium and, therefore, impairing implantation due to the reduction of its important functions previously mentioned (60).

8.5.5 Fetuin-A

Fetuin-A is a protein released by the liver into blood stream that has a role in adipocyte disfunction (consequently, reduction of adipokines), insulin resistance, metabolic syndrome, and atherosclerosis. In addition, inhibits the tyrosine kinase activity of insulin receptors, the same ones used by growth factors to control the growth and invasion of the trophoblast. This protein has been associated to pregnancy complications such as pre-eclampsia, diabetes and intrahepatic cholestasis (65).

A pioneer case control study revealed that serum fetuin-A levels were significantly higher in a cohort consisting of women with previous RIF compared to a cohort of fertile women (247.77 ± 32.18 vs. 219.59 ± 48.86, respectively; $p<0.001$). This discovery might be explained by the inhibition of the tyrosine kinase activity of insulin receptors which impairs trophoblast development and invasion (65). Further larger and randomized studies are needed to investigate if serum fetuin-A levels indeed underlie implantation failure and if they can be used as a therapeutic target or prognostic factor.

8.6 Angiopoietin-like protein 4

As said before, successful decidualisation is essential for embryo implantation. Furthermore, to achieve the adequate endometrial receptivity it is also fundamental a good blood supply that can be evaluated through uterine arterial impedance (47).

Angiopoietin-like protein 4 (ANGPTL4) is a glycoprotein involved in lipid and glucose metabolisms, angiogenesis, vascular permeability, and wound healing. Li *et al.* found that in RIF women with increased uterine arterial impedance ANGPTL4 mRNA and protein levels were significantly decreased when compared with controls. The study group also presented lower mRNA levels of decidualisation markers (IGFBP-1 and PRL), suggesting that this process was disrupted. In the same study they demonstrated *in* vitro that ANGPTL4 increases decidualisation on human ESCs and enhances proliferation and migration of human umbilical vein endothelial cells, indicating that this protein has a key role in endometrial decidualisation and angiogenesis during WOI (47).

The specific cellular mechanisms behind ANGPTL4 action in ESC and endothelial cells are yet to be discovered, but alterations in this protein abundance in endometrium and serum might be a possible cause of RIF and even a treatment target (47).

8.7 Relaxin classical receptor – LGR7

Relaxin (RLX) is a hormone produced during the luteal phase by granulosa cells of corpus luteum and during the first trimester of pregnancy. This peptide belongs to the insulin-like growth factor family and is thought to have a key role in the preparation of endometrium for receiving the embryo. RLX, along with cAMP, are the only molecules known to stimulate ESCs independently from progesterone action. It is also responsible for the stimulation of hormones and growth factors secretion, such as PRL and IGFBP, both markers of decidualisation. The RLX classical receptor is named LGR7 and its expression suffers a sudden increase during the early luteal phase of menstrual cycle, suggesting that RLX might have an important role in implantation (66).

In fact, women with history of at least three failed IVF cycles were found to have a significantly lower endometrial expression of LGR7 in both mRNA and protein levels ($p=0.02422$ and $p=0.05$, respectively) (66). This decrease could be responsible for structural and functional alterations of the endometrium impairing endometrium receptivity. However, there are scarce studies about RLX and LGR7 functions in humans *in vivo* and thus far no correlation between decreased RLX function and implantation failure was explored.

8.8 Prokineticins

Prokineticins (PROK) are two different proteins: PROK1 (endocrine gland VEGF) and PROK2, whose function depends on binding to their G-protein attached receptors (PROKR1

and PROKR2) with consequent production of an intracellular signal. PROK and their receptors are expressed in tissues where steroidogenesis occurs like ovary, uterus, and placenta. They are responsible for tissue angiogenesis and modulate inflammatory response and haematopoiesis, thus are essential for embryo implantation and for placental development (67). PROK1 expression in females occurs only during reproductive age and fluctuates throughout the menstrual cycle, is low during follicular phase, reaches a peak in the WOI during mid-luteal phase and, ultimately, decreases during late luteal phase (67).

Karear *et al.* wanted to explore new mechanisms that could underlie RIF pathogenesis, so he and his partners designed a case-control study to explore if the PROK and PROKR expression was altered in the endometrium of women suffering from RIF (67). They concluded that PROK1 mRNA levels were significantly higher in women with RIF ($p=0.006$), however at protein levels this difference was not present. On the other side, PROKR1 mRNA and protein levels in the luminal epithelium were both significantly lower in women with RIF ($p=0.02$ and $p=0.04$, respectively). In conclusion, although this study has small sample it brings new insights about RIF aetiopathogenesis. The disruption of the PROK1/PROKR1 signalling system can be one of the multiple alterations that result in repeated implantation failure, however further larger studies are needed to reach a stronger conclusion (67).

***Table 3* – Altered protein expression: main characteristics of the studies considered.**

Authors and years	Country	Study design	Participants and inclusion criteria	Samples, timing and methods	Main outcomes
Lédée, 2011	France	Case control study	30 RIF patients (≥10 embryos transferred), 25 RM or RPL patients (≥3 pregnancy losses 6-12 weeks), 15 fertile controls	Endometrial ultrasound and biopsy 7-9 days after ovulation. RT-PCR.	Both conditions have deregulated cellular functions: — RIF: cell morphology, cellular development, cell cycle, and cellular assembly — RM or RPL: cell signaling and maintenance.
Mariee, 2012	United Kingdom	Case control study	45 RIF patients (≥3 fresh IVF cycles or ≥2 fresh and ≥2 frozen IVF cycles), 15 control women	Blood samples collected at the early follicular phase and at the time of endometrial biopsy. Endometrial samples collected 7-9 days after LH peak. Immunohistochemistry for LIF and IL-15.	LIF staining intensity in glandular epithelium was significantly lower (p=0.01) in RIF patients compared with controls. IL-15 staining intensity in the stroma of endometrium was significantly higher (p=0.009) in women with RIF compared with controls IL-15 staining in the stroma was significantly positively correlated with uNK cell number.
Dos Santos, 2012	France	Case control study	31 RIF patients (>10 failed embryo transfers), 19 fertile controls	Endometrial ultrasound. Endometrial samples collected with a Cornier pipelle during a natural cycle. qRT-PCR, Western Blotting and immunohistochemistry for adiponectin, leptin and their receptors.	ER-α and PR mRNA levels were significantly increased in RIF patients compared with controls. Lower expression of β3 integrin and IL-15 in RIF patients' endometrium. Leptin mRNA was decreased and leptin receptor mRNA was increased in RIF patients. Same results were found at protein level. Adiponectin mRNA levels were not significantly different, but its

					receptors were lower at both mRNA and protein levels in RIF patients.
Coughlan, 2013	Australia	Prospective nonrandomized study	45 RIF patients (≥4 good-quality embryos in ≥3 failed transfers), 6 fertile controls; <40 years old	Endometrial samples collected 7-9 days after LH peak. Immunohistochemistry for integrins α_1, α_4, and $\alpha_v\beta_3$.	Significantly increased expression of integrins α_1 and α_4 in the glandular epithelium compared with the luminal epithelium. No differences in integrins expression were found between the two groups. None of the examined integrins appears to have prognostic value.
Koot, 2016	Netherlands	Case control study	43 RIF patients (≥3 failed IVF/ICSI cycles or ≥10 embryos transferred), 72 controls that conceived within three ART cycles	Endometrial samples collected 6 or 7 days after LH peak. Microarray hybridization.	Downregulated genes in RIF are associated with cell cycle regulation and cell proliferation. Upregulated genes are associated with extracellular organization and cell motility. Gene expression profile can be used to determine RIF prognosis based on misclassification rate.
Campitiello, 2016	Italy	Prospective observational study	23 RPL patients, 23 patients with ≥3 failed IVF cycles, 23 fertile women; 18-35 years old.	Endometrial samples collected during the secretory phase. Immunohistochemistry and PCR for RLX and LGR7).	Immunohistochemistry showed significantly lower levels of LGR7 expression in abnormal fertility outcome groups compared with fertile women.

Cheng, 2017	China	Prospective case control study	15 RIF patients (≥4 good-quality embryos in ≥3 failed transfers), 13 fertile controls	Endometrial samples collected during secretory phase and 10 more samples were collected during proliferative phase. Immunohistochemistry for ATF3 expression patterns. qRT-PCR, Western blotting and immunofluorescence for ATF3 after oestrogen and MPA treatment. Western blotting for LIF.	ATF3 expression was higher in the secretory phase than in the proliferative phase. ATF3 expression was decreased in RIF patients compared with fertile women. ATF3 regulates endometrial receptivity and embryo attachment in vitro via up-regulation of LIF.
Guo, 2018	China	Prospective case control study	22 RIF patients (≥4 good-quality embryos in ≥3 failed transfers), 18 women with successful index IVF cycle, 18 women with tubal factor infertility	Endometrial samples. qRT-PCR, Western blotting, immunohistochemistry, and immunofluorescence for PECAMI and TGF-β1 expression. Short hairpin RNA plasmid transfection for PECAMI and TGF-β1 expression in Ishikawa cells, hEECs and hESCs.	PECAM1 and TGF-β1 expression was significantly lower in the mid-secretory phase in RIF patients. Reduction of PECAM1 significantly declined the expression of TGF-β1 in Ishikawa cells, hEECs and hESCs. PECAM1 and TGF-β1 might have a key role in regulating endometrial receptivity.
Li, 2020	China	Prospective case control study	18 RIF patients with elevated uterine arterial impedance; 18 control women who achieved clinical pregnancy in the following IVF cycle	Peripheral blood and endometrial samples collected 7 days after LH peak. Serum ANGPTL4 concentration. Immunohistochemistry, RT-PCR, and Western blotting for ANGPTL4 and PPARγ. Cell culture and proliferation assays of hESCs and HUVECs.	ANGPTL4 mRNA levels were lower in RIF women. ANGPTL4 enhanced hESCs decidualisation and HUVECs proliferation, migration and angiogenesis.

Ozgu-Erdinc, 2020	Turkey	Case control study	42 RIF patients (≥2 consecutive failed ART), 36 healthy; <40 years old	Peripheral blood samples collected in the first three days of menstruation. ELISA to assess fetuin-A serum concentration.	Fetuin-A serum levels of RIF group were significantly higher than those of control group ($p<0.001$). Fetuin-A serum concentration was not correlated with the number of oocytes and embryos.
Hviid Saxtorph, 2020	Denmark	Case-control	84 RIF patients (≥3 embryo transfers), 37 women starting their first ART cycle due to male infertility	Endometrial and blood samples collected on day-6 of progesterone intake of a hormone-substituted regimen. Plasma steroids measurement. Noyes criteria, ERA, immunohistochemistry.	Association between uNK density and ERA was found. Progesterone levels were not associated with ERA maturation phases nor uNK cell counts. Oestradiol levels were associated with ERA maturation phases but not with uNK cell counts.
Karaer, 2020	Turkey	Case control study	15 RIF patients (≥4 good-quality embryos in ≥3 failed transfers), 15 fertile controls	Endometrial samples collected during WOI. qRT-PCR and immunohistochemistry for PROK1, PROK2 and their receptors (PROKR1 and PROKR2).	PROK1 mRNA and protein levels were higher and PROKR1 mRNA and protein levels were lower in RIF patients compared with the controls. No differences regarding PROK2 and PROKR2 expression were found between the two groups.

ANGPTL4 angiopoietin-like protein 4, *ART* assisted reproductive technique, *ATF* activating transcription factor 3, *ELISA* enzyme-linked immunosorbent assay, *ER-*α oestrogen receptor alpha, *ERA* endometrial receptivity array, *hEECs* human endometrial epithelial cells, *hESCs* human endometrial stromal cells, *HUVECs* human umbilical vein endothelial cells, *IL* interleukin, *IVF* In vitro fertilisation, *LGR7* endometrial RLX receptor, *LH* luteinizing hormone, *LIF* leukaemia inhibitory factor, *MPA* medroxyprogesterone acetate, *PECAMI* platelet and endothelial cell adhesion molecule I, *PPAR-*γ peroxisome proliferator-activated receptor gamma, *PR* progesterone receptor, *PROK* prokineticin, qRT-PCR quantitative real-time polymerase chain reaction, RIF recurrent implantation failure, RLX relaxin, *RM* recurrent miscarriage, *RPL* recurrent pregnancy loss, *RT-PCR* real-time polymerase chain reaction, *TGF-*β*1* Transforming growth factor beta-1, *uNK* uterine natural killer, *WOI* window of implantation.

9 Thrombophilic conditions

For a long time, thrombophilia has been associated to RIF because it exacerbates the pregnancy's typical hypercoagulability and increases the risk of complications such as pre-eclampsia, late and early recurrent miscarriage, intrauterine death, and stillbirth (68). However, the association between thrombophilic conditions and RIF is controversial (2). Thrombophilia can be classified as inherited or as acquired (2, 68).

9.1 Hereditary thrombophilia

Hereditary thrombophilia can result, for example, from methylene tetrahydrofolate reductase (MTHFR) deficiency, factor V Leiden, prothrombin deficiency, or antithrombin III deficiency (9). Pregnancy complications associated to factor V Leiden and prothrombin deficiency have maternal origin and usually are RPL, pre-eclampsia, small for gestational age new-borns or placental abruption (68). RIF is more expected to be associated to polymorphisms of MTHFR or annexin A5 genes.

In detail, MTHFR is an enzyme involved in folate metabolism crucial for the conversion of 5,10-methylenetetrahydrofolate into folate circulating form (5-methylenetetrahydrofolate). Folic acid is a key vitamin for amino-acid metabolism, nucleic acid synthesis and methylation. These functions are essential for DNA synthesis and repair, consequently folate deficiency leads to DNA disruption, slower DNA replication and chromosome damage. It is thought that MTHFR gene is one of the few genes capable of influencing embryo aneuploidy because interferes, for instance, with chromosome non-disjunction (69). Furthermore, folic acid deficiency impairs oocyte development, endometrial receptivity, embryo implantation and placental vascularisation (69). Enciso *et al.* conducted a study with the aim of exploring the incidence of two of the most investigated MTHFR polymorphisms (c.677C>T and c.1298A>C, both translated into a MTHFR with reduced activity) in infertile couples and their role in infertility. Secondly, they aimed do explore these polymorphisms influence in the production of aneuploid embryos (69). They concluded that the c.1298A>C genotype was four times more prevalent in couples with at least three failed previous IVF attempts than in fertile controls; on the other side the c.677C>T genotype showed no difference in frequency between groups, however these alleles where not distributed according to Hardy-Weinberg equilibrium in the infertile population on study. In addition, it was found that carriers of these polymorphisms had a 20% higher rate of embryo aneuploidy when compared to homozygous patients for the completely functional alleles. This aneuploidy is mostly due to meiotic errors during oogenesis, however the mechanisms behind those errors still need further investigation. At last, this study demonstrated that homozygous embryos for MTHFR 677T have significantly higher chance (20%) of implantation failure The author defends that MTHFR polymorphisms impair embryo implantation not through coagulation alterations but rather through reduction of folate

concentration, which has a negative impact on trophoblast invasion and on cell division and differentiation, or through induction of aneuploid embryos (69). Oppositely, a recent meta-analysis of case-control studies investigated the association between MTHFR polymorphisms and RIF and concluded that they were not associated (70). Thus, we cannot affirm if MTHFR polymorphisms are a cause of RIF alone, but it is possible that in combination with polymorphisms from other genes they would increase the odds of suffering from RIF (70).

Another hereditary thrombophilic condition more recently discovered is the M2 haplotype of the annexin A5 gene (M2/ANXA5). ANXA5 is an anticoagulant protein expressed at the placental surface of the syncytiotrophoblast's membrane that owes its function to its capacity to bind phospholipids making them unavailable for coagulation reactions and, consequently, preventing thrombosis (71). This protein has a key role on maintaining the pro- and anticoagulatory balance needed for implantation and the haemodynamic balance at the placenta (68, 72). In addition, ANXA5 is essential for the fusion of trophoblast cells to form the syncytiotrophoblast and for its membrane repair, which is the epithelial covering of embryonic placental villi that allows nutrients and gases exchange and produces progesterone, leptin, hCG and human placental lactogen. The M2/ANXA5 haplotype is a polymorphism in the core promoter of the gene that reduces ANXA5 expression, thus it could preclude implantation by disturbing syncytiotrophoblast formation (72). In fact, Rogenhofer *et al.* demonstrated, after comparing 63 RIF couples with two independent fertile control groups, that M2/ANXA5 haplotype was more frequent in patients suffering from RIF than in the control groups and that its carriers had 1.8 times more chance for RIF. They also concluded that both maternal and paternal alleles had similar chance of causing implantation failure (72).

9.2 Acquired thrombophilia

Antiphospholipid syndrome (APS) is the most common form of acquired thrombophilia (68). This syndrome is an autoimmune disease whose diagnosis is based on laboratorial criteria such as the presence of anti-phospholipid antibodies (anti-cardiolipin antibodies, lupus anticoagulant or anti-β2-glycoprotein I antibodies) measured in two different occasions,12 weeks apart, and clinical criteria such as vascular thrombosis or poor pregnancy outcomes (9, 68). Late pregnancy complications are shown to be more common in the presence of anti-phospholipid antibodies (68), however there is lack of evidence connecting them directly to RIF (9). There is some evidence that the presence of antiphospholipid antibodies reduces the expression of ANXA5 (68) and that patients suffering from RIF produce anti-annexin A5 antibodies more frequently than their fertile counterparts (8.3% vs. 1.1%, $p<0.05$) (72). Therefore, it is possible that couples in this situation may have their embryo implantation precluded by the mechanisms previously mentioned.

Prothrombotic states can be also associated to circulating cell-derived microparticles (cMPs) that are derived from vesiculation of pre-apoptotic or activated cells' cytoplasm. These particles are highly thrombogenic and are involved in placental haemostatic equilibrium, trophoblast invasion and angiogenesis. When they are increased, they promote inflammation, formation of thrombus, and endothelial alterations. A pioneer case-control study identified increased levels of cMPs in women with RIF compared to women with previous successful IVF cycles or to fertile women ($p<0.05$ and $p<0.01$, respectively). Further larger studies are needed to ascertain if there is a correlation between cMPs levels and RIF. A possible hypothesis is that these particles impair embryo implantation by altering syncytiotrophoblast invasion capacity and angiogenesis and by promoting inflammatory and apoptotic processes, rather than altering the coagulation state (73).

Although it appears that thrombophilic conditions and RIF have no cause-effect relation, there is evidence that in those cases anti-thrombotic therapy with enoxaparin or unfractionated heparin (UFH) reduces the risk of implantation failure and ameliorates the IVF cycle outcome (68).

10 Vaginal microbiota

Vaginal microbiota consists of commensal bacteria that evolved to maintain balance and prevent invasion of pathogens. The dominant microorganism on healthy women is *Lactobacillus,* responsible for the acidic and anaerobic environment of the vagina through the production of lactate acid (26).

A higher diversity of microorganisms was found in RIF patients in comparison to women who achieved pregnancy after their first FET cycle, and most important *Lactobacillus* was significantly decreased in the first group. Furthermore, samples with relative abundance of *Lactobacillus* ≤90% were associated to poorer pregnancy rates when compared to samples with >90% (34.723% vs. 72.723% respectively; $p = 0.006$) (26). This alteration on vaginal microbiota structure might underlie implantation failure once it is related to changes on metabolite profile. After vaginal metabolome evaluation, Fu *et al*. reported significant changes of 37 metabolites between the two groups. In the study cohort, 2',3-cyclic UMP and inositol phosphate were increased by four times or more, while benzopyran and glycerophospholipid were decreased by four times or more. Inositol phosphate high levels could lead to uterine contraction through Ca^{2+} influx, which impairs embryo implantation. In addition, glycerophospholipid has several metabolites being inositol phosphate one of them. Its elevation could mean lack of glycerophospholipid to produce other metabolites such as lysobisphosphatidic acid (LPA) and arachidonic acid, that will later be turned into prostaglandin (PG) under COX-2 action. LPA and PG are both key players in embryo implantation and

decidualisation (26). In mice, LPA maintains the normal size and spacing of the embryo and controls PGs levels through regulation of COX-2 activity. As a result, LPA downregulation was associated to delayed implantation and foetal development, embryo crowding and altered PGs production, which could directly impair the implantation and decidualisation processes (26). Benzopyran is a selective oestrogen receptor modulator and its downregulation, associated with higher levels of androgens in RIF patients, was thought to diminish oestrogens activity. This could result in low endometrium thickness, reduced ovarian and pituitary function and, thus, in failed embryo implantation. However, the exact role of benzopyran in implantation process is still unknown (26).

At last, glycerophospholipids and benzopyran were found to increase with the amount of *Lactobacillus* (26), reinforcing the hypothesis that vaginal microbiota alterations could possibly underlie RIF.

DISCUSSION AND CONCLUSION

RIF is a financial burden and a cause of stress for both couples and physicians. Although this pathology has been the centre of multiple investigations in the last ten years and even before, its underlying mechanisms still are not fully understood. This is related to the high complexity of the implantation process itself that relies on multiple molecules and signalling pathways to ensure an effective communication between the embryo and the endometrium.

The complexity of RIF aetiopathogenesis is summarized in *Figure 2.* There are maternal risk factors that interfere with oocyte quality and with the WOI timing. Male-caused RIF is especially due to higher rates of sperm aneuploidy. Embryo factors are related to aneuploidy, zona hardening and stage of development since a successful implantation requires a 5 or 6-day blastocyst. Uterine factors include anatomical anomalies, CE, and endometriosis, all of them modifying vascularisation and decidualisation, cytokine profile, and thus endometrial receptivity. In addition, hydrosalpinx is a known cause of RIF through fluid's toxic effect and endometrial receptivity alteration. Vaginal microbiota alterations might also underlie RIF because of metabolome changes.

After exclusion of the previous causes, immunological alterations, genetic factors, and altered expression of specific molecules should be considered. These alterations underlie implantation failure, since they impair embryo development and invasion, endometrial receptivity, vascular remodelling, decidualisation, and endometrial proliferation. Thrombophilia role in RIF aetiopathogenesis is still controversial due to the number of contradictory studies.

The lack of certainties about RIF pathogenesis results in the lack of guidelines from both the European Society of Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM). A recent guideline from the Canadian Fertility and Andrology Society recommends transvaginal ultrasound and karyotype testing for RIF couples. However, it does not routinely recommend thrombophilia testing, sperm DNA fragmentation index, CE screening or PTG-A due to lack of good quality evidence. Furthermore, they recommend immune testing and ERA only for research purposes (74).

RIF's aetiopathogenesis establishment is fundamental to clinical practice. This allows the identification of potential biomarkers and prognosis factors, and the development of directed complementary diagnostic methods. An easier diagnosis and the distinction of women who really benefit from continuing ART cycles from those who do not would turn this pathology less stressful and more sustainable. Furthermore, the development of new therapeutic approaches relies on the comprehension of aetiopathogenesis.

After studying the potential cause of RIF, a personalised therapeutic approach is defended. There are several different options available, some with more evidence than the others (2, 9), such as endometrial scratching, low molecular weight heparin, antibiotics for

infection, intravenous immunoglobulin, intravenous Intralipid, infusion of peripheral blood mononuclear cells, and granulocyte colony stimulating factor. The last four mentioned therapies are components of immunotherapy (9, 74).

The present review exposes the current knowledge about RIF aetiopathogenesis that appears to be multifactorial. It is important to note that the studies included are essentially retrospective case-controls studies with a small sample size and cohort studies. For this reason, it is impossible to discard that their results might be influenced by multiple biases. Therefore, in order to better understand this pathology and the specific mechanisms behind it, further, larger and randomized controlled trials are needed.

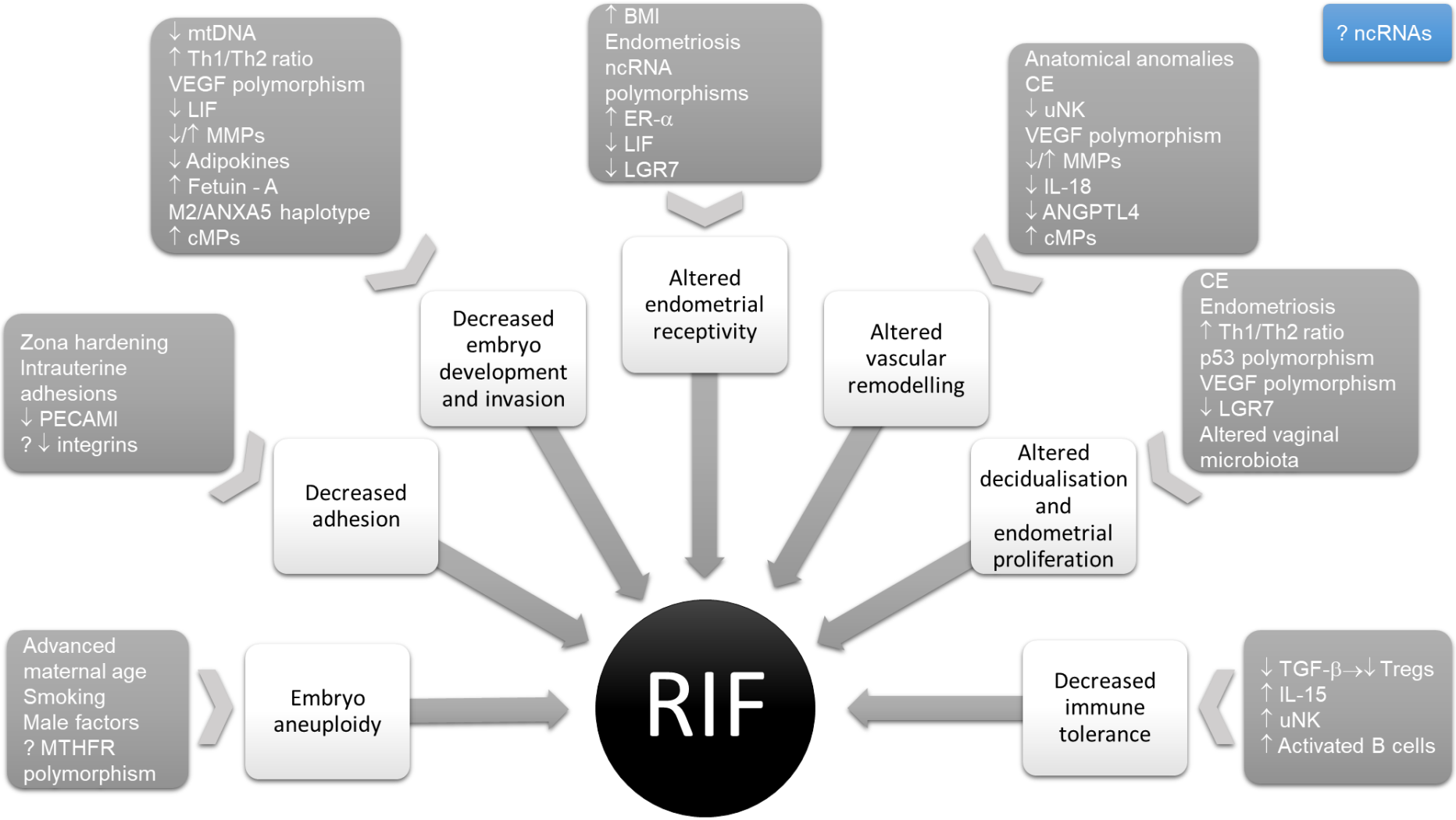

Figure 2 - Aetiopathogenesis of RIF.

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