Potential innate immunity-related markers of endometrial receptivity and recurrent implantation failure (RIF)

BASATVAT, Shaghayegh, RUSSELL, J.M., SAARE, M., THURSTON, L.M., SALUMETS, A. and FAZELI, A.

Title: Potential innate immunity-related markers of endometrial receptivity and recurrent implantation failure (RIF)

Authors: Shaghayegh Basatvat[1,2], Jean M Russell[3], Merli Saare[4,5], Lisa Marie Thurston[1,6], Andres Salumets[4,5,7,8], Alireza Fazeli[1,7, 9]

[1] Academic Unit of Reproductive and Developmental Medicine, Department of Oncology and Metabolism, The Medical School, University of Sheffield, Sheffield, UK.

[2] Biomolecular Science Research Centre, Department of Bioscience and Chemistry, Sheffield Hallam University, Sheffield, UK.

[3] Corporate Information and Computing Services, University of Sheffield, Sheffield, UK.

[4] Institute of Clinical Medicine, Department of Obstetrics and Gynaecology, University of Tartu, Tartu, Estonia.

[5] Competence Centre on Health Technologies, Tartu, Estonia.

[6] Department of Comparative Biomedical Sciences, Royal Veterinary College, University of London, London, UK.

[7] Institute of Veterinary Medicine and Animal Sciences, Estonian University of Life Sciences, Kreutzwaldi 62, 51006 Tartu, Estonia.

[8] Institute of Genomics, University of Tartu, Tartu, Estonia.

[9] Institute of Biomedicine and Translational Medicine, Department of Pathophysiology, University of Tartu, Tartu, Estonia.

Correspondences: Prof. Alireza Fazeli
Institute of Biomedicine and Translational Medicine, Department of Pathophysiology, University of Tartu, Ravila St. 14b, 50411, Tartu, Estonia.
Email address: Fazeli@UT.EE

Acknowledgement
Authors would like to thank Dr Triin Laisk for her help with design of the experiments and manuscript edition.

Abstract

The successful implantation of the embryo into a receptive endometrium is essential for the establishment of a viable pregnancy while recurrent implantation failure (RIF) is a real challenge in assisted reproduction. The maternal innate immune system, specifically the Toll-like receptors (TLRs), are involved in maintaining immunity in the female reproductive tract (FRT) required for fertility. In this study, we aimed to investigate the importance of innate immunity-related gene expression in the regulation of human fertility and as a prediction of potential outcome of *in vitro* fertilization - embryo transfer (IVF-ET), thus, we assessed the gene expression levels of TLR signalling molecules using quantitative real-time PCR between endometrial biopsies of healthy fertile women, and the patients experiencing RIF. Interestingly, our results showed that, *TRIB2* and *TLR9* genes were differentially expressed between the endometrial biopsies of healthy women and those with RIF. However, comparing expression levels of same genes between pre-receptive and receptive healthy endometrial biopsies showed different genes (*ICAM1*, *NFKBIA*, *VCAM1*, *LIF*, *VEGFB*, *TLR5*) had significantly altered expression, suggesting their involvement in endometrial receptivity. Thus, further investigations will enable us to better understand the role of these genes in the biology of FRT and as a possible target for the improvement of infertility treatments and/or development of non-hormonal contraception.

Key words: innate immune system, embryo implantation, recurrent implantation failure, toll-like receptors, endometrial receptivity.

Declaration of interests: None

Authors' contribution

S. B contributed to the experimental design and implementation of the research, performance of the experiments, analysis of the results, preparation of the manuscript and designing figures. **J. M. R** contributed to data analysis. **M. S** contributed to experimental design and to the perfomance of early experiments. **L. M. T** contributed to the edition of the manuscript. **A.S** contributed to experimental design and supervision of the project. **A. F** contributed to experimental design and leading and supervision of the project.

Ethical approval

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia (Ethical permission: 276/M-15).

Funding

This research was funded by grants from the European Union through the Corporation in Science and Technology (COST) Action CA16119 'In vitro 3-D total cell guidance and fitness'; the COST Action FA1201 'Epigenetics and periconception environment'; the Horizon 2020 programme, grant No. 692299 'Scientific Excellence in Animal Reproductive Medicine and Embryo Technology; SEARMET' and grant No. 857418, "COMBIVET" ERA Chair project. This work was also financially supported by grants from the Estonian Ministry of Education and Research (grant IUT34-16); Enterprise Estonia (grant EU48695); the European Commission Horizon 2020 research and innovation program under grant agreement 692065 (project WIDENLIFE) and MSCA-RISE-2015 program MOMENDO (691058).

1. Introduction

Recurrent implantation failure (RIF) is a significant problem during *in vitro* fertilization- embryo transfer (IVF-ET) [1]. RIF is defined as the lack of embryo implantation after transferring at least three high quality embryos [2]. Poor endometrial receptivity, insufficient endometrial thickness, advanced maternal age, hormonal imbalances, implantation-related gene mutations and genetic or developmental abnormalities of the embryo are associated with implantation failure [3-6]. The causes of RIF can either be immunological or inflammatory factors since maternal innate immune system plays a major role during embryo-maternal communication [7]. Accordingly, investigating immunity factors that influence endometrial receptivity and embryo implantation are significant in improving pregnancy success rates for IVF-ET.

The maternal innate immune system has an important function during pregnancy by protecting the female reproductive tract (FRT) against infections while providing a tolerance towards the semi-allogenic foetus [8]. Toll-like receptors (TLRs) are the most documented family of pattern recognition receptors, playing a key role in innate immune system. Once stimulated by their specific ligands, they commence an intracellular cascade of signals through various adaptor proteins that end up in the expression of anti-inflammatory cytokines and chemokines [9, 10]. Innate immune cells such as natural killer (NK) cells, macrophages, and dendritic cells which express TLRs, are abundant at the site of embryo implantation [11-15].

Earlier investigations, have revealed that stimulation of TLRs with their specific ligands at the time of embryo implantation undesirably affects the outcome of embryo implantation *in vivo* [16, 17], and *in vitro* [17-20]. While different mechanisms were used to explain this failure of embryo implantation, all hypotheses identified a disruption of endometrial receptivity, due to the involvement of abnormal activation of innate immunity.

In order to address the role of innate immunity in endometrial receptivity and RIF, we firstly compared the gene expression profile of innate immunity-related molecules in endometrial biopsies obtained from healthy/fertile women during endometrial transition between the non/pre-receptive to the receptive stages; and secondly, between the endometrial biopsies from healthy/fertile women, and the RIF patients both with the endometrial biopsies representing the receptive stages.

Candidate genes were coding for receptors, adaptor molecules, cytokines and regulatory proteins selected from the KEGG pathway https://www.kegg.jp/ (Kyoto Encyclopaedia for Gene and Genome). of TLR signalling cascade including both MAPK signalling and NFKB signalling arms https://www.genome.jp/pathway/hsa04620 and based on their potential to influence endometrial receptivity and/or embryo implantation. These included

Tribbles-2 (*TRIB2*) https://www.genome.jp/entry/hsa:28951, Toll-like receptor 5(*TLR5*) https://www.genome.jp/entry/hsa:7100, Toll-like receptor 9 (*TLR9*) https://www.genome.jp/entry/hsa:54106, Myeloid differentiation primary response gene 88 (*MyD88*) https://www.genome.jp/entry/K04729, Mucin 1 (*MUC1*), Mucin 16 (*MUC16*), Leukemia inhibitory factor (*LIF*) https://www.genome.jp/entry/K05419, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (*NFKBIA*) https://www.genome.jp/entry/K04734, Intracellular adhesion molecule 1 (*ICAM1*) https://www.genome.jp/entry/K04734, Vascular cell adhesion molecule 1 (*VCAM1*) https://www.genome.jp/entry/K06527, Vascular endothelial growth factor B (*VEGFB*) https://www.genome.jp/entry/K16858 and Interleukin-8 (*IL-8*) https://www.genome.jp/entry/hsa:3576.

2. Materials and Methods:

2.1 Ethical approval and sample collection

The study was approved by the Research Ethics Committee of the University of Tartu, Estonia (Ethical permission: 276/M-15) and written informed consent form was obtained from all participants. Endometrial biopsies were obtained from 10 healthy and fertile volunteers of reproductive age (≤35 years) with a normal BMI (within the range 19-25), who had no previous infertility record, and had at least one live-born child. Endometrial biopsies were obtained using a Pipelle catheter (Laboratoire CCD, Paris, France) on day 2 and 8 after the luteinizing hormone (LH) surge (LH+2 and LH+8, respectively). The LH surge was determined using a commercial urine LH kit (BabyTime hLH urine cassette, Pharmanova). Endometrial biopsies were also acquired 8 days after the LH surge, from an additional group of 10 individuals of fertile age (≤42 years) with a normal BMI (within a range of 19-25), who had undergone at least 3 unsuccessful IVF-ET or 3 ICSI (intracytoplasmic sperm injection)-ET cycles. This RIF group consisted of women diagnosed with primary or secondary infertility. All women selected for the study had regular menstruation and were clinically examined for the absence of hormonal aberrations and/or uterine pathologies. All the 20 women were non-smokers and did not take any hormonal treatments for three months prior to sample collection. The endometrial tissue recovered at LH+8 from both groups, was histologically validated according to the Noyes' criteria [21] in order to confirm the receptive status of endometrial maturation. Endometrial tissue was frozen after biopsy at -80°C in RNAlater (Ambion Inc., Austin, TX) for further analysis.

2.2 RNA extraction, cDNA synthesis and quantitative real-time PCR analysis

Endometrial total RNA was isolated using the Qiagen All Prep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA quality and concentration were assessed using the Agilent 2100 Bioanalyzer, (Agilent Technologies, Santa Clara, California, USA) and the RNAs with an RNA Integrity Number (RIN) values ≥7 was used for subsequent complementary DNA (cDNA) reactions. The first-strand cDNA was synthesized from 2µg of DNase treated RNA using the RNA to cDNA kit (Applied Biosystems, Life Technologies; Paisley, UK) according to the manufacturer's instruction.

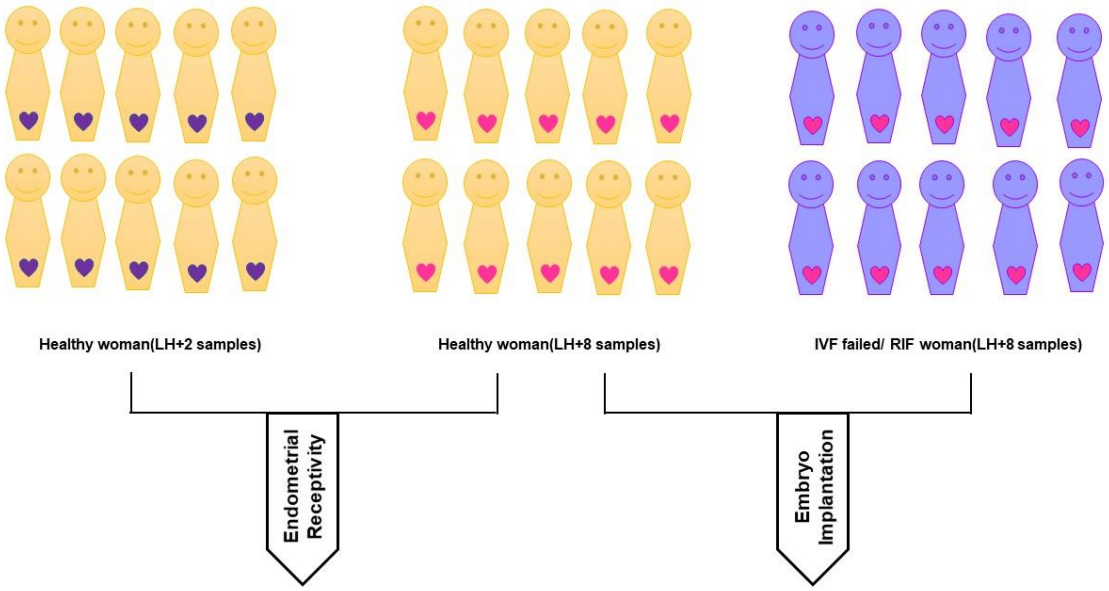
The forward (fwd) and reverse (rev) primers (Integrated DNA Technology Company, Leuven, Belgium) for all the genes investigated in this study, were created with the Primer-Blast tool (National Centre for Biotechnology Information website; NCBI) (Table 1). According to the MIQE guidelines (minimum information for publication of quantitative real-time PCR experiments) [22] three housekeeping genes were used as reference genes for normalization. These genes were human β-Actin (*BACT*), succinate dehydrogenase subunit A (*SDHA*) and mitochondrial ribosomal protein L19 (*MRPL19*).

The quantitative real-time PCR (qPCR) reaction was performed using SYBR Green Jump Start *Taq* Ready mix® (Sigma, UK). Quantitative real-time PCR products were compared to a MiniSizer ladder (Norgen Biotek; Ontario, Canada) to confirm the expected size according to Table 1. The experimental design for the comparison of gene expression in endometrial biopsies is summarised in Figure 1.

Table 1. Sequence of Forward and reverse primers

GENE	FORWARD 5'—3'	REVERSE 5'–3'	PRODUCT SIZE	ACCESSION NUMBER
*B-ACTIN**	CAAGATCATTGCTCCTCCTG	ATCCACATCTGCTGGAAGG	90 bp	NM_001101.3
*SDHA**	ACTGTTGCAGCACAGCTAGAA	TCCAAACTTGAGGCTCTGTCC	102 bp	NM_001294332.1
*MRPL19**	ATCGAAGGACAAGGTGTCGAG	TAGCAAGCTATCATCCACCG	121 bp	NM_014763.3
TRIB2	GAGCTGGTGTGCAAGGTGTT	CCCAGGATAATTTCAGTGATTTGGT	110 bp	NM_021643.3
TLR5	CCTCATGACCATCCTCAC AGTCAC	GGCTTCAAGGCACCAGCCATCTC	355 bp	NM_003268
MUC1	CCGCCGAAAGAACTACGG	CCTGCAGAAACCTTCTCATAG	179 bp	NM_001204296.1
MUC16	GCCTCTACCTTAACGGTTACAATGAA	GGTACCCCATGGCTGTTGTG	114 bp	NM_024690.2
IL-8	GAACTGAGAGTGATTGAGAGTGGA	CTCTTCAAAAACTTCTCCACAACC	134 bp	NM-000584.3
IKBA	CCCTACACCTTGCCTGTGAG	CGTGTGGCCATTGTAGTTGG	116 bp	NM_020529.2
TLR9	CTGGAAGGCCTTGGTTTTAGT	CGTCTTGAAGGCCTGGTGTTG	141 bp	NM_017442.3
LIF	CCACCCATGTCACAACAACC	CCCTGGGCTGTGTAATAGAGAA	102 bp	NM_002309.4
VCAM1	TGTTTGCAGCTTCTCAAGCTTTT	GATGTGGTCCCCTCATTCGT	181 bp	NM_001078.3
ICAM1	ATGGCAACGACTCCTTCTCG	GCCGGAAAGCTGTAGATGGT	142 bp	NM_000201.2
MYD88	GACCCAGCATTGAGGAGGAT	CTGCACAAACTGGATGTCGC	212 bp	NM_001172567.1
VEGF B	CCACCAGAGGAAAGTGGTGT	ATCTGCATCCGGACTTGGTG	213 bp	NM_001243733.1

*Housekeeping genes used as a reference gene in normalisation for calculation of relative mRNA expression level



179

180 **Figure 1. The endometrial biopsies used for this study were obtained from 20 individuals divided into 3**
181 **groups.** The endometrial Biopsies were obtained from 10 healthy women at day LH+2 (yellow dolls with purple
182 heart) and day LH+8 (yellow dolls with pink heart) representing the pre-receptive and receptive endometrium
183 respectively. Endometrial biopsies were collected at day LH+8 from 10 other women who experienced at least 3
184 rounds of unsuccessful IVF/ICSI (purple dolls with pink heart) considered as RIF women. Relative expression
185 level of 12 selected genes was compared between pre-receptive and receptive endometrium from healthy
186 woman to investigate gene expression alterations during endometrial receptivity. Relative expression level of 12
187 selected genes was compared between healthy woman and RIF patients both at day LH+8 to investigates gene
188 expression changes at embryo implantation.

189

190

191

192

193

194

195

196

197

198

2.3 Statistical analysis

The $\Delta\Delta C_t$ method was used to analyse the relative gene expression data. The C_t value of the gene of interest ($\Delta C_{t\ sample}$) was normalized to the standard sample (pool of the cDNA of all the samples) and to the C_t of the reference genes ($\Delta C_{t\ reference}$). The $\Delta\Delta C_t$ is calculated as:

$$\Delta\Delta C_t = \Delta C_{t\ sample} - \Delta C_{t\ reference}$$

The relative expression of a particular gene, for each sample, was calculated as $2^{(-\Delta\Delta Ct)}$.

The results are shown as mean ± SEM. Statistical analysis was performed using GraphPad Prism software (V6, San Diego, California). Paired two-tailed Wilcoxon test was used to compare gene expression levels between biopsies from day LH+2 and biopsies from day LH+8 of healthy woman and unpaired two-tailed Mann-Whitney test was used to compare gene expression analysis between biopsies from day LH+8 of healthy women and biopsies from day LH+8 of RIF patients. P value ≤ 0.05 was considered to be significant.

To compare between the differentiation ability of each gene in fertile woman and RIF patients, a logistic regression was carried out using forwards stepwise selection. This would take the most significant terms first and add it to the model and then the next most significant term.

3. Results

3.1 Expression patterns of selected genes differed with the phase of endometrial receptivity in fertile woman

To investigate the difference in gene expression level of selected genes at the time of endometrial receptivity, their expression level was compared between pre-receptive (LH+2) and receptive (LH+8) endometrial biopsies of healthy women. Quantitative real-time PCR analysis of gene expression demonstrated that *LIF* and *VCAM1* genes were significantly up regulated in biopsies obtained at LH+8 days compared to biopsies from the same women at LH+2 (Figure 2). Conversely, analysis of gene expression levels identified a significant decline in *ICAM1*, *TLR5*, *IKB* alpha and *VEGFβ* in the receptive endometrial biopsies (LH+8) as compared to samples collected from the pre-receptive endometrial biopsies (LH+2) (Figure 2).

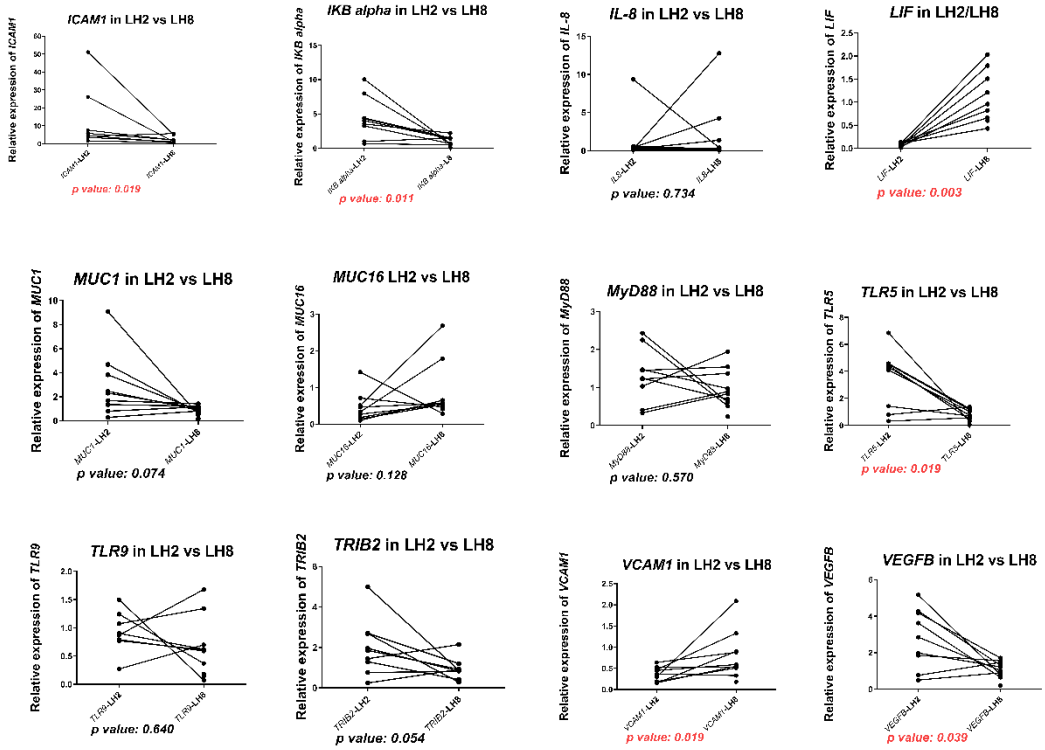


Figure 2. Different gene expression levels from biopsies of healthy women compared at two different stages of their menstrual cycles. Biopsies from day LH+2 represent the pre-receptive endometrium and from day LH+8 represent the receptive endometrium. Relative expression of *ICAM1*, *NFKBIA*, *IL-8*, *LIF*, *MUC1*, *MUC16*, *MyD88*, *TLR5*, *TLR9*, *TRIB2*, *VCAM1* and *VEGFB* was analysed using qPCR. Paired two-tailed test (Wilcoxon) was used to analyse the difference between the two groups and $P \leq 0.05$ was considered to be significant. The *p* value of genes with significantly different expression levels are shown in red.

3.2 TRIB2 and *TLR9* genes were significantly upregulated in the endometrial biopsies from RIF women compared with biopsies from healthy women

To understand which of the selected genes could be important during failed embryo implantation, expression level of these genes was compared between healthy and RIF endometrial biopsies both obtained at supposedly receptive stage of endometrium (LH+8). Quantitative real-time PCR analysis of gene expression demonstrated that *TRIB2* and *TLR9* genes were with significantly increased expression in the endometrial samples collected from RIF patients compared with their healthy endometrial biopsies' counterparts (Figure 3). Other investigated genes did not show any remarkable expression differences between RIF patients and healthy women (Figure 3).

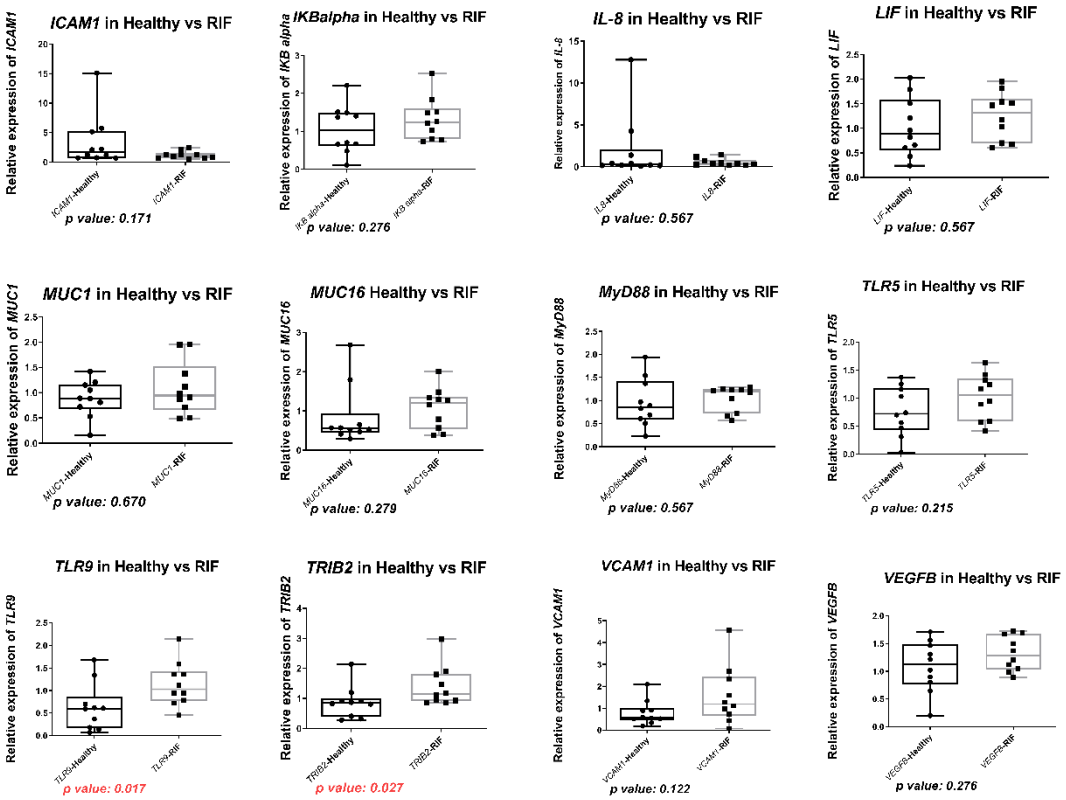


Figure 3. Different gene expression levels from biopsies of healthy women compared to the biopsies from IVF-failed patients. Biopsies from both groups were collected on day LH+8 representing the receptive endometrium. Relative expression of *ICAM1*, *NFKBIA*, *IL-8*, *LIF*, *MUC1*, *MUC16*, *MyD88*, *TLR5*, *TLR9*, *TRIB2*, *VCAM1 and VEGFB* was analysed using qPCR between the two types of samples. Unpaired two-tailed test (Mann-Whitney) was used to analyse the difference between the two groups and $P \leq 0.05$ was considered to be significant. The *p* value of genes with significantly different expression levels are shown in red.

Interestingly, binary logistic regression analysis of gene expression patterns demonstrated that the *TLR9* gene expression can be utilized as a reliable marker/predictor of whether an endometrial tissue sample was originated from a healthy woman or RIF individual (Table2). Hence, binary logistic regression analysis of *TRIB2* gene expression patterns did not show that this gene can be utilized as a reliable predictor of an endometrial tissue origin. Although, *TRIB2* expression was significantly different between the endometrial tissue of healthy women and RIF patients, its inclusion along with *TLR9*, did not add any value to the discriminatory power of *TLR9* to identify healthy women and RIF individuals (Table 2). It is important to note that this analysis is based on a sample size of 10 individuals per group (10 healthy women and 10 RIF patients).

	Term	Odds Ratio	Confidence Intervals	Healthy Percentage Correct	RIF Percentage Correct	Overall Percentage Correct
Base Model	Constant	1	NA	100%	0%	50%
Model 1	Constant	0.155				
	TLR9	8.633	(0.929, 80.266)	80%	70%	75%
Model 2	Constant	0.018				
	TLR9	9.699	(0.688, 136.6370)	70%	80%	75%
	TRIB2	5.579	(0.538, 57.866)			

Table 2. Binary logistic regression analysis of dependent variables (healthy and IVF failed/RIF women) and independent variables (*TLR9* and *TRIB2* different gene expression levels) The classification table is a method to evaluate the logistic regression model. In this table the observed values for the dependent outcome (Healthy or RIF women) and the predicted values are cross classified. The Model 1 (*TLR9* only) shows the prediction of the dependent variable based on the differential expression level of *TLR9*. Binary logistic regression analysis of gene expression patterns demonstrated that *TLR9* expression can be utilized as a reliable marker/predictor of whether an endometrial tissue samples originates from a healthy or RIF individual. The prediction of the dependent variable based on the differential expression of *TLR9* and *TRIB2* combined (Model 2) shows there is no difference between the percentage of correct predictions when one gene or both genes are considered.

3.3 Age distribution of healthy controls and RIF patients:

The age distribution of healthy controls (32.3 ±3.0, years ± SD) and patients with RIF (34.4±4.0) was statistically significantly different (p value: 0.0058) however, the BMI of healthy group and women with RIF was not statistically different (p value: 0.121). The age and BMI of our control and patients are shown in table 3. Also, we performed regression analysis to see whether gene expression values of specific genes were influenced by the age, but there was no significant effect of age observed for any of the 12 genes analysed in this study. The graphs for linear regression analysis are shown in Figure 4.

Patient code	*Group*		*Age*	*Hight*	*Weight*	*BMI*
NOTNV25	Healthy		33	1.63	66	24.84098
NOTNV15	Healthy		27	1.68	72	25.5102
NOTNV40	Healthy		30	1.67	57	20.43817
NOTNV48	Healthy		33	1.7	68	23.52941
NOT12013	Healthy		32	1.69	80	28.01022
NOTNV09	Healthy		29	1.63	51.6	19.42113
NOTNV01	Healthy		30	1.67	104	37.29069
NOTNV03	Healthy		23	1.62	60	22.86237
NOTNV04	Healthy		32	1.65	48	17.63085
NOTNV06	Healthy		33	1.76	75.3	24.30914
NOTNV27	IVF		32	1.74	72	23.78121
NOTNV29	IVF		39	1.6	62	24.21875
NOTNV30	IVF		35	1.61	53	20.44674
NOTNV32	IVF		30	1.7	52	17.99308
NOTNV35	IVF		32	1.67	62	22.23099
NOTNV36	IVF		37	1.68	59	20.9042
NOTNV38	IVF		35	1.64	54	20.07733
NOTNV39	IVF		37	1.76	62	20.0155
NOTNV43	IVF		33	1.7	70	24.22145
NOTNV44	IVF		34	1.64	53	19.70553
		Healthy average	32.3			22.8719
		STDEV	3.059412			5.19678
		RIF average	34.4			21.35948
		STDEV	3.975232			5.303306
		Unpaired T-test between healthy vs. RIF	*P value:* 0.0058			*P value:* 0.121414

Table 3: Age and BMI distribution of healthy controls and RIF patients. The age, BMI, and sample type (Healthy or IVF patient groups) for each woman recruited in this study was shown in this table. The age and BMI distribution has been compared between both groups using unpaired T-test. The age distribution of healthy controls (32.3 ±3.0, years ± SD) and patients with RIF (34.4±4.0) was statistically significantly different (*p value: 0.0058)* however, the BMI of healthy group and women with RIF was not statistically different (*p value: 0.121*).

414
415
416
417
418
419
420
421
422
423
424
425
426
427
428
429
430
431
432
433
434
435
436
437
438
439
440
441
442
443
444
445
446
447

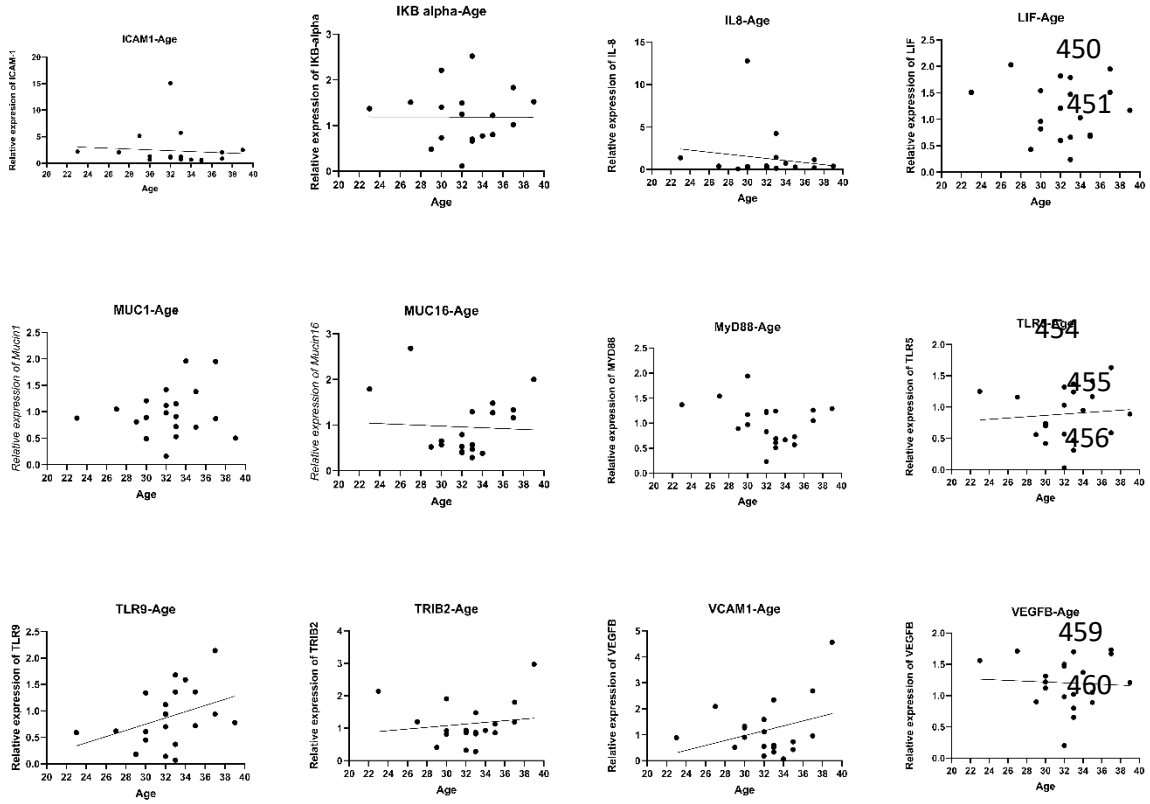


Figure 4. Linear Regression analysis. Linear regression analysis for age-dependant gene expression for all 12 genes analysed in this study showed no significant correlation between individual's age (both healthy controls and RIF patients) and the level of gene expression.

4. Discussion

Human fecundity rate as compared to other mammalian species is quite low. In spite of being fertile, healthy couples would only have 25-30% chance of becoming pregnant during one menstrual cycle. Despite the fact that, assisted reproductive techniques (ART) are helping infertile couples to carry their own babies, the rate of successful pregnancy through these techniques are still poor mainly due to embryo implantation failure [23]. This demands more investigation being conducted to increase our knowledge of embryo implantation process and different molecules determining its accomplishment.

The conceptus as a non-self-entity to the mother, is expected to be repelled by the maternal immune system. However, during a healthy pregnancy, the embryo is protected from the maternal immune response and allowed to thrive. This protective mechanism denotes the fundamental importance of the maternal innate immune system in allowing embryo development, implantation, and parturition [24]. Since, alterations in gene expression result in differences in the cell function [25], in this study, we aimed to investigate the transcription level of innate immune-related genes during the endometrial receptivity and window of implantation in human endometrial biopsies using quantitative real-time PCR. Transcriptomic studies could help to recognise the important markers of endometrial receptivity and embryo implantation. Recent publication by Bastu and his team, reported that innate immune system is one of the key pathways in the pathogenesis of RIF [25].

All the genes selected for our study except for *TRIB2* are part of the TLR signalling pathway according to KEGG https://www.kegg.jp/ (Kyoto Encyclopaedia for Gene and Genome). The role of some of the selected genes in RIF and implantation failure was previously studied by others. For instance, *IL-8 [26-28]*, *MUC1* [29], *MUC16* [30], *VEGF* [31] and *LIF* [32, 33] while other genes were firstly investigated here. Despite the mean age values between the healthy controls and the RIF patients recruited to our investigation being different, our analysis showed that the expression of these selected genes was not age-dependent.

At the implantation site, several cytokines, chemokines, and adhesion molecules form part of the innate immune system and are differentially expressed to facilitate communication between the mother and the implanting embryo [34, 35]. Hence, any unwelcome modifications to the gene expression levels of these molecules might lead to implantation failure or pregnancy loss [36]. It is possible that these molecules can be utilized as potential biomarkers of endometrial receptivity and embryo implantation. Thus, studying the pattern of gene expression

in order to identify those endometrial receptivity biomarkers, and further discovering their role during implantation would be advantageous for the diagnosis and treatment of infertility [37]. An optimal endometrial biomarker of embryo implantation, would be localised at the site of embryo implantation, exhibit differential expression across the menstrual cycle and would be present during the window of implantation but absent before and thereafter [38].

It is noteworthy to consider the fact that, immune system is rather steady in one individual over the time as compared to being variable between individuals. Since the immune response is a collection of immune stimulators and immune regulators, which are both dependent on the different immune cell population. Accordingly, the response to a single stimulation is dissimilar between diverse persons [39] and this would explain the observed inter-individual difference in gene expression level, which is a well-acknowledged weakness of transcriptomic studies. Here, we tried to overcome this limitation by including the paired endometrial samples from the same fertile women from pre-receptive and receptive stages.

TLR5 and *TLR9* were selected for this investigation as *TLR5* signalling pathway is under the control of the *TRIB2* gene [40] and its stimulation by Flagellin would have negative effect on outcome of embryo implantation *in* vitro [18]. *TLR9*, as a ligand for the recognition of *Chlamydia trachomatis* [41, 42] is linked to infection-related infertility [43]. In the current study, significant downregulation of *TLR5* gene expression in LH+8-day receptive endometrial samples were observed compared to LH+2-day pre-receptive samples in healthy women. In contrast, *TLR9* expression did not show any difference between pre-receptive and receptive endometrial samples in fertile women. However, *TLR9* expression was significantly higher in the endometrial samples from RIF patients compared to healthy individuals. Earlier investigations from our laboratory, using different endometrial biopsies, have shown that both *TLR5* and *TLR9* genes have significantly higher expression during the secretory phase of the menstrual cycles in healthy samples. [44, 45].

There are limited studies investigating the pattern of *TLR* gene expression in the female reproductive tract during the menstrual cycles. Investigation of *TLR* 1-6 expression, in the human fallopian tube cell line (OE-E6/E7), showed that *TLR5* expression is higher in response to a combination of oestrogen and progesterone treatment during the window of implantation, compared to other stages of the menstrual cycle [46]. The window of implantation corresponds to the LH+8-day receptive endometrium in our study, and so surprisingly, both studies by Aflatoonian [44] (in human endometrial tissue) and Zandieh [46] (in human fallopian tube cell line), contradicted our findings regarding *TLR5* expression in the receptive endometrium. The reason for these

opposite results could be the use of different primers for *TLR9* gene analysis and size of the samples for each study.

TRIB2 gene expression, showed no significant variation related to the stage of endometrial receptivity analysis but its expression was significantly higher in samples collected from RIF patients compared to healthy individuals. One may conclude that the *TRIB2* gene is not affected by hormonal changes during the female menstrual cycle as its expression was not significantly altered between the pre-receptive and receptive endometrial biopsies in fertile women in the current study. However, *TRIB2* expression is known to be an essential factor in the establishment of the receptive endometrium. Previous data from *Trib2* knockout mice, showed that an absence of *Trib2* gene expression, resulted in a prevention of embryo implantation (Unpublished data from our lab). Given that, we observed higher expression levels of the *TRIB2* gene, in IVF-failed women we can suggest that, as a scaffold protein [47] a delicately controlled expression of *TRIB2* is essential for the success of embryo implantation [48].

NFκβI α (IKB alpha), is the main inhibitor of members of the NFκβ transcription factor family. Here, we observed a significant decrease in NFκβIα mRNA expression in the receptive endometrial (LH+8) biopsies as compared to the biopsies from the pre-receptive (LH+2) endometrium. We might speculate that, progesterone dominancy in the LH+8 samples may have influenced the expression of NFκβIα. Ross et al [49] have investigated the regulation of NFκβ subunits and NFκβI α mRNA expression in the endometrium during oestrous cycle in pig. Consistent with our observations, Ross et al. observed that, the expression of NFκβIα is high in the oestrous state and is downregulated during the rest of the cycle (associated with the high levels of progesterone). This lower level of NFκβIα mRNA expression was also observed in pregnant gilts [49] Therefore, it can be argued that NFκβIα is downregulated by progesterone during the receptive state to facilitate the activation of the NFKβ signalling pathway, whose end products are involved in the implantation process [50].

In our analysis of gene expression during endometrial receptivity, *ICAM-1* expression was significantly down regulated in the receptive endometrium (LH+8) compared to the pre-receptive endometrium (LH+2). Thomson et al have stated that *ICAM-1* expression is up regulated at the time of menstruation in endometrial stromal cells [51] and Wu et al. have reported that *ICAM-1* is involved in the pathology of the endometrium, exhibiting increased expression in women with endometriosis [52]. Considering these findings, it is not unexpected to observe a down-regulation of *ICAM-1* during the transition from a pre-receptive to a receptive endometrium.

We also, observed a significant up-regulation of *VCAM-1* gene expression in the receptive endometrial biopsies compared with the pre-receptive endometrial samples. Interestingly, Bai et al. have also proposed that endometrial expression of *VCAM-1* is crucial for the attachment of the bovine conceptus [53]. Indeed, Konac et al. have observed that a decline in the expression of VCAM-1 mRNA is associated with unexplained infertility [54]. Even though, we did not observe any significant difference in endometrial *VCAM-1* expression between healthy and RIF patients, the remarkably higher *VCAM-1* expression in the receptive endometrium compared to its pre-receptive counterpart, indicates a significant role for VCAM-1 in endometrial receptivity.

Vascular endothelial growth factor (VEGF) is a necessary cytokine for embryonic development, formation of the placenta, vascularization and angiogenesis [55] during the invasion of the embryonic cells into the endometrial stromal cells [56, 57]. Measuring the concentrations of *VEFG-α* in human uterine fluids, Hannan et al. have found significantly higher levels of *VEGF-α* in uterine fluids collected from the mid-secretory phase. In addition, Hannan et al. have used these mid-secretory phase human uterine secretions to significantly increase mouse embryo outgrowth *in vitro* [58]. Further studies of the *VEFG-α* isoform in mouse embryo implantation, have verified that this cytokine, significantly increases the blastocyst cell number and outgrowth, as well as improving the rate of embryo implantation [59]. In contrast to these published data, during our investigations of *VEGF-β* isoform, we observed a significant decline in *VEGF-β* expression in endometrial biopsies in the receptive state as compared with the pre-receptive endometrial samples. In addition, we did not detect any significant differences in *VEGF-β* expression between endometrial samples obtained from healthy women or IVF-failed individuals. It is possible that the *VEGF* α and β isoforms have a variable influence on endometrial receptivity and blastocyst implantation, however, it is more likely that the absence of a viable embryo in our receptive state endometrial samples is the cause of our contradictory findings.

Leukemia inhibitory factor (LIF), a glycoprotein member of IL-6 cytokine family has a key role in embryo implantation by preparing endometrial receptivity, embryo-endometrium interaction [60], decidualization of stromal cell [61], trophoblast invasion, uterine leukocyte infiltration, blastocyst growth and the development and modulation of prostaglandin synthesis. We have observed an up regulation in *LIF* mRNA expression in endometrial biopsies obtained from the receptive endometrium in comparison with biopsies from the pre-receptive endometrium in healthy women. However, unlike previous investigations reporting significantly lower concentrations of *LIF* in uterine flushing from infertile women compared to their fertile counterparts [62, 63],

we observed no significant changes in *LIF* mRNA between the endometrial biopsies obtained from fertile women and IVF-failed individuals.

In this investigation, we may conclude that, the establishment of endometrial receptivity in order to facilitate embryo implantation, involves significant changes to the expression of genes relating the maternal innate immune system. Furthermore, by comparing the expression patterns of genes between healthy women and RIF patients, we have identified two genes which may have major roles in ensuring successful implantation of the embryo. Further research with larger sample size would establish the predictive values of these genes in identifying if the endometrium is of optimal condition to support embryo implantation and a subsequent viable pregnancy, as well as identifying additional factors that are involved in the rejection or acceptance of the embryo by the female reproductive tract.

References

1. Kupka, M.S., et al., *Assisted reproductive technology in Europe, 2010: results generated from European registers by ESHREdagger.* Hum Reprod, 2014. **29**(10): p. 2099-113.
2. Koot, Y.E., et al., *Molecular aspects of implantation failure.* Biochim Biophys Acta, 2012. **1822**(12): p. 1943-50.
3. Achache, H. and A. Revel, *Endometrial receptivity markers, the journey to successful embryo implantation.* Hum Reprod Update, 2006. **12**(6): p. 731-46.
4. Sharkey, A.M. and S.K. Smith, *The endometrium as a cause of implantation failure.* Best Pract Res Clin Obstet Gynaecol, 2003. **17**(2): p. 289-307.
5. Tersoglio, A.E., et al., *Regenerative therapy by endometrial mesenchymal stem cells in thin endometrium with repeated implantation failure. A novel strategy.* JBRA Assist Reprod, 2020. **24**(2): p. 118-127.
6. Moustafa, S. and S.L. Young, *Diagnostic and therapeutic options in recurrent implantation failure.* F1000Res, 2020. **9**.
7. Comins-Boo A, G.-S.Á., Núñez del Prado N, de la Fuente L, and A.J.a.S.-R. S, *Evidence-based update: Immunological evaluation of recurrent implantation failure.* Reproductive Immunology, 2016. **1**(4).
8. Zenclussen, A.C., et al., *Immunology of pregnancy: cellular mechanisms allowing fetal survival within the maternal uterus.* Expert Rev Mol Med, 2007. **9**(10): p. 1-14.
9. Newton, K. and V.M. Dixit, *Signaling in Innate Immunity and Inflammation.* Cold Spring Harbor Perspectives in Biology, 2012. **4**(3).
10. Janeway, C.A., Jr. and R. Medzhitov, *Innate immune recognition.* Annu Rev Immunol, 2002. **20**: p. 197-216.
11. Bulmer, J.N., et al., *Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies.* Hum Reprod, 1991. **6**(6): p. 791-8.
12. Bulmer, J.N., D. Pace, and A. Ritson, *Immunoregulatory cells in human decidua: morphology, immunohistochemistry and function.* Reprod Nutr Dev, 1988. **28**(6B): p. 1599-613.
13. Ratsep, M.T., et al., *Uterine natural killer cells: supervisors of vasculature construction in early decidua basalis.* Reproduction, 2015. **149**(2): p. R91-102.
14. Canavan, T.P. and H.N. Simhan, *Innate immune function of the human decidual cell at the maternal-fetal interface.* J Reprod Immunol, 2007. **74**(1-2): p. 46-52.

15. Krikun, G., et al., *Expression of Toll-like receptors in the human decidua.* Histol Histopathol, 2007. **22**(8): p. 847-54.
16. Chin, P.Y.T., J. G. Roberston, S. A. , *A modest inflammatory insult in the pre-implantation period alters oviduct cytokine expression and programs fetal development* Reproduction, Fertility and Development, 2010. **22**(9): p. 90.
17. Sanchez-Lopez, J.A., et al., *Local activation of uterine Toll-like receptor 2 and 2/6 decreases embryo implantation and affects uterine receptivity in mice.* Biol Reprod, 2014. **90**(4): p. 87.
18. Aboussahoud, W., et al., *Activation of Toll-like receptor 5 decreases the attachment of human trophoblast cells to endometrial cells in vitro.* Hum Reprod, 2010. **25**(9): p. 2217-28.
19. Montazeri, M., et al., *Interleukin-1 receptor antagonist mediates toll-like receptor 3-induced inhibition of trophoblast adhesion to endometrial cells in vitro.* Hum Reprod, 2016. **31**(9): p. 2098-107.
20. Montazeri, M., et al., *Activation of Toll-like receptor 3 reduces actin polymerization and adhesion molecule expression in endometrial cells, a potential mechanism for viral-induced implantation failure.* Hum Reprod, 2015. **30**(4): p. 893-905.
21. Noyes, R.W., A.T. Hertig, and J. Rock, *Dating the endometrial biopsy.* Am J Obstet Gynecol, 1975. **122**(2): p. 262-3.
22. Bustin, S.A., et al., *The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.* Clin Chem, 2009. **55**(4): p. 611-22.
23. Weimar, C.H., et al., *In-vitro model systems for the study of human embryo-endometrium interactions.* Reprod Biomed Online, 2013. **27**(5): p. 461-76.
24. Mor, G., *Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction.* Ann N Y Acad Sci, 2008. **1127**: p. 121-8.
25. Bastu, E., et al., *Potential Marker Pathways in the Endometrium That May Cause Recurrent Implantation Failure.* Reprod Sci, 2018: p. 1933719118792104.
26. Arici, A., et al., *Interleukin-8 in the human endometrium.* J Clin Endocrinol Metab, 1998. **83**(5): p. 1783-7.
27. Arici, A., et al., *Interleukin-8 induces proliferation of endometrial stromal cells: a potential autocrine growth factor.* J Clin Endocrinol Metab, 1998. **83**(4): p. 1201-5.
28. Rajaei, S., et al., *Cytokine profile in the endometrium of normal fertile and women with repeated implantation failure.* Iran J Immunol, 2011. **8**(4): p. 201-8.
29. Wu, F., et al., *Decreased MUC1 in endometrium is an independent receptivity marker in recurrent implantation failure during implantation window.* Reprod Biol Endocrinol, 2018. **16**(1): p. 60.
30. Constantinou, P.E., M. Morgado, and D.D. Carson, *Transmembrane Mucin Expression and Function in Embryo Implantation and Placentation.* Adv Anat Embryol Cell Biol, 2015. **216**: p. 51-68.
31. Shim, S.H., et al., *Association between vascular endothelial growth factor promoter polymorphisms and the risk of recurrent implantation failure.* Exp Ther Med, 2018. **15**(2): p. 2109-2119.
32. Mariee, N., T.C. Li, and S.M. Laird, *Expression of leukaemia inhibitory factor and interleukin 15 in endometrium of women with recurrent implantation failure after IVF; correlation with the number of endometrial natural killer cells.* Hum Reprod, 2012. **27**(7): p. 1946-54.
33. Aghajanova, L., *Leukemia inhibitory factor and human embryo implantation.* Ann N Y Acad Sci, 2004. **1034**: p. 176-83.
34. Paria, B.C., et al., *Deciphering the cross-talk of implantation: advances and challenges.* Science, 2002. **296**(5576): p. 2185-8.
35. van Mourik, M.S., N.S. Macklon, and C.J. Heijnen, *Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment.* J Leukoc Biol, 2009. **85**(1): p. 4-19.

36. Granot, I., Y. Gnainsky, and N. Dekel, *Endometrial inflammation and effect on implantation improvement and pregnancy outcome.* Reproduction, 2012. **144**(6): p. 661-8.
37. Cavagna, M. and J.C. Mantese, *Biomarkers of endometrial receptivity--a review.* Placenta, 2003. **24 Suppl B**: p. S39-47.
38. Zhu, L.J., et al., *Calcitonin is a progesterone-regulated marker that forecasts the receptive state of endometrium during implantation.* Endocrinology, 1998. **139**(9): p. 3923-34.
39. Kaczorowski, K.J., et al., *Continuous immunotypes describe human immune variation and predict diverse responses.* Proc Natl Acad Sci U S A, 2017. **114**(30): p. E6097-E6106.
40. Wei, S.C., et al., *Tribbles 2 (Trib2) is a novel regulator of toll-like receptor 5 signaling.* Inflamm Bowel Dis, 2012. **18**(5): p. 877-88.
41. Joyee, A.G. and X. Yang, *Role of toll-like receptors in immune responses to chlamydial infections.* Curr Pharm Des, 2008. **14**(6): p. 593-600.
42. Ouburg, S., et al., *TLR9 KO mice, haplotypes and CPG indices in Chlamydia trachomatis infection.* Drugs Today (Barc), 2009. **45 Suppl B**: p. 83-93.
43. den Hartog, J.E., S.A. Morre, and J.A. Land, *Chlamydia trachomatis-associated tubal factor subfertility: Immunogenetic aspects and serological screening.* Hum Reprod Update, 2006. **12**(6): p. 719-30.
44. Aflatoonian, R., et al., *Menstrual cycle-dependent changes of Toll-like receptors in endometrium.* Hum Reprod, 2007. **22**(2): p. 586-93.
45. Aflatoonian, R. and A. Fazeli, *Toll-like receptors in female reproductive tract and their menstrual cycle dependent expression.* J Reprod Immunol, 2008. **77**(1): p. 7-13.
46. Zandieh, Z., et al., *The Effect of Estradiol and Progesterone on Toll Like Receptor Gene Expression in A Human Fallopian Tube Epithelial Cell Line.* Cell J, 2016. **17**(4): p. 678-91.
47. Hegedus, Z., A. Czibula, and E. Kiss-Toth, *Tribbles: a family of kinase-like proteins with potent signalling regulatory function.* Cell Signal, 2007. **19**(2): p. 238-50.
48. Ferrell, J.E., Jr., *What do scaffold proteins really do?* Sci STKE, 2000. **2000**(52): p. pe1.
49. Ross, J.W., et al., *Activation of the transcription factor, nuclear factor kappa-B, during the estrous cycle and early pregnancy in the pig.* Reprod Biol Endocrinol, 2010. **8**: p. 39.
50. Nakamura, H., et al., *NF-kappaB activation at implantation window of the mouse uterus.* Am J Reprod Immunol, 2004. **51**(1): p. 16-21.
51. Thomson, A.J., et al., *Expression of intercellular adhesion molecules ICAM-1 and ICAM-2 in human endometrium: regulation by interferon-gamma.* Mol Hum Reprod, 1999. **5**(1): p. 64-70.
52. Wu, M.H., et al., *The differential expression of intercellular adhesion molecule-1 (ICAM-1) and regulation by interferon-gamma during the pathogenesis of endometriosis.* Am J Reprod Immunol, 2004. **51**(5): p. 373-80.
53. Bai, R., et al., *Involvement of VCAM1 in the bovine conceptus adhesion to the uterine endometrium.* Reproduction, 2014. **148**(2): p. 119-27.
54. Konac, E., et al., *Endometrial mRNA expression of matrix metalloproteinases, their tissue inhibitors and cell adhesion molecules in unexplained infertility and implantation failure patients.* Reprod Biomed Online, 2009. **19**(3): p. 391-7.
55. Torry, D.S., et al., *Angiogenesis in implantation.* J Assist Reprod Genet, 2007. **24**(7): p. 303-15.
56. Cross, J.C., Z. Werb, and S.J. Fisher, *Implantation and the placenta: key pieces of the development puzzle.* Science, 1994. **266**(5190): p. 1508-18.
57. Huang, H.Y., *The cytokine network during embryo implantation.* Chang Gung Med J, 2006. **29**(1): p. 25-36.
58. Hannan, N.J., et al., *Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation.* Endocrinology, 2011. **152**(12): p. 4948-56.

59. Binder, N.K., et al., *Endometrial signals improve embryo outcome: functional role of vascular endothelial growth factor isoforms on embryo development and implantation in mice.* Hum Reprod, 2014. **29**(10): p. 2278-86.
60. Tapia, A., et al., *Leukemia inhibitory factor promotes human first trimester extravillous trophoblast adhesion to extracellular matrix and secretion of tissue inhibitor of metalloproteinases-1 and -2.* Hum Reprod, 2008. **23**(8): p. 1724-32.
61. Shuya, L.L., et al., *Leukemia inhibitory factor enhances endometrial stromal cell decidualization in humans and mice.* PLoS One, 2011. **6**(9): p. e25288.
62. Laird, S.M., et al., *The production of leukaemia inhibitory factor by human endometrium: presence in uterine flushings and production by cells in culture.* Hum Reprod, 1997. **12**(3): p. 569-74.
63. Ledee-Bataille, N., et al., *Concentration of leukaemia inhibitory factor (LIF) in uterine flushing fluid is highly predictive of embryo implantation.* Hum Reprod, 2002. **17**(1): p. 213-8.