Altered Expression of C4BPA and CXCL1 Genes in the Endometrium of Patients with Recurrent Implantation Failure after In Vitro Fertilization and Thin Endometrium

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Posted Date: 13 August 2024

doi: 10.20944/preprints202408.0869.v1

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 doi:10.20944/preprints202408.0869.v1

Article

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Gaukhar Kurmanova [1], Yeldar Ashirbekov [2], Almagul Kurmanova [1,*], Nagima Mamedaliyeva [1], Gaukhar Moshkalova [1], Gaini Anartayeva [1], Damilya Salimbayeva [3] and Aidana Tulesheva [1]

[1] Al Farabi Kazakh National University, 71, Al-Farabi avenue, 050040 Almaty, Kazakhstan;
[2] M. Aitkhozhin Institute of Molecular Biology and Biochemistry, 86, Dosmukhamedov street, 050012 Almaty, Kazakhstan;
[3] Scientific Center of Obstetrics, Gynecology and Perinatology, 125, Dostyk Ave., 050010, Almaty, Kazakhstan
* Correspondence: Kurmanova.Almagul@kaznu.kz; 050040 Almaty, Kazakhstan

Abstract: Nowadays recurrent implantation failure (RIF) after invitro fertilization is common problem that reproductive specialists usually faced. The phenomenon of thin endometrium in RIF patients has yet not completely understood and properly treated. This study aimed to reveal significantly dysregulated selected genes expression between RIF patients with thin endometrium and fertile women. Endometrial samples were collected in the implantation window days (LH+7 – LH+10) of the natural menstrual cycle from RIF patients (n=20) and fertile women (n=14). Ten genes were chosen as target genes regarding their possible relations with the implantation process. Endometrial gene expression levels showed differences in RIF samples compared to fertile samples. The significant downregulation was observed for CXCL1 (p=0.005) and C4BPA (p=0.03) genes. There was no statistically significant difference between the RIF groups and the fertile group in the expression of 8 genes: CXCL8, HPRT1, MMP10, INFG, VEGFB, HAND2, IL15 and TNC (p>0.05). The use of a combination of two markers (C4BPA + CXCL1) allows for good discrimination of RIF patients from fertile women (AUC 0.806).

Keywords: endometrial receptivity; recurrent implantation failure; thin endometrium; transcriptomics

1. Introduction

Recurrent implantation failure (RIF) after in vitro fertilization-embryo transfer (IVF-ET) is diagnosed when high-quality embryos fail to implant in at least three consecutive IVF attempts, and is common challenge encountered by reproductive specialists [1–3].

One of the critical factors for implantation failure is considered to be thin endometrium, in which the thickness of the endometrium is less than 7 mm during the "implantation window". Pathophysiological features of "thin endometrium" include insufficient growth of the glandular epithelium, decreased expression of vascular endothelial growth factor (VEGF), and depletion of blood vessels [4]. This is especially true for pathological conditions associated with thin endometrium. In the thin endometrium, in contrast to normal, local microenvironment in the proliferation phase characterized by a decreasing of stromal, epithelial, natural killers, T cells and increasing of cellular senescence in perivascular cells [5]. The thin endometrium observed in patients with RIF remains a challenge in understanding and effectively addressing these conditions.

Studies over the past decade have shed light on structural changes in the endometrium, including the development of pinopode, morphological changes, shifts in the expression of molecular markers of endometrial receptors and the secretion of regulatory factors [6,7]. These changes have been associated with gene transcriptional activity, highlighting the intricate link between compromised receptivity due to a thin endometrium and impaired implantation [8]. The studies

 doi:10.20944/preprints202408.0869.v1

using transcriptomic and molecular analysis of the endometrium have shed light on deciphering of the implantation processes and on the identifying potential molecular biomarkers of endometrial receptivity [9,10].

A significant number of genes are differentially expressed between the pre-receptive and receptive endometrial phases [11–14]. However, differentially expressed genes varied from 107 to 2878, due to the diversity of patients included in different studies [15]. Due to the significant role of numerous immune pathways in the pathogenesis of unexplained RIF, it is difficult to identify a limited number of genes that predict endometrial receptivity [16].

The bioinformatics prediction identifies 30 genes associated with endometrial receptivity, meta-signature genes highlight the importance of immune responses, the complement cascade pathway in endometrial mid-secretory functions [17]. A panel for determining the human endometrial receptivity ER Map®/ER Grade® showed that in healthy women and patients undergoing infertility treatment, the expression of 85 genes significantly changed in the pre-receptive and receptive endometrium [13].

Various mechanisms have been proposed to explain embryo implantation failure, but most involve disruption of endometrial receptivity due to abnormal activation of the innate immune system [18–20]. Gene ontology analysis showed that the most widely represented endometrial receptivity genes were the group responsible for vascular proliferation [21] and immunological activity [22,23]. Therefore, the genes identified in several studies may become potential biomarkers of endometrial receptivity [17].

In this study, we focused on 10 genes also identified and showed up-regulation in the receptive phase in healthy women and responsible for the pro-inflammatory signaling cascade - C-X-C motif chemokine ligand 8 (CXCL8) and C-X-C motif chemokine ligand 1 (CXCL1), complement cascade - Complement Component 4 Binding Protein Alpha (C4BPA), breakdown of extracellular matrix in the in normal physiological processes, such as embryonic development, reproduction, and tissue remodeling - Matrix metallopeptidase 10 (MMP10), generation of purine nucleotides - Hypoxanthine phosphoribosyltransferase 1 (HPRT1), cell adhesion - Tenascin C (TNC), vascular proliferation pathway - VEGF-B, innate and adaptive immune systems - interferon gamma (INFG), Heart- and neural crest derivatives-expressed transcript 2 (HAND2), interleukin 15 (IL-15). HAND2 regulates IL15, which is required for the activation and survival of uterine natural killer cells [22,23].

Another reason for the failure of implantation is associated with a decrease in the activity of the tenascin C (TNC) gene, which is involved in reducing cell adhesion on the surface of the endometrium, facilitating the attachment of the embryo to the endometrium. TNC gene expression level is significantly ($p < 0,05$) down-regulated in the RIF samples compared to the control group [19,24].

Thus, 10 immune response genes were selected (CXCL8, CXCL1, HPRT1, MMP10, INFG, C4BPA, TNC, VEGFB, HAND2 and IL15), the activities of which were increased during the receptive endometrial phase in healthy women. In addition, two of them (C4BPA and TNC) showed down-regulation upon implantation failure in women. It is assumed that a decrease in the expression of these transcriptomes will indicate implantation failure in assisted pregnancies with thin endometrium. The research question whether the selected genes would show downregulation in the case of thin endometrium in patients with recurrent implantation failures was posed in the present study.

2. Materials and Methods

2.1. Subjects

In accordance with the purpose of the study, two groups were formed from participants who applied to the Scientific Center of Obstetrics, Gynecology and Perinatology (Almaty, Kazakhstan) in 2023. An individual follow-up chart was compiled for each woman, including physical examination findings, complaints, obstetric and gynecological history data, lab tests, as well as diagnostic tests where indicated: pelvic ultrasound, blood hormone level testing, genetic counseling and karyotyping.

The inclusion criterion for RIF group was the presence of 3 or more implantation failures after the IVF procedure, as well as the presence of thin endometrium (less than 7 mm during the

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implantation window during ultrasound examination). Fertile women had at least one term and healthy newborn and was without any reproductive losses in anamnesis.

Demographic and clinical characteristics of the study groups are presented in Table 1.

Table 1. Demographic and clinical characteristics of the study groups.

Characteristics	RIF patients (N = 20)	Fertile women (N = 14)
Age (years): Mean±SD	34.65 ± 5.29	32.71 ± 5.47
BMI (kg/m2): Mean±SD	23.36 ± 4.24	23.98 ± 3.81
Endometrium thickness (mm): Mean±SD	6.51 ± 1.27	7.74 ± 0.88
Fibrinogen, g/L: Mean±SD	3.05 ± 0.51	2.71 ± 0.17
Protrombin index, %: Mean±SD	99.16 ± 8.22	97.62 ± 7.81
Protrombin time, sec: Mean±SD	13.04 ± 2.35	12.47 ± 3.27
Activated Partial Thromboplastin Time, sec: Mean±SD	34.04 ± 3.61	25.15 ± 13.87
International Normalized Ratio: Mean±SD	1.04 ± 0.12	1.06 ± 0.03
Luteinizing hormone, mIU/mL: Mean±SD	8.34 ± 2.13	-
Follicle stimulating hormone, mIU/mL: Mean±SD	6.19 ± 1.43	-
Prolactin, mIU/L: Mean±SD	325.0 ± 152.4	-
Thyroid stimulating hormone, mIU/mL: Mean±SD	2.84 ± 0.74	-
Chronic endometritis, yes/no	20/0	0/14
Chronic salpingoophoritis, yes/no	10/10	0/14
Pelvic organs surgeries, yes/no	10/10	0/14
Ectopic pregnancy, yes/no	2/18	0/14
Endometriosis, yes/no	5/15	1/13
Uterine fibroids, yes/no	1/19	0/14
Polyps, yes/no	1/19	6/8

When clinical characteristics were compared between two groups, no statistical differences were found by age and BMI. All participants were under 40 years of age. In gynecological history, the presence of chronic salpingoophoritis and operations on the pelvic organs were more often noted in patients of RIF group compared to fertile group. There were no significant differences between the groups in hemostasis parameters, blood hormone levels.

2.2. Ethics Approval

This study was approved by the Ethical Committee of Al Farabi Kazakh National University, Kazakhstan (Code: IRBA400/IRB 00010790). All participants provided written informed consent for the use of biomaterials in this study.

2.3. Sample Processing

Endometrial samples were collected in the implantation window days (LH+7 – LH+10) of the natural menstrual cycle from RIF patients with thin endometrium (n=20) and fertile women (n=14). Endometrial tissue was collected using Pipelle biopsy with Goldstein catheter (SonoBiopsyTM J-GSBX-072026 size (Fr) 7.2 Cook Incorporated, USA). Tissue samples were transferred from the catheter into a cryotube with 1 ml RNA-later stabilization solution (Thermo Fisher Scientific) to store in a refrigerator 4°C for 12 h. The next day, samples were transferred to freezer −20°C. After sample collection was completed, they were transferred to M. Aitkhozhin Institute of Molecular Biology and Biochemistry (Almaty, Kazakhstan).

2.4. RNA Isolation from Endometrial Samples

Isolation of total RNA from endometrial samples was performed using Dynabeads™ mRNA DIRECT™ Purification Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. The isolated RNA was immediately subjected to further analysis.

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2.5. cDNA Synthesis and Quantitative Polymerase Chain Reaction (PCR)

Reverse-transcription and quantitative PCR was performed using primers and probes from TaqMan™ Gene Expression Assay (Applied Biosystems, USA). cDNA was obtained using High-capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Quantitative PCR was performed in duplicates using TaqMan Fast Advanced Master Mix (Applied Biosystems) under the conditions recommended by the manufacturer on the StepOnePlus Real-Time PCR System (Applied Biosystems). Primary processing was performed using StepOnePlus 2.2.2 and ExpressionSuite v1.3 programs. Relative quantification of gene expression is carried out using the comparative Ct (ΔΔCt) method with modifications as described by Königshoff M. et al. (2009) [25]. Relative transcript abundance is expressed in ΔCt values (ΔCt = Ctreference − Cttarget). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta (YWHAZ) housekeeping genes was used as a reference, in accordance with previous study [13]. ΔΔCt value (ΔΔCt = ΔCtcase − ΔCtcontrol) was considered as log2 fold change.

2.6. Statistical Analysis

Most of the statistics were performed in the Jamovi program (https://www.jamovi.org). Statistical significance of the differences in ΔCt between the groups was calculated using the two-tailed Mann–Whitney U test. Spearman's rank correlation method was used to examine the relationship between quantitative variables. $P<0.05$ was considered statistically significant. For multiple comparisons, the online calculator of FDR correction was used to adjust the p-values (https://www.sdmproject.com/utilities/?show=FDR). For a comparative visualization of gene expression levels, box plots were constructed using a web tool BoxPlotR (http://shiny.chemgrid.org/boxplotr). The characteristics of the markers were evaluated by ROC analysis using the web-tool easy ROC [26], and Jamovi. Youden's index method was used to calculate optimal cut-off points. Evaluation of classifiers by interpretation of the area under the ROC curve (AUC) was performed as described by [27].

3. Results

3.1. *The level of mRNA in endometrial samples of RIF patients in comparison with the fertile group*

Comparative gene expression statistics between RIF and fertile women are presented in Table 2. The study showed that the expression of 2 out of 10 genes differs significantly between the studied groups. It was found, that the expression of the CXCL1 and C4BPA were significantly decreased in RIF with thin endometrium patients compared to the fertile women (log_2 fold change=-1.95 and -2.09, p = 0.005 and 0.030, respectively) (Figure 1). However, after adjustment for multiple comparisons only the difference in CXCL1 expression remained significant (pFDR = 0.050 and 0.150, respectively). There were not significant differences in expression of CXCL8, HPRT1, MMP10, INFG, VEGFB, HAND2, IL15 and TNC genes between groups.

Table 2. Comparative gene expression statistics between RIF and fertile women.

Gene	Ct mean ± SD		ΔCt mean ± SE		ΔΔCt (95% CI)	P value
	RIF	Fertile	RIF	Fertile	log2 fold change	
CXCL1	27.84 ± 4.27	27.76 ± 3.84	-6.82 ± 0.29	-4.87 ± 0.58	-1.95 (-3.45; -0.60)	0.005**
C4BPA	29.69 ± 3.29	30.28 ± 4.21	-9.65 ± 0.38	-7.39 ± 0.81	-2.09 (-3.99; -0.38)	0.030*
IL15	25.89 ± 3.89	26.86 ± 2.94	-4.87 ± 0.22	-3.97 ± 0.32	-0.73 (-1.65; 0.00)	0.051
IL8	28.16 ± 4.00	28.60 ± 4.44	-7.55 ± 0.54	-5.71 ± 1.00	-1.67 (-3.47; 0.18)	0.060

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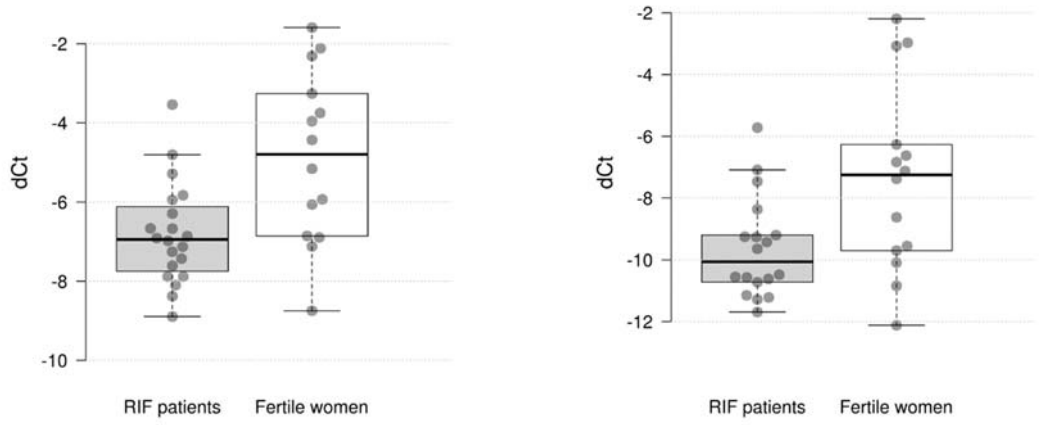
TNC	25.07 ± 4.49	28.20 ± 4.21	-4.04 ± 0.40	-5.30 ± 0.60	1.48 (-0.45; 3.27)	0.112
VEGFB	24.20 ± 3.41	25.60 ± 2.50	-3.18 ± 0.21	-2.71 ± 0.27	-0.45 (-1.06; 0.18)	0.148
IFNG	33.58 ± 2.85	34.86 ± 3.11	-13.52 ± 0.36	-12.51 ± 0.52	-0.89 (-2.17; 0.30)	0.157
HPRT1	24.77 ± 4.23	26.31 ± 3.29	-3.75 ± 0.13	-3.42 ± 0.33	-0.32 (-1.13; 0.12)	0.180
HAND2	22.90 ± 3.52	24.48 ± 2.73	-1.88 ± 0.17	-1.59 ± 0.31	-0.31 (-0.87; 0.23)	0.231
MMP10	29.76 ± 5.55	31.22 ± 4.82	-8.74 ± 0.66	-8.33 ± 1.11	-0.63 (-2.90; 1.95)	0.904
GAPDH	21.24 ± 4.45	23.19 ± 3.60	-	-	-	-
YWHAZ	20.81 ± 3.89	22.59 ± 2.74	-	-	-	-



(**a**) (**b**)

Figure 1. Differences in gene expression levels (ΔCt values) in endometrial tissue between RIF patients and fertile women: (a) CXCL1 gene; (b) C4BPA gene.

3.2. Associations with Clinical and Laboratory Characteristics

Next, we analyzed differences in gene expression among groups with different clinical characteristics. Among fertile women, C4BPA expression was significantly increased, HAND2 expression was significantly decreased in the endometrium of women with polyps compared to those without them (in both cases, p=0.020). Among RIF women, there were no significant differences in gene expression levels between patients with and without endometriosis.

Spearman's rank correlation method was used to examine the relationship between expression of studied genes and quantitative laboratory characteristics in two groups. The correlations are presented in Table 3. In RIF patients with thin endometrium group significant inverse moderate correlations have been established: between HAND2 and TSH levels (rho = -0.671, p = 0.001), HAND2 and fibrinogen levels (rho = -0.465, p = 0.038), VEGFB and prolactin levels (rho = -0.446, p = 0.049), VEGFB and TSH levels (rho = -0.526, p = 0.017). In the fertile group an inverse moderate relationship

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between HAND2 and BMI (rho = -0.688, p = 0.008). It should be noted, however, that after adjustment for multiple comparisons none correlations remained significant.

Table 3. Detected correlations between gene expression levels and clinical and laboratory parameters.

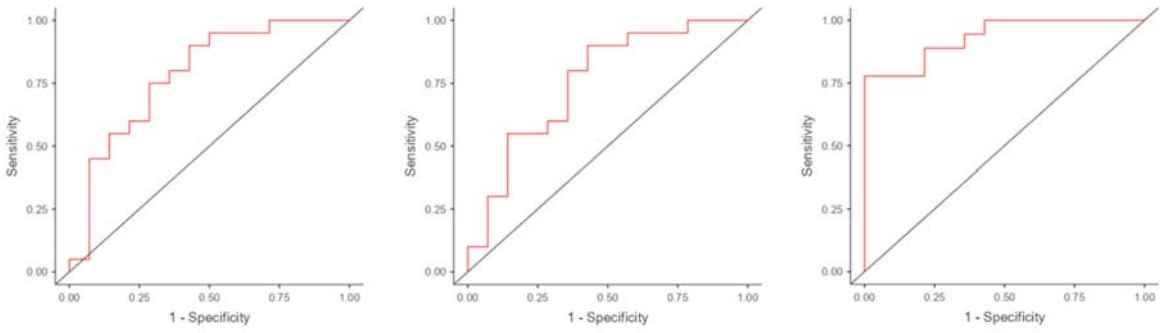
Group	Parameter 1	Parameter 2	Spearman rho	P value
RIF group	VEGFB	Prolactin	-0.446	0.049
	VEGFB	Thyroid stimulating hormone	-0.526	0.017
RIF group	VEGFB	Prolactin	-0.446	0.049
	VEGFB	Thyroid stimulating hormone	-0.526	0.017
	HAND2	Thyroid stimulating hormone	-0.671	0.001
	HAND2	Fibrinogen	-0.465	0.038
Fertile group	HAND2	Body mass index	-0.688	0.008

3.3. ROC Analysis

We performed ROC analysis to evaluate the potential of using mRNA of genes differentially expressed in the endometrium of RIF patients as markers for predicting RIF. The results are presented in Table 4. The areas under the ROC curve (AUC) were obtained for CXCL1 (0.782) and C4BPA (0.726) (Figure 2). The use of combination of C4BPA and CXCL1 resulted in an increase in AUC to 0.806 (with a specificity of 64.3% and a sensitivity of 83.3%).

Table 4. ROC analysis results.

Potential markers and combinations	AUC (95% CI)	Optimal cut-off point	Sensitivity (95% CI)	Specificity (95% CI)
CXCL1	0.782 (0.613-0.951)	-5.286	0.900 (0.683-0.988)	0.571 (0.289-0.823)
C4BPA	0.726 (0.535–0.918)	-7.462	0.889 (0.653–0.986)	0.571 (0.289–0.823)
C4BPA + CXCL1	0.806	-	0.833	0.643



(**a**) (**b**) (**c**)

Figure 2. ROC curves for potential markers and combinations of markers in discrimination RIF patients from fertile women: (a) CXCL1; (b) C4BPA; (c) combination of CXCL1 and C4BPA.

4. Discussion

Patients suffering from thin endometrium are more susceptible to fertility impairments [28,29]. RIF caused by thin endometrium may be associated with abnormal activation of the inflammatory environment together with an abnormally reduced response to oxidative stress [30]. Therefore,

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studying the cellular and molecular mechanisms of the thin endometrium with the elucidation of dysfunctional signaling pathways is important.

We focused on 10 genes involved in immune pathways of endometrial receptivity, which have shown up-regulations in receptive endometrial phase of healthy women.

In this study, gene expression of RIF patients with thin endometrium along with the matched endometrial tissues from fertile women were explored, and we revealed the abnormal activation of the inflammatory environment in thin endometrium. It was found, that the expression C4BPA and CXCL1 genes were significantly decreased in RIF with thin endometrium patients compared to the fertile women ($p=0.030$ and $p=0.005$, respectively).

Complement Component 4 Binding Protein Alpha (C4BPA) gene codes an inhibitor of complement system activation, which reduce of a misdirected complement attack to the embryo [31]. Abnormally decreased levels of C4BPA have been detected in the mid-secretory endometrium of women with implantation failure [18] and endometriosis [32]. Moreover, in the thin endometrium, the ligand-receptor pair genes in CD45 and complement signals were upregulated [30]. In this study also highlighted that the mis-activation of immune cells and disorder of cell dialogue to other cell types may contribute to the pathology to thin endometrium. Through activation of the immune system and enhancement of signaling pathways associated with lymphoid cells, the expression of chemokine family genes can be examined for the risk of thin endometrium [30].

In our study, the down-regulation of C-X-C motif chemokine ligand 1 (CXCL1) gene indicates a proinflammatory signaling cascade activation in RIF patients with thin endometrium. At the same time, there were not significant differences in concentrations for CXCL8, HPRT1, MMP10, INFG, VEGFB, HAND2, IL15 and TNC genes expression between groups. Our findings indirectly indicate "silent endometrium" is observed in thin endometrium. Pathologies that directly affect the uterine mucosa, including the thin endometrium, can provoke a refractory endometrium, characterized by the absence of proliferation and abnormal thickness [33]. When examining intercellular communication in the thin endometrium using CellChat, it was shown that the number and strength of interactions between stromal cells and proliferating stromal cells were reduced [30]. In the late-proliferative phase, suppressed cell proliferation was detected in women with thin endometrium and reduced expression of PDZ-binding kinase in endometrial stromal cells, which was facilitated by the presence of an aberrantly proinflammatory environment [34].

In our study, in RIF patients with thin endometrium significant ($p<0.05$) inverse moderate correlations were shown. It was noted that the decreasing of HAND2 gene level was associated to increasing of TSH and fibrinogen levels, and in fertile women the decreasing of HAND2 gene level was associated with increasing of BMI. Heart- and neural crest derivatives-expressed transcript 2 (HAND2) regulates interleukin 15 (IL-15), a key immune factor required for the activation and survival of uterine natural killers, briefly induces immunotolerance. During the secretory phase, levels of HAND2 and IL-15 are significantly increased in the healthy human endometrium [23]. The same trends were observed in VEGFB with TSH and prolactin levels. VEGF-B has been shown to be a survival factor for vascular endothelial cells [21]. These finding confirmed that RIF population is highly heterogenous, and numerous risk factors for RIF have been identified lifestyle factors, including BMI, uterine and ovarian factors (endometriosis, polyps, adenomyosis, polycystic syndrome), thrombophilia [35,36]. The following studies should be performed taking these factors into account, depending on the clinical forms. The endometrial receptivity is characterized by complex dynamics of interactive networks and regulatory functions in different aspects of the implantation process, and high phenotype variability between individuals implicate them as central gatekeepers of receptivity for embryo implantation [37]. However, our studies showed the prognostic potential of a combination of C4BPA + CXCL1 genes to predict RIF.

Our studies had limitations that need to be addressed in future studies. It was a small sample sizes, it is recommended to increase the observational cohorts according to clinical forms. Transcriptional analysis of thin endometrial tissue should be validated using multi-omics data, including proteomics (cell immunophenotyping) or metabolomics.

5. Conclusions

Our study revealed differential expression of C4BPA and CXCL1 genes in RIF patients with thin endometrium compared with the endometrium of fertile women. These data allowed us to evaluate

 doi:10.20944/preprints202408.0869.v1

the predictive value of the combined model for predicting implantation failure depending on endometrial thickness. The data also provide potential therapeutic targets for the treatment of thin endometrium in patients undergoing ART programs.

Author Contributions: Conceptualization, G.K., Y.A., A.K. and N.M.; Funding acquisition, G.M., G.A. and A.T.; Investigation, Y.A.; Methodology, G.K., Y.A., A.K., N.M., D.S.; Resources, A.K. and G.M.; Supervision, N.M.; Visualization, A.K. and G.M.; Writing – original draft, Y.A. and A.K.; Writing – review & editing, G.K., Y.A., A.K., N.M., G.M., G.A., D.S. and A.T. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Science Committee of the Ministry of Science and High Education of the Republic of Kazakhstan (Grant No. AP 14870089).

Institutional Review Board Statement: This study was approved by the Ethical Committee of Al Farabi Kazakh National University, Kazakhstan (Code: IRBA400/IRB 00010790).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: Experimental and clinical-pathological data is available.

Acknowledgments: The authors are appreciative to all clinicians of Scientific Center of Obstetrics, Gynecology and Perinatology, and scientists of M. Aitkhozhin Institute of Molecular Biology and Biochemistry.

Conflicts of Interest: The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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