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Oxidative stress, inflammatory settings, and microRNA regulation in the recurrent implantation failure patients with metabolic syndrome

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Abstract

Problem: Increased oxidative stress (OS) and inflammatory factors in metabolic syndrome (MS) patients are considered as risk factors for recurrent implantation failure (RIF). The aim of this study was to investigate OS markers, inflammatory factors, related microRNAs (miRNA) expression, and cytokine and transcription factors RNA expression.

Method of study: We evaluated the frequency of helper T (Th) 17 and regulatory T (Treg) cells in recurrent implantation failure (RIF) women with or without MS. miRNA expression, an inflammatory cytokine, and transcription factors were measured by real-time PCR. The level of interleukin (IL)-1β, IL-6, IL-17, tumour necrosis factor-alpha (TNF-alpha) and chemokine (C-C motif) ligand 2 (CCL-2), and C-X-C motif chemokine ligand 8 (CXCL-8) were measured by enzyme-linked immunosorbent assay (ELISA). OS markers were evaluated by spectrophotometric assay. Th17 and Treg cell frequencies were determined by flow cytometry.

Results: The expression of AP1, NF-κB, FOXP3, miRNA-21; serum or plasma level of OS markers (ie, nitric oxide, total oxidant status, and myeloperoxidase); serum level of inflammatory factors (ie, IL1-β, IL-6, IL-17, TNF-alpha, CXCL-8, and CCL-2); and frequency of Th17 cells were increased in RIF-MS patients in comparison with RIF women without MS (RIF-NMS) and control group. The expression of miRNA-223 and 146a, antioxidant enzymes, namely superoxide dismutase (SOD) and catalase (CAT), and frequency of Treg also declined in RIF-MS patients.

Conclusion: Overall, our findings suggest that MS in RIF patients causes increased inflammatory factors and OS, which in turn leads to implantation failure.

KEYWORDS

inflammatory factors, metabolic syndrome, microRNA, oxidative stress, oxidative stress markers, recurrent implantation failure

1 | INTRODUCTION

A metabolic syndrome (MS) is recognized by metabolic abnormalities such as body mass index >30 kg/m^2, a HDL cholesterol level <50 mg/dL, a triglyceride level >150 mg/dL, a fasting glucose level >100 mg/dL, and a blood pressure level >130/85 mm Hg. Metabolic syndrome diagnosis occurs when an individual shows at least three of these risk factors, which are provided by the recent National Cholesterol Education Program.[1] This syndrome has adverse effects, such as increased cardiovascular disease, type 2 diabetes mellitus, increased inflammation, and preterm birth.[1-3] Obesity is introduced as the main cause and causal component of metabolic syndrome development.[4] In adipose tissue, decreased and increased activities of antioxidative enzymes and NADPH oxidase lead to increased generation of reactive oxygen species (ROS), which results in oxidative stress (OS).[5] OS refers to conditions in which surplus oxygen radicals could overwhelm the normal antioxidant capacity.[6] OS deregulates the adipocytokine production, including monocyte chemotactic protein-1 (MCP-1) or CCL-2, tumour necrosis factor-α (TNF-alpha), plasminogen activator inhibitor-1 (PAI-1), leptin, interleukin (IL)-6, and adiponectin.[7-10] In adipose tissue, macrophage infiltration is induced by obesity, leading to pro-inflammatory cytokine productions such as IL-1β, IL-6, and TNF-alpha.[11] In addition, the adaptive immune system in adipose tissue plays an important role in macrophage modifications. In lean adipose tissue, M2 macrophage polarization occurs through interaction with helper T (Th) 2 and regulatory T (Treg) lymphocytes. In contrast, the polarization of adipose tissue toward M1 macrophages in obesity is stimulated by Th1, Th17, and cytotoxic CD8+ T cells, which result in obesity-induced inflammation.[12-14] As reported, OS is the underlying cause of various disease pathogeneses. Several studies have shown a correlation between oxidative stress and reproductive diseases, such as endometriosis, polycystic ovarian syndrome, unexplained infertility, and pregnancy complications including spontaneous abortion, recurrent pregnancy loss (RPL), preeclampsia, intrauterine growth restriction, and preterm labor.[14-17] However, to the best of our knowledge, oxidative stress approximately has not been investigated in RIF patients. Nowadays, the application of the assisted reproductive techniques is very common because of increased infertility problem. RIF is defined as implantation failure after at least three in vitro fertilization (IVF) cycles with the transfer of high-quality embryos, which is still a challenging issue in the IVF success. The main causes of RIF are embryonic defects, decreased endometrial receptivity, and multifactorial effectors.[18] Endometrial receptivity and embryo implantation are mainly affected by immunological reasons, especially inflammatory cytokines that are produced by Th1 and Th17.[19] miRNAs, as non-coding RNA molecules, have critical functions in control pathways including organogenesis, hematopoiesis, cell proliferation, apoptosis, and inflammation.[20,21] Dysregulation of some microRNAs such as miR-21, miR-223, and miR-146a plays a role in increasing inflammation.[22-24] As mentioned earlier, increased inflammation has been seen in metabolic syndrome patients and recurrent implantation failure. Therefore, the expression of these microRNAs can be changed in these patients. This study aimed at investigating and comparing the levels of inflammatory factors and OS markers and the Treg and Th17 frequencies in peripheral blood of RIF patient suffering from MS, as well as expression analysis of miRNAs involved in inflammation and OS.

2 | MATERIALS AND METHOD

2.1 | Study population

This study was approved by the Research Ethics Committee of Tabriz University of Medical Sciences (IR.TBZMED.REC.1396.943). The

TABLE 1 General characteristics of individuals

Variable	Control (n = 40)	RIF-NMS patients (n = 26)	RIF-MS patients (n = 21)
Age (y)	26.82 ± 3.64	28.14 ± 3.32	28.39 ± 4.26
Blood pressure systolic mm Hg	109.12 ± 15.35	116.91 ± 20.42	138.46 ± 26.03
Blood pressure diastolic mm Hg	70.23 ± 12.92	78.34 ± 13.36	91.96 ± 15.41
BMI, kg/m^2	24.02 ± 4.26	25.13 ± 4.68	28.89 ± 5.86
Basic biochemical parameters			
Fasting blood sugar, mg/dL	84.35 ± 12.35	87.01 ± 13.11	123.49 ± 25.44*
Total cholesterol, mg/dL	181.21 ± 12.61	223.30 ± 32	230.26 ± 40.5**
HDL-C, mg/dL	53.17 ± 3.23	53.32 ± 3.12	41.05 ± 3.89*
Triacylglycerides, mg/dL	92.66 ± 10.02	100.01 ± 19.18	261.57 ± 52.16*

Abbreviations: BMI, body mass index; HDL, low-density lipoprotein-cholesterol; HDL-C, high-density lipoprotein-cholesterol.

*($P < .05$) vs RIF-NMS and Healthy Proven-Fertile Women.

**($P < .05$) vs Healthy Proven-fertile Women.

TABLE 2 Used primer sequences for studied genes

Gene	Primer strand	Sequence (5′→3′)
FoxP3	Forward	TCATCCGCTGGGCCATCCTG
	Reverse	GTGGAAACCTCACTTCTTGGTC
IL-17	Forward	CATAACCGGAATACCAATACCAAT
	Reverse	GGATATCTCTCAGGGTCCTCATT
IL-6	Forward	ACTCACCTCTTCAGAACGAATTG
	Reverse	CCATCTTTGGAAGGTTCAGGTTG
IL-1β	Forward	CCAGGATGAGGACCCAAGCA
	Reverse	TCCCGACCATTGCTGTTTCC
TNF-alpha	Forward	GCATGATCCGAGATGTGGAA
	Reverse	GGCTGACTTTCTCCTGGTATG
NF-κB	Forward	GGATGACAGAGGCGTGTATAAG
	Reverse	CCGTAAGCAGGAAATCCATAGT
CXCL-8	Forward	CAGTTTTGCCAAGGAGTGCTAA
	Reverse	GGTGGAAAGGTTTGGAGTATGTC
CCL-2	Forward	CAGCCAGATGCAATCAATGCC
	Reverse	TGGAATCCTGAACCCACTTCT

Abbreviations: CCL-2, C-C motif chemokine ligand 2; CXCL-8, C-X-C motif chemokine ligand 8; FoxP3, forkhead box P3; IL-17, interleukin 17; IL-1β, interleukin 1 beta; IL-6, interleukin 6; NF-κB, nucleate factor-kappa B; TNF-α, necrosis factor-alpha.

population of this study is comprised of 87 women referred to Valiasr hospital of Tabriz between September 2017 and October 2018. Of these subjects, 47 women with at least three implantation failures transferred more than three high-quality embryos after IVF without any anatomic abnormalities, endocrine disorders, infectious, and genetic etiologies disorder. Of these 47 patients, 26 women had metabolic syndrome (MS) while 21 women did not show MS presentations. In addition, 40 healthy proven-fertile women were enrolled as the control group. Baseline data of the study participants are listed in Table 1. Written informed consent forms were signed by all the participants before participation in the study.

2.2 | Separation of peripheral blood mononuclear cells (PBMCs) and T-cell subset isolation

About 15 mL of peripheral blood was collected by venipuncture in sterile conditions from RIF patients and normal proven-fertile women. For PBMCs isolation, 8 mL of blood samples was heparinized. Isolation of PBMCs in blood samples was done using standard Ficoll (lymphosep) 1.077 g/mL (Biosera Inc) gradient centrifugation (25 minutes, 450 *g*). The cells were washed twice with Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma-Aldrich), and 5×10^6 of PBMCs was cultured in 5 mL of medium supplemented with 10% heat-inactivated fetal calf serum (FCS) and also 10 ng/mL Phorbol myristate acetate (PMA) as activator (eBioscience). Cultured samples were incubated for 48 hours at 37°C and 5% CO_2 until further experiments.

2.3 | Serum and plasma preparation

About 4 mL of the peripheral blood was used for serum collection. Next, 3 mL of this blood sample was added into a heparinized tube and then was centrifuged for 15 minutes at 400 *g* for plasma preparation. Plasma and serum samples were kept at −70°C for further assessments.

2.4 | RNA extraction and complementary DNA (cDNA) synthesis

Total RNA extraction was accomplished using a Total RNA Purification Mini kit (YTA). NanoDrop spectrophotometer was used for RNA purity and concentration analysis. Reverse transcription of extracted RNA into cDNA with oligo dT and random hexamer primers was done by M-MLV Reverse Transcriptase kit (Thermo Fisher). Conversion of miRNA to cDNA was performed using MicroRNA Reverse Transcription Kit (Thermo Fisher) and High Capacity RNA-to-cDNA Master Mix.

2.5 | Quantitative real-time PCR

The gene expression analysis was performed by real-time PCR test. Expression levels of IL-1β, IL-6, IL-17, TNF-alpha, CXCL-8, CCL-2, FOXP3, NF-κB, activator protein 1 (AP1), miR-223, miR-21, and miR-146a were measured using specific primers and SYBER Green Master Mix (Roche). The primer sequences are summarized in Tables 2 and 3. The housekeeping genes (ie, 18sRNA and RNU6) were used for normalization. Then, data were presented as relative mRNA expression using the $2^{-\Delta\Delta C_t}$ method proposed by Schmittgen and Livak.[25] In order to confirm the amplification, an electrophoretic analysis in 2% agarose gel was conducted.

TABLE 3 Primer sequences for miRNAs expression evaluation

miRNA	Primer strand	Sequence (5′→3′)
miR-223	Forward	ATGGTTCGTGGGTGTCAGTTTGTCAAAT
	Reverse	GCAGGGTCCGAGGTATTC
miR-21	Forward	ACACTCCAGCTGGGTAGCTTATCAGACTGA
	Reverse	TGGTGTCGTGGAGTCG
miR-146a	Forward	CTAGCCTGCAGGCTGCCCTTGGACCAGCAGTC
	Reverse	ATCCGGCCGGCCGCTCTCTTTTTCTTCTTGAC

2.6 | Flow cytometry analysis

To detect Th17 and Treg cells from whole-blood samples, flow cytometry method was used. To analyze the Treg cells percentage, surface staining with phycoerythrin-labeled anti-human CD25, FITC-labeled anti-human CD4 (BD Biosciences), and PerCP-Cy5.5-conjugated anti-human CD127 (BD Biosciences) antibodies was carried out for 45 minutes at 4°C. After twice washing with PBS, the fluorescence-activated cell sorting (FACS) solution was used for re-suspending the cells. For Th17 detection, 1×10^6 cells were incubated and stimulated with 10 ng/mL Phorbol myristate acetate (PMA) and 0.5 µmol/L ionomycin (eBioscience) for 5 hours at 37°C in a 5% CO_2 humidified incubator following Monensin (eBioscience) addition. Afterward, to obtain a cell pellet, the cell suspensions were centrifuged at 300 *g* for 10 minutes and washed with FACS buffer, and then were incubated for 15 minutes at 4°C with the anti-CD4-FITC (BD Biosciences). After washing, the permeabilization process was performed with fixation or permeabilization buffer (eBioscience). Subsequently, intracellular staining of samples was conducted by PE-labeled anti-human IL-17 for 20 minutes at room temperature. Flow cytometric analysis was performed using a FACSCalibur, and data were analyzed by FlowJo software (Tree Star).

2.7 | Enzyme-linked immunosorbent assay (ELISA)

IL-1β, IL-6, IL-17, TNF-alpha, CXCL-8, and CCL-2, NO levels in serum of study subjects were measured using ELISA according to the manufacturer's instructions (Biosource). To increase the accuracy, all samples were analyzed in duplicate.

2.8 | Biochemistry assays

2.8.1 | Catalase and superoxide dismutase activity assay

The catalase (CAT) activity was measured in accordance with the method proposed by Aebi.[26] For sample preparation, H_2O_2 in the phosphate buffer was added to the requisite volume of a serum sample, and then, the absorbance reduction was read at 240 nm. The superoxide dismutase (SOD) activity was determined by Oyanaguis method.[27] The result showed that there is a positive and negative proportion between the concentration of products and the amount and activity of SOD, so the absorbance was measured at 550 nm using PerkinElmer analyzer. The SOD activity was expressed in the nitric unit (NU)/mg.

2.8.2 | Total antioxidant activity and total oxidant activity assay

Total antioxidant activity (TAS) and total oxidant activity (TOS) in the plasma were measured in accordance with the method proposed by Erel.[28] The experiment was conducted in an automated analyzer (PerkinElmer) in which calibration was done with hydrogen peroxide and Trolox for evaluating TOS and TAS, respectively. The results were expressed as µmol equivalent/L.

2.8.3 | Myeloperoxidase activity assay

Myeloperoxidase (MPO) activity was determined according to Bradley et al.[29] Briefly, in the presence of H_2O_2, the MPO-induced yellowish-orange product formation of o-dianisidine oxidation was read at 460 nm and activity of MPO in plasma was expressed in U/L.

2.8.4 | Advanced oxidation protein products measurement

The level of advanced oxidation protein products (AOPPs) was determined by spectrophotometric assay.[30] Briefly, after plasma dilution with PBS, potassium iodide (KI) was added to diluted plasma and glacial acetic acid was added after 2 minutes. Finally, the mixed reaction absorbance was read immediately at 340 nm. Chloramine-T and PBS were used as a calibration and blank, respectively.

2.8.5 | Nitric oxide evaluation

NO_2^- and NO_3^-, as the metabolites of NO, were measured as the source of NO production because of the short half-life of NO. Determination of NO was done based on Griess reaction.[31] Strong absorbance of the red-pink color complex—which is formed by NO_2^- and mixture of naphthylethylenediamine and sulfanilamide reaction—was analyzed at 540 nm wavelength.

2.9 | Statistical analysis

Statistical analysis was done by SPSS software (version 23.0, SPSS Inc). To determine the normal distribution of data, the Kolmogorov-Smirnov test was applied. Data analysis of three groups was carried out by one-way analysis of variance (ANOVA) and Tukey's post hoc test. For graph illustration, GraphPad Prism software version 7.00 (GraphPad Software, www.graphpad.com) was used. A probability level of $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | The expression level of inflammatory cytokines, chemokine, and transcription factors

The mRNA expression levels of IL-6 and IL-1β ($P = .0001$ and $P < .0001$, respectively), IL-17 ($P < .0001$), TNF-alpha ($P < .0001$), CXCL-8 ($P < .0001$), CCL-2 ($P < .0001$), NF-κB ($P = .0014$ and $P < .0001$), and AP1 ($P < .0001$) were significantly increased in RIF-MS patients compared with RIF-NMS patients and healthy individuals. However, the mRNA expression level of FOXP3 was significantly decreased in PBMCs from the RIF-MS women compared

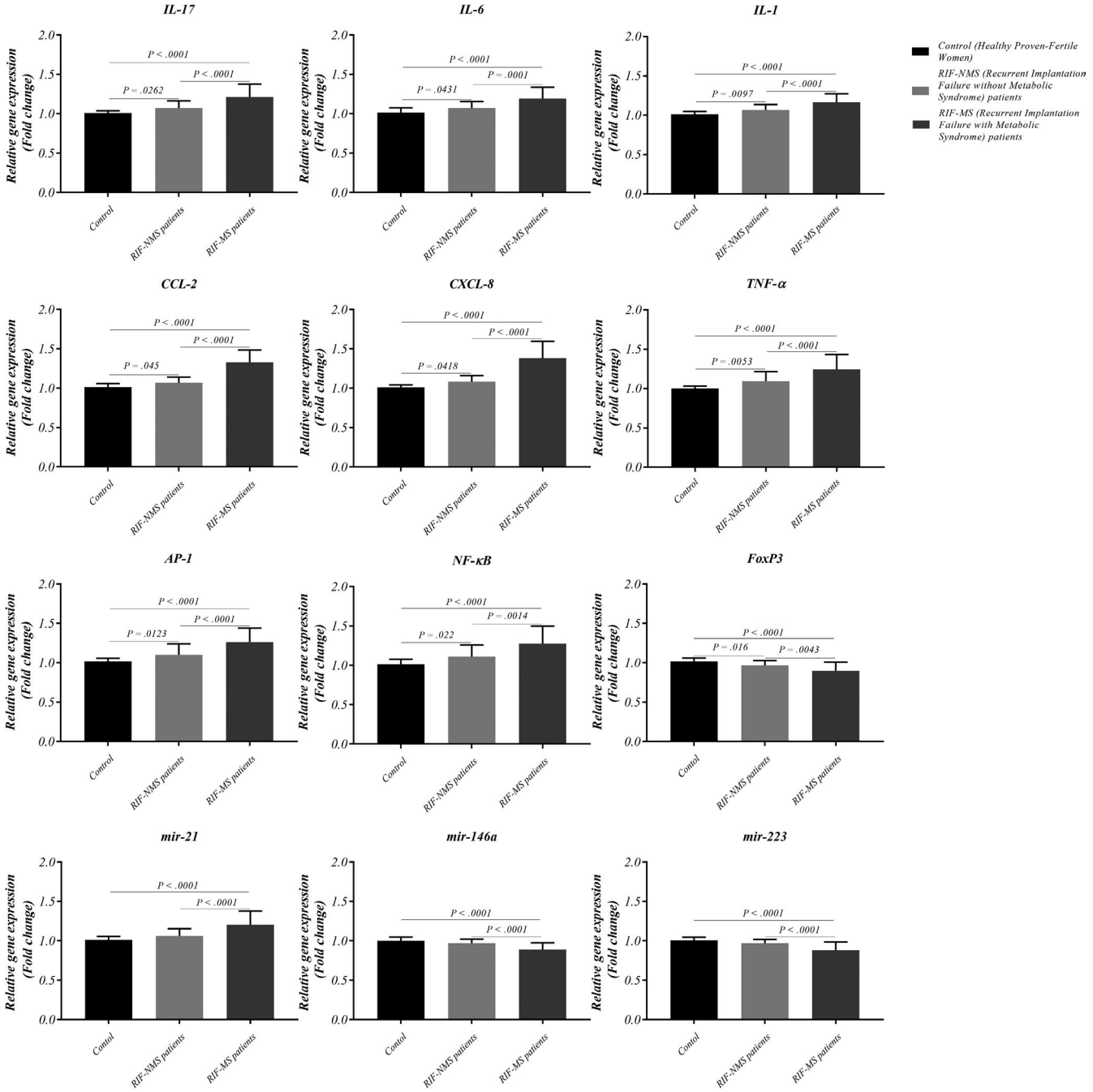

FIGURE 1 The mRNA expression of cytokines, chemokines, transcription factors, and miRNAs in the studied groups. The expression levels of inflammatory cytokines and chemokines, AP1 and NF-κB transcription factors, and *miR-21* were significantly increased in the RIF-MS in comparison with the RIF-NMS women and the control group. The expression levels of *FOXP3*, *miR-146a*, and *miR-223* were significantly decreased in RIF-MS women compared with the control group. Results are given as, mean ± SD (RIF-MS, n = 26; RIF-NMS, n = 21; healthy control group, n = 40)

with the RIF-NMS women and the control group (P = .0043 and P < .0001, respectively; Figure 1).

3.2 | miRNA expression levels

Evaluation of miRNA expression demonstrated significantly increased expression of miR-21 in PBMCs of RIF-MS patients compared with the RIF-NMS patients and the control group (P < .0001). However, miR-146a and miR-223 expression were significantly downregulated in PBMCs of RIF-MS patients in comparison with the RIF-NMS patients and the control group (P < .0001; Figure 1).

3.3 | Serum cytokines and chemokines level

IL-1β (P < .0001), IL-6 (P < .0001), IL-17 (P < .0001), TNF-alpha (P < .0001), CXCL-8 (P = .0008 and P < .0001), and CCL-2 (P < .0001)

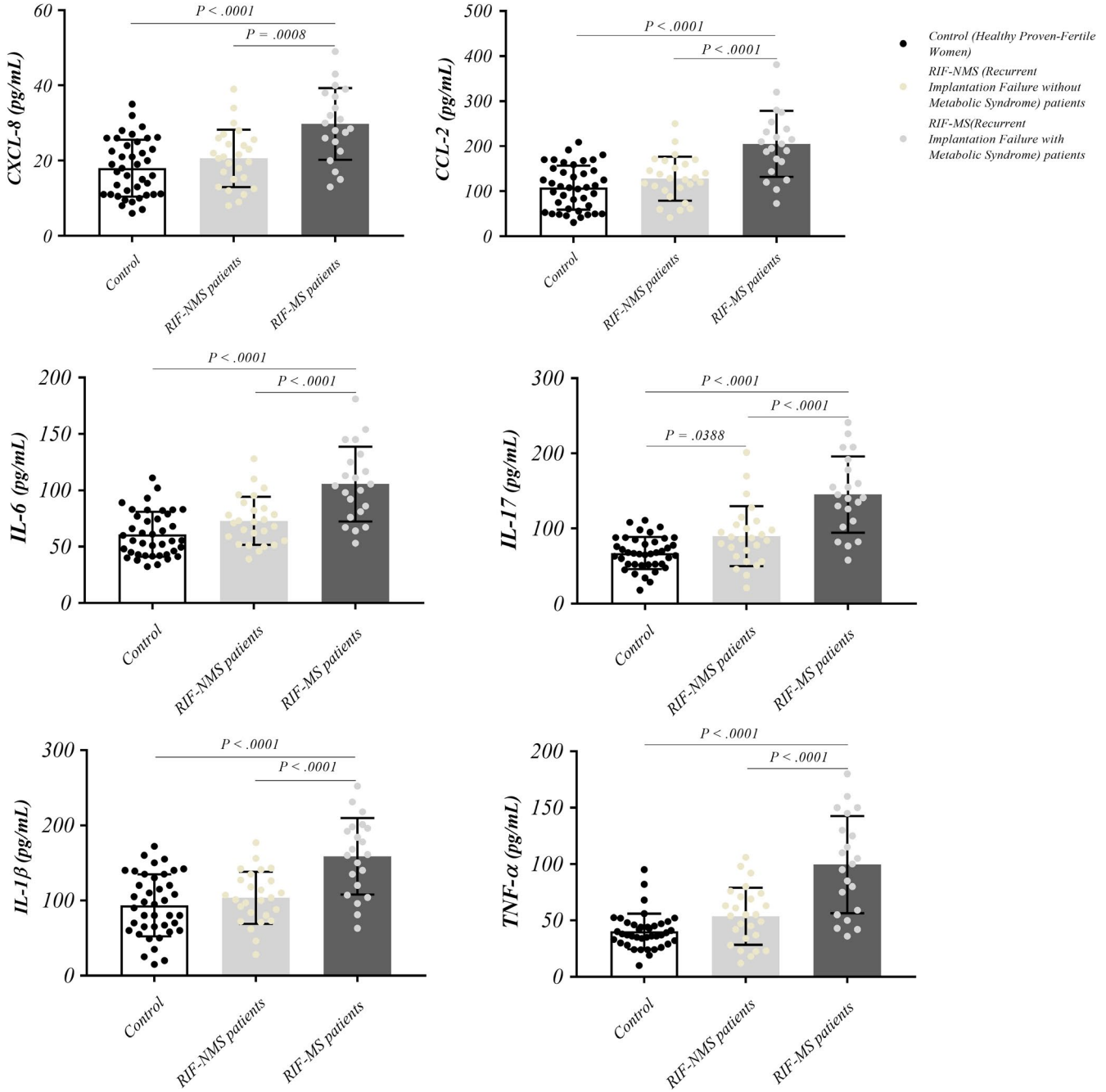

FIGURE 2 Serum cytokine and chemokine levels in the studied groups. The serum level of the inflammatory cytokines and chemokines were higher in RIF-MS women compared with the RIF-NMS patients and the control group. Results are given as mean ± SD (RIF-MS, n = 26; RIF-NMS, n = 21; healthy control group, n = 40)

serum levels were significantly increased in RIF-MS patients compared with the RIF-NMS patients and the control group (Figure 2).

3.4 | Oxidative stress status

According to Figure 3, the serum NO level of the RIF-MS patients significantly increased compared with the RIF-NMS patients and the control group ($P = .0001$ and $P < .0001$, respectively). Moreover, the level of AOPPs was significantly increased in the RIF-MS patients compared with the control group ($P < .0032$). The TOS level indicated a statistically significant increase in RIF-MS patients serum compared with the RIF-NMS and the control group ($P < .0117$ and $P < .0004$, respectively). No significant differences were observed in the TAS level between RIF-MS and RIF-NMS women. However, compared to control subjects, the TAS level was higher in the RIF-MS patients ($P = .0003$). SOD ($P = .023$ and $P = .0428$) and CAT ($P = .0178$ and $P = .0181$) levels decreased in the RIF-MS patients compared with the control group and also in RIF-NMS patients compared with the control group. However, the MPO level was significantly increased in the RIF-MS patients compared with the RIF-NMS and the control group ($P < .0001$; Figure 3).

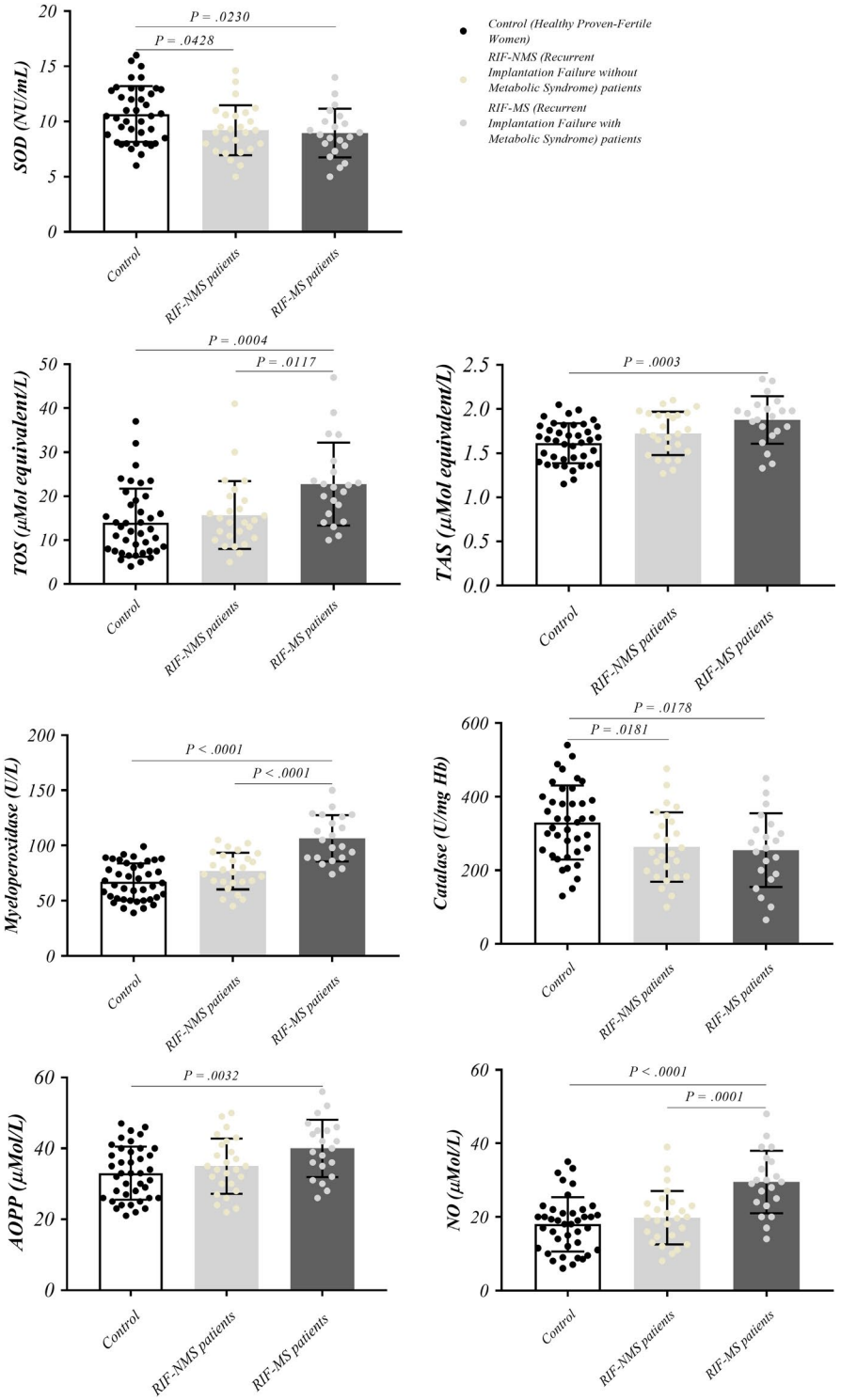

FIGURE 3 Serum oxidative stress markers evaluation in the studied groups. The SOD and CAT levels were lower in RIF-MS patients in comparison with the RIF-NMS patients and the control group. The NO, AOPPs, TOS, and MPO levels were higher in the RIF-MS women compared with the RIF-NMS patients and the control group. Results are given as mean ± SD (RIF-MS, n = 26; RIF-NMS, n = 21; healthy control group, n = 40)

3.5 | Th17 and Treg cell frequencies

As shown in Figure 4, Th17 frequency was significantly higher in RIF-MS patients (5.671%) compared with the RIF-NMS women (4.185%) and the control group (3.495%) ($P = .0006$ and $P < .0001$, respectively). Furthermore, Treg frequency in the RIF-MS women (2.710%) was significantly lower compared with RIF-NMS patients (3.900%) and control group (4.528%) ($P = .0033$ and $P < .0001$, respectively). The obtained results are shown in Table 4.

4 | DISCUSSION

In this study, female RIF-MS patients showed an increased OS and inflammatory factors compared with the RIF-NMS and the healthy proven-fertile women. MS is a multiple risk factor syndrome consisting of hyperglycemia, dyslipidemia, and hypertension with a high incidence in developed countries.[32,33] The prevalence of MS has been reported to be 15% in women and 25% in adult men.[34] Studies have shown different components of metabolic aberrations

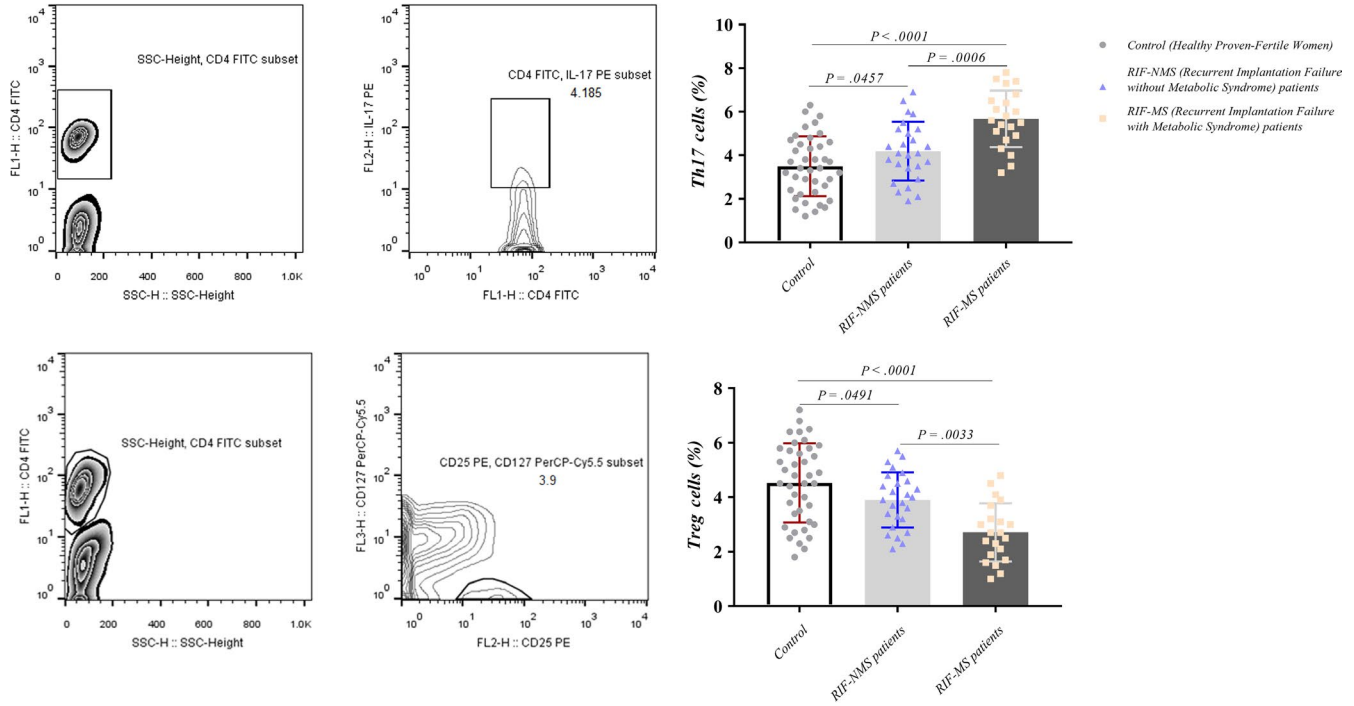

FIGURE 4 Determination of Th17 and Treg cell frequencies by flow cytometric analysis in the studied groups. Th17 was significantly higher in the RIF-MS patients compared with the RIF-NMS women ($P = .0006$) and the control group ($P < .0001$). Treg cell frequency was also significantly lower in RIF-MS patients compared with the RIF-NMS women ($P < .0033$) and the control group ($P < .0001$). Results are given as mean ± SD (RIF-MS, n = 26; RIF-NMS, n = 21; healthy control group, n = 40)

resulting in increased inflammation.[35] Increased type 2 diabetes mellitus (T2DM) and cardiovascular diseases have been reported in metabolic syndrome, which is an obesity-related metabolic disorder.[2] Evaluation of immune cells and low-grade inflammation plays a role in the pathogenesis of these diseases.[35-37] In some studies, white blood cell count, chemokines, and inflammatory cytokines have been identified as a predictor of T2DM.[38,39] According to some researches, the concentration of pro-inflammatory cytokines including tumour necrosis factor (TNF)-α, interleukin1β and IL-6, and chemokines increases in T2DM and obese patient.[3,40] These cytokines play a role in the activation of the c-JUN N-terminal kinase (JNK)–nuclear factor-kappa B (NF-κB) pathways that have an important role in promoting inflammation.[36] Moreover, macrophage infiltration has been reported in adipose tissues; phagocytosis of oxidized LDL by macrophage and formation of foam cells occur in this tissue. In this event, macrophages produce ROS and pro-inflammatory factors, and further increasing the OS condition and inflammation.[41] Generation of the superoxide radicals results in the activation of nuclear factor κB (NF-κB) and activator protein 1 (AP-1) that has a central role in increasing inflammation.[42] Considering these studies, inflammation might be an underlying factor for an association between metabolic syndrome and OS with RIF. In this regard, excessive inflammation has shown a function in the pathogenesis of RIF. Implantation is a crucial step in reproduction that starts with attachment to uterine endometrium. Increased Th1 cell and pro-inflammatory cytokines have been reported in early implantation.[43,44] In successful pregnancy trophoblast, cells produce regulatory cytokine and modulate immune cells and inflammatory responses.[45] Misbalance between immune cells and increased pro-inflammatory cytokines has been reported in RIF patients.[46] Therefore, it was decided to investigate the increased OS markers, inflammatory microRNAs, and also inflammatory cytokines and chemokines that augment in MS and OS conditions and can result in RIF disorder. A previous study has reported an obesity-induced OS, deregulated adipocytokines, and developed metabolic syndrome in the accumulated fat tissues.[5] Chatzi et al[1] also demonstrated the association between MS and preterm birth in early pregnancy.

Oxidative stress is detected in spontaneous abortion, impaired placental development, and syncytiotrophoblast degeneration.[47] Spontaneous abortion occurs if the OS is developed in 8 and 9 weeks after pregnancy.[48,49] Additionally, early development of maternal circulation and consequently OS augmentation in early pregnancy usually happens following increased natural killer (NK) cells frequency, which leads to increased pre-implantation angiogenesis in RPL patients.[50] In this study, the correlation between MS and OS was investigated and the OS markers and inflammatory factors were evaluated in the RIF-MS and the RIF-NMS subjects. Moreover, the levels of NO, AOPPs, TOS, and TAS were evaluated as OS markers. Except for TAS, our data indicated increased levels of oxidative stress markers in RIF-MS patients compared with the RIF-NMS patients and the control group. It has been reported that an imbalance between antioxidant and oxidant factors leads to OS development.[51] SOD and CAT are

TABLE 4 Cellular and molecular analysis of RIF-MS and RIF-NMS patients and the control group

Measurement	A Control N = 40	*P* value A vs B	B RIF-NMS patients N = 26	*P* value B vs C	C RIF-MS patients (N = 21)	*P* value A vs C
Mean level of gene expression in the PBMCs						
IL-1β	1.013 ± 0.03605	.0097	1.067 ± 0.07098	<.0001	1.164 ± 0.1108	<.0001
IL-6	1.014 ± 0.06226	.0431	1.072 ± 0.08275	.0001	1.192 ± 0.1447	<.0001
IL-17	1.009 ± 0.02833	.0262	1.073 ± 0.08991	<.0001	1.21 ± 0.1654	<.0001
TNF-α	1.002 ± 0.0304	.0053	1.095 ± 0.1203	<.0001	1.245 ± 0.19	<.0001
CXCL-8	1.013 ± 0.02953	.0418	1.084 ± 0.07553	<.0001	1.38 ± 0.2155	<.0001
CCL-2	1.014 ± 0.04431	.0450	1.07 ± 0.06934	<.0001	1.328 ± 0.1581	<.0001
FoxP3	1.016 ± 0.04454	.0160	0.9665 ± 0.06105	.0043	0.9 ± 0.1074	<.0001
NF-κB	1.012 ± 0.06562	.0220	1.11 ± 0.148	.0014	1.277 ± 0.2228	<.0001
AP1	1.015 ± 0.04261	.0123	1.103 ± 0.139	<.0001	1.263 ± 0.1777	<.0001
miR-21	1.012 ± 0.0442	NS	1.063 ± 0.09159	<.0001	1.203 ± 0.1747	<.0001
miR-146a	1 ± 0.04758	NS	0.9688 ± 0.05203	<.0001	0.889 ± 0.08432	<.0001
miR-223	1.005 ± 0.04032	NS	0.9677 ± 0.05062	<.0001	0.8805 ± 0.1036	<.0001
Level of cytokines and chemokines in the serum (pg/mL)						
IL-1β	93.58 ± 41.24	NS	103.6 ± 34.7	<.0001	158.8 ± 50.83	<.0001
IL-6	60.99 ± 19.98	NS	72.93 ± 21.33	<.0001	105.6 ± 33.17	<.0001
IL-17	67.47 ± 21.43	.0388	90.07 ± 39.82	<.0001	145.3 ± 50.73	<.0001
TNF-α	40.34 ± 15.81	NS	53.76 ± 25.3	<.0001	99.52 ± 43.06	<.0001
CCL-2	108.5 ± 49.05	NS	128.2 ± 48.76	<.0001	205.1 ± 73.31	<.0001
CXCL-8	18.03 ± 7.537	NS	20.62 ± 7.625	.0008	29.78 ± 9.561	<.0001
Stress oxidative status parameter in the serum or plasma						
NO (μmol/L)	18.01 ± 7.365	NS	19.8 ± 7.286	.0001	29.5 ± 8.5	<.0001
AOPPs (μmol/L)	33 ± 7.466	NS	35 ± 7.756	NS	40 ± 8.081	.0032
TOS (μmol equivalent/L)	13.96 ± 7.744	NS	15.69 ± 7.733	.0117	22.75 ± 9.445	.0004
TAS (μmol equivalent/L)	1.613 ± 0.2256	NS	1.725 ± 0.2444	NS	1.877 ± 0.2707	.0003
SOD (NU/mL)	10.68 ± 2.522	.0428	9.215 ± 2.264	NS	8.957 ± 2.2	.0230
CAT (U/mg Hb)	329.6 ± 100.5	.0181	263.2 ± 94.52	NS	254.5 ± 100.5	.0178
MPO (U/L)	67 ± 16.67	.0003	76.92 ± 16.65	<.0001	106.6 ± 21.01	<.0001
T-cell frequency (%)						
Th17	3.495 ± 1.373	.0457	4.185 ± 1.35	.0006	5.671 ± 1.302	<.0001
Treg	4.528 ± 1.454	.0491	3.900 ± 1.011	.0033	2.710 ± 1.066	<.0001

Note: $P < .05$ was considered as statistically significant.

Abbreviations: AOPPs, advanced oxidation protein products; AP1, activator protein 1; CAT, catalase; CCL-2, chemokine C-C motif ligand 2; CXCL-8, chemokine C-X-C motif ligand 8; FoxP3, forkhead box P3; IL, interleukin; miR, microRNA; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; NO, nitric oxide; NS, non-significant; PBMC, peripheral blood mononuclear cells; RIF-MS, recurrent implantation failure-metabolic syndrome; RIF-NMS, recurrent implantation failure-without metabolic syndrome; SOD, superoxide dismutase; TAS, total antioxidant activity; Th, helper T; TNF, tumour necrosis factor; TOS, total oxidant activity.

the antioxidant enzymes that regulate this balance.[52] The activity levels of these enzymes are decreased in different OS-related diseases. Tatone et al[53] reported that the protein levels of SOD and CAT declined in granulosa cells of older women suffering from age-dependent oxidative stress injury.[3,37-39] MPO, which is produced by leukocyte, is also an oxidant enzyme that increases the ROS production.[54] Our results showed decreased SOD and CAT levels in RIF-MS patients in comparison with control group. In contrast, the MPO level was significantly increased in this group compared with the other groups. Previous studies have also reported that white blood cells count, coagulation factors, acute-phase proteins, pro-inflammatory cytokine (TNF-alpha, IL-1β, and IL-6), and chemokines are increased in obese patients.[3,55] During elevated OS and inflammation, there is an up-regulation of AP1 and NF-κB. Both AP1 and NF-κB are transcription

factors that regulate the expression of pro-inflammatory cytokines and antioxidant protective genes. While pro-inflammatory mediators are increased, an imbalance between pro-inflammatory and antioxidant protective mediators occurs in inflammation and OS situation.[56] In line with our results, Kalem et al[57] reported an increased CCL-2 level in peripheral blood of RIF women. In our study, higher upregulation of NF-κB and AP1 genes was observed in RIF-MS and RIF-NMS patients compared with the control group. In addition, the inflammatory factors such as cytokines (ie, IL-1β, IL-6, and TNF-alpha) and chemokines (ie, CCL-2 and CXCL-8) increased in RIF-MS patients' serum compared with the RIF-NMS patients and the control group. Imbalance of different kinds of immune cells in early pregnancy has also been reported.[58] Immune cells (ie, Th1 and Th17) produced pro-inflammatory and inflammatory cytokines that play an important role in successful implantation. The excessive response of the immune system can lead to implantation failure and embryo rejection by the hostile maternal immune responses. Hence, the balance between immune responses is necessary for successful pregnancy.[59] Extensive studies have shown dysregulated immune responses and inflammatory factors in RIF patients. On one hand, increased Th17/Treg and Th1/Th2 ratios and IL-1, IL-6, 1L-17, and TNF-alpha cytokines have been observed in RIF patients. On the other hand, decreased numbers of Treg and Th2 have been reported in RIF patients.[46,59] Treg and Th2 immune responses are the causes of the fetus protection from the immune system attack.[60] In the present study, the frequencies of Treg and Th17 cells were investigated. The results showed decreased Treg and increased Th17 frequencies in the RIF-MS and the RIF-NMS patients compared with the control group. Furthermore, increased gene expressions of IL-1β, IL-17, IL-6, and TNF-alpha cytokines were detected in the PBMCs of the 2 groups. The cytokine gene expression in the RIF-MS patients was higher than that of the RIF-NMS patients and the control group. $CD4^+CD25^+$ Treg cells express a specific transcription factor called FOXP3, which is required for Treg development.[61] In line with previous reports,[46] both Treg number and *FOXP3* gene expression were decreased in the RIF-MS compared with the RIF-NMS patients and the control group.

miRNAs are endogenous and small non-coding RNAs that have an important role in the gene expression regulation by targeting the coding mRNAs. The dysregulation of miRNA levels has also been reported to be involved in the pathogenesis of inflammatory diseases.[22,62] In the current study, miR-146a, miR-223, and miR-21 expressions in the PBMCs of the MS-RIF and NMS-RIF patients were evaluated. miR-146a is one of the miRNAs that participate in inflammation regulation. miR-146a inhibits IRAK1-TRAF6-NF-κB pathway as a result of this, and miR-146a downregulation leads to increase in pro-inflammatory cytokines and enhances the inflammatory responses.[22] In parallel with previous studies,[46] the miR-146a expression level was reduced in RIF-MS patients compared with the RIF-NMS patients and the healthy controls. Various functions of miR-223 during hematopoiesis, inflammation, infection, and cancer have previously been described. miR-223 overexpression has shown a role in hematopoietic progenitor cells differentiation into NK cells or erythrocytes. However, it is downregulated during differentiation to monocyte, neutrophils, and eosinophils. Moreover, downregulation of miR-223 can increase the IL-1β and IL-6 production.[23] In the present study, we observed a decreased miR-223 expression in RIF-MS compared with the RIF-NMS patients and the control group. Besides, the levels of IL-1β and IL-6 increased in the RIF patients as previously reported in other diseases. miR-21 is highly expressed in different kinds of mammalian cells. This gene plays a dynamic role in the inflammatory responses and characterizes the immunosuppressive or pro-inflammatory state.[63] Kim et al[24] showed that miR-21 stimulated inflammation via inhibiting the transforming growth factor (TGF)-β signaling pathway in human adipose tissue-derived mesenchymal stem cells (AD-MSCs). The other function of miR-21 is repression of SOD2 production in the adipose tissue, which results in OS.[64] In our study, the upregulation of miR-21 was observed in RIF-MS patients. Nevertheless, further studies must be conducted to confirm the adverse effects of MS on RIF patients and fetus implantation outcomes.

5 | CONCLUSION

Considering all the evaluations performed in this study, we suggest that MS in RIF patient causes increased inflammatory factors and OS, which in turn leads to implantation failure. As a consequence, implantation failure might be hopefully prevented through controlling these factors after IVF and also by MS treatment before IVF cycle performance.

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CONFLICT OF INTEREST

None.

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