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# Sirolimus as a new drug to treat RIF patients with elevated Th17/Treg ratio: A double-blind, phase II randomized clinical trial

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ABSTRACT

*Background:* RIF is clinically defined as the failure of good quality embryos to implant into the uterus following at least three cycles of In Vitro Fertilization/Embryo Transfer (IVF/ET). During human pregnancy, a genetically different fetus is allowed to survive within the uterus despite the maternal recognition of fetal alloantigens. Compared with normal pregnant women, early loss of embryo is associated with systemic lower levels of Treg cells in IVF. Moreover, several lines of evidence have indicated that differentiation of naive T cells into Th17 is deleterious for normal pregnancy and may cause implantation failure. Sirolimus as the most common mTOR (mammalian target of Rapamycin) inhibitor is able to effectively prevent allograft rejection. Here we aimed to evaluate Sirolimus effects on Th17/Treg axis and subsequently on pregnancy outcome.

*Methods and materials:* 121 patients with a history of at least 3 implatation failures were selected and enrolled in this clinical trial. Blood was drawn between days 5 and 10 of the cycle prior to the index IVF/ET cycle to assess baseline value of Th17 cells and regulatory T cells ratios using flowcytometry. A Th17/Treg cell ratio equal or > 0.74 was considered to be the elevated Th17/Treg cell ratio. In 76 patients with elevated Th17/Treg ratios, 43 individuals were treated with Sirolimus and 33 remained untreated.

*Results:* Our results demonstrated that Sirolimus treatment led to an increase in Treg cells number and function in treated group and reduced the frequency and function of Th17 cells. Moreover Th17/Treg cell ratio, significantly reduced from 1.18 ± 0.46% to 0.9 ± 0.45% following Sirolimus intervention ($P = 0.024$). In contrast, no significant difference in Th17 and Treg cell frequencies and Th17/Treg cell ratio was observed in untreated control subjects before and after ET. Finally our data showed a significantly higher clinical pregnancy rate (55.81%) in Sirolimus-treated patients compared with control group (24.24%) ($P < 0.0005$). We also found a significantly increased live birth rate (48.83%) in RIF women who received Sirolimus compared with control group (21.21%) ($P < 0.0001$).

*Conclusion:* The findings of the current study revealed the fact that Sirolimus exhibit potent immunosuppressive effects by blocking intracellular immune responses downstream of co-stimulatory signals, also is able to improve reproductive outcome in RIF women with imbalanced Th17/Treg ratio by modulate of Th17 /Treg axis, thus representing a new approach for the potential treatment of patients with embryo implantation failure.

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## 1. Introduction

Within the recent decades, there has been a dramatic increase in the number of infertile couples who need to undergo In Vitro Fertilization (IVF). Despite substantial progresses in Assisted Reproductive Technology (ART), no obvious increase was observed in pregnancy rate in IVF cycles [1]. Embryo implantation failure in patients with Recurrent Implantation Failure (RIF) is a major and critical barrier to achieve success in IVF process [2]. RIF is clinically defined as the failure to implant high or good-quality embryos into the uterus following at least three cycles of IVF/ET [3]. Implantation, as a complex physiological process that involves the blastocyst invasions into the endometrium, is critical and essential for the continued embryonic development [3], protection of the growing embryo, and establishment of pregnancy. A successful implantation and pregnancy depends on the activity of various factors including adhesion molecules, cytokines and immune cells [4]. Cytokines and adhesion molecules are known as key players in the embryonic attachment to the maternal endometrium [3]. In addition, various immune cells infiltrating the site of implantation lead to different impacts on the fetal development and pregnancy [3]. Numerous studies supported the concept that normal pregnancy is different state that requires dynamically changes in maternal immune system especially in fetus implantation site [5]. Several immunological mechanisms have been proposed for tolerance to the semiallogeneic fetus. It is suggested that implantation failure and miscarriage occurs in mice and humans when these mechanisms become dysregulated [5–7].

Sirolimus is a macrolide antibiotic and immune modulator that was first discovered in the 1960s and eventually Food and Drug Administration (FDA) approved for the prevention of solid organ transplant rejection in 1999. Sirolimus as the most common mTOR inhibitor is able to effectively prevent allograft rejection and possesses significant antitumor properties [8]. These effects are mainly mediated by the inhibition of the mTOR kinase pathway. Sirolimus was first discovered to be an antifungal agent produced by the bacterium Streptomyces hygroscopicus. Shortly afterward, this compound was found to elicit antiproliferative and immunosuppressive effects on mammalian cells [8]. Sirolimus exerts immunosuppressive function by preventing the proliferation of interleukin-2-stimulated T cells [9], a biological function which is highly desired for allograft survival. Furthermore, this agent has been previously shown to attenuate inflammatory responses [10]. It has been reported that > 14,000 women with organ transplants have had successful pregnancies worldwide [11]. The occurrence of successful gestation in female transplant recipients exposed to Sirolimus indicates that this agent does not seem to represent an absolute contraindication for pregnancy. Also, recent studies have revealed that the rate of congenital abnormalities in the progeny of transplant recipients treated with Cyclosporine, Tacrolimus and Sirolimus is similar to that of general population [11]. Pregnancy is a state of immunosuppression and the dysregulated immune responses has been observed in women with RIF. Accordingly, modulation of the immune system by an immunosuppressant drug may present an approach to overcome implantation failure. This is important that sirolimus promoted generation of $CD4^+$ CD25+ FoxP3+ Tregs while strongly inhibiting the differentiation of Th17 cells in peripheral tissue in humans, animal model and in vitro. Therefore sirolimus prevent acute graft rejection in human and animal model [12–14]. Animal model studies have demonstrated that adoptive transfer of Tregs after depletion of $FoxP3^+$ Tregs resulted in improved pregnancy outcomes in abortion-prone mice [15–17]. Royster et al. have recently elucidated that Sirolimus is able to induce the expansion of Treg cells and improve pregnancy outcome back to normal state following knockdown of Treg cells in a murine model of embryo implantation failure [18]. In this context, utilization of Sirolimus might offer a promising approach to achieve a better pregnancy outcome among women with implantation failure who undergo IVF. Based on previous findings, we hypothesized that Sirolimus may be efficient in improving pregnancy rate in women with IVF failure. In the current study, we performed a phase II randomized clinical trial to determine whether Sirolimus can be used as a bona fide treatment to increase the success rate of IVF among women with RIF of immune etiologies.

## 2. Materials & method

### 2.1. Study population

A total of 121 patients with a history of at least three implantation failure after IVF/ET cycles referred to East Azerbaijan ACECR ART center, infertility center of Al-Zahra Hospital of Tabriz University of Medical Sciences (TUOMS); Iran and infertility treatment center of ACER of city of Qom; Iran, were selected and enrolled in this multicenter, randomized, double-blind, phase II study.

All subjects were assessed by transvaginal ultrasound, hysterosalpingography, and hysteroscopy prior to the index ART cycle. None of the participants had endometrial polyps, submucosal fibroids, intrauterine adhesions, congenital anomalies of uterus or hydrosalpinges. Women with any of infectious, endocrine, genetic abnormalities and acquired or inherited thrombophilia were excluded. Women's partners were also evaluated for semen parameters according to World Health Organization criteria (WHO, 2012). Randomization was done according to a computer-generated list. Also, the randomization code and allocation group were blinded to the participants and hospital staff until the last participant gave birth. A signed informed consent was obtained from all participants before entering the study. The study was approved by the Committee of Research Ethics of TUOMS (IR.TBZMED.REC.1395.429). Moreover, the trial was registered in *ClinicalTrials.gov*. (ClinicalTrials.gov Identifier: NCT03161340).

Blood samples was drawn between days 5 and 10 of a cycle prior to the index IVF/ET cycle to assess the baseline value of $CD4^+IL\text{-}17^+$ T cells and $CD4^+$ $CD25^+$ $CD127^-$ regulatory T cells ratios by flow cytometry. Normal ranges for Th17/Treg cell ratios were established using 50 normal fertile women who had a history of normal delivery by natural conception. The Th17/Treg cell ratio equal to or > 0.74 was classified to be an elevated Th17/Treg cell ratio, which was determined by the mean plus SD of Th17/Treg cell ratio levels. 76 patients (62.8%) had the elevated ratios Th17/Treg cell (≥0.74) and 45 subjects (38.2%) had normal Th17/Treg cell ratio (< 0.74).

Those 76 patients with elevated ratios of Th17/Treg were divided in two groups. 43 were treated with Sirolimus (Rapamune®; Pfizer, UK) (treatment group) and 33 patients were not treated with the same drug (control group). The patients in the treatment group began Sirolimus 2 days prior to ET and it continued until the pregnancy testing day (15 days after ET). The procedure was lasted for a total of 17 days. Sirolimus was administered at a dose of 2 mg/day.

### 2.2. Separation of peripheral blood mononuclear cells

Blood sampling was performed to assess the immunologic parameters in RIF patients and normal fertile women before the treatment in luteal phase. Blood samples were also taken from RIF patients (treated and control groups) 15 days after ET. A sample of 10 mL of peripheral blood was harvested and collected in heparinized tubes. Peripheral Blood Mononuclear Cells (PBMCs) were isolated using the Ficoll separation technique. After adding Ficoll (lymphosep) (Biosera, UK) with the density of 1.077 g/mL to the cells and centrifugation process (25 min, 450 *g*), Cells were washed twice with Phosphate Buffered Saline (PBS) (Sigma, Germany). $5 \times 10^6$ cells were cultured in 1 mL of a medium containing 10% heat-inactivated Fetal Calf Serum (FCS), 100 U/mL penicillin and 200 mM L-glutamine. Subsequently, 10 ng/mL Phorbol Myristate Acetate (PMA) (eBioscience, San Diego, CA, USA) was added to the medium, and cells were incubated for 48 h at 37 °C with 5% $CO_2$. Finally, the cultured cells were used for RNA extraction and the supernatant was exploited for cytokine assessment using Enzyme Linked Immunosorbent Assay (ELISA).

### 2.3. Analyss of Treg and Th17 cells

The frequency of $CD4^+IL\text{-}17^+$ T cells, $CD4^+CD25^+CD127^-$ T cells and Th17/Treg ratio in RIF patients and normal fertile women were demonstrated in the isolated PBMCs using flow cytometry technique and different monoclonal antibodies against surface and intracellular antigens. For Th17 enumaration $1 \times 10^6$ of PBMCs were incubated with 10 ng/mL PMA (Sigma) and 0.5 μM ionomycin (Sigma) for 5 h at 37 °C in a humidified incubator of 5% $CO_2$. Cells were washed with PBS and 0.09% *w/v* sodium azide (Sigma), and then stained with anti-CD4 APC (BD Biosciences), (eBioscience) for 15 min at 4 °C. Afterward, cells were washed with PBS and get fixed using fixation buffer (eBioscience) followed by washing twice with 1 × permeabilization buffer (eBioscience) and incubation with 0.25 μg conjugated anti-human IL-17A-PE antibody (eBioscience) for 20 min at room temperature. For Treg cells, surface stained with FITC-labeled anti-human CD4 (BD Biosciences, San Jose, CA, USA), phycoerytrin (PE)-labeled anti-human CD25 (BD Biosciences, San Jose, CA, USA) and PerCP-Cy5.5-conjugated anti-human CD127 (BD Biosciences, San Jose, CA, USA) antibodies for 45 min at 4 °C carried out.

FITC, PE, APC and PerCP-Cy5.5 rat IgG2a were used as isotype controls. Viable lymphocytes were gated based on forward and side scatter profiles.

### 2.4. Real-time PCR

Expression levels of the transcription factor of Treg (FoxP3) and Th17 (RORγt) were evaluated by Real-time PCR using SYBR Green method. Total RNA was isolated using RNX-PLUS solution (SinaClon, Tehran, Iran), and complementary DNA (cDNA) was synthesized using a RevertAid Reverse Transcriptase Kit (Thermo Fisher, Waltham, MA, USA). Six standard solutions were prepared using 10-fold serial dilutions of a concentrated sample of the reference gene (beta-actin) and the genes of interest. The standard curves were generated for both target and reference genes. The relative gene expression was normalized to beta-actin (described as relative fold changes in gene expression).

The polymerase chain reaction conditions for SYBR Green method were as follows: initial denaturation at 95 °C for 10 s, followed by 40 cycles of denaturation at 95 °C for 10 s, and annealing/extension at 60 °C for 30 s. Amplification was confirmed using electrophoresis analysis on 2% agarose gel followed by DNA sequencing performed by Applied Biosystems (SEQLAB, Germany).

### 2.5. Enzyme linked immunosorbent assay (ELISA)

The secretion of Treg- and Th17-associated cytokines (IL-10, TGF-β, IL-17, IL-23) was measured in the culture supernatant of PBMCs using ELISA, according to the manufacturer's instructions (Biosource, Nivelles, Belgium). This analysis was performed to compare the cytokines profile of RIF patients to the normal fertile women. All measurements were conducted in duplicate.

### 2.6. Ovarian stimulation, IVF-ET treatment, and embryo transfer

Mild stimulation of ovarian was started for 5 days from days 3 to 7, and recombinant human follicle stimulating hormone (rFSH; Sereno, Switzerland) was administrated on the third day of menstrual cycle. The daily rFSH doses ranged from 150 to 300 IU, depending on body mass index (BMI), age, and the anticipated ovarian response. Dose adjustment was done according to follicular development and serum estradiol levels. On day 10, when the average diameters of three preovulatory follicles reached 18-20 mm and the dominant follicles reached 17–20 mm in diameter, 10,000 IU of human Chorionic Gonadotropin (hCG) (1000 IU, Choriomon, Meizler, Brazil) was injected.

Ovum Pickup (OPU) was done 36 h after hCG administration by vaginal ultrasound-guided puncture of the ovarian follicles. The collected oocytes were incubated in 37 °C with 6% CO2 for 3–4 h and then were used for IVF and Intracytoplasmic Sperm Injection (ICSI) depending on the semen parameters. For fresh embryo transfer, patients received a transfer of one or two morphologically Good-quality Embryos (MGEs) 5 days after oocyte retrieval. Then, 3 day-old frozen/thawed embryos were transferred 3 days after ovulation in either of the natural ovulatory cycle or 3 days after progesterone administration in Hormonal Replacement Cycle (HRC). MGEs were defined to have more than seven or more blastomeres and to possess < 10% of fragmentation 3 days after oocyte retrieval [19].

A pregnancy test was performed 15 days after ET, and a positive β-hCG was defined as a pregnancy. Clinical pregnancy was recognized when, 21 days after ET, the development of a gestational sac was detected by transvaginal ultrasound. Ongoing pregnancy was recognized when the normal fetus was detected by transvaginal ultrasound at the 12th gestational week. The primary and secondary endpoints of this study were clinical pregnancy and giving birth to live infant, respectively.

### 2.7. Statistical analysis

Results were expressed as mean ± SD. Statistical analysis was performed using SPSS PC Statistics software (version 19.0; SPSS Inc.). We applied unpaired *t*-test for multiple comparisons. Also, paired t-test was used to compare the results of immunologic studies before and after embryo transfer in control and Sirolimus treated groups. Linear-by-linear association chi-square test was used to determine linear trend among the groups of immune variables, response rate of variables and pregnancy outcome. To plot the graphs, the GraphPad Prism for Windows (version 7.00) (GraphPad Software, La Jolla, CA, USA, www.graph pad.com) was used. $P$ values < 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Study population

Among the 76 RIF patients with an elevated Th17/Treg cell ratio, 43 were treated with Sirolimus and 33 patients were remained untreated with the same drug (control group). The age, obstetrical and infertility histories of these two groups were compared and are summarized in Table 1. Maternal age, BMI, pregnancy history, previous live birth, the number of primary and secondary infertility, the numbers of previous ET attempts, the numbers of previously transferred embryos, the numbers of previous transferred MGEs in this study and endometrial lining of the study RIF patients were homogenous between the two groups.

### 3.2. Th17/Treg cell ratios in RIF and fertile women

Normal ranges for Th17/Treg cell ratios were demonstrated using 50 normal fertile women who had a history of normal delivery by natural conception. The cellular proportion of CD4+ CD25+ CD127- Treg cells and CD4+ IL-17A+ Th17 cells and Treg/Th17 ratio in RIF patients were determined using flow cytometry, 2 days before embryo transfer and the outcomes were compared to that of normal fertile women to demonstrate the normal ranges for Th17/Treg cell ratios. Moreover, the prevalence of Treg and Th17 cells in RIF patients were measured in both treated and untreated groups 15 days after ET and were compared with the pre- treatment phase. Our results showed decrease in the percentage of Treg cells in RIF patients (3.16 ± 1.6%) when compared to the normal pregnant women (3.87 ± 1.48%) and with a statistically significant difference ($P = 0.0079$) (Table 1 and Fig. 1A). An increased level of Th17 cells was also observed in RIF

**Table 1**
.Demographic data of patients.

| Variable | Treated group (n = 43) | Untreated group (n = 33) | *p*-value | Fertile women |
|---|---|---|---|---|
| No. of patient | 43 | 33 | _ | 50 |
| Maternal age (years, range) | 35.7 ± 3.7(23–41) | 36.1 ± 2.8(23–40) | NS | 36.6 ± 2.4(22–40) |
| Body mass index (kg/m2) | 27.3 ± 3.2 | 26.2 ± 3.1 | NS | 26.3 ± 1.9 |
| No. of smoking patient | 0 | 1 | NS | 1 |
| No. of smoking partners | 9 | 8 | NS | 13 |
| No. of previous pregnancy history (percent) | 12 (27.9%) | 10 (30.3%) | NS | 50(100%) |
| No. of previous live birth (percent) | 7 (16.27%) | 6 (18.18%) | NS | 50(100%) |
| No. of primary infertility (percent) | 29(67.44%) | 22 (66.66%) | NS | _ |
| No. of secondary infertility (percent) | 14 (32.56%) | 11 (33.33%) | NS | _ |
| No. of previous ET attempts (range) | 4.32 ± 2.2 (3−11) | 4.45 ± 2.64 (3−10) | NS | _ |
| Total No. of previous transferred embryos | 8.4 ± 3.2 | 9.6 ± 2.8 | NS | _ |
| No. of MGEs transfers in this study (range) | 1.64 ± 0.26 (1–2) | 1.78 ± 0.18 (1–2) | NS | _ |
| No. of Frozen-thawed embryo transfer (percent) | 31 (72.1%) | 24(72.72%) | NS | _ |
| No. of Fresh embryo transfer (percent) | 12 (27.9%) | 9(27.28%) | NS | _ |
| Endometrial lining (mm) | 10.6 ± 1.6 | 10.5 ± 1.4 | NS | _ |
| Progesterone levels (ng/ml) | 8.65 ± 7.92 | 7.17 ± 8.49 | NS | 13.24 ± 4.98 |
| Treg cell levels (percent) | 2.96 ± 1.6% | 3.09 ± 1.6% | NS | 3.87 ± 1.48 |
| Th17 cell levels (percent) | 4.61 ± 1.31% | 4.52 ± 1.23% | NS | 2.9 ± 1.29 |
| T17/Treg cell ratio | 1.18 ± o.46 | 1.22 ± 0.45 | NS | 0.74 ± 0.37 |

NS: not significant, ET: embryo transfer, MGEs: morphologically good-quality embryos.

patients and the percentage of Th17 cells was notably higher in RIF patients (4.57 ± 1.27%) when compared to the normal controls (2.9 ± 1.29%) ($P$ =0.0005) (Table 1 and Fig. 1B). In addition, Th17/Treg ratio in RIF patients was 1.19 ± 0.45 that was significantly higher than fertile women 0.74 ± 0.37 ($P$ = 0.0008) (Table 1 and Fig. 1C).

### 3.3. mRNA expression level of transcription factors in RIF and fertile women

The mRNA expression levels of the transcription factors of Treg (FoxP3) and Th17 (RORγt) were evaluated and compared between RIF patients and normal fertile women. As shown in Fig. 1D, the mRNA level of FoxP3 decreased in RIF patients ($P$ =0.0001). By contrast, the mRNA level of RORγt demonstrated an increase in RIF patients when compared to the normal healthy controls ($P$ = 0.0015).

### 3.4. Cytokine secretion levels of Treg and Th17 cells in RIF and fertile women

Measuring Treg- and Th17-associated cytokines by ELISA showed that there was a significant difference in the cytokine secretion profile of these cells between RIF patients and normal subjects. The results indicated that the level of IL-10 secretion was significantly lower in RIF patients when compared to the normal fertile women ($P$ = 0.019). It is while, the level of cytokine secretion by Th17 cells, including IL-17 and IL-23 increased significantly in RIF patients in comparison to the controls ($P$ = 0.006 and $P$ = 0.015, respectively) (Fig. 2).

### 3.5. Th17/Treg cell ratio before and after Sirolimus treatment

Flow cytometric analysis was applied to enumerate the peripheral Th17 and Treg cells among RIF women treated and untreated with Sirolimus. The results revealed that the frequency of Treg and Th17 cells, and Th17/Treg cell ratio were significantly affected following Sirolimus treatment in RIF patients.

Sirolimus treatment led to the increased Treg cell number from 2.96 ± 1.54% to 3.82 ± 1.54% in treated group ($P$ = 0.0001), as well as remarkable reduction of Th17 cells frequencies from 4.54 ± 1.34% to 3.28 ± 1.19% ($P$ = 0.0002) (Fig. 3A).

Th17/Treg cell ratio, was 1.18 ± 0.46% at the pre-treatment phase while, it reduced significantly to 0.9 ± 0.45% following Sirolimus treatment ($P$ = 0.024) (Fig. 3B). In contrast, no significant difference in Th17 and Treg cell frequencies and Th17/Treg cell ratio was observed in untreated control subjects pre and post ET (Fig. 3A and B).

### 3.6. mRNA expression level of transcription factors after sirolimus treatment

The results of gene expression analysis demonstrated that the mRNA level of FoxP3 increased significantly in Sirolimus group after 17 days treatment ($P$ = 0.0021) while, the mRNA level of RORγt decreased in treated group; however it was not significant. There was no significant difference in the mRNA levels of FoxP3and RORγt in control group pre and 15 day post ET (Fig. 3C).

### 3.7. Pregnancy outcome and live birth rate

The ART outcomes in both groups are summarized in Table 2. The numbers of the morphologically good-quality embryos transfers per cycle in treatment and control groups were 1.64 ± 0.26 and 1.78 ± 0.18, respectively. Pregnancy outcome, defined as a positive serum β-hCG test, was assessed in participants 15 days post embryo transfer and was followed up to 28–30 weeks of gestation. Results revealed that there was a significant difference in the success rate of pregnancy and live birth between Sirolimus-treated and untreated control groups. Clinical pregnancy of fresh ET and frozen-thawed ET were (5/12) 41.66% and (19/31) 61.29% respectively in test groups, while they were respectively (2/9) 22.2% and (6/24) 25% in control groups. These findings indicated that the pregnancy rate was significantly higher in Sirolimus-treated patients compared to the controls in both fresh and frozen-thawed ETs ($P$ < 0.0001). Furthermore, we found that clinical pregnancy rate was (24/43) 55.81% in treated group, while it was (8/33) 24.24% in control group. These results revealed a remarkable increase in pregnancy rate in treated patients compared to the controls ($P$ < 0.0001). The same results were observed for live birth rate in treated and control groups ((21/43) 48.83% and (7/33) 21.21%, respectively), suggesting a considerable increase in the live birth rate in RIF women who received Sirolimus in comparison to the controls ($P$ < 0.0001) (Fig. 3D and Table 2). Also, spontaneous abortion rate was (3/24) 12.5% in treated group while it was (1/8) 12.5% in control group, ($p$ = NS). The side effects reported in this study were mild (such as diarrhea, constipation, nausea and headache) and so, there was no need to stop medication. Nineteen of 21 babies were normal, and only two babies showed a little low weight.

## 4. Discussion

To the best of our knowledge, this study is the first report describing the positive effects of Sirolimus on pregnancy outcomes in women who

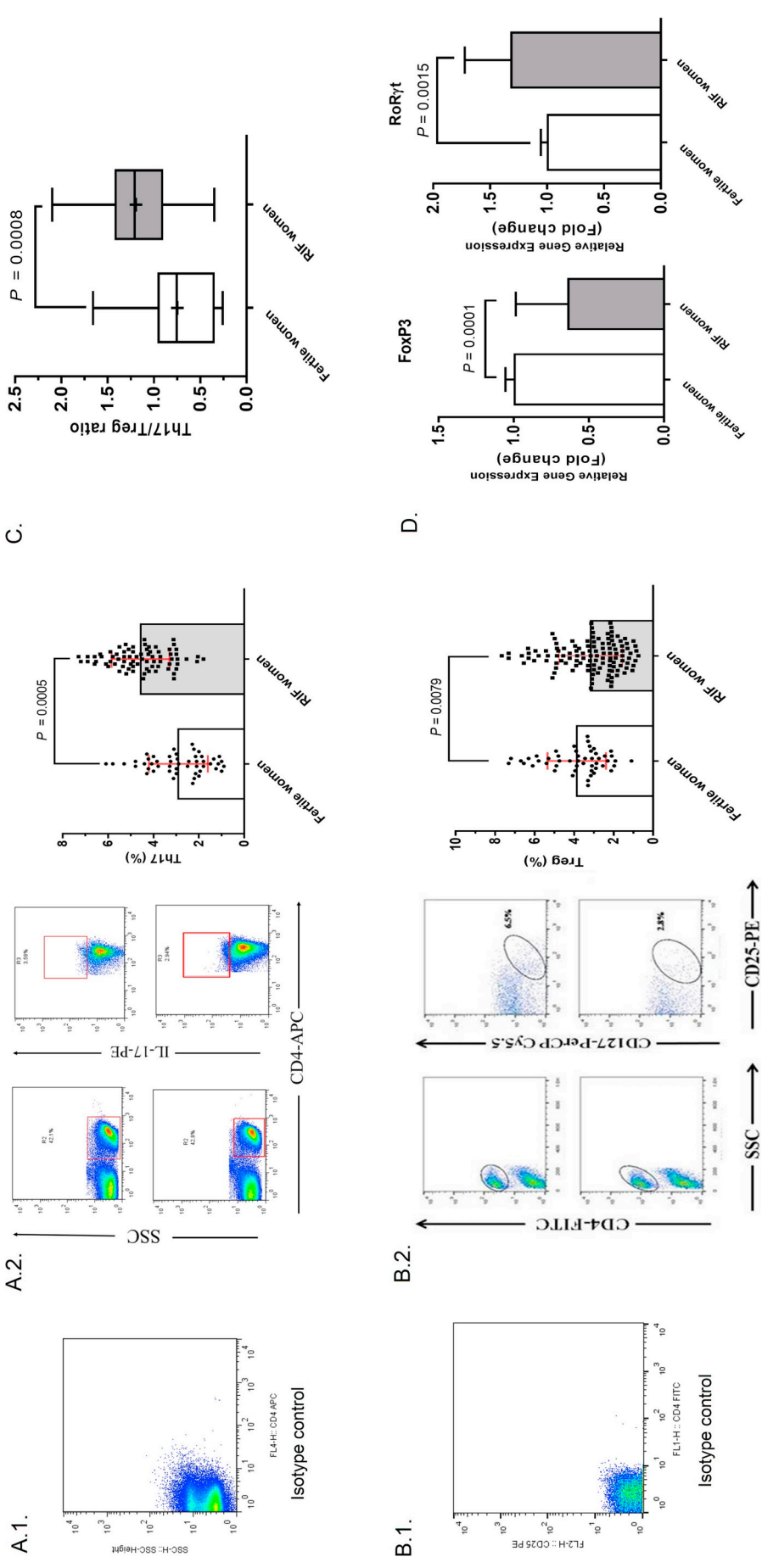


**Fig. 1.** Th17/Treg cells ratio in RIF patients compared to the normal fertile women. A.1. The isotype control for the flow cytometric strategy of $CD4^+$ IL-$17^+$ cells. A.2. Pre-treatment of Th17 prevalence evaluation in normal fertile women ($n = 50$) and RIF patients ($n = 121$). Th17 prevalence was increased in RIF patients when compared to the normal fertile women ($P = 0.0005$). B.1. The isotype control for the flow cytometric strategy of $CD4^+$ IL-$25^+$ $CD127^-$ cells. B.2. Pre-treatment of Treg prevalence evaluation in normal fertile women (n = 50) and RIF patient (n = 121). Treg prevalence was decreased in RIF patients when compared to the normal fertile women ($P = 0.0079$). C. Th17/Treg cells ratio in RIF patients and normal fertile women. Th17/Treg ratio in RIF patients was 1.19 ± 0.45 and it was significantly higher than fertile women 0.74 ± 0.37 ($P = 0.0008$). D. The results of gene expression analysis demonstrated that FoxP3 is highly expressed in normal fertile women than the RIF patients respectively ($P = 0.0001$). In contrast, we found higher expression levels of RORγt in RIF patients compared with normal fertile women ($P = 0.0015$). Results are given as mean ± SD.

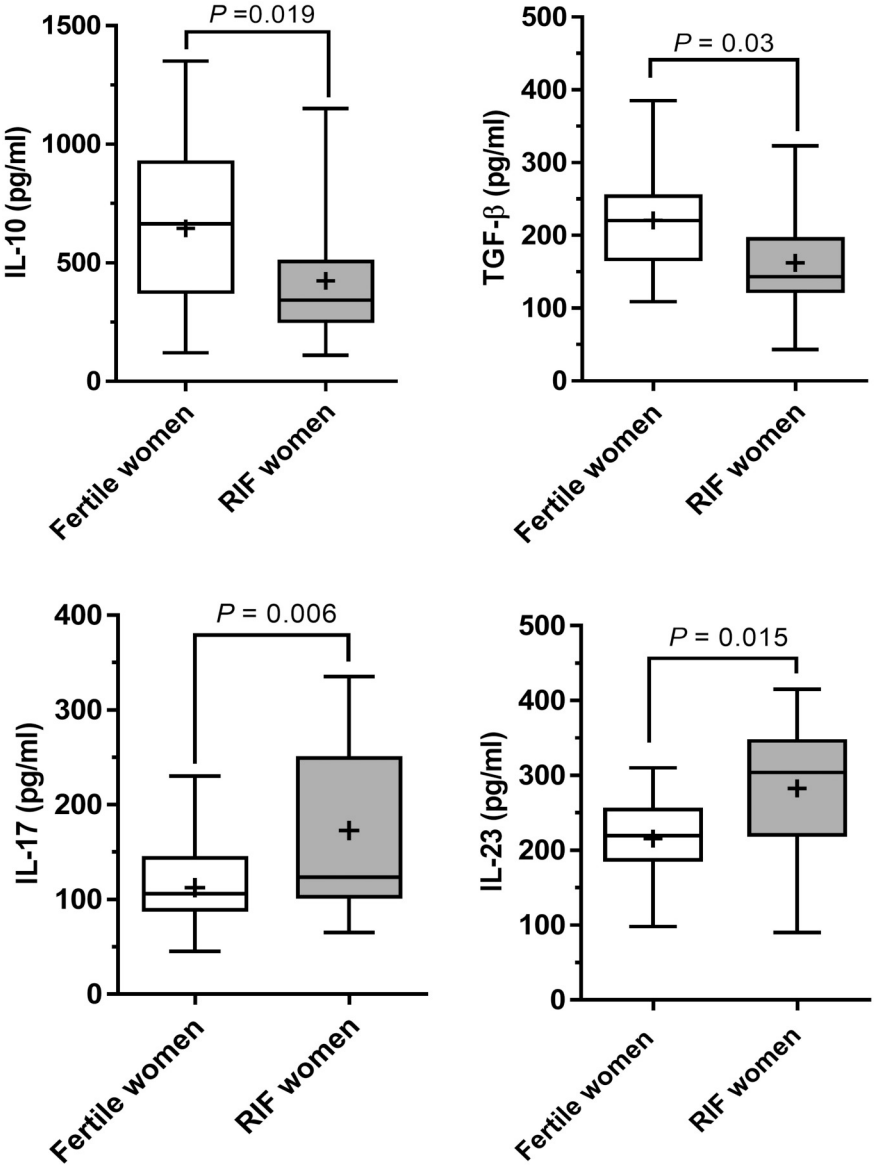


**Fig. 2.** Treg and Th17 associated cytokine secretion levels. Evaluation of Treg and Th17 associated cytokine secretion levels in RIF women (n = 121). The evaluation of Treg-associated cytokine secretion indicated significantly lower levels of IL-10 in RIF patients than the normal pregnant women (n = 50) ($P = 0.019$). There was no significant difference in the rate of TGF-β secretion between the two groups. With regard to Th17-associated cytokines, the secretion of IL-17 and IL-23 was higher in RIF patients ($P = 0.006$ and $P = 0.015$, respectively) when compared to the control. Obtained data showed minimum, maximum and median of cytokine secretion levels in participants.

have experienced RIF after IVF and embryo transfer cycles. We demonstrated that women with RIF and imbalanced immune profiles can benefit from Sirolimus-based immunosuppressive therapy. Implantation failure accounts for 75% of unsuccessful pregnancies, and is considered as a major limitation in IVF success [20]. Successful implantation is the consequence of a well-orchestrated sequence of events and it requires many factors related to either embryo or mother [21]. The adhesion of blastocyst to the maternal uterine mucosa is accompanied by the transition of endometrium to a receptive state in response to ovarian hormones [22]. The local paracrine factors including cytokines and growth factors are produced by decidual stromal cells, endometrium and immune cells. All of these secreted factors contribute into the physical interactions between the trophoblast and the endometrial lining [23]. Uterine anomalies, non-receptive endometrium and immune dysregulations are maternally originated disorders that adversely affect the attachment of the embryo to the uterus [24]. Immunological or inflammatory disorders and metabolic dysfunctions such as autoimmune thyroid disease, diabetes mellitus, and production of antiphospholipid antibodies have been implicated in RIF [25].

In the present study, evaluating the proportion of Treg cells in peripheral blood of non-pregnant women with a history of RIF and normal fertile women revealed that a decreased number of Treg cells is associated with implantation failure [26]. Women with RIF had significantly higher Th17/Treg ratio than fertile controls. In addition, gene expression analysis demonstrated that FoxP3 gene is highly expressed in fertile women when compared to RIF patients. In contrast, high level of RORγt expression was observed in RIF patients when compared to the normal controls. We also observed that IL-10 and TGF-β secretion was significantly lower in RIF patients while; IL-17 and IL-23 secretion was considerably higher in these patients than the normal controls. Several previous studies have reported the elevated level of Th17/Treg ratio during implantation window in RPL patients [27–29]. These findings imply that Th17/Treg imbalance is correlated with the pathogenesis of RIF and can be considered as a predictive factor for pregnancy outcome in women undergoing IVF. Evaluating the levels of several cytokines in the maternal blood has demonstrated that patients with high ratio of Th17/Treg-related cytokines yield higher risk of negative pregnancy outcome and lower implantation rate in IVF [30,31].

Despite substantial progress in our understanding of the role of immune system in normal pregnancy and the contribution of immunologic factors in RIF etiology, it has remained as a serious unsolved problem in IVF procedure. Heterogeneity in RIF patients with diverse immunological and non-immunological factors highlights the need to

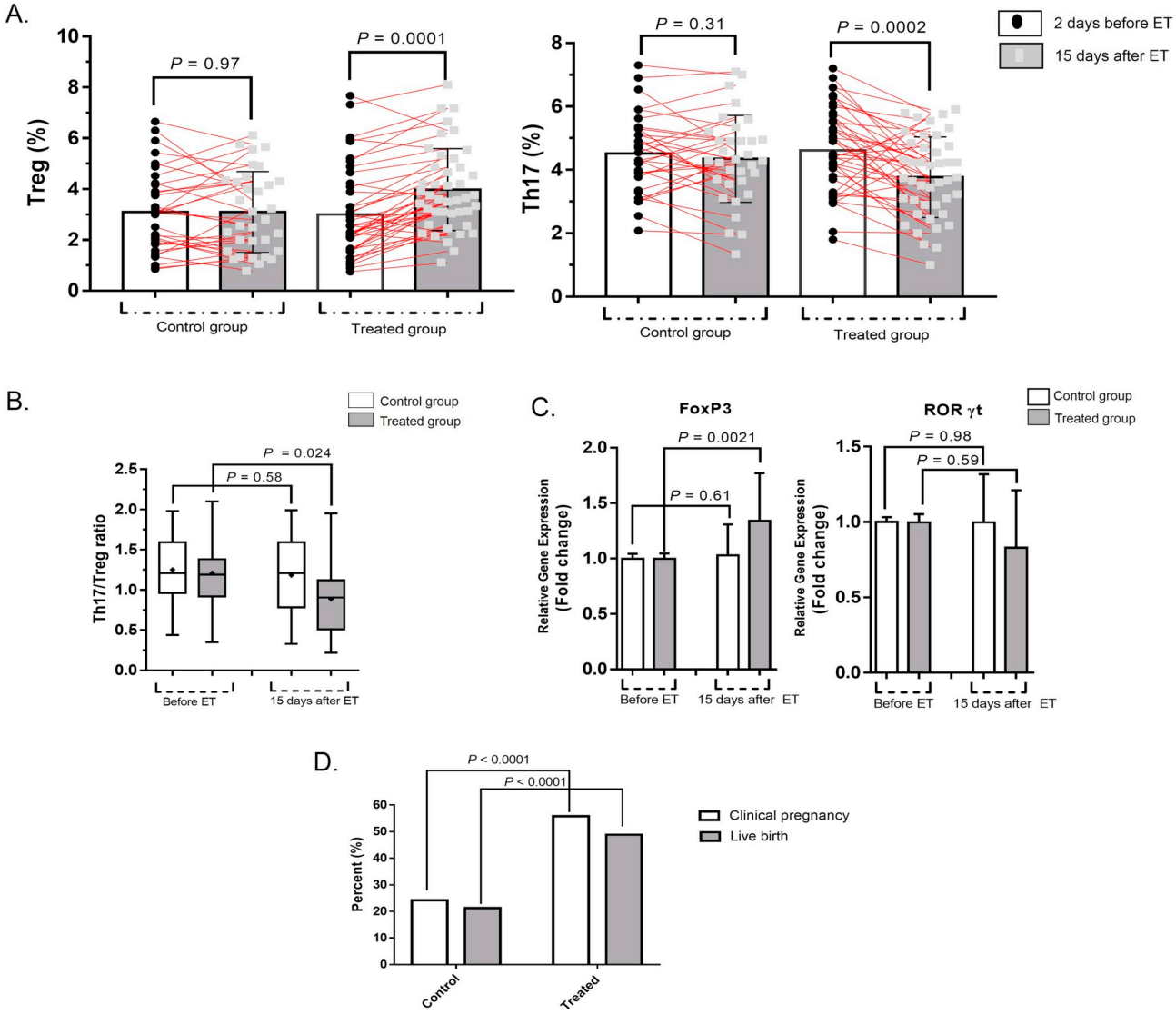


**Fig. 3.** The effects of Sirolimus therapy on the Th17/Treg cells ratio in RIF patients. A. The effects of Sirolimus therapy on the frequency of peripheral Treg and Th17 cells in RIF patients. Sirolimus treatment significantly increased the frequency of Treg cells in treated patients ($n = 43$) ($P = 0.0001$). Sirolimus treatment also reduced Th17 cells in treated patients ($P = 0.0002$). In contrast, no significant difference in Th17 and Treg cell frequencies was observed in untreated control subjects ($n = 33$) 2 day pre- ET and 15 days post- ET. B. Th17/Treg cell ratio, was 1.18 ± 0.46% in treated group before Sirolimus treatment and significantly reduced to 0.9 ± 0.45% following the intervention ($P = 0.024$). In contrast, no significant difference in Th17/Treg cell ratio was observed in untreated control subjects pre and post ET. C. The mRNA expression levels of FoxP3 and RORγt, 2 days before and 15 days after ET were quantitated by Real-time PCR. mRNA level of FoxP3 increased significantly in Sirolimus treated group when compared to the untreated control group after 17 days Sirolimus treatment ($P = 0.0021$). Also the mRNA level of RORγt decreased in treated group after Sirolimus treatment though it was, not significant. There was no significant difference in the mRNA rate of FoxP3 and RORγt in untreated group pre and 15 days post ET. D. The evaluation of pregnancy outcome and live birth rate in RIF women. As shown in the figure, there is a significant difference in the success rate of pregnancy in Sirolimus-treated patients (n = 43) (24/43; 55.81%) when compared to the control group (n = 33) (8/33; 24.24%) ($P < 0.0001$). Live birth rate was also significantly higher in Sirolimus-treated RIF women (21/43; 48.83%) than the control group (7/33; 21.21%) ($P < 0.0001$).

stratify patients to design treatment approaches and achieve better outcomes. In the recent decade, given the progresses in elucidating and understanding of immune etiologies of pregnancy complication, administration of immunomodulatory and immunosuppressive drugs such as IVIG, Prednisolone, and Tacrolimus are elevated [32].

In current study, we selected RIF patients with immunological etiologies including increased levels of Th17 cells and decreased levels of Treg cells in the peripheral blood. We also investigated the expression level of key transcription factors and associated cytokines in patients prior to entering the study. Several treatment approaches have previously been reported for RIF patients with increased Th1/Th2 levels. More recently, It has been suggested that tacrolimus is efficient in modulation of immune reactivity and improvement of the pregnancy rate in women with history of recurrent implantation failures after IVF/ET cycles [33]. The administration of Intravenous immunoglobulin G (IVIg) has been indicated to reduce Th1/Th2 ratio and effectively boost reproductive outcome in women with RIF and enhance Th1/Th2 levels [32]. In our previous study, we evaluated the effect of IVIG administration on the cellular proportion of Th1 and Th2 in RPL women. We observed that IVIG was an effective therapeutic option to treat reproductive failures such as RPL particularly in patients with immune cell abnormalities such as an elevated rate of NK cells. Moreover, IVIG is able to improve pregnancy outcome and enhance live birth rate [34]. We demonstrated that the administration of IVIG during pregnancy in RM women with cellular immune cells abnormalities, influenced the Th17/Treg ratio in peripheral blood and enhanced the Treg as well as decreased Th17 responses [35].

In current study, we investigated the use of Sirolimus (Rapamycin)

**Table 2**
Pregnancy outcome in treated and control groups.

| Variable | Treated group (n = 43) | Control group (n = 33) | P-value |
|---|---|---|---|
| No. of transferred cycles | 43 | 33 | _ |
| No. of MGEs transfers in this study (range) | 1.64 ± 0.26 (1–2) | 1.78 ± 0.18 (1–2) | NS |
| No. of positive hCG (percent) | 24 (55.81%) | 8 (24.24%) | < 0.0001 |
| No. of biochemical pregnancy rate | 0 | 0 | NS |
| No. of clinical pregnancy (percent) | 24 (55.81%) | 8 (24.24%) | < 0.0001 |
| No. of clinical pregnancy of fresh ET (percent) | 5 (41.66%) | 2 (22.2%) | < 0.0001 |
| No. of clinical pregnancy of cryo-thaw ET (percent) | 19 (61.29%) | 6 (25%) | < 0.0001 |
| No. of spontaneous abortion rate (percent) | 3 (12.5%) | 1(12.5%) | NS |
| No. of live birth rate of fresh ET (percent) | 4 (33.3%) | 2 (22.2%) | 0.009 |
| No. of live birth rate of thawed ET (percent) | 17 (54.83%) | 5 (20.83%) | < 0.0001 |
| No. of live birth rate (percent) | 21 (48.83%) | 7 (21.21%) | < 0.0001 |

Values are mean _ standard deviation unless otherwise specified.

as a treatment modality in RIF patients with elevated Th17/Treg levels. Our results demonstrated that the success rate of IVF was enhanced (55.81%) in Sirolimus-treated patients when compared to the control group (21.21%). The live birth rate was also increased from 18.18% in control group to 48.83% in Sirolimus-treated group. The difference in reproductive outcome and the live birth rate between two groups was statistically significant ($p < 0.0001$), indicating the clinical efficacy of Sirolimus treatment in these patients. Although Sirolimus is capable of preventing embryo rejection and supporting implantation through hypothetically immunomodulatory action, but the abortion rate came to be same for both treated and control groups despite the treatment after 15 days. Our recent study [36] suggested that IVIG therapy can be an appropriate strategy in treating implantation failure in RIF women especially with an immunologic basis. IVIG can improve the implantation and pregnancy rate by affecting immunoregulatory mechanisms specially Tregs whereas in current study, Sirolimus increases the implantation and pregnancy rate by affecting both Tregs and Th17 cells. Our results showed that sirolimus promoted generation of Treg cells while strongly inhibiting the differentiation of Th17 cells in peripheral tissue in humans, Therefore prevent implantation failure in RIF patients [12–14]. Sirolimus exerts its biological function via forming toxic complex with FK506 binding proteins, (FKBPs) a family of intracellular proteins [37]. The FKBP-Sirolimus complexes are indicated to interfere with specific components of intracellular metabolic pathways. The molecular target of this inhibitory complex is mTOR, which is the catalytic subunit of two multiprotein complexes participating in some essential biological functions in eukaryotic cells [38,39]. In addition to inhibitory effects on mTOR-mediated signalling, Sirolimus shows its potent immunosuppressive impacts by blocking intracellular immune responses downstream of co-stimulatory signals as well as inhibiting signal transduction pathways by binding to cytokine receptors during G1 phase. Moreover, Sirolimus is shown to suppress interleukin-2- and interleukin-4-induced proliferation of B and T cells [40]. The IL-2-mediated proliferation and survival of activated T cells requires a coordinated effort between multiple signalling pathways downstream of the IL-2R. After IL-2 binding, the transcription factor STAT5 (Signal Transducer and Activator of Transcription 5) is activated and in turn, the recruitment of PI3K (phosphoinositide 3-kinase) and Ras-MAPK (Ras-Mitogen Activated Protein Kinase). This complex signalling system finally results in the up-regulation of critical genes for cell cycle progression and cell survival. It is now clear that the IL-2-IL-2R signalling pathway is essential in the development and peripheral activity of Treg cells [41]. Therefore, it is unlikely that Sirolimus, which inhibits signal transduction delivered by IL-2 allows expansion of functional Treg cells. It has been shown that the engagement of the IL-2R on CD4+ CD25+ Treg cells fails to activate downstream targets of the PI3K signalling pathway, that is functional in CD4+ CD25-T cells [42]. So, we can assume that Sirolimus, through binding mammalian target of Rapamycin and selectively blocking PI3K-mediated signalling, specifically targets PI3K-sensitive CD4+ CD25- T effector cells while spare CD4+ CD25+ Tregs. A recent study also showed that the IL-2-mediated up-regulation of FoxP3 in purified CD4+ CD25+ T cells involves STAT3/5 proteins binding to a highly conserved STAT binding site located in the first intron of the FoxP3 gene [43]. It is therefore possible that Sirolimus, by sparing the JAK/STAT-IL-2 signalling pathway, consequently maintains/induces high FoxP3 expression.

In this study, we have tried to explain that Sirolimus increase the Treg frequency and decrease Th17 in RIF patient after 17-days administration. Concerning the mechanism of action of Sirolimus in immune regulation in RIF. Several studies have emphasize the further profits of mTOR inhibition on the increase of Tregs in peripheral tissue, thus linking the known profits of mTOR inhibition with upcoming therapies related to dysregulation of Tregs and embryo implantation [14,44–46]. Royster et al. [18] designed an animal model study, and hypothesized that depletion of regulatory T cell (DEREG) with one dose of diphtheria toxin (DT) reduces litter sizes and treatment with Rapamycin will increase Tregs and return litter sizes back to normal levels, results of above study showed that Rapamycin (sirolimus; Pfizer) through the time of embryo implantation will increase Tregs and return litter sizes almost to normal levels. These data provides us with the mechanism by which Sirolimus affects implantation process and subsequent gestation might potentially be the expansion of Treg cells that are known to be key players in the development of maternal tolerance to the fetus. It is explained that Sirolimus can stimulate anergy even in the presence of costimulation [47]. It was initially thought that it was due to the ability of Sirolimus to inhibit proliferation. More studies strongly implicated mTOR in regulating activation versus anergy [48]. Thus, the induction of anergy and regulatory T cells were two additional explanations for the ability of Sirolimus to suppress immune responses.

Furthermore, it is depicted that everolimus, a derivative of Sirolimus that works similarly to Sirolimus, may increase the levels of human leukocyte antigen-G (HLA-G). HLA-G is a protein generally expressed during pregnancy contributing in the maintenance of maternal immune tolerance of the fetus [49].

According to our expectations the side effects reported in this study were mild and did not necessitate discontinuation of treatment. Fortunately, Sirolimus is previously approved by the United States Food and Drug Administration (FDA) (pregnancy category C) and several reports of successful pregnancies have been described in birth registries for mothers exposed to sirolimus or its analogues throughout their pregnancy because they received a solid organ transplant and needed life-long immunosuppression [50,51]. Many of the benefits of Sirolimus are related to mTORC1 inhibition, with many of the side effects related to the lack of inhibition of mTORC2 by Rapamycin [52,53].

Despite promising results of the current study that showed Sirolimus decreased Th17/Treg ratio, the other probable mechanisms by which Sirolimus enables RIF patients with immune cell abnormalities to respond better to IVF treatment have remained to be elucidated. Since We were some limitation in this study further studies are needed to

investigate and validate the clinical value of Th17/Treg ratio as a therapeutic approach to predict ART outcomes in women with RIF. One of the potential limitation of this study was the lack of placebo use for control group patient, also small sample size was another weakness of our study.

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## References

[1] E. Cicinelli, M. Matteo, R. Tinelli, A. Lepera, R. Alfonso, U. Indraccolo, S. Marrocchella, P. Greco, L. Resta, Prevalence of chronic endometritis in repeated unexplained implantation failure and the IVF success rate after antibiotic therapy, Hum. Reprod. 30 (2) (2014) 323–330.
[2] C. Boomsma, A. Kavelaars, M. Eijkemans, E. Lentjes, B. Fauser, C. Heijnen, N. Macklon, Endometrial secretion analysis identifies a cytokine profile predictive of pregnancy in IVF, Hum. Reprod. 24 (6) (2009) 1427–1435.
[3] M.S. Van Mourik, N.S. Macklon, C.J. Heijnen, Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment, J. Leukoc. Biol. 85 (1) (2009) 4–19.
[4] N. Mariee, T. Li, S. 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. 27 (7) (2012) 1946–1954.
[5] P. Luppi, How immune mechanisms are affected by pregnancy, Vaccine 21 (24) (2003) 3352–3357.
[6] J. Shojaeian, S.M. Moazzeni, S. Nikoo, M. Bozorgmehr, M. Nikougoftar, A.H. Zarnani, Immunosuppressive effect of pregnant mouse serum on allostimulatory activity of dendritic cells, J. Reprod. Immunol. 75 (1) (2007) 23–31.
[7] A. Makrigiannakis, G. Petsas, B. Toth, K. Relakis, U. Jeschke, Recent advances in understanding immunology of reproductive failure, J. Reprod. Immunol. 90 (1) (2011) 96–104.
[8] B.K. Law, Rapamycin: an anti-cancer immunosuppressant? Crit. Rev. Oncol. Hematol. 56 (1) (2005) 47–60.
[9] D. Benjamin, M. Colombi, C. Moroni, M.N. Hall, Rapamycin passes the torch: a new generation of mTOR inhibitors, Nat. Rev. Drug Discov. 10 (11) (2011) 868.
[10] M. Attur, R. Patel, G. Thakker, P. Vyas, D. Levartovsky, P. Patel, S. Naqvi, R. Raza, K. Patel, D. Abramson, Differential anti-inflammatory effects of immunosuppressive drugs: cyclosporin, rapamycin and FK-506 on inducible nitric oxide synthase, nitric oxide, cyclooxygenase-2 and PGE 2 production, Inflamm. Res. 49 (1) (2000) 20–26.
[11] L. Coscia, S. Constantinescu, M. Moritz, A.M. Frank, C.B. Ramirez, W.R. Maley, C. Doria, C.H. McGrory, V.T. Armenti, Report From the National Transplantation Pregnancy Registry (NTPR): Outcomes of Pregnancy after Transplantation, (2010).
[12] H. Kopf, M. Gonzalo, O.Z. Howard, X. Chen, Rapamycin inhibits differentiation of Th17 cells and promotes generation of FoxP3+ T regulatory cells, Int. Immunopharmacol. 7 (13) (2007) 1819–1824.
[13] K.W. Kim, B.H. Chung, B.M. Kim, M.L. Cho, C.W. Yang, The effect of mammalian target of rapamycin inhibition on T helper type 17 and regulatory T cell differentiation in vitro and in vivo in kidney transplant recipients, Immunology 144 (1) (2015) 68–78.
[14] C. Procaccini, V. De Rosa, M. Galgani, L. Abanni, G. Calì, A. Porcellini, F. Carbone, S. Fontana, T.L. Horvath, A. La Cava, An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness, Immunity 33 (6) (2010) 929–941.
[15] R.J. Heitmann, R.P. Weitzel, Y. Feng, J.H. Segars, J.F. Tisdale, E.F. Wolff, Maternal T regulatory cell depletion impairs embryo implantation which can be corrected with adoptive T regulatory cell transfer, Reprod. Sci. 24 (7) (2017) 1014–1024.
[16] W.-J. Wang, F.-J. Liu, C.-F. Hao, H.-C. Bao, Q.-L. Qu, X.-M. Liu, Adoptive transfer of pregnancy-induced CD4+ CD25+ regulatory T cells reverses the increase in abortion rate caused by interleukin 17 in the CBA/J× BALB/c mouse model, Hum. Reprod. 29 (5) (2014) 946–952.
[17] C. Scottà, M. Esposito, H. Fazekasova, G. Fanelli, F.C. Edozie, N. Ali, F. Xiao, M. Peakman, B. Afzali, P. Sagoo, Differential effects of rapamycin and retinoic acid on expansion, stability and suppressive qualities of human CD4+ CD25+ FOXP3+ T regulatory cell subpopulations, Haematologica 98 (8) (2013) 1291–1299.
[18] G.D. Royster, J.C. Harris, A. Nelson, Y. Castro, R.P. Weitzel, J. Tisdale, R.J. Heitmann, A.H. DeCherney, E.F. Wolff, Rapamycin corrects T regulatory cell depletion and improves embryo implantation and live birth rates in a murine model, Reprod. Sci. (2019) (1933719119828110). Article first published online: February 19, 2019.
[19] K. Nakagawa, J. Kwak-Kim, K. Kuroda, R. Sugiyama, K. Yamaguchi, Immunosuppressive treatment using tacrolimus promotes pregnancy outcome in infertile women with repeated implantation failures, Am. J. Reprod. Immunol. 78 (3) (2017).
[20] O.W. Griffith, A.R. Chavan, S. Protopapas, J. Maziarz, R. Romero, G.P. Wagner, Embryo implantation evolved from an ancestral inflammatory attachment reaction, Proc. Natl. Acad. Sci. (2017) 201701129.
[21] C. Boomsma, N. Macklon, What can the clinician do to improve implantation? Reprod. BioMed. Online 13 (6) (2006) 845–855.
[22] C. Finn, L. Martin, The control of implantation, J. Reprod. Fertil. 39 (1) (1974) 195–206.
[23] O. Guzeloglu-Kayisli, U.A. Kayisli, H.S. Taylor, The role of growth factors and cytokines during implantation: endocrine and paracrine interactions, Seminars in Reproductive Medicine, © Thieme Medical Publishers, 2009, pp. 062–079.
[24] N. Laufer, A. Simon, Recurrent implantation failure: current update and clinical approach to an ongoing challenge, Fertil. Steril. 97 (5) (2012) 1019–1020.
[25] A. García-Segovia, N.N. del Prado, L. de la Fuente, J. Alonso, S. Sanchez-Ramon, Evidence-based update: immunological evaluation of recurrent implantation failure, Reproductive Immunology: Open Access 1 (4) (2016).
[26] H.-H. Chen, M.-J. Chen, D.-Y. Chen, J.-L. Lan, Decreased peripheral blood CD4+ CD25bright T cells in women with repeated implantation failures after in vitro fertilization and embryo transfer, Formos. J. Rheumatol 23 (2009) 28–36.
[27] Y.S. Liu, L. Wu, X.H. Tong, L.M. Wu, G.P. He, G.X. Zhou, L.H. Luo, H.B. Luan, Study on the relationship between Th17 cells and unexplained recurrent spontaneous abortion, Am. J. Reprod. Immunol. 65 (5) (2011) 503–511.
[28] S. Lee, J. Kim, S. Hur, C. Kim, B. Na, M. Lee, A. Gilman-Sachs, J. Kwak-Kim, An imbalance in interleukin-17-producing T and Foxp3+ regulatory T cells in women with idiopathic recurrent pregnancy loss, Hum. Reprod. 26 (11) (2011) 2964–2971.
[29] S. Abdolrahim, n.d. T Regulatory (Treg)/T Helper 17 Imbalance with Emphasis on Treg Factors during Implantation Window in Unexplained Recurrent Spontaneous Miscarriage.
[30] J. Zhou, Z. Wang, X. Zhao, J. Wang, H. Sun, Y. Hu, An increase of Treg cells in the peripheral blood is associated with a better in vitro fertilization treatment outcome, Am. J. Reprod. Immunol. 68 (2) (2012) 100–106.
[31] S. Rajaei, A.H. Zarnani, M. Jeddi-Tehrani, M. Tavakoli, A. Mohammadzadeh, A. Dabbagh, M. Mirahmadian, Cytokine profile in the endometrium of normal fertile and women with repeated implantation failure, Iranian Journal of Immunology 8(4) (2011) 201.
[32] S. Abdolmohammadi-Vahid, S. Danaii, K. Hamdi, F. Jadidi-Niaragh, M. Ahmadi, M. Yousefi, Novel immunotherapeutic approaches for treatment of infertility, Biomed. Pharmacother. 84 (2016) 1449–1459.
[33] K. Nakagawa, J. Kwak-Kim, K. Ota, K. Kuroda, M. Hisano, R. Sugiyama, K. Yamaguchi, Immunosuppression with tacrolimus improved reproductive outcome of women with repeated implantation failure and elevated peripheral blood TH1/TH2 cell ratios, Am. J. Reprod. Immunol. 73 (4) (2015) 353–361.
[34] M. Ahmadi, S. Abdolmohammadi-Vahid, M. Ghaebi, L. Aghebati-Maleki, A. Afkham, S. Danaii, S. Abdollahi-Fard, L. Heidari, F. Jadidi-Niaragh, V. Younesi, Effect of intravenous immunoglobulin on Th1 and Th2 lymphocytes and improvement of pregnancy outcome in recurrent pregnancy loss (RPL), Biomed. Pharmacother. 92 (2017) 1095–1102.
[35] M. Ahmadi, M. Nouri, Z. Babaloo, L. Farzadi, A. Ghasemzadeh, K. Hamdi, A.A. Movassaghpour, F. Jadidi-Niaragh, A. Afkham, M. Motallebnezhad, Intravenous immunoglobulin (IVIG) treatment modulates peripheral blood Th17 and regulatory T cells in recurrent miscarriage patients: non randomized, open-label clinical trial, Immunol. Lett. 192 (2017) 12–19.
[36] M. Ahmadi, S. Abdolmohammadi-Vahid, M. Ghaebi, L. Aghebati-Maleki, S. Dolati, L. Farzadi, A. Ghasemzadeh, K. Hamdi, V. Younesi, M. Nouri, Regulatory T cells improve pregnancy rate in RIF patients after additional IVIG treatment, Syst Biol Reprod Med 63 (6) (2017) 350–359.
[37] C.J. Sabers, M.M. Martin, G.J. Brunn, J.M. Williams, F.J. Dumont, G. Wiederrecht, R.T. Abraham, Isolation of a protein target of the FKBP12-rapamycin complex in mammalian cells, J. Biol. Chem. 270 (2) (1995) 815–822.
[38] R. Loewith, E. Jacinto, S. Wullschleger, A. Lorberg, J.L. Crespo, D. Bonenfant, W. Oppliger, P. Jenoe, M.N. Hall, Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control, Mol. Cell 10 (3) (2002) 457–468.
[39] D.-H. Kim, D.D. Sarbassov, S.M. Ali, J.E. King, R.R. Latek, H. Erdjument-Bromage, P. Tempst, D.M. Sabatini, mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery, Cell 110 (2) (2002) 163–175.
[40] A. Mehrabi, H. Fonouni, A. Kashfi, B. Schmied, C. Morath, M. Sadeghi, P. Schemmer, J. Encke, P. Sauer, M. Zeier, The role and value of sirolimus administration in kidney and liver transplantation, Clin. Transpl. 20 (s17) (2006) 30–43.
[41] T.R. Malek, A.L. Bayer, Tolerance, not immunity, crucially depends on IL-2, Nat. Rev. Immunol. 4 (9) (2004) 665.
[42] S.J. Bensinger, P.T. Walsh, J. Zhang, M. Carroll, R. Parsons, J.C. Rathmell, C.B. Thompson, M.A. Burchill, M.A. Farrar, L.A. Turka, Distinct IL-2 receptor signaling pattern in CD4+ CD25+ regulatory T cells, J. Immunol. 172 (9) (2004) 5287–5296.
[43] E. Zorn, E.A. Nelson, M. Mohseni, F. Porcheray, H. Kim, D. Litsa, R. Bellucci, E. Raderschall, C. Canning, R.J. Soiffer, IL-2 regulates FOXP3 expression in human CD4+ CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo, Blood 108 (5) (2006) 1571–1579.
[44] G.M. Delgoffe, K.N. Pollizzi, A.T. Waickman, E. Heikamp, D.J. Meyers, M.R. Horton, B. Xiao, P.F. Worley, J.D. Powell, The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2, Nat. Immunol. 12 (4) (2011) 295.
[45] L. Gabryšová, J.R. Christensen, X. Wu, A. Kissenpfennig, B. Malissen, A. O'Garra, Integrated T-cell receptor and costimulatory signals determine TGF-β-dependent differentiation and maintenance of Foxp3+ regulatory T cells, Eur. J. Immunol. 41 (5) (2011) 1242–1248.
[46] S. Haxhinasto, D. Mathis, C. Benoist, The AKT–mTOR axis regulates de novo differentiation of CD4+ Foxp3+ cells, J. Exp. Med. 205 (3) (2008) 565–574.
[47] Y.M. Oh, H.B. Park, J.H. Shin, J.E. Lee, H.Y. Park, D.H. Kho, J.S. Lee, H. Choi,

T. Okuda, K. Kokame, Ndrg1 is a T-cell clonal anergy factor negatively regulated by CD28 costimulation and interleukin-2, Nat. Commun. 6 (2015) 8698.
[48] S. Colombetti, V. Basso, D.L. Mueller, A. Mondino, Prolonged TCR/CD28 engagement drives IL-2-independent T cell clonal expansion through signaling mediated by the mammalian target of rapamycin, J. Immunol. 176 (5) (2006) 2730–2738.
[49] R. Sheshgiri, F. Gustafsson, J. Sheedy, V. Rao, H.J. Ross, D.H. Delgado, Everolimus but not mycophenolate mofetil therapy is associated with soluble HLA-G expression in heart transplant patients, J. Heart Lung Transplant. 28 (11) (2009) 1193–1197.
[50] H. Margoles, V. Gomez-Lobo, J. Veis, M. Sherman, J. Moore Jr, Successful maternal and fetal outcome in a kidney transplant patient with everolimus exposure throughout pregnancy: a case report, Transplant. Proc., Elsevier, 2014, pp. 281–283.
[51] P. Carta, L. Caroti, M. Zanazzi, Pregnancy in a kidney transplant patient treated with everolimus, Am. J. Kidney Dis. 60 (2) (2012) 329.
[52] S.I. Arriola Apelo, J.C. Neuman, E.L. Baar, F.A. Syed, N.E. Cummings, H.K. Brar, C.P. Pumper, M.E. Kimple, D.W. Lamming, Alternative rapamycin treatment regimens mitigate the impact of rapamycin on glucose homeostasis and the immune system, Aging Cell 15 (1) (2016) 28–38.
[53] K. Makki, S. Taront, O. Molendi-Coste, E. Bouchaert, B. Neve, E. Eury, S. Lobbens, M. Labalette, H. Duez, B. Staels, Beneficial metabolic effects of rapamycin are associated with enhanced regulatory cells in diet-induced obese mice, PLoS One 9 (4) (2014) e92684.