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Recurrent miscarriages and the association with regulatory T cells; A systematic review

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ABSTRACT

Regulatory T cells (Tregs) are essential in tolerizing the maternal immune system toward the semi-allogeneic embryo. In this systematic review, we evaluated the association of levels and function of Tregs in peripheral blood and decidua with recurrent miscarriage (RM), defined as two unexplained miscarriages. We included 18 studies. Ten studies showed a significantly decreased level of Tregs in peripheral blood of non-pregnant women with RM, compared to controls ($p < 0.05$). In pregnant women with RM, levels of Tregs in the peripheral blood were significantly lower compared to control groups ($p = 0.0004$), as shown in nine studies. Moreover, seven studies described a decrease of Treg levels in the placenta of pregnant women with RM ($p < 0.0001$) compared to controls. Accordingly, the median of the relative changes (MRC) between cases and controls in the non-pregnant group (peripheral blood), and the two pregnant groups (peripheral blood and decidua) were -0.18 (-0.27-0), -0.26 (−0.35 to −0.17), and -0.52 (0.63–0.31), respectively. In addition to the assessment of Tregs by phenotype, six out of the 18 included studies investigated the functionality of these cells. These studies showed a lower inhibitory effect of Tregs cells on the proliferation of effector T cells of women with RM compared to fertile women. Also, the expression of IL-10 and TGF-beta was diminished. This systematic review shows that Treg levels and their function are significantly decreased in peripheral blood and decidua of pregnant and non-pregnant women with RM. This underlines the hypothesis that Tregs play a role in the pathogenesis of RM.

1. Introduction

Recurrent miscarriages (RM) is a diagnosis that is defined as two or more consecutive miscarriages before 24 weeks of gestation. (Christiansen, 2013) In 1–3 % of the couples trying to conceive, this condition is diagnosed. (Stirrat, 1990) Several potential causal factors are known, including uterine abnormalities, endocrine disorders, acquired thrombophilia as antiphospholipid syndrome, and balanced translocations in the maternal and paternal karyotype. (Ke, 2014) However, in 50–70 % of couples no cause can be established. (Branch et al., 2010)

The embryo is a semi-allograft, which requires modulation of the maternal immune system in order to tolerate the embryo. Indeed, immune suppressing T cells, and in particular, CD4 + CD25^{bright} regulatory T cells (Tregs) (Guerin et al., 2009) are essential in modulating this maternal immune response in pregnancy. (Tilburgs et al., 2008) In 2004, Aluvihare et al. showed that Tregs play a pivotal role in the tolerance of fetal allograft in mice. Adoptive transfer experiments were performed by administering lymphocytes depleted of CD25 + Tregs into pregnant mice, that were T cell-deficient, leading to miscarriage. (Aluvihare et al., 2004) Transfer of anti-CD25 + monoclonal antibodies led to implantation failure in mice. (Shima et al., 2010) Also, the administration of pregnancy-induced CD4 + CD25 + Tregs protected abortion-prone pregnant mice from fetal rejection. (Zenclussen et al., 2005, 2006)

In humans, several studies showed a decrease of the level of Tregs in the decidua and the peripheral blood of women with recurrent miscarriages compared to women with healthy pregnancies. (Mei et al., 2010; Inada et al., 2013) However, these studies considered different markers for phenotypic characterization of Tregs, different control groups, and different definitions of miscarriages.

The aim of this study was to systematic review the literature to

Abbreviations: Tregs, regulatory T cells; RM, recurrent miscarriages; FoxP3, transcription factor forkhead P3; CD, cluster of differentiation; PBMC, peripheral blood mononuclear cells; NS, non-significant

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explore the role of Tregs in women with unexplained RM. A second aim of this study was to set a cut-off value for normal levels of Tregs, which might be used as biomarker to select women with RM and immune etiology for further studies.

2. Method

2.1. Search strategy

The databases of Embase, Medline Ovid, Web of Science, Cochrane CENTRAL and Google Scholar were searched for studies evaluating the level and/or function of Tregs and their association with RM. The search was performed on October 26, 2018, and the complete search protocol is shown in supplementary text A. As a search limit English-language publications and human studies were used.

2.2. Eligibility criteria

Titles and abstracts were screened for eligibility independently by the authors (CK and LL). Studies included in the systematic review had to meet the following criteria:

- the case group included women with two or more unexplained miscarriages.
- the design of the study was a case control study.
- cells were stained for CD4+ and CD25+, in combination with an additional marker, such as FoxP3 (transcription factor forkhead P3), $CD127^{dim}$or $CD25^{bright}$, providing a more specific marker for Tregs.

2.3. Risk of bias assessment

Risk of bias was assessed following the Newcastle-Ottawa scale by two observers (CK and LE) (http://www.ohri.ca/programs/clinical_epidemiology/nosgen.pdf). The Newcastle-Ottawa scale (NOS) refers to three aspects: selection, comparability, and exposure criteria.

Selection bias was taken into consideration if cases were not selected from the same hospital or in a different period of time. Also, if the control group was drawn from another population than the case group, there was a risk of selection bias. Controls must have had a history of healthy pregnancy. An inadequate case definition would be considered as information bias. The clinical examination should have ruled out causes that could explain the miscarriages, such as uterus anomalies, coagulation disorders, and endocrine causes. Confounders factors must have been equally assessed for both groups.

2.4. Data extraction

Data extracted independently by the authors (CK and LL) comprised design of the study, definition of the case and control group, number of case and control subjects, whether the case and control group were pregnant, tissue collected (decidua, peripheral or menstrual blood), time of collection, inclusion and exclusion criteria of the cases and control group, method used for measuring the percentage of Tregs (such as flow cytometry), percentages of Tregs and unit of measurement. In addition, functional analysis of the Tregs, such as the suppressive effect of Tregs on effector T cells or the expression of IL-10 and TGF-beta in Tregs, was evaluated in the included articles.

Authors were contacted if data was missing. In case of multiple articles published by one author, overlap was checked to prevent using the same data.

2.5. Statistical analyses

Analysis was performed by using SPSS Statistics 23 (IBM SPSS Software, New York, USA). To test the difference between the paired values of the percentages of the Tregs in the systematic review, the Wilcoxon signed rank test was used. While the dataset of the studies did not follow a normal distribution, non-parametric methods were used for analyzing this data. The relative difference between case and control group were characterized using the median in three groups (non-pregnant/peripheral blood, pregnant/peripheral blood and pregnant/decidua). The relative difference was calculated by dividing the difference of levels of the case and control group by the level of the control group. A p-value of < 0.05 was considered significant.

3. Results

3.1. Literature search

The main search identified 1162 potentially relevant studies. After removal of 34 duplicates, 1128 articles were screened on title and abstracts, and 55 articles were selected for a full-text read. Reasons for exclusion were different study design (n = 4), animal studies (n = 1), a limited set of markers to define Tregs (no FoxP3, $CD127^{dim}$, or $CD25^{bright}$ as an extra marker) (n = 3), divergent case or control group definition (n = 13), no data available for analysis for the review, even after contacting the authors (n = 14). For two articles a full-text was not available. In total, 18 case control studies were included, as shown in Fig. 1.

3.2. Risk of bias assessment

In supplementary text B the assessment of methodological quality according to the Newcastle Ottawa Scale is summarized. Six studies were rated as high quality, scoring seven out of nine points (Mei et al., 2010; Kwiatek et al., 2015; Yang et al., 2009; Zhang et al., 2015; Zhu et al., 2017; Wang et al., 2010) and four studies six out of nine points. (Abdolmohammadi Vahid et al., 2018; Liu et al., 2010; Quan and Yang, 2017; Wu et al., 2015) Five studies showed average quality with five points. (Arruvito et al., 2007; Hosseini et al., 2016; Lee et al., 2011; Sereshki et al., 2014; Yang et al., 2018) One study was rated with four points (Bao et al., 2011) and two studies with three points (Mahmoud et al., 2008; Sasaki et al., 2004) indicating low quality. None of the studies adjusted in analysis or design for confounding factors. All studies were rated three points for exposure. Four articles showed no clear case definition. (Bao et al., 2011; Mahmoud et al., 2008; Liu et al., 2010; Sasaki et al., 2004). In four studies, definition of controls was not clear. (Mahmoud et al., 2008; Quan and Yang, 2017; Sasaki et al., 2004; Wu et al., 2015)

3.3. Overview of the selected articles and the Treg markers employed

The characteristics of all included studies are summarized in Table 1. The results of the studies were divided into four groups:

1 4 Non-pregnant women with levels of Tregs in peripheral blood.
2 5 Non-pregnant women with levels of Tregs in menstrual blood.
3 6 Pregnant women with levels of Tregs in peripheral blood.
4 7 Pregnant women with levels of Tregs in decidua.

A full overview of the results of the markers per group is shown in Table 2. Markers included are listed in supplementary text C.

In group 1, 14 out of 23 different Treg markers were significantly decreased in the case group compared to controls. In group 2, only one study used one marker to identify Tregs in menstrual blood, which showed no significant difference in non-pregnant women with RM compared to controls. In group 3, 13 different Treg markers were significantly lower than the levels in the control group. Group 4 showed in 15 markers (out of a total of 17 markers) a significantly decreased level.

Different markers were used to identify the proportion of Tregs. Therefore, several subgroups were designed per marker (Table 3 and supplementary text D). The marker for Tregs in menstrual blood was evaluated in only one study, hence this was not included in the

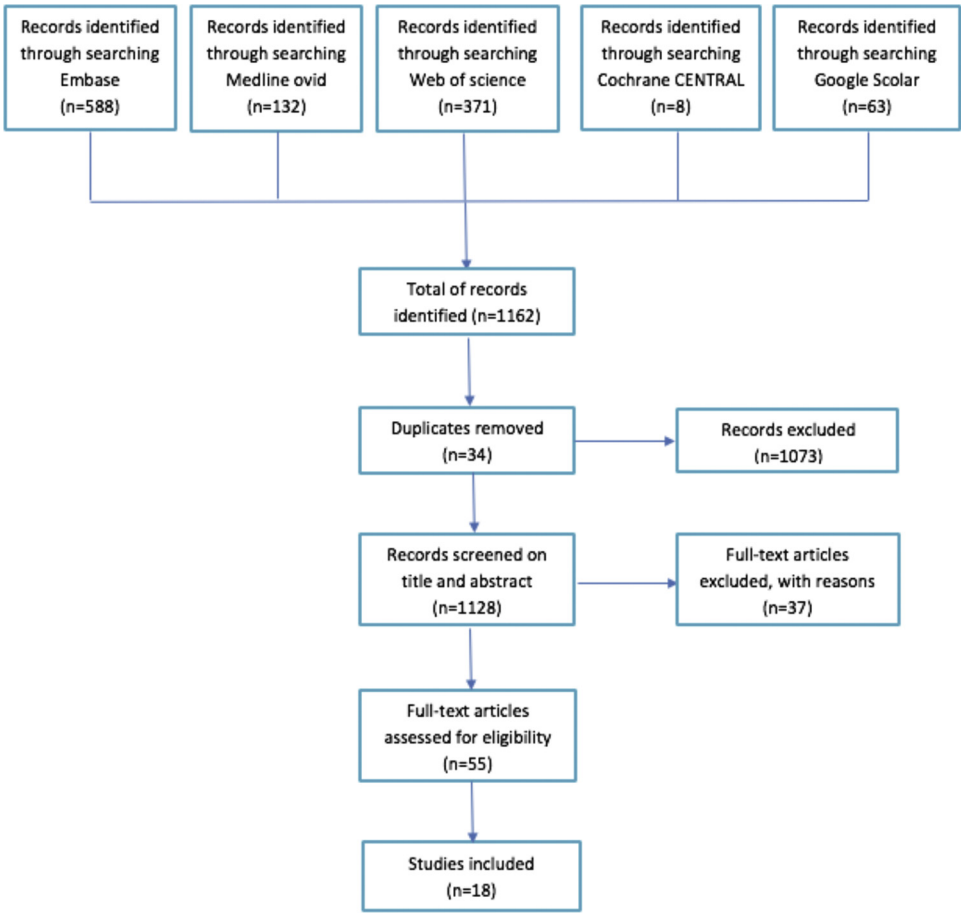

Fig. 1. Flowchart of the selection of the studies of the systematic review.

subgroups.

3.4. Median of the relative changes between cases and controls per subgroup

We calculated the median of the relative changes (MRC) between case and control group in the three groups (group 1, 3, and 4). In the non-pregnant group (peripheral blood) the MRC (IQR) was -0.18 (-0.27-0) (n = 23). In the pregnant group (peripheral blood) MRC (IQR) was -0.26 (−0.35 to −0.17) (n = 18). In the pregnant group (decidua) MRC (IQR) was -0.52 (−0.63 to −0.31) (n = 17). We did not calculate the MRC for the non-pregnant group (menstrual blood) as only one study measured level of Tregs in the menstrual blood.

3.5. Wilcoxon signed rank test

In group 1, 3, and 4 a Wilcoxon signed rank test was used to test an overall difference between levels of the Tregs in case and control group. Indeed, in each groups a significantly decreased level of Tregs between cases and controls was observed: for group 1 ($p = 0.0430$), for group 3 ($p = 0.0004$), and for group 4 ($p < 0.0001$) (Fig. 2).

3.6. Functional analysis of Tregs

In addition to the level of Tregs assessed according to phenotype, several studies examined the functionality of Tregs in women with RM, and fertile women. Arruvito et al. (Arruvito et al., 2007) evaluated the ability of Tregs in peripheral blood to inhibit the proliferation of CD4 + CD25- effector cells, derived from both controls and RM patients, in response to paternal allo-stimulation in a mixed lymphocyte reaction. They showed that higher numbers of CD4 + CD25 + FoxP3 Tregs from women with RM were necessary to accomplish a similar level of suppression compared to such cells from women without a history of RM. Bao et al. (Bao et al., 2011) observed a lower inhibitory effect of CD4 + CD25 + $CD127^{dim/neg}$ Tregs cells on the proliferation of effector T cells in decidua of women with RM ($p < 0.05$). In addition, multiple studies showed that the expression of IL-10 and TGF-beta was diminished in Tregs of women with RM compared to controls in. (Bao et al., 2011; Sereshki et al., 2014; Zhang et al., 2015; Zhu et al., 2017). These cytokines mediate the inhibitory effect of Tregs.

4. Discussion

In this systematic review, our aim was to investigate the current literature and see whether there is an association between the levels of the Tregs in women with unexplained RM compared to pregnant and non-pregnant women. We observed a significant diminished level of Tregs in both pregnant and non-pregnant women, both in peripheral blood and in the decidua. In addition, both the functional effect of Tregs on effector T cells, as well as the expression of IL-10 and TGF-beta of the Tregs, was seen to be diminished.

One earlier study analyzed the levels of Tregs in relation to RM in a meta-analysis. Teshnizi et al. (2019) showed a significant correlation between two single nucleotide polymorphisms of FoxP3 and immune-related pregnancy complications. (Teshnizi et al., 2019) In our study, the focus was exclusively on recurrent miscarriage, whereas Teshnizi also included pregnancy complications such as pre-eclampsia and infertile women with or without endometriosis. Another difference is, that not only FoxP3, but also other markers were included to define the Tregs in this study. Here, CD4 + CD25 + was used to define Tregs, but with an additional marker such as FoxP3, $CD25^{bright}$ or $CD127^{dim}$, in

Table 1
overview of included studies.

	Author (year)	Study	Pregnant case group	Pregnant control group	Tissue collected	Time of collection	Size case group	Characteristics cases group	Size control group	Characteristics control group
1	Vahid et al. (2018)	Case control	No	No	PBMC	Luteal phase of the menstrual cycle	50	Women with a history of two miscarriages before 20th week of gestation. Semen quality test: no abnormalities. Age: 18–41 years. Elevated Th1/Th2 balance, elevated frequency and cytotoxicity of CD3−CD56+ natural killer (NK) cell.	50	Women with a history of successful pregnancy.
2	Arruvito et al. (2007)	Case control	No	No	PBMC	Luteal phase of the menstrual cycle (days 20–24)	75	Women with a history of three or more consecutive miscarriages before week 12 of gestation. Age: 22–45 years. None of the women was taking oral anticonceptives.	60	Women with a history of one successful pregnancy (and no previous abortions) Regular menstrual cycles. Age: 22–42 years. None of the women was taking oral anticonceptives.
3	Bao et al. (2011)	Case control	Yes	Yes	Decidual samples	Not mentioned	21	Women with a history of at least three successive spontaneous early miscarriages of unexplained etiology. The diagnosis of 'unexplained' miscarriage was made in a previous report.	30	Pregnant women who were undergoing elective termination during the first trimester. No significant differences in age and gestational length.
4	Hosseini et al. (2016)	Case control	No	No	PBMC, menstrual blood	Second day of menstrual bleeding. The last abortion was at least three months before participating in the study.	15	Women with a history of at least two consecutive unexplained miscarriages before 20 weeks of gestation. No irregular menstrual cycle or history of hormonal or immunomodulatory (vitamin D3 and prednisolone) therapy during the last 2 months.	15	The women were enrolled from fertile healthy employees in Avicenna Research Institute (ARI) and Avicenna Infertility and Recurrent Abortion Clinic (AIC).
5	Kwiatek et al. (2015)	Case control	Yes	Yes	PBMC	Patients were examined within 24 hours after the start of blood loss. The control group was examined during their first obstetrical evaluation.	33	Women with a history of two or more miscarriages. Women had not been given birth to a healthy child.	20	Women with normally progressing pregnancy.
6	Lee et al. (2011)	Case control	No	No	PBMC	Peripheral blood was sampled between 9 a.m. and 11 a.m. during the early to mid-follicular phase of the menstrual cycle.	42	Women with a history of at least two or more unexplained miscarriages.	24	Healthy women without a history of miscarriages.
7	Liu et al. (2011)	Case control	Not clear whether the case group was pregnant. Probably not.	The control groups consisted of a pregnant and a non-pregnant group.	PBMC	Blood samples were taken from all the participants in a fasting state. Not clear at what moment in the menstrual cycle peripheral blood was taken.	22	Women with a history of at least three consecutive unexplained miscarriages (7–12 weeks of gestation) with the same partner. Age: 25–38. Mean number of miscarriages was 3.88 ± 1.04 (range, 3–7).	17 and 19	17 non-pregnant healthy women, who had at least one living child and had no history of spontaneous abortion, ectopic pregnancy, preterm delivery, or stillbirth. Age: 24–35. 19 normal early pregnant patients; Age: 22–35.
8	Mahmoud et al. (2008)	Case control	No	No	PBMC	Blood was obtained within three to six weeks after the	16	Women with a history of at least three miscarriages.	8	Multiparous healthy women with no history of miscarriages.

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Table 1 (*continued*)

	Author (year)	Study	Pregnant case group	Pregnant control group	Tissue collected	Time of collection	Size case group	Characteristics cases group	Size control group	Characteristics control group
						miscarriage of the patient. Not mentioned in wat time during the menstrual cycle.		Two groups of eight cases were composed, one group (1) with elevated plasma AC antibodies and one group (2) with normal blood levels. Age range group 1: 25–40 years; mean 30.3 ± S.E. 1.6. Age range group 2: 22–42 years; mean 31.4 ± S.E. 2.2.		Age range, 20–43 years; mean 29.5 ± 1.8)
9	Mei et al. (2010)	Case control	A pregnant and non-pregnant case group	A pregnant and non-pregnant control group	PBMC and decidua	Heparinized venous blood and decidual samples during dilation and evacuation procedure. For the non-pregnant women, it is not mentioned when tissue collected.	125	Women with a history of at least three consecutive miscarriages (7–12 weeks of gestation). Group 1 consisted of 107 non-pregnant women. Group 2 consisted of 18 women who had an early miscarriage, confirmed by ultrasound.	35 pregnant women and 28 nonpregnant women	Group 3 consisted of 35 healthy women aged 34.1 +- 3.2 years Mean gestational age: 7.94 +- 1.63 weeks). Group 4 consisted of 28 healthy non-pregnant women aged 33.7 +- 2.3 years who had had at least one live birth.
10	Quan et al. (2017)	Case control	Both	Both.	PBMC and decidua	Not mentioned when blood samples were taken	35 non pregnant RM patients (Group A) and 41 RM patients with early abortion (Group B).	Women with a history of at least two unexplained miscarriages.	30 (pregnant) and 30 (non-pregnant)	30 pregnant patients (group C) and 30 non-pregnant healthy women (Group D)
11	Sasaki et al. (2004)	Cross sectional study	Yes	A pregnant and a non-pregnant group	PBMC and decidua	Not mentioned when the blood samples were taken from the non-pregnant group. Decidual tissue was obtained in the fifth to ninth gestational week.	9	Patients with early spontaneous abortion.	10 (non-pregnant) and 19 (pregnant with induced abortions)	Non-pregnant healthy women and woman in early pregnancy with induced abortions.
12	Sereshki et al. (2014)	Case control study	No	No	PBMC	The proliferative and secretory phases of the menstrual cycle.	25	Women with a history of at least three consecutive miscarriages in the first trimester. Mean age: 29.45 years (range: 21–43 years). The partners had normal semen status, according to criteria from the World Health Organization. The last pregnancy was at least three months before participating in the study.	35	Non-pregnant healthy women. Mean age: 30.5 years (range: 22–42 years) who had at least one successful pregnancy. The last pregnancy was at least three months before participating in the study.
13	Wang et al. (2009)	Case control study	Yes	Yes	PBMC and decidua	Blood and decidua were taken when participants underwent dilation and evacuation.	15	Women with unexplained recurrent miscarriages. The partners had normal semen status, according to criteria from the World Health Organization. Mean days of gestation: 63.1 +- 11.4 days of gestation. Mean number of miscarriages: 4.0 +-1.0. Mean maternal age: 29.2 +- 3.3 years.	15	Healthy pregnant women Mean gestation: 60.2+-9.7 days. Mean age of 31.6 +-2.8 years. At least one successful pregnancy.
14	Wu et al. (2015)	Case control study	Yes	Both	PBMC	Blood samples were taken from the participants during dilation and evacuation.	20	Women with a history of at least three unexplained consecutive miscarriages. Seven to twelve weeks of gestation.	20 induced abortion (IA group) and 20 non-pregnant	Healthy early pregnant women and non-pregnant women.

(*continued on next page*)

Table 1 (*continued*)

	Author (year)	Study	Pregnant case group	Pregnant control group	Tissue collected	Time of collection	Size case group	Characteristics cases group	Size control group	Characteristics control group
									women (control group).	
15	Yang (2008)	Prospective, case–control study	Yes	Yes	PBMC and decidua	Blood and decidua samples were taken from the participants during dilation and evacuation.	25	Women with at least three consecutive unexplained miscarriages. Seven tot twelve weeks of gestation. Age of 30.2 +- 5.60 (range, 23–41) years. Mean number of miscarriages: 4.04+- 1.24 (range, 3–9).	34	Healthy pregnant women. Mean gestation: seven to twelve weeks. The women had at least one successful pregnancy. Mean age: 29.7 +- 4.32 (range, 18–35) years.
16	Yang et al. (2018)	Case control study	No	No	PBMC	Blood samples were taken from both groups during the luteal phase (at days 19–23 of the menstrual cycle, particularly on day 21).	28	Women with a history of at least three consecutive unexplained first trimester miscarriages. Mean Age (± standard deviation): 30.0 ± 4.1 (range: 23–40) years Gestation: seven to twelve weeks Mean number of abortions was 3.9 ± 1.1 (range: 3–7). The last pregnancy was at least three months before participating in the study.	30	Non-pregnant healthy women. Mean age: 30.3 ± 4.8 (range: 22–41) years. The women had at least one successful pregnancy. The last pregnancy was at least three months before participating in the study.
17	Zhang et al. (2015)	Case control	Yes	Yes	PBMC and decidua	At the early stages of pregnancy	20	Women with a history of RM. Mean age: 29.6 ± 2.5 years. Mean of miscarriages: 3.1 ± 0.5 miscarriages. Mean days of gestation: 50.1 ± 9.5.	20	Pregnant women. Mean age: 28.6 ± 3.4 years. All women had at least one successful pregnancy. Mean gestation: 52.8 ± 14.8 days.
18	Zhu et al. (2017)	Case control	Yes	Yes	PBMC	Blood samples were taken before operation.	25	Women with a history of at least three consecutive miscarriages in the first trimester. Only normal embryo karyotypes were included. Mean age: 29.08 +- 2.90 years (range: 25–35 years).	25	Healthy pregnant women. All the women had at least one successful pregnancy and no previous abortions.

All studies analyzed the cells by flow cytometry.

Table 2
Treg levels in the non-pregnant group (peripheral blood and menstrual blood) and the pregnant group (peripheral blood and decidua).

Author	Treg marker	Case group	N	Control group	N	Value	P value
Not pregnant	Peripheral blood						
Vahid et al.	Tregs (cell frequency in PBMC), anti CD4, anti CD25, anti CD127	3.37 +-0.223	50	4.26 +-0.252	50	Mean+-SEM	0.009
Arruvito et al.	CD4+CD25+ (% of CD4 + T lymphocytes)	15.24+-0.36 %	75	17.62+-0.47 %	60	Mean+-SEM	0.0001
	CD4+CD25high (% of CD4 + T lymphocytes)	0.77+-0.06 %		0.99+-0.05 %		Mean+-SEM	0.007
	FoxP3+ (% of CD4 + T lymphocytes)	3.16 +-0.39 %		4.36+-0.42 %		Mean+-SEM	0.046
Hosseini et al.	CD4+CD25+FoxP3+ (Peripheral blood) (% in CD4+ cells)	%0.2 (0.8–0.1)	15	%0.2 (1.6–0.1)	15	Mean+-SD	NS
Lee et al.	FoxP3+ lymphocytes (% of lymphocytes)	2.0 +-0.6	42	2.5 +-0.8	24	Mean+-SD	0.035
	% of FoxP3 low (% of lymphocytes)	1.8+-0.6		2.2+-0.8		Mean+-SD	0.032
	% CD4+FoxP3+ cells (% of lymphocytes)	1.7+-0.5		2.1+-0.8		Mean+-SD	0.037
	%FoxP3high (% of lymphocytes)	0.2+-0.1		0.2+-0.1		Mean+-SD	NS
	%CD4-FoxP3+Treg cells (% of lymphocytes)	0.3+-0.2		0.3+-0.1		Mean+-SD	NS
Liu et al.	CD4+ CD25brightFoxP3+/CD4 + T cells	4.08 ± 0.29 %	22	4.79 ± 0.19 %	17	Mean+-SD	P < 0.05
Mahmoud et al.	CD4+CD25+ frequencies (% of CD4+)	11.3	16	6.0	8	Median	p = 0.003
	CD4+CD25+high (%CD4+)	0.53		2.3		Median	0.005
	CD4+CD25+low (%CD4+)	10.5		3.5		Median	0.003
Mei et al.	Proportions of CD4+CD25high lymphocytes in PBL (%)	0.68 +-0.26	107	1.05+-0.26	28	Mean+-SD	*P < .01*
	CD4+CD25low in PBL	6.41 +- 1.54		6.23 +- 1.45		Mean+-SD	*NS*
	CD4+CD25high CD4+ in PBL	2.35 +- 0.73		3.27 +-0.84		Mean+-SD	P < 0.01
Quan et al.	CD4+CD25+Treg/CD4+	4.77 ± 1.22	35	5.33 ± 1.46	30	Mean+-SD	NS.
	FoxP3+/CD4+CD25+Treg percentage	19.85 ± 8.63		20.34 ± 7.84		Mean+-SD	NS.
Sereshki et al.	CD4+FoxP3+ (% of CD4+ lymphocytes)	0.9 ± 1.1* (proliferative phase/day 1–14, no menses)	25	0.07 (0.821)	35	Mean+-SD	0.010
	CD4+FoxP3+ (% of CD4+ lymphocytes)	1.3 ± 1.2 (secretory phase/day14–28)		1.4 ± 1.5		Mean+-SD	0.410
	CD4+FoxP3+ (% of CD4+ lymphocytes)	1.1 ± 1.1 (without considering phase)		1.9 ± 1.7		Mean+-SD	0.030
Yang et al., 2018	CD3+CD8−CD25+ FoxP3+ Treg cells	4.32 ± 1.52 %	28	6.23 ± 1.41 %	30	Mean+-SD	P < 0.01
Not pregnant	Menstrual blood						
Hosseini et al.	CD4+CD25+FoxP3+ (% in CD4+ cells)	%0.5 (2.1-0.2)	15	%0.3 (2.3-0.1)	15	Mean+-SD	NS

Author	Treg marker	Case group	N	Control group	N	Value	P value
Pregnant	Peripheral blood						
Kwiatek et al.	CD4+CD25+FoxP3+regulatory T cells (in CD4+ cells)	1.33 ± 0.76	33	1.80 ± 0.51	20	Mean+-SD	p = 0.003
Mei et al.	CD4+CD25high lymphocytes	1.82 +- 0.43	18	2.84 +-1.17	35	Mean+-SD	P < .01
	CD4+CD25low	9.81 +- 2.34		10.46 +- 1.57		Mean+-SD	NS
	CD4+CD25high CD4+	5.61 +- 1.94		7.82 +- 2.35		Mean+-SD	P < .01
Quan et al.	CD4+CD25+Treg/CD4+	4.86 ± 1.32	41	5.62 ± 1.54	30	Mean+-SD	NS
	FoxP3+/CD4+CD25+Treg percentage	21.52 ± 8.52		25.69 ± 7.95		Mean+-SD	NS
Sasaki et al.	CD4+CD25bright/ CD4+ (%)	5.66+-1.58	9	8.51+-2.48	19	Mean+-SD	P < 0.001
	CD4+CD25bright/ lymphocytes	2.39+-0.65		3.26+-0.95		Mean+-SD	P < 0.05
Wang et al.	proportion of CD4+CD25+CD127low/- T cell of the lymphocyte population	2.8 % (IQR 1.8–4.0)	15	3.8% (IQR 2.6–4.7)	15	Median	P < 0.05
	proportion of CD4+CD25+CD127low/- T cell in CD4+	7.3 % (IQR 6.3–9.1)		9.8 % (IQR 8.6–12.2)		Median	P < 0.01
Wu et al.	The percentage of CD4+CD25+FoxP3+ Tregs	0.77 ± 0.31	20	1.00 ± 0.35	20	Mean+-SD	P < 0.05
	CD4+CD25+FoxP3+/CD4+	0.029 ± 0.012		0.044 ± 0.020		Mean+-SD	P < 0.05
Yang et al 2008	CD4+CD25bright (%)	1.55 +- 0.77	25	2.65 +- 1.10	34	Mean+-SD	P < .05.
	CD4+CD25dim (%)	10.70 +- 1.86		9.43 +- 1.34		Mean+-SD	NS
	CD4+CD25bright/CD4+ (%)	4.64 +- 2.07		5.59 +- 2.62		Mean+-SD	NS
Zhang et al.	Proportions of CD4+FoxP3+ T cells	1.76 ± 0.93 %	20	13.4 ± 2.6 %	20	Mean+-SD	P < 0.001
	Proportions CCR6+CD4+FoxP3+ T cells	1.01 ± 0.9 %,		14.6 ± 1.6 %		Mean+-SD	P < 0.001
Zhu et al.	Proportion of CD4+CD25+FoxP3+ Treg cells among CD4 + T cells	5.64 % +- 1.15 %	25	7.18% +- 1.49 %	25	Mean+-SD	P = .0002
Pregnant	Decidua						
Bao et al.	CD4+CD25+FoxP3+/lymphocytes (%)	1.36+-0.66	21	2.02+-0.83	30	Not mentioned	0.003
	CD4+CD25+CD127dim/- /lymphocytes %	2.09 +-0.86		2.97+-1.19		Not mentioned	0.005
	FoxP3+ /CD4+CD25+CD127 dim- (%)	61.11+-8.02		72.63+-12.78		Not mentioned	0.02
Mei et al.	CD4+CD25high lymphocytes	0.57 +- 0.21	18	1.36 +- 0.29	35	Mean+-SD	P < .01
	CD4+CD25low	3.92 +- 1.26		3.51 +- 1.38		Mean+-SD	NS
	CD4+CD25high CD4+	6.26 +- 2.43		16.73 +- 5.71		Mean+-SD	P < .01
Quan et al.	CD4+CD25+Treg/CD4+	12.67 ± 6.49	41	24.52 ± 12.32	30	Mean+-SD	P < 0.05
	FoxP3+/CD4+CD25+Treg percentage	52.95 ± 25.85		89.69 ± 35.57		Mean+-SD	p < 0.05
Sasaki et al.	CD4+CD25bright/ CD4+ (%)	7.14+-1.85	9	21.84+-2.92	19	Mean+-SD	P < 0.0001
	CD4+CD25bright/ lymphocytes	0.35+-0.09		1.31+-0.17		Mean+-SD	P < 0.0001
Wang et al.	Proportion of CD4+CD25+CD127low/- T cell of the lymphocyte population	0.7 % (IQR 0.5–1.0)	15	1.7% (IQR 1.1–2.3)	15	Median	P < 0.05
	Proportion of CD4+CD25+CD127low/- T cell in CD4 + T cells	9.0 % (IQR 8.4–11.1)		24.0 % (IQR 21.4–25.9)		Median	P < 0.01
Yang et al 2008	CD4+CD25bright (%)	0.59 +- 0.23	25	1.24 +- 0.55	34	Mean+-SD	P < .01
	CD4+CD25dim (%)	4.23 +- 1.52		3.75 +- 1.88		Mean+-SD	NS
	CD4+CD25bright/CD4+ (%)	5.16 +- 2.83		13.10 +- 10.25		Mean+-SD	P < .01.
Zhang et al.	Proportions of CD4+FoxP3+ T cells /CD4+	3.4 ± 0.4 %,	20	6.2 ± 0.6	20	Mean+-SD	P = 0.0016
	CCR6+CD4+FoxP3+ T cells	6.8 ± 0.6 %		24.2 ± 1.0		Mean+-SD	P < 0.001

Table 3
Non-pregnant and pregnant subgroups per marker in peripheral blood and decidua.

Author	Treg marker	Case group	N	Control group	N	P value
Non-pregnant peripheral blood						
CD4 + CD25high (% of CD4 + T cells)						
Arruvito et al.	CD4 + CD25high (% of CD4 + T lymphocytes	0.77 + -0.06 %	75	0.99 + -0.05 %	60	0.007
Mei et al.	CD4 + CD25high CD4 +	2.35 + - 0.73	107	3.27 + -0.84	28	P < 0.01
Non-pregnant peripheral blood						
CD4 + CD25 + FoxP3 (% of CD4 + T cells)						
Hosseini et al.	CD4 + CD25 + FoxP3 + (% in CD4 + cells)	%0.2 (0.8–0.1)	15	%0.2 (1.6–0.1)	15	NS
Yang et al., 2018	CD3 + CD8 − CD25 + FoxP3 + Treg cells	4.32 ± 1.52 %	28	6.23 ± 1.41 %	30	P < 0.01
Liu et al.	CD4 + CD25brightFoxP3 + /CD4 + T cells	4.08 ± 0.29 %	22	4.79 ± 0.19 %	17	P < 0.05
Non-pregnant peripheral blood						
FoxP3 (% in CD4 + cells)						
Arruvito et al.	FoxP3 + (% of CD4 + T lymphocytes)	3.16 + -0.39 %	75	4.36 + -0.42 %	60	0.046
Sereshki et al.	CD4 + FoxP3 + (% of CD4 + lymphocytes)	1.3 ± 1.2 (secretory phase/day 14–28)	25	1.4 ± 1.5	35	p = 0.410

Author	Treg marker	Case group	N	Control group	N	P value
Pregnant peripheral blood						
CD4 + CD25 + FoxP3 + /CD4 +						
Kwiatek et al.	CD4 + CD25 + FoxP3 + regulatory T cells (in CD4 + cells)	1.33 ± 0.76	33	1.80 ± 0.51	20	p = 0.003
Wu et al.	CD4 + CD25 + FoxP3 + /CD4 +	0.029 ± 0.012	20	0.044 ± 0.020	20	P < 0.05
Zhu et al.	Proportion of CD4 + CD25 + FoxP3 + among CD4 + T cells	5.64 % + - 1.15 %	25	7.18% + - 1.49 %	25	P = .0002
Pregnant peripheral blood CD4 + CD25high/CD4 +						
Mei et al.	CD4 + CD25high CD4 +	5.61 + - 1.94	18	7.82 + - 2.35	35	*P < .01*
Sasaki et al.	CD4 + CD25bright/ CD4 + (%)	5.66 + - 1.58	9	8.51 + - 2.48	19	P < 0.001
Yang et al 2008	CD4 + CD25bright/CD4 + (%)	4.64 + - 2.07	25	5.59 + - 2.62	34	NS
Pregnant decidua						
CD4 + CD25high % in CD4 cells						
Mei et al.	CD4 + CD25high CD4 +	6.26 + - 2.43	18	16.73 + - 5.71	*35*	*P < .01*
Sasaki et al.	CD4 + CD25bright/ CD4 + (%)	7.14 + -1.85	9	21.84 + -2.92	19	P < 0.0001
Yang et al 2008	CD4 + CD25bright/CD4 + (%)	5.16 + - 2.83	25	13.10 + - 10.25	34	P < .01
Pregnant decidua						
FoxP3 + /CD4 + CD25 +						
Bao et al.	FoxP3 + /CD4 + CD25 + CD127 dim- (%)	61.11 + -8.02	21	72.63 + -12.78	30	0.02
Quan et al.	FoxP3 + /CD4 + CD25 + Treg percentage	52.95 ± 25.85	41	89.69 ± 35.57	30	p < 0.05

order to define Tregs more specifically and enlarge the probability that these cells actually function as Tregs. The expression of CD127 is inversely correlated with FoxP3 expression and the suppressive function of human Tregs. (Liu et al., 2006) In addition, in our study we incorporated previous analyses on the function of Tregs. Regulatory CD4 + CD25 + $CD127^{dim}$ cells produce IL-10 and TGF-beta, which are cytokines that control harmful immune responses against the embryo. (Jutel et al., 2003)

We previously have shown a diminished suppressive capacity of Tregs of women who suffered from repeated implantation failure compared to fertile women (Lashley et al., 2015). The current review study showed an association of the impaired functional response of Tregs with a higher allo-immune response of peripheral mononuclear blood cells (PBMC). This emphasizes that an inhibited function of Tregs might play a role during conception and implantation. Though it is not clear whether the lower levels and impaired function of the Tregs are a cause or a consequence of the miscarriage or implantation failure.

To further explore the causality of Tregs, a cohort study could be conducted that includes women with a known cause for the RM (such as uterine abnormality) as a control group and women with idiopathic RM. If Tregs are shown to be diminished in this control group, then these lower Tregs may rather represent a consequence of the miscarriage.

In our review, the quality of the included studies varied, which is summarized in Supplementary text B. It is remarkable that none of the studies corrected for confounding factors. Apart from the methodological quality other factors could be taken into consideration that influenced the heterogeneity between the studies. For example, the moment of obtaining blood and decidua was not always indicated. This could explain why the levels of the Tregs differed greatly between the studies. It is known that levels of Tregs fluctuate during the menstrual cycle and during pregnancy. (Arruvito et al., 2007; van Mourik et al., 2009) Also the gating strategy by which percentages of the specific markers were determined by flow cytometry differed in the included studies. Most markers were set as a percentage of CD4 + cells, but some markers were established in the lymphocyte gate, which makes it difficult to compare certain outcomes. The levels of the Tregs were significantly lower in RM groups than in control groups. However, in some studies with particular markers, the levels of the Tregs were surprisingly higher in the control group than in the case groups. It is remarkable that this was only the case when CD4 + $CD25^{low}$ was studied as marker. Finally, most included studies were performed in Asian cohorts and this could raise questions on the external validity of the results. As some studies showed immunological differences between ethnic groups (Happe et al., 2019; Gillespie et al., 2016; Paulucci et al., 2017), other populations might differ immunologically from Asian populations.

Our second aim was to set a cut-off value for Tregs, to use as a biomarker that might be implemented for future studies. In the absence of raw data, it was not possible to establish a cut-off value. In most studies, means were used to describe the levels of the Tregs. However, no individual data of patients could be extracted from the articles. Kwiatek et al. (2015) was able to set a cut-off value based on their own individual data. The marker used was the percentage of CD4 + CD25 + FoxP3 + regulatory T cells in CD4 + cells. The cut-off value was 1.50. According to that study, the level of Tregs was the best predictive marker, with a sensitivity of 70 % and a specificity of 75 %. (Kwiatek et al., 2015) It was not possible to apply this cut-off point in our systematic review, due to varying values of the Treg markers. To set a cut-off value an Individual Patient Data (IPD) review is recommended. In contrast to conventional meta-analysis, IPD meta-analysis uses the IPD of the original studies and permits synthesis at an individual level, which enables the calculation of a cut-off value. Though the values of Tregs among different cohorts may still vary, data

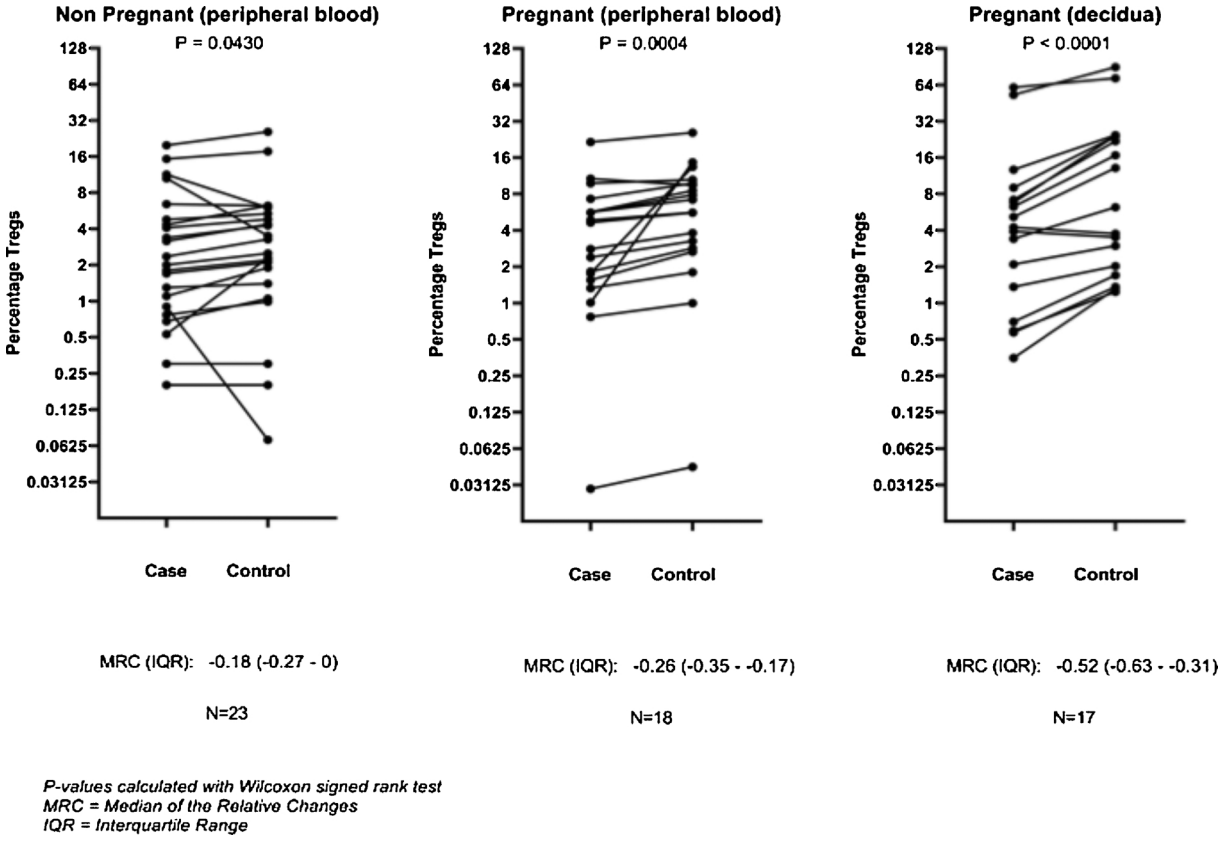

Fig. 2. Wilcoxon signed rank test and median of the relative changes (MRC) of percentages of Tregs between cases and controls per subgroup of women. Most values of Tregs were set as a percentage of CD4+ cells, but some markers were established in the lymphocyte gate. For a specific overview of the gates used per study, we refer to Table 2. MRC (IQR) between case and control group in the three groups was in the non-pregnant group (peripheral blood) -0.18 (-0.27-0) (n = 23). In the pregnant group (peripheral blood) MRC (IQR) was -0.26 (−0.35 to −0.17) (n = 18). In the pregnant group (decidua) MRC (IQR) was -0.52 (−0.63 to −0.31) (n = 17). In each group a significantly decreased level of Tregs between cases and controls was observed: for group 1, the non-pregnant group (peripheral blood) (p = 0.0430), for group 2, the pregnant group (peripheral blood) (p = 0.0004), and for group 3, the pregnant group (decidua) (p < 0.0001).

must be extracted from cohorts that have values of Tregs in the same range, for setting a cut-off value. This cut-off value could be used in prospective cohort studies, to include certain women suffering from RM based on the level of their Tregs in a case group, for testing a therapeutic intervention.

5. Conclusion

In this review study, the levels and function of the Tregs of women with RM were diminished. This effect was most pronounced for Tregs analyzed in the placenta. Tregs might serve as new targets for intervention studies, such as Tregs suppletion. (Craenmehr et al., 2016) Several promising trials have been performed to use Tregs for treating graft-versus-host disease in patients with stem cell transplant. (Tang et al., 2012) The implementation of Tregs in human pregnancy complications however has not yet been applied. For this, more studies are needed.

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Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jri.2020.103105.

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