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Relationship between leukocytospermia, reproductive potential after assisted reproductive technology, and sperm parameters: a systematic review and meta-analysis of case–control studies

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ABSTRACT

Background: The association of leukocytospermia with male fertility is still under debate.

Objective: To evaluate the impact of leukocytospermia (≥1 × 10^6 white blood cells/mL of semen, according to the World Health Organization) in men attending a fertility clinic for couple subfertility, on fertility outcomes after assisted reproductive technology (ART) and on semen quality.

Materials and Methods: A systematic review with meta-analysis of case–control studies reporting mean ± standard deviation for values of different seminal parameters (sperm concentration, progressive motility, sperm morphology, sperm DNA fragmentation, semen volume, and Ph) and fertilization rate (FR), or the odds ratio (OR) for clinical pregnancy rate (PR) per cycle after ART in leukocytospermic and non-leukocytospermic patients was performed. A literature search was carried out in MEDLINE and SCOPUS for English-language studies published till June 2018.

Results: Twenty-eight case-controlled retrospective studies met the inclusion criteria, comparing fertility outcomes after ART or semen parameters in men with or without leukocytospermia. FR and PR after ART were not significantly different in the two groups. Leukocytospermic samples showed a lower sperm concentration (pooled SMD = −0.14; 95% CI: −0.28, −0.01, I^2 = 71%, $p_{\text{for heterogeneity}} < 0.00001$) and a lower progressive motility (pooled SMD = −0.18; 95% CI: −0.29, −0.06; I^2 = 59%, $p_{\text{for heterogeneity}} < 0.0001$). However, the significant differences disappeared, along with the large inter-study heterogeneity, when analyses were restricted to studies clearly reporting the inclusion of men without clinical evidence of seminal tract infection.

Discussion and Conclusion: Leukocytospermia in men seeking consultation for couple subfertility is not associated with a reduced fertility after ART and with altered semen quality in populations asymptomatic for genital tract infection. Therefore, the current clinical criteria for definition of leukocytospermia should be re-assessed in subfertile couples attending a fertility clinic.

INTRODUCTION

Leukocytospermia is defined by the World Health Organization (WHO) as the presence of white blood cells (WBCs) in the ejaculate at the concentration of ≥1 × 10^6/mL (WHO, 1992, 2010). Although it is considered a possible marker of seminal tract infection (Comhaire *et al.*, 1980; WHO, 1992), a high concentration of semen leukocytes is not predictive for actual microbial infection (Trum *et al.*, 1998). The prevalence of leukocytospermia in men attending fertility clinic ranges from 2% to 40%, depending on the study populations, detection methods, and threshold values used (Keck *et al.*, 1998). Leukocytes are present throughout the human male reproductive tract and are found in the ejaculate of almost every male (El-Demiry *et al.*, 1987). Most leukocytes are suggested to originate from the testis

or the epididymis (Anderson *et al.*, 1991) and are thought to play a key role in both immunosurveillance (Pudney & Anderson, 1993; Kiessling *et al.*, 1995) and phagocytic clearance of abnormal spermatozoa (Tomlinson *et al.*, 1992). Granulocytes are the predominant leukocyte in the semen (50% to 60%), followed by macrophages (20–30%) and T lymphocytes (2–5%) (Aitken *et al.*, 1995). Activated WBCs secrete reactive oxygen species (ROS), proteases, and cytokines, which may result in sperm damage via lipid peroxidation and DNA fragmentation (Wolff, 1995; Ochsendorf, 1999; Whittington & Ford, 1999; Henkel *et al.*, 2005; Agarwal *et al.*, 2014). Therefore, an association between leukocytospermia and altered semen quality and/or subfertility has long been hypothesized. However, the relationship between high seminal leukocyte count and semen quality is ambiguous at present (reviewed by Keck *et al.*, 1998), as is the association between seminal leukocytes and fertility. The concentration of seminal leukocytes was not associated with the conception rate *in viv*o (Tomlinson *et al.*, 1993), or with *in vitro* fertilization (IVF) (Tomlinson *et al.*, 1992; De Geyter *et al.*, 1994; Seshadri *et al.*, 2012; Ricci *et al.*, 2015) or with intracytoplasmic sperm injection (ICSI) outcome (Ricci *et al.*, 2015). Nevertheless, a negative association with IVF results was reported in some studies (Talbert *et al.*, 1987; Sukcharoen *et al.*, 1995; Moilanen *et al.*, 1998). Conflicting results may arise from the different methods used to assess the leukocyte count. The WHO-recommended peroxidase method (WHO, 1992, 2010) only detects the granulocytes, the number of which may be underestimated as some of them are in an activated state in the semen and therefore might have already released their peroxidase-positive granules (Aitken & West, 1990). Monoclonal antibody-mediated targeting of the surface antigens of WBC subpopulations, such as the differentiation cluster (CD)-45, is the gold standard to detect all semen leukocytes, although its use is limited by the high cost (Wolff, 1998; Ricci *et al.*, 2000).

We conducted a meta-analysis of the available case–control studies to determine: (i) whether leukocytospermia is associated with a reduced fertilization rate (FR) and a reduced odd for clinical pregnancy after assisted reproductive technology (ART) and (ii) whether, and to what extent, the presence of leukocytospermia negatively affects the semen quality in men attending fertility clinics.

MATERIAL AND METHODS

The study was conducted according to the Cochrane Collaboration and PRISMA statement (Moher *et al.*, 2009). The PRISMA Checklist has been presented as Table S1. The study protocol has been registered in the 'PROSPERO international prospective register of systematic reviews' at https://www.crd.york.ac.uk/PROSPERO/, with the registration number CRD42018099599.

Systematic search strategy

We conducted a systematic search in the MEDLINE and SCOPUS databases to identify all relevant studies published till June 2018 in English language with the terms: (leukocytospermia* OR leukocytes OR macrophages OR "white blood cells" OR WBC*) AND (semen OR seminal OR sperm* OR infertility OR insemination OR ART OR pregnancy OR fertilization). In case the title and abstract were not informative, the full paper was retrieved. The references cited in all full-text articles were also searched manually to identify additional studies.

Inclusion and exclusion criteria

The outcome was the correlation of leukocytospermia with FR or occurrence of pregnancies after ART and with semen quality. The eligibility criteria for the selected studies were as follows: (i) observational case–control studies conducted on subjects aged 18 years or older with leukocytospermia ($\geq 1 \times 10^6$ WBC/mL) and comparable control groups without leukocytospermia ($<1 \times 10^6$ WBC/mL); (ii) availability of FR and/or number of clinical pregnancies per cycle achieved after ART and/or mean values of semen parameters in both groups. Commentaries/letters to the editor, reviews, and *in vitro* studies, as well as studies with missing or unsuitable data, wrong design, and wrong populations (e.g., patients symptomatic for genital tract infections), were excluded. When the same population sample was used for multiple publications, the study with the largest number of cases was included. Two independent reviewers (AB and CC) evaluated the full text of all selected studies to determine eligibility, and any disagreements were resolved by a third reviewer (SF).

Data extraction

The data on sperm parameters were extracted from the total number of cases (men with leukocytospermia) and controls (age-matched non-leukocytospermic men). The mean ± standard deviation (SD) of FR and the number of clinical pregnancies per cycle after ART in cases and controls were extracted from the studies evaluating FR and/or pregnancy rate (PR). In all studies assessing FR, we considered the presence of two pronuclei (2PN) in the zygote as the fertilization criterion. When the summary statistics (mean ± SD) were not fully reported, they were calculated whenever possible (Bland, 2015). When, in the original papers, leukocytospermic and non-leukocytospermic samples were divided into subgroups according to leukocyte concentration, means and SD were regrouped using the formulas $(n_1 * M_1 + n_2 * M_2 + n_n * M_n)/(n_1 + n_2 + n_n)$ and $\sqrt{[2]\{[(n_1 - 1) * \mathrm{SD}_1^2] + [(n_2 - 1) * \mathrm{SD}_2^2] + [(n_1 - 1) * \mathrm{SD}_n^2]\}/(n_1 + n_2 + n_n - 2)}$, respectively (where n is the sample size, M the mean, and SD the standard deviation). When, in the original paper, leukocytospermic patients were divided into subgroups according to semen culture results, only data from leukocytospermic group with negative semen culture were extracted. When data were missing or inconsistent, the authors were contacted to obtain the necessary information.

Quality assessment

The quality of studies was assessed through the 'star system' of the Newcastle–Ottawa Quality Assessment Scale (NOS) (Deeks *et al.*, 2003) and scored on a scale ranged from 0 to 9. Studies scoring ≥7 were considered high quality. The quality assessment was performed by two reviewers (AB and SF), and any disagreements were resolved by a re-evaluation of the original study with an open discussion.

Statistical analysis

The relationship between leukocytospermia and occurrence of pregnancies after ART was assessed using odds ratio (OR) with a 95% coefficient interval (CI) and Mantel–Haenszel estimates. The relationship of leukocytospermia with FR and with different semen parameters [sperm concentration, total sperm count (TSC), sperm progressive motility (%), normal sperm morphology

(%), sperm viability (%), percentage of spermatozoa with DNA damage] was assessed by calculating the standardized mean difference (SMD). In the presence of significant heterogeneity, data were combined using random effect models which assumed that the included studies have varying effect sizes, thus providing a more conservative estimate of the overall effect compared to the fixed effect model.

The Cochrane chi-square (Cochrane Q) and the I^2 tests were used to quantify the statistical heterogeneity between the results of different studies: I^2 values > 50% and/or p values < 0.05 indicated substantial heterogeneity. The forest plot assessing the relationship between leukocytospermia and occurrence of pregnancies was subjected to a sensitivity analysis by sequential omission of individual studies to determine the contribution of each study to the pooled estimate and evaluate the stability of the results (Barbonetti *et al.*, 2019b). Subgroup analyses were conducted to investigate the causes of the inter-study heterogeneity. Publication bias was graphically determined using funnel plots: a symmetric inverted funnel shape arises from a 'well-behaved' data set, in which case the publication bias is unlikely (Sterne & Egger, 2001).

Funnel plots were also subjected to the Duval and Tweedie's 'trim-and-fill' analysis which, in the presence of asymmetric shape, detects putative missing studies to re-balance the funnel distribution; this analysis also provides an adjusted pooled estimate taking into account these additional studies, thus correcting the analysis for publication bias (Borenstein *et al.*, 2009; Weinhndl & Duval, 2012; Barbonetti *et al.*, 2019a; Barbonetti *et al.*, 2019b). The extracted data were analyzed using the R statistical software (version 3.0.3; R Foundation for Statistical Computing, Vienna, Austria) and the Review Manager (RevMan) of the Cochrane Library (version 5.3. Copenhagen: The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark).

RESULTS

Study selection

The initial database search yielded 6543 studies, of which 5414 were shortlisted after removing the duplicate articles. Another 5215 were excluded based on titles and abstracts, and out of the remaining 199 studies (Figure S1), the following 28 met the inclusion criteria: Wolff *et al.*, 1990; Gonzales *et al.*, 1992; Kortebani *et al.*, 1992; Chan *et al.*, 1994; Kiessling *et al.*, 1995; Yanushpolsky *et al.*, 1995; Omu *et al.*, 1999; Kaleli *et al.*, 2000; Ricci *et al.*, 2000; Sharma *et al.*, 2001; Aziz *et al.*, 2004; Yilmaz *et al.*, 2005; Bezold *et al.*, 2007; Lackner *et al.*, 2008; Lackner *et al.*, 2008; Ziyyat *et al.*, 2008; Fariello *et al.*, 2009; Lackner *et al.*, 2010; Barraud-Lange *et al.*, 2011; Vidya *et al.*, 2011; Cavagna *et al.*, 2012; Aghazarian *et al.*, 2013; Agarwal *et al.*, 2014; Flint *et al.*, 2014; Moretti *et al.*, 2014; Ricci *et al.*, 2015; Barbonetti *et al.*, 2017; and Micillo *et al.*, 2016. Characteristics of the selected articles are summarized in Table 1.

Quality of the included studies

The NOS scores of the studies are reported in Table 2. Eleven articles were considered of high quality scoring ≥ 7 (Gonzales

Table 1 Main characteristics of the selected studies

Study	Geographic region	Reported absence of genital tract infection symptoms	Leukocyte detection method	Mean abstinence days	Mean age (years)	Mean leukocyte count in leuko ($\times 10^6$/mL)	Mean leukocyte count in non-leuko ($\times 10^6$/mL)
Agarwal *et al.* (2014)	Saudi Arabia	NA	Peroxidase	3.4	39.05	3.78	0.0
Aghazarian *et al.* (2013)	Austria	Asymptomatic	Peroxidase	NA	36.2	1.95	0.03
Aziz *et al.* (2004)	USA	NA	Peroxidase	NA	NA	3.80	0.1
Barbonetti *et al.* (2017)	Italy	NA	CD45	NA	NA	4.20	0.0
Barraud-Lange *et al.* (2011)	France	NA	Peroxidase	NA	36.9	NA	NA
Bezold *et al.* (2007)	USA	Asymptomatic	Peroxidase	NA	NA	NA	NA
Cavagna *et al.* (2012)	Brazil	NA	Morphological evaluation	NA	NA	2.4	0.4
Chan *et al.* (1994)	USA	Asymptomatic	Peroxidase	NA	NA	4.40	0.0
Fariello *et al.* (2009)	Brazil	NA	Peroxidase	4.8	37.36	3.56	0.15
Flint *et al.* (2014)	South Africa	NA	Peroxidase	NA	NA	1.34	0.74
Gonzales *et al.* (1992)	Argentina	NA	Morphological evaluation	NA	NA	NA	NA
Kaleli *et al.* (2000)	Turkey	NA	Peroxidase	NA	33.1	NA	NA
Kiessling *et al.* (1995)	USA	NA	CD45	NA	38.7	6.35	0.19
Kortebani *et al.* (1992)	Argentina	NA	Peroxidase	NA	NA	NA	NA
Lackner *et al.* (2008)	Austria	NA	Peroxidase	NA	NA	1.91	0.25
Lackneret *al.* (2008)	Austria	Asymptomatic	Peroxidase	NA	NA	1.60	0.1
Lackner *et al.* (2010)	Austria	Asymptomatic	CD45	NA	NA	1.40	0.0
Micillo *et al.* (2016)	Italy	Asymptomatic	Peroxidase	5.0	NA	3.24	0.26
Moretti *et al.* (2014)	Italy	NA	Morphologic evaluation	NA	NA	NA	NA
Omu *et al.* (1999)	Kuwait	Asymptomatic	Peroxidase and CD45	NA	NA	NA	NA
Ricci *et al.* (2000)	Italy	Asymptomatic	Peroxidase and CD45	NA	36	NA	NA
Ricci *et al.* (2015)	Italy	NA	Peroxidase	NA	40.0	1.8	0.19
Sharma *et al.* (2001)	USA	NA	Peroxidase	NA	NA	NA	NA
Vidya *et al.* (2011)	India	NA	Peroxidase	NA	NA	NA	NA
Wolff *et al.* (1990)	USA	NA	Peroxidase	NA	NA	NA	NA
Yanushpolsky *et al.* (1995)	USA	Asymptomatic	Peroxidase	NA	NA	2.2	0
Yilmaz *et al.* (2005)	Turkey	NA	Peroxidase	NA	NA	3.40	0.1
Ziyyat *et al.* (2008)	France	NA	Peroxidase	5.5	36.2	5.87	0.0

ART, assisted reproductive technology; CD-45, differentiation cluster-45; Leuko, leukocytospermic group; Non-leuko, non-leukocytospermic group; NA, not available.

et al., 1992; Kiessling *et al.*, 1995; Yilmaz *et al.*, 2005; Ziyyat *et al.*, 2008; Fariello *et al.*, 2009; Lackner *et al.*, 2010; Barraud-Lauge *et al.*, 2011; Agarwal *et al.*, 2014; Moretti *et al.*, 2014; Ricci *et al.*, 2015; Micillo *et al.*, 2016), and the remaining 17 papers were assessed to be of low to moderate quality (Wolff *et al.*, 1990; Kortebani *et al.*, 1992; Chan *et al.*, 1994; Yanushpolsky *et al.*, 1995; Omu *et al.*, 1999; Kaleli *et al.*, 2000; Ricci *et al.*, 2000; Sharma *et al.*, 2001; Aziz *et al.*, 2004; Bezold *et al.*, 2007; Lackner *et al.*, 2008; Lackner *et al.*, 2008; Vidya *et al.*, 2011; Cavagna *et al.*, 2012; Agazarian *et al.*, 2013; Flint *et al.*, 2014; Barbonetti *et al.*, 2017). Comparability could not be ensured for most studies by adjusting either for the abstinence period or other variables. Furthermore, a selection bias could not be ruled out in the studies by Omu *et al.* (1999) and by Flint *et al.* (2014), as they also enrolled volunteers lacking a well-documented history of subfertility.

Analysis of results

Leukocytospermia and fertility outcomes after ART

Fertilization rate after ART was available for 254 leukocytospermic cases and 3613 non-leukocytospermic controls (Fig. 1A). Three studies evaluated FR after ICSI (Yilmaz *et al.*, 2005; Cavagna *et al.*, 2012; Ricci *et al.*, 2015), one study evaluated FR after IVF (Ricci *et al.*, 2015), and two studies evaluated FR after pooling together results of ICSI and IVF (Lackner *et al.*, 2008; Barraud-Lange *et al.*, 2011). The number of pregnancies achieved after ART was available for 254 leukocytospermic and 3613 non-leukocytospermic controls (Fig. 1B). Two studies reported pregnancies after ICSI (Yilmaz *et al.*, 2005; Cavagna *et al.*, 2012) and three studies pooling together results of ICSI and IVF (Lackner *et al.*, 2008; Barraud-Lange *et al.*, 2011; Ricci *et al.*, 2015). Although no difference was seen between the leukocytospermic cases and non-leukocytospermic controls in terms of FR (pooled SMD = −0.12; 95% CI: −0.39, 0.16; $p = 0.41$, $I^2 = 55\%$, $p_{\text{for heterogeneity}} = 0.05$, Fig. 1A), the overall odd for the occurrence of pregnancy was significantly lower in the absence of leukocytospermia (pooled OR = 1.72; 95% CI: 1.26, 2.34; $p = 0.0005$, $I^2 = 0\%$, $p_{\text{for heterogeneity}} = 0.66$, Fig. 1B). At the sensitivity analysis, the statistical significance of the positive association between leukocytospermia and occurrence of pregnancies after ART was lost when the largest study, by Barraud-Lange *et al.* (2011), was excluded from the estimation of the pooled OR (Figure S2).

Table 2 Newcastle–Ottawa Assessment Scale for case–control studies

Study	Selection				Comparability		Exposure			Total
	Definition of cases	Representativeness	Selection of controls	Definition of controls	Main risk factor[a]	On other risk factors	Assessment of exposure	Same methods of ascertainment for cases and controls	Non-response rate	
Agarwal *et al.* (2014)	★	★	★	★	★	★	★	★	★	9
Aghazarian *et al.* (2013)	★	★	★	★	☆	☆	★	★	☆	6
Aziz *et al.* (2004)	★	★	★	★	☆	☆	★	★	☆	6
Barbonetti *et al.* (2017)	★	★	★	★	☆	☆	★	★	☆	6
Barraud-Lauge *et al.* (2011)	★	★	★	★	☆	★	★	★	☆	7
Bezold *et al.* (2007)	★	★	★	★	☆	☆	★	★	☆	6
Cavagna*et al.* (2012)	★	☆	★	★	★	☆	★	★	☆	6
Chan *et al.* (1994)	★	★	★	★	☆	☆	★	★	☆	6
Fariello *et al.* (2009)	★	★	★	★	★	★	★	★	☆	8
Flint *et al.* (2014)	★	☆	★	★	☆	☆	★	★	☆	5
Gonzales *et al.* (1992)	★	★	★	★	☆	☆	★	★	★	7
Kaleli *et al.* (2000)	★	★	★	★	☆	☆	★	★	☆	6
Kiessling *et al.* (1995)	★	★	★	★	☆	★	★	★	☆	7
Kortebani *et al.* (1992)	★	★	★	★	☆	☆	★	★	☆	6
Lackner *et al.* (2008)	★	★	★	★	☆	☆	★	★	☆	6
Lackner *et al.* (2008)	★	★	★	★	☆	☆	★	★	☆	6
Lackner *et al.* (2010)	★	★	★	★	☆	★	★	★	☆	7
Micillo *et al.* (2016)	★	★	★	★	★	☆	★	★	☆	7
Moretti *et al.* (2014)	★	★	★	★	☆	★	★	★	☆	7
Omu *et al.* (1999)	★	☆	★	★	☆	☆	★	★	☆	5
Ricci *et al.* (2000)	★	★	★	★	☆	☆	★	★	☆	6
Ricci *et al.* (2015)	★	★	★	★	★	★	★	★	☆	8
Sharma *et al.* (2001)	★	★	★	★	☆	☆	★	★	☆	6
Vidya *et al.* (2011)	★	★	★	★	☆	☆	★	★	☆	6
Wolff *et al.* (1990)	★	★	★	★	☆	☆	★	★	☆	6
Yanushpolsky *et al.* (1995)	★	☆	★	★	☆	★	★	★	☆	6
Yilmaz *et al.* (2005)	★	★	★	★	☆	★	★	★	☆	7
Ziyyat *et al.* (2008)	★	★	★	★	★	★	★	★	★	9

[a]Main risk factor: 'abstinence' for studies evaluating semen parameters and 'female factor' for studies evaluating fertilization rate or pregnancy rate after assisted reproductive technology.

Leukocytospermia and semen parameters

The meta-analysis provided information from 1320 leukocytospermic cases and 4856 non-leukocytospermic controls for sperm count, 460 cases and 2586 controls for TSC, 1294 cases and 4810 controls for progressive motility (%), 1235 cases and 4680 controls for normal sperm morphology (%), 560 cases and 2822 controls for sperm viability (%), 84 cases and controls for sperm DNA damage (%), 966 cases and 3870 controls for semen volume, and 328 cases and 712 controls for semen pH. In the presence of leukocytospermia, pooled estimates indicated lower, albeit not significantly different, TSC, percentage of spermatozoa with normal morphology, percentage of viable spermatozoa and semen volume, while higher, albeit not significantly different, percentage of spermatozoa with DNA damage and semen pH value (Table 3). As shown in Fig. 2, the sperm concentration and the percentage of spermatozoa with progressive motility were significantly lower in leukocytospermic compared to non-leukocytospermic samples (sperm count: pooled SMD = –0.14; 95% CI: −0.28, −0.01; $p = 0.03$, $I^2 = 71\%$, $p_{\text{for heterogeneity}} < 0.00001$; Fig. 2A; sperm motility: pooled SMD = −0.18; 95% CI: −0.29, −0.06; $p = 0.003$, $I^2 = 59\%$, $p_{\text{for heterogeneity}} < 0.0001$; Fig. 2B).

Publication bias evaluation

As shown in Fig. 3A, the reasonably symmetrical shape of funnel plot of the studies analyzing sperm concentration suggested the absence of obvious publication bias. Accordingly, the trim-and-fill analysis did not identify putative additional missing studies. On the contrary, the asymmetrical shape of the funnel plot indicated publication bias among studies analyzing progressive motility: the trim-and-fill analysis identified two putative missing studies on the left side of the distribution (Fig. 3B). However, when the funnel distribution was re-balanced by including these additional studies, at the adjusted pooled estimate, sperm motility (%) remained significantly lower in the leukocytospermic group (adjusted pooled SMD = −0.21; 95% CI: −0.35, −0.07; $p = 0.003$).

Heterogeneity evaluation

As we found a significant inter-study heterogeneity in the pooled analysis of both sperm concentration ($I^2 = 71\%$, $p_{\text{for heterogeneity}} < 0.00001$ Fig. 2A) and progressive motility ($I^2 = 59\%$, $P_{\text{for heterogeneity}} < 0.0001$, Fig. 2B), a subgroup analysis was conducted wherein the studies were categorized into three groups according to the leukocyte detection method: CD45-based microscopic immunocytochemistry or flow-cytometry, peroxidase staining, and a morphological evaluation of stained semen smears. The difference in sperm concentration and in sperm progressive motility between leukocytospermic cases and non-leukocytospermic controls disappeared when leukocytes were assessed by immunocytochemistry (sperm count: pooled SMD = −0.08; 95% CI: −0.50, 0.35; $p = 0.71$, $I^2 = 53\%$, $p_{\text{for heterogeneity}} = 0.09$, Fig. 2A; progressive motility: pooled SMD = 0.17; 95% CI: −0.38, 0.73; $p = 0.54$, $I^2 = 71\%$, $p_{\text{for heterogeneity}} = 0.02$, Fig. 2B). The difference in sperm concentration between leukocytospermic cases and non-leukocytospermic controls was reduced, resulting no more statistically significant when leukocytes were detected by peroxidase test (pooled SMD = −0.14; 95% CI: −0.31, 0.03; $p = 0.11$, $I^2 = 77\%$, $p_{\text{for heterogeneity}} < 0.00001$, Fig. 2A), whereas it was still significantly lower in leukocytospermic compared to non-leukocytospermic samples using the morphological evaluation of stained semen smears (pooled SMD = −0.18; 95% CI: −0.34, −0.02; $p = 0.03$, $I^2 = 0\%$, $p_{\text{for heterogeneity}} = 0.41$, Fig. 2A). Sperm progressive motility was still significantly reduced in leukocytospermic compared to non-leukocytospermic samples when leukocytes were detected by peroxidase test (pooled SMD = −0.18; 95% CI: −0.31, −0.06; $p = 0.005$, $I^2 = 58\%$, $p_{\text{for heterogeneity}} = 0.0008$, Fig. 2B) as well as by morphological evaluation (pooled SMD = −0.30; 95% CI: −0.51, −0.09; $p = 0.005$, $I^2 = 33\%$, $p_{\text{for heterogeneity}} = 0.22$, Fig. 2B). In a subsequent subgroup analysis restricted to studies including patients with a declared no evidence of genital tract infections, the association between leukocytospermia and both lower sperm concentration and lower progressive motility was no longer significant (sperm concentration: pooled SMD = −0.02; 95% CI: −0.13, 0.09; $p = 0.72$, Fig. 4A; sperm progressive motility: pooled SMD = −0.04; 95% CI: −0.15, 0.07; $p = 0.48$, Fig. 4B), and no inter-study heterogeneity was observed ($p_{\text{for heterogeneity}} = 0.89$; $I^2 = 0\%$; $p_{\text{for heterogeneity}} = 0.82$; $I^2 = 0\%$, for sperm concentration and for sperm progressive motility, respectively, Fig. 4A,B).

DISCUSSION

A high seminal leukocyte count is a potential factor contributing to subfertility, possibly because of its detrimental effect on the spermatozoa (Wolff, 1995; Keck *et al.*, 1998). Therefore, determination of granulocytes, the most prevalent cell type in

Table 3 Results from meta-analysis of semen parameters not significantly different between leukocytospermic and non-leukocytospermic group

Semen parameter	Leuko group (N)	Non-leuko group (N)	SMD, IV, Random, 95% CI	Heterogeneity
Volume (ml)	966	3870	−0.03 [−0.11, 0.05]	I^2: 0% $P = 0.45$
pH	267	426	0.27 [−0.03, 0.58]	I^2: 69% $P = 0.01$
TSC ($\times 10^6$/ejaculate)	460	2586	−0.11 [−0.26, 0.03]	I^2: 37% $P = 0.14$
Normal morphology (%)	1235	4680	−0.08 [−0.23, 0.06]	I^2: 73% $P < 0.00001$
Viability (%)	560	2822	−0.15 [−0.37, 0.07]	I^2: 75% $P = <0.0001$
Sperm DNA damage (%)	84	304	0.20 [−0.05, 0.45]	I^2: 0% $P = 1.00$

CI, confidence interval; IV, inverse variance; Leuko, leukocytospermic; Non-leuko, non-leukocytospermic; SMD, standardized mean difference; TSC, total sperm count.

the ejaculate (Wolff, 1995, 1998), is routinely included in semen analysis of men attending a fertility clinic, and a cell count $\geq 1 \times 10^6$/mL is considered leukocytospermia (WHO, 1992, 2010).

The correlation between seminal leukocytes and fertility potential/semen quality has been largely ambiguous. We therefore conducted a meta-analysis of twenty-eight carefully selected case–control studies, to analyze the relationship of leukocytospermia with fertilization rate and with the occurrence of pregnancies after ART, as well as with semen quality in couples attending a fertility clinic. We found that leukocytospermia did not have an adverse effect on the reproductive potential after ART. Meta-analysis showed no differences in oocyte fertilization after IVF or ICSI with ejaculates with or without leukocytospermia (Fig. 1A). Four out of six studies excluded from the quantitative analysis confirmed no effect on FR after IVF (van der Ven *et al.*, 1987; Tomlinson *et al.*, 1992; Moilanen *et al.*, 1998; Seshadri *et al.*, 2012), while the other two reported a negative association between leukocytospermia and oocyte fertilization after IVF (Talbert *et al.*, 1987; Sukcharoen *et al.*, 1995).

Meta-analyzed trials showed that the occurrence of pregnancies after IVF and after ICSI was higher in leukocytospermic ejaculates (Fig. 1B). Results were greatly influenced by the largest trial which pooled together results of ICSI and of IVF (Barraud-Lange *et al.*, 2011), and statistical relevance of the positive association between leukocytospermia and occurrence of pregnancies after ART was lost when that study (Barraud-Lange *et al.*, 2011) was excluded from the estimation of the pooled OR (Figure S2). Therefore, although the effect of leukocytospermia on pregnancies after ART deserves to be better assessed, according to available studies it seems wise to suggest that leukocytospermia, according to WHO definition (WHO, 1992, 2010), has no detrimental effect on pregnancy rate after ART. Interesting is the observation that the putative effect of leukocytospermia on pregnancy rate after ART was balanced by a higher pregnancy loss and ectopic pregnancies, so that the actual chance of having a successful pregnancy was not significantly different in leukocytospermic compared to non-leukocytospermic couples (Barraud-Lange *et al.*, 2011). Taken together, the fertility potential of spermatozoa after ART is not affected by leukocyte infiltration into the semen although very high concentrations of peroxidase-positive round cells in semen ($>4\times10^6$/mL) are reported to have a negative effect in fertility outcomes after IVF-ET (De Geyter *et al.*, 1994). The ROS secreted by leukocytes inadvertently co-cultured with spermatozoa in assisted conception may lead to sperm DNA damage, which could negatively affect oocyte fertilization and embryo development (Aitken & De Iuliis, 2010) in case of very high level of semen leukocyte infiltration.

Here we found that leukocytospermia is associated to a significantly lower sperm progressive motility (%) and to a lower sperm concentration at a limit of statistical relevance, compared to non-leukocytospermic samples. As the epididymis is an established leukocyte reservoir (Flickinger *et al.*, 1997), possible leukocyte-induced alterations in spermatozoa maturation during epididymal migration might explain the lower sperm motility observed in leukocytospermic men (Aziz *et al.*, 2004). Leukocytospermia-induced sperm damage is a likely result of the high levels of leukocyte-derived ROS and inflammatory mediators (Aitken & West, 1990; Plante *et al.*, 1994; Whittington & Ford, 1999; Sharma *et al.*, 2001; Henkel *et al.*, 2005; Agarwal *et al.*, 2014; Hagan *et al.*, 2015). The high polyunsaturated fatty acid (PUFA) content of the spermatozoa membrane increases their susceptibility to lipid peroxidation by both the intrinsic (mitochondrial) and extrinsic (leukocyte)-derived ROS (Aitken *et al.*,

Figure 1 Forest plots depicting (A) standardized (Std) mean differences in fertilization rate (FR) and (B) odds ratio for the occurrence of clinical pregnancies after assisted reproductive technology (ART) in case of men with and without leukocytospermia. The diamonds indicate the overall summary estimates for the analyses (the width of the diamonds represents the 95% CI); the boxes indicate the weight of the individual studies in the pooled analyses. Studies reporting outcomes of intracytoplasmic sperm injection (ICSI), *in vitro* fertilization (IVF) or ICSI and IVF pooled together, were indicated by 'a', 'b', and 'c', respectively. CI, confidence interval; df, degrees of freedom; IV, inverse variance; M–H, Mantel–Haenszel.

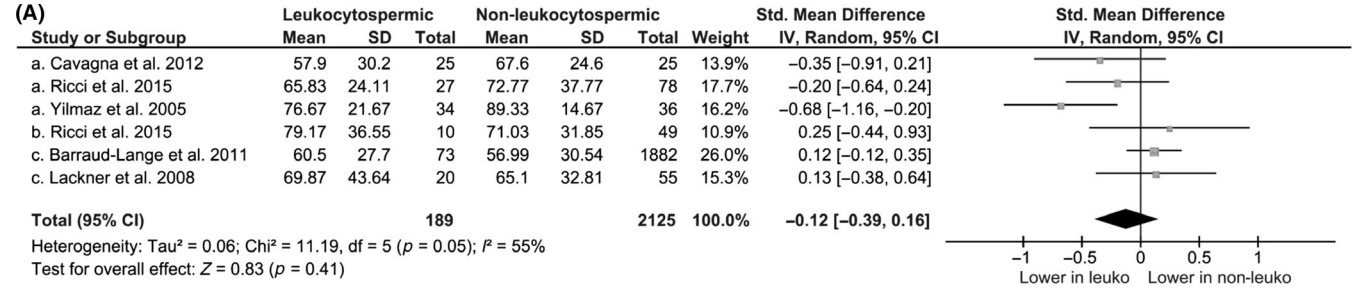
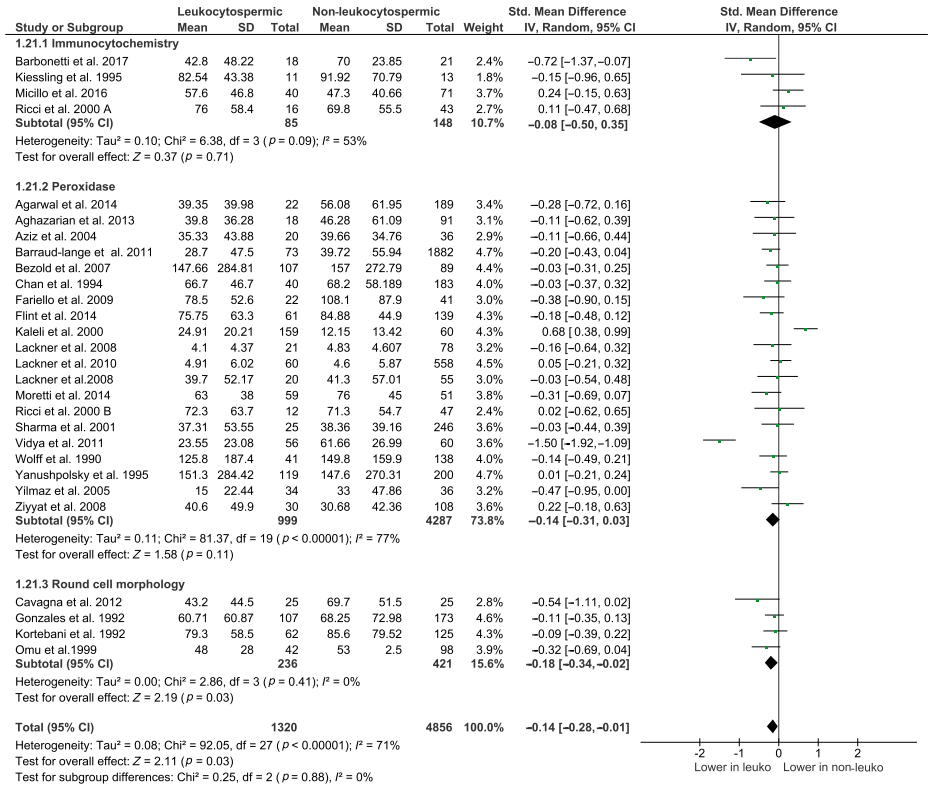


Figure 2 Forest plots depicting standardized (Std) mean differences in sperm concentration (A) and percentage of spermatozoa with progressive motility (B) between men with and without leukocytospermia. In the subgroup analysis, studies were categorized into three groups according to the leukocyte detection method: CD45-based cytochemistry or flow-cytometry, peroxidase staining, and morphological evaluation of smeared and stained semen round cells. The diamonds indicate the summary estimates for the overall and subgroup analyses (the width of the diamonds represents the 95% CI); the boxes indicate the weight of the individual studies in the pooled analyses. The paper by Ricci et al. (2000) provided information on two populations where semen leukocytes were assessed by CD45-based flow-cytometry (Ricci et al., 2000; A) and peroxidase staining (Ricci et al., 2000; B), respectively. CI, confidence interval; df, degrees of freedom; IV, inverse variance.

(A)



(B)

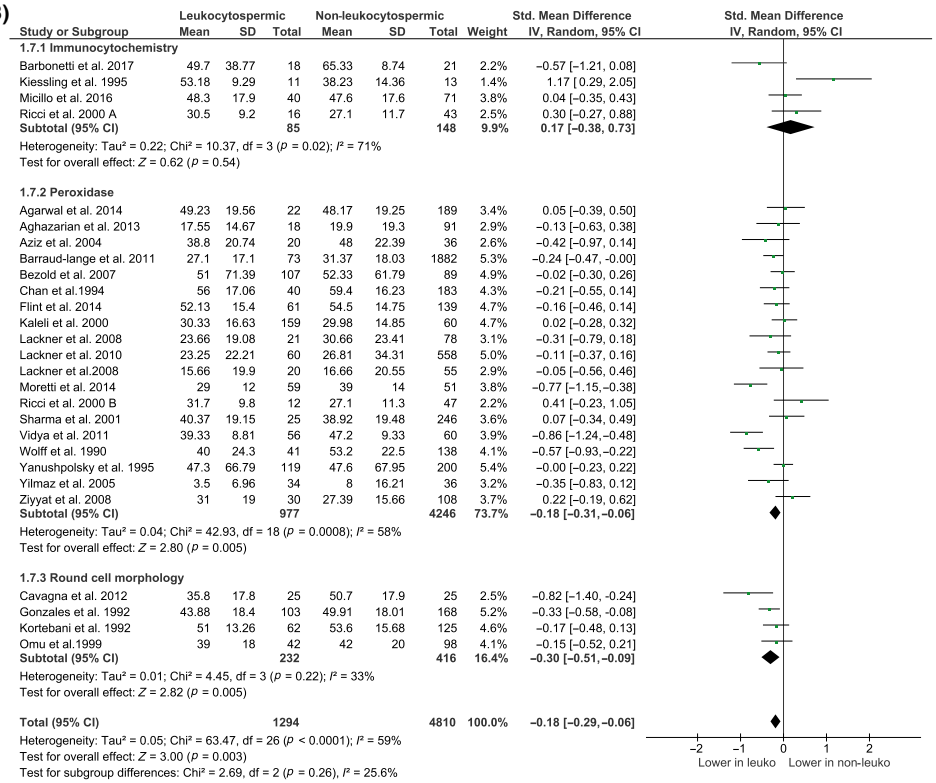


Figure 3 Funnel plots for the analysis of the relationship of leukocytospermia with sperm concentration (A) and percentage of spermatozoa with progressive motility (B). In the analysis of progressive motility (B), the trim-and-fill test identified two putative missing studies (white circle) on the left side of the distribution.

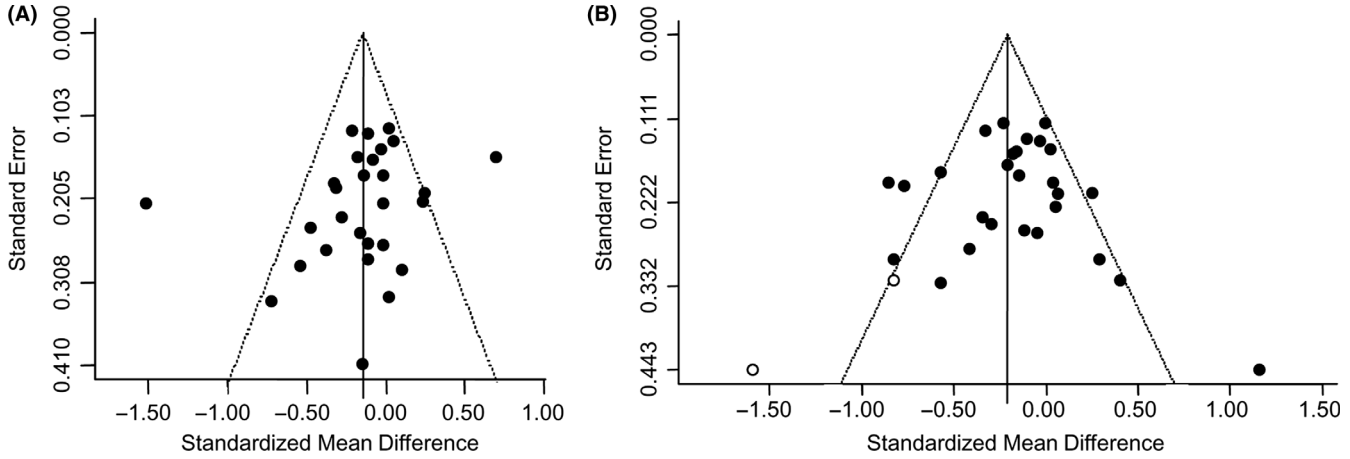
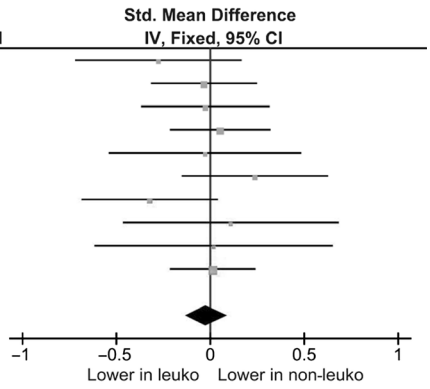


Figure 4 Forest plot depicting the results of the subgroup analyses for sperm concentration (A) and progressive motility (B) only including studies enrolling asymptomatic men with leukocytospermia. The diamonds indicate the overall summary estimates for the analyses (the width of the diamonds represents the 95% CI); boxes indicate the weight of the individual studies in the pooled analyses. CI, confidence interval; df, degrees of freedom; IV, inverse variance.

(A)

Study or Subgroup	Leukocytospermic Mean	SD	Total	Non-leukocytospermic Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Agarwal et al. 2014	39.35	39.98	22	56.08	61.95	189	6.1%	−0.28 [−0.72, 0.16]
Bezold et al. 2007	147.66	284.81	107	157	272.79	89	15.2%	−0.03 [−0.31, 0.25]
Chan et al.1994	66.7	46.7	40	68.2	58.18	183	10.3%	−0.03 [−0.37, 0.32]
Lackner et al. 2010	4.91	6.02	60	4.6	5.87	558	16.9%	0.05 [−0.21, 0.32]
Lackner et al.2008	39.7	52.17	20	41.3	57.01	55	4.6%	−0.03 [−0.54, 0.48]
Micillo et al. 2016	57.6	46.8	40	47.3	40.66	71	7.9%	0.24 [−0.15, 0.63]
Omu et al.1999	48	28	42	53	2.5	98	9.1%	−0.32 [−0.69, 0.04]
Ricci et al. 2000 A	76	58.4	16	69.8	55.5	43	3.6%	0.11 [−0.47, 0.68]
Ricci et al. 2000 B	72.3	63.7	12	71.3	54.7	47	3.0%	0.02 [−0.62, 0.65]
Yanushpolsky et al. 1995	151.3	284.42	119	147.6	270.31	200	23.3%	0.01 [−0.21, 0.24]
Total (95% CI)			**478**			**1533**	**100.0%**	**−0.02 [−0.13, 0.09]**

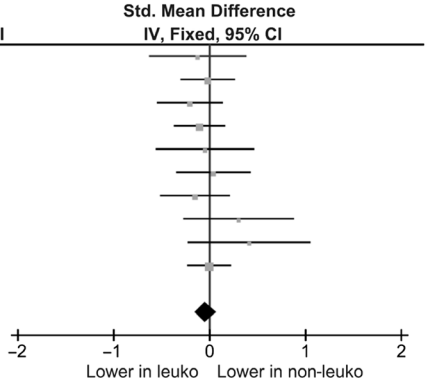
Heterogeneity: Chi² = 6.25, df = 9 (p = 0.72); I^2 = 0%
Test for overall effect: Z = 0.36 (p = 0.72)



(B)

Study or Subgroup	Leukocytospermic Mean	SD	Total	Non-leukocytospermic Mean	SD	Total	Weight	Std. Mean Difference IV, Fixed, 95% CI
Aghazarian et al. 2013	17.55	14.67	18	19.9	19.3	91	4.8%	−0.13 [−0.63, 0.38]
Bezold et al. 2007	51	71.39	107	52.33	61.79	89	15.4%	−0.02 [−0.30, 0.26]
Chan et al. 1994	56	17.6	40	59.4	16.23	183	10.4%	−0.21 [−0.55, 0.14]
Lackner et al. 2010	23.25	22.21	60	26.81	34.31	558	17.2%	−0.11 [−0.37, 0.16]
Lackner et al.2008	15.66	19.9	20	16.66	20.55	55	4.6%	−0.05 [−0.56, 0.46]
Micillo et al. 2016	48.3	17.9	40	47.6	17.6	71	8.1%	0.04 [−0.35, 0.43]
Omu et al.1999	39	18	42	42	20	98	9.3%	−0.15 [−0.52, 0.21]
Ricci et al. 2000 A	30.5	9.2	16	27.1	11.7	43	3.7%	0.30 [−0.27, 0.88]
Ricci et al. 2000 B	31.7	9.8	12	27.1	11.3	47	3.0%	0.41 [−0.23, 1.05]
Yanushpolsky et al. 1995	47.3	66.79	119	47.6	67.95	200	23.6%	−0.00 [−0.23, 0.22]
Total (95% CI)			**474**			**1435**	**100.0%**	**−0.04 [−0.15, 0.07]**

Heterogeneity: Chi² = 5.18, df = 9 (p = 0.82); I^2 = 0%
Test for overall effect: Z = 0.70 (p = 0.48)



1993; Barbonetti *et al.*, 2011), finally impairing tail motion (Tremellen, 2008; Barbonetti *et al.*, 2011). This could be the likely mechanism underlying the significant association often seen between leukocytospermia and poor sperm motility (Wolff *et al.*, 1990; Gonzales *et al.*, 1992; Aziz *et al.*, 2004; Vidya *et al.*, 2011; Moretti *et al.*, 2014). However, numerous studies did not find any association between leukocytospermia and reduced sperm motility (Chan *et al.*, 1994; Kaleli *et al.*, 2000; Ricci *et al.*, 2000; Sharma *et al.*, 2001; Agarwal *et al.*, 2014; Flint *et al.*, 2014), and these divergent results could be attributed to the different methods used for quantifying semen leukocytes. Indeed, stratifying the meta-analyzed studies on the basis of the leukocyte detection method revealed a significant association between lower sperm progressive motility and leukocytospermia in studies using peroxidase staining and morphological evaluation as opposed to immunostaining, which is the gold standard for leukocyte detection (Ricci *et al.*, 2000). However, demarcating data according to the methods of detecting leukocytospermia

did not significantly reduce the heterogeneity between the studies analyzing the association of leukocytospermia with reduced progressive motility and sperm concentration in men attending fertility clinics (Fig. 2). Heterogeneity was eliminated when the analysis was restricted to the 10 studies that clearly reported, as an inclusion criterion, the absence of clinical evidence for seminal tract infection (Fig. 4). This subgroup analysis eliminated any difference in the sperm progressive motility as well as in sperm concentration between men with or without leukocytospermia. Therefore, in selected populations of men with no clinical evidence of seminal tract infections attending a fertility clinic, the presence of leukocytospermia, as defined by the WHO criteria (WHO, 1992, 2010), does not seem to negatively affect semen parameters including sperm DNA damage.

The main limitation of this meta-analysis is that most of the selected studies did not analyze potential factors influencing semen parameters, for example, the age of participants and abstinence period. Therefore, we could not determine their contribution to the inter-studies heterogeneity. In addition, changes in the seminal parameters do not necessarily reflect pathological conditions but can be indicative of physiological intra-individual variability (Francavilla *et al.*, 2007) or inappropriate collection and/or storage. Finally, the diagnosis of leukocytospermia and the selection of comparison groups were not uniform across the studies, and some of the included trials did not differentiate fertilization rate and pregnancy rate after IVF or after ICSI (see Fig. 1). Further studies are needed to elucidate the potential impact of leukocytospermia on reproductive outcomes after ART by a more accurate selection of study populations.

In conclusion, leukocytospermia in men seeking consultation for couple subfertility is not associated with a reduced fertility after ART and also with altered semen quality at least in selected populations asymptomatic for genital tract infections. Therefore, the current clinical criteria for definition of leukocytospermia should be re-assessed in subfertile couples attending a fertility clinic.

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DISCLOSURES

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHORS' CONTRIBUTIONS

CC was involved in the systematic search to identify all relevant studies, participated in the assessment of the eligibility of each selected study, and helped draft the manuscript; SDA, AM, and EM participated in the systematic search to identify all relevant studies; SN participated in performing statistical analysis; FF contributed to conception and critically revised the manuscript; SF contributed to conception, participated in the assessment of the eligibility and quality of each selected study, and wrote and critically revised the manuscript; AB participated in the assessment of the eligibility of each selected study, assessed the quality of the studies, participated in performing statistical analysis, and helped draft the manuscript; All authors read and approved the final manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Flow diagram showing an overview of the study selection process.
Figure S2. Sensitivity analysis of the relationship between leukocytospermia and occurrence of clinical pregnancies: influence of each individual study on the pooled odds ratio (OR) 95% Confidence Interval (CI) for the occurrence of clinical pregnancies after assisted reproductive technology.
Table S1. PRISMA checklist.