Human Reproduction, pp. 1–11, 2023
https://doi.org/10.1093/humrep/dead072

human reproduction

ORIGINAL ARTICLE *Reproductive endocrinology*

Recent clomiphene citrate exposure does not impact subsequent clinical outcomes in single euploid frozen embryo transfer cycles

Carlos Hernandez-Nieto [1,*], Joseph Lee[1], Tamar Alkon-Meadows[1], Luz Soto-Cossio [1], Benjamin Sandler[1,2], Tanmoy Mukherjee [1,2], and Alan Copperman[1,2]

[1]Reproductive Medicine Associates of New York, New York, NY, USA [2]Department of Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, New York, NY, USA

*Correspondence address. Reproductive Medicine Associates of New York, New York, NY, USA and Department of Obstetrics, Gynecology and Reproductive Science, Icahn School of Medicine at Mount Sinai, 635 Madison Ave, Floor 10th, New York, NY 10022, USA. Tel: +1-212-756-5777; E-mail: chernandez@rmany.com https://orcid.org/0000-0002-6703-1341

Submitted on October 18, 2022; resubmitted on March 27, 2023; editorial decision on April 03, 2023

STUDY QUESTION: Do infertile couples who recently utilized clomiphene citrate (CC) for ovulation induction or ovarian stimulation (<90 days previously) followed by a single euploid embryo transfer (SEET) have lower implantation potential compared with patients who were not exposed to CC within 90 days before embryo transfer (ET)?

SUMMARY ANSWER: There does not appear to be an association between recent CC exposure and lower implantation potential in patients who undergo a frozen embryo transfer (FET) of euploid embryos.

WHAT IS KNOWN ALREADY: Clomiphene has been found to be associated with lower pregnancy rates when compared against other ovarian stimulation medications. The majority of published research about the effects of CC on implantation potential suggest an anti-estrogenic effect on the endometrium. Quality evidence and information about utilization of CC and its effect on implantation potential after euploid ETs is lacking in the literature.

STUDY DESIGN, SIZE, DURATION: A retrospective cohort study with propensity score matching was carried out. We included all patients that underwent an autologous SEET from September 2016 to September 2022 at a single academic-private ART center.

PARTICIPANTS/MATERIALS, SETTING, METHODS: The study group included patients that had utilized CC during either ovulation induction cycles and/or controlled ovarian stimulation at least 90 days before FET. A propensity score-matched control group of patients that were unexposed to CC within 90 days prior to SEET was used for comparisons. The primary outcome was positive pregnancy test (defined as a positive serum β-hCG measured 9 days after ET), with other outcomes including clinical pregnancy, ongoing pregnancy, biochemical pregnancy loss, and clinical pregnancy loss rates per SEET. Multivariate regression analyses fitted with generalized estimating equations were utilized to analyze if there was an association between CC utilization and IVF outcomes. Furthermore, the study evaluated the cumulative effect of CC and endometrial receptivity *in vivo* and subsequent IVF outcomes.

MAIN RESULTS AND THE ROLE OF CHANCE: A total of 593 patients with utilization of CC in <90 days before ET were compared with 1779 matched controls. Positive pregnancy test rates were comparable among the control group and the CC exposed groups, respectively (74.3% versus 75.7%, $P=0.79$), as were clinical pregnancy (64.0% versus 65.0%, $P=0.60$), ongoing pregnancy (51.8% versus 53.2%, $P=0.74$), biochemical pregnancy loss (15.7% versus 14.03%, $P=0.45$), and clinical pregnancy loss rates were also comparable among cohorts (17.1% versus 18.1%, $P=0.71$). No association was found between utilization of clomiphene and lower implantation rates (adjusted odds ratio 0.95, 95% CI 0.76–1.18). Also, no differences were observed in sub-analyses based on multiple CC utilization periods. Finally, no association was found between the number of consecutive cumulative clomiphene cycles and sub-optimal IVF outcomes.

LIMITATIONS, REASONS FOR CAUTION: The study has inherent bias that originated from its retrospective design. Serum levels of CC were not measured and sample size for the sub-analyses was small.

WIDER IMPLICATIONS OF THE FINDINGS: There does not appear to be an association between recent CC exposure and lower implantation potential in patients who undergo a FET of euploid embryos. This finding remains consistent, even in patients who undergo multiple, consecutive clomiphene cycles prior to ET. There were no long-term effects of CC on endometrial development and clinical characteristics examined in this study. Patients that utilized CC medication prior to a SEET cycle for either ovarian stimulation or ovulation induction, can be assured that there is no evidence of a residual effect of recent CC administration that could jeopardize their pregnancy probability.

STUDY FUNDING/COMPETING INTEREST(S): No funding was received for the realization of this study. A.C. is advisor and/or board member of Sema4 (stakeholder in data) and Progyny. The other authors have no conflicts of interest to declare.

TRIAL REGISTRATION NUMBER: N/A.

Key words: clomiphene citrate / frozen embryo transfer / IVF / preimplantation genetic testing / controlled ovarian hyperstimulation / ovulation induction

Introduction

Clomiphene citrate (CC) is a non-steroidal synthetic ovulatory stimulant categorized as a selective estrogen receptor modulator (SERM) that is used in the treatment of female infertility of endocrine origin and can be used as a coadjutant during ovarian stimulation for IVF (Kamath *et al.*, 2017). CC is a triphenyl-ethylene derivative that yields both estrogenic agonist and estrogenic antagonist properties. Its mechanism of action is by competitive binding to estrogen receptors in the hypothalamus and pituitary, followed by a reduced signaling of estrogen via its receptors. In this way, CC is interfering with the feedback mechanism of endogenous estrogen resulting in an increase in FSH and LH secretion that subsequently will stimulate ovarian follicular recruitment and development (Kerin *et al.*, 1985; Maruncic and Casper, 1987).

Despite its effectiveness in achieving ovulation since its development in the late 1960s, CC has been found to be associated with lower pregnancy rates when compared against other ovarian stimulation medications (Bonhoff *et al.*, 1996; Kamath *et al.*, 2017; Zhang *et al.*, 2020). The majority of published research about the effects of CC on implantation potential suggest an anti-estrogenic effect on the cervical mucus (Marchini *et al.*, 1989; Randall and Templeton, 1991), endometrial vascular resistance alterations (Kupesic and Kurjak, 1993), and, more importantly, direct effects on endometrial structural development and thinning of the endometrial lining during stimulation as potential causes of altered endometrial receptivity (Eden *et al.*, 1989).

Commercially available CC is a racemic mixture of two stereoisomers with different pharmacodynamics and pharmacokinetics: Enclomiphene, which is completely anti-estrogenic and the isomer Zuclomiphene, which is mildly estrogenic, as well as anti-estrogenic (Mikkelson *et al.*, 1986). In general, CC has a half-life of 5–7 days but metabolites have been detected in humans up to 6 weeks after administration. This extended bioavailability is possible because of extensive plasma protein binding, enterohepatic cycling, and accumulation in fatty tissues. This aspect of CC is thought to be clinically important, as the effect after therapy could continue for several months (Young *et al.*, 1999; Ghobadi *et al.*, 2009). Based on these observations, the CC pharmacologic profile and its undesirable adverse effect on pregnancy rates during ART, clinicians have pointed toward the necessity for patients to utilize a freeze-all strategy after the utilization of CC for ovarian stimulation by way of avoiding fresh embryo transfers (ETs) and optimizing pregnancy rates (Reed *et al.*, 2018).

Quality evidence and information about utilization of CC and its effect on implantation potential after ET is lacking in the literature. Evidence-based recommendations of the optimal wash out time for transferring an embryo after utilization of CC remain controversial. A study by Nakagawa *et al.* (2014) demonstrated patients who utilized CC within 90 days of frozen/thawed ET experienced significantly lower implantation rates than those who were unexposed to CC. Conversely, a more recent study by Kato *et al.* (2018) showed that CC administration <60 days from last dose exhibited a detrimental effect on fresh ET although subsequent vitrified/thawed ETs were not associated with impaired implantation regardless of the timing of embryo replacement and the CC exposure.

Currently published studies about CC exposure and implantation potential have some methodological shortcomings that could limit their generalizability. Moreover, no study has analyzed CC exposure and subsequent transfer of a chromosomally screened euploid embryo, which is of the utmost importance when analyzing the characteristics of early implantation *in vivo*. Therefore, the objective of our study was to analyze infertile couples who utilized CC for ovulation induction and/or ovarian stimulation followed by a single euploid blastocyst transfer and assess the association between CC utilization and sustained implantation rates. Furthermore, the study evaluated the cumulative effect of CC and endometrial receptivity *in vivo* and subsequent IVF outcomes.

Material and methods

Study design and patient populations

The retrospective propensity score-matched cohort analysis was performed at a single academic-private ART center and included infertility patients who underwent an IVF cycle with preimplantation genetic testing for aneuploidy (PGT-A) with a subsequent single euploid embryo transfer (SEET) from September 2016 to September 2022. All patients underwent controlled ovarian stimulation (COS), ICSI, extended embryo culture, and trophectoderm (TE) biopsy. All PGT-A analyses were performed with next-generation sequencing technology. All ovarian stimulation protocols and laboratory methods used in the study had been previously described (Hernandez-Nieto *et al.*, 2020).

As a study group, all patients who underwent a SEET and had utilized CC before their ET preparation cycle either during ovulation

induction cycles and/or COS including CC were included. Only embryos generated during the COS cycle that utilized CC were included in this group. Dosage, duration of CC, and number of consecutive prior CC cycles were recorded. Time from exposure to CC was defined from the date of last ingestion of pill until the day of the ET procedure. Control patients were not exposed to CC before the ET nor had exposure to CC within 90 days of treatment. All control cases were selected from a matched 3:1 cohort of subjects that were identified using a propensity score matching algorithm based on clinical parameters including: oocyte age at retrieval, BMI, and serum anti-Müllerian hormone (AMH) levels. Cases with incomplete information were excluded from the analysis, also cases involving multiple TE biopsies, multiple thaw/freeze procedures, and cases with patients utilizing donor oocytes or testicular sperm extraction. Similarly, patients with recurrent implantation failure, recurrent pregnancy losses, or patients with known chromosomal rearrangements were excluded from analysis.

For the ETs, all cases underwent synthetic endometrial preparation, as previously described (Hernandez-Nieto *et al.*, 2019). For all cases, thawing and transfer of the embryos were carried out on the sixth day of progesterone supplementation regardless of the day of embryo development at time of vitrification. Euploid embryos with the top morphology grade were selected for transfer. In gender selection for family balancing cases, the highest graded embryo of the preferred genetic sex was transferred. When patients have multiple embryos frozen that share the same morphological grades, Day 5 embryos were preferentially selected over biopsied Day 6 or 7 embryos. Among embryos biopsied on the same day of development, inner cell mass grade was prioritized in embryo selection, followed by expansion grade, and then TE grade, as described previously (Nazem *et al.*, 2019).

For the main analysis, the cohorts were created using a cutoff value of 90 days since last CC utilization and compared with control cases that includes patients with more than 90 days since last CC utilization and/or patients unexposed to the medication. This criterion was based on the reports from Nakagawa *et al.* (2014) of lower pregnancy rates when ETs were performed in <90 days from the last administration of CC. Consequently, a sensitivity analysis considering multiple periods of CC exposure was performed, with cohorts for this analysis separated in periods of <30 days of CC utilization (Group A); 31–60 days (Group B); 61–90 days (Group C); >90 days (Group D); and a different group of patients unexposed to CC (Group E). Finally, another sub-analysis was performed observing the total number of consecutive cumulative CC cycles prior to the ET cycle. Cohorts were categorized as: <3 cumulative cycles (Group 1); 4–6 cycles (Group 2); >6 cycles (Group 3); and unexposed patients (Group 4).

Outcome measures

The primary outcome of the study was implantation rate, determined here by the positive pregnancy test rate and defined as a positive serum β-hCG measured 9 days after ET. Secondary outcomes included implantation rate in the setting of a SEET, defined as the number of gestational sacs observed at vaginal ultrasound 3–5 weeks after ET divided by the number of embryos transferred; clinical pregnancy rate (CPR): the proportion of patients with ultrasonographically detectable fetal cardiac activity; biochemical pregnancy loss rate (BPL): pregnancy loss occurring after the presence of a positive pregnancy test followed by a decrease or lack of increase of β-hCG serum levels in serial measurements 48 h after the first measurement and/or without detection of a gestational sac visualized by vaginal ultrasound at the fifth week of pregnancy; clinical pregnancy loss rate (CPL): pregnancy loss occurring after the presence of a confirmed gestational sac; and ongoing pregnancy rate (OPR), a sustained pregnancy after detected fetal heart beat on a vaginal ultrasound and/or complete delivery of a product of fertilization after ≥22 completed weeks of gestational age, which breathes or shows evidence of life (Zegers-Hochschild *et al.*, 2017). Secondary analysis objectives are to assess if multiple time periods between last dose of CC and ET are associated with differences in IVF outcomes previously described. Last, we analyzed if there was a cumulative effect of CC on IVF outcomes based on the total cumulative number of cycles that utilized CC before the ET cycle.

Statistical methods

Statistical analyses were performed using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA). Demographic and embryological data were registered for all participants. Medians, means, SD, interquartile ranges (IQRs), and frequencies were calculated for all variables. Descriptive and comparative unadjusted analysis was performed by Student's *t*-test, Mann–Whitney *U* test, Fisher's exact, and χ^2 tests, as appropriate. A multivariate logistic regression fitted with generalized estimating equations (GEE) with an exchangeable correlation structure to account for patients who underwent multiple frozen embryo transfer (FET) cycles was utilized. Adjusted odds ratio (aOR) with 95% CI were calculated. All variables that showed significance on the unadjusted analysis and/or variables that were thought to be clinically relevant were encompassed and adjusted for as covariates in the models. All *P*-values were two-sided with a clinical significance level determined at $P < 0.05$. For the primary outcome, on a power analysis calculation, a sample size of 329 patients per group was required to detect a difference of 10% in positive pregnancy test rates and to have an 80% power with an alpha of 0.05.

Regulatory approval

This retrospective analysis was approved by an academic Institutional Review Board (HS #: STUDY-18-00441). Patient information was de-identified before data analysis.

Results

A total of 2372 SEET cycles from 2162 infertile couples were included in the analysis. A total of 593 patients who utilized CC in <90 days prior to ET day were compared against 1779 propensity score-matched controls. Demographic and baseline characteristics of cohorts, including embryological and IVF outcomes, were compared among cohorts (Table I).

In the unadjusted analysis, significant differences were found in the days from last CC utilization to the ET date in controls versus patients that used CC <90 days from ET (median days 302.5 IQR 613, versus 64 IQR 31, $P \leq 0.0001$), and differences in the total number of prior CC cycles (mean 0.7 SD ± 1.4, versus 2.5 SD ± 1.7, $P \leq 0.0001$) and cumulative dose of CC (median 0 IQR 500 mg, versus 1000 IQR 1500 mg, $P \leq 0.0001$) were found when comparing controls against

Table I Demographic characteristics, IVF outcomes, and data comparison between groups categorized by clomiphene citrate exposure.

	Control group		Clomiphene citrate (<90 days)		
	n = 1779		n = 593		
	Mean/median	SD/IQR	Mean/median	SD/IQR	*P*-value
Time between CC and embryo transfer (days)	302.5	613.0	64.0	31.0	<0.0001*
Prior CC cycles	0.7	1.4	2.5	1.7	<0.0001*
Cumulative dose of CC (mg)	0.0	500.0	1000.0	1500.0	<0.0001*
Age at oocyte retrieval (years)	36.7	3.9	36.5	3.8	0.73**
Age at ET (years)	37.5	4.0	36.6	3.8	<0.001**
Anti-Müllerian hormone (ng/ml)	2.9	3.2	2.8	3.7	0.72**
BMI (kg/m^2)	23.7	4.2	23.7	4.1	0.83**
Baseline serum estradiol (pg/ml)	37.7	26.4	37.3	26.0	0.08*
Baseline serum progesterone (ng/ml)	0.4	0.2	0.4	0.2	0.31*
Baseline LH (IU/l)	4.8	3.0	4.8	2.9	0.96*
Baseline FSH (IU/l)	6.5	3.7	6.5	3.6	0.96*
Estradiol at conversion (pg/ml)	271.6	204.4	264.1	183.4	0.62*
Serum progesterone at exogenous progesterone start (ng/ml)	0.3	0.2	0.3	0.2	0.34*
Endometrial thickness at transfer (mm)	9.2	3.0	9.4	3.0	0.15*
Previous euploid ETs	0.7	1.0	0.2	0.7	<0.0001*
Top embryo quality ET	1259/1779	70.77%	446/593	75%	0.40$^{\chi}$
Positive pregnancy test (N/%)	1322/1779	74.3%	449/593	75.7%	0.79$^{\chi}$
Clinical pregnancy (N/%)	1114/1779	64.0%	386/593	65.0%	0.60$^{\chi}$
Ongoing pregnancy (N/%)	923/1779	51.8%	316/593	53.2%	0.74$^{\chi}$
Biochemical pregnancy loss (N/%)	208/1322	15.7%	63/449	14.03%	0.45$^{\chi}$
Clinical pregnancy loss (N/%)	191/1114	17.1%	70/386	18.1%	0.71$^{\chi}$

Data presented as mean and SD, or median and interquartile range (IQR) and frequencies unless stated otherwise. Statistical significance, $P < 0.05$.
N: sample size; CC: clomiphene citrate; ET: embryo transfer.
*Student's *t*-test.
**Mann–Whitney *U*-test.
$^{\chi}$Chi-squared test.

exposed patients, respectively. Female patients age at the moment of ET was younger in the CC exposed group compared with controls (mean 37.4 ± 3.9, versus 36.6 ± 3.7, $P \leq 0.001$) and moreover, a difference was found in the total number of prior euploid ETs among the exposed group compared with controls (mean 0.7 SD ± 1.0, versus 0.2 SD ± 0.7, $P \leq 0.0001$, respectively). No differences were found in age at oocyte retrieval, baseline AMH, BMI, baseline serum estradiol, progesterone, FSH, and LH levels among cohorts. Additionally, maximum estradiol and progesterone levels measured at conversion (start of exogenous progesterone) were similar among groups. Notably, endometrial thickness was similar among groups (9.2 mm ± 3.0 in controls versus 9.4 mm ± 3.0, $P = 0.15$). Regarding IVF outcomes: no differences were found in the total number of good quality embryos transferred. Positive pregnancy test rates were comparable among the control group and the CC exposed group, respectively (74.3% versus 75.7%, $P = 0.79$), also clinical pregnancy (64.0% versus 65.0%, $P = 0.60$), ongoing pregnancy (51.8% versus 53.2%, $P = 0.74$), biochemical pregnancy loss (15.7% versus 14.03%, $P = 0.45$), and clinical pregnancy loss rates were comparable among cohorts (17.1% versus 18.1%, $P = 0.71$) (Table I and Fig. 1). In a sensitivity analysis, after assessing IVF outcomes exclusively on patients that underwent their first ET after the oocyte extraction cycle, we observed no significant differences between the control group (n = 1507) and <90 days of matched CC exposed patients (n = 532), and the positive pregnancy test, clinical pregnancy, ongoing pregnancy, and pregnancy loss rates were comparable among cohorts (Supplementary Table SI).

A subsequent analysis was performed of IVF outcomes of patients with exposure to CC in <90 days prior to ET according to the type of stimulation in which CC was utilized prior to the FET preparation cycle. CC was used for COS in 334 patients and CC was utilized for ovulation induction (OI) in 259 cases. When compared with patients unexposed or patients that utilized CC >90 days from the ET, no differences were observed in positive pregnancy test, clinical pregnancy, ongoing pregnancy, biochemical pregnancy loss, and clinical pregnancy loss rates among groups (Supplementary Table SII).

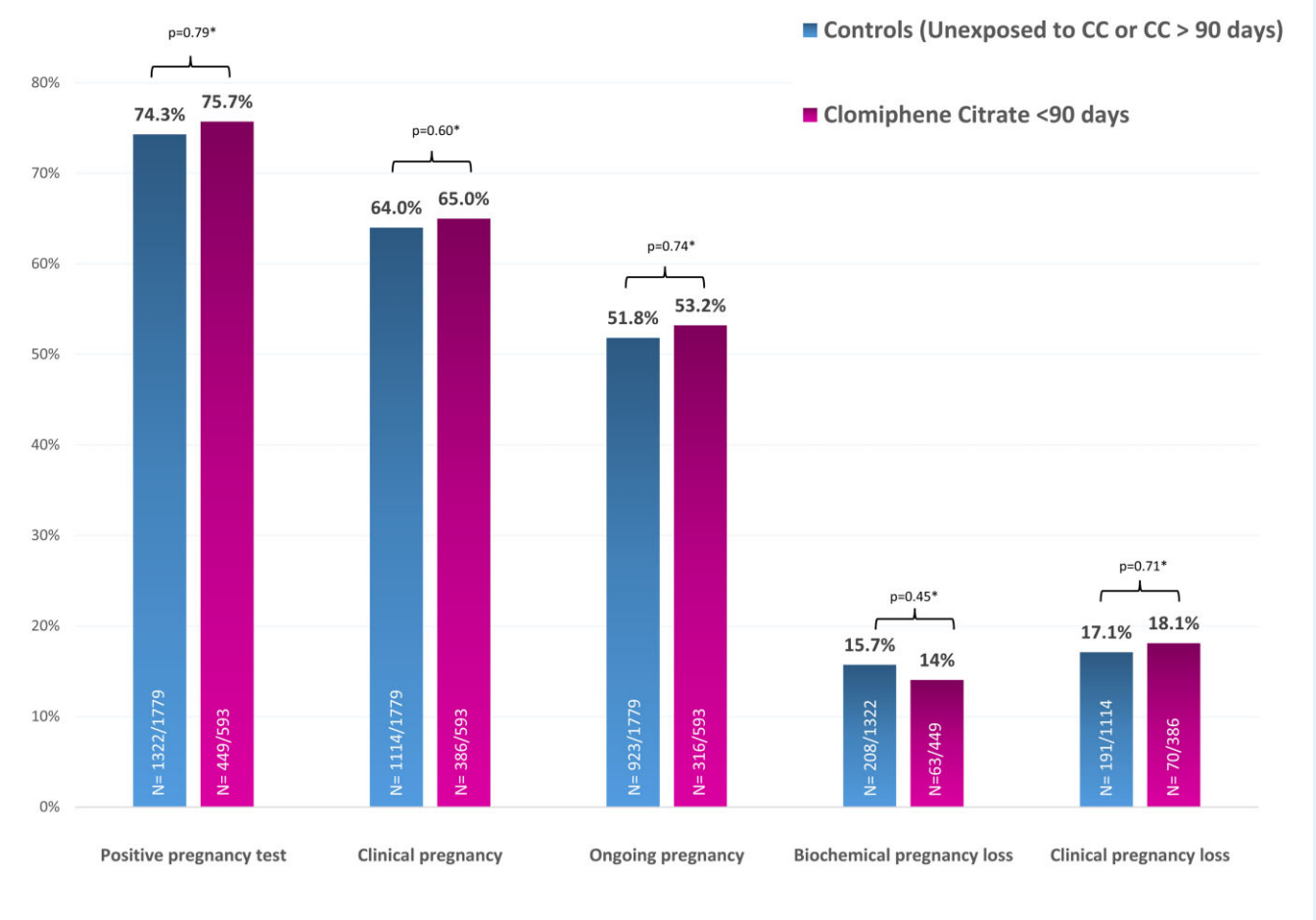

Figure 1. IVF outcomes comparison between groups categorized by clomiphene citrate exposure. N: sample size; CC: clomiphene citrate. *Chi-squared test.

In a multivariate regression analysis fitted with a GEE, after adjusting for patient age at ET, embryonic grade, prior number of CC cycles, and prior number of euploid ETs per patient, no association was found between utilization of CC in <90 days from the ET and lower positive pregnancy test rates (aOR 0.95, CI 95% 0.76–1.18). Similarly, no association was found with lower CPR (aOR 0.99, CI 95% 0.80–1.21) and OPR (aOR 0.96, CI 95% 0.79–1.16). Furthermore, no association was found between CC exposure and higher odds of biochemical pregnancy loss (aOR 1.09, CI 95% 0.81–1.48) or clinical pregnancy loss (aOR 1.10, CI 95% 0.82–1.49).

Another sub-analysis was performed analyzing multiple periods of days since the last CC utilization and ET of a single euploid blastocyst. Cohorts for this analysis were separated into periods of <30 days of CC utilization (Group A, n = 6); 31–60 days (Group B, n = 223); 61–90 days (Group C, n = 365); >90 days (Group D, n = 534); and patients unexposed to CC (Group E, n = 1244). IVF outcomes are depicted in Fig. 2. No significant differences were found in positive pregnancy test, CPR, OPR, BPL, and CPL rates among all groups. No differences were found in endometrial thickness at the time of ET among cohorts analyzed ($P = 0.14$).

A final sub-analysis was performed to assess the impact of cumulative and consecutive cycles of CC and CC dosages on implantation rates after a SEET. Cohorts were categorized as: <3 cumulative cycles (Group 1), n = 856; 4–6 cycles (Group 2), n = 240; >6 cycles (Group 3), n = 31; and unexposed patients (Group 4), n = 1245. No difference was found in endometrial thickness at ET among cohorts (Group 1: mean 9.7 SD 2.3 mm; Group 2: 9.7 ± 2.2; Group 3: 10.2 ± 2.7; and Group 4: 9.5 ± 2.1, $P = 0.09$). A significant difference was found among the median and IQR dosage of cumulative CC utilized (Group 1: 1000 mg IQR 1000; Group 2: 2000 mg IQR 1000; Group 3: 4000 mg IQR 1500 and Group 4: 0 mg IQR 0, $P = <0.0001$). No differences were found in positive pregnancy test rates, CPR, OPR, BPL, and CPL rates among the cohorts (Fig. 3).

In a multivariate analysis fitted with a GEE and using as a reference Group 4 (patients unexposed to CC) after adjusting for age, BMI, embryo grading, endometrial thickness at ET, and prior euploid ETs per patient, no association was found between higher number of cumulative CC cycles and lower odds of implantation, clinical pregnancy, and ongoing pregnancy rates. Finally, no association was found with

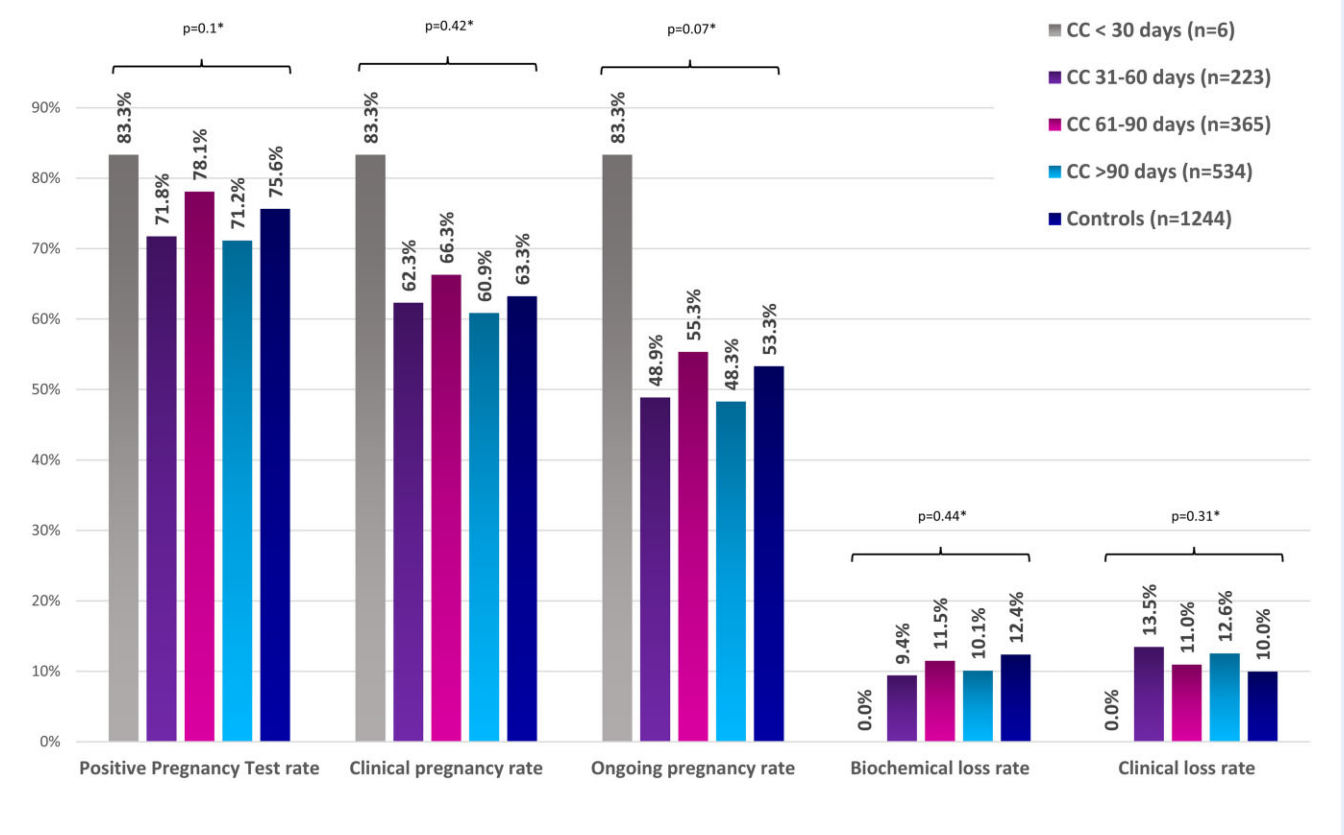

Figure 2. IVF outcome comparisons of different clomiphene citrate exposure time categories and unexposed patients. N: sample size; CC: clomiphene citrate. *Chi-squared test.

increased odds of biochemical pregnancy loss and clinical pregnancy loss rates (Table II).

Discussion

CC has been utilized in different ART treatment regimens for many decades. Despite its effectiveness and safety profile, some concerns surrounding its anti-estrogenic effects have kept clinicians cautious toward CC use and its potentially adverse effects on pregnancy outcomes. Our study findings suggest that patients who were exposed to CC are not at risk of impaired implantation, clinical pregnancy, and ongoing pregnancy rates. Even after adjusting for some subtle clinical differences found among both cohorts, such as age at ET, and other important covariates, such as embryo grading and prior euploid ETs, we found that there is no association of CC utilization with lower implantation, clinical pregnancy, and ongoing pregnancy rates. Also, there was no association with increased biochemical and clinical pregnancy loss rates. Furthermore, while looking only at the first ET in this same populations, we observed no significant differences in positive pregnancy test and pregnancy loss rates among cohorts, and finally, no differences were found regardless of whether CC was used for COS or as a ovulation induction agent.

Clinical scrutiny of the pharmacological properties of CC has been highly controversial. Prior studies have suggested that CC may cause lower pregnancy rates in distinct protocols of ART treatment (Dickey *et al.*, 1965; Garcia *et al.*, 1977; Franks *et al.*, 1985; Gadalla *et al.*, 2018) and increased pregnancy loss rates (Goldfarb *et al.*, 1968). These undesirable outcomes have been attributed to a direct anti-estrogenic effect of clomiphene on the endometrium. Moreover, some authors had hypothesized that the utilization of CC is associated with altered structural development of the endometrium, clinically observed as a thinning of the endometrial lining (Randall and Templeton, 1991; Practice Committee of the American Society for Reproductive Medicine, 2003; Zollner *et al.*, 2003; Chaube *et al.*, 2005; Cha *et al.*, 2012; Mahajan and Sharma, 2016; Weiss *et al.*, 2017). This clinical effect was likely caused by an inhibition of epithelial cell proliferation and estrogen response element transactivation in the endometrial tissue (Amita *et al.*, 2010), as well as decreased pinopode cell formation (Creus *et al.*, 2003). Besides the endometrial lining thinning effect, other researchers had proposed that CC could alter endometrial receptivity by different mechanisms such inhibition of prostaglandin synthesis (Lerner *et al.*, 1975). Furthermore, CC also diminishes the expression of estrogen and progesterone receptors in the endometrium, and downregulates several proteins, growth factors, and cytokines related to epithelial function, adhesion, cell invasion, and

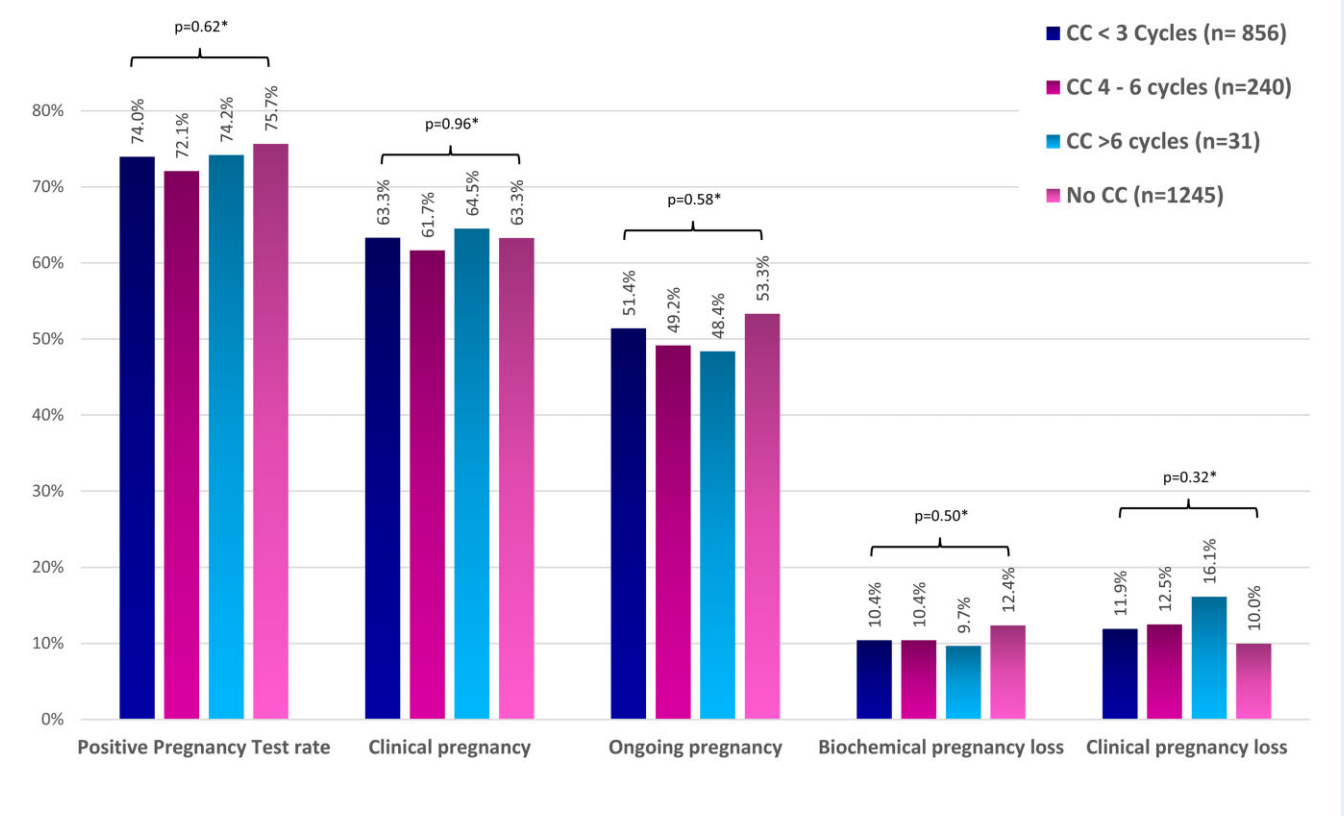

Figure 3. IVF outcomes based on different clomiphene citrate cumulative cycles before embryo transfer. N: sample size; CC: clomiphene citrate. *Chi-squared test.

Table II Multivariate regression analysis for IVF outcomes based on different clomiphene citrate cumulative cycles before embryo transfer.

	Group 1 (CC <3 cycles)	Group 2 (CC 4–6 cycles)	Group 3 (CC >6 cycles)	Group 4 (unexposed)
	aOR (95% CI)	aOR (95% CI)	aOR (95% CI)	
Positive pregnancy test rate	0.90 (0.7–1.1)	0.80 (0.5–1.1)	0.70 (0.2–1.8)	Reference
Clinical pregnancy rate	1.003 (0.8–1.2)	0.88 (0.6–1.2)	0.73 (0.3–1.60)	Reference
Ongoing pregnancy rate	0.94 (0.7–1.15)	0.8 (0.6–1.1)	0.71 (0.2–1.8)	Reference
Biochemical loss rate	0.80 (0.5–1.09)	0.83 (0.5–1.38)	1.008 (0.3–3.3)	Reference
Clinical pregnancy loss rate	1.18 (0.8–1.7)	1.25 (0.7–2.14)	1.15 (0.3–3.9)	Reference

Data presented as adjusted odds ratios (aORs) with 95% CI.
CC: clomiphene citrate.

decidualization during the implantation window (Palomino *et al.*, 2005; Bao *et al.*, 2009; Wallace *et al.*, 2011; Valdez-Morales *et al.*, 2015; Chen *et al.*, 2016; Mehdinejadiani *et al.*, 2019). Moreover, CC has been associated with suboptimal uterine vascular flows (Ng *et al.*, 2006; Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group, 2008; Omran *et al.*, 2018), abnormal endometrial tissue maturation (Yeko *et al.*, 1992; Reed *et al.*, 2018; Montenegro *et al.*, 2021), and detrimental effects on cervical mucus (Marchini *et al.*, 1989; Massai *et al.*, 1993).

Our main findings demonstrate no detrimental effect of recent CC exposure on IVF outcomes. Our findings contrast with the results published by Nakagawa *et al.*, in which they observed that patients who were exposed to CC within 90 days of ET experienced significantly lower pregnancy rates than those who were not exposed to CC. Those authors recommended a 90-day wash-out window before attempting the transfer of a frozen embryo in patients utilizing CC medication for ovarian stimulation (Nakagawa *et al.*, 2014); it is important to mention that the study findings were based on analysis of a

relatively small sample size (219 CC exposure patients (<90 days prior to ET); 159 control patients). Also, the study included multiple cleavage stage ETs that were performed in fresh endometrial stimulated cycles. Those factors that could explain the relatively low pregnancy rates found (control group 36.7% and exposed patient cohort 25.1%, ($P < 0.05$)). As described previously, cleavage stage transfers and fresh ETs could yield lower implantation rates than blastocyst transfers and FETs (Zaat et al., 2021; Glujovsky et al., 2022). In contrast, the results of our study showed comparable positive pregnancy test rates after the transfer of single euploid FETs over synthetically prepared endometrium (74.3% control group versus 75.7% in patients exposed to <90 days of CC, $P = 0.49$). Our IVF outcomes and findings correlate with another study by Kato et al. (2018) in which the authors found no differences in pregnancy rates and neonatal outcomes in patients exposed to <60 days of CC after a single vitrified-warmed blastocyst transfer. Furthermore, no association was found for the duration between the last day of CC administration and the day of the blastocyst transfer (Kato et al., 2018). Those findings agree with our adjusted analysis in which we observed no associations with decreased positive pregnancy test, clinical and ongoing pregnancy rates, or increased rates of pregnancy losses after adjusting for potential cofounders and covariates.

Another important circumstance to consider is the possible detrimental effect of cumulative CC exposure. This question is paramount as it is common for patients to undergo multiple consecutive cycles of CC prior to high complexity ART. To date, no study has analyzed the impact of consecutive CC cycles on implantation rates in vitrified-warmed euploid blastocyst transfer cycles. This is relevant for any reproductive endocrinologist and clinical data are lacking. Commercially available CC is a mixture of two diastereoisomers: enclomiphene (62%) and zuclomiphene (38%) (Ernst et al., 1976). The trans-isomer, enclomiphene, is described as having a higher response on receptors and is primarily responsible for the ovulation-inducing actions of CC (Clark and Markaverich, 1981). In pharmacokinetic studies, enclomiphene levels are observed to rapidly rise after administration and fall to undetectable concentrations soon thereafter, with a half-life: 10.5 h (Mikkelson et al., 1986). The other cis-isomer, zuclomiphene, is eliminated in a slower period of time. The serum levels of zuclomiphene remain detectable for more than 30 days after administration and may accumulate over consecutive cycles of treatment (Young et al., 1999). Based on these properties, some authors had hypothesized that long-lasting estrogen receptor occupancy by CC might alter endometrial cell function, thus disturbing the implantation window (Palomino et al., 2005). In our study, we performed a sub-analysis including multiple cohorts of patients exposed to CC over consecutive cycles before ET. We observed that the positive pregnancy test, clinical pregnancy, and ongoing pregnancy rates were similar among all the cohorts analyzed. Also, there were no differences in biochemical pregnancy and clinical pregnancy losses. Even in an adjusted analysis controlling for important covariates, we observed no association between cumulative CC cycles and a detrimental effect on embryo implantation rates. So, besides a potential cumulative effect of clomiphene isomers and their pharmacokinetic/pharmacodynamic properties impacting on endometrial and uterine tissues, no evidence surfaced to display impairing effects on clinical IVF outcomes in this study.

Finally, the main analysis and sub-analysis showed that endometrial thickness at the time of the ET was similar among all compared cohorts, suggesting the effect of CC on impaired endometrial growth or receptivity, if present, to be short-lasting and associated only with the time throughout cycle when the medications are being utilized, especially during the course of fresh ET cycles or ovulation induction cycles (Palomino et al., 2005; Nakagawa et al., 2014; Gadalla et al., 2018; Hawkins Bressler et al., 2021). Therefore, our findings suggest that there is no clinical long-lasting or cumulative effect of CC on endometrial development and implantation rates during a vitrified/thawed SEET cycle.

Above and beyond our best efforts to avoid biases, this study is not without limitations. Primarily, the inherent bias originated from its retrospective design. Also, CC serum and tissue levels were not measured and not incorporated into the study design since measuring drug levels is not part of our standard clinical operating procedures. An important limitation to consider is that in the main analysis subtle population differences can be found mostly owing to the difference in time between CC exposure and the ET in the CC exposed group, and the longer interval time to ET in the control group; differences that can be attributed because some patients in the dataset had a failed ETs or different outcomes in prior cycles, although a sub-analysis showed that the majority of the included cases was patients undergoing their first ET after the COS cycle. In the sensitivity analysis, while exclusively observing patients that underwent the first ET after COS we observed no significant differences in the analyzed outcomes, and, furthermore, in the multivariate analysis, we adjusted for these differences between the populations and for other important confounding variables. The final analysis showed that these differences did not significantly affect the outcomes analyzed. Another limitation is that the populations analyzed could be heterogeneous in nature because of their multiple infertility diagnoses, although we excluded patients with a diagnosis of recurrent implantation failure, recurrent pregnancy loss, patients with uterine factor, patients with known chromosomal rearrangements and cases involving multiple TE biopsies, and/or multiple thaw/freeze procedures. Likewise, we utilized a propensity score-matched control group to improve the comparability of the controls and interest populations, and we utilized an adjusted multivariate analysis with a GEE model trying to control for potential confounders and covariables, including the same patient appearing multiple times in the same dataset. Lastly, of all the studies analyzing CC and its pharmacological consequences on FETs, our study includes the largest population analyzed to date of chromosomally screened embryos via next-generation sequencing that were transferred in a SEET protocol. This approach helps us to aptly analyze the potential effect of the CC on implantation rates by excluding the potential failure caused by embryonic aneuploidy that exists in published studies and, thus, potentially confounding the theoretical clinical consequences of CC on implantation rates after IVF. Nonetheless, our sub-analysis findings must be taken with caution owing to the increased risk of bias caused by the limited sample sizes, the consequence of creating multiple groups for data exploration.

Further clinical and pharmacogenomic research is needed and should focus on prospectively analyzing serum and tissue CC concentrations along with multiomic profiles of endometrial cellular sub-types or endometrial organoids in order to advance our understanding and provide foundational information on the deep mechanisms of endometrial proliferation, receptivity, and the potential pharmacological anti-estrogenic effects of CC.

In conclusion, there does not appear to be an association between recent CC exposure and lower implantation potential in patients who undergo FET of euploid embryos. This finding remains consistent even when patients who undergo multiple, consecutive CC cycles prior to ET are included. There were no long-term effects of CC on endometrial development and the clinical characteristics assessed in this study. Patients that utilized CC medication prior a SEET cycle, either for ovarian stimulation or ovulation induction, can be assured that there is no evidence of a residual effect of recent CC administration that could jeopardize their pregnancy probability.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Data availability

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author upon reasonable request.

Acknowledgements

The authors thank all the physicians, fellows, embryologists, research, and staff members for all the valuable work and help in realizing this article.

Authors' roles

All authors have made substantial contributions to the conception, design, performance, analysis, and writing of the study. C.H.-N. participated in the conception, design, acquisition of data, analysis of data, drafting of article, and final approval. J.L. participated in the drafting of article and final approval. T.A.-M. participated in acquisition of data and the drafting of article and final approval. L.S.-C. participated in the drafting of article and final approval. B.S. participated in the drafting of article and final approval. A.C. participated in conception, drafting of article, and final approval. T.M. participated in conception, design, drafting of article, and final approval.

Funding

No funding was obtained for the realization of this article.

Conflict of interest

A.C. is advisor and/or board member of Sema4 and Progyny. The remaining co-authors do not have any conflicts of interest.

References

Amita M, Takahashi T, Tsutsumi S, Ohta T, Takata K, Henmi N, Hara S, Igarashi H, Takahashi K, Kurachi H. Molecular mechanism of the inhibition of estradiol-induced endometrial epithelial cell proliferation by clomiphene citrate. *Endocrinology* 2010;**151**: 394–405.

Bao SH, Sheng SL, Peng YF, Lin QD. Effects of letrozole and clomiphene citrate on the expression of HOXA10 and integrin alpha v beta 3 in uterine epithelium of rats. *Fertil Steril* 2009;**91**: 244–248.

Bonhoff AJ, Naether OG, Johannisson E. Effects of clomiphene citrate stimulation on endometrial structure in infertile women. *Hum Reprod* 1996;**11**:844–849.

Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med* 2012;**18**:1754–1767.

Chaube SK, Prasad PV, Thakur SC, Shrivastav TG. Estradiol protects clomiphene citrate-induced apoptosis in ovarian follicular cells and ovulated cumulus-oocyte complexes. *Fertil Steril* 2005;**84**(Suppl 2): 1163–1172.

Chen C, Yan Q, Liu K, Zhou X, Xian Y, Liang D, Zhao X, Guo X, Quan S. Endometrial receptivity markers in mice stimulated with raloxifene versus clomiphene citrate and natural cycles. *Reprod Sci* 2016;**23**:748–755.

Clark JH, Markaverich BM. The agonistic-antagonistic properties of clomiphene: a review. *Pharmacol Ther* 1981;**15**:467–519.

Creus M, Ordi J, Fábregues F, Casamitjana R, Carmona F, Cardesa A, Vanrell JA, Balasch J. The effect of different hormone therapies on integrin expression and pinopode formation in the human endometrium: a controlled study. *Hum Reprod* 2003;**18**: 683–693.

Dickey RP, Vorys N, Stevens VC, Besch PK, Hamwi GJ, Ullery JC. Observations on the mechanism of action of clomiphene (MRL-41). *Fertil Steril* 1965;**16**:485–494.

Eden JA, Place J, Carter GD, Jones J, Alaghband-Zadeh J, Pawson ME. The effect of clomiphene citrate on follicular phase increase in endometrial thickness and uterine volume. *Obstet Gynecol* 1989;**73**: 187–190.

Ernst S, Hite G, Cantrell JS, Richardson A Jr, Benson HD. Stereochemistry of geometric isomers of clomiphene: a correction of the literature and a reexamination of structure-activity relationships. *J Pharm Sci* 1976;**65**:148–150.

Franks S, Adams J, Mason H, Polson D. Ovulatory disorders in women with polycystic ovary syndrome. *Clin Obstet Gynaecol* 1985; **12**:605–632.

Gadalla MA, Huang S, Wang R, Norman RJ, Abdullah SA, El Saman AM, Ismail AM, van Wely M, Mol BWJ. Effect of clomiphene citrate on endometrial thickness, ovulation, pregnancy and live birth in anovulatory women: systematic review and meta-analysis. *Ultrasound Obstet Gynecol* 2018;**51**:64–76.

Garcia J, Jones GS, Wentz AC. The use of clomiphene citrate. *Fertil Steril* 1977;**28**:707–717.

Ghobadi C, Mirhosseini N, Shiran MR, Moghadamnia A, Lennard MS, Ledger WL, Rostami-Hodjegan A. Single-dose pharmacokinetic study of clomiphene citrate isomers in anovular patients with polycystic ovary disease. *J Clin Pharmacol* 2009;**49**:147–154.

Glujovsky D, Quinteiro Retamar AM, Alvarez Sedo CR, Ciapponi A, Cornelisse S, Blake D. Cleavage-stage versus blastocyst-stage embryo transfer in assisted reproductive technology. *Cochrane Database Syst Rev* 2022;**5**:CD002118.

Goldfarb AF, Morales A, Rakoff AE, Protos P. Critical review of 160 clomiphene-related pregnancies. *Obstet Gynecol* 1968;**31**: 342–345.

Hawkins Bressler L, Fritz MA, Wu SP, Yuan L, Kafer S, Wang T, DeMayo FJ, Young SL. Poor endometrial proliferation after clomiphene is associated with altered estrogen action. *J Clin Endocrinol Metab* 2021;**106**:2547–2565.

Hernandez-Nieto C, Lee JA, Alkon-Meadows T, Luna-Rojas M, Mukherjee T, Copperman AB, Sandler B. Late follicular phase progesterone elevation during ovarian stimulation is not associated with decreased implantation of chromosomally screened embryos in thaw cycles. *Hum Reprod* 2020;**35**:1889–1899.

Hernandez-Nieto C, Lee JA, Slifkin R, Sandler B, Copperman AB, Flisser E. What is the reproductive potential of day 7 euploid embryos? *Hum Reprod* 2019;**34**:1697–1706.

Kamath MS, Maheshwari A, Bhattacharya S, Lor KY, Gibreel A. Oral medications including clomiphene citrate or aromatase inhibitors with gonadotropins for controlled ovarian stimulation in women undergoing in vitro fertilisation. *Cochrane Database Syst Rev* 2017; **11**:CD008528.

Kato K, Ezoe K, Yabuuchi A, Fukuda J, Kuroda T, Ueno S, Fujita H, Kobayashi T. Comparison of pregnancy outcomes following fresh and electively frozen single blastocyst transfer in natural cycle and clomiphene-stimulated IVF cycles. *Hum Reprod Open* 2018;**2018**: hoy006.

Kerin JF, Liu JH, Phillipou G, Yen SS. Evidence for a hypothalamic site of action of clomiphene citrate in women. *J Clin Endocrinol Metab* 1985;**61**:265–268.

Kupesic S, Kurjak A. Uterine and ovarian perfusion during the periovulatory period assessed by transvaginal color Doppler. *Fertil Steril* 1993;**60**:439–443.

Lerner EJ, Carminati P, Schiatti P. Correlation of anti-inflammatory activity with inhibition of prostaglandin synthesis activity of nonsteroidal anti-estrogens and estrogens (38532). *Proc Soc Exp Biol Med* 1975;**148**:329–332.

Mahajan N, Sharma S. The endometrium in assisted reproductive technology: how thin is thin? *J Hum Reprod Sci* 2016;**9**:3–8.

Marchini M, Dorta M, Bombelli F, Ruspa M, Campana A, Dolcetta G, Radici E. Effects of clomiphene citrate on cervical mucus: analysis of some influencing factors. *Int J Fertil* 1989;**34**:154–159.

Maruncic M, Casper RF. The effect of luteal phase estrogen antagonism on luteinizing hormone pulsatility and luteal function in women. *J Clin Endocrinol Metab* 1987;**64**:148–152.

Massai MR, de Ziegler D, Lesobre V, Bergeron C, Frydman R, Bouchard P. Clomiphene citrate affects cervical mucus and endometrial morphology independently of the changes in plasma hormonal levels induced by multiple follicular recruitment. *Fertil Steril* 1993;**59**:1179–1186.

Mehdinejadiani S, Amidi F, Mehdizadeh M, Barati M, Pazhohan A, Alyasin A, Mehdinejadiani K, Sobhani A. Effects of letrozole and clomiphene citrate on Wnt signaling pathway in endometrium of polycystic ovarian syndrome and healthy women. *Biol Reprod* 2019; **100**:641–648.

Mikkelson TJ, Kroboth PD, Cameron WJ, Dittert LW, Chungi V, Manberg PJ. Single-dose pharmacokinetics of clomiphene citrate in normal volunteers. *Fertil Steril* 1986;**46**:392–396.

Montenegro IS, Kuhl CP, Schneider RA, Zachia SA, Durli ICLO, Terraciano PB, Rivero RC, Passos EP. Use of clomiphene citrate protocol for controlled ovarian stimulation impairs endometrial maturity. *JBRA Assist Reprod* 2021;**25**:90–96.

Nakagawa K, Kaneyama M, Nishi Y, Sugiyama R, Motoyama H, Sugiyama R. Clomiphene citrate affects the receptivity of the uterine endometrium. *Reprod Med Biol* 2014;**14**:73–78.

Nazem TG, Sekhon L, Lee JA, Overbey J, Pan S, Duke M, Briton-Jones C, Whitehouse M, Copperman AB, Stein DE. The correlation between morphology and implantation of euploid human blastocysts. *Reprod Biomed Online* 2019;**38**:169–176.

Ng EH, Chan CC, Tang OS, Yeung WS, Ho PC. Relationship between uterine blood flow and endometrial and subendometrial blood flows during stimulated and natural cycles. *Fertil Steril* 2006; **85**:721–727.

Omran E, El-Sharkawy M, El-Mazny A, Hammam M, Ramadan W, Latif D, Samir D, Sobh S. Effect of clomiphene citrate on uterine hemodynamics in women with unexplained infertility. *Int J Womens Health* 2018;**10**:147–152.

Palomino WA, Fuentes A, González RR, Gabler F, Boric MA, Vega M, Devoto L. Differential expression of endometrial integrins and progesterone receptor during the window of implantation in normo-ovulatory women treated with clomiphene citrate. *Fertil Steril* 2005;**83**:587–593.

Practice Committee of the American Society for Reproductive Medicine. Use of clomiphene citrate in women. *Fertil Steril* 2003; **80**:1302–1308.

Randall JM, Templeton A. Cervical mucus score and in vitro sperm mucus interaction in spontaneous and clomiphene citrate cycles. *Fertil Steril* 1991;**56**:465–468.

Reed BG, Wu JL, Nemer LB, Carr BR, Bukulmez O. Use of Clomiphene Citrate in minimal stimulation in vitro fertilization negatively impacts endometrial thickness: an argument for a freeze-all approach. *JBRA Assist Reprod* 2018;**22**:355–362.

Thessaloniki ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group. Consensus on infertility treatment related to polycystic ovary syndrome. *Hum Reprod* 2008;**23**:462–477.

Valdez-Morales FJ, Gamboa-Domínguez A, Vital-Reyes V, Cruz JC, Chimal-Monroy J, Franco-Murillo Y, Cerbón M. Changes in receptivity epithelial cell markers of endometrium after ovarian stimulation treatments: its role during implantation window. *Reprod Health* 2015;**12**:45.

Wallace KL, Johnson V, Sopelak V, Hines R. Clomiphene citrate versus letrozole: molecular analysis of the endometrium in women with polycystic ovary syndrome. *Fertil Steril* 2011;**96**:1051–1056.

Weiss NS, van Vliet MN, Limpens J, Hompes PGA, Lambalk CB, Mochtar MH, van der Veen F, Mol BWJ, van Wely M. Endometrial thickness in women undergoing IUI with ovarian stimulation. How thick is too thin? A systematic review and meta-analysis. *Hum Reprod* 2017;**32**:1009–1018.

Yeko TR, Nicosia SM, Maroulis GB, Bardawil WA, Dawood MY. Histology of midluteal corpus luteum and endometrium from clomiphene citrate-induced cycles. *Fertil Steril* 1992;**57**:28–32.

Young SL, Opsahl MS, Fritz MA. Serum concentrations of enclomiphene and zuclomiphene across consecutive cycles of clomiphene citrate therapy in anovulatory infertile women. *Fertil Steril* 1999;**71**:639–644.

Zaat T, Zagers M, Mol F, Goddijn M, van Wely M, Mastenbroek S. Fresh versus frozen embryo transfers in assisted reproduction. *Cochrane Database Syst Rev* 2021;**2**:CD011184.

Zegers-Hochschild F, Adamson GD, Dyer S, Racowsky C, de Mouzon J, Sokol R, Rienzi L, Sunde A, Schmidt L, Cooke ID *et al*. The international glossary on infertility and fertility care, 2017. *Hum Reprod* 2017;**32**:1786–1801.

Zhang Y, Zhang C, Shu J, Guo J, Chang HM, Leung PCK, Sheng JZ, Huang H. Adjuvant treatment strategies in ovarian stimulation for poor responders undergoing IVF: a systematic review and network meta-analysis. *Hum Reprod Update* 2020;**26**:247–263.

Zollner U, Zollner KP, Blissing S, Pöhls U, Steck T, Dietl J, Müller T. Impact of three-dimensionally measured endometrial volume on the pregnancy rate after intrauterine insemination. *Zentralbl Gynakol* 2003;**125**:136–141.