



# Risk factors for monozygotic twinning after in vitro fertilization: a systematic review and meta-analysis

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**Objectives:** To establish the risk factors for monozygotic twin (MZT) and monochorionic twin (MCT) pregnancies after in vitro fertilization (IVF).

**Design:** Systematic review and meta-analysis.

**Setting:** Not applicable.

**Patient(s):** Women who achieved MZT and non-MZT pregnancies through IVF.

**Intervention(s):** Systematic search of Medline from January 1995 to October 2018 with cross-checking of references from relevant articles in English.

**Main Outcome Measure(s):** Possible risk factors for MZT or MCT pregnancies after IVF, comprising extended embryo culture, insemination method (conventional IVF and intracytoplasmic sperm injection [ICSI]), embryo biopsy for preimplantation genetic testing for aneuploidies or for monogenic/single-gene defects (PGT-A or PGT-M) programs, assisted hatching (AH), oocytes donation, female age, and embryo cryopreservation.

**Result(s):** A total of 40 studies were included. Blastocyst transfer compared with cleavage-stage embryo transfer, and female age <35 years were associated with a statistically significant increase in the MZT and MCT pregnancy rate after IVF: (23 studies, OR 2.16, 95% CI, 1.74–2.68,  $I^2=78\%$ ; 4 studies, OR 1.29; 95% CI, 1.03–1.62,  $I^2=62\%$ ; and 3 studies, OR 1.90, 95% CI, 1.21–2.98,  $I^2=59\%$ ; 2 studies, OR 2.34; 95% CI, 1.69–3.23,  $I^2=0$ , respectively). Conventional IVF compared with ICSI and assisted hatching were associated with a statistically significantly increased risk of MZT pregnancy (9 studies, OR 1.19, 95% CI, 1.04–1.35,  $I^2=0$ ; 16 studies, OR 1.17, 95% CI, 1.09–1.27,  $I^2=29\%$ , respectively). Embryo biopsy for PGT-A or PGT-M, embryo cryopreservation, and oocytes donation were not associated with MZT pregnancies after IVF.

**Conclusion(s):** Blastocyst transfer is associated with an increased risk of both MZT and MCT pregnancies after IVF. Further evidence is needed to clarify the impact of female age, insemination method and AH on the investigated outcomes. (Fertil Steril® 2019;111:302–17.

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**El resumen está disponible en Español al final del artículo.**

**Key Words:** Blastocyst, IVF, monochorionic, monozygotic

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**M**ultiple pregnancy is considered a serious complication of assisted reproductive technology (ART) (1). Extended culture with embryo selection and elective single-embryo transfer is currently

recognized as the most effective means of reducing the incidence of multiple pregnancies (2). Nonetheless, the risks have yet to be completely eliminated, even with this strategy (3). In fact, elective single-embryo transfer cannot pre-

vent concurrent natural conception and embryo splitting, which are only seemingly noniatrogenic occurrences. Indeed, several publications have raised concern over the increased rate of monozygotic twin (MZT) pregnancies after ART when compared with natural conception (4). In an attempt to reach an estimation of the incidence of MZT pregnancies after ART, Vitthala et al. (5) conducted a meta-analysis that included 27 studies published between 1993 and 2007. The overall incidence of MZT pregnancies was 0.9% (95%

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confidence interval [CI], 0.8–0.9%). However, the majority of studies included were underpowered to estimate such an uncommon event. It is thus not surprising that many investigators subsequently have tried to provide a more realistic estimate by analyzing larger cohorts of patients who had undergone in vitro fertilization (IVF), including conventional IVF, intracytoplasmic sperm injection (ICSI), and fresh and frozen embryo transfer. It is interesting that almost all the studies reported higher incidences, ranging from 0.97% (95% CI, 0.47–1.99%) to 2.35% (95% CI, 2.07–2.67%) (4–20).

These results have raised considerable clinical concern. Monozygotic twin pregnancies carry a much higher risk of maternal and fetal complications than singleton and dizygotic pregnancies, including increased rates of premature delivery, growth discordance, developmental anomalies, and perinatal morbidity and mortality (19). Monochorionic placentation is associated with additional risks due to the specific angioarchitecture of monochorionic placentas, which can lead to the formation of intertwin anastomoses and thus to the development of pathologic conditions such as twin-twin transfusion syndrome, twin anemia-polycythemia sequence, single intrauterine fetal demise, and selective intrauterine growth restriction (11, 21, 22).

Many investigators have hypothesized that the aforementioned increased MZT rate is determined by factors associated with the IVF techniques themselves. Because MZT and monochorionic twin (MCT) pregnancies have worrying complications, we conducted a systematic review and meta-analysis to identify risk factors for their onset after IVF. In particular, our systematic review with meta-analysis addressed the question of whether extended embryo culture, the insemination method (conventional IVF and ICSI), embryo biopsy for preimplantation genetic testing for aneuploidies or for monogenic/single gene defects (PGT-A or PGT-M) programs, assisted hatching (AH), oocytes donation, female age, or embryo cryopreservation represented a risk factor for MZT/MCT pregnancies in couples undergoing IVF.

## MATERIALS AND METHODS

Our literature overview was conducted according to the PRISMA guidelines for systematic reviews (23, 24). Because published deidentified data were used, this study was exempt from institutional review board approval. Most of the published studies did not distinguish between monochorionic and dichorionic twin pregnancies. For this reason, we used the generic acronym “MZT” to refer to the monozygotic pregnancies reported in these studies. When the investigators specifically referred to monochorionic twin pregnancies, we used the acronym “MCT.”

### Sources

Our review was restricted to published research articles that investigated possible risk factors for MZT or MCT pregnancies after IVF. We searched the Medline database for publications dating from January 1995 to July 2018. The searches were limited to studies in humans and were conducted using the following terms: monozygotic AND in vitro fertilisation OR IVF OR intracytoplasmic sperm injection OR ICSI OR assisted

hatching OR blastocyst OR cleavage stage embryos OR preimplantation genetic testing for aneuploidies OR PGT-A OR preimplantation genetic testing for monogenic/single gene defects OR PGT-M OR female age OR maternal age OR oocytes donation OR eggs donation OR frozen embryos OR thawed embryos. We repeated the same search replacing the term “monozygotic” with the term “monochorionic.” The last search was performed on October 15, 2018.

Published cohort (retrospective or prospective), case control studies, and randomized clinical trials were eligible for inclusion. All pertinent articles were retrieved, and the relative reference lists were systematically reviewed to identify further reports that could be included in the meta-analysis. Moreover, review articles and meta-analysis published on MZT pregnancies during the same time span were consulted as well, we searched their reference lists for potential additional studies. No attempt was made to identify unpublished studies.

## Study Selection and Quality Assessment

Two authors (A.B. and C.D.) independently performed an initial screening of all the titles and abstracts to exclude any citations deemed irrelevant by both observers. In cases of doubt, the studies were discussed in consensus meetings with two other authors (L.F. and A.P.). Studies were excluded if [1] the clinical pregnancy rate was not reported, [2] ART procedures were used other than IVF (which comprises conventional IVF, ICSI, fresh and frozen embryo transfer), [3] crude or adjusted effect estimates with corresponding 95% CI or results allowing calculation of odds ratios (OR) were not reported, or [4] data overlap between two studies was observed. Case reports, letters to the editor, and reviews were also excluded.

The diagnosis of chorionicity was considered adequate if it was determined before 13 + 6 weeks of gestation by identification of the “T” sign or the “lambda” sign, measurement of the intertwin membrane thickness, or determination of the number of placental masses (25). Studies reporting different or less accurate diagnostic methods were excluded.

Reports were classified according to the study design into case control studies, prospective and retrospective cohort studies, or randomized clinical trials. The quality of case control and cohort studies was evaluated by means of the Newcastle-Ottawa scale, a validated tool for assessing the quality of observational and nonrandomized studies (26). The scale uses a score system based on three major criteria: selection of participants, comparability of study groups, and assessment of exposure. The quality checklist includes eight items with a score of either 0 or 1 for each item except for “comparability of cohorts,” where a score of 0, 1, or 2 can be awarded. Therefore, the quantitative appraisal of the overall quality of each individual study ranged from 0 to 9. No cutoff score was set for inclusion in the meta-analysis. To gain insight into the methodological quality and validity of the trials we used the CONSORT 2010 checklist (27).

## Data Extraction and Analysis

Three authors (A.B., E.S., and M.R.) independently evaluated all articles and extrapolated the data on standardized forms.

A final abstraction form was compiled from the three evaluation forms, after resolution of all the discrepancies among reviewers through a discussion with the two remaining authors. The year of publication, location, study design, study period, type of placentation, MZT pregnancies diagnosis method, embryo culture media used, and investigated risk factors were recorded.

The investigated risk factors were as follows: embryo extended culture (blastocyst transfer vs. cleavage-stage embryo transfer), AH, insemination method (conventional IVF and ICSI), embryo biopsy for PGT-A or PGT-M, female age, oocytes donation, and frozen-thawed embryo transfer. The risk factors for monochorionic pregnancies were investigated separately.

From each selected article we extracted the information on study characteristics, incidence of MZT or MCT pregnancies, and quality of the evidence. Data were used to construct  $2 \times 2$  tables reporting the investigated risk factor and the number of MZT or MCT pregnancies and singleton pregnancies. So that we could compare the data from various studies, we harmonized the definitions and cutoffs of risk factors among the studies whenever possible.

Irrespective of the method used in the original publications, our results are expressed as OR with 95% CI (28). Risk estimates greater than 1 indicate an increased risk of the defined outcome; risk estimates less than 1 indicate a decreased risk of the defined outcome. We assessed statistical significance using 95% CI: if the 95%CI did not include the neutral value 1, we considered the risk statistically significant.

The inconsistency of the studies' results was measured using Cochran Q and the  $I^2$  statistic (29). Negative values of  $I^2$  are set equal to 0 so that  $I^2$  lies between 0 and 100%. According to the *Cochrane Handbook for Systematic Reviews of Intervention*, an  $I^2$  value of 0 indicates no observed heterogeneity, whereas  $I^2$  values from 30% to 60% may represent moderate heterogeneity,  $I^2$  values from 50% to 90% may represent substantial heterogeneity, and  $I^2$  values from 75% to 100% represent considerable heterogeneity (24, 29). If the  $I^2$  values indicated moderate, substantial, or considerable heterogeneity, we conducted sensitivity analyses to verify whether any one of the included studies unduly influenced the pooled effect size.

The OR were combined in a meta-analysis using a fixed-effects model when the heterogeneity found among the studies was absent to moderate ( $0 \leq I^2 < 30\%$ ). When heterogeneity was moderate, substantial, or considerable ( $I^2 \geq 30\%$ ), we used the DerSimonian and Laird method (30, 31) for a random-effects model (28).

Funnel plots, which graph OR on a log scale (effect) against standard error of log-OR (precision), were generated and visually inspected for asymmetry to determine whether the included studies were nonrepresentative of the body of possible studies on the subject (as could result from a small-study effect or other biases, such as publication and poor-quality bias). The approach by Egger et al. (32) was used to test the significance of funnel plot asymmetry (32). All analyses were performed using Review Manager 5 (RevMan 5; Cochrane Collaboration) or Stata, version 13 (StataCorp).

## RESULTS

### Results of Search and Description of Studies

Supplemental Figure 1 (available online) summarizes the process of literature identification and selection of studies for the risk factors assessment. Our literature searches yielded 264 studies, from which 16 duplicates were removed. After a review of the titles and abstracts, 68 studies were identified as potentially eligible for inclusion. After a full review, we excluded four systematic reviews or meta-analysis (33–36), 12 case reports (2, 33, 37–46), two letters to the editor (47, 48) five publications because the data were not extractable (11, 17, 49–51), three publications because the monozygotic clinical pregnancy rates were not reported (3, 52), and one publication because ART techniques other than conventional IVF and ICSI were used (i.e., subzonal insemination) (53, 54). The two studies conducted by Knopman et al. (8, 13) featured considerable overlap, so the study published in 2010 was excluded (8). Data on the risk factors for MZT after IVF or ICSI were extracted from the remaining 40 articles, all of which were published in peer-reviewed journals between 1998 and 2018 (4, 6, 7, 9, 12–16, 19, 20, 55–82).

Details of the characteristics of the selected studies are shown in Table 1. One of the included studies was a prospective cohort study, 30 were retrospective cohort studies, one was a nested case-control study, and eight were prospective randomized trials. The potential risk factors that could be pooled included embryo extended culture (blastocyst transfer vs. cleavage-stage transfer), assisted hatching, fertilization technique used (ICSI vs. conventional IVF), oocyte donation, embryo biopsy for PGT-A or PGT-M, and female age.

### Extended Embryo Culture

Twenty-three of the included studies investigated whether prolonged embryo culture may affect the risk of MZT (4, 6, 7, 9, 12–15, 19, 60, 61, 63–67, 71, 72, 76–78, 81, 82). Pooling of results from the studies showed that blastocyst transfer was associated with a statistically significant increase in MZT pregnancy risk when compared with cleavage-stage embryo transfer. Considering the  $I^2$  value (78%) indicating considerable heterogeneity and the high clinical heterogeneity between the studies, the pooled OR was derived using a random-effects model (OR 2.16; 95% CI, 1.74–2.68;  $P < .00001$ ) (Fig. 1). A funnel plot showed no indication of asymmetry among the studies (Supplemental Fig. 2, available online). The association between blastocyst transfer and MZT pregnancy risk was also confirmed after limiting the analysis to high-quality cohort (Newcastle-Ottawa scale score  $>7$ ) and case control studies (random-effects model, OR 1.88; 95% CI, 1.54–2.30;  $P < .00001$ ;  $I^2 = 62\%$ ).

### Assisted Hatching

Sixteen of the included studies investigated a possible association between AH and MZT after IVF (4, 12, 13, 18, 19, 55–58, 72, 73, 77–79, 81, 82). The pooling of results from the studies showed a statistically significant association between AH and MZT pregnancies after IVF (OR 1.17; 95% CI, 1.09–1.27;

TABLE 1

## Included studies investigating potential risk factors for monozygotic twinning after in vitro fertilization.

Study	Year	Country	Study design	Study period	Twinning	Method of diagnosis	Embryos culture media	Fresh and/or frozen ET	Risk factors investigated	Quality of evidence <sup>a</sup>
Meldrum et al. (55)	1998	USA	Retrospective	1990–1996	MZT	NR	Modified Ham's F10	NR	AH (acidified Tyrode's solution)	6
Hershlag et al. (56)	1999	USA	Retrospective	1990–1996	MZT	US and placental histology	NR	NR	AH (Mechanical)	8
Hurst et al. (72)	1998	USA	Prospective randomized trial	NR	MZT	NR	HTF with HEPES and 15% SSS overlain with oil	Fresh	AH (acidified Tyrode's solution)	NA
Lanzendorf et al. (73)	1998	USA	Prospective randomized trial	1995–1996	MZT	NR	Ham's F10	NR	AH (acidified Tyrode's solution)	NA
Schieve et al. (57)	2000	USA	Retrospective	1996	MZT	US	NR	Fresh	AH (specific procedure not reported)	7
Saito et al. (54)	2000	Japan	Retrospective	1994–1995	MZT	NR	NR	NR	Microinsemination procedures	7
Sills et al. (58)	2000	USA	Retrospective	1995–1998	MZT	US and placental examination at delivery	NR	Fresh and frozen	AH (acidified Tyrode's solution), insemination method, oocytes donation	8
Schachter et al. (59)	2001	Israel	Retrospective	1997–1999	MZT	US	NR	NR	Insemination method, AH (acidified Tyrode's solution)	6
Da Costa et al. (60)	2001	Brasil	Retrospective	1996–1999	MZT	US	S1 and S2 medium (Scandinavian IVF Science)	NR	Embryo extended culture	7
Sheiner et al. (61)	2001	Israel	Prospective	1998–1999	MZT	NR	NR	NR	Embryo extended culture	7
Karaki et al. (74)	2002	Jordan	Prospective randomized trial	1999–2000	MZT	NR	IVF medium (MediCult), G1.2 and G2.2 media (Scandinavian IVF Sciences)	Fresh	Embryo extended culture	NA
Tarlatzis et al. (62)	2002	Greece	Retrospective	1999–2000	MZT	US	NR	NR	Insemination method	7
Alikani et al. (63)	2003	USA	Retrospective	1995–2002	MZT <sup>b</sup>	US	NR	Fresh and frozen	Insemination method, embryo extended culture	8
Emiliani et al. ()	2003	Belgium	Prospective randomized trial	NR	MZT	US	In-house sequential media	Fresh and frozen	Embryo extended culture	NA

Busnelli. Monozygotic twinning after IVF. Fertil Steril 2018.

TABLE 1

Continued.

Study	Year	Country	Study design	Study period	Twinning	Method of diagnosis	Embryos culture media	Fresh and/or frozen ET	Risk factors investigated	Quality of evidence <sup>a</sup>
Milki et al. (64)	2003	USA	Retrospective	1998–2002	MZT	US	P1 and blastocyst medium (Irvine Scientific)	NR	Embryo extended culture, AH (acidified Tyrode's solution), insemination method	8
Jain et al. (65)	2004	USA	Retrospective	1997–2000	MZT	US	P-1 media (Irvine Scientific) and Blastocyst Media (Irvine Scientific)	NR	Embryo extended culture	7
Wright et al. (66)	2004	USA	Retrospective	1999–2000	MZT	US	NR	Fresh	Embryo extended culture	7
Kolibianakis et al. (76)	2004	Belgium	Prospective randomized trial	2001–2003	MZT	US	Sequential media (Vitrolife, Göthenburg, Sweden)	NR	Embryo extended culture	NA
Ng et al. (77)	2005	China	Prospective randomized trial	2003–2004	MZT	NR	NR	Frozen	AH (laser)	NA
Ma et al. (78)	2006	Canada	Prospective randomized trial	1999–2003	MZT	US	NR	NR	AH (acidified Tyrode's solution)	NA
Moayeri et al. (67)	2007	USA	Retrospective	2002–2005	MZT	US and follow-up information	Quinn's Advantage cleavage-stage medium	NR	Embryo extended culture	8
Skiadas et al. (68)	2008	USA	Retrospective	1998–2004	MZT-MC	US	P1 (Irvine Scientific) or IVF-500, G1.2 or G1.3 (Scandinavian IVF Science/Vitrolife); from days 3–5: sequential media marketed for use with the corresponding medium for days 1–3	NR	Embryo extended culture, AH (acidified Tyrode's solution), insemination method	8
Balakier et al. (79)	2009	Canada	Prospective randomized trial	2005–2006	MZT	US and records review	NR	NR	AH (laser)	NA
Verpoest et al. (69)	2009	Belgium	Retrospective	2001–2006	MZT	US	MediCult BlastAssist system, Vitrolife G2 and G3 series, Cook Medical Sydney IVF medium	NR	PGT-M	8

Busnelli. Monozygotic twinning after IVF. *Fertil Steril* 2018.

TABLE 1

Continued.

Study	Year	Country	Study design	Study period	Twinning	Method of diagnosis	Embryos culture media	Fresh and/or frozen ET	Risk factors investigated	Quality of evidence <sup>a</sup>
Sharara and Abdo (7)	2010	USA	Retrospective	2003–2008	MZT	US	P1 (Irvine Scientific), Global Medium (Life Global), Blastocyst Medium (Irvine Scientific)	Fresh	Embryo extended culture	8
Papanikolaou et al. (6)	2010	Belgium	Retrospective	2003–2005	MZT	US and follow up information	Media A (MediCult) and Media B (Vitrolife)	Fresh	Embryo extended culture	8
Kawachiya et al. (9)	2011	Japan	Retrospective	2002–2008	MZT	US	Cleavage-stage medium (SAGE), blastocyst medium (Quinn's Advantage; SAGE)	Fresh and frozen	Embryo extended culture	7
Kang et al. (70)	2012	South Korea	Retrospective	2008–2009	MZT	US	MRC D16 medium (YS medium)	Fresh	Embryo culture (morula vs. blastocyst transfer)	7
Nakasuji et al. (12)	2014	Japan	Retrospective	2010	MZT	US	NR	Fresh and frozen	AH (specific procedure not reported), embryo extended culture	7
Knopman et al. (13)	2014	USA	Nested case control	2000–2009	MZT <sup>c</sup>	US, genetic testing and placental pathology	Quinn's cleavage and blastocyst media	Fresh and frozen	Oocytes donation, AH (acidified Tyrode's solution), insemination method, embryo extended culture, PGT-A/PGT-M, female age, estrogen peak level, cycle year	8
Wu et al. (71)	2014	Taiwan	Retrospective	2001–2011	MZT	US	Cleavage culture medium equilibrated with 6% CO <sub>2</sub> in air and blastocyst culture medium (Cook IVF)	Fresh	Embryo extended culture, insemination method, AH (laser)	8
Franasiak et al. (14)	2015	USA	Retrospective	1999–2014	MZT	US and records review	Quinn's Advantage (CooperSurgical) followed by BlastAssist (Origio)	Fresh	Embryo extended culture, female age, transfer order, embryology parameters	8

Busnelli. Monozygotic twinning after IVF. *Fertil Steril* 2018.

TABLE 1

Continued.

Study	Year	Country	Study design	Study period	Twinning	Method of diagnosis	Embryos culture media	Fresh and/or frozen ET	Risk factors investigated	Quality of evidence <sup>a</sup>
Sotiroska et al. (15)	2015	Macedonia	Retrospective	2008–2013	MZT	US	Quinn's Advantage sequential media under mineral oil (SAGE, Cooper Surgical)	Fresh	Embryo extended culture	8
Tocino et al. (16)	2015	Spain	Retrospective	1995–2013	MZT <sup>c</sup>	US	NR	Fresh and frozen	Embryo extended culture	8
Kanter et al. (18)	2015	USA	Retrospective	2003–2012	MZT	US and delivery data	NR	Fresh	Female age, ethnicity, infertility diagnosis, obstetric and ART history, embryo extended culture, insemination method, AH (specific procedure not reported), supernumerary embryos, number of oocytes retrieved	8
Vaughan et al. (19)	2016	USA	Retrospective	2002–2013	MZT	US and records review	NR	Fresh	Ovarian stimulation, insemination method, embryo extended culture, cycle year, female age, oocytes donation, embryo biopsy, AH (specific procedure not reported), number of embryos transferred	8
Mateizel et al. (4)	2016	Belgium	Retrospective	2004–2013	MZT <sup>c</sup>	US	Irvine HTF medium, Cook IVF media, Vitrolife sequential media, MediCult, EmbryoAssist, BlastAssist Medium, Sage Quinn's Advantage Protein Plus cleavage and blastocyst media	Fresh and frozen	Female age, embryo extended culture, oocyte donation, insemination method, PGT-M, AH (specific procedure not reported)	8

Busnelli. Monozygotic twinning after IVF. *Fertil Steril* 2018.

TABLE 1

Continued.

Study	Year	Country	Study design	Study period	Twinning	Method of diagnosis	Embryos culture media	Fresh and/or frozen ET	Risk factors investigated	Quality of evidence <sup>a</sup>
Song et al. (20)	2017	China	Retrospective	2011–2016	MZT-MC-DA	US	NR	Fresh and frozen	Female age, embryo extended culture, insemination method, transfer order	8
Liu et al. (82)	2018	China	Retrospective	2014–2015	MZT	US	G1 and G2 Plus medium (Vitrolife)	Fresh and frozen	Embryo extended culture, insemination method, AH (laser), stimulation protocol, number of transferred embryos	8
Ikemoto et al. (81)	2018	Japan	Retrospective	2007–2014	MZT	US	NR	Fresh and frozen	Embryo extended culture, insemination method, AH (specific procedure not reported)	8

Note: AH = assisted hatching; ART = assisted reproductive technology; DA = diamniotic; HTF = human tubal fluid; IVF = in vitro fertilization; MC = monochorionic; MZT = monozygotic; NA = not applicable; NR = not reported; PGT-A = preimplantation genetic testing for aneuploidies; PGT\_M = preimplantation testing for monogenic/single gene defects; US = ultrasound.

<sup>a</sup> Based on the Newcastle–Ottawa scale. Wells et al. 2000 (26).

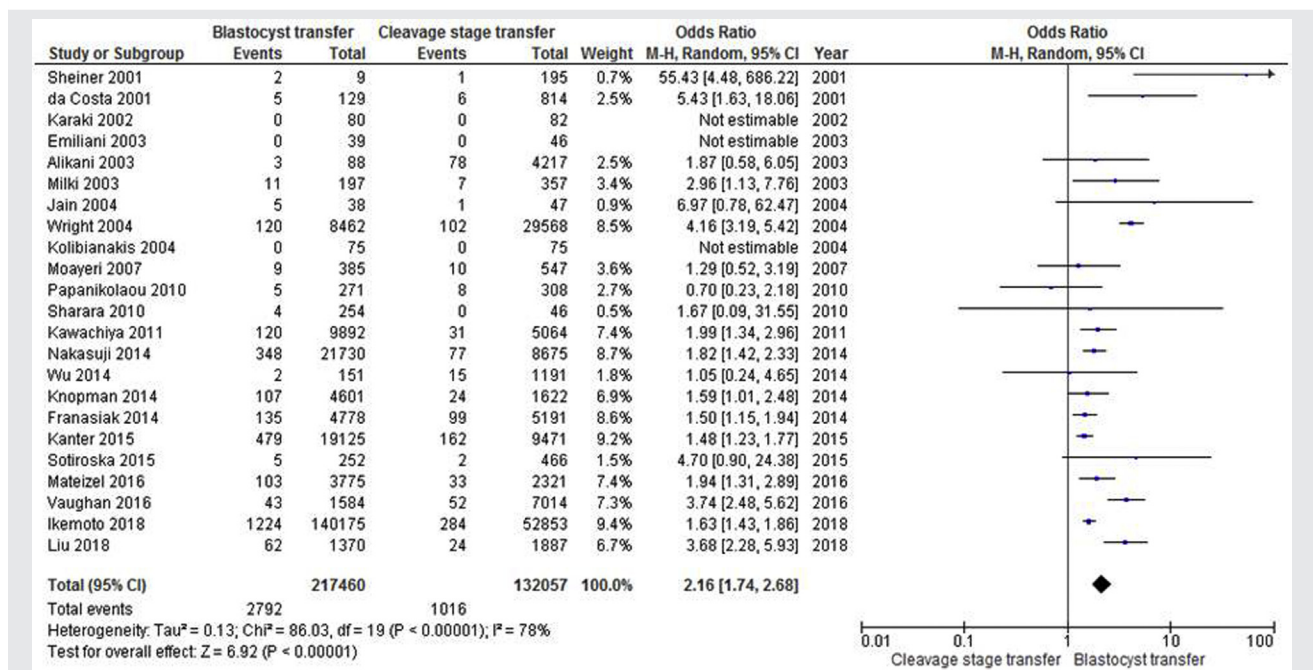
<sup>b</sup> Chorionicity specified.

<sup>c</sup> Chorionicity and amnionicity specified.

Busnelli. Monozygotic twinning after IVF. *Fertil Steril* 2018.



FIGURE 1



Embryo extended culture and risk of monozygotic twinning.

Busnelli. Monozygotic twinning after IVF. Fertil Steril 2018.

$P < .0001$ ) (Supplemental Fig. 3, available online). The  $I^2$  value was 29%. A funnel plot showed no indication of asymmetry among the studies (Supplemental Fig. 4, available online). The association was not confirmed after limiting the analysis to high-quality cohort (Newcastle-Ottawa scale score  $>7$ ) and case control studies (random-effects model, OR 1.00; 95% CI, 0.81–1.24,  $P = .99$ ,  $I^2 = 53\%$ ).

### Insemination Method

Ten studies investigated the possible impact of the insemination method (conventional IVF or ICSI) on the rate of MZT pregnancies (4, 13, 18, 19, 38, 58, 59, 62, 65, 81). Our meta-analysis showed a statistically significant increase of this risk after the use of classic IVF (OR 1.13; 95% CI, 1.02–1.26;  $P = .02$ ) (Supplemental Fig. 5, available online). The  $I^2$  value was 0. Alikani et al. (63) analyzed this risk factor by reporting the number of IVF-ICSI cycles as a denominator, so this study could not be included in the meta-analysis. The association was also confirmed after limiting the analysis to high-quality cohort (Newcastle-Ottawa scale score  $>7$ ) and case control studies (fixed-effects model, OR 1.14; 95% CI, 1.02–1.26;  $P = .02$ ;  $I^2 = 0$ ). Alikani et al. (63) analyzed this risk factor by reporting the number of IVF-ICSI cycles as a denominator, so this study could not be included in the meta-analysis. Results showed the lack of an association (OR 1.50; 95% CI, 0.95–2.37;  $P = .08$ ).

### Embryo Biopsy for PGT-A or PGT-M

Four studies evaluated the effect of embryo biopsy for PGT-A or PGT-M on the MZT risk (4, 13, 19, 69). The meta-analysis

showed the lack of an association between these genetic tests and MZT pregnancies. Considering the  $I^2$  value (79%) indicating considerable heterogeneity, the high clinical heterogeneity between studies, and the low number of included studies, the pooled OR was derived using a random-effects model (OR 1.52; 95% CI, 0.76–3.02;  $P = .23$ ) (Supplemental Fig. 6, available online). A funnel plot showed no indication of asymmetry among the studies (Supplemental Fig. 7, available online). All the included studies were high quality. Two studies investigated exclusively the effect of PGT-M (4, 69). Pooling of results showed no association between PGT-M and monozygotic twinning after IVF (random-effects model, OR 1.04; 95% CI, 0.56–1.93;  $P = .90$ ;  $I^2 = 51\%$ ). Knopman et al. (13) also investigated the additive effect of ICSI and embryo biopsy (PGT-M/PGT-A) and failed to demonstrate any association (OR 0.92; 95% CI, 0.22–3.77;  $P = 1.00$ ).

### Frozen-thawed Embryos

Eight studies evaluated the impact of frozen-thawed embryo transfer on the risk of MZT (4, 9, 12, 13, 58, 63, 81, 82). The meta-analysis did not show any association between frozen cycles and MZT pregnancies. Considering the  $I^2$  value (74%) indicating considerable heterogeneity and the high clinical heterogeneity between the studies, the pooled OR was derived using a random-effects model (OR 1.18; 95% CI, 0.91–1.52;  $P = .21$ ) (Supplemental Fig. 8, available online). A funnel plot showed no indication of asymmetry among the studies (Supplemental Fig. 9, available online). The lack of an association was also confirmed after limiting the analysis to high-quality cohort (Newcastle-Ottawa scale score  $>7$ ) and case

control studies (random-effects model, OR 1.10; 95% CI, 0.72–1.69;  $P=.66$ ;  $I^2=79\%$ ).

### Female Age

Four studies evaluated the effect of oocyte age on the MZT risk by estimating the incidence of this event among women aged 35 or older and younger women (4, 13, 14, 18). The pooling of the results from studies showed a statistically significantly increased risk among women younger than 35 years (Fig. 2). Considering the  $I^2$  value (62%) indicating substantial heterogeneity, the clinical heterogeneity between studies, and the low number of included studies, the pooled OR was derived using a random-effects model (OR 1.29; 95% CI, 1.03–1.62;  $P=.03$ ). All the included studies were high quality. A funnel plot showed no indication of asymmetry among the studies (Supplemental Fig. 10, available online).

### Oocyte Donation

Five studies evaluated the effect of the use of donor oocytes on the risk of MZT (4, 13, 19, 58, 63). The meta-analysis showed no association (OR 1.10; 95% CI, 0.82–1.50;  $P=.52$ ) (Supplemental Fig. 11, available online). The  $I^2$  value was 8%.

### Miscellaneous

Knopman et al. (13) hypothesized a possible effect of the estrogen peak level on the risk of MZT but failed to document an association (OR 1.08; 95% CI, 0.76–1.55;  $P=.64$ ). Two studies analyzed the period of time in which the IVF cycle was performed and its association with the risk of MZT. Knopman et al. (13) reported a statistically significantly lower risk in cycles performed after 2004 (OR 0.68; 95% CI, 0.47–0.96;  $P=.02$ ). By contrast, Vaughan et al. (19) observed a statistically significantly higher risk in cycles performed after 2005 (OR 1.56; 95% CI, 0.84–2.91;  $P=.16$ ). Franasiak et al. (14) reported a statistically significantly higher risk of MZT among patients with supernumerary embryos available (risk ratio 1.59; 95% CI, 1.23–2.06;  $P=.0004$ ).

### Monochorionic Twinning

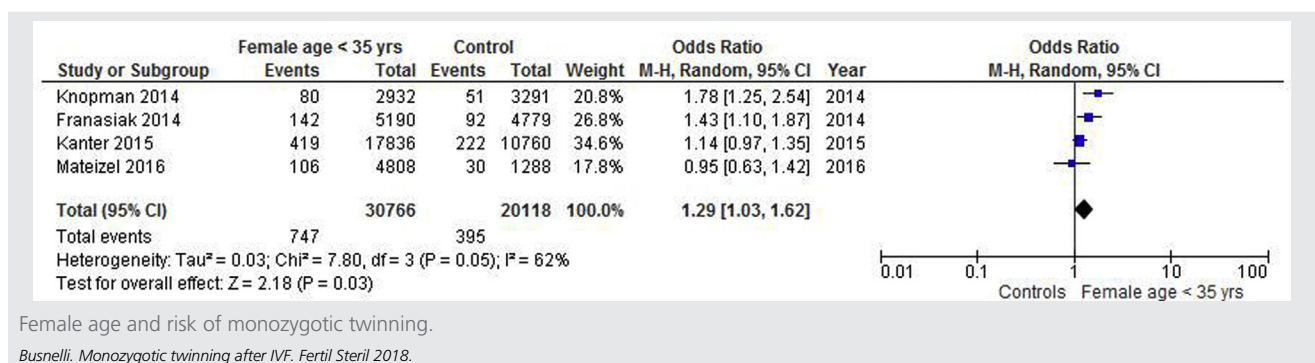
Three studies specifically provided data on the risk factors for monozygotic monochorionic twinning (MCT) after IVF (13, 20, 68). Again, the meta-analysis showed a statistically significantly increased risk for this event after extended embryo culture (Fig. 3). Considering the  $I^2$  value (59%) indicating substantial heterogeneity, the clinical heterogeneity between studies, and the low number of included studies, the pooled effect estimate was derived using a random-effects model (OR 1.90; 95% CI, 1.21–2.98;  $P=.005$ ). All the included studies were high quality. The meta-analysis also showed an association between young female age and risk of MCT pregnancies after IVF (fixed-effects model, OR 2.34; 95% CI, 1.69–3.23;  $P<.00001$ ;  $I^2 = 0$ ) (see Fig. 3). The pooling of results from the studies did not show an association between ICSI, assisted hatching, and frozen-thawed embryo transfer and MCT after IVF (see Fig. 3).

### DISCUSSION

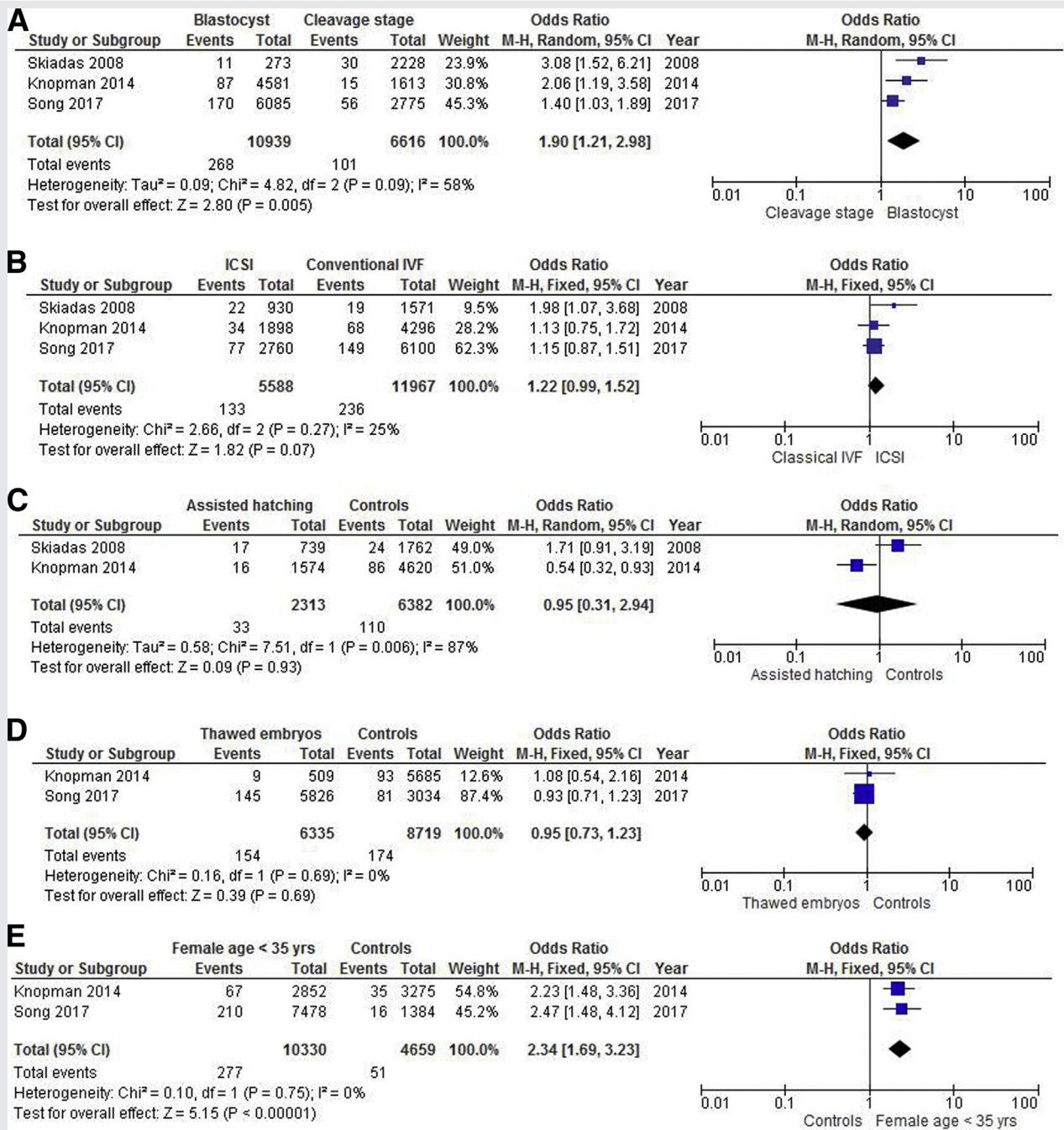
The data from our systematic review and meta-analysis indicated that blastocyst transfer and younger female age (i.e., age <35 years) are associated with an increased risk of both MZT and MCT pregnancies after IVF. The meta-analysis also showed a milder but still statistically significant association between conventional IVF and AH and the risk of MZT pregnancies. Of note, we failed to document any association between all the other risk factors extrapolated from the literature (i.e., frozen-thawed embryo transfer, PGT-M or PGT-A, or oocyte donation) and the occurrence of MZT pregnancies after IVF. Importantly, after limiting the analysis to high-quality cohort (Newcastle-Ottawa scale score >7) and case control studies, all our results were confirmed with the exception of the increased risk of MZT pregnancies after AH.

Our results are in line with those reported in a recent meta-analysis by Hviid et al. (36) which showed a statistically significantly higher rate of MZT pregnancies after blastocyst transfer than after cleavage-stage embryo transfer (fixed-effects meta-analysis: OR 2.18; 95% CI, 1.93–2.48 and random-effects meta-analysis: OR 2.00; 95% CI, 1.48–2.70). The basis of the association between blastocyst transfer and MZT and

FIGURE 2



**FIGURE 3**



Risk factors for monozygotic monochorionic twinning after IVF. (A) Embryo extended culture and risk of monozygotic monochorionic twinning. (B) Insemination method and risk of monozygotic monochorionic twinning. (C) Assisted hatching and risk of monozygotic monochorionic twinning. (D) Frozen-thawed embryo transfer and risk of monozygotic monochorionic twinning. (E) Female age and risk of monozygotic monochorionic twinning.

Busnelli. Monozygotic twinning after IVF. *Fertil Steril* 2018.

MCT pregnancies after IVF is not fully understood, but several not-mutually-exclusive theories have been proposed. For instance, it has been hypothesized that extended exposure to culture media may determine the hardening of the zona pellucida, an acellular area of mucopolysaccharides and spe-

cific proteins surrounding the ovum (11). On this basis, it has been speculated that spontaneous or assisted hatching of the blastocyst through a less flexible zona pellucida might increase the risk of splitting of the inner cell mass (ICM) and the consequent development of two fetal plates (5, 80, 81).

The components of the culture media and their concentrations might also play a role. The long exposure of the embryo to low levels of calcium might destabilize the intracellular bonds and consequently predispose the ICM to the division (6, 15, 81). At the same time, the medium used to grow blastocysts may lead to an overstimulation of apoptosis through free radical formation due to the excessive glucose levels. Linear polarization of apoptotic cells in the ICM could lead to splitting during or before the hatching process (83). Similarly, growth factors such as insulin-like growth factors 1 and 2 might induce changes in signaling pathway, cytoplasmic shifting, or polarity changes in the embryo, which may increase the MZT risk (15). According to other investigators, it is possible that culture media devoid of growth factors or cytokines may cause a metabolic stress to embryos, which is exacerbated by extended culture. This may translate into higher rates of MZT pregnancies partly due to increased apoptosis and weaker cell-to-cell adhesion (64).

The characteristics of the blastocyst itself might also be involved. It has been hypothesized that the cause of increased twinning in this situation could be related to blastocyst-stage embryos being more sensitive to the effects of mechanical manipulation in the laboratory or transient changes in temperature or pH during monitoring or embryo transfer (11).

It is interesting that data from our meta-analysis also demonstrate that embryos derived from younger oocytes (i.e., female age at time of retrieval <35 years) are statistically significantly more likely to result in a MZT or MCT pregnancy. According to Knopman et al. (13), MZT production is another means by which younger, presumably healthier oocytes demonstrate their superior reproductive potential. This theory, even if fascinating, is still too vague, and further data are warranted to deepen the reasons behind this association. Furthermore, it must be considered that blastocyst transfer may act as a confounding factor. In fact, it is well known that embryos derived from young oocytes are more likely to be transferred at an advanced blastocyst stage. Unfortunately, only Knopman et al. (13) controlled for the association with extended culture, and they reported that the twinning increase in younger women remained statistically significant. The available data are thus insufficient to control the entire meta-analysis investigating the impact of female age on the risk of MZT for the stage of the transferred embryo. As a consequence, age might not be an independent risk factor for MZT but rather a proxy for blastocyst transfer.

The protective effect of ICSI on the risk of MZT pregnancies was unexpected. In fact, the majority of studies on this topic have speculated that this insemination method could be encouraging an increase in the frequency of MZT due to the splitting of the ICM after its herniation through a compromised zona pellucida (52). Hypothesizing a biological mechanism that could justify the association between conventional IVF and MZT pregnancy risk is thus very difficult. A possible explanation could be sought in the expansion of ICSI indications to non-male-factor infertility causes and the consequent preferential use of classic IVF in patients with good-quality oocytes with a high fertilization potential (84). In contrast with these results, Hviid et al. (36), on the basis

of more than 10 high-quality studies, concluded that there is no clear consensus regarding the influence of the methods of fertility treatment on the frequency of MZT. Even if they had not performed a meta-analysis, this discrepancy was probably due to the fact that they chose not to include the study by Kanter et al. (18), which accounts for the highest weight (61%) in our quantitative analysis and shows an association between conventional IVF and MZT pregnancy risk (OR 1.21, 95% CI, 1.03–1.43). Considering the weak though still statistically significant association and the lack of a rational justification, more studies designed to investigate this specific aspect and its biological basis are warranted.

According to the most accepted theory, AH might lead to embryo splitting in two ways: first, a premature disruption in the zona pellucida can interfere with signaling mechanisms within the embryo; second, an artificial hole can allow for blastomere separation and division. Both processes, either individually or collectively, might thus enhance the risk for MZT (13). A first analysis seemed to confirm this hypothesis. However, the pooling of results extrapolated exclusively from high-quality studies failed to confirm the association. The available evidence is thus insufficient to draw reliable conclusions regarding the impact of this technique on the investigated outcomes.

The results from our meta-analysis clearly highlight reasons for clinical concern and may have important implications in the IVF shared decision-making process. Embryo transfer at the blastocyst stage leads to higher live-birth rates per embryo transfer episode (85). As a consequence, patients tend to perceive this strategy as the best for achieving their goal. However, they often ignore the possible associated midterm and long-term drawbacks (33, 83–88). In this regard, our results provide a precise estimate of the increased risk not only of MZT pregnancies but also of MCT pregnancies after blastocyst transfer. Considering the significant potential complications associated with these events, this information should be communicated to patients. On the other hand, a critical vision of the whole picture should not be lost. First, the extent of the association is not so great as to hypothesize a certain cause-effect relationship between embryo transfer at blastocyst stage and MZT or MCT pregnancies (89). Second, there are other aspects that need to be considered during the risk-benefit assessment when we must choose whether to transfer an embryo at the cleavage or blastocyst stage. In particular, the numerous advantages of blastocyst transfer should not be forgotten, such as the large reduction in the rate of multiple pregnancies after the worldwide introduction of elective single-embryo transfer after extended embryo culture (90). It is thus necessary to perform studies specifically focused on evaluating the risks, benefits, and costs associated with each procedure and to develop a decisional algorithm to help choose the best option.

### Strengths and Limitations

The present systematic review and meta-analysis included many studies with heterogeneous populations and provides the largest sample of women in whom the risk factors for

MZT pregnancies after IVF have been examined. Furthermore, it constitutes the first systematic attempt to establish risk factors for monochorionic placentation after IVF.

Nevertheless, some limitations of our meta-analysis deserve to be mentioned. First, in the majority of the included studies the investigators used ultrasound criteria to diagnose MZT pregnancies. However, it is well known that the only reliable way to diagnose zygosity is to perform a DNA profiling of all multiple same-sex deliveries (16). Second, the heterogeneity of the included studies partially limits the findings of our meta-analysis. Third, the association between conventional IVF and MZT pregnancies is doubtful. This is more likely a spurious finding attributable to the heterogeneity and/or to the quality of available studies. There are several unconvincing aspects. From a statistical perspective, the OR magnitude is low and the CI nearly crosses 1. Furthermore, there are no rational biological bases to support this association. Finally, the last study that investigated this aspect was conducted 18 years after the first one. That the included studies cover such a long period of time also limits the reliability of the other associations investigated in our meta-analysis. In this field of medicine innovation is very fast, and many practices have drastically changed. On the other hand, although they were not able to fully control this limit, the subanalyses we performed on the basis of the study period did not alter our results.

## CONCLUSION

The available data on the relationship between characteristics of ART and MZT, explored here via meta-analysis, primarily indicated a robust association between extended culture and MZT, while finding a lack of association between embryo biopsy, embryo cryopreservation, and oocytes donation. Our analysis suggests the possibility of increased MZT among younger patients, but we found a weak association, with the lower bound of the 95% CI as 1.03 for OR. In addition, the available data do not enable us to control appropriately for extended culture in our analysis, which may be an important confounder; in fact, the increase in MZT with younger age may be entirely attributable to the overrepresentation of blastocyst transfer among younger patients. So we caution that these data do not definitively show younger age to be associated with MZT. The relative increase in the odds of MZT with blastocyst transfer was large (116% increase) and highly statistically significant ( $P < .00001$ ), but the absolute increase in MZT attributable to blastocyst culture would be expected to be quite small (<2%), given the overall low reported incidence of MZT after ART. Therefore, this analysis should in no way discourage extended culture performed with the goal of elective single-embryo transfer, a practice that greatly decreases twinning from ART.

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**Factores de riesgo relacionados con el origen de gemelos monocigóticos tras fecundación in vitro: revisión sistemática y meta-análisis**

**Objetivos:** Estudiar los factores de riesgo relacionados con el origen de gemelos monocigóticos (GMZ) y monocoriónicos (GMC) tras fecundación in vitro (FIV).

**Diseño:** Revisión sistemática y meta-análisis.

**Escenario:** No aplica.

**Pacientes:** Mujeres que gestaron GMZ y no GMC tras FIV.

**Intervenciones:** Búsqueda sistemática en *Medline* de artículos en inglés publicados entre enero de 1995 y octubre de 2018.

**Medida de los resultados principales:** Posibles factores de riesgo relacionados con el embarazo de GMZ y GMC tras FIV, tales como el cultivo prolongado, el método de inseminación (FIV convencional y microinyección intracitoplasmática [ICSI]), la biopsia embrionaria para el diagnóstico genético preimplantación para la selección de aneuploidías o para las enfermedades monogénicas/ de un solo gen (DGP-A, DGP-M), la eclosión asistida (EA), la donación de ovocitos, la edad materna y la criopreservación embrionaria.

**Resultados:** Un total de 40 estudios fueron incluidos. Se observó un incremento estadísticamente significativo de embarazos de GMZ y GMC tras FIV cuando se comparó la transferencia en estadio de blastocisto vs. la transferencia en estadio de células, así como cuando la edad materna era <35 años (23 estudios, OR 2.16, 95% IC, 1.74–2.68,  $I^2=78\%$ ; 4 estudios, OR 1.29; 95% IC, 1.03–1.62,  $I^2=62\%$ ; y 3 estudios, OR 1.90, 95% IC, 1.21–2.98,  $I^2=59\%$ ; 2 estudios, OR 2.34; 95% IC, 1.69–3.23,  $I^2=0$ , respectivamente). La biopsia embrionaria para DGP-A o DGP-M, la criopreservación embrionaria y la donación de ovocitos no se asociaron con los embarazos de GMZ y GMC tras FIV.

**Conclusiones:** La transferencia en estadio blastocisto está asociada con un incremento de riesgo de embarazos de GMZ y GMC tras FIV. Para esclarecer el impacto de la edad materna, el método de inseminación y la EC en los resultados investigados, son necesarios más evidencias.