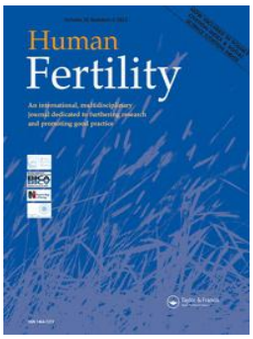

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RESEARCH ARTICLE

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Effect of vitamin D supplementation on frozen embryo transfer cycle outcomes

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ABSTRACT

The role of serum 25-OH D3 (D3) in the physiology and regulation of the female genital system has gained significant research interest. Recent data have suggested that sufficient D3 levels are associated with improved outcomes of *in vitro* fertilization (IVF), although other studies failed to confirm so. Screening for D3 levels before IVF is becoming common practice in many IVF centres, and D3 insufficiency is treated with supplements before treatment. However, little is known about the effect of this intervention on D3 levels during endometrial preparation for frozen-embryo transfer (FET) cycles, especially regarding clinical outcomes. To examine the effect of vitamin D supplementation and the impact of vitamin D status in women undergoing FET cycles, a prospective study of infertile women undergoing FET cycles was carried out. Initial screening of D3 levels was performed in 252 infertile women before a FET cycle, and where insufficiency was found (<30 ng/mL) [115 (45.6%)], supplements were prescribed according to a standardized protocol. Serum D3 levels were measured at three distinct time-points: at the initiation of endometrial preparation (T1), embryo transfer (T2), and beta-hCG testing (T3). We found no significant difference in ongoing pregnancy [40 (34.8%) *vs.* 51 (37.2%); odds ratio (OR) 0.90, 95% confidence interval (CI) 0.54–1.51; adjusted OR 1.04, 95% CI 0.58–1.83], live birth, positive β-hCG, clinical pregnancy, miscarriage, and ectopic pregnancy rates between D3-insufficient participants at T1 receiving vitamin D and D3-replete ones not receiving any supplementation (*p*-values >0.05). We also found no significant difference in ongoing pregnancy [21 (30.9%) *vs.* 66 (40.2%), and 17 (34.0%) *vs.* 51 (41.5%)] and the rest of the outcomes between D3-insufficient and replete participants at T2 and T3, respectively (*p*-values >0.05). In conclusion, this prospective cohort study of women undergoing FET cycles found no significant difference in ongoing pregnancy rates between D3-insufficient participants receiving supplementation at the beginning of endometrial preparation and replete ones receiving no supplementation. Large, high-quality trials are required to further investigate this hypothesis and compare the IVF outcomes between replete participants, insufficient participants receiving no supplementation, and insufficient participants receiving supplementation.

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Serum vitamin D; vitamin D supplementation; frozen embryo transfer; assisted reproductive techniques; infertility; vitamin D insufficiency

Introduction

Serum 25-OH D3 (D3) is an important steroid hormone involved in various physiological processes. Its primary source is cutaneous synthesis following sun exposure, which results in its D2 (ergocalciferol) and D3 (cholecalciferol) forms. Following a hydroxylation process in the liver, it is converted into 25-hydroxyvitamin D and, subsequently, into 1,25-dihydroxyvitamin D in the kidneys, which is the active form of the vitamin (Bikle, 2021; Janoušek et al., 2022). The main endocrine effects of D3, relate to the regulation of calcium and phosphorus metabolism (Moretti et al., 2018). In contrast, a wider endocrine action of D3 is further suggested by the presence of D3 receptors in several tissues, including the brain, immune system cells, and pancreatic beta cells (Fung et al., 2017; Institute of Medicine (US) Committee to Review Dietary Reference Intakes for Vitamin D and Calcium et al., 2011). Of note, D3 receptors are present in the ovaries, the endometrium, and the placenta, indicating an extra

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role of the hormone in regulating reproductive function. Gene expression of D3 receptors shows cyclicity during the menstrual cycle with D3 being involved in ovarian steroidogenesis (Parikh et al., 2010). Serum D3 insufficiency has been shown to affect reproductive function in various ways. Animal studies suggest an association of compromised D3 receptor expression with disorders of folliculogenesis, and uterine hypoplasia (Halloran & Deluca, 1980; Kinuta et al., 2000; Yoshizawa et al., 1997), while, more recently, D3 insufficiency in humans has been associated with infertility, polycystic ovary syndrome and endometriosis (Bikle, 2021; He et al., 2015; Janoušek et al., 2022; Somigliana et al., 2007).

The association of serum D3 status with *in vitro* fertilization (IVF) outcomes remains unclear, as data suggests lower clinical pregnancy rates in women with D3 insufficiency (Paffoni et al., 2014; Polyzos et al., 2014) at the same time, other studies failed to confirm the negative effect (Banker et al., 2017; van de Vijver et al., 2016). Of note, the rationale of the positive impact of vitamin D is mainly on the endometrium, rather on oocyte quality (Abedi et al., 2019; Iliuta et al., 2022) as it has been shown that it could influence endometrial receptivity through the regulation of ovarian and endometrial cell proliferation and the expression of genes involved (Cozzolino et al., 2020; Du et al., 2005). In addition, it may particularly benefit the metabolic profile of some categories of infertile women, such as women with PCOS, in lowering hyperandrogenism (androgen excess) (Showell et al., 2020). Finally, vitamin D is reported to affect embryogenesis and follicle development and modulate primary follicle recruitment through the regulation of anti-Müllerian hormone production (Dennis et al., 2012; Yoshizawa et al., 1997).

Interestingly, despite the increasing use of vitamin D supplementation in infertile women, there is only a small number of trials focusing specifically on its effect on clinical pregnancy rates in IVF, with so far, conflicting results (Abedi et al., 2019; Doryanizadeh et al., 2021; Somigliana et al., 2021). Moreover, data from meta-analyses have associated D3 insufficiency with a significantly lower probability of live birth -but not clinical pregnancy (Lv et al., 2016; Zhao et al., 2018). Another has shown a significant benefit of sufficient D3 levels on implantation, biochemical, clinical, and ongoing pregnancy rates (Iliuta et al., 2022), but the robustness of the findings has been doubted due to the exclusion of certain studies (Cozzolino et al., 2020). Regarding vitamin D3 supplementation, the SUNDRO randomized controlled trial (RCT) did not find a significant improvement in clinical pregnancy, following a single oral dose of 600,000 IU of vitamin D in women with normal weight, preserved ovarian reserve, and low D3 levels undergoing IVF (Somigliana et al., 2021). A 2020 Cochrane review including two RCTs also found no significant difference in live birth and clinical pregnancy rates between vitamin D supplementation and placebo (Showell et al., 2020). In contrast, a 2023 meta-analysis suggested that moderate daily dosing of vitamin D supplementation significantly increased clinical pregnancy (Meng et al., 2023), while a 2022 study found an improvement only in biochemical pregnancy (Zhou et al., 2022).

Of note, there is scarce data in the existing literature considering frozen-embryo transfer (FET) cycles. Given concerns have been raised regarding implantation due to low Vit-D and existing controversy on the value of D3 deficiency and its supplementation, we designed the current prospective cohort study aiming to address the effect of vitamin D supplementation on the IVF outcomes of women with D3 insufficiency undergoing FET cycles.

Materials and methods

This is a single-centre prospective cohort study conducted at the Assisted Reproductive Unit of Embryolab Fertility Clinic, Thessaloniki, Greece, with the scientific collaboration of the Assisted Reproductive Unit, Second Department of Obstetrics and Gynecology at the 'Aretaieion' University Hospital, Athens, Greece, between January 2020 and November 2022. The study protocol was approved by the Embryolab Fertility Clinic Ethics and Research Committee (10-12/2019/1). The study was performed in accordance with good clinical practice guidelines and the ethical standards of the Helsinki Declaration. Written informed consent was obtained from all patients.

Patient population/eligibility criteria

Women scheduled for a FET cycle were enrolled in the present study. Patient demographics were obtained following clinical evaluation, as well as medical and gynaecological history assessment.

Inclusion criteria for the study entry were: infertile women who were eligible to undergo endometrial preparation (hormone replacement cycles) for embryo transfer of homologous frozen-thawed embryos at the blastocyst stage following IVF/intracytoplasmic sperm injection (ICSI) treatment; age 25–43, regular menstrual cycles (22–35 days). Exclusion criteria were: women undergoing donor oocyte IVF, pre-implantation testing,

uterine and/or endometrial pathology; pelvic inflammatory disease; BMI $> 35\ kg/m^2$.

Serum D3 measurement

Serum D3 levels were measured at 3 distinct time-points during endometrial preparation:

1. At the initiation of endometrial preparation (T1).
2. On the day of embryo transfer (T2).
3. On the day of serum beta-human chorionic gonadotropin (β-hCG) testing (T3).

Serum samples were taken at each time-point, as defined above, and assayed for serum D3 concentration, using electrochemiluminescence (ECL) technology for immunoassay analysis (Cobas e411 analyzer, Roche Diagnostics). D3 insufficiency was diagnosed when serum D3 levels were <30 ng/mL. In cases of D3 insufficiency at T1, a vitamin D supplement (Lecalcif®, SMB Technology S:A, Marche en Famenne, Belgium) at a dose of 25.000–50.000 IU weekly was administered. In other words, the allocation of participants to receiving vitamin D supplementation or not was decided according to their serum vitamin D measurement status at T1. Insufficient participants at T1 received supplementation, whereas replete participants at T1 did not. No further supplementation was administered based on the vitamin D status at T2 or T3.

Protocol of endometrial preparation and frozen-thawed embryo transfer

Endometrial preparation was initiated when basal E2 levels were <50 ng/mL and endometrial thickness at ultrasound was <4 mm.

Endometrial preparation was carried out with the administration of oral oestradiol valerate (Cyclacur®; Bayer Weimar GmbH und Co. KG, Weimar, Germany), with a starting dose of 4 mg to a gradual increase to 6 mg daily, or higher if required, until an endometrial thickness of >7 mm was achieved after at least 10 days of administration of oestrogen treatment. At that point, vaginal progesterone 3 times daily 200 mg (Utrogestan®; Capsugel Ploermel, Ploërmel, France) or subcutaneous progesterone 25 mg (Prolutex, IBSA Farmaceutici Italia Srl, Lodi, Italia) was administered 5 days before embryo transfer of blastocysts. All embryos thawed for transfer were previously cryopreserved at the blastocyst stage through vitrification. Embryo transfer was performed under transabdominal ultrasound guidance with a soft catheter (Guardia Embryo Transfer Catheter, Cook Medical). Women were allowed to be transferred at most two blastocysts, but single blastocyst transfer was strongly encouraged if the woman was <35 years old and had two or more good-quality blastocysts. Oestrogen and progesterone treatment were continuously co-administered until β-hCG results were obtained, and in the case of pregnancy, until 10 weeks of gestation.

Outcomes

The primary study endpoint was ongoing pregnancy per embryo transfer. Ongoing pregnancy was defined as a positive foetal heartbeat on ultrasound from a gestational age of 12 weeks onwards. The secondary outcomes were live birth, clinical pregnancy, miscarriage, positive β-hCG, and ectopic pregnancy per embryo transfer. Live birth was defined as the birth of a live foetus from 20 weeks onwards. We defined clinical pregnancy as the presence of a gestational sac on ultrasound at 6–7 weeks of gestation, miscarriage as a pregnancy loss before 20 weeks of gestation, and ectopic pregnancy as a clinical pregnancy outside the uterine cavity (ACOG, 2013; Clement et al., 2019; Kolte et al., 2015; NICE, 2019).

Statistical analysis

Assuming an ongoing pregnancy rate of 25% in the study population to be included as per the inclusion criteria, and a drop-out rate of 5%, we wanted to investigate whether vitamin D supplementation in D3-insufficient patients could increase the ongoing pregnancy rate by 15% (i.e. to 40% in the vitamin D supplementation group) with 80% power at an alpha level of 0.05. The 15% absolute increase in vitamin D3 replete women was based upon empirical data from our unit during a 3-year period prior to this study and, also, on observational data, previously published in a similar context (Franasiak et al., 2015; Ozkan et al., 2010; Rudick et al., 2014; van de Vijver et al., 2016). The power calculation analysis showed that a minimum of 252 participants is required to investigate this hypothesis.

We presented continuous variables as median [interquartile range (Q1, Q3)] and categorical variables as frequencies (%). We tested the normality of continuous data using the Shapiro-Wilk test. If the data was normally distributed, we compared the means of independent samples *via* the independent-samples *t*-test. If not, we used the Mann-Whitney U (Wilcoxon rank-sum) test. We performed Chi-square/Fisher's

exact tests to compare categorical data. We compared the participants' D3 values at the three time-points between one another using the Friedman test. We created an alluvial plot to visualize the changes in D3 status across the three time-points (T1, T2, and T3, as defined above). We compared the primary and secondary outcomes between women with insufficiency status at T1 (<30 ng/mL) who received supplementary vitamin D and D3-replete participants at T1 (≥30 ng/mL) who received no supplementary vitamin D *via* Chi-square/Fisher's exact tests. To further examine the impact of serum D3 status per se on clinical outcomes, we compared the outcome rates between D3-replete and insufficient participants at the other two time-points (T2 and T3) regardless of whether they received vitamin D supplementation or their D3 status at T1. Although the allocation of participants to receiving vitamin D supplementation or not was decided according to their serum vitamin D measurement and status at T1, and no further supplementation was administered based on the vitamin D status at T2 or T3, we decided to conduct these post-hoc analyses for exploratory purposes. The T2 and T3 analyses aimed to investigate a potential association between the insufficiency status at these additional time-points and the pre-specified IVF outcomes. We calculated crude odds ratios (ORs) and their 95% confidence intervals (95% CIs) *via* univariate binary logistic regression with each primary or secondary outcome serving as the dependent variable and the vitamin D supplementation at T1 (yes or no) or the D3 status (replete or insufficient) at each time-point being the independent variable. We further fitted multivariable logistic regression models, adjusting for age and body mass index (BMI). We performed the analyses using Stata version 15 (StataCorp. LLP, College Station, TX, USA). The alluvial plot was created with R version 4.4.1. We considered a *p*-value <0.05 as statistically significant.

Results

Study characteristics

A total of 285 participants were scheduled for FET and were screened for participating in the study. Of these, 32 were excluded from the study as per its protocol: 11 were excluded due to BMI > 35 kg/m^2, one due to age >43, 16 because of preimplantation genetic testing, and four because they underwent FET for reasons other than infertility. One of the remaining participants cancelled the cycle and withdrew from the study. Therefore, 252 participants were finally included. Of these, 115 (45.6%) were D3-insufficient at T1 and, therefore, received vitamin D supplementation, and 137 (54.4%) were D3-replete. Additional serum D3 measurements were available in 232 participants at T2 [164 (70.7%) replete, and 68 (29.3%) insufficient], and 173 participants at T3 [123 (71.1%) replete, and 50 (28.9%) insufficient]. Twenty-six (10.3%) individuals had D3 deficiency at T1, 5 (2.2%) at T2, and 3 (1.7%) at T3.

Demographic data were similar between D3-insufficient participants at T1 receiving vitamin D supplementation and D3-replete participants who did not receive vitamin D (Table 1). As expected, serum D3 levels at T1, T2, and T3 differed significantly from one another [median (Q1, Q3) at T1: 30.5 ng/mL (25.7, 35.7), T2: 33.6 ng/mL (29.4, 39.0), and T3: 34.4 ng/mL (29.6, 39.8), Friedman test $p < 0.001$]. The serum D3 levels at the three time-points also differed significantly from one another in participants who were given supplemental vitamin D [median (Q1, Q3) at T1: 25.5 ng/mL (21.2, 27.6), T2: 30.1 ng/mL (26.3, 33.9), and T3: 31.6 ng/mL (28.4, 36.0), Friedman test $p < 0.001$]. However, the difference was not significant in those who did not receive supplemental vitamin D [median (Q1, Q3) at T1: 35.2 ng/mL (32.0, 41.8), T2: 37.8 ng/mL (32.3, 41.9), and T3: 36.9 ng/mL (31.4, 42.3), Friedman test $p = 0.147$]. The serum D3 values also significantly differed at all time-points between those receiving and those not receiving vitamin D supplementation [T1: 25.5 ng/mL (21.2, 27.6) *vs.* 35.2 ng/mL (32.0, 41.8); T2: 30.1 ng/mL (26.3, 33.9) *vs.* 37.8 ng/mL (32.3, 41.9); T3: 31.6 ng/mL (28.4, 36.0) *vs.* 36.9 ng/mL (31.4, 42.3), respectively; all Mann-Whitney *U* test *p*-values <0.001]. Figure 1 is the participants' flow diagram. Figure 1 and Table S1 also depict the serum D3 status transitions. The alluvial plot (Figure 2) illustrates the changes in D3 status across the three time-points between participants reaching ongoing pregnancy and those not.

Outcomes

Ongoing pregnancy rates did not significantly differ between D3-insufficient participants at T1 receiving vitamin D and D3-replete ones not receiving any supplementation [40 (34.8%) *vs.* 51 (37.2%); $p = 0.687$; OR 0.90, 95% CI 0.54–1.51; adjusted OR 1.04, 95% CI 0.58–1.83]. The analysis of live births led to similar findings [18 (19.4%) *vs.* 15 (14.9%); $p = 0.404$] (Table 2). Similarly, positive β-hCG [70 (60.9%) *vs.* 85 (62.0%); $p = 0.849$], clinical pregnancy [54 (47.0%) *vs.* 68

Table 1. Characteristics of study participants.

	Overall study population ($n = 252$)		Vitamin D supplementation (D3-insufficient at T1) ($n = 115$)		No vitamin D supplementation (D3-replete at T1) ($n = 137$)		
	Characteristic	*n*	Characteristic	*n*	Characteristic	*n*	*p*-Value
Age (years)	37 (34, 40)	252	37 (34, 40)	115	38 (34, 40)	137	0.485
BMI (kg/m^2)	22.4 (20.7, 25.0)	212	22.8 (20.9, 26.1)	100	22.2 (20.4, 24.3)	112	0.142
Infertility duration (years)	2 (1, 4)	235	2 (1, 4)	108	2 (1, 4)	127	0.706
AMH (ng/mL)	1.89 (0.93, 2.82)	175	1.71 (0.89, 2.61)	83	2.08 (0.98, 2.95)	92	0.261
AFC	12 (8, 17)	179	14 (10, 17)	81	12 (7, 16)	98	0.083
FSH (IU/L)	6.49 (5.00, 8.36)	72	6.13 (4.51, 8.00)	35	6.89 (5.89, 8.90)	37	0.053
LH (IU/L)	4.73 (3.37, 6.55)	236	4.71 (3.11, 6.30)	108	4.92 (3.50, 6.67)	128	0.486
Oestradiol (pg/mL)	34.0 (22.0, 46.0)	237	34.3 (25.7, 45.8)	108	33.0 (20.5, 47.0)	129	0.418
Oocytes	5 (0, 11)	224	6 (0, 11)	104	3 (0, 11)	120	0.355
Metaphase II oocytes	4 (0, 9)	225	5 (1, 10)	104	3 (0, 9)	121	0.282
Fertilized oocytes	2 (1, 6)	230	2 (2, 6)	106	2 (1, 6)	124	0.653
Embryos transferred	Single: 92 (39.3%) Double: 142 (60.7%)	234	Single: 44 (40.7%) Double: 64 (59.3%)	108	Single: 48 (38.1%) Double: 78 (61.9%)	126	0.680
Cryopreserved embryos	1 (0, 3)	230	1 (0, 3)	106	1 (0, 3)	124	0.323
Ovulatory dysfunction	38 (16.6%)	229	15 (14.4%)	104	23 (18.4%)	125	0.421
Endometriosis	19 (8.3%)	229	8 (7.7%)	104	11 (8.8%)	125	0.762
Uterine factor	4 (1.8%)	229	2 (1.9%)	104	2 (1.6%)	125	1.000*
Tubal factor	44 (19.2%)	229	20 (19.2%)	104	24 (19.2%)	125	0.995
Female factor	104 (45.4%)	229	41 (39.4%)	104	63 (50.4%)	125	0.097
Male factor	99 (43.2%)	229	48 (46.2%)	104	51 (40.8%)	125	0.415
Unexplained infertility	47 (20.5%)	229	26 (25.0%)	104	21 (16.8%)	125	0.126
Other infertility factor	8 (3.5%)	229	2 (1.9%)	104	6 (4.8%)	125	0.297*
Unknown infertility factor	23 (9.1%)	252	11 (9.6%)	115	12 (8.8%)	137	0.825

T1: time-point 1 (i.e. initiation of endometrial preparation); *n*: number of participants (with available data).
Comparisons of baseline characteristics between participants receiving (i.e. serum D3-insufficient at T1) and those not receiving vitamin D supplementation (i.e. serum D3-replete at T1). All continuous variables were compared *via* the Mann-Whitney *U* test.
*Fisher's exact test.

(49.6%); $p = 0.672$], miscarriage [14 (12.2%) *vs.* 17 (12.4%); $p = 0.955$], and ectopic pregnancy rates [1 (0.9%) *vs.* 0%; Fisher's exact $p = 0.456$] were not significantly different between the two groups (Table 2). The univariate and multivariable logistic regression provided similar results (Table 2).

The ongoing pregnancy rates did not significantly differ between D3-insufficient and D3-replete participants at T2 [21 (30.9%) *vs.* 66 (40.2%), respectively; $p = 0.180$] and between D3-insufficient and D3-replete participants at T3 [17 (34.0%) *vs.* 51 (41.5%), respectively; $p = 0.362$], regardless of whether they received vitamin D supplementation before embryo transfer (Tables S2(a,b)). The same applied to the rest of the outcomes. The univariate and multivariable logistic regression led to similar findings (Tables S2(a,b)).

Discussion

Main findings

This prospective cohort study of infertile women scheduled for FET found no significant difference in both primary (ongoing pregnancy), and secondary outcomes (live birth, positive β-hCG, clinical pregnancy, miscarriage, and ectopic pregnancy) between D3-insufficient participants at the initiation of endometrial preparation (Time-Point 1—T1) receiving vitamin D supplementation and D3-replete ones who did not receive any supplementation. The univariate and multivariable (adjusting for age and BMI) logistic regression led to similar findings. We also found no significant difference in all outcomes between D3-replete and insufficient participants at the time-points of embryo transfer (T2) and β-hCG measurement (T3).

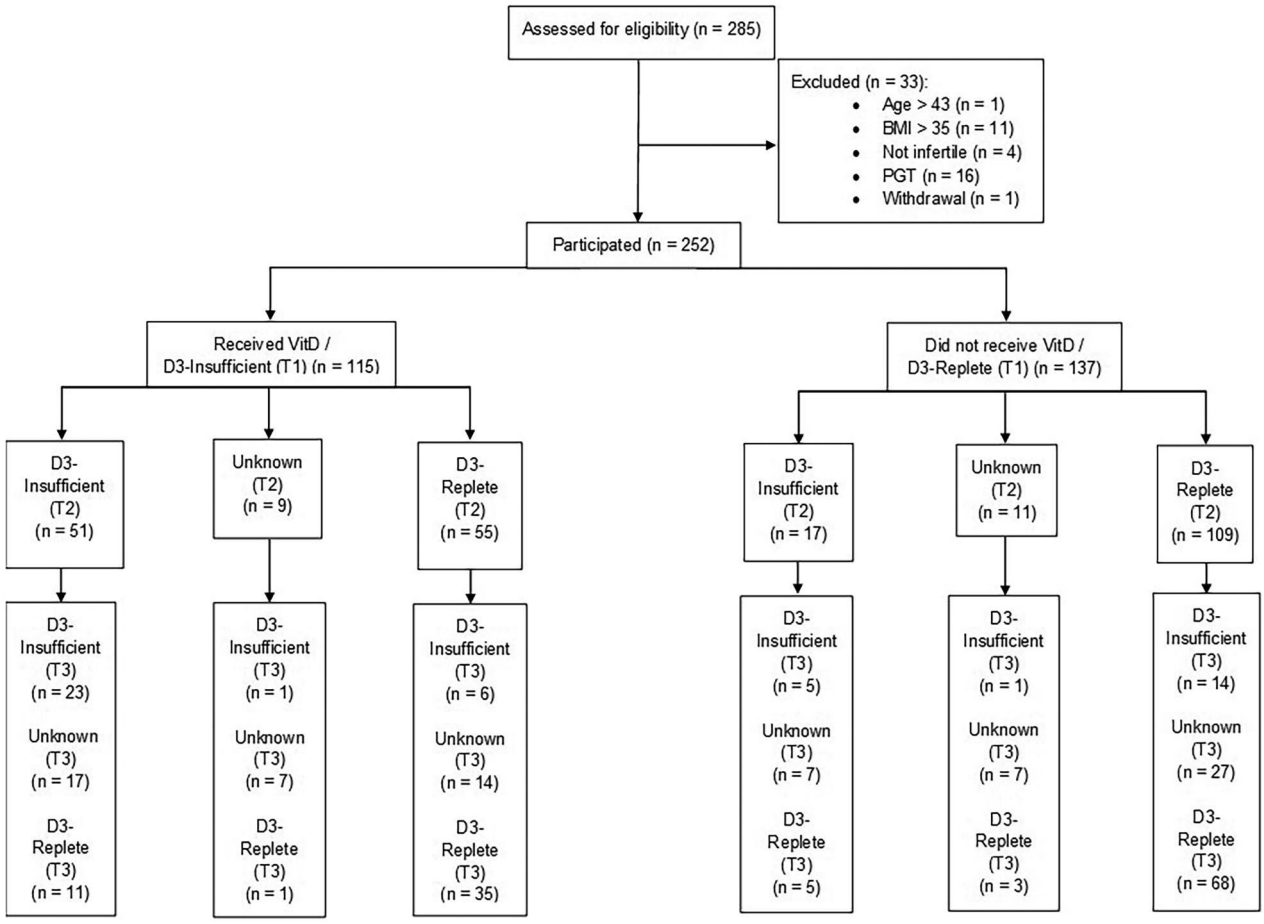

Figure 1. Participants' flow diagram. Adapted from the CONSORT flow diagram. An 'unknown' vitamin D status refers to missing data. VitD: vitamin D supplementation; D3: 25-OH vitamin D3/cholecalciferol; T1: time-point 1 (initiation of endometrial preparation); T2: time-point 2 (embryo transfer); T3: time-point 3 (beta-human chorionic gonadotropin measurement); *n*: number of participants; BMI: body mass index; PGT: preimplantation genetic testing.

Strengths and limitations

Regarding the strengths, and to the best of our knowledge, this study includes the largest sample of patients undergoing an FET cycle studied for the effects of vitamin D supplementation on IVF outcomes in the literature so far. It also prospectively measured serum D3 levels at three distinct time-points during a frozen cycle. We compared IVF outcomes between insufficient patients receiving vitamin D and replete patients receiving no supplement.

The main limitation of our study includes the lack of randomization during patient recruitment. While we could have included a control group of patients with insufficiency receiving no vitamin D supplementation, we designed the study so that all patients with insufficiency could receive vitamin D and compared the cycle outcomes between serum D3-replete participants receiving no supplement and insufficient ones receiving supplementation. We also assessed the potential effect of remaining insufficiency at embryo transfer and the time-point of β-hCG measurement. It is important to highlight that the main aim of this more 'pragmatic' prospective cohort study was to compare insufficient participants at T1 receiving vitamin D supplementation and replete participants at T1 who did not receive supplementation. Therefore, the allocation to vitamin D supplementation or not was decided based on the serum D3 status at T1, which was known in all patients. The analyses on T2 and T3 were supplementary, *post-hoc*, and aimed to potentially stimulate further research. Due to missing data on vitamin D status at T2 and T3 and for the reasons presented above, the findings of the exploratory analyses investigating a potential association between the vitamin D status at T2 and T3 and the pre-specified IVF outcomes should be interpreted with extreme caution. Additionally, the total number of women whose status was measured as replete at T3 (123) seems less than those whose status was measured as replete at T1 (137). However, women whose status was measured as insufficient at T3 (50) were much less than those whose status was insufficient at T1 (115). Therefore,

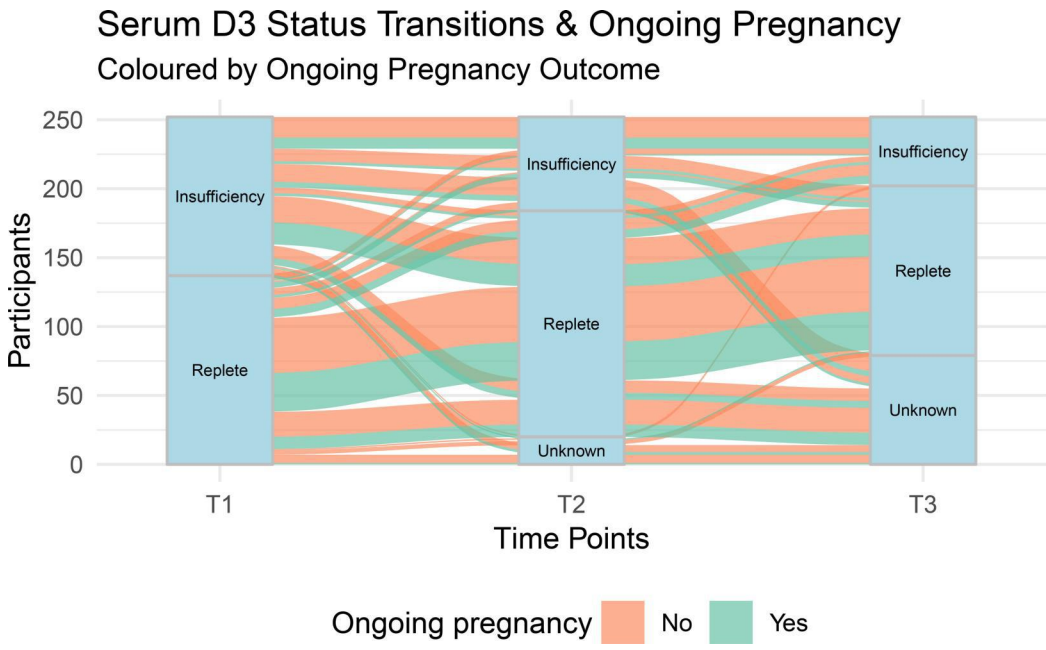

Figure 2. Alluvial plot depicting the changes in serum D3 status (replete, insufficient, or unknown) from time-point 1 (initiation of endometrial preparation) to time-point 2 (embryo transfer), and time-point 3 (beta-human chorionic gonadotropin measurement). Participants who reached ongoing pregnancy are represented by green lines, while those not reaching it are represented by red lines. An 'unknown' vitamin D status refers to missing data. T1: time-point 1 (initiation of endometrial preparation); T2: time-point 2 (embryo transfer); T3: time-point 3 (beta-human chorionic gonadotropin measurement).

Table 2. Comparisons of outcomes between participants receiving (i.e. serum D3-insufficient at T1) and those not receiving vitamin D supplementation (i.e. serum D3-replete at T1).

	Overall study population ($n = 252$)	Vitamin D supplementation (D3-insufficient at T1) ($n = 115$)	No vitamin D supplementation (D3-replete at T1) ($n = 137$)	OR (95% CI)**	Adjusted OR (95% CI)**	*p*-Value
Ongoing pregnancy	91 (36.1%)	40 (34.8%)	51 (37.2%)	0.90 (0.54–1.51)	1.04 (0.58–1.83)	0.687
Live birth	33 (17.0%)***	18 (19.4%)	15 (14.9%)	1.38 (0.65–2.92)	1.82 (0.80–4.17)	0.404
Clinical pregnancy	122 (48.4%)	54 (47.0%)	68 (49.6%)	0.90 (0.55–1.48)	1.00 (0.58–1.73)	0.672
Miscarriage	31 (12.3%)	14 (12.2%)	17 (12.4%)	0.98 (0.46–2.08)	0.93 (0.41–2.14)	0.955
Ectopic pregnancy	1 (0.4%)	1 (0.9%)	0 (0%)	NA	NA	0.456*
Positive β-hCG	155 (61.5%)	70 (60.9%)	85 (62.0%)	0.95 (0.57–1.58)	1.10 (0.62–1.94)	0.849

T1: time-point 1 (initiation of endometrial preparation); OR: odds ratio; 95% CI: 95% confidence interval; *n*: number of participants (with available data); β-hCG: beta-human chorionic gonadotropin; NA: not applicable.
The univariate and multivariable (adjusted for age and body mass index) logistic regression results are also provided.
*Fisher's exact test.
**The D3-replete group at T1 that did not receive vitamin D supplementation served as the reference group.
***The outcome of live birth was known in 194 participants.

this picture of less replete participants at T3 than at T1 might be created by the missing data at T3. Furthermore, all the main conclusions drawn from this study consider ongoing pregnancy, the pre-specified primary endpoint, rather than live birth, a secondary outcome. The findings of the analyses on live births should be interpreted with caution due to missing data on this outcome.

Comparison with other studies

Based on previous studies, it appears that D3 has a pivotal role in reproductive function, and therefore, D3 status assessment and vitamin D supplementation are increasingly applied in clinical practice (Grundmann & von Versen-Höynck, 2011). However, the association of serum D3 levels with IVF outcomes is still controversial, as clinical studies of vitamin D supplementation in patients undergoing IVF treatment have been inconsistent so far in their findings (Meng et al., 2023; Somigliana et al., 2021; Zhou et al., 2022). While a previous systematic review and meta-analysis on the relationship between vitamin D3 levels and IVF outcomes failed to show any significant association between vitamin D3 levels and clinical pregnancy, live birth, and miscarriage rates (Cozzolino et al., 2020), a recent

systematic review and meta-analysis on the influence of vitamin D3 supplementation on reproductive outcomes of infertile patients has suggested an improvement in clinical pregnancy rates of infertile women (Meng et al., 2023). Regarding the effect of vitamin D3 levels on frozen embryo transfer cycle outcomes, previous studies have shown no significant association (Ko et al., 2025; van de Vijver et al., 2016).

So far, five RCTs have examined the effect of vitamin D supplementation before IVF, with only three of those looking at the exclusive effects of vitamin D (Abedi et al., 2019; Aflatoonian et al., 2022; Bezerra Espinola et al., 2021; Fatemi et al., 2017; Kermack et al., 2020). A study by Abedi et al. (2019) using the same scheme of vitamin D supplementation showed a significantly higher clinical pregnancy rate in women undergoing a complete ICSI cycle. More recently, an RCT by Somigliana et al. (2021) examining the effect of a single dose of 600.000 IU of vitamin D before the initiation of IVF in women with serum D3 deficiency or insufficiency showed no significant benefit on the chances of a clinical pregnancy.

Our study showed that ongoing pregnancy rates were similar between women with insufficient levels receiving vitamin D supplementation and replete serum D3 levels receiving no supplement. A possible explanation could be that D3 levels may not be the principal parameter in reproductive outcomes but rather an association with other undetected confounders. In this context, D3 status may be considered a surrogate marker of the general state of health (Manson et al., 2019; Manson & Bassuk, 2015; Preiss & Sattar, 2019). Another possible explanation of this study's findings could be that supplementary vitamin D in insufficient women at T1 may lead to comparable outcomes with replete women at T1 who did not receive any supplementation.

Moreover, the current definition of D3 insufficiency is based on peripheral levels of D3, as proposed within the research setting of endocrine studies on bone and calcium metabolism. However, we still lack evidence to support that the same reference values apply to the field of reproductive physiology, with a recent study by Cozzolino et al. (2020) failing to identify a cut-off point associated with infertility. Lastly, it cannot be ruled out that a paracrine effect of D3 may still be adequate at a local level in the endometrium and the ovaries despite insufficient serum levels. As we are not able to assess the local paracrine action of D3, inevitably, we use serum levels as a surrogate marker, a potential source of bias in the interpretation of peripheral D3 levels.

Implications for clinical practice

This prospective cohort study of patients undergoing FET found no significant differences in ongoing pregnancy and other important IVF outcomes between serum D3-insufficient participants receiving vitamin D supplementation before embryo transfer and D3-replete participants receiving no supplement. Taking this study's and the previous studies' findings into consideration, no robust conclusions regarding vitamin D supplementation in women undergoing IVF or ICSI, and in this case, those scheduled for FET, can be drawn at this point. There is currently inadequate evidence to support routine vitamin D supplementation.

Implications for future research

Future larger, high-quality trials with more standardized vitamin D regimens are required to investigate potentially smaller effects of vitamin D supplementation on IVF outcomes, especially in women scheduled for FET. Future studies could further compare the IVF outcomes between replete participants, insufficient participants receiving no supplementation, and insufficient participants receiving a vitamin D supplement. They could also focus on the effect of vitamin D on IVF outcomes of patients with serum D3 deficiency (serum D3 levels <20 ng/mL) and the outcomes of participants remaining insufficient at all time-points despite receiving supplementation. This study contained little data on these categories and could not perform such analyses.

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Author contributions

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Data availability statement

The data that support the findings of this study are available from the corresponding author, NC, upon reasonable request.

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