

Vitamin D deficiency and pregnancy rates in women undergoing single embryo, blastocyst stage, transfer (SET) for IVF/ICSI

Nikolaos P. Polyzos^{1,*}, Ellen Anckaert², Luis Guzman^{2,3},
Johan Schiettecatte², Lisbet Van Landuyt¹, Michel Camus¹,
Johan Smitz², and Herman Tournaye¹

¹Centre for Reproductive Medicine, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Laarbeeklaan 101, 1090 Brussels, Belgium

²Laboratory of Hormonology and Tumormarkers, Universitair Ziekenhuis Brussel, Vrije Universiteit Brussel, Brussels, Belgium

³Grupo PRANOR, Lima, Perú

*Correspondence address. Tel: +32-2-477-66-60; Fax: +32-2-477-66-49; E-mail: n.polyzos@gmail.com; nikolaos.polyzos@uzbrussel.be

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STUDY QUESTION: What is the influence of vitamin D deficiency on pregnancy rates among women undergoing IVF/ICSI and Day 5 (blastocyst stage) single embryo transfer (SET)?

SUMMARY ANSWER: Vitamin D deficiency results in significantly lower pregnancy rates in women undergoing single blastocyst transfer.

WHAT IS KNOWN ALREADY: Preliminary experiments have identified the presence of vitamin D receptors in the female reproductive system. However, results regarding the effect of vitamin D deficiency on clinical outcomes are conflicting. None of the previous studies adopted a SET strategy.

STUDY DESIGN, SIZE, DURATION: Serum vitamin D concentration was measured retrospectively in patients who underwent SET on Day 5. Overall 368 consecutive infertile women treated within a period of 15 months were included in the study.

PARTICIPANTS/MATERIALS, SETTING, METHODS: All patients underwent ovarian stimulation for IVF/ICSI and Day 5 SET. Serum samples were obtained 7 days prior to embryo transfer and stored frozen at -20°C . Samples were collectively analyzed for their 25-OH vitamin D content. Vitamin D deficiency was defined as serum 25-OH vitamin D levels <20 ng/ml in accordance with the Institute of Medicine and the Endocrine Society clinical practice guidelines.

MAIN RESULTS AND THE ROLE OF CHANCE: Clinical pregnancy rates were significantly lower in women with vitamin D deficiency compared with those with higher vitamin D values (41 versus 54%, $P = 0.015$). Logistic regression analysis was performed to identify whether vitamin D deficiency is independently associated with clinical pregnancy rates after controlling for 16 potential confounding factors. According to our results vitamin D deficiency was independently associated with lower clinical pregnancy rates, odds ratios [ORs (95% confidence interval (CI)) 0.61 (0.39–0.95)] for vitamin D deficiency (deficient versus non-deficient women), $P = 0.030$. Finally, even when restricting our analysis to women undergoing elective SET (274 patients), vitamin D deficiency was again independently associated with pregnancy rates [OR (95% CI) 0.56 (0.33–0.93), $P = 0.024$].

LIMITATIONS, REASONS FOR CAUTION: Our results refer only to patients undergoing Day 5 SET. Although vitamin D deficiency appears to compromise pregnancy rates in this population, no guidance can be provided regarding a potential relationship between vitamin D deficiency and ovarian reserve or response to ovarian stimulation.

WIDER IMPLICATIONS OF THE FINDINGS: Vitamin D deficiency impairs pregnancy rates in women undergoing single blastocyst transfer. Future prospective confirmatory studies are needed to validate our results and examine the exact underlying mechanism by which vitamin D levels may impair pregnancy rates in infertile women undergoing IVF/ICSI.

STUDY FUNDING/COMPETING INTEREST(S): None declared.

Key words: vitamin D deficiency / IVF / single embryo transfer / pregnancy

Introduction

Vitamin D has a prominent role in the homeostasis of calcium and phosphorus and its deficiency has been proved to increase the risk of osteoporosis, fractures and muscle weakness in elderly people (Holick, 2007). However, in addition to its beneficial effects in the bone and muscular system [Bischoff-Ferrari *et al.*, 2005, 2009, 2012; DIPART (Vitamin D Individual Patient Analysis of Randomized Trials) Group, 2010], there is an increased scientific interest regarding non-skeletal actions of vitamin D (Holick, 2007; Bouillon *et al.*, 2008). Data from experimental research suggest that, among others, vitamin D receptors are present in brain, prostate, breast and colon tissues, with latest reports suggesting the presence of these receptors in immune cells (Holick, 2007; Bouillon *et al.*, 2008).

Female reproduction has been a research field in which the role of vitamin D has not been extensively examined. However, early evidence from basic research strongly indicates a potential role of vitamin D in human reproduction. Vitamin D receptors are present and differentially expressed in murine endometrium and ovary throughout the estrous cycle (Zamani *et al.*, 2010), whereas knock-out experiments have shown that vitamin D receptor null mice experience uterine hypoplasia and impaired folliculogenesis (Yoshizawa *et al.*, 1997). Finally, a study in cell cultures confirmed the expression of vitamin D receptors in human endometrial cells and demonstrated that the expression 1 α -hydroxylase, an enzyme which catalyzes the hydroxylation of calcidiol to calcitriol (the bioactive form of vitamin D) is up-regulated in the human endometrial stromal cells of early pregnant versus cycling endometria (Vigano *et al.*, 2006).

Nonetheless, despite the data derived from basic research, up to date, only a few cohort studies have attempted to examine the role of vitamin D levels in infertile patients (Anifandis *et al.*, 2010; Ozkan *et al.*, 2010; Aleyasin *et al.*, 2011; Rudick *et al.*, 2012). Results from these studies are strongly contradictory, with some findings showing that maternal vitamin D deficiency is associated with lower pregnancy rates (Ozkan *et al.*, 2010; Rudick *et al.*, 2012) and others demonstrating that vitamin D deficiency does not affect the final reproductive outcome (Anifandis *et al.*, 2010; Aleyasin *et al.*, 2011). In addition, the number of patients enrolled in these studies is fairly small, whereas the major shortcoming of all of the studies is the fact that, no single embryo transfer policy has been adopted, with patients receiving up to four embryos per transfer. This indeed may severely bias the results, since it might be a strong confounding factor for estimating differences in pregnancy rates between vitamin D deficient and replete women. Finally, a recent retrospective study postulated that vitamin D deficiency may negatively affect pregnancy rates with an effect mediated through the endometrium, given that vitamin D deficiency was not correlated with ovarian stimulation characteristics or with markers of embryo quality in this study (Rudick *et al.*, 2012).

Taking into account the above evidence, our aim was to design a large cohort study in order to examine, in a selected good prognosis population, whether vitamin D may impair pregnancy rates in women undergoing a single Day 5 embryo transfer. For this reason we measured serum

25-OH vitamin D levels 7 days prior to the embryo transfer in consecutive patients who underwent ovarian stimulation for IVF/ICSI and had a single blastocyst embryo transfer.

Materials and Methods

Institutional review board approval was obtained for the conduction of the study from the Ethical Committee of Universitair Ziekenhuis Brussel (decision number B.U.N. 143201214016, date 26 April 2012).

Patients' eligibility criteria

All infertile women between 18 and 36 years old who underwent an ovarian stimulation cycle for IVF/ICSI and had single embryo, blastocyst stage (Day 5), transfer (SET) within a period of 15 months (February 2011–May 2012) were eligible.

Patients were considered eligible irrespective of the stimulation protocol used. Ovulation triggering was performed with s.c. injections of hCG (5000 or 10 000) as soon as three follicles of 17 mm were visible in transvaginal ultrasound.

Women who were planned to have a Day 3 embryo transfer or women who had their cycle canceled were excluded from the study, since the purpose of the study was to examine whether vitamin D levels may affect the pregnancy rates in women undergoing an embryo transfer with a single blastocyst. Women undergoing ovarian stimulation for IVF/ICSI for preimplantation genetic diagnosis were also excluded from the study due to the fact that these women are not infertile but are patients undergoing IVF/ICSI for the prevention of hereditary genetic disease.

Serum 25-OH vitamin D measurement

Li-heparin samples from all patients had been obtained 7 days prior to embryo transfer (on the day of hCG administration) and were kept frozen at -20°C until the measurement of 25-OH vitamin D levels. Plasma total 25-OH vitamin D was measured with the Elecsys Vitamin D Total immunoassay on a Cobas6000 immunoanalyzer (Roche Diagnostics, Mannheim, Germany). Total imprecision coefficients of variance were 9.7% at a concentration level of 16.5 ng/ml and 5.9% at 31.7 ng/ml.

Vitamin D deficiency was defined as serum 25-OH vitamin D levels < 20 ng/ml in accordance with the Institute of Medicine (IOM) and the Endocrine Society clinical practice guidelines (Holick *et al.*, 2011; Rosen *et al.*, 2012).

Main outcome measures

The primary outcome was clinical pregnancy rates defined as the presence of an intrauterine sac with an embryonic pole demonstrating cardiac activity at 7 weeks of gestation.

Secondary outcomes were positive hCG rates and live birth rates.

Pregnancy rates were assessed between vitamin-D deficient women (< 20 ng/ml) and those with vitamin D levels ≥ 20 ng/ml. In addition, subgroup analyses were performed for vitamin D insufficient patients (Vitamin D 20–30 ng/ml) and vitamin D replete patients (≥ 30 ng/ml) as defined by the Endocrine Society (Holick *et al.*, 2011).

Assessment of confounding factors

To eliminate the likelihood of confounding bias, owing to the retrospective study design, we examined additional variables that could be potential confounders for the association between vitamin D levels and pregnancy outcomes. For this reason we recorded 16 variables that might be related with the final outcome (clinical pregnancy).

These variables were related to the following:

- (1) Patients' characteristics (age, previous IVF attempts and type of infertility).
- (2) Ovarian stimulation characteristics [type of treatment protocol (GnRH agonist or antagonist), type of gonadotrophin used (rFSH, hpHMG, corifollitropin alfa), gonadotrophins starting dose, E2 levels on the day of hCG and the number of oocytes retrieved].
- (3) Type of procedure (IVF or ICSI).
- (4) Embryo quality [elective SET (eSET) (yes/no) (patients in whom one blastocyst (Day 5 embryo) was selected from a larger cohort of available Day 5 embryos, suitable for embryo transfer), the quality of the embryo transferred (top quality or not) and the percentage of top quality embryos (the percentage of top quality embryos among the total number of 2PN embryos)].
- (5) Endometrial receptivity [endometrial thickness on the day of hCG, and serum progesterone levels on the day of hCG administration (≥ 1.5 ng/ml versus < 1.5 ng/ml)].
- (6) Vitamin D levels [vitamin D deficiency (yes or no)].
- (7) Season of blood sampling and embryo transfer (winter, spring, summer or autumn).

The season of blood sampling and embryo transfer was included in the model because it has been previously demonstrated that serum vitamin D levels significantly vary according to season owing to the different exposure to sun (Pittaway et al., 2013).

Blastocyst quality was evaluated on Day 5 at the moment of transfer using the grading system of Gardner and Schoolcraft (1999). Embryos were selected for fresh transfer on Day 5 if they had at least reached the stage of full compaction, early blastocyst (BI1 or BI2), full (BI3), expanded (BI4) or hatching (BI5-6) blastocyst. Full, expanded and hatching blastocysts were eligible for transfer if they had at least an inner cell mass (ICM) type C and trophoctoderm (TE) quality type B. Top quality embryos on Day 5 were considered to have reached at least the full blastocyst stage (BI3) with an ICM and TE quality type AA or AB.

Seasons were defined prior data processing according to the calendar definitions of the seasons for Europe with each season lasting 3 months: autumn: September 21–December 20; winter: December 21–March 20; Spring: March 21–June 20; summer: June 21–September 20, as previously described (Wunder et al., 2005).

Finally, we assessed fertilization rates and percentage of top quality embryos in order to identify potential differences between the different groups according to their serum 25-OH vitamin D levels. Normal fertilization was identified by the presence of two pronuclei (2PN) at the time of fertilization assessment, 16–19 h after ICSI or conventional insemination. Fertilization rate was defined as the percentage of 2PN embryo divided by number of MII oocytes. The percentage of top quality embryos was defined as the percentage of top quality embryos among the total number of 2PN embryos. Both outcomes were expressed as mean percentages with 95% confidence intervals (CIs).

Statistical analysis

Continuous variables were presented as means and standard deviations and categorical variables as percentages. Analysis was performed with the use of an independent *t*-test or a Mann–Whitney *U*-test for continuous variables and with the use of the χ^2 test for categorical variables.

Stepwise logistic regression was applied to identify independent variables associated with clinical pregnancy rates.

In the logistic regression model, clinical pregnancy was set as the dependent variable, with independent variables being age, previous IVF attempts, type of infertility, the type of treatment protocol (GnRH agonist or antagonist), type of gonadotrophin used (rFSH, hpHMG, corifollitropin alfa), gonadotrophins starting dose, E2 levels on the day of hCG, number of oocytes retrieved, type of procedure (IVF or ICSI), elective SET (eSET) (yes/no) was performed [patients in whom one blastocyst (Day 5 embryo) was selected from a larger cohort of available Day 5 embryos, suitable for embryo transfer), quality of the embryo transferred (top quality or not), the percentage of top quality embryos (the percentage of top quality embryos among the total number of 2PN embryos), endometrial thickness, serum progesterone levels on the day of hCG administration (≥ 1.5 ng/ml versus < 1.5 ng/ml), the season of the embryo transfer (winter, spring, summer, autumn) and vitamin D deficiency (yes or no).

The significance level of the candidate predictive variables to enter the model was set to 0.05 and to stay in the model it was set to 0.10. After selection of the candidate predictive factors, the final model included those prognostic factors with statistical significance according to the Wald statistic test at a threshold of 0.05. The goodness of fit of the normal regression models was assessed by the Hosmer–Lemeshow goodness-of-fit test.

Similarly, logistic regression analysis was performed only in women undergoing eSET.

Analyses were performed using SPSS 20 statistical software (SPSS version 20, Chicago, IL, USA).

Sample-size calculation

Based on a previous study from our group the clinical pregnancy rates following single blastocyst transfer was 38.7% per embryos transfer (Papanikolaou et al., 2006). Our hypothesis was that vitamin D deficient patients should have 15% lower pregnancy rates compared with those with vitamin D levels > 20 ng/ml. Based on this hypothesis we made the assumption that a total sample size of 366 patients has 80% power to detect a difference between the group proportions of 15%. The proportion in Group 1 (vitamin D levels > 20 ng/ml) is assumed to be 35% under the null hypothesis and 50% under the alternative hypothesis. The proportion in Group 2 (vitamin D deficiency) is 35%. The test statistic used is the two-sided Fisher's exact test. The significance level of the test was targeted at 0.05.

Results

Patients' and stimulation characteristics

Overall, 368 patients were included in this study (Fig. 1), with a mean (SD) age of 30.5 ± 3.7 and BMI of 23.2 ± 4.0 . As shown in Table I, patients' characteristics and stimulation protocol characteristics were comparable between vitamin-D deficient women and those with vitamin D levels ≥ 20 ng/ml.

Embryological data

No significant differences were observed between vitamin-D deficient women and those with vitamin D levels ≥ 20 ng/ml regarding the number of oocytes, number of mature oocytes, fertilization rates, percentage of top quality embryos and quality of transferred embryo (top quality or not) Table II. However, vitamin-D deficient women had a borderline significantly lower cycles with eSET compared with the remaining population (81 versus 71%, $P = 0.046$) Table II.

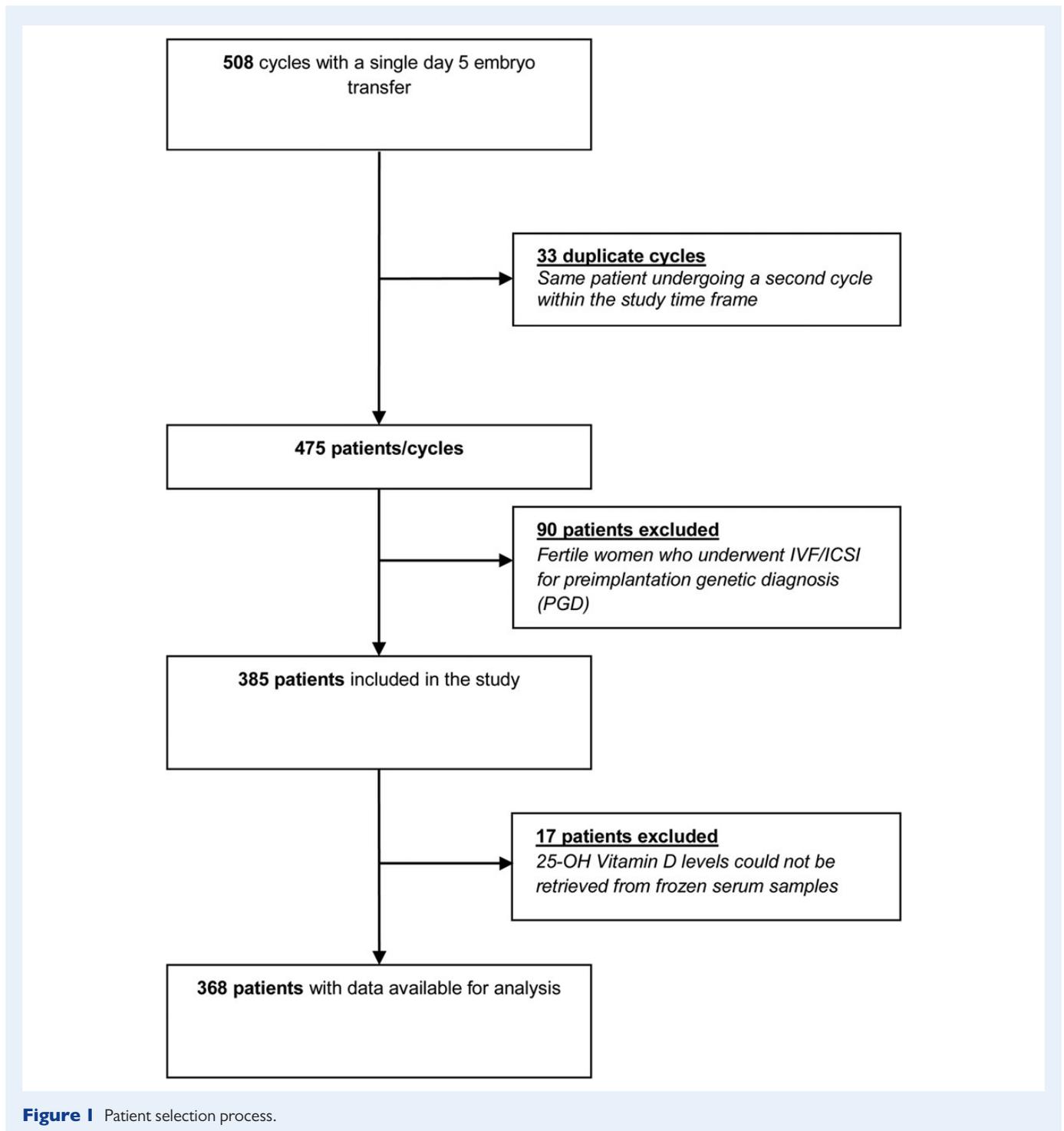


Figure 1 Patient selection process.

Pregnancy rates

Overall, 57% of the patients had a positive pregnancy test, 46% a clinical pregnancy and 40% had a live birth. As shown in Table II, positive hCG, clinical pregnancy and live birth rates were significantly lower in vitamin-D deficient women compared with those with 25-OH vitamin D values exceeding 20 ng/ml.

Clinical pregnancy rates were significantly lower in women with vitamin D deficiency (<20 ng/ml) compared with those with higher

vitamin D values [41% (98/239) versus 54% (70/129), $P = 0.015$]. When analyzing the results according to different thresholds, as proposed by the Endocrine society, clinical pregnancy rates were comparable between vitamin D insufficient (20–30 ng/ml) and vitamin D replete women (≥ 30 ng/ml) [53.3% (49/92) versus 56.7% (21/37) $P = 0.845$]. Vitamin D-deficient patients demonstrated significantly lower clinical pregnancy rates compared with vitamin D-insufficient women [41% (98/239) versus 53.3% (49/92), $P = 0.044$] and

Table I Patients' and ovarian stimulation characteristics according to vitamin D status.

	Vitamin D < 20 ng/ml	Vitamin D ≥ 20 ng/ml	P value
Patients characteristics			
Number of patients	239	129	
Age, mean (SD)	30.3 (3.8)	30.9 (3.5)	0.13
BMI, mean (SD)	23.4 (4.3)	23.0 (3.6)	0.38
Type of infertility, <i>n</i> (%)			
Male factor	134 (56)	55 (43)	0.094
Idiopathic	53 (22)	41 (32)	
Tubal factor ovulatory	24 (10)	11 (8)	
Endometriosis	19 (8)	15 (12)	
Other	9 (4)	7 (5)	
Ethnicity, <i>n</i> (%)			
Caucasian	223 (93.3)	120 (93)	0.652
Other	16 (6.7)	9 (7)	
Previous IVF/ICSI attempts, <i>n</i> (%)			
0	183 (77)	93 (72)	0.34
≥ 1	56 (23)	26 (28)	
Stimulation protocol characteristics			
Type of GnRH analog, <i>n</i> (%)			
Antagonist	215 (90)	120 (93)	0.33
Agonist	24 (10)	9 (7)	
Type of gonadotrophins, <i>n</i> (%)			
rFSH	161 (67)	93 (72)	0.63
hp HMG	44 (18)	21 (16)	
Corifollitropin alfa	34 (14)	15 (12)	

BMI, body mass index; rFSH, recombinant follicle stimulating hormone; hpHMG, highly purified human menopausal gonadotrophin.

borderline non-significant lower pregnancy rates compared with vitamin D-replete women [41% (98/239) versus 56.7% (21/37), $P = 0.07$].

Although, the incidence of vitamin D deficiency differed among different seasons ($P < 0.0001$) (Table I), seasonality did not significantly affect clinical pregnancy rates ($P = 0.17$). Logistic regression analysis was performed to determine variables independently associated with clinical pregnancy. Although 16 independent variables (patients' and stimulation characteristics, type of IVF procedure and factors related with embryo quality and implantation) were included in the logistic regression model, vitamin D deficiency, eSET and elevated progesterone on the day of hCG administration were the only independent variables associated with clinical pregnancy rates (Table III).

Vitamin D deficiency was independently associated with reduced pregnancy rates with an odds ratio (OR, 95% CI) of 0.61 (0.39–0.95) for vitamin D deficiency (deficient versus non-deficient women), $P = 0.030$, (Table III). According to these figures, women with vitamin D deficiency had a 39% lower odds ratio for a clinical pregnancy compared with those with 25-OH vitamin D levels ≥ 20 ng/l.

Finally, logistic regression analysis including only women undergoing eSET (274 patients) has shown that the only variables associated with clinical pregnancy rates were vitamin D deficiency, endometrial thickness and progesterone elevation on the day of hCG administration (Table III). As shown, vitamin D deficiency was again independently associated with

lower pregnancy rates with an OR (95% CI) for vitamin D deficiency (deficient versus non-deficient women) of 0.56 (0.33–0.93), $P = 0.024$. According to the figures presented in Table III, among women undergoing eSET, vitamin D-deficient patients had a 44% lower odds for a clinical pregnancy compared with those with 25-OH vitamin D levels ≥ 20 ng/ml.

Discussion

To our knowledge this is the largest study examining the correlation of serum vitamin D levels with pregnancy rates in an infertile population undergoing IVF/ICSI. Furthermore, it is the first study examining the effect of vitamin D deficiency among patients who underwent a single blastocyst embryo transfer and had their vitamin D levels determined shortly prior to embryo replacement.

Our study suggests that serum vitamin D levels, prior to the transfer of a single Day 5 (blastocyst) embryo, are significantly associated with clinical pregnancy rates and vitamin D deficiency appears to compromise pregnancy rates, among women undergoing Day 5 SET.

A potential explanation for the deleterious effect of vitamin D deficiency on the final reproductive outcome may be associated with a negative effect of low vitamin D levels on endometrial receptivity. The presence of vitamin D receptors in the endometrium of mice has been

Table II Embryological and clinical outcomes.

	Vitamin D < 20 ng/ml	Vitamin D ≥ 20 ng/ml	P value
Number of patients	239	129	
Number of oocytes retrieved, mean (SD)	12.1 (6.4)	12.0 (5.5)	0.91
Number of MII oocytes, mean (SD)	8.5 (5.5)	8.2 (4.8)	0.58
Fertilization rate (%), mean (95% CI)	78.7 (76.4–81.0)	78.2 (75.2–81.4)	0.81
Percentage of top quality embryos (%), mean (95% CI)	9.0 (7.2–10.9)	10.2 (7.4–12.9)	0.48
Elective SET (eSET) ^a , n (%)	170 (71)	104 (81)	0.046
Top quality transferred embryo, n (%)	121 (51)	65 (50)	0.97
Season of embryo transfer, n (%)			
Winter	73 (31)	24 (19)	<0.0001
Spring	93 (39)	44 (34)	
Summer	29 (12)	39 (30)	
Autumn	44 (18)	22 (17)	
Positive hCG, n (%)	124 (52)	86 (67)	0.006
Clinical pregnancy, n (%)	98 (41)	70 (54)	0.015
Live birth, n (%) ^b	78 (35)	61 (48)	0.015

^aElective single transfer (eSET) is the selection of one Day 5 embryo from a larger cohort of available Day 5 embryos suitable for embryo transfer.

^bData for live births were not available in 17 patients who were lost in follow-up. These patients were not calculated for the live birth analysis.

Table III Logistic regression models.

	B ^a	SE	Wald χ^2	P value	OR ^a	95% CI
Whole study population ^a						
Elective SET	0.722	0.257	7.912	0.005	2.059	1.245–3.407
Progesterone elevation on the day of hCG	−1.069	0.435	6.046	0.014	0.343	0.146–0.805
Vitamin D deficiency	−0.492	0.227	4.704	0.030	0.612	0.392–0.954
Only patients with eSET ^b						
Endometrial thickness on the day of hCG	0.135	0.053	6.360	0.012	1.144	1.030–1.270
Progesterone elevation on the day of hCG	−0.891	0.449	3.939	0.047	0.410	0.170–0.989
Vitamin D deficiency	−0.588	0.261	5.092	0.024	0.556	0.333–0.926

B, regression coefficient; SE, standard errors of regression coefficient; OR, odds ratios; 95% CI, 95% confidence intervals; (e)SET (elective), single embryo transfer.

^aB < 0 and OR < 1 mean clinical pregnancy outcome is inclined to be negative.

^bThe modified Hosmer–Lemeshow goodness-of-fit χ^2 test statistics was 2.862 ($P = 0.413$) for the analysis including the whole study population and 10.697 ($P = 0.219$) for the analysis including only women undergoing eSET.

previously described (Zamani *et al.*, 2010), whereas knock-out experiments have proved that vitamin D receptor null mice experience uterine hypoplasia and infertility (Yoshizawa *et al.*, 1997). Thus, this evidence from basic research indirectly suggests a potential role of vitamin D in female reproduction. This is further supported by reports demonstrating the up-regulation of HOXA10 mRNA and protein expression, an essential protein for embryo implantation and fertility, after 1,25(OH)₂D₃ administration by binding vitamin D receptor in human endometrial stromal cells (Du *et al.*, 2005). Consequently, considering that experiments in mice have shown that HOXA10 is expressed in endometrial cells and that its expression in human endometrium rises dramatically at the time of implantation (Bagot *et al.*, 2000), it may be suggested that vitamin D levels may indirectly affect implantation rates in infertile

women undergoing IVF/ICSI. Evidence from human endometrial cell lines further support such a hypothesis, given that the enzyme 1-alpha-hydroxylase, which catalyzes the hydroxylation of calcidiol (25-OH vitamin D) to calcitriol (1,25(OH)₂ vitamin D, the bioactive form of Vitamin D) is up-regulated in the human endometrial stromal cells of early pregnant compared with cycling endometrium (Vigano *et al.*, 2006). Our results are in line with the above-mentioned evidence given that we demonstrated that low serum vitamin D levels, shortly prior to embryo transfer, is an independent variable compromising pregnancy rates in women undergoing single blastocyst embryo transfer.

This hypothesis is further enhanced by the design we adopted in the specific study. According to our protocol, only women who reached the Day 5 embryo transfer stage were included in the study. In this

regard, we excluded patients who either demonstrated suboptimal response or had few oocytes and patients who might have not reached the blastocyst stage due to poor embryo quality. The rationale behind this study design was to minimize the biases related to oocyte and embryo quality and actually evaluate whether vitamin D deficiency independently compromises the chances of pregnancy in women reaching the blastocyst transfer stage. According to our results, this hypothesis appears to be plausible, although great caution is needed given that endometrial receptivity markers have not been tested in any of the patients included. Thus, future studies examining the effect of vitamin D levels on endometrial receptivity markers are mandatory prior to generating such a hypothesis.

Although, our results may suggest a detrimental effect of vitamin D deficiency on implantation of Day 5 embryos, we need to highlight that we cannot exclude an additional detrimental effect on oocyte or embryo quality. Even though we failed to identify significant differences in oocyte retrieval and fertilization rates between women with low and normal vitamin D levels, the percentage of cycles with eSET (selection of one Day 5 embryo from a larger number of available Day 5 embryos) was significantly higher in women with higher serum 25-OH vitamin D levels, suggesting that vitamin D may indeed play a role in human folliculogenesis, oogenesis and embryo quality. Previous experiments have shown that vitamin D receptor null mice, except from uterine hypoplasia, also exhibit impaired folliculogenesis (Yoshizawa et al., 1997). In addition, indirect evidence from previous studies support that circulating vitamin D correlates with serum anti-Müllerian hormone (AMH) levels in late-reproductive-aged women (Merhi et al., 2012), while others suggested that vitamin D may be a positive regulator of AMH production in adults (Dennis et al., 2012). Therefore, although a recent retrospective cohort study failed to demonstrate a correlation of vitamin D levels with stimulation characteristics (Rudick et al., 2012) future prospective cohorts are essential prior to establishing or rejecting a potential role of vitamin D level on folliculogenesis, oogenesis and embryo quality.

An interesting finding of our study was that pregnancy rates were comparably high among women with vitamin D levels between 20 and 30 ng/ml and patients with levels >30 ng/ml. Over the last years, a debate has arisen regarding the optimal level above which serum 25-OH vitamin D levels should be maintained (Holick, 2011), with the IOM supporting an optimal value exceeding 20 ng/ml (Rosen et al., 2012) and the Endocrine Society supporting that this level should be 30 ng/ml (Holick et al., 2011). Unfortunately, no solid scientific evidence is available at the moment to support a specific target value above which serum 25-OH vitamin D levels should be maintained among infertile patients. Our study demonstrates that for infertile patients undergoing single blastocyst transfer, pregnancy rates are compromised only in vitamin D-deficient women (<20 ng/ml), although future research with larger cohorts of patients is needed to validate our findings.

In our study, vitamin D deficiency was strongly associated with seasonality. Given this strong relationship, one would expect to find significant differences in pregnancy rates among different seasons; nonetheless, we failed to find any relationship between season and clinical pregnancy rates, and vitamin D deficiency was independently associated with pregnancy rates, irrespective of the season. Our results may be in contrast with early reports suggested a potential seasonal variability in fertilization and embryo quality rates in women undergoing IVF (Rojansky et al., 2000); however, we are in total agreement with more recent reports,

which failed to identify seasonal variations in fertilization, pregnancy and implantation rates in women undergoing IVF (Wunder et al., 2005).

A major strength of the current study is that we included a large number of patients, with almost as many patients as cumulatively in all the previous cohort studies examining the role of vitamin D in infertile patients undergoing IVF/ICSI. In addition, we attempted to examine the effect of vitamin D levels in a clearly defined good prognosis infertile population undergoing IVF/ICSI. Women included in the study were patients between 18 and 36 years old; thus given that analysis of >20 000 ICSI cycles in our center has shown that pregnancy rates remain fairly stable up to the age of 37 (Stoop et al., 2012), we attempted to eliminate the confounder of age, which might have otherwise affected our results. Furthermore, given that vitamin D-deficient patients and those with vitamin D levels >20 ng/ml did not significantly differ in any of the baseline and ovarian stimulation characteristics, it is highly unlikely that any of these variables might have biased our results. Our final regression model appears to justify our approach given that age was not significantly associated with clinical pregnancy rates, and only vitamin D deficiency and elevation of progesterone levels on the day of hCG and eSET were associated with the final reproductive outcome.

Another important strength of the study is that we included only women undergoing single embryo transfer with a Day 5 embryo. This constitutes a substantial difference compared with all the previous published studies, which included women irrespectively of the number of embryos transferred. Thus, our study design not only ensured that the likelihood of bias related to the confounding effect of multiple transferred embryos is absent, but also allowed us to include the embryo quality on the day of embryo transfer as an independent variable in our regression models, and therefore, minimize the confounding effect of embryo quality on the relationship between vitamin D deficiency and pregnancy outcome.

An important limitation of our study is that, although we provide results regarding lower live birth rates in vitamin D-deficient patients, this should be interpreted with great caution. The reason for caution is mainly the lack of any data regarding maternal vitamin D levels until delivery and potential vitamin D intake during this period. Owing to this, we decided to limit our logistic regression analysis only to clinical pregnancy rates. Although a recent meta-analysis demonstrated that vitamin D insufficiency is associated with an increased risk of gestational diabetes, pre-eclampsia and small for gestational age infants (Aghajafari et al., 2013), the lack of patients' vitamin D level follow-up during the second and third trimester of pregnancy and the lack of information regarding vitamin D supplementation in the current study do not allow us to make any safe conclusions regarding live births rates, and therefore, our results can only be indicative.

In addition, although previous groups have examined the effect of vitamin D deficiency in infertile women in relation to their race (Rudick et al., 2012), we did not examine the effect of vitamin D in women with different ethnic origins in order to identify differences between races. The reason for not proceeding in such an analysis was the fact that the vast majority of enrolled patients (93%) were of Caucasian origin and thus we could not examine differences in relation to patients' race. Although a previous study has shown that the relationship between vitamin D status and pregnancy rates differed by race, with vitamin D deficiency leading to lower pregnancy rates in non-Hispanic whites, but not in Asians, results from this study need to be validated by future cohort studies, given that only 49 patients of Asian origin were included (Rudick et al., 2012).

Finally, we need to highlight that our study was not designed to provide evidence regarding the effect of vitamin D levels on response to ovarian stimulation or in relation to ovarian reserve. Patients included in this cohort were only women who underwent Day 5 SET. Consequently, our study provides evidence that vitamin D deficiency significantly compromises the pregnancy rates in women undergoing single blastocyst transfer. However, the specific study design does not allow us to provide any guidance regarding a potential relationship between vitamin D deficiency and ovarian response.

In conclusion, although our study suggests a potential detrimental effect of vitamin D deficiency on the pregnancy rates of women undergoing Day 5 SET, we have to highlight that validation studies are needed, mainly due to the retrospective study design adopted. Therefore, future studies with a prospective study design should be performed in order to validate whether vitamin D deficiency may have a substantial impact on the final reproductive outcome and whether this is mediated through a detrimental effect in the endometrial receptivity or in the oocyte and embryo quality. At the moment two ongoing prospective studies by our group aim to examine the potential underlying mechanism, by which vitamin D deficiency might affect pregnancy rates in women undergoing ART treatment. The first aims to evaluate the pregnancy rates in women undergoing embryo transfer of frozen-thawed embryos as an attempt to eliminate confounding factors related to ovarian stimulation (Polyzos and van de Vijver, 2013a), whereas the other is a prospective cross-sectional analysis of 500 patients aiming to examine the relationship between serum 25-OH vitamin D levels and ovarian reserve markers (AMH and AFC) in order to evaluate the relationship between vitamin D deficiency and ovarian reserve (Polyzos and van de Vijver, 2013b). If data from such studies confirm our results, and provide reassuring evidence that vitamin D deficiency negatively affects pregnancy rates, the next step would be to examine whether vitamin D supplementation and restoration of vitamin D levels may actually improve the prognosis of infertile women undergoing IVF/ICSI.

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Authors' roles

N.P.P. is the primary investigator of the study and he is responsible for the study conception, design, statistical analysis and writing of the manuscript. L.G. participated in the statistical analysis and writing of the manuscript. All the authors substantially contributed to the interpretation of the results and editing of the manuscript.

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Conflict of interest

None declared.

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