Single oral dose of vitamin D_3 supplementation prior to *in vitro* fertilization and embryo transfer in normal weight women: the SUNDRO randomized controlled trial

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CONDENSATION

In women scheduled for *in vitro* fertilization, a single oral dose of 600,000 IU of vitamin D_3 does not improve the chances of clinical pregnancy.

SHORT TITLE

Vitamin D supplementation prior to IVF

AJOG AT A GLANCE

Why was the study conducted?

Observational studies suggested that women with deficient or insufficient serum vitamin D undergoing *in vitro* fertilization (IVF) have lower chances of success, but whether vitamin supplementation improves clinical pregnancy rate is unclear.

What are the key findings?

In this randomized clinical trial that included 630 women with deficient or insufficient peripheral levels of vitamin D, a single oral dose of 600,000 IU of vitamin D_3 prior to initiate IVF did not improve the chances of pregnancy.

What does this study add to what is already known?

In women scheduled for IVF, preliminary administration of a single high oral dose of vitamin D_3 is not justified.

KEY WORDS

in vitro fertilization; IVF; vitamin D; cholecalciferol; infertility

ABSTRACT

Background: Improving *in vitro* fertilization (IVF) success is an unmet need. Observational studies suggested that women with deficient or insufficient vitamin D have lower chances of success, but whether supplementation improves clinical pregnancy rate is unclear.

Objective: To determine whether oral vitamin D_3 supplementation may improve clinical pregnancy in women undergoing an IVF cycle

Study design: The SUNDRO trial is a two-centers randomized superiority double-blind placebo controlled trial. Subjects were recruited between October 2016 and January 2019. Participants were women aged 18-39 years with low vitamin D (peripheral 25-hydroxyvitamin D < 30 ng/ml), serum calcium ≥ 10.6 mg/dl, body-mass index (BMI) 18-25 Kg/m^2, anti-mullerian hormone (AMH) levels > 0.5 ng/ml, starting their first, second, or third treatment cycle of conventional IVF or intracytoplasmic sperm injection (ICSI). The primary outcome was the cumulative clinical pregnancy rate per cycle. Pregnancies obtained with both fresh or frozen embryo transfers were included. Clinical pregnancy was defined as intrauterine gestational sac with viable fetus. The primary analysis was performed according to the intention to treat principle and could also include natural conceptions. Secondary outcomes included total dose of gonadotropins used, embryological variables (number of oocytes retrieved, number of suitable oocytes retrieved, fertilization rate and rate of top quality embryos) and clinical outcomes (miscarriage rate and live birth rate).

Results: 630 women were randomized 2-12 weeks prior to initiate the IVF cycle to receive either a single dose of 600,000 IU of vitamin D_3 (n=308) or placebo (n=322). One hundred thirteen (37%) and 130 (40%) women achieved a clinical pregnancy in the treatment and placebo groups, respectively (p=0.37). The risk ratio (RR) of clinical pregnancy in women receiving vitamin D_3 was 0.91 (95% confidence interval-CI: 0.75 – 1.11). Compared to placebo, vitamin D_3 supplementation did not significantly improve the secondary outcomes. Exploratory subgroup analyses for BMI, age, indication to IVF, ovarian reserve, interval between drug administration and initiation of the cycle

and basal levels of 25-hydroxyvitamin D failed to highlight any clinical situation that could benefit from the supplementation.

Conclusions: In normal weight women with preserved ovarian reserve and low vitamin D levels undergoing IVF cycles, a single oral dose of 600,000 IU of vitamin D_3 does not improve the chances of clinical pregnancy. Although the findings do not support the use of vitamin D_3 supplementation to ameliorate IVF success, further studies are required to rule out milder but potentially still interesting benefits as well as exploring the effectiveness of alternative modalities of supplementation.

Introduction

Across the world, more than 8 million babies have been born after *in vitro* fertilization (IVF) procedures. However, despite the recent refinements in ovarian stimulation protocols and the introduction of new technologies in the laboratory, the live birth rate per initiated cycle remains between 19% and 22% and does not overcome 50% even in couples with optimal prognosis. [1] Improving the effectiveness of IVF is an unmet need.

Several observational studies have correlated low serum 25-hydroxyvitamin D (henceforth, 'vitamin D') levels to a reduction of both natural fertility and *in vitro* fertilization (IVF) success. [2,3] Such findings may be explained by the targeted action elicited by Vitamin D in reproductive physiology and mediated by the binding of the active form to its receptor identified in the ovary, uterus, endometrium and placenta. [2,4,5] The enzyme 1α-hydroxylase, which converts the inactive into the active form 1,25-dihydroxyvitamin D, is as well expressed in the ovary and endometrium supporting a local paracrine role of the vitamin. Epidemiological evidence further fuelled this hypothesis of the hormone as a regulator of human reproductive function. Even if evidence is not univocal, low vitamin D has been associated with endometriosis, polycystic ovary syndrome, uterine fibroids and adverse obstetrics outcomes, including preeclampsia, gestational diabetes, low birth weight and preterm birth. [2,5-8]

A recent meta-analysis assessed the reproductive outcomes of 3711 women undergoing IVF cycles and found favourable outcomes correlated with vitamin D replete status. Women with sufficient vitamin D levels had significantly higher clinical pregnancy rate in autologous oocyte IVF cycles with an Odds Ratio (OR) of 1.47 (95%CI: 1.20-1.69).[3] A second more selective meta-analysis confirmed this finding, showing an OR of 1.47 (95%CI: 1.02-2.08) for women with peripheral levels ≥ 30 ng/ml.[9] However, these authors failed to highlight any effect when focusing on the threshold of 20 ng/ml. Moreover, causality of these observations has only been tested in few underpowered interventional studies investigating the possible beneficial effects of vitamin D_3

supplementation in improving the IVF success and results were inconsistent.[10-14] In addition, it is worth pointing out that the cut-off points advocated for vitamin D insufficiency (20-29.9 ng/ml) or deficiency (< 20 ng/ml) are based on studies investigating bone health. None of the guidelines for vitamin D was actually validated for fertility outcomes.[9] To note, in a recent large (n=2,569) cohort of women undergoing embryo transfer, Cai et al. failed to identify any cut-off point associated with lower chances of success.[15]

Motivated by the evidence suggesting a possible effect of vitamin D on IVF success and by the appealing profile of vitamin D_3 supplementation in terms of costs and safety, the primary hypothesis for this RCT was that oral supplementation with a high dose of vitamin D_3 would have improved clinical pregnancy rates in women with low peripheral vitamin D undergoing IVF. We also assessed the effect on gonadotropins doses needed to induce the ovarian follicular growth and the embryological variables which were pre-specified secondary outcomes.

MATERIALS AND METHODS

Trial design

This pragmatic two-center randomized superiority double-blind placebo controlled clinical trial evaluated the efficacy of a single dose of oral administration of vitamin D_3 (cholecalciferol 600,000 IU) in improving the chances of clinical pregnancy in women scheduled for IVF. The protocol of the study has been previously reported in details and is herein succinctly described.[16] The study was approved by the Institutional Review Boards of the two participating centres. Patients provided a written informed consent to participate. The EudraCT registration number is 2015-004233-27. The trial was initially registered with Agenzia Italiana del Farmaco on March 8, 2016, and the date of initial participant enrollment was October 10, 2016. Trial registration in EudraCT was delayed due to administrative issues and occurred on November 17, 2017.

The data were collected by trial-site personnel and stored in an electronic data capture database. The statistician of the coordinating centre analyzed the data. All the authors vouched for the accuracy and completeness of the data and for the fidelity of the trial to the protocol. All of them contributed to the interpretation of the results and the preparation of the manuscript. No pharmacological manufacturers contributed to the planning, design or conduct of the trial. Trial pills were manufactured by the hospital pharmacological service of the coordinating centre. None of the authors neither the participating Units received any financial support from manufacturers with economical interests in vitamin D products during the last five years.

Participants

Women scheduled for initiating IVF with or without intracytoplasmic sperm injection (ICSI) at the Infertility units of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and IRCCS San Raffaele Scientific Institute between October 2016 and January 2019 were considered for study entry. Recruitment was consecutive but was interrupted during the summer period (June to September) to prevent a potentially relevant confounding effect of transient intense sun exposure during summertime. Only women scheduled for controlled ovarian stimulation aimed at embryo transfer were included while those who were scheduled for using frozen oocytes/embryos as well as those undergoing oocyte retrieval for fertility preservation were excluded. Additional inclusion criteria were as follows: age 18-39 years, body-mass index (BMI) 18-25 Kg/m^2, no more than two previous completed IVF cycles (i.e. no more than two oocyte retrievals regardless of the number of embryo transfers), preserved ovarian reserve as documented by a level of serum anti-mullerian hormone (AMH) > 0.5 ng/ml and availability of ejaculated semen (couples requiring surgical retrieval of the spermatozoa were excluded). Women who were already taking vitamin D_3 and those with contra-indications to vitamin D_3 administration (hyper-calcemia or conditions leading to high peripheral calcium levels such as sarcoidosis, tubercolosis or parathyroid diseases) were excluded.

Women that, for any reason, could not initiate the cycle within the following 12 weeks were not included but were invited to refer again for inclusion at a later time when they were about to initiate.

Eligible women who agreed to participate underwent a peripheral blood test to assess serum concentration of vitamin D and calcium. Women were excluded and not randomized if vitamin D levels exceeded 30 ng/ml or calcium was below 10.6 mg/dl. Eligibility for these two dosages was checked by an external researcher not involved in patients' management. Researchers, caregivers and participants were unaware of the results of these dosages.

Intervention and procedures

Supplementation was given as a single administration of oral 600,000 IU of vitamin D_3 in order to ensure maximal adherence. This modality is expected to properly maintain peripheral levels of vitamin D above 30 ng/ml for three months [17], i.e., a period of time that in most cases properly cover a complete IVF cycle (including both fresh and frozen cycles). In general, infertile women embarking in IVF are overburden by complex treatments and ensuring simplicity was considered a priority at the time of study design.

Recruited subjects were planned to initiate IVF cycle within 2-12 weeks after administration of the drug. Eligible women were randomized 1:1 into two arms. The treatment group received a single oral administration of 600,000 IU of vitamin D_3 diluted in olive oil. The control group received placebo (only the olive oil preparation). The two potions (vitamin D_3 and placebo) were visually indistinguishable. Randomized patients were given the drug at the time of randomization by researchers in the hospital. They consumed the drug in front of the researcher. During the trial, research staff, caregivers (both physicians and embryologists) and participants were unaware of treatment allocation. Randomization was centralized to the pharmaceutical service of the hospital of the organizing Center. The allocation sequence was computer-generated and hidden to participants, caregivers and embryologists. The randomization list was stratified for the two participating centers.

To maximise the ability of the trial to observe a treatment effect, participants were invited to refrain from using supplements with vitamin D_3 outside ~~of~~ the trial context. They were also invited to initiate the cycle within the scheduled period (2-12 weeks) and to promptly perform the subsequent transfers in case of cryopreservation of supernumerary embryos.

An internal validation of the treatment was done on the day of oocyte retrieval by testing the peripheral levels of vitamin D. For women who had their ovarian stimulation cycle interrupted, this assessment was made on the day the cycle was stopped.

Women underwent their IVF cycle according to the clinical standards of the participating centers. The policies used in these centers were recently described in detail elsewhere.[18,19] They include the possibility of embryo transfer cancellation because of elevated risk of developing ovarian hyper-stimulation syndrome (OHSS) or premature raise of serum progesterone (documented by the presence of peripheral progesterone above 1.5 ng/ml on the day of ovulation trigger) as well as the adoption of elective single embryo transfer in the vast majority of cases. Apart from the above-mentioned assessment of peripheral vitamin D on the day of ovulation trigger or cycle interruption, no additional or particular interventions were introduced.

Outcome measures

The primary outcome was the cumulative clinical pregnancy rate per randomized woman. Clinical pregnancies obtained with the transfer of supernumerary frozen embryos were included in the outcome. The study was designed as a pragmatic intention-to-treat trial and, therefore, natural pregnancies recorded prior to oocytes retrieval were included in the primary outcome. Clinical pregnancy was defined as the presence of at least one intrauterine gestational sac with viable fetus. Clinical pregnancy rate rather than live birth rate was chosen as primary outcome because the single administration of a high dose of vitamin D_3 allowed to significantly increase peripheral levels of vitamin D for about three months. This timeframe is generally sufficient to complete a full IVF cycle (including fresh and frozen transfers) but cannot efficiently cover the whole duration of

pregnancy. However, for women achieving pregnancy, the follow-up was prolonged up to the time of delivery or pregnancy termination and data on live birth rate are presented. More specifically, secondary outcomes included total dose of gonadotropins used, embryological variables (number of oocytes retrieved, number of suitable oocytes retrieved, fertilization rate and rate of top quality embryos) and clinical outcomes (time to pregnancy, miscarriage rate, termination of pregnancy, stillbirth, live birth, gestational age, birth weight and neonatal health). In addition, data are presented according to the recently implemented core outcomes set for infertility research.[20,21] Natural pregnancies occurring after the oocyte retrieval were not included. For women achieving clinical pregnancies, additional pregnancies that could be subsequently obtained with frozen transfers (transfers after a miscarriage or a live birth) were not included in the analysis. Recruitment was stopped once the scheduled sample size was reached. Follow-up was prolonged up to the end of August 2020.

Given the well-known pharmacological and safety profile of vitamin D_3 [17,22], we did not deem necessary a strict monitoring of the included women and only severe adverse events were recorded (death, life-threatening conditions, new or prolonged hospitalization, persistent or significant disability, congenital anomalies or birth defects). Only maternal events occurring within three months from randomization were considered because a single dose of 600,000 IU is expected to raise peripheral levels for no more than three months.[17] However, for congenital anomalies or birth defects, all pregnancies obtained using the oocytes retrieved during the index cycle could be considered independently from the time passed from randomization because the origin of these defects may take place early in pregnancy. Conversely, we did not make efforts to identify cases of neonatal hypercalcemia [23] because, given the months interval from vitamin administration, relatedness of such events to pre-IVF supplementation would have been implausible.

Sample size calculation and statistical analyses

The sample size was calculated based on the following assumptions: 1) Expected cumulative clinical pregnancy rate in women with insufficient vitamin D levels: 20%, 2) Improvement of the absolute rate of cumulative clinical pregnancy rate in supplemented women: 10% (success rate thus raising from 20% to 30%), 3) Type I and II errors set at 0.05 and 0.20, 4) expected proportion of women with insufficient vitamin D: 90%. These assumptions were made based on evidence emerged from previous contributions from our groups.[24,25] Specifically, we observed a clinical pregnancy rate in women with peripheral values of vitamin $D_3 < 20$ and ≥ 20 ng/ml of 20% and 31%, respectively.[24] Excluding the summer season, the rate of women with non sufficient levels varied between 8 and 27%.[25] On these bases, we predicted a 18-month-period of recruitment and a total number of women to be recruited of 700. Taking into consideration the expected rate of excluded women of 10% because of serum vitamin D levels ≥ 30 ng/ml, this scheduled sample size could allow us to randomize at least 300 women per arm.

Statistical analysis was made per intention to treat. No adjustment for the primary outcome was foreseen and the result is presented as crude Relative Risk (RR) and 95% Confidence Interval (95%CI). Data are reported as mean ± standard deviation (SD), median [interquartile range-IQR] and number (%) and were compared using Student *t* test, non parametric Mann-Whitney test, Chi squared test or Fisher Exact test, as appropriate. The Shapiro-Wilk test was preliminary performed to assess the consistency of the data with normal distribution. P values below 0.05 were considered statistically significant. Exploratory subgroup analyses were done for basal levels of vitamin D (<20 ng/ml versus 20-29.9 ng/ml), race (Caucasian versus others), indication (unexplained infertility versus others), ovarian reserve, BMI, interval between drug administration and initiation of the cycle and age. For the last four variables, subjects were divided into two groups based on the median values of the whole cohort of randomized women.

RESULTS

The flow-chart of the study is shown in Figure 1. Seven-hundred thirty-eight women were initially eligible; twenty subsequently declined to be recruited prior to randomization and 88 were excluded because peripheral levels of vitamin D and calcium did not fulfil our selection criteria. Six-hundred thirty women were randomized; 308 received cholecalciferol 600,000 IU and 322 were allocated to placebo. Baseline characteristics of the study groups are shown in Table 1. Median [IQR] serum levels of vitamin D at study entry were 20.0 [15.5 - 23.6] and 19.9 [14.6 - 23.9] ng/ml, (p=0.66) respectively for treated and control subjects. Twenty-three women in the treatment arm (7%) and 34 in the control group (11%) did not perform oocyte retrieval (p=0.21). Reasons for drop-out are depicted in Figure 1 and did not significantly differ between the study groups.

IVF outcomes for women who underwent oocyte retrieval (285 and 288 women) are shown in Table 2. No significant difference emerged. The dose of gonadotropins needed, the total number of developed follicles, the number of oocytes retrieved, the number of suitable oocytes, the fertilization rate, the total number of embryos and the total number of top quality embryos were similar in both treatment groups. Serum levels of vitamin D at the time of oocyte retrieval or cycle cancellation were 52.2 [41.1 – 64.8] and 19.8 [14.1 – 24.6] ng/ml in women who did and did not receive vitamin D_3, respectively ($p<0.001$).

IVF outcome in the subgroups of women performing fresh embryo transfer (169 + 159 women) is summarized in Table 3. None of the studied variables was found to significantly differ.

The cumulative clinical pregnancy rate per oocyte retrieval, the primary outcome of the study, did not differ between the study groups. One hundred thirteen (37%) and 130 (40%) women achieved a clinical pregnancy in the treatment and placebo groups, respectively (p=0.37). The RR of clinical pregnancy in women receiving vitamin D_3 was 0.91 (95%CI: 0.75 - 1.11). The core clinical outcomes are shown in Table 4.

Severe adverse events were observed in four cases. They were all amenable to the group of congenital anomalies and birth defects. They included two pregnancy terminations (one for Down syndrome in the Cholecalciferol arm and one for hydrocephalus in the placebo arm) and major congenital abnormalities at birth (one Down syndrome and one Prader Willi syndrome, both belonging to the placebo arm).

Finally, we performed exploratory subgroup analyses for the primary outcome to investigate whether vitamin D_3 supplementation could be of some benefit in specific situations. We focussed on BMI, age, indications to IVF, biomarkers of ovarian reserve, interval between drug administration and initiation of the cycle and basal levels of vitamin D (Figure 2). We failed to identify any subgroup that could benefit from the supplementation.

COMMENT

Principal findings

In this RCT involving reproductive age women with deficient or insufficient levels of vitamin D undergoing IVF cycles, supplementation with a single oral dose of 600,000 IU of vitamin D_3 compared to placebo did not result in an increased chance of clinical pregnancy. Surrogate markers of IVF success such as ovarian responsiveness or embryo number and quality were not improved, as well. Subgroup analyses failed to identify any condition that might benefit from supplementation. Albeit secondary, the surrogate findings and the subgroups analyses tend to reinforce the general conclusion emerging from the trial, i.e., that oral vitamin D_3 supplementation is ineffective.

Results

To our knowledge, five RCTs investigated the possible benefits of vitamin D_3 supplementation prior to IVF. [10-14] Three of them showed some benefits but results were difficult to interpret because vitamin D_3 was part of a multiple intervention. Vitamin D_3 was administered together with vitamin

E in the first RCT [11], with folic acid, alpha-lactalbumin, myo-inositol and melatonin in the second one [14] and with omega-3 and olive oil in the third.[13] Conversely, two studies investigated the exclusive effects of vitamin D_3. Aflatoonian et al. investigated the effectiveness of 50,000 IU of vitamin D_3 weekly for 6-8 weeks in a specific group of women scheduled for frozen embryo transfer but failed to show any effect.[10] In a previous study by Abedi et al., the same scheme of treatment, i.e. vitamin D_3 50,000 IU weekly for 6-8 weeks, was associated with a significantly higher clinical pregnancy rate in women undergoing a complete ICSI cycle.[12] Regardless of the inconsistencies and the differences in study designs, it is noteworthy that all these five studies were underpowered for robust conclusions; the total number of included women varied between 85 and 114.

Our study was designed to detect a 10% absolute increase in the chance of success of the procedure. This target was chosen on the basis of previous epidemiological evidence of our group showing a 11% absolute difference between women with peripheral vitamin D < 20 and ≥ 20 ng/mL.[24] The present trial, however, cannot rule out milder but still interesting benefits from a clinical and economical point of view. Future studies and meta-analyses are warranted. In addition, it has to be underlined that the idea that vitamin D_3 supplementation prior to IVF may be valuable regardless of the possible benefits on the success of the procedure because of its favorable effects on pregnancy and perinatal outcomes is also not supported. We failed to observe any difference in obstetrics outcome in our study. In line, recent systematic reviews of studies testing the potential effects of vitamin D supplementation in pregnancy failed to show a significant benefit.[26,27] Overall, even if our study did not document any adverse effect of vitamin D_3 supplementation thus confirming the excellent safety profile of this treatment, it did not provide encouraging evidence for recommending vitamin D assessment and vitamin D_3 treatment prior to initiate IVF cycles.

Research implications

Two main explanations can be postulated to explain the inconsistency between molecular, epidemiological and interventional findings. Firstly, vitamin D deficiency or insufficiency may be associated with other undetected confounders, thus not providing an essential role *per se*. Vitamin D status may be a surrogate marker of general state of health. For example, outdoor physical activity, adiposity, smoking, ethnicity, inflammation status, general nutritional conditions and other unknown factors may produce spurious protective associations.[28-30] We would also like to call attention to the current definitions of vitamin D insufficiency and deficiency which are based on peripheral levels of vitamin D and are derived from endocrine studies on bone and calcium metabolism. No evidence, however, supports the idea that peripheral levels of vitamin D necessary for bone and calcium homeostasis overlap those needed to ensure optimal functioning also in the uterus and ovaries. As above mentioned, a large cohort study aimed at identifying a cut-off point for infertility problems failed to provide any meaningful information.[9] To note, vitamin D appears to have a paracrine action in these districts, being locally transformed into the active form. We cannot exclude that vitamin D function may be adequately ensured in these districts even with extremely low levels of peripheral vitamin D. Unfortunately, we could not assess whether or not vitamin D_3 supplementation could be effective only in women with extremely low peripheral levels (< 10 ng/ml) due to an insufficient number of these cases (we observed 13 clinical pregnancies out of 30 among women allocated to vitamin D_3 group and 9 out of 24 among controls, p=0.78).

A second explanation for the lack of effectiveness of vitamin D_3 in our trial may be related to the modality of supplementation.[31] We opted for a single oral high dose of cholecalciferol (600,000 IU) as the simplest and most friendly modality of administration. This choice has guaranteed full adherence to the protocol of treatment (all randomized women received the drug) and had previously shown to result in sufficient peripheral levels of vitamin-D for up to 3 months, the duration of time needed to cover the oocyte retrieval and up to two embryo transfers. Notably, if the

benefit had been exerted on oocyte and embryo quality, the impact on the success of the procedure would have lasted beyond the timeframe of peripheral vitamin D sufficiency (frozen embryos would have benefited from supplementation even if transferred several months later). We cannot, however, exclude that opting for a daily (2,000 IU) or weekly (50,000 IU) administration or starting supplementation significantly earlier would have resulted in better outcomes. For example, initiating 6 months before the IVF cycle rather than 2-12 weeks would have allowed the whole process of folliculogenesis (lasting about 6 months) to take place in a vitamin D sufficient milieu. Our decision was taken on pragmatic bases, taking also into utmost consideration the significant burden of treatments that women embarking in IVF have to face. Demonstrating that a single 600,000 IU dose of cholecalciferol few weeks prior to initiate IVF was effective would have facilitated prompt adoption of this modality into everyday clinical practice. Importantly, dose, modality and duration of assumption of cholecalciferol may not be the unique reasons for failure. In general, in several clinical and research settings, we may have excessively simplified our vision of vitamin D metabolism. The vitamin D production in subcutaneous tissues (which represents 90% of our reserve) is accompanied by the production of several other metabolites whose importance may have been neglected.[32]

In this regard, it has also to be clarified that we measured peripheral levels of vitamin D in randomized women at the time of oocytes retrieval showing remarkably higher levels in women allocated to the active treatment compared to placebo (52.2 [41.1 – 64.8] versus 19.8 [14.1 – 24.6] ng/ml). However, measurement of total vitamin D after stimulation may be less reliable because, as a consequence of the high estrogen levels, the synthesis of vitamin D binding protein increases and the bound vitamin D is not biologically active.[33] A similar confounding effect was demonstrated in pregnancy.[34] Measuring free vitamin D would have provided more robust evidence in support of our strategy of supplementation.

Strengths and limitations

Strengths include the pragmatic approach, the large sample size, the completeness of data and the availability of information for all core outcomes recently advocated for studies in infertility. An additional interesting strength of the study is the scant diffusion among the studied population of over the counter vitamin D_3 supplementation , which is conversely very common in the United States where the recent RCTs on the preventive effects of vitamin D for type 2 diabetes, cardiovascular diseases and cancer were run.[29,35] In contrast to United States, peripheral assessment of vitamin D and subsequent tailored supplementation is infrequently done in Italy, allowing us to study a practically naive population. Accordingly, basal average levels of vitamin D in our population were 19-20 ng/ml, thus markedly lower than the averages of 28-31 ng/ml observed in the two RCTs carried out in the United States. [29,35]

Limitations include the selection criteria that excluded women who were older, overweight or with a compromised ovarian reserve. One may postulate that vitamin D_3 could be particularly efficacious in these groups of subjects. Exclusion of older women and those with AMH levels < 0.5 ng/ml was decided in order to enhance the power of the study while exclusion of obese women was decided to overcome the complex and still unclear confounder role of adiposity on vitamin D bioavailability.[28] Inferences on our findings should take into consideration this limitation. On the other hand, albeit exploratory and of limited value, the subgroup analyses performed based on age (younger and older than 35 years), BMI (higher and lower than 20.9 Kg/m^2) and AMH levels (higher and lower than 2.2 ng/ml) do not provide support to this concern. A second limitation is the choice of the primary outcome. We opted for clinical pregnancy rate rather than live birth rate. The choice of this outcome was based on the selected modality of supplementation ensuring optimal levels of vitamin-D for three months, and not for the entire duration of pregnancy. Data on live birth is however available and reported in the study. Thirdly, there is an important discrepancy between the basal assumption of 20% success rate in the non-supplemental arm used to calculate the sample size and the results

observed in the trial (40%, in the non-supplemented group). The progressive technological improvements of the procedure in the participating centers since the time of the study design (2012) may have caused a meaningful change in success rate. Nonetheless, the upper limit of the 95%CI of the RR for clinical pregnancy (1.11) allows us to rule out an absolute benefit of more than 4.4% (0.40 x 0.11). Fourthly, we limited our safety evaluation to the record of severe adverse events. We observed two severe adverse events in the cholecalciferol arm (one Down syndrome and one stillbirth) and three in the control arm (one hydrocephalus, one Down syndrome and one with Prader willi syndrome). Relatedness of the two adverse events in the active arm was deemed unlikely. Even if the studied mode of vitamin D administration (600,000 IU in a single dose) is well-established and considered safe, the significance or our study would have been improved by the performance of more accurate safety evaluations such as serial measurements of calcium, phosphate, parathyroid hormone, and estimated Glomerular Filtration Rate.

Conclusions

In conclusion, we failed to document a benefit of cholecalciferol supplementation with a single oral administration of 600,000 IU of vitamin D on IVF-mediated clinical pregnancy rate. Further studies are required to rule out milder but potentially still interesting benefits as well as to explore the effectiveness of alternative modalities of supplementation.

References

1. Kamath MS, Mascarenhas M, Franik S, Liu E, Sunkara SK. Clinical adjuncts in in vitro fertilization: a growing list. Fertil Steril. 2019;112(6):978-986.
2. Pilz S, Zittermann A, Obeid R, et al. The Role of Vitamin D in Fertility and during Pregnancy and Lactation: A Review of Clinical Data. Int J Environ Res Public Health. 2018;15(10):2241.
3. Iliuta F, Pijoan JI, Lainz L, Exposito A, Matorras R. Women's vitamin D levels and IVF results: a systematic review of the literature and meta-analysis, considering three categories of vitamin status (replete, insufficient and deficient). Hum Fertil (Camb). 2020:1-19. [Online ahead of print]
4. Franasiak JM, Lara EE, Pellicer A. Vitamin D in human reproduction. Curr Opin Obstet Gynecol. 2017;29(4):189-194.
5. Chen Y, Zhi X. Roles of Vitamin D in Reproductive Systems and Assisted Reproductive Technology. Endocrinology. 2020;161(4):bqaa023.
6. Palacios C, Kostiuk LK, Peña-Rosas JP. Vitamin D supplementation for women during pregnancy. Cochrane Database Syst Rev. 2019;7(7):CD008873.
7. Rizzo G, Garzon S, Fichera M, et al. Vitamin D and Gestational Diabetes Mellitus: Is There a Link? Antioxidants (Basel). 2019;8(11):511.
8. Kalaitzopoulos DR, Lempesis IG, Athanasaki F, et al. Association between vitamin D and endometriosis: a systematic review. Hormones (Athens). 2020;19(2):109-121.
9. Cozzolino M, Busnelli A, Pellegrini L, et al. How vitamin D level influences in vitro fertilization outcomes: results of a systematic review and meta-analysis. Fertil Steril. 2020;114(5):1014-1025
10. Aflatoonian A, Arabjahvani F, Eftekhar M, Sayadi M. Effect of vitamin D insufficiency treatment on fertility outcomes in frozen-thawed embryo transfer cycles: A randomized clinical trial. Iran J Reprod Med. 2014;12(9):595-600.
11. Fatemi F, Mohammadzadeh A, Sadeghi MR, et al. Role of vitamin E and D₃ supplementation in Intra-Cytoplasmic Sperm Injection outcomes of women with polycystic ovarian syndrome: A double blinded randomized placebo-controlled trial. Clin Nutr ESPEN. 2017;18:23-30.

12. Abedi S, Taebi M, Nasr Esfahani MH. Effect of Vitamin D Supplementation on Intracytoplasmic Sperm Injection Outcomes: A Randomized Double-Blind Placebo-Controlled Trial. Int J Fertil Steril. 2019;13(1):18-23.

13. Kermack AJ, Lowen P, Wellstead SJ, et al. Effect of a 6-week "Mediterranean" dietary intervention on in vitro human embryo development: the Preconception Dietary Supplements in Assisted Reproduction double-blinded randomized controlled trial. Fertil Steril. 2020;113(2):260-269.

14. Bezerra Espinola MS, Bilotta G, Aragona C. Positive effect of a new supplementation of vitamin D₃ with myo-inositol, folic acid and melatonin on IVF outcomes: a prospective randomized and controlled pilot study. Gynecol Endocrinol. 2020:1-4.

15. Cai S, Li J, Zeng S, Hu L, et al. Impact of vitamin D on human embryo implantation-a prospective cohort study in women undergoing fresh embryo transfer. Fertil Steril. 2020 Oct 7:S0015-0282(20)32206-8.

16. Paffoni A, Somigliana E, Sarais V, et al. Effect of vitamin D supplementation on assisted re production technology (ART) outcomes and underlying biological mechanisms: protocol of a randomized clinical controlled trial. The "supplementation of vitamin D and reproductive outcome" (SUNDRO) study. BMC Pregnancy Childbirth. 2019 Nov 1;19(1):395.

17. Cipriani C, Romagnoli E, Scillitani A, et al. Effect of a single oral dose of 600,000 IU of cholecalciferol on serum calciotropic hormones in young subjects with vitamin D deficiency: a prospective intervention study. J Clin Endocrinol Metab. 2010;95(10):4771-7.

18. Leonardi M, Papaleo E, Reschini M, et al. Risk of miscarriage in women with endometriosis: insights from in vitro fertilization cycles. Fertil Steril. 2016;106(2):386-392.

19. Vanni VS, Somigliana E, Reschini M, et al. Top quality blastocyst formation rates in relation to progesterone levels on the day of oocyte maturation in GnRH antagonist IVF/ICSI cycles. PLoS One. 2017;12(5):e0176482.

20. Duffy JMN, AlAhwany H, Bhattacharya S, et al. Developing a core outcome set for future infertility research: an international consensus development study. Hum Reprod. 2020;35(12):2725-2734.

21. Duffy JMN, Bhattacharya S, Bhattacharya S, et al. Standardizing definitions and reporting guidelines for the infertility core outcome set: an international consensus development study. Hum Reprod. 2020;35(12):2735-2745.

22. Vieth R. Vitamin D and cancer mini-symposium: the risk of additional vitamin D. Ann Epidemiol. 2009;19(7):441-5.

23. Schlingmann KP, Kaufmann M, Weber S, et al. Mutations in CYP24A1 and idiopathic infantile hypercalcemia. N Engl J Med. 2011;365(5):410-21.

24. Paffoni A, Ferrari S, Viganò P, et al. Vitamin D deficiency and infertility: insights from in vitro fertilization cycles. J Clin Endocrinol Metab. 2014;99(11):E2372-6.

25. Pagliardini L, Vigano' P, Molgora M, et al. High Prevalence of Vitamin D Deficiency in Infertile Women Referring for Assisted Reproduction. Nutrients. 2015;7(12):9972-84.

26. Roth DE, Leung M, Mesfin E, Qamar H, Watterworth J, Papp E. Vitamin D supplementation during pregnancy: state of the evidence from a systematic review of randomised trials. BMJ. 2017;359:j5237.

27. Bialy L, Fenton T, Shulhan-Kilroy J, Johnson DW, McNeil DA, Hartling L. Vitamin D supplementation to improve pregnancy and perinatal outcomes: an overview of 42 systematic reviews. BMJ Open. 2020;10:e032626.

28. Manson JE, Bassuk SS. Vitamin D research and clinical practice: at a crossroads. JAMA. 2015;313(13):1311-1312.

29. Manson JE, Cook NR, Lee IM, et al; VITAL Research Group. Vitamin D Supplements and Prevention of Cancer and Cardiovascular Disease. N Engl J Med. 2019;380(1):33-44.

30. Preiss D, Sattar N. Research digest: vitamin D supplementation. Lancet Diabetes Endocrinol. 2019;7(2):91.

31. Karras SN, Anagnostis P, Naughton D, Annweiler C, Petroczi A, Goulis DG. Vitamin D during pregnancy: why observational studies suggest deficiency and interventional studies show no improvement in clinical outcomes? A narrative review. J Endocrinol Invest. 2015;38(12):1265-1275.

32. Hossein-nezhad A, Holick MF. Vitamin D for health: a global perspective. Mayo Clin Proc. 2013;88(7):720-755.

33. Tsuprykov O, Chen X, Hocher CF, et al. Why should we measure free 25(OH) vitamin D? J Steroid Biochem Mol Biol. 2018;180:87-104.

34. Tsuprykov O, Buse C, Skoblo R, Hocher B. Comparison of free and total 25-hydroxyvitamin D in normal human pregnancy. J Steroid Biochem Mol Biol. 2019 Jun;190:29-36.

35. Pittas AG, Dawson-Hughes B, Sheehan P, et al; D2d Research Group. Vitamin D Supplementation and Prevention of Type 2 Diabetes. N Engl J Med. 2019;381(6):520-530.

539 **Figure legends**

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541 **Figure 1:** Flow-chart of the study.

542 **Figure 2:** Exploratory subgroup analyses. The reported relative risks refer to the chances of
543 achieving clinical pregnancy in women randomized to cholecalciferol in every subgroup of women.
544 The number of subjects was lower for the analysis on the interval between drug administration and
545 IVF because those who did not initiate the cycle were excluded.