RESEARCH

100 YEARS OF VITAMIN D

Effect of serum vitamin D level before ovarian stimulation on the cumulative live birth rate of women undergoing *in vitro* fertilization: a retrospective analysis

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Abstract

Objective: Vitamin D receptors are present in the female reproductive tract. Studies on the association between serum vitamin D level and pregnancy rate of *in vitro* fertilization (IVF) showed inconsistent results and focused on a single fresh or frozen embryo transfer cycle. The objective of our study was to evaluate if serum vitamin D level before ovarian stimulation was associated with the cumulative live birth rate (CLBR) of the first IVF cycle.

Design: Retrospective cohort study.

Methods: Women who underwent the first IVF cycle from 2012 to 2016 at a university-affiliated reproductive medicine center were included. Archived serum samples taken before ovarian stimulation were analyzed for 25(OH)D levels using liquid chromatography-mass spectrometry.

Results: In total, 1113 had pregnancy outcome from the completed IVF cycle. The median age (25th–75th percentile) of the women was 36 (34–38) years and serum 25(OH)D level was 53.4 (41.9–66.6) nmol/L. The prevalence of vitamin D deficiency (less than 50 nmol/L) was 42.2%. The CLBR in the vitamin D-deficient group was significantly lower compared to the non-deficient group (43.9%, 208/474 vs 50.9%, 325/639, *P* = 0.021, unadjusted), and after controlling for women's age, BMI, antral follicle count, type and duration of infertility. There were no differences in the clinical/ongoing pregnancy rate, live birth rate and miscarriage rate in the fresh cycle between the vitamin D deficient and non-deficient groups.

Conclusions: Vitamin D deficiency was prevalent in infertile women in subtropical Hong Kong. The CLBR of the first IVF cycle in the vitamin D-deficient group was significantly lower compared to the non-deficient group.

Introduction

Vitamin D is a key hormone in the regulation of calcium and phosphorus metabolism and hence is important for the maintenance of bone health (1). Its role in human reproduction is suggested by the expression of vitamin D receptor and vitamin D-metabolizing enzymes in various human reproductive tissues including ovarian granulosa cells, placenta, pituitary, endometrium, testis, sperm, epididymis, seminal vesicle and prostate (2, 3). Vitamin D is principally synthesized in the skin upon sun exposure (4). In one study, significant seasonal variation of the pregnancy rate of *in vitro* fertilization (IVF), which was lower in spring despite higher fertilization rate and better embryo quality in those undergoing IVF in this season, implied an effect of light hours on the endometrium (5).

In basic research, vitamin D has been found to alter Anti-Mullerian hormone (AMH) signaling, follicle-stimulating hormone, sensitivity as well as progesterone production and release in granulosa cells with possible effect on steroidogenesis and follicular development (6, 7, 8). Vitamin D deficiency can also lead to calcium deficiency, which is associated with abnormalities in oocyte maturation and development (9), sperm motility and fertilization (10). Cohort studies have not shown a consistent relationship between serum vitamin D level and IVF outcomes (11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21), but meta-analyses of these cohort studies have shown lower live birth rate in women with vitamin D deficiency undergoing IVF (22, 23, 24). The latest meta-analysis however still showed inconsistent evidence supporting the positive impact of serum vitamin D levels on IVF outcomes after sensitivity analysis (25).

Existing reports mainly focused on a single fresh or frozen embryo transfer cycle. With the increasing practice of elective embryo freezing, cryopreservation of surplus embryos and subsequent transfer of frozen embryos in the modern-day IVF program, the cumulative live birth rate (CLBR) per stimulated cycle is a more meaningful outcome measure to the women and the clinicians (26). The CLBR refers to live birth from transfer of fresh and all frozen embryos from an index stimulation cycle (26, 27). This retrospective analysis was designed to evaluate the effect of serum 25(OH)D level prior to ovarian stimulation on the CLBR of the first IVF cycle.

Methods

This was a retrospective study carried out at the Centre of Assisted Reproduction and Embryology, The University of Hong Kong – Queen Mary Hospital, Hong Kong. Clinical details were prospectively entered into a computerized database and retrieved for analysis. The study was approved by the Institutional Review Board of the University of Hong Kong/Hospital Authority Hong Kong West Cluster and was registered under the Hong Kong Clinical Trial Registry (HKCTR-2361).

Patients

Women undergoing the first IVF cycle in the Centre between December 2012 and November 2016 were included for analysis. Those undergoing donor oocyte IVF, *in vitro* maturation, pre-implantation genetic testing and women whose archived serum sample could not be retrieved were excluded (Fig. 1).
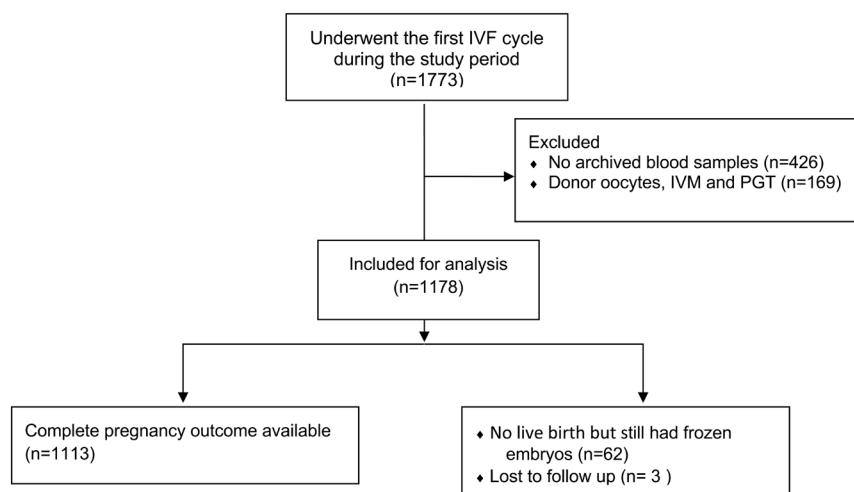
Figure 1
Flow of selection of the study population.

Serum 25-OH vitamin D measurement

Serum samples were taken in the early follicular phase of the cycle for measurement of estradiol and progesterone at the commencement of ovarian stimulation. The surplus serum samples were routinely archived and kept frozen at −20°C in our laboratory. The archived serum samples were retrieved and assayed for serum 25(OH)D concentration using liquid chromatography-mass spectrometry (LC-MS) at the Guangzhou KingMed Centre for Clinical Laboratory, which is accredited by the Vitamin D External Quality Assessment Scheme (DEQAS) in the United Kingdom for 25(OH)D and 1,25(OH)$_2$D assays.

Vitamin D deficiency was defined as serum 25(OH)D levels <50 nmol/L and vitamin D insufficiency as serum 25(OH)D > 50 and <75 mmol/L in accordance with the Endocrine Society criteria (28). Serum 25(OH)D levels of >75 nmol/L were considered replete. Women who were vitamin D insufficient and replete were grouped together as the non-deficient group and compared with those who were vitamin D deficient in the primary analysis. The three groups (vitamin D deficient, insufficient and replete) were separately compared in further analysis.

Ovarian stimulation and embryo transfer (ET)

The details of the procedures for ovarian stimulation, oocyte retrieval, handling of the gametes, cryopreservation of the embryos and frozen embryo transfer (FET) were previously described (29).

All women were treated either with the long gonadotrophin-releasing hormone (GnRH) agonist protocol or the GnRH antagonist protocol for pituitary downregulation. In the long GnRH agonist protocol, Buserelin (Suprecur®, Hoechst, Frankfurt, Germany) nasal spray was administered at 150 μg four times per day starting from the mid-luteal phase of the cycle preceding ovarian stimulation. Ovarian stimulation was accomplished by human menopausal gonadotrophin (HMG) or recombinant FSH. In the GnRH antagonist protocol, the women received ganirelix (Orgalutran®, NV Organon, The Netherlands) or cetrorelix (Cetrotide®, Merck) 250 μg daily starting from the sixth day of stimulation. The initial dose of stimulation was determined according to the baseline antral follicle count (AFC). Human chorionic gonadotrophin (hCG) (Pregnyl® 5000 or 10,000 units or Ovidrel® 250 μg) was injected when the mean diameter of the leading follicle reached 18 mm and more than three follicles reaching a mean diameter of 16 mm or above, followed by transvaginal ultrasound-guided oocyte

retrieval 36 h later. Fertilization was carried out either by conventional insemination or intracytoplasmic sperm injection (ICSI) depending on semen parameters.

Women were allowed to have replacement of at most two embryos or blastocysts, but single embryo or blastocyst transfer was strongly encouraged if the woman was <35 years old and had two or more good quality embryos (embryo of ≥4 cells, grade 1 or 2). ET was performed under transabdominal ultrasound guidance with a soft catheter (Sydney IVF Embryo Transfer Catheter®, Cook, Indiana, USA). If the woman was considered at risk of ovarian hyperstimulation syndrome (OHSS) or the serum estradiol concentration on the day of hCG injection was > 20,000 pmol/L, fresh embryo transfer was canceled. Cryopreservation of day 2 early cleaving embryos was performed by a slow freezing protocol using a programmable freezer (Planer Products Ltd.; Sunbury-On-Thames, UK), while that of blastocysts was carried out by vitrification. Frozen-thawed embryos or blastocysts were transferred in natural cycles in ovulatory women and in clomiphene-induced or hormone replacement cycles for anovulatory women. Urine pregnancy test was performed 18 days after the hCG trigger in IVF cycles or the LH surge in frozen embryo transfer cycles. If the urine pregnancy test was positive, transvaginal scanning was performed at 6 and 8 weeks of gestation to confirm fetal viability and ongoing pregnancy. Pregnancy outcome was routinely tracked and recorded for all cases.

Main outcomes

The primary outcome was CLBR per initiated cycle including live birth from the fresh embryo transfer and replacement of all frozen embryos that resulted from the first IVF cycle (26, 27). Secondary outcomes included: (i) duration of ovarian stimulation; (ii) dose of gonadotrophin used for ovarian stimulation; (iii) number of oocytes collected; (iv) clinical pregnancy rate (per cycle started and per transfer in the fresh cycle); (v) ongoing pregnancy rate (per transfer in the fresh cycle); (vi) miscarriage rate (in the fresh cycle) and (vii) live birth rate (per transfer in the fresh cycle).

Live birth was defined as the delivery of an infant born alive after 24 weeks' gestation (the definition of fetal viability adopted in this locality). Clinical pregnancy was defined as the presence of a gestational sac by ultrasonography at 6 weeks of gestation and ongoing pregnancy as viable pregnancy beyond 8 weeks of gestation.

Pregnancy outcomes were tracked from the Hospital Authority electronic patient record system or self-returned

reply slips from the women or their obstetricians. If the woman did not deliver within the public hospital system and no reply letter was received 2–3 months after the expected date of delivery (40 weeks by date of embryo transfer), they were contacted by our nurses to update the database.

Statistical analysis

Data were entered and analyzed using IBM SPSS 25.0 (IBM Corporation, NY, USA). Continuous and categorical variables were compared between groups using Mann–Whitney U test, Kruskal–Wallis test and chi-square test, respectively. Odds ratio was calculated with the vitamin D replete group as the comparison group. Logistic regression analysis was performed to examine the predictive performance of vitamin D on pregnancy outcomes controlling for women's age at the start of the stimulated cycle, BMI, antral follicle count, type and duration of infertility. The two-tailed P value of <0.05 was considered statistically significant.

Results

Patient characteristics

A total of 1178 women were included for analysis, of which 1113 had pregnancy outcomes available. In the whole cohort, the median age of the women (interquartile range) was 36 (34–38) years and serum 25(OH)D level was 53.4 (41.9–66.6) nmol/L. The prevalence of vitamin D deficiency (less than 50 nmol/L) was 42.2% (497/1178), and vitamin D insufficiency and deficiency (less than 75nmol/L) was 86.7% (1021/1178). Serum 25(OH)D was significantly lower in women undergoing IVF in spring and winter compared to those in summer and autumn (Fig. 2).

Table 1 shows the patient characteristics in the vitamin D deficient and non-deficient groups. There were no significant differences in the women's age, duration, type and cause of infertility, AFC and anti-Mullerian hormone between the two groups. Women in the deficient group had a significantly higher BMI when compared to the non-deficient group although the difference in absolute magnitude was small.

Main outcomes

When analyzing the results based on the threshold in the Endocrine Society guideline of 50 nmol/L for vitamin D
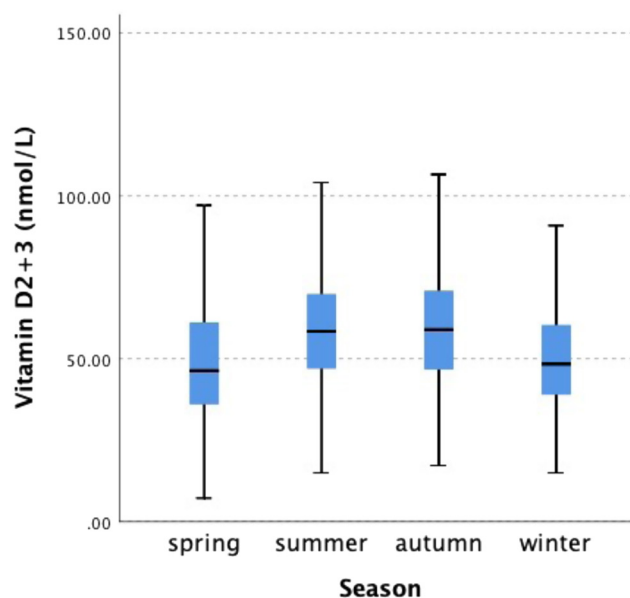
Figure 2
Box–whisker plot showing seasonal variations in serum 25(OH)D levels. The boxes represent the median and interquartile range and the whiskers represent the minimum and maximum. Spring (March–May), Summer (June–August), Autumn (September–November), Winter (December–February).

deficiency, the CLBR in the vitamin D-deficient group was significantly lower compared to the non-deficient group (43.9%, 208/474 vs 50.9%, 325/639, OR 0.755, 95% CI 0.595–0.959, $P = 0.021$, unadjusted) and after controlling for women's age, BMI, antral follicle count, type and duration of infertility (adjusted odds ratio (95% CI) 0.752 (0.586–0.964), $P = 0.024$). The clinical/ongoing pregnancy rate, live birth rate and miscarriage rate in the fresh cycle did not show significant differences between the vitamin D deficient and non-deficient groups (Table 2). When vitamin D replete (≥75 nmol/L), insufficient (50–75 nmol/L) and deficient (<50 nmol/L) groups were analyzed separately, there was a trend of higher CLBR in the vitamin D replete group compared to the vitamin D insufficient group, which was in turn higher compared to the vitamin D-deficient group (51.7% (74/143), 50.6% (251/496) and 43.9% (208/474), $P = 0.031$) (Table 3).

Serum vitamin D level was similar in those who achieved live birth in the fresh cycle compared to those who did not (54.5 (41.7–66.9) vs 53.7 (40.9–65.6) nmol/L, respectively, $P = 0.783$), as well as in those who had cumulative live birth from the IVF cycle compared to those who did not (54.5 (42.4–66.9) vs 52.2 (40.2–66.1) nmol/L, respectively, $P = 0.084$). The live birth rate per fresh transfer in the different seasons was: spring 109/269 (40.5%), summer 84/234 (37.0%), autumn 88/251 (35.1%) and winter 50/144 (30.1%), respectively.

Table 1 Patient characteristics. Data shown represent the median (interquartile range) for continuous variables and number (%) for categorical variables.

Parameters	Deficient group < 50 nmol/L (n = 497)	Non-deficient group ≥ 50 nmol/L (n = 681)	P[a]
Age of women (year)	36 (34–38)	36 (34–38)	0.686
BMI (kg/m^2)	21.8 (20.2–24.4)	21.5 (19.9–23.5)	0.028[b]
Duration of infertility (years)	4.0 (2.0–5.0)	4.0 (2.0–5.0)	0.826
Cause of infertility, n (%)			0.457
Endometriosis	28 (5.6)	36 (5.3)	
Tuboperitoneal factor	75 (15.1)	96 (14.1)	
Male factor	193 (38.9)	276 (40.6)	
Anovulation	25 (5.0)	35 (5.1)	
Unexplained	104 (20.9)	148 (21.7)	
Mixed factors	72 (14.5)	90 (13.2)	
Type of infertility			0.159
Primary	359 (72.2)	466 (68.4)	
Secondary	138 (27.8)	215 (31.6)	
Antral follicle count	10 (5–15)	10 (6–15)	0.536
Anti-Mullerian hormone (ng/mL)	1.4 (0.7–2.6)	1.6 (0.8–2.7)	0.248
Ovarian stimulation protocols (%)			
Antagonist	423/497 (85.1)	613/681 (90.0)	0.034[b]
Long agonist	72/497 (14.5)	67/681 (9.8)	
Others	2/497 (0.4)	1/681 (0.2)	

[a]Analyzed by Mann–Whitney *U* test except cause and type of infertility, which was by chi-square test; [b]Statistically significant.

Other secondary outcomes

There was a higher total dosage of gonadotropins used in women who were vitamin D deficient compared to those who were non-deficient. The vitamin D-deficient group had statistically longer duration of stimulation, less oocytes retrieved and less normally fertilized oocytes compared to the vitamin D non-deficient group although the magnitude of absolute difference was small (Table 2).

Discussion

Our study showed that the CLBR of the first IVF cycle in the vitamin D-deficient group was significantly lower compared to the non-deficient group, although there was

Table 2 Outcomes of IVF in the vitamin D-deficient group and the vitamin D non-deficient group. Data shown represent the median (*interquartile range*) or number (%).

	Vitamin D-deficient group < 50 nmol/L	Vitamin D non-deficient group ≥ 50 nmol/L	Odds ratio (95% CI)	P[a]	Adjusted odds ratio (95% CI)[b]	P
Total dose of gonadotrophins (IU)	2400 (1500–3225)	2250 (1350–3000)	–	0.018[c]	–	–
Duration of stimulation (days)	11 (10–13)	11 (10–12)	–	0.032[c]	–	–
Endometrial thickness on trigger day (mm)	11.9 (10.3–13.2)	11.7 (10.1–13.3)	–	0.859	–	–
Number of oocytes retrieved	8 (4–13)	8 (5–14)	–	0.045[c]	–	–
Number of normally fertilized oocytes	4 (2–8)	5 (3–8)	–	0.004[c]	–	–
Fresh CPR per cycle started	163/497 (32.8%)	236/681 (34.7%)	0.920 (0.720–1.175)	0.506	0.927 (0.724–1.188)	0.551
Fresh CPR per transfer	163/375 (43.5%)	236/523 (45.1%)	0.935 (0.716–1.221)	0.622	0.967 (0.737–1.270)	0.811
Fresh ongoing pregnancy rate per transfer	143/375 (38.1%)	199/523 (38.0%)	1.004 (0.764–1.319)	0.980	1.060 (0.802–1.401)	0.684
Fresh LBR per transfer	139/375 (37.1%)	192/523 (36.7%)	1.015 (0.772–1.337)	0.913	1.074 (0.811–1.423)	0.616
CLBR	208/474 (43.9%)	325/639 (50.9%)	0.755 (0.595–0.959)	0.021[c]	0.752 (0.586–0.964)	0.024[c]
Miscarriage rate per fresh transfer	32/178 (18.0%)	50/252 (19.8%)	0.886 (0.541–1.449)	0.628	0.853 (0.512–1.421)	0.542

[a]Analyzed by chi-square test for categorical variables and Mann–Whitney *U* test for continous variables; [b]Adjusted for age of women, BMI, antral follicle count, type and duration of infertility; [c]Statistically significant.

Table 3 Outcomes of IVF using the Endocrine Society Guidelines cut-off. Data shown represent the number (%).

	Vitamin D < 50 nmol/L	50 nmol/L ≤ vitamin D < 75 nmol/L	Vitamin D ≥75 nmol/L	*P*[a]
Fresh CPR per cycle started (%)	163/497 (32.8)	183/524 (34.9)	53/157 (33.8)	0.644
Fresh CPR per transfer (%)	163/375 (43.5)	183/411 (44.5)	53/112 (47.3)	0.496
Fresh ongoing pregnancy rate per transfer (%)	143/375 (38.1)	151/411 (36.7)	48/112 (42.9)	0.600
Fresh LBR per transfer (%)	139/375 (37.1)	148/411 (36.0)	44/112 (39.3)	0.843
CLBR (%)	208/474 (43.9)	251/496 (50.6)	74/143 (51.7)	0.031[b]
Miscarriage rate per fresh transfer (%)	32/178 (18.0)	44/197 (22.3)	6/55 (10.9)	0.646

[a]Analyzed by chi-square test for trend; [b]Statistically significant,.

no difference in live birth rate per fresh transfer between both groups. Women with vitamin D deficiency had a higher BMI and had less oocytes retrieved and normally fertilized oocytes despite requiring a higher dosage of gonadotrophin for ovarian stimulation compared to the vitamin non-deficient group, although the magnitude of absolute difference was small. As vitamin D is fat-soluble, this could be related to reduced bioavailability of vitamin D in women with higher BMI owing to a larger volume of distribution and higher deposition of vitamin D into the adipose tissue (30). There were slight differences in ovarian stimulation protocols used in the vitamin D deficient and non-deficient groups in our cohort, but existing data suggest that there are no differences in live birth rate per transfer and CLBR between the antagonist and agonist protocols so this is unlikely to affect the outcomes (31, 32).

The mechanism underlying the relationship between vitamin D deficiency and IVF outcomes is still unclear. In animal models, vitamin D receptor knockout female mice were unable to reproduce due to defects in uterine development and impaired folliculogenesis (6, 33). Conversely, direct injection of 1,25(OH)2D3 into the uterine lumen of female rats increased uterine weight and promoted endometrial decidual differentiation (34). Vitamin D is a known anti-proliferative, anti-inflammatory and immunomodulatory agent and could affect embryo implantation via several pathways: regulating myometrial contraction and myometrial cell proliferation; regulating the expression of the homeobox gene HOXA10 (35), which is a well-known molecule involved in the mechanism of implantation; promoting immunosuppression, extravillous trophoblast invasion and inducing decidualization; regulating the production of human chorionic gonadotrophin, human placental lactogen, estradiol and progesterone; and influencing uterine natural killer cells, dendritic cells, macrophages and T-cells, inhibition of Th1 cytokines and promotion of Th2 cytokines to promote anti-microbial, anti-inflammatory and anti-migratory functions (36). Some studies have

demonstrated that the metabolite of 1,25(OH)2D3 plays a role in modifying ovarian activity (37).

Existing studies suggest that in women who become pregnant, increased vitamin D concentration before conception, but not in early pregnancy, was associated with reduced pregnancy loss, which implies that the effects of vitamin D on gametes are more important than the effects on early pregnancy (38, 39) and that earlier replacement is better. Furthermore, a retrospective study using donor oocytes in IVF showed an improvement in the clinical pregnancy rate and live birth rate in recipient women who were vitamin D replete compared to those who were vitamin D deficient (12). By eliminating the impact of oocytes, studies among donor egg recipients suggest a role on endometrial receptivity being the key biologic mediator of the relation between vitamin D and ART outcomes (12, 18). Although two other cohort studies using donor oocytes have not shown the same improved outcomes in women replete in vitamin D (18, 40), some studies have found associations between vitamin D and endometrial thickness even in the absence of improved live birth rates (41, 42). In our study, there was no significant difference in the endometrial thickness between the vitamin D deficient and non-deficient groups.

There was an alarmingly high prevalence of vitamin D deficiency and insufficiency in our cohort of women in reproductive age, consistent with several publications citing vitamin D deficiency as a global epidemic. The high prevalence of vitamin D deficiency in subtropical Hong Kong has been attributed to sun avoidance, use of sun protection lotion for cosmetic reasons, shift work and indoor lifestyle (43, 44). Yet controversies remain as to what defines vitamin D deficiency or insufficiency. The recommended serum 25(OH)D thresholds for vitamin D deficiency, insufficiency and sufficiency differ among different international societies (45). The Institute of Medicine (IOM) report proposing the use of serum vitamin D level of 30 and 50 nmol/L as cut-off for vitamin D deficiency and insufficiency respectively focused

only on bone health and was not supported by evidence that 25(OH)D concentration above this concentration has beneficial effect for non-skeletal outcomes (46). Regarding bone health, achieving a serum 25(OH)D concentration of greater than 50 nmol/L was the primary treatment goal, and serum 25(OH)D concentration of less than 30 nmol/L was associated with higher mortality, infections, osteomalacia and nutritional rickets (47). The Endocrine Society defined vitamin D deficiency as a serum 25(OH)D as <50 nmol/L and insufficiency as a serum 25(OH)D between 50 and 75 nmol/L (28). For reproductive health, the cut-off may be different. Previous data from women with polycystic ovary syndrome have suggested that these women are more likely to be vitamin D deficient, and it is associated with metabolic abnormalities. In our study, women with anovulation (presumably mostly polycystic ovary disease) seemed evenly divided between groups. There is evidence that even higher thresholds of vitamin D beyond 75 nmol/L are beneficial for reproduction in women with polycystic ovary syndrome undergoing ovulation induction (48). In addition to using the Endocrine Society criteria, we analyzed the pregnancy and live birth rates in our cohort using the IOM definitions and there was a similar significant trend of increasing CLBR with serum 25(OH)D, with an even lower CLBR in those who had serum 25(OH)D less than 30 nmol/L (shown in Supplementary Table, see section on supplementary materials given at the end of this article). However, the interpretation is likely limited by the small numbers in the group with vitamin D levels below 30 nmol/L. As an overwhelming proportion of women in our cohort were vitamin D deficient/insufficient whichever criteria we adopted and there is no consensus on an adequate cut-off, it is not known if IVF outcome would improve if all women were supplemented with vitamin D. To complicate matters, despite high prevalence of vitamin D deficiency, the threshold of 25(OH)D for maximal suppression of PTH in the Chinese population has been found to be lower than the suggested threshold of vitamin D deficiency in the literature, possibly related to ethnic variations in vitamin D metabolism (49, 50, 51). Black and Asian women have been found to have lower serum vitamin D levels compared to women of other ethnic groups, so ethnic-specific variations and cut-off may indeed exist (25).

Previous studies focused on the association between vitamin D and various IVF outcomes in the fresh IVF cycle. We assessed the effect of vitamin D on CLBR of the first IVF cycle after transfer of all fresh plus frozen embryos. As embryo freezing has become an integral part of IVF programs in the modern day, the CLBR provides a more

comprehensive and meaningful outcome measure of IVF to the patients and the clinicians by taking into account the results of the fresh and all the frozen embryo transfers resulting from the index IVF cycle rather than the fresh cycle outcome alone (26). However, there are still challenges in the use of different numerators and denominators in the definition of CLBR, as well as the factor of time taken for couples to use up their embryos (26). Despite efforts to trace the IVF outcomes in our women, 62 women who have not achieved a live birth have not used up their embryos resulting from their IVF cycle more than 2 years from the initiation of their IVF cycle and 3 women were lost to follow-up. In Hong Kong, couples are allowed to store their embryos for a maximum of 10 years. There are various social and medical reasons for not returning for embryo transfer. From our experience, some women came back after several years for FET, but this group of women may also represent those with poorer prognosis. Nevertheless, these women made up 5.3% of the cohort and are unlikely to cause significant change in the findings of the study.

The main limitation of our study was its retrospective design. Serum 25(OH)D was taken at the start of the IVF cycle before ovarian stimulation, while the entire IVF cycle could span over several months and may not have reflected the serum 25(OH)D at the time of FET or during the course of pregnancy. This may not have important influence on our main finding regarding the association between vitamin D status and ovarian response which impacts CLBR quantitatively. It could be postulated that women who were vitamin D deficient continued to be vitamin D deficient overtime when they returned for FET, owing to persistence of lifestyle factors that led to vitamin D deficiency in the first place, but this would worth further exploration to confirm. Vitamin D supplementation is widespread in women having assisted reproduction, but we did not have information on what proportion of women were on some form of vitamin D supplementation. We have not studied the effect of vitamin D on pregnancy or neonatal complications. In the clinical setting, vitamin D status can be assessed by measuring the serum 25 (OH)D concentration via immunoassays, LC-MS and HPLC. Inter-assay variations exist between the different assay methodologies. In our study, we used the LS-MS technique, which is the gold standard, but immunoassays are widely used in many other centers. It is important to be aware of potential limitations when interpreting the results obtained by different assay methodologies. Although 25(OH)D is currently accepted as the best indicator of vitamin D stores, 25(OH)D is bound to vitamin D-binding protein (DBP) in the circulation (52). Measurements of bioavailable 25(OH)D and other vitamin D metabolites in

the serum or follicular fluid are currently only available as research tool and were not performed in this study. Recently, one conference abstract reported the degradation of 25(OH)D concentrations in serum and follicular fluid in frozen stored samples involving 35 patients over a 7-month period, which can impact the assessment of vitamin D in archived samples and is worth further exploration (53).

Conclusion

Vitamin D deficiency/insufficiency was prevalent in infertile women in subtropical Hong Kong. The CLBR of the first IVF cycle in the vitamin D-deficient group (<50 nmol/L) was significantly lower compared to the non-deficient groups (≥50 nmol/L), even after controlling for women's age, BMI, antral follicle count, type and duration of infertility. The ovarian response in the vitamin D-deficient group was significantly lower compared to the non-deficient group although by a small magnitude.

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