

Increased bioavailability of Vitamin D improved pregnancy outcomes in *in vitro* fertilization cycles, only in patients over 36 years: a cross-sectional study

D. BALDINI¹, A. MALVASI¹, I. KOSMAS², G.M. BALDINI¹, E. SILVESTRIS³

¹Momò Fertilfe Clinic, Bisceglie, Italy

²Department Obstetrics and Gynecology, Ioannina State General Hospital, Ioannina, Greece

³Gynecologic Oncology Unit, IRCCS Istituto Tumori "Giovanni Paolo II", Bari, Italy

Abstract. – **OBJECTIVE:** Vitamin D (VitD) is a secosteroid hormone showing both antiproliferative and immunomodulatory effects. Its involvement in placental steroidogenesis and endometrial decidualization even plays a role in other gynecological functions, including assisted reproductive technology (ART). However, controversial data have been reported on its implication in pregnancy outcomes during In Vitro Fertilization (IVF) program. In order to elucidate the VitD role in ART success, we evaluated serum and follicular fluid (FF) VitD levels in infertile women concerning the pregnancy rate.

PATIENTS AND METHODS: In our IVF center, 446 patients, under the age of 42 years old, were evaluated in the period between January 2018 and December 2019. It is here important to clarify that, in order to respect the exclusion criteria, only 103 of them were enrolled for the study concerning the VitD evaluation in serum and follicular fluid at the time of the egg retrieval. This took place both in 34 pregnant patients (Group 1) and 69 non-pregnant ones (Group 2). Furthermore, the collection of these data gave us the opportunity to assess a possible correlation between the VitD levels and the achievement of pregnancy in the performed IVF cycles.

RESULTS: The studied group included 103 eligible women. The average age for Group 1 was 33.12 ± 3.72 yrs whereas 33.72 ± 3.99 yrs ($p=0.467$) for Group 2. The main differences were observed concerning follicle numbers of 17-21 mm ($p=0.0043$), the number of retrieved oocytes ($p=0.0207$), as well as the number of mature oocytes ($p=0.0233$) among the different groups. Different reference ranges, established according to the pregnancy outcomes, revealed that pregnant women with >36 yrs showed significantly higher levels of VitD.

CONCLUSIONS: Increased serum and FF-VitD levels in women undergoing IVF with age ≥ 36

yrs, were significantly associated with a favorable outcome to achieve and carry on with the pregnancy.

Key Words:

Assisted reproductive technology, Follicular fluid, Pregnancy rate, Vitamin D.

Introduction

Vitamin D Metabolism

Vitamin D (VitD) is a fat-soluble secosteroid hormone produced in the skin by ultraviolet irradiation of 7-dehydrocholesterol¹; its function is to mediate both antiproliferative and immunomodulatory effects². VitD is biologically inert and must be converted in 25-hydroxyvitamin D3 in the liver, and then, to $1\alpha,25$ -dihydroxy vitamin D3 in the kidney as an active form able to exert its effect in bone mineralization, as well as in other biologic functions, thanks to the link with its nuclear receptor (VitDR).

The VitDR is a ligand-dependent transcription factor mainly localized in nuclei of target cells of several human tissues, including the hypothalamus, pituitary gland, epithelial cells, and female reproductive organs as the ovary, endometrium, fallopian tube, placenta, and decidual cells³⁻⁶. It is estimated that the VitDR binding by $1\alpha,25$ -dihydroxyvitamin D3 ligand, drives the transcription of more than 900 genes. These genes are involved in the regulation of calcium absorption⁷, phosphate uptake by enterocytes, calcium mobilization from bone tissues, and subsequent calcium reabsorption by kidneys.

Vitamin D Levels

ESNA's (Endocrine Society of North America) last revised guidelines claim VitD insufficiency when serum levels range among 21-29 ng/mL. Its deficiency, also known as hypovitaminosis, includes concentrations lower than 20 ng/mL and can be correlated with increased risk for clinical adverse events⁸. This condition, indeed, is often associated with severe osteoporosis conditions, as well as with increased incidence of autoimmune disorders, diabetes, and cardiovascular diseases⁹⁻¹¹. Moreover, higher recurrence of prostate, colon, ovarian, and breast cancers is also reported in subjects with VitD hypovitaminosis¹⁰. Males of infertile couples who undergo IVF cycles also have a reduced pregnancy rate if they have a VitD deficiency¹².

Vitamin D Effects in Females

Several authors¹³⁻¹⁶ have identified A VitD involvement in placental steroidogenesis and endometrial decidualization. This discovery allowed them to show that this hormone plays a critical role in a few gynecological functions. A lot of conditions, such as the endometriosis onset and/or its severity, as well as the pathogenesis of polycystic ovary syndrome (PCOS), preeclampsia, preterm delivery, gestational diabetes, and bacterial vaginosis are discovered to be correlated with low levels of VitD¹⁷. Furthermore, reduced fertility rates related to clinical conditions as hypogonadism, uterine hypoplasia, or impaired folliculogenesis and endometrial receptivity, have been reported¹⁸⁻²⁰ in VitD-deficient animal models, supporting its functional role even in the reproduction's physiopathology.

However, despite these results from experimental animal models, there are still controversial opinions: whereas several authors emphasize the need of normal levels of VitD in order to get satisfactory pregnancy outcomes in sub or infertile women undergoing assisted reproductive technologies (ART)²¹, others refuse its functional effects in *In Vitro* Fertilization (IVF) procedures²². At present, only two meta-investigations explored the association between VitD serum levels and IVF-outcomes.

Vanni et al²² underline the adverse effect of VitD hypovitaminosis on ART results, thus disagreeing about the putative beneficial influence of VitD supplementation before the IVF. Later, some subsequent meta-analyses have reexamined the correlation between serum VitD status and IVF procedures resulting in pregnancies and live

birth rate (LBR) in infertile women. Their authors, in fact, described no correlation between a VitD deficiency and the pregnancy outcomes, although the lower LBR was attributed to a previous hypovitaminosis condition^{23,24}. Finally, these meta-analysis are both insufficient to provide any crucial information concerning the role of this hormone in human reproduction, and other observations and clinical studies are needed to elucidate its pathways and the potential effects in infertility and IVF outcomes.

With the purpose to contribute to this debate, here we compared serum and follicular VitD levels in a large cohort of infertile women candidates to ART procedures to achieve pregnancy. In particular, we investigated the relation of VitD concentration concerning both the number and quality of the oocyte pool retrieved as well as with the pregnancy rate obtained.

Patients and Methods

Study Population

In this cross-sectional study, female patients aged under the age of 42 and candidate to IVF/intracytoplasmic sperm injection (ICSI) at "Momò Fertilife – Center for Reproductive Medicine" in Bisceglie (Italy), from January 2018 to December 2019 were enrolled to investigate the correlation of VitD levels in serum and follicular fluid (FF) of the retrieved oocyte pool with the fertility procedures outcome.

The exclusion criteria included premature ovarian failure (POF), previous evidence of poor hormonal response according to Bologna's criteria²⁵, endometriosis, glucidic, calcium, and/or VitD metabolic disorders, VitD exogenous assumption, and presence of blood in follicular fluid retrieved at the egg retrieval. The eligible group of patients included only 103 out of 446 women evaluated at the center who were treated with a controlled standard ovarian stimulation (COS) protocol using the recombinant follicle-stimulating hormone (FSH) preparation (GONAL-f., Merck Serono, Darmstadt, Germany) in association with conventional gonadotropin-releasing hormone (GnRH) antagonist (Cetrotide, Merck Serono, Germany) and human chorionic gonadotropin (hCG) (Gonasi, IBSA, Lugano, Switzerland) to induce ovulation.

Some particular attention was paid in setting up our study; actually we had previously evaluated the concentration of the content of VitD in the follicular serum of each follicle so to show that

the concentration in the serum does not change with the size of the follicle. Subsequently, our investigation turned to the evaluation of the concentration of VitD in each patient's follicular pool, but unlike the other studies, the dosages were immediately made without storing the biological material.

Ethical Consideration

All patients were voluntarily enrolled in the study and signed their informed consent at the first clinical evaluation. In the informed consent, they were informed both of the risks of the procedure and of those potentially linked to metabolic abnormalities²⁶. The study was regularly approved by the Local Ethical Committee of the Momò Fertility Institute (number approbation 07/2018).

Serum and Follicular Fluids (FF)

VitD was measured at the beginning of treatment in each patient enrolled.

Serum and FF from each patient were collected on the day of ovarian pick up (OPU). The FF was gathered with a 17G oocyte aspiration needle (Cook Medical, IN, USA) through a transvaginal ultrasound probe guide (VOLUSON S8, GE Healthcare, Boston, MA, USA), using a closed vacuum system tube commonly used for the follicle aspiration.

After the oocyte collection, obtained by a single ovarian puncture, an exposition to a hyaluronidase solution (25 IU/ml) and the corona radiata removal were performed by repeated pipetting. Metaphase II (MII) oocytes were selected by a stereomicroscope (Nikon SMZ 1500, Singapore, Japan), incubated in LGGF medium (Fertilization, Global, CooperSurgical, Trumbull, CT, USA), and injected with sperms (spermatic count eligible lower than 5 million/mL) by an inverted microscope (Nikon Eclipse TE 200, Singapore, Japan) at 400x magnification²⁷. Subsequently, the fertilized oocytes were cultured in LGGG medium (Global, CooperSurgical, Trumbull, CT, USA) for 3-5 days until their implantation. Then, luteal support was performed after the transfer with intramuscular progesterone or vaginal micronized^{28,29}.

After the oocyte recovery, the FF was collected into sterile tubes for a total amount of approximately 30 mL for the patient concerning the ovarian response to stimulation (max 14 follicles). Both plasmas collected before the follicle aspiration and FFs were immediately delivered to the laboratory to complete the VitD measurement.

Vitamin D (VitD) Measurement

Serum from each patient and FF were collected the same day of the OPU, and then immediately sent to the laboratory for an investigation about their VitD concentration by VIDAS[®] 25OH VitD Total (BioMérieux SA, France). This test is described as a quantitative test combining an ELISA method with a final fluorescent detection (ELFA). Each determination of VitD was performed in triplicate in order to ensure the best accuracy.

Statistical Analysis

Demographic features and ovarian stimulation data were registered in a Statistical Package for the Social Sciences (SPSS, Chicago, IL, USA, version 2018) database. Numerical data were presented as an average (SD) and compared between groups using independent *t*-tests. All *p*-values reported are 2-sided and *p*<0.05 were considered statistically significant. The pairwise correlation was performed in order to examine multiple variables of the study.

Reference ranges for VitD were established, using the Lambda-Mu-Sigma (LMS) method, from measurements in VitD (serum and FF pool). The LMS method summarizes the changing distribution by three curves representing the skewness expressed as a Box-Cox power (L), the median (M), and the coefficient of variation (S). The resulting L-, M- and S-curves contain the needed information to draw any percentile curve. Degrees of freedom for each curve (L, M, and S) were selected according to the changes in the model deviance. Centile curves reporting the average and the 90% interval of VitD (5th and 95th percentiles) for each age interval were created; the LMS Chartmaker software (LMS Chartmaker Light version 2.54; Medical Research Council, Cambridge, UK) was used.

Results

In the first phase of the study we wanted to measure the vitD levels of each follicle for each patient. The aim was to demonstrate that the vitD levels in each follicle are the same regardless its size. After evaluating 17 patients, we completed this first study as there was absolute correspondence between the vitD values of follicles with different sizes and the serum Vit D value.

The eligible cohort included only 103 women aged under 42 since from the initial 446 evaluated patients, 228 of them were out of inclusion crite-

ria and the other 118 of them were excluded due to blood within the recovered FFs.

The average age for clinical pregnancy patients (Group 1) was 33.12 ± 3.72 (M \pm SD) yrs while for patients with no pregnancies (Group 2) was 33.72 ± 3.99 yrs ($p=0.467$). As reported in Table I, significant differences were observed among the number of follicles 17-21 mm, the number of oocytes retrieved, and the MII oocytes developed between the two groups, contrarily to other parameters, where no differences were found. Numerical data are presented as an average (SD) and compared between groups using independent *t*-tests. All *p*-values reported are 2-sided and $p < 0.05$ and were considered statistically significant.

As reported in Table II, concerning the demographic characteristics, there is a remarkable correlation between the age and the antral follicle count (AFC) evaluated during the enrollment into the study, as well as for the anti-Mullerian hormone (AMH) levels.

During the ovarian controlled stimulation (COS) the Estradiol (E2), Progesterone (P), Follicle-stimulating hormone (FSH), and Luteinizing hormone (LH) levels were reported. A correlation was described between E2, the body mass index (BMI), and the serum glucose value and between P and E2 as well as for P and AMH and also for LH and FSH.

Finally, the number of follicles of 17-21 mm was related not only with age and BMI, but also with AFC and AMH as well as with P during COS.

Regarding the embryological features, also described in Table II, the number of oocytes retrieved reflected the AFC and the AMH detected, but also with the E2, and P during cycle and the number of 17-21 mm sized follicles of diameter.

Also, the number of the MII oocyte retrieved was correlated with AFC, AMH, E2, P, and the number of follicles 17-21 mm on the day of OPU. Moreover, the number of developed embryos was related not only with the number of follicles of 17-21 mm and the MII developed but also with age, BMI, AFC, AMH, and E2 values.

In conclusion, the patients who achieved pregnancy showed higher concentrations of VitD in sera and FF, but not statistically significant. In patients who achieved pregnancy with an age > 36 years (Table I), however, a statistically significant correlation was observed between serum and FF levels of VitD (Table II), so it appears important that beyond this age limit the concentration of VitD could play an essential role.

In this regard, reference ranges for serum and follicular pool values of VitD have been es-

tablished, using the LMs method. Two different types of reference ranges have been constructed according to clinical pregnancy (positive Figures 1, 2, 3 or negative Figures 4, 5, 6). From this figure, it is evident that the patients aged over 36 years old who become pregnant presented higher levels of vitD.

Discussion

Several studies³⁰⁻³⁴ have been investigating the association between IVF outcome and VitD levels in FFs to analyze its role in reproduction, particularly in folliculogenesis but also in the implantation rate. In this regard, it has been recently reported that this secosteroid hormone could probably be considered a potential biomarker in the prognosis of successful pregnancy following IVF, although many results are still contradictory and its mechanisms have not been closely elucidated²¹.

Anifandis et al³² and Ozkan et al²⁰ observed a direct link between VitD levels and IVF outcome in terms of a clear association of favorable pregnancy outcomes in the presence of high serum bioavailability of VitD. Furthermore, Antunes et al³³ investigated the VitD levels in FFs demonstrating that in patients with distinctive infertility diagnosis the relative VitD concentration was unaltered. Two population clusters were identified with higher (35 ng/ml) and conversely lower (17 ng/ml) intrafollicular concentrations. Although differences in follicular VitD levels did not appear to influence the numbers of total oocytes and MII oocytes after ovarian stimulation, the subgroup

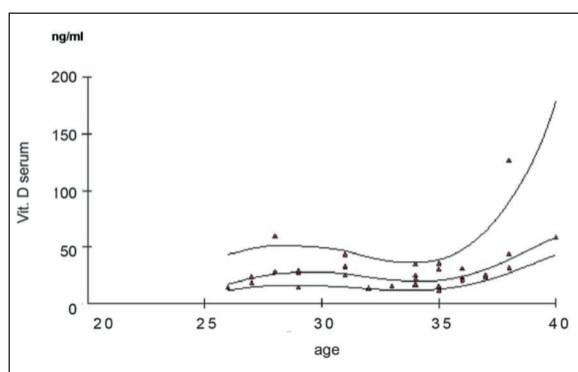


Figure 1. Vitamin D reference ranges (from our sample-serum starting day of ovarian stimulation) in patients who presented positive clinical pregnancy rates, according to their age, with lines indicating the 5th, 50th and 95th percentiles.

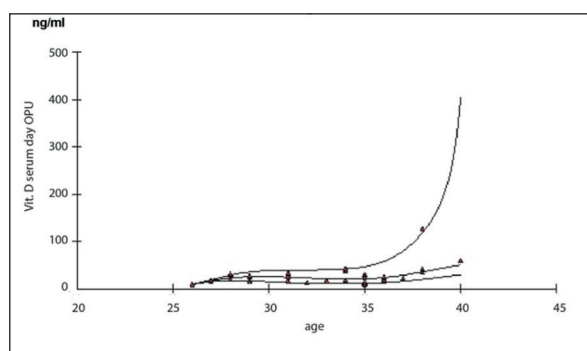


Figure 2. Vitamin D reference ranges (from our sample-serum on the day of OPU) in patient who presented positive clinical pregnancy rates according to age with lines indicating the 5th, 50th, and 95th percentiles.

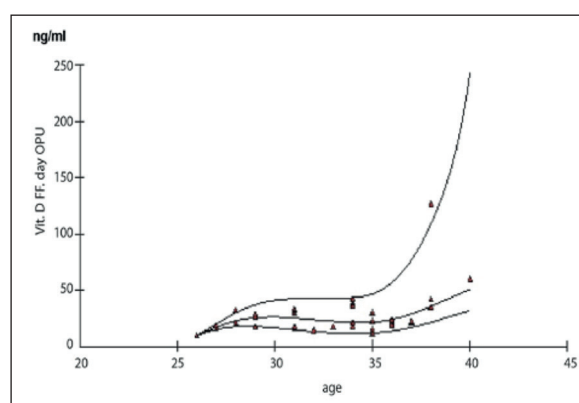


Figure 3. Vitamin D reference ranges (from our sample-Follicular Fluid on the day of OPU) in patient who presented positive clinical pregnancy rates according to age with lines indicating the 5th, 50th, and 95th percentiles.

with lower levels underwent the formation of a significantly greater number of larger follicles, suggesting that follicular VitD plays a role in the follicle development³⁵. In a parallel study, Rudick et al³⁵ also reported that the VitD status in women undergoing IVF may directly influence the success of the procedure. Pregnancy rates were found to range up to 37% in IVF receiving women with VitD hypovitaminosis in contrast with 78% detected in patients with physiologic VitD levels, while the live births were 31% and 59% respectively. Thus, the authors concluded that VitD interacted with endometrium to influence IVF success, and this interpretation was supported by the biological evidence that VitD signaling is enrolled in cross-talk between the embryo and endometrium in response to interleukin (IL)-1B secreted by the blastocyst. On the other hand, en-

dometrial dendritic cells and macrophages have been also described to secrete 1-alpha hydroxylase and calcitriol, known as the active form of vitamin D, to improve the hormonal homeostasis for embryo grafting³⁶.

Further observation provided additional evidence that VitD hypovitaminosis related to structural defects of the relative receptor is also responsible for low pregnancy rates since its deficiency may severely affect the follicle integrity as well as oocyte maturation and the general ovarian functions, especially in estradiol synthesis. Therefore, based on these observations, it is consequently possible that high levels of VitD would improve pregnancy outcomes by counteracting effects on its deprivation status³⁷⁻⁴⁰.

Table I. Comparison of VitD concentration, number of follicles and oocytes detected into the two study groups (pregnant: Group 1 and not-pregnant: Group 2).

All participants	Group 1 (n=34) pregnant	Group 2 (n=69) not-pregnat	p-value
Serum VitD concentration (ng/ml)	28.27 ± 21.07	24.02 ± 8.04	0.1442
Follicular fluid VitD concentration (ng/ml)	28.7 ± 20.66	24.067 ± 7.67	0.1021
17-21 mm follicles (n)	8.87 ± 4.86	6.24 ± 3.93	0.0043*
Oocytes detected (n)	12.78 ± 7.49	9.69 ± 5.51	0.0207*
MII oocytes (n)	9.39 ± 5.97	7.07 ± 4.06	0.0233*
Women with age > 36 yrs old	Group 1 (n=9)	Group 2 (n=20)	p-value
Serum VitD concentration (ng/ml)	42.02 ± 34.92	24.98 ± 7.88	0.0444*
FF concentration (ng/ml)	41.76 ± 34.63	25.22 ± 7.75	0.0486*

All data are presented as average ± SD. VitD: Vitamin D; MII: Metaphase II. *All p-values reported are 2-sided and p < 0.05 and were considered statistically significant. The pairwise correlation was performed to examine multiple variables of the study

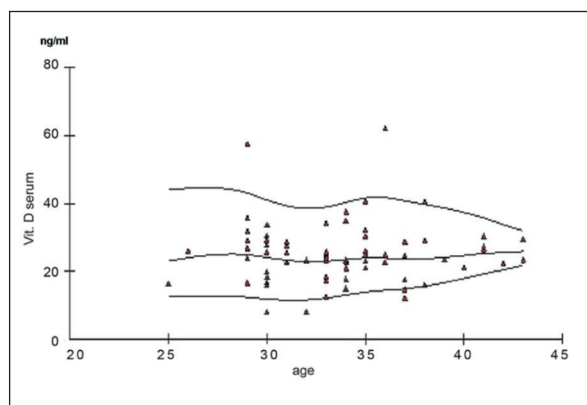


Figure 4. Vitamin D reference ranges (from our sample-serum starting day of ovarian stimulation) in patients who presented negative clinical pregnancy rates, according to their age, with lines indicating the 5th, 50th and 95th percentiles.

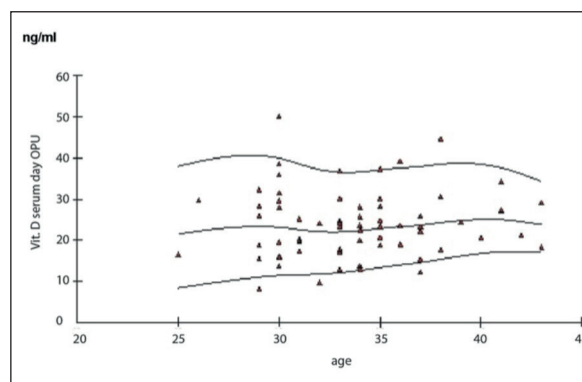


Figure 5. Vitamin D reference ranges (from our sample-serum on the day of OPU) in patient who presented negative clinical pregnancy rates according to their age with lines indicating the 5th, 50th, and 95th percentiles.

Table II. Comparative evaluation of different variables.

Variable	By variable	Correlation	Number	Lower 5 %	Upper 95%	Significance
AFC	Age	-0.3912	101	-0.5450	-0.2120	<0.0001
AMH	Age	-0.2778	102	-0.4480	-0.0880	0.0047
AMH	AFC	0.7397	101	0.6363	0.8171	<0.0001
LH	FSH	0.2801	102	0.0906	0.4501	0.0044
E2	BMI	-0.2177	102	-0.3955	-0.0243	0.0279
E2	Glucose	-0.3406	102	-0.5018	-0.1565	0.0005
P	E2	0.4052	102	0.2287	0.5558	<0.0001
N. of follicles (17-21 mm)	Age	-0.1993	102	-0.3791	-0.0051	0.0446
	BMI	-0.1961	102	-0.3762	-0.0016	0.0483
	AFC	0.3067	101	0.1183	0.4737	0.0018
	AMH	0.2609	102	0.0700	0.4334	0.0081
	E2	0.4963	102	0.3341	0.6300	<0.0001
No. of oocytes	P	0.4103	102	0.2346	0.5601	<0.0001
	AFC	0.4116	101	0.2351	0.5618	<0.0001
	AMH	0.2261	102	0.0330	0.4028	0.0223
	E2	0.3401	102	0.1560	0.5014	0.0005
	P	0.2704	102	0.0801	0.4417	0.0060
No. of MII oocytes	N. follicles (17-21 mm)	0.3908	102	0.2124	0.5439	<0.0001
	AFC	0.3843	101	0.2042	0.5393	<0.0001
	AMH	0.2037	102	0.0096	0.3830	0.0400
	E2	0.3710	102	0.1903	0.5274	0.0001
	P	0.2035	102	0.0094	0.3828	0.0402
N embryos	N. follicles (17-21 mm)	0.3053	102	0.1178	0.4717	0.0018
	Age	-0.1976	102	-0.3775	-0.0032	0.0465
	BMI	-0.2100	102	-0.3886	-0.0162	0.0341
	AFC	0.3878	101	0.2081	0.5421	<0.0001
	AMH	0.2127	102	0.0190	0.3910	0.0319
	E2	0.2616	102	0.0707	0.4340	0.0079
N embryos	No. of MII oocytes	0.7144	102	0.6038	0.7980	<0.0001

AFC: Antral follicle count; E2: Estradiol; AMH: Anti-Mullerian hormone; P: Progesterone; LH: Luteinizing hormone FSH: Follicle-stimulating hormone; MII: Metaphase II; BMI: Body Mass Index.

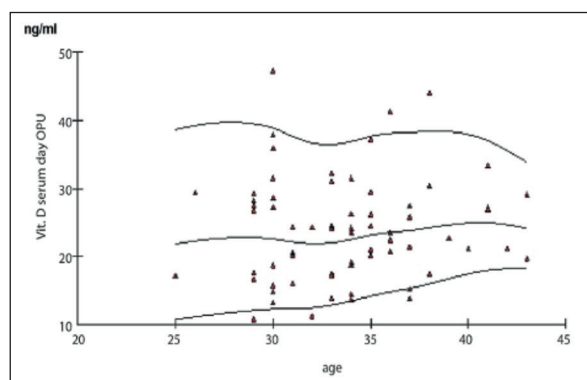


Figure 6. Vitamin D reference ranges (from our sample-Follicular Fluid on the day of OPU) in patient who presented negative clinical pregnancy rates according to age with lines indicating the 5th, 50th, and 95th percentiles.

In our study, we investigated a cohort of 103 infertile women undergoing IVF the concentration of VitD in sera and FFs as well as the relation of FF-VitD levels with the number and quality of oocytes retrieved after OPU, in term of MII, the number of fertilized oocytes, and successful pregnancy rates. We found that increased serum and FF VitD levels on the OPU day in IVF undergoing females over 36 yr, were significantly associated with favorable pregnancy outcomes. Since at present there are no data concerning the centile curves in this set of women, our work is the first attempt to correlate IVF outcomes and VitD from a general and unselected adult population in Italy. Since VitD concentrations change between different latitude located populations, the centile curves presented in our report are not suitable for all European populations but should be considered as southern-Italy-related data.

Moreover, it has been described⁴¹ that there are seasonal variations of VitD bio-availability related to the sun exposition. We have not investigated so far the relationship between the improvement of the pregnancy rate and the pregnancy seasons although we considered that high VitD bio-availability induced by either external supplement or increased seasonal related synthesis was unequivocally suitable to induce favorable IVF outcomes. This study puts a point on the dispute whether VitD levels can favor pregnancies in assisted fertilization treatment. In our opinion, only patients aged over 36 have a benefit in achieving pregnancy with normal Vitamin D values.

Conclusions

In conclusion, although further studies are necessary to assess the favorable role of VitD in IVF procedures, our work represented a contribution to this topic and suggests that at least in a southern region of our country, in contrast with women showing VitD hypovitaminosis, those with VitD increased levels in both and FFs, and aged over 36, are protected in pursuing their IVF programs.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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