25-HYDROXYVITAMIN D SERUM LEVELS AND ENDOMETRIOSIS: RESULTS OF A CASE-CONTROL STUDY

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The study has been conducted in Milan, Italy.

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ABSTRACT

The immunomodulatory, anti-inflammatory and anti-proliferative properties of vitamin D have laid the basis for a possible function of this prohormone in the pathogenesis of endometriosis. The aim of this case-control study was investigating vitamin D status, by measuring 25-hydroxyvitamin D [25(OH)D] serum levels, in women with and without endometriosis. Only Italian women of Caucasian origin aged between 18 and 45 years were deemed eligible. Enrollment was limited to the period October-May. Cases and controls were matched for month of recruitment, and secondarily for age and parity. Overall, 434 women were enrolled (endometriosis $n = 217$; controls $n = 217$). The group of cases included 127 women with ovarian endometrioma and 90 patients with deep endometriosis. Mean ± SD levels of 25(OH)D in women with and without endometriosis were 17.9 ± 7.0 ng/ml and 18.4 ± 7.6, respectively ($P = 0.46$). Analyzing the two endometriosis sub-group separately no statistically significant differences emerged (18.7 ± 7.4 ng/ml in deep endometriosis group versus 17.3 ± 6.6 ng/ml in women with ovarian endometrioma; $P = 0.14$). Comparing the sub-group of women with deep endometriosis with paired controls no differences occurred (18.7 ± 7.4 ng/ml versus 18.5 ± 7.7 ng/ml, $P = 0.80$). Similar data emerged when performing the same analysis for ovarian endometriomas (17.4 ± 6.6 ng/ml versus 18.3 ± 7.6 ng/ml, $P = 0.23$). The results of the present case-control study do not support an association between serum vitamin D levels and different phenotypes of endometriosis.

KEY WORDS: endometriosis; vitamin D; 25-hydroxyvitamin D; deep endometriosis; ovarian endometrioma

INTRODUCTION

The immunomodulatory, anti-inflammatory and anti-proliferative properties of vitamin D have laid the basis for a possible function of this prohormone in the pathogenesis of endometriosis.[1] In fact, a dysfunction of the immune system responsible for a state of chronic inflammation has been claimed to play a role in the multifactorial pathogenesis of the disease.[2] Indeed, endometriosis is characterized by a reduced T-cell cytotoxicity, a functional deficit of natural-killer lymphocytes and higher concentration of activated macrophages in the peritoneal fluid, which generate a cascade of cytokines and vascular endothelial growth factors favoring the proliferation of endometrial cells and angiogenesis.[3,4] Along with this theory, abnormal levels of pro-inflammatory cytokines have been detected in the peritoneal fluid and serum of affected women,[5] and murine models suggested the potential role of interleukin-6 and tumor necrosis factor α through their effect on inflammatory angiogenesis.[6]

Moreover, vitamin D receptor is expressed in ovarian tissue, endometrium, and fallopian epithelial cells,[1] and both eutopic and ectopic endometrium express the enzyme 1α-hydroxylase, responsible for the conversion of 25-hydroxyvitamin D [25(OH)D] into the biologically active form of vitamin D, calcitriol.[7] Of relevance here is a recent study in a murine model of endometriosis showing that calcitriol is able to both prevent ectopic implantation of endometrium and to reduce already established lesions.[8] Finally, numerous *in vitro* and *in vivo* studies have demonstrated that vitamin D deficiency could increase the risk of several cancer and autoimmune diseases, which tend both to be more common in women with endometriosis.[9-11]

Given this background, the potential role of vitamin D is of increasing interest and, in the last two decades, various studies have investigated the relation between endometriosis and vitamin D serum levels, with inconsistent results (Table 1).[12-17] Therefore, the influence of vitamin D on endometriosis development and progression remains to be clarified. To shed more light on this

potential association, we have compared serum concentrations of 25(OH)D in a large case-control study of women with and without endometriosis.

MATERIAL AND METHODS

This case-control study was performed in an academic hospital, the Fondazione Ca' Granda Ospedale Maggiore Policlinico that includes a tertiary referral center for the study and management of endometriosis. Participants were recruited during the period October 2014 – January 2017. Only Italian women of Caucasian origin aged between 18 and 45 years were deemed eligible. Cases were women with a surgical diagnosis of endometriosis in the previous 24 months or with a current non-surgical diagnosis of endometriosis. Non-surgical diagnoses were based on previously published criteria.[18-21] Affected women were sub-categorized in two groups, namely deep invasive endometriosis and ovarian endometrioma. The former included women with rectovaginal plaques, bladder detrusor nodules, bowel lesions, intrinsic ureteral endometriosis, and deep endometriosis infiltrating the pouch of Douglas and parametria. Cases with iatrogenic, post-Caesarean bladder detrusor endometriosis were excluded. In the same time span, women attending our outpatient clinics for periodic well-woman visits, contraception, cervical cancer screening program, or attending the blood bank of our hospital for blood donation, and without a previous clinical or surgical diagnosis of endometriosis, were enrolled as control group. Endometriosis was excluded based on gynecological history, pelvic transvaginal ultrasound, gynecological bimanual examination and visual inspection of the posterior vaginal fornix.

Patients reporting malignancy, uterine leiomyomas, hypertension, diabetes, multiple sclerosis, autoimmune disorders, and coronary, hepatic, or renal diseases were excluded from both study groups. Other exclusion criteria include vitamin D supplementation and full body sun exposure during the month before study enrollment. Cases and controls were matched for month of recruitment, and secondarily for age and parity. Moreover, to prevent the impact of seasonality and

the likely inter-participant variable degree of sun exposure, the recruitment was limited to the period October-May.

In women who agreed to participate a blood sample was drawn. Blood samples were allowed to clot at room temperature and then centrifuged at 2,000 g for 10 minutes. The resulting serum was stored at -20 °C until assayed. The quantitative detection of total 25(OH)D levels were obtained using a commercially available kit based on a chemiluminescence technology (DiaSorin, Inc. Corp., Stillwater, MN, USA). The assessments were performed in three distinct experiments thawing a similar number of blood samples from matched case and controls. The intra- and interassay coefficients of variations were 10% and 15%, respectively. The biologists engaged in the 25(OH)D assessment were blinded to the condition of the patients and to the study aims.

Data were collected on standardized forms including demographic information and clinical characteristics. In addition, enrolled women filled out a detailed questionnaire evaluating current and previous sun exposure habits, phenotypic characteristics, and skin phototype; the latter has been assessed according to the Fitzpatrick classification,[22] that reflects, to some extent, the degree of skin color intensity and the extent of skin sensitivity to damage generated by ultraviolet radiation.

In the current study, the concentration of vitamin D is expressed as ng/ml, and severe deficiency, deficiency, insufficiency, and sufficiency were defined when values were <10, <20, ≥20 - <30, and ≥30 ng/ml, respectively.[23] The competent Institutional Review Board approved the study, and patients gave written informed consent (Comitato di Etica Milano Area B; determination #1940/2014, approval date September 5, 2014).

Data were archived using Excel 2003 (Microsoft Corporation, Redmond, Washington, U.S.A.) and exported in SPSS 18.0 (SPSS, Inc, Chicago, IL, U.S.A.) for statistical analysis. Data were compared using unpaired or paired Student-*t* test, Fisher Exact test or McNemar test, as appropriate. *P* values below 0.05 were considered statistically significant. As previously adopted by

Paffoni *et al.*,[24] the choice of the sample size was calculated based on an expected serum concentration of 25(OH)D in controls of 20.4±11.8 ng/mL.[13] A difference of -20% in serum 25(OH)D in women with endometriosis was deemed clinically important. Setting type I and II errors to 0.05 and 0.05, the calculated number of women to be recruited was 434, 217 per study group.

RESULTS

Recruitment continued until the pre-planned number of participants was reached (endometriosis n = 217; controls n = 217). The group of cases included 127 women with ovarian endometriomas and 90 patients with deep lesions. The deep endometriosis group comprised 51 patients with rectovaginal endometriotic plaques, 18 with full-thickness bladder detrusor nodules, 11 with deep lesions infiltrating the pouch of Douglas and parametria, 7 with full-thickness bowel lesions, and 3 with intrinsic ureteral endometriosis. Baseline clinical and gynecological characteristics of cases and controls are shown in Table 2. The distribution of the demographic variables is similar between the two study groups. Regarding gynecological characteristics, parity did not differ (as expected based on the study design) whereas, as predictable, use of hormonal therapies and pain symptoms (dysmenorrhea, deep dyspareunia, non-menstrual pelvic pain, and dyschezia) were significantly more frequent in women with endometriosis (Table 2).

Serum levels of 25(OH)D were 17.9 ± 7.0 ng/ml in the endometriosis group and 18.4 ± 7.6 ng/ml in the control group (P = 0.46). The monthly distribution of serum 25(OH)D in the two study groups is illustrated in Figure 1. When analyzing the two endometriosis sub-groups separately, no statistically significant differences emerged (18.7 ± 7.4 ng/ml in the deep endometriotic lesions group versus 17.3 ± 6.6 ng/ml in the ovarian endometrioma group; P = 0.14). At subgroup analysis, no statistically significant differences were observed when comparing separately women with deep endometriotic lesions and those with ovarian endometriomas with their matched controls (18.7 ± 7.4 ng/ml versus 18.5 ± 7.7 ng/ml, P = 0.80 and 17.4 ± 6.6 ng/ml versus 18.3 ± 7.6 ng/ml, P = 0.23,

respectively). In addition, no statistically significant differences emerged after subdividing 25(OH)D serum concentrations of the two study groups into the four categories (severe deficiency, deficiency, insufficiency, and sufficiency) ($P = 0.29$; Table 3). A total of 13% of women with deep endometriotic lesions ($n = 12$) had severely deficient 25(OH)D serum levels, 49% ($n = 44$) deficient concentrations, 31% ($n = 28$) insufficient levels, and, 7% ($n = 6$) adequate levels; these figures were not statistically significantly different from those observed in their matched controls ($P = 0.50$). The corresponding frequencies for women with ovarian endometriomas were, 16% ($n = 20$), 68% ($n = 53$), 28% ($n = 35$), and 3% ($n = 4$), respectively, again without statistically significant differences with their matched controls ($P = 0.38$).

The phenotypic characteristics, current and previous sun exposure habits, and cutaneous reaction to UV are shown in Table 4. No statistically significant differences emerged between the two study groups. Current and past global UV exposure were comparable between women with and without the disease, being 23.2 ± 18.7 days and 20.9 ± 14.1 days during adulthood ($P = 0.14$) and 43.3 ± 35.3 days and 45.9 ± 32.3 days during adolescence ($P = 0.44$), respectively. The median [interquartile range, IQR] duration of last sun exposure prior to enrollment was 14 [7-15] days in endometriosis group and 10 [7-15] days in control group ($P = 0.75$), whereas the number of days elapsed between last UV exposure and recruitment was, respectively, 195 [135-236] - in the former group and 202 [142-258] in the latter group ($P = 0.26$).

COMMENT

In this study, statistically significant differences in 25(OH)D serum levels were not observed when comparing women with and without endometriosis. In addition, no differences emerged after subdividing patients into the phenotypic categories of deep endometriotic lesions and ovarian endometriomas. Lacks of differences in UV exposure habits further support our findings. In both study groups, median 25(OH)D serum concentrations were below the limit of normalcy established by the Endocrine Society guidelines.[23] This questions the validity of this categorization scheme, at

least in the Northern Italian context, and supports the opportunity of reconsidering its discriminatory cut-off limits.[25] Indeed, particularly in our Mediterranean country, an insufficient degree of sun exposure may not be deemed the cause of low 25(OH)D serum concentrations.

Our findings are in line with those obtained by Agic *et al.*,[14] whereas in other observational studies higher[13] or lower[15-17] vitamin D serum levels were observed in women with endometriosis (Table 1). Noteworthy, the present results are also at odds with previous evidence from our own group.[13] Among the reasons that may explain these inconsistencies, differences in study design and sample size presumably play a role. Unfortunately, studying the impact of vitamin D on the pathogenesis of endometriosis is methodologically challenging. Confounding may bias observational studies, and the disease itself might also theoretically lower vitamin D concentrations (reverse causality bias).[26] Indeed, low 25(OH)D serum levels could result from the inflammatory process, peculiar of endometriotic disease.[27] Environmental factors, such as sun exposure, smoke, obesity and dietary intake also influence the levels of vitamin D.[28]

To disentangle whether vitamin D may play a role in the pathogenesis of endometriosis, it would be more interesting to test women before disease development, i.e. in the adolescent period or even earlier,[29] rather than when the disease is diagnosed at a later age. To this aim, a long-term cohort design would be more appropriate, but also much more costly and cumbersome to conduct. In order to obtain some information on this aspect, we included in our study some questions regarding UV exposure during adolescence and, again, we failed to show any difference between the study groups. This type of evidence is however exposed to a significant risk of recall bias.

One of the advantages of our study design is represented by the large sample size; in fact, in none of the previously published observational studies, more than 400 subjects had been enrolled. Only the study from Harris *et al.*[15] reporting on the Nurses' Health Study II included a larger sample size but the study exclusively focused on the estimated nutritional intake of the vitamin that

generally represents only 10% of the human needs. In humans, the most relevant source of vitamin D is provided by UV exposure.[9,30.31]

Matching cases and controls for month of recruitment allowed to elude the relevant potential confounding effect of seasonality. Moreover, to prevent the impact of variable sun exposure, we decided to limit enrollments to the October – May period and to exclude the women that reported a global UV radiations exposure in the month before blood collection. Noteworthy, this latter exclusion criterion should not have biased our results considering that only a minority of patients were excluded on this basis, and this proportion did not differ between the study groups (less than 5% in both groups). In addition, women with disease potentially related to vitamin D deficiency, such as uterine leiomyomas, cancer, multiple sclerosis or taking vitamin D supplements were excluded from the study. Finally, the two study groups had similar basal characteristics (geographic origin, BMI, cigarette smoke, phenotypic and sun exposure characteristics).

As for any case-control study, the choice of controls may be cause of concern. In our study, we decided to include women without known endometriosis presenting to the gynecologic unit for routine well-woman visit, contraception, cervical cancer screening or to the blood bank of our hospital for blood donation. Endometriosis was ruled out based on gynecological and ultrasonographic examination but we cannot exclude to have inadvertently included some cases among controls. However, the impact of this potential inaccuracy would be presumably modest, given the limited prevalence of asymptomatic endometriosis in the general population.[32] Moreover, mis-diagnoses are more likely for early superficial peritoneal endometriosis, a condition of doubtful clinical relevance.[33]

Another potential limitation of our study could be represented by the lack of a food questionnaire investigating the dietary habits of recruited women. However, as mentioned earlier, vitamin D reserve is mostly due to sunlight exposure (90%) rather than dietary intake (10%).[9]

In conclusion, the results of the present case-control study do not support an association between serum vitamin D levels and endometriosis. If these findings will be confirmed the potential role of vitamin D in the development of endometriosis should be challenged.

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DECLARATION OF AUTHORS' ROLE:

L. Buggio conceived, drafted, revised the article, and acquired the data;

E. Somigliana performed statistical analysis, participated in conceiving the article, drafted a part and revised it;

MN. Pizzi, D. Dridi, E. Roncella acquired the data;

P. Vercellini participated in conceiving the article and revised it;

all authors approved the final version of the article.

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306 FIGURE LEGEND

307 Figure 1. Serum levels of 25(OH)D in women with endometriosis (red columns) and in control
308 participants (blue columns) according to month of recruitment. None of the differences is
309 statistically significant.

Table 1. List of studies evaluating 25(OH)D serum levels in women with and without endometriosis (literature data 1990-2017).

Source	Year	Country	Study design	N° of patients enrolled (endometriosis; controls)	Dosage evaluation	Mean ± DS 25(OH)D serum levels (endometriosis; controls)	*P*
Hartwell, *et al.*[12]	1990	Denmark	Case-control	155 (*n* = 42; *n* =113)	Serum	32.2 ± 0.6 ng/ml; 30.5 ± 14.1 ng/ml	0.43
Somigliana *et al.*,[13]	2007	Italy	Cross-sectional in women scheduled for gynecologic surgery	140 (*n* = 87; *n* = 53)	Serum	24.9 ± 14.8 ng/ml; 20.4 ± 11.8 ng/ml	0.05
Agic *et al.*,[14]	2007	Germany	Case-control in women scheduled for benign gynecologic surgery	79 (*n* = 46; *n* = 33)	Serum	25.7 ± 2.1 ng/ml; 22.6 ± 2.0 ng/ml	0.31
Harris *et al.*,[15]	2013	United States	Prospective cohort study (Nurses' Health Study II)	70.556 (*n* = 1385; *n* = 69.171)	Predicted serum levels based on daily food intake	Predicted plasma 25(OH)D levels were inversely associated with endometriosis risk	0.004[a]
Miyashita *et al.*,[16]	2016	Japan	Cross-sectional in women scheduled for laparoscopy for endometriosis or benign ovarian tumor (patients were negative for uterine fibroids).	76 (*n* = 39[b]; *n* =37)	Serum	Severe endometriosis: 17.2 ± 1.1 ng/ml;[c] Mild endometriosis: 21.5 ± 1.4 ng/ml; Controls: 21.8 ± 1.3 ng/ml	<0.01[c] <0.05[c]
Anastasi *et al.*,[17]	2017	Italy	Case-control	194 (*n* = 104; *n* = 90)	Serum	21.3 ± 8.9 ng/ml; 32.3 ± 2.7 ng/ml	<0.01

25(OH)D = 25-hydroxy vitamin D

[a] Women in the highest quintile of predicted vitamin D level had a 24% lower risk of endometriosis than women in the lowest quintile (rate ratio = 0.76, 95% confidence interval: 0.60, 0.97; p trend = 0.004).

[b] Mild endometriosis (*n* = 17, stage 1 and 2), severe endometriosis (*n* = 22; stage 3 and 4)

[c] Serum levels of 25(OH)D in samples from patients with severe endometriosis were significantly lower than those detected in samples from women with mild endometriosis (*P* < 0.01) and controls (P <0.05)

Table 2. Baseline demographic and clinical characteristics of participants in the two study groups.

Characteristics	Endometriosis	Controls	*P*
Age (years)	34,2 ± 6,5	33,2 ± 6,5	0.14
Italian area of origin			
North	175 (81%)	164 (76%)	0.31
Center	11 (5%)	10 (4%)	
South	31 (14%)	43 (20%)	
BMI (Kg/m^2)	21,7 ± 3,3	22,0 ± 3.0	0.29
Smoking			
Yes	57 (26%)	59 (27%)	0.47
No	143 (66%)	134 (62 %)	
Previous smoker	17 (8%)	24 (11%)	
Marital status			
Married	77 (35%)	60 (28%)	0.10
Unmarried	140 (65%)	157 (72%)	
Working status			0.13
Employed	181 (83%)	193 (89%)	
Unemployed (or student)	36 (17%)	21 (11%)	
Previous deliveries			0.67
None	155 (72)	159 (73)	
1	33 (15)	35 (16)	
≥ 2	29 (13)	23 (11)	
Hormonal therapies			<0.001
None	89 (41)	153 (71)	
Estroprogestins	61 (28)	60 (28)	
Progestins	63 (29)	4 (2)	
GnRH analogues	4 (2)	0 (0)	
Dysmenorrhea [a]	112 (52)	42 (20)	<0.001
Dyspareunia [a]	79 (36)	7 (3)	<0.001
Non menstrual pelvic pain [a]	71 (33)	11 (5)	<0.001
Dyschezia [a]	48 (22)	7 (3)	<0.001

BMI = body mass index.
Data are expressed as mean ± SD or number (percentage).
[a] The presence of pain symptoms was determined using the NRS (numeric rating scale) and considering the symptom present if NRS was >5.

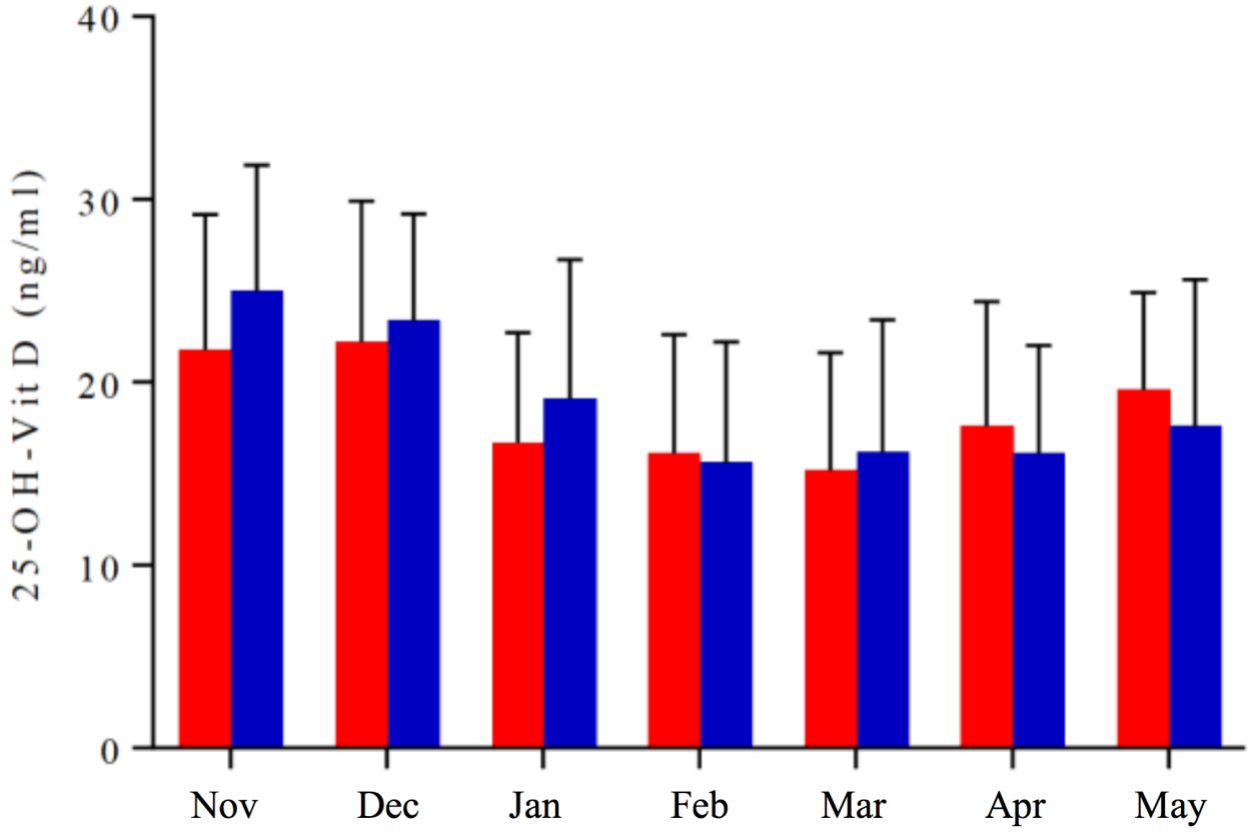
Table 3. Categorization of serum concentrations of 25(OH)D in women with and without endometriosis.

Serum levels (ng/ml)	**Endometriosis (%)**	**Controls (%)**	***P***
			0.29
Severe deficiency (<10 ng/ml)	32 (15)	31 (14)	
Deficiency (10-19.9 ng/ml)	112 (52)	107 (49)	
Insufficiency (20-29.9 ng/ml)	63 (29)	60 (28)	
Sufficiency (≥30 ng/ml)	10 (4)	19 (9)	

Table 4. Phenotypic characteristics, sun exposure habits and cutaneous reaction to UV in patients with endometriosis and in control participants.

	Endometriosis (%) (*n* = 217)	**Controls (%) (*n* = 217)**	P_{trend}
Hair color			0.26
Black	5 (2)	6 (3)	
Dark brown	107 (49)	97 (44)	
Light brown	77 (36)	80 (37)	
Blonde	25 (12)	28 (13)	
Red	3 (1)	6 (3)	
Skin phototype			0.45
Type 1	9 (4)	8 (4)	
Type 2	49 (23)	39 (18)	
Type 3	86 (39)	96 (44)	
Type 4	49 (23)	52 (24)	
Type 5	24 (11)	17 (8)	
Type 6	0 (0)	5 (2)	
Sun exposure during adolescence (16-18 aa)			0.42
Never	1 (1)	0 (0)	
Rare	15 (6)	19 (9)	
Occasional	82 (38)	82 (38)	
Frequent	82 (38)	88 (40)	
Very frequently	37 (17)	28 (13)	
Sun exposure during work activity			0.11
Never	152 (70)	165 (76)	
Rare	35 (16)	29 (13)	
Occasional	19 (9)	18 (8)	
Frequent	8 (4)	4 (2)	
Very frequently	3 (1)	1 (1)	
Sun exposure during leisure			0.23
Never	5 (2)	1 (1)	
Rare	38 (17)	39 (18)	
Occasional	95 (44)	120 (55)	
Frequent	69 (32)	52 (24)	
Very frequently	10 (5)	5 (2)	
Use of UV tanning lamps during adolescence			0.85
Si	112 (52)	115 (53)	
No	105 (48)	102 (47)	

	Endometriosis (%) (*n* = 217)	**Controls (%) (*n* = 217)**	P_{trend}
Current use of UV tanning lamps			0.81
Yes	44 (20)	41 (19)	
No	173 (80)	176 (81)	
Cutaneous reaction after 1h of sun exposure			0.36
None	68 (31)	63 (29)	
Occasional burns	116 (54)	113 (52)	
Always burns	33 (15)	41 (19)	
Do you like to catch the sun?			1.00
Yes	168 (77)	169 (78)	
No	49 (23)	48 (22)	
Use of protective tanning cream			0.73
Never	11 (5)	8 (4)	
Occasionally	51 (24)	61 (28)	
Always	155 (71)	148 (68)	


40
30
20
10
0
25-OH-Vit D (ng/ml)
Nov
Dec
Jan
Feb
Mar
Apr
May