ORIGINAL ARTICLE

Direct detection of free vitamin D as a tool to assess risk conditions associated with chronic plaque psoriasis

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Keywords

Psoriasis • Free vitamin D • Direct immunoassay • Cross-sectional study

Summary

Introduction. *Psoriasis is a major public health problem that results in high social and health costs. New approaches and methods are required to identify any conditions related to the disease and comorbidity development. The vitamin D deficiency is associated to psoriasis and could play an important role in its pathogenesis. However, the serum level of vitamin D is currently measured as total vitamin D, which is affected by wide variability. Therefore, the determination of the free form could be more significant, since it is independent of confounding factors. A cross-sectional study was performed to assess the association between chronic plaque psoriasis and serum level of free vitamin D, detected by a direct analytical method.*
Methods. *The levels of bioavailable vitamin D, total vitamin D and other metabolic parameters whose homeostasis is regulated by vitamin D were evaluated in 72 psoriasis patients and in 48 healthy controls. A direct immunoassay method was used to measure serum free vitamin D level. Analysis of covariance was performed to calculate estimated marginal means (EMM) and 95% confidence interval (CI), after adjustment for age, sex and BMI, within the two groups.*
Results. *Patients showed an EMM of 5.526 ± 0.271pg/ml, 95% CI 4.989-6.063; while controls an EMM of 6.776 ± 0.271 pg/ml, 95% CI 6.115-7.437.*
Conclusions. *Chronic plaque psoriasis patients exhibited a serum level of free vitamin D lower than controls. The direct immunoassay method could represent a useful tool to assess vitamin D status and identify a risk condition associated with the onset of the pathology.*

Introduction

Psoriasis is a chronic inflammatory skin disease characterized by raised, well-demarcated, erythematous oval plaques with adherent silvery scales, resulting from a hyperproliferative epidermis with abnormal differentiation [1, 2].
It is a major public health problem, affecting approximately 125 million people worldwide [3]. Globally, the prevalence of psoriasis varies considerably across different populations, with higher levels in northern countries and in Caucasians. Overall, reported prevalence rates were about 1-3%, up to 8.5 and 11.8% in Norway and the Arctic, respectively. In Italy, the prevalence of psoriasis ranged from 1.8 to 3.1% in the overall general Italian population, varying across regions [4].
The majority of cases were diagnosed in individuals younger than 30 years of age with more than 10 000 cases per year arising in children less than 10 years old [5]. The prevalence of psoriasis among children was up to 2% in Europe and 2.1% in Italy [6].
The cutaneous manifestations of psoriasis have a strong negative impact on quality of life and social relations. In addition to the skin lesions, several adverse effects and comorbid conditions can affect psoriasis patients, including arthritis (which can be severe and deforming), bowel inflammatory disease, cardiovascular diseases, metabolic syndrome, mental distress, depression, which all contribute to the disease burden [7-10].
Psoriasis also places a substantial economic burden on both patient and society [11, 12]. Psoriasis patients are more likely to require urgent care, have greater hospitalization rates and more frequent outpatient visits, and incur greater costs than other patients [13]. In addition, comorbidities result in higher overall pharmacy and medical costs [14]. The financial burden of this disease in the USA has been estimated to be as high as $11.25 billion annually [9].
The pathogenesis of psoriasis involves autoimmune, genetic and environmental factors. In addition, the vitamin D altered pathway may play a role in the pathogenesis of psoriasis since the skin acts as both a site of its biosynthesis and a target organ for its activity [15, 16].
Deficiency of the active form of vitamin D was hypothesized to be implicated in the onset of psoriatic plaques and, for this reason, drugs based on vitamin D and its analogues have been used as standard therapy for decades [17, 18].
Recently, therapies based on biotechnological drugs have been developed; their action is directly performed against specific parts of the immune system associated with psoriasis without altering the serum levels of vitamin D.
The biosynthesis of vitamin D requires that ultraviolet solar rays penetrate the skin and transform 7-dehydro-

cholesterol into pre-vitamin D, which is rapidly converted to vitamin D.
Vitamin D is transported to the liver by Vitamin D Binding Protein (DBP), where it undergoes a first hydroxylation of carbon 25 thus forming 25 hydroxyvitamin D (25(OH) D) which is the major circulating form of vitamin D and whose serum levels are commonly used for the evaluation of vitamin D status. [19]. The 25(OH)D can be found for about 85-90% bound with high affinity to DBP, for 10-15% weakly bound to albumin and less than 1% exists in free form, which is able to perform physiological functions; the sum of free and albumin-bound vitamin D represents the bioavailable vitamin D [20].
Several studies have shown that the serum levels of 25(OH)D were significantly lower among patients with psoriasis compared to healthy controls [21-23]. However this association was often unclear because it took into account the plasma level of total 25(OH) vitamin D which is affected by numerous individual, genetic, environmental and physiological factors which result in a wide variability in the general population.
The level of vitamin D could be more significant if the free form were detected, since it is independent of confounding factors and much better correlates with some pathological conditions. However, in most of the published studies the dosage of free 25(OH)D was carried out by indirect and low accurate methods. Moreover, there are no current studies on the measurement of this parameter in psoriasis patients.
A cross-sectional study was performed to assess the association between chronic plaque psoriasis and serum level of free 25(OH)D, detected by a direct analytical method.
The levels of total vitamin D, bioavailable vitamin D and other metabolic parameters whose homeostasis is regulated by vitamin D were also evaluated either in psoriasis patients and in healthy controls.

Methods

Study design

The study was performed in a three-months period (January-March 2018) in order to avoid the variability of vitamin D levels due to seasons.
The patients were recruited from the 128 subjects diagnosed with chronic plaque psoriasis who, during the study period, went to the Psoriasis Centre of the Dermatology Unit of the Vito Fazzi General Hospital in Lecce for a check-up. The healthy controls were healthcare workers of the hospital who voluntary accepted to participate in the study after an informed invitation. Information about age, sex, drugs or any phototherapy treatments of subjects were obtained from their medical records and were used to select the individuals eligible for the study.
All participating subjects had to be at least 18 years old and of Caucasian race. In addition, for patients the following criteria of inclusion were set: a clinical diagnosis of chronic plaque psoriasis (lasting at least 6 months) regardless of the actual score of the Psoriasis Area and Severity Index (PASI), the absence of systemic or topical anti-psoriasis treatments, including phototherapy and/or topical vitamin D derivatives, for at least 3 months before the study investigations. The only systemic treatment allowed was the therapy with biotechnological drugs. Patients with other types of psoriasis (guttate psoriasis, erythrodermic and pustular), those with concomitant inflammatory bowel disease (i.e. Crohn's disease and ulcerative colitis) and those receiving therapeutic interventions that could affect the status of vitamin D, including bisphosphonates, systemic corticosteroids, vitamin D and calcium supplements were also excluded. The subjects recruited as controls were free from current or previous pathologies, did not take vitamin D or calcium supplements and were not subjected to phototherapy.
Overall, after exclusion of not eligible subjects, seventy-two patients and forty-eight controls were included in the study.
The measurement of analytical parameters was performed on blood samples taken from patients who are subjected to the laid down checks for their pathology and from healthcare workers who routinely undergo health surveillance. Each subject, after being informed about the research purposes, gave his/her consent for processing personal data and the execution of the analytical investigations provided by the present study.
Data regarding anthropometric measurements (weight and height) from both patients and controls, taken according to World Health Organization (WHO) recommendations [24], were used to calculate the body mass index (BMI) (weight [kg]/height [m]squared).
The study was conducted in compliance with Helsinki Declaration and with the Italian Laws concerning the rights of the study participants.

PASI score

The most frequently adopted measure to define the severity of skin involvement is the Psoriasis Area and Severity Index (PASI), a numerical index that combines the extension of the damage with other clinical signs [25]. This index is based on the quantitative assessment of three typical signs of psoriatic lesions: erythema (E), infiltration (I), and desquamation (D), on a scale of 0-4, combined with the skin surface involved (A = affected area), which is divided in four separate body areas: head (h), trunk (t), and upper (u) and lower extremities (l). To calculate PASI, the formula is:

$$0,1\,(Eh + Ih + Dh)\,Ah + 0,2\,(Eu + Iu + Du)\,Au + 0,3\,(Et + It + Dt)\,At + 0,4\,(El + Il + Dl)\,Al$$

A numerical value is given to the extent of the lesions in each area: 1 = < 10%; 2 = 10-30%; 3 = 30-50%; 4 = 50-70%; 5 = 70-90%; and 6 = 90-100%. E, I and D are assessed according to a five-point scale (0 = no symptoms; 1 = slight; 2 = moderate; 3 = marked; and 4 = very marked) to obtain a final value between 0 and 72.

Measurement of analytical parameters

After sampling, blood was promptly centrifuged (3000 rpm at 4°C for 10 minutes) and serum was immediately stored at -80°C until the measurement of the analytical parameters. Blood samples from both patients and controls were dosed in a single analytical session carried out in the Clinical Pathology Unit of the Vito Fazzi General Hospital in Lecce and the following parameters were assessed: total 25(OH)D, free 25(OH)D, parathyroid hormone (PTH), albumin, calcium, phosphorus; the bioavailable 25(OH)D was calculated on the basis of total 25(OH)D and albumin levels and the albumin affinity constant for vitamin D [26]. The laboratory uses standard operating procedures, carries out the required internal quality controls and participates in external quality checks for all the services provided.

The method used to measure the free 25(OH)D is described in the following section.

For the total 25(OH)D measurement was used Abbott Architect 25-OH D reagent on i2000 Architect analyzer (Abbott Laboratories, Abbott Park, IL 60064 USA) with a chemiluminescent competitive delayed phase immunoassay (Chemiflex) standardized according to the NIST SRM 2972 (National Institute of Standard & Technology Standard Reference Material 2972).

PTH 1-84 measurement was performed with a two-step chemiluminescent immunoassay on DiaSorin LIAISON XL analyzer (DiaSorin, Stillwater, Mn, USA).

Albumin was measured with an immunoturbidimetric method (Roche Tina-quant Albumin) standardized using reference material ERM-DA470k/IFCC of the IRMM (Institute for Reference Materials and Measurements) on Roche/Hitachi Modular P analyzer (Roche Diagnostic GmbH, Mannheim, Deutschland). The same analyzer was also used to measure calcium and phosphorus levels with a spectrophotometric method, respectively Roche CA2 and Roche Phosphate inorganic ver.2.

Measurement of free 25(OH)D

The measurement of free 25(OH)D was performed with a direct quantitative immunoassay on a microplate developed by Future Diagnostics Solutions (Wijchen, The Netherlands), based on patented monoclonal antibodies from DIAsource ImmunoAssays (Louvain-la-Neuve, Belgium). Briefly, calibrators, controls and samples diluted 1:10 were transferred into the microplate wells coated with the anti-vitamin D antibody and incubated for 90 min at 37°C with shaking at 650 RPM. After washing, 100 µL of biotinylated 25(OH)D were added to each well and the plate was incubated for 30 min at 37°C with shaking at 650 RPM and washed again. Then, a 100 µL aliquot of the streptavidin-peroxidase reagent was added to the wells and further incubated for 20 min at 37°C with shaking at 650 RPM. In the next step, the plate was washed, added with 100 µL of tetramethylbenzidine and incubated for 15 min at room temperature, stationary and protected from light. Finally, 100 µL of HCl 1 M were added to stop the reaction and the absorbance was read on the spectrophotometer at a wavelength of 450 nm. According to the producers, this test has a sensitivity of 0.5-1.0 pg/mL.

Statistical analysis

All data were entered into a Microsoft Excel database and statistically processed using MedCalc Software version 14.8.1 (MedCalc Software bvba, Ostend, Belgium). The chi-square test was used to detect any difference in the distribution of female and male subjects among patients and controls; while the t-student test was used for age and BMI. For all analytical parameters (dependent variable) estimated marginal means (EMM), standard error (SE) and 95% confidence interval (CI), after adjustment for age, sex and BMI, were calculated within the two considered groups by analysis of covariance. In all cases, differences were considered significant when $p < 0.05$.

Results

The study population consisted of 72 patients affected by psoriatic plaque disease (41 males and 31 females) and 48 controls (22 males and 26 females) (Tab. I). On average patients were 50.5 ± 13.0 years old and control 50.0 ± 12.9 years old. The BMI average value was 27.8 ± 5.98 kg/m^2 for patients and 25.4 ± 5.45 kg/m^2 for controls ($p = 0.024$). PASI score calculated for cases was

Tab. I. Descriptive characteristics of patients and controls.

Variables		Unit of measure	Patients (n = 72)	Controls (n = 48)	*p-values*
Age		Average ± DS	50.5 ± 13.0	50.0 ± 12.9	***0.846****
Sex	Male	N (%)	41 (56.9%)	22 (46%)	***0.233*** **
	Female		31 (43%)	26 (54%)	
BMI		Kg/m^2 ± DS	27.8 ± 5.98	25.4 ± 5.45	***0.024****
PASI		Score ± DS	1.88 ± 3.34	0	-
Drugs	Ustekinumab	N (%)	32 (43.7%)	0	-
	Adalimumab		11 (16.6%)	0	-
	Etanercept		14 (18.7%)	0	-
	Secukinumab		9 (12.5%)	0	-
	Golimumab		6 (9%)	0	-

*Differences between groups evaluated by Student t-test; ** differences between groups evaluated by chi square test.

Tab. II. Estimated margin means (EMM) ± standard error (SE) and 95% confidence interval (CI) of serum analytical parameters after adjustment for age, sex and BMI among patients affected by psoriasis and controls.

Variables	Groups	EMM ± SE	95% CI	*p-values*
Total Vitamin D (ng/ml)	Patients Controls	23.051 ± 1.195 25.286 ± 1.473	20.684-25.418 22.369-28.203	***0.2477***
Free Vitamin D (pg/ml)	Patients Controls	5.526 ± 0.271 6.776 ± 0.271	4.989-6.063 6.115-7.437	***0.0049***
Albuminemia (g/dl)	Patients Controls	4.146 ± 0.041 4.197 ± 0.050	4.065-4.227 4.098-4.297	***0.4379***
Calcemia (mg/dl)	Patients Controls	9.665 ± 0.068 9.614 ± 0.083	9.531-9.799 9.449-9.780	***0.6409***
Phosphoremia (mg/dl)	Patients Controls	3.570 ± 0.076 3.728 ± 0.093	3.420-3.720 3.543-3.913	***0.1969***
PHT (pg/ml)	Patients Controls	22.539 ± 1.030 22.633 ± 1.269	20.500-24.579 20.120-25.147	***0.9549***
Bioavailable Vitamin D (ng/ml)	Patients Controls	2.232 ± 0.128 2.522 ± 0.158	1.979-2.486 2.210-2.834	***0.1613***

on average 1.88 ± 3.34. Patients involved in the study were treated only with biotech drugs, among which Ustekinumab was the most used (43.7%).

The measurement of analytical parameters showed that in patients the serum concentration of Total and Free 25(OH)D was on average 22.781 ± 11.410 ng/ml and 5.487 ± 2.238 pg/ml respectively while in controls 25.692 ± 7.482 ng/ml and 6.642 ± 2.379 pg/ml.

The estimated marginal mean (EMM), adjusted for age, sex and BMI, of Total 25(OH)D in patients was 23.051 ± 1.195 ng/ml while in controls was 25.286 ± 1.473 ng/ml (Tab. II). However, differences seemed to be not significant ($p > 0.05$). Overall, 58 (80,5%) patients and 36 (75,0%) controls ($p < 0.05$) had a level of total 25(OH)D lower than the reference threshold of 30 ng/ml (not in table). Also the bioavailable 25(OH)D was detected lower, with non-significant differences, among patients respect to controls. Instead, significant differences ($p < 0.05$) were observed for the free 25(OH)D, whose concentration appeared lower in patients (EMM 5.526 ± 0.271pg/ml; 95% CI 4.989-6.063) compared to controls (EMM 6.776 ± 0.271 pg/ml; 95% CI 6.115-7.437).

PTH, albuminemia, calcemia and phosphoremia showed similar levels both for patients and controls.

Discussion

This study evaluated serum concentration of free 25(OH) vitamin D by a direct immunoassay method in a group of patients with chronic plaque psoriasis compared to a control group. Bioavailable and total 25(OH) vitamin D as well as other metabolic parameters, whose homeostasis is regulated by vitamin D, were also evaluated.

The results showed that the level of free 25(OH)D was significantly lower in patients compared to controls, while total and bioavailable 25(OH)D levels appeared lower but not significantly. The other parameters were similar in the two study groups.

In addition, most of subjects (78.3%) exhibited a level of total vitamin D lower than the value of 30 ng/ml accepted as the reference threshold to define the vitamin D deficiency. In this case, the proportion of subjects with vitamin D deficiency was higher among patients with psoriasis than healthy controls.

Many studies compared serum level of vitamin D in psoriasis patients with corresponding controls and found that the former had significantly lower serum concentrations of 25(OH)D [27, 28]. In addition, the prevalence of subjects with high deficiency of vitamin D (serum 25(OH)D levels < 20 ng/mL) was found to be higher in psoriasis patients (57.8%) than in healthy controls (29.7%) ($p < 0.001$) [21]. Similar results were obtained in our study where the prevalence of 25(OH)D levels < 20 ng/mL was 51.4% among patients and 22.9% among controls.

Although it is not the most active metabolite, the concentrations of total 25(OH) vitamin D in the serum are currently routinely used in clinical practice to assess vitamin D status [20]. To date, no studies compared serum free 25(OH)D levels in psoriasis patients with healthy subjects.

Due to the wide variability of total 25(OH)D, several studies agree that free 25(OH)D is more significant in assessing vitamin D levels, especially when there are physiological or pathological alterations [20]. In fact, liver functions, estrogens, kidney functions and genetic background as well as environmental exposure, might influence total circulating vitamin D levels. Moreover, diseases or conditions that affect the synthesis of DBP or albumin have a huge impact on the amount of total 25(OH)D. DBP and albumin are synthesized in the liver, therefore people with an impairment of liver functions have alterations in their total vitamin D concentrations, while free 25(OH)D levels remain mostly constant [29]. Estrogens stimulate the synthesis of DBP and this explains why total vitamin D concentrations are higher during pregnancy as compared to non-pregnant women, while the concentrations of free 25(OH)D remain similar in both groups of women [30]. The vitamin D-DBP and vitamin D-albumin complexes are filtered through the kidney, hence acute and chronic kidney diseases characterized by a tubular damage, are associated with a loss of

vitamin D-DBP complexes in the urine [31, 32]. Finally, the gene encoding DBP protein is highly polymorphic in different human racial groups [26, 33].
To improve accuracy and precision of vitamin D measurements, free vitamin D could be measured, because it is independent of these confounding factors and thus are much better correlated with pathological conditions, particularly, with liver, kidney, tumor, and allergic diseases as well as in pregnancy [20]. Findings of this study showed that free 25(OH)D is also related to chronic plaque psoriasis.
In addition, the thresholds set to identify the "normal" level of circulating total vitamin D as well as its deficiency [34] are often controversial, therefore, many authors agree that a different set of normal values for total vitamin D should be developed considering all confounding factors. This is also confirmed by the large amount of people who has lower levels of total 25(OH) D in respect of the threshold of 30 ng/ml established to indicate the vitamin D deficiency [35, 36].
From a methodological point of view, free 25(OH)D concentrations can be either measured directly or calculated based on total 25(OH)D, DBP, and albumin serum levels [37, 38].
The indirect method is the most used. However, it presents several problems. The weakness of the calculation method is its relative inaccuracy due to many factors included into the formula. For instance, the binding constants for DBP are known to vary depending on several physiologic and pathologic conditions (for example, in pregnancy or in hyperlipemic conditions, when the binding capability of DBP to vitamin D metabolites can be lowered, since DBP may bind fatty acids as well) [38-42].
Free vitamin D levels obtained by calculation were reported to be overestimated compared to directly measured vitamin D concentrations [38]. A direct free 25(OH)D measurement can be performed either by centrifugal ultrafiltration or by a recently established immunoassay method (ELISA). However, centrifugal ultrafiltration did not become commonly used due to its high costs and technical difficulties in application [39].
ELISA kit for a direct measurement of free 25(OH)D in serum, such that used in our study, is instead characterized by ease of use, cost-effectiveness and sensitivity [43].
However, it is important to underline that the direct measurement of free 25(OH)D by immunoassay method was sometimes reported to have a lower affinity to the vitamin D2 metabolite compared to vitamin D3 (from 60% [42] to 77% [43]), which might result in underestimation of real values of free 25(OH)D.
This study has various strengths. To our knowledge, this is the first study concerning the association of free 25(OH)D with chronic plaque psoriasis. In addition, the methodology used for the detection of free 25(OH)D is innovative since it was carried out by a recently established commercially ELISA kit.
This study has some limits too. First, the control group could be few representative of the general population, since it consisted of healthcare workers recruited in the same hospital where the patients were treated. Second, the model used to assess the association between free 25(OH)D and psoriasis took into account only few factors, while many other variables could influence that relation (i.e. nutrition, work, physical activity, sun exposure, etc.).
Therefore, a lot of work still remains to be done in order to overcome those limits and clearly establish the relationship between free 25(OH)D and disease. It would be interesting to select a cohort of patients and follow the temporal trend of free 25(OH)D levels in respect of the disease status, therapy, seasonality, lifestyles and individual characteristics.

Conclusions

Patients affected by chronic plaque psoriasis exhibited a lower serum level of free 25(OH) vitamin D than healthy controls. In addition, this parameter seemed to be more sensitive than total and bioavailable vitamin D to identity abnormalities in vitamin D pathways in chronic plaque psoriasis patients.
The direct detection of free 25(OH)D by immunoassay method could represent a useful tool to assess vitamin D status and identify a risk condition associated with the onset of chronic plaque psoriasis.

Acknowledgements

Funding sources: this research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The authors declare no conflict of interest.

Authors' contributions

All authors made substantial contributions to the conception and design of the study and were involved in drafting and critically revising the manuscript in terms of intellectual content. In particular, FB conceived the study and performed the statistical analysis. TG, GL and MC interpreted the results. FB, TG and ADD were involved in the study design and methodology. AP, GI, IG and DL were involved in collection and management of data. ADD, GL and MC were involved in planning and supervising the work.

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Received on January 31, 2020. Accepted on June 15, 2020.

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How to cite this article: Grassi T, Panico A, Bagordo F, Imbriani G, Gambino I, Lobreglio D, Lobreglio G, Congedo M, De Donno A. Direct detection of free vitamin D as a tool to assess risk conditions associated with chronic plaque psoriasis. J Prev Med Hyg 2020;61:E489-E495. https://doi.org/10.15167/2421-4248/jpmh2020.61.3.1482