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Correlation between Vitamin D, Some Circulating Micro RNA with CRP and Faecal Calprotectin in Patients with Crohn's Disease

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ABSTRACT

Introduction: The mal-absorption and the inflammation participate in the pathogenesis of Crohn's disease (CD) and influence the vitamin D levels. Serum microRNAs (miRNAs) are secreted during the inflammation in the gastrointestinal tract and therefore could affect the regulatory functions of vitamin D and its signal pathways via negative feedback.

Objective: This study aims to assess the relationship between the Vitamin D serum levels and the expression of several circulating miRNAs in patients with CD and to correlate their levels with the activity of the disease.

Methods: 15 miRNAs expression was assessed using reverse transcriptase quantitive real-time PCR in 27 consecutive CD patients and then correlated with the serum level of 25(OH)D, C-reactive protein (CRP), faecal calprotectin (FCP) and clinical Crohn's disease assessment index (CDAI).

Results: Activity of CD is a risk factor for a decrease in the 25(OH)D serum levels (OR=7.5 (1, 09-51, 52; p<0.05). There is a strong inverse correlation between the levels of FCP and 25(OH)D (r=-0.641; p<0.001). The results indicate that 25(OH)D deficiency induces the highest risk for a change in the miRNA-96_1 serum expression.

Conclusion: The 25(OH)D serum levels and serum expression of miRNA-96_1, miRNA-142-5p_1, miRNA-191_1 and miRNA-199a_1 open an opportunity for optimization of the initial assessment of CD and potential as a prognostic tool.

Key Words: Crohn's disease, Micro-RNA, Vitamin D, CRP, Fecal calprotectin

INTRODUCTION

Crohn's disease (CD) is a chronic recurrent disease, involving different segments of the gastrointestinal tract.[1]Its pathogenesis is characterized by multilayer interaction between genetic predisposition, environmental factors, gut microbiome and imbalanced immune homeostasis, leading to intestinal inflammation.[2,3] The precise molecular pathophysiology, leading to the development of CD, is still obsolete. The activity of the disease is currently measured with different clinical indexes and the most widely used is the Crohns's Disease Activity Index (CDAI).[4]

The serum C-reactive protein (CRP) and the faecal calprotectin (FCP) are widely used serum biomarkers in CD. Unfortunately, their specificity and sensitivity are not high. This indicates the need for other biomarkers, that on one side would correlate with the molecular mechanisms during the early development of the disease and on the other – with the disease progression, the development of complications and would be valuable in the prediction of treatment response.

Vitamin D (25-hydroxyvitamin D; [25 (OH) D]) is an immune modulator of the innate and the acquired immune response. Levels of 25(OH)D in patients with CD correlate the activity of their disease[5]: low levels (<30 mg/ml) are a risk factor for loss or no response to anti-TNF treatment (HR = 3, 49, 95% CI: 1, 34–9,09).[6] 25(OH)D deficiency in patients with inflammatory bowel disease (IBD) is also reported to increase the risk of disease relapse, frequent hospitalizations and disease-related surgeries.[7] The activity of CD correlates with the 25(OH)D levels, but until the present, it is not clear whether a decrease in 25(OH)D levels is a primary triggering the disease relapse event or a secondary consequence of the activated disease. These define the levels of 25(OH)D as a new non-invasive biomarker, that is related to the pathogenesis of the disease. It also reflects the changes in the im-

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mune system and the effect of the treatment of the disease. The activity of the disease could be bridged to the pathogenetic mechanisms that are specific to the IBD as it influences the micro-RNA (miRNA) expression of all immune cells.[8] Patients with CD frequently have 25(OH)D deficiency and the levels of 25(OH)D are significantly lower as compared to healthy controls.[9]

miRNAs are small non-coding RNA molecules with a length of 18–23 nucleotides. They participate in many cell processes as cell differentiation, growth, apoptosis and autophagy as well as in proliferation. miRNAs function as regulators of the protein synthesis via induction of the degradation of the mRNA or silencing the translation.[10] The circulating miRNAs can be found in a stable form in many-body liquids, incl. in the serum.[11] Recent research reports that the miRNA expression profiles in blood of patients with CD may be different.[12,13]

Serum miRNAs are secreted in the process of inflammation of the gastrointestinal tract and they mediate the 25(OH) D signalling capacity via feedback control.[8,12,14] Paraskevi et al. identified that the expression of 11 serum miRNAs is increased in patients with CD (MiR-16, miR-23a, miR-29a, miR-106a, miR-107, miR-126, miR-191, miR-199a-5p, miR-200c, miR-362-3p, miR-532-3p) 12. Other publications assessed the serum expression of 11 miRNAs in children with CD (MiR-16, miR-484, miR-30e, miR-106a, miR-195, miR-20a, miR-21, miR-140, let-7b, miR-192, miR-93), and report high specificity and sensitivity.[14]

In the present study we aimed to assess the relationship between the 25(OH)D serum levels and the expression of several circulating miRNAs in patients with CD and to try to correlate their levels with the clinical or biochemical activity of the disease.

MATERIALS AND METHODS

The study was initiated after approval and permission №82 / 28.03.2019 of the Ethics Commission for scientific research at the Medical University – Varna, Bulgaria. Before study entry, all patients signed an Informed consent form. Vitamin D was measured in 27 consecutive patients with CD, who attended the Gastroenterology Clinic during a period of one year (from April 2019 to April 2020). 25(OH)D serum concentrations were measured by a commercial paramagnetic particle chemiluminescent immunoassay for the quantitative determination of the total 25-hydroxyvitamin D [25(OH) vitamin D] levels use on Access 2 Immunoassay Systems. 25(OH)D deficiency is defined as a serum level of 25OHD lower than 50 nmol/L. Serum level above 50 nmol/L but lower than 75 nmol/L are classified as 25(OH)D insufficiency.

All patients were classified according to the Montreal classification. The clinical course, treatment regimens and the occurrence of extraintestinal manifestations (EIMs) were recorded.

Disease activity was evaluated by clinical symptoms, biochemical inflammatory parameters (CRP, FCP) and validated clinical indices (CDAI). In the evaluation of FCP, the criteria used were normal, <50 mg/g and for CRP were considered as reagents for values >5 mg/l.

Levels of miRNAs were assessed in blood serum. 5 ml of blood was obtained via peripheral venous puncture with closed system BD Vacutainer™ SST™ II Advance (Becton Dickinson, USA). After withdrawal, the blood sample was held for 30 minutes at room temperature for clothing. Subsequently, it was centrifuged at 500×g for 15 minutes at room temperature and the serum was separated and divided into aliquots of 500 µl that were immediately stored at − 80°C until the moment of the analysis.

miRNAs were isolated from 200 µl serum using a pre-existing commercial miRNeasy Serum/Plasma Kit (50), catalogue №217184 (QIAGEN, Germany) as per the protocol of the manufacturer. 3,5 µl (1,6×108 копия на µl) control miRNA C. Elegans miR-39: miRNeasy Serum/Plasma Spike-In Control, catalogue №219610 (QIAGEN, Germany), was added to each sample for normalization control; the samples were afterwards eluted in 14 µl RNA-ase free water.

Each of the samples was subsequently submitted to reverse transcription via ready-to-use commercial kit miScript II RT Kit (50), catalogue №218161 (QIAGEN, Germany) as per manufacturer's protocol from 2,5 µl eluted miRNA in a final volume of 10 µl with HiFlex buffer and it was incubated at 37°C for 60 minutes and the enzyme was inactivated at 95 °C for 5 minutes.

Each of the samples was then submitted to quantitative real time polymerase chain reaction (rt-PCR) via a ready-to-use commercial kit miScript SYBR Green PCR Kit (200), catalogue № 218073 (QIAGEN, Germany) and prepared primers miScript Primer Assay (100), catalogue № 218300 (QIAGEN, Germany) as per manufacturer's protocol: 1 µl complementary DNA (cDNA) in 10 µl reactions in 3-times repetitions for 15 target miRNA in 384 well plates. The used miScript Primer Assay primers(100), catalogue № 218300 (QIAGEN, Germany) are as follows (the reference number is in the brackets): Hs_miR-28_1 (MS00003255), Hs_miR-29c_1 (MS00003269), Hs_miR-96_1 (MS00003360), Hs_miR-191_1 (MS00003682), Hs_miR-451_1 (MS00004242), Hs_miR-142-5p_1 (MS00006671), Hs_miR-199a_1 (MS00006741), Hs_miR-363_1 (MS00009576), Ce_miR-39_1 (MS00019789), Hs_miR-144_4 (MS00020328), Hs_miR-142-3p_2 (MS00031451), Hs_miR-155_2 (MS00031486), Hs_miR-16_2 (MS00031493), Hs_RNU6-

2_11 (MS00033740), Hs_miR-1228-3p_1 (MS00042385). The used temperature parameters are as follows: maintenance for 15 minutes at 95 °C for enzyme activation; 40 cycles of 15 seconds at 94 °C; 30 seconds at 70 °C with fluorescent reading; analysis of the melting curve in order to prove the specificity of the amplification: primary denaturation for 15 seconds at 95 °C and cooling to 55°C for 60 seconds with an increase to 95 °C with velocity of +0,05 °C per second and fluorescent reading. The analysis was done by QuantStudio Dx instrument of Applied Biosystems (USA) company; a threshold cycle (Ct) was assessed for each sample.

Receiver Operating Characteristic Curve (ROC) was made to detect the diagnostic performance of the test, sensitivity, specificity, positive and negative predictive values. The significance of the obtained results was judged at the 5% level.

Analyses

Serum levels of 25(OH)D were measured and correlated with serum CRP, FCP, CDAI and the expression of different miRNAs. Additional co-variation models, including stratification factors as age, the sex were assessed. Correlation of levels of miRNAs with some clinical factors as 25(OH)D deficiency, treatment with corticosteroids or anti-TNF antibodies was also done. The Spearman correlation coefficient was used for analyzing the correlation between the serum concentration of 25 (OH) D and the serum expression of some microRNAs.

The results were calculated with SPSS, v. 20.0 for Windows. We used variation, correlation, regression analyses as well risk assessment and comparative analyses (χ^2, t-test), $p<0.05$ was used as a level of significance.

RESULTS

Table 1 shows a summary of the characteristics of the patients with Crohn's disease. The mean 25(OH)D expression levels in patients with CD is 42.97 nmol/L ±16.75 nmol/L, with variations from 2,76 nmol/L to 73,17 nmol/L. There is no correlation between the 25(OH)D serum levels and the sex or age of the patients. The 25(OH)D serum expression levels differ significantly (p=0.043) between patients with activity (39.45 nmol/L ± 16.65 nmol/L) vs patients in remission (53.02 nmol/L ± 16.76 nmol/L). The activity of the CD is a risk factor for a decrease in the 25(OH)D serum levels (OR=7.5 (1,09-51,52; p<0.05). The relative rate of patients with CD and 25(OH)D serum levels below 50 nmol/L is 63.0 %. There is an inverse relationship between 25(OH)D and the activity of the disease, measured by CDAI (r=-0.421; p=0.029) (Figure 1), showing that 25(OH)D serum levels decrease with the increase of CDAI.

The most widely clinically used parameters as CRP and faecal calprotectin (FCP) were correlated with the levels of 25(OH)D. There is an inverse correlation, showing that the 25(OH)D serum levels decrease in patients with increased levels of CRP and FCP. There is a weak inverse correlation between CRP and 25(OH)D (r=-0.202; p<0.05) (Figure 2).

The FCP is a widely used biomarker, used to monitor patients with CD. We found a strong inverse correlation between the levels of FCP and the 25(OH)D serum levels (r=-0.641; p<0.001) (Figure 3).

The expression of some microRNAs and their correlation with the levels of 25(OH)D is presented in Table 2. The expression of the different miRNAs is different as compared to the serum levels of 25(OH)D. The expression of two miRNAs - miRNA-28_1 and miRNA-1228-3p_1, is increased in patients with normal 25(OH)D serum levels. The expression of all other miRNAs is increased in 25(OH)D deficiency.

Table 3 shows the risk analysis of the expression of miRNAs and the 25(OH)D levels. The results indicate that 25(OH)D deficiency induces the highest risk for a change in the miRNA-96_1 serum expression.

There is no correlation between 25(OH)D expression and the tested miRNAs in patients with CD and intestinal complications. There is an increased expression of all tested miRNAs in patients with either 25(OH)D deficiency, treatment with corticosteroids or anti-TNFantibodies.

DISCUSSION

Our results confirm some previously reported data that there is a high prevalence of 25(OH)D deficiency among patients with CD. In Ireland, 63% of the patients with CD have levels of 25(OH)D <50 nmol/L.[15] The 25(OH)D deficiency rates in Canada are 22% (<40 nmol/L).[16] A Japanese trial reported that 27, 3% of the patients with CD are 25(OH)D deficient (<25 nmol/L).[17]

There is already data that significantly lower 25(OH)D levels are frequently seen among patients with IBD; it is also reported that lower levels correlate with the activity of the disease. Our results and the correlation analysis of 25(OH)D serum levels and activity of CD confirm previously reported data by Cheong-Lee C et. al. and Chatu et al. in IBD patients, the levels of CRP inversely correlate with the levels of 25(OH)D.[18,19] Chat et al. do not find differences between the mean 25(OH)D levels in patients with CD or ulcerative colitis. Despite this, the mean 25(OH)D levels in Asian and Afro-American people are significantly lower in comparison with Caucasians.[19]

It was proved that the levels of 25(OH)D inversely correlate with the disease activity, assessed as per CDAI.[20] Joseph et al. show that patients with CD have significantly lower 25(OH)D levels as compared to healthy controls.[21] Refer-

ringtodiseaseactivity, some authors report that patients with mild CD have 25(OH)D levels, comparable to healthy controls. In moderately severe disease, the 25(OH)D levels are significantly lower.[21] Additionally, Suibhneet al. report that 25(OH)D deficiency is a frequent event in patients with CD even in clinical remission.[15] The localization of the disease or its activity, together with the presence or not of the previous resection might not be the only factors, influencing the bioavailability of 25(OH)D.[22]

The number of publications, assessing the potential relation between 25(OH)D levels and bowel inflammation, using FC Pisscarce.[23] In Australia, Garg et al. study 40 patients with Crohn's disease and conclude that there is a strong inverse correlation between 25(OH)D and FCP levels.[23] These results are also confirmed in our study.

The levels of the differently expressed miRNAs serum profiles as IBD diagnostic and monitoring biomarkers is largely studied. Data about different miRNAs expression and serum 25(OH)D levels in immune-mediated diseases is scarce. A. Hsieh published in 2016 that there is a correlation between miRNA-142-3p and 25(OH)D in mice models.[24] The author concludes that the 25(OH)D deficiency changes the miRNA-142-3p –miRNA, targeted to the ATG16L1 autophagy gene, contributes to the increased sensitivity in IBD.[24] While assessing the 25(OH)D and some miRNAs levels in patients with rheumatoid arthritis, another author reports the inverse correlation between the two biomarkers.[25] Despite the design, methods and population heterogeneity (in mice or men) in the cited publications, an inverse correlation between 25(OH)D and the tested miRNAs levels are consistently noted. Our results also confirm that in most miRNAs (miRNA-96_1, miRNA-142-5p_1, miRNA-191_1 and miRNA-199a_1) the correlation is inverse and only in two of the tested miRNAs (miRNA-28_1 and miRNA-1228-3p_1), it is proportional.

Our study reports the first results of correlation between 25(OH)D and some miRNAs levels in patients with CD in Bulgaria. Worldwide, the number of similar scientific data in patients with CD is also very scarce, limiting the possibility to compare our results or test their validity with others. Larger prospective trials in patients with CD are needed to prove definitively the validity of the reported results.

CONCLUSION

The 25(OH)D serum levels inversely correlate with the levels of the serum biomarkers CRP and FCP, as well as with the CD activity index CDAI, used to measure the activity of CD. On the other hand, the low 25(OH)D serum levels correlate with increased serum expression of miRNA-96_1, miRNA-142-5p_1, miRNA-191_1 and miRNA-199a_1. These data open an opportunity for optimization of the initial assessment of CD and potential for prediction of the activity of the disease if these biomarkers are combined as a prognostic tool.

Approval: Approval and permission №82 / 28.03.2019 of the Ethics Commission for scientific research at the Medical University – Varna, Bulgaria.

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Table 1: Characteristics of the patients with Crohn's disease according the Montreal classification

Baseline characteristics		Crohn's disease(n=27)
Age, years (mean± SD, range)	Current	41.51±13.55 (18-75)
	Onset of complaints	33.97±13.45 (11-75)
	Age at diagnosis	36.11±13.02 (14-75)
Sex	Male	14/51.8 %
	Female	13/48.2 %
Location	L1	17/62.9 %
	L2	3/11.1 %
	L3	7/26.0 %
Behavior	B1	11/40.7 %
	B2	9/33.3 %
	B3	6/22.2 %
	B2-B3	1/3.8 %
Duration of IBD, months	(mean± SD, range)	66.91±59.67 (3-204)
Treatment	5ASA	2/7.4 %
	Corticosteroids	7/25.9 %
	Immune modulators	16/59.3 %
	Biological treatment	2/7.4 %

Table 2: Expression of miRNA inpatients with Crohn's disease and correlation with the 25(OH)D serum levels

miRNA	25(OH)D < 50nmol/L	25(OH)D ≥ 50 nmol/L	P value	Expression of miRNA	Expression of 25(OH)D
miRNA -16_2	3.59±2.17	3.31±2.24	0.739	-	-
miRNA-28_1	2.52±1.88	4.54±8.14	0.05	↑	↑
miRNA -29c_1	2.08±1.04	1.50±0.68	0.139	-	-
miRNA-96_1	4.04±4.38	0.82±0.65	0.024	↑	↓
miRNA -142-5p_1	3.22±2.69	1.84±0.85	0.012	↑	↓
miRNA -142-3p_2	3.33±2.22	2.71±1.69	0.188	-	-
miRNA -144_4	2.54±1.32	3.64±2.06	0.345	-	-

Table 2: ***(Continued)***

miRNA	25(OH)D < 50nmol/L	25(OH)D ≥ 50 nmol/L	P value	Expression of miRNA	Expression of 25(OH)D
miRNA -155_2	3.14±2.69	2.80±3.73	0.687	-	-
miRNA -191_1	2.75±2.15	1.97±0.88	0.067	↑	↓
miRNA -199a_1	3.40±5.41	1.77±0.99	0.008	↑	↓
miRNA -363_1	2.48±1.72	2.88±1.37	0.958	-	-
miRNA -451_1	2.25±1.06	2.96±2.36	0.087	-	-
miRNA -1228-3p_1	1.68±1.74	2.66±3.42	0.05	↑	↑

Table 3: Risk analysis of miRNAs expression in patients with Crohn's disease according to the 25(OH)D serum levels

miRNAs	OR	95%CI	P value
miRNA-28_1	2.77	0.264-29.047	0.037
miRNA-96_1	2.92	0.952-8.934	0.049
miRNA-142-5p_1	1.22	0.431-3.476	0.052
miRNA-191_1	1.67	0.257-10.792	0.047
miRNA-199a_1	2.77	0.264-29.047	0.037
miRNA-1228-3p_1	1.39	0.505-3.844	0.053

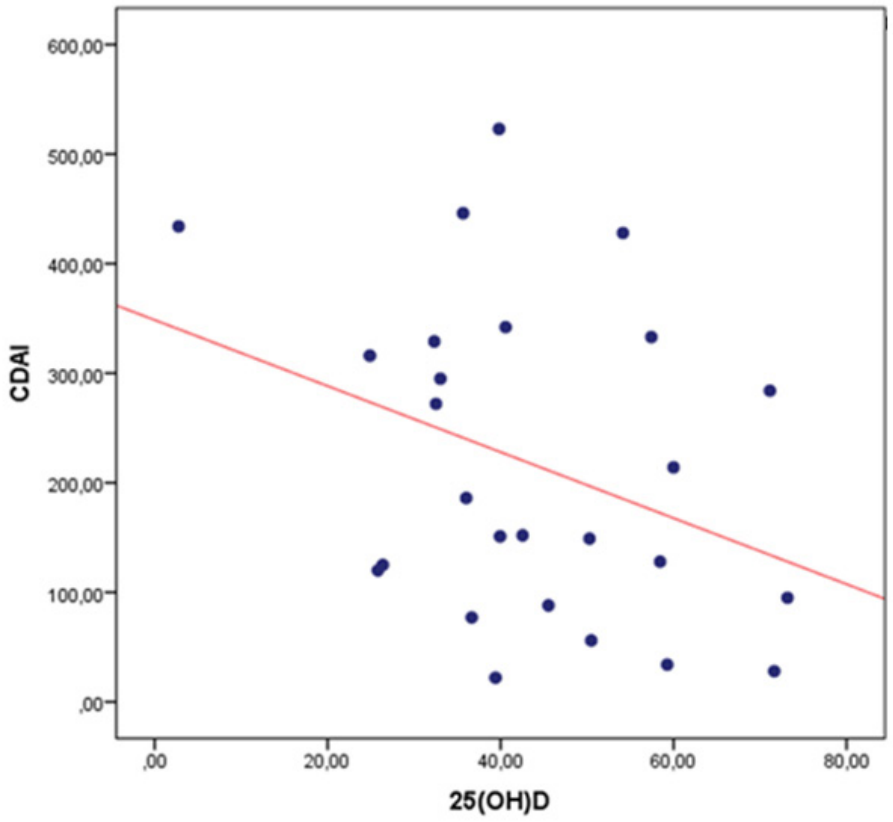

Figure 1: Correlation between CDAI and 25(OH)D levels.

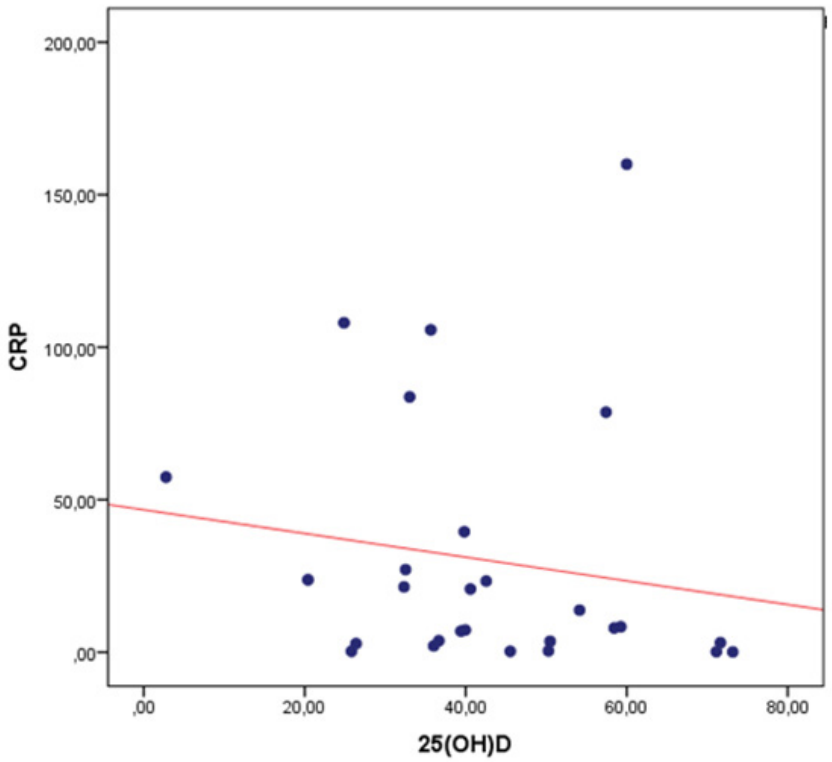

Figure 2: Correlation betweenCRPand 25(OH)D.

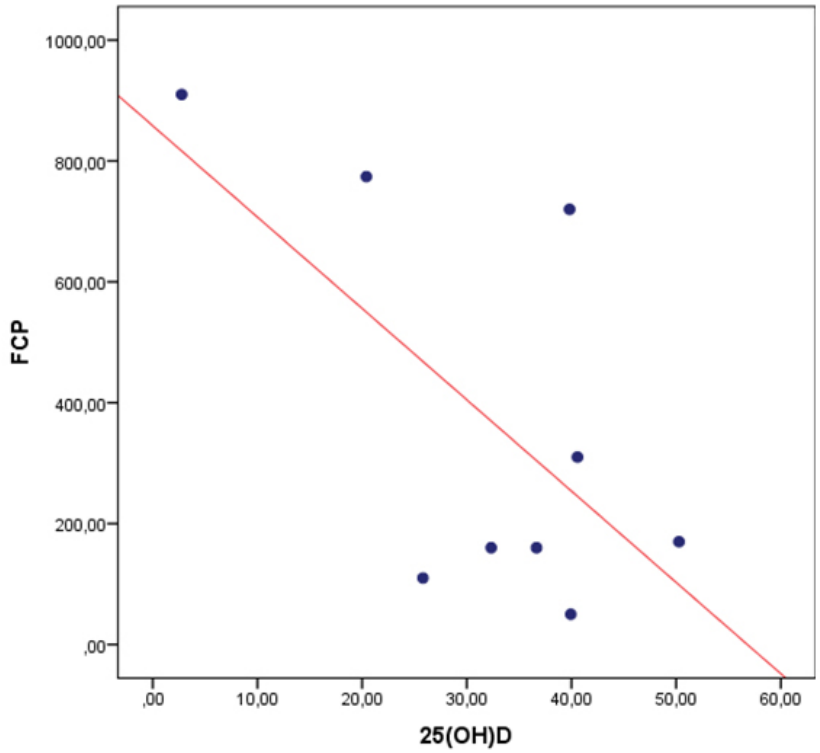

Figure 3: Correlation betweenFCPand 25(OH)D.