Maria Paula de Paiva et al. JBC Genetics. 2021;4(2):76-84
https://doi.org/10.24911/JBCGenetics/183-1613502878

ORIGINAL ARTICLE

Effect of vitamin D3 supplementation upon the metabolic and DNA methylation profile of cystic fibrosis patients

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ABSTRACT

Background: Cystic fibrosis (CF) is a genetic disease that affects the transmembrane conductance regulator gene responsible for modulating chloride ion transportation in the cell membrane. Hypovitaminosis D is frequently observed among fibrocystic disease patients. Therefore, this study was aimed to evaluate the effect of vitamin D3 supplementation in patients with CF concerning their metabolic and deoxyribonucleic acid (DNA) methylation profiles.

Methods: A clinical trial involving 12 CF patients was carried out in João Pessoa. After assessment of hypovitaminosis D prevalence in the studied population, four patients with vitamin D3 insufficiency/deficiency were administered cholecalciferol megadose supplementation in addition to biochemical examinations and analysis of inflammatory and epigenetic indicators. The DNA methylation profile of the studied genes' promoter regions was determined through a qualitative methylation restriction enzyme technique. Data were analyzed using the Statistical Package for the Social Sciences 25.0 software for *T*-tests, Mann-Whitney, and Wilcoxon test calculations.

Results: Hypovitaminosis D was observed in 58%, 33% of the studied individuals. Patients with hypovitaminosis D reported blood sugar, glutamic-pyruvic transaminase (ALT), and uric acid levels significantly higher (p = 0.02; p = 0.05; p = 0.02, respectively) compared to individuals with sufficient 25-hydroxyvitamin D (25(OH)D), as well as elevated inflammatory values. Supplementation did not influence epigenetic nor metabolic parameters significantly, although the mean 25(OH)D serum concentration value increased from 18.3 ng/dl to 34.1 ng/dl (p = 0.06).

Conclusion: Cholecalciferol megadose elevated 25(OH)D serum levels, although it did not alter inflammatory, glycemic, or epigenetic parameters. This encourages future studies on the matter since significant differences were found in blood sugar, uric acid, and ALT serum levels for the vitamin D3 insufficiency/deficiency group despite this study's small sample size.

Keywords: Epigenetics, cystic fibrosis, supplementation, vitamin D, DNA methylation.

Introduction

Cystic fibrosis (CF-OMIM 219700) is an autosomal recessive disease caused by a mutation in the transmembrane conductance regulator gene (*CFTR*), located in chromosome 7. This mutation results in deficient transportation of chloride ions through the cellular membrane, leading to thick mucus secretion

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Received: 16 February 2021 | **Accepted:** 26 May 2021

in respiratory, digestive, and reproductive systems (1). Mucous accumulation in the pancreas might result in pancreatic insufficiency (PI), with absorption impairments and deficiency of some vitamin types, since fat-soluble ones (A, D, E, and K) are co-absorbed with fat (2). Vitamin D deficiency stands out for it reaches a 61% (3) prevalence in children and adolescents with CF due to hepatic damage (4) among other factors, such as low nutritional intake, decrease in solar exposure, flawed vitamin D hydroxylation, and non-adherence to the prescribed vitamin D regimens. These might contribute to hypovitaminosis cases (5). Likewise, vitamin D metabolism is coupled with its receptor, vitamin D receptor (*VDR)*, a ligand-dependent transcription factor that mediates *1*,25-dihydroxycholecalciferol activation and is located predominately in target cell nuclei. *VDR* target cells are spread along with many tissues and organs (6). This receptor is acknowledged in the literature as an essential factor in gene activating/inhibiting complex formation, with implications for various biological functions (7-8). Pulmonary function and inflammatory profiling (9-10) are the main study themes in CF. Still, there are no research studies on the epigenetic aspects [deoxyribonucleic acid (DNA) methylation] and vitamin D status and intervention effects for various age groups. DNA methylation is an epigenetic process involved in gene expression control and is a biomarker for inflammatory and tumor diseases (11). The literature reveals that nutritional interventions may modulate the DNA methylation profile in different contexts (12-13). Therefore, the present study aims to evaluate the effect of Vitamin D3 supplementation upon the metabolic and methylation profiles *VDR* gene of patients with the fibrocystic disease.

Subjects and Methods

The study protocol was approved by the Hospital Universitário Lauro Wanderley (HULW) Research Ethics Committee under protocol number 87354018.1.0000.5183. A clinical trial including CF patients from both sexes without pancreatic or hepatic insufficiency; children older than 5 years old and adolescents who gave informed consent, both under parental consent; and adults aged more than 18 years old, were recruited for study at the HULW, João Pessoa-Paraíba, Brazil. The study had two stages: 1-Characterization; and 2-Intervention. A convenience sample of 15 subjects with CF was recruited with patients regularly seen at HULW and further downsized to 12 subjects after inclusion and exclusion criteria were considered. Individuals ranged from 8 to 12 years old subjects and were submitted to stage 1. In stage 2 (Intervention), four male patients between 15 and 18 years old completed the experimental protocol. Participants in the experimental group were on similar medications, with no history of infection or diagnosis of CF complications (pancreatitis, diabetes, among others).

Questionnaires were used to obtain demographic information and the time of sun exposure, Vitamin D and calcium intake, and anthropometric data (body weight/ height/age) according to the World Health Organization criteria (14). Following the biochemical examination of 25-hydroxyvitamin D (25(OH)D) serum levels, all subjects were classified into 3 groups according to the reference values established by the Endocrine Society (15): vitamin D sufficiency (>30 ng/dl) and insufficiency (20-30 ng/dl) deficiency (<20 ng/dl) participants with hypovitaminosis D (insufficiency/ deficiency) were considered for the intervention stage (Figure 1).

Patients classified with vitamin D insufficiency/ deficiency were supplemented with daily vitamin D3 (cholecalciferol) megadoses for 8 weeks, according to the dosage per age recommended by the CF Foundation (16): 4,000 UI/day for children up to 10 years old; and 10,000 UI/day for children over 10 years old. The supplement was formulated in a specialized pharmaceutical facility, and a certificate of quality control analysis was provided. During the intervention period, 24-hours dietary recalls and telephone monitoring were carried out. Four weeks after supplementation completion, patients were submitted to blood collections once more, and new analysis concerning biochemical, hematological, inflammatory, and epigenetic parameters was carried out. Subjects who presented severe pulmonary infections, renal or hepatic insufficiency, or received indication of/ were submitted to lung transplants during the sample collection period, resulting in data loss, were excluded from the sample set (Figure 1).

Biochemical analysis: 15 ml of blood were collected from patients after 8-12 hours of fasting, both before and after supplementation. Blood was fractioned in anticoagulant-free tubes (5 ml) to obtain the serum for complete blood count tests, as well as analysis of 25(OH) D (evaluation of serum vitamin D dosage); parathyroid hormone (PTH), calcium (bone metabolism markers); glutamic-oxaloacetic transaminase (AST), glutamic-pyruvic transaminase (ALT) (hepatic markers); glutamic-oxaloacetic transaminase (AGPA), C-reactive protein (CRP) (inflammatory markers); creatinine, uric acid, and urea levels (renal markers). 4 ml were transferred to tubes containing EDTA and stored at -20ºC for genetic analysis. Calcium and 25(OH)D serum concentrations and blood counts were measured through chemiluminescent immunoassays (UniCel DxI 800-Beckman Coulter). All other previously mentioned analyses were performed in the biochemistry laboratory of HULW using specific kits.

Epigenetic analysis

DNA methylation

VDR Gene. For DNA extraction, a previously published protocol was used (17). Methylation analysis of the *VDR* gene promoter was performed through the COBRA method (combined bisulfite restriction analysis) (18). Each sample's DNA amount was initially quantified in a spectrophotometer (NanoDrop™ 2000/2000 c spectrophotometers), followed by bisulfite treatment with the EZ DNA Methylation-Gold™ Kit (Zymo Research). Afterward, DNA amplification was carried out through polymerase chain reaction (PCR) with specific primers (F: 5' ATTGTTGGATGATTTTGTGAGT 3' ; and R: 5' TCTCAACTTCCCTAATCCCTAA 3;), resulting in a 325 bp fragment containing seven CpG sites. Primer sequences were obtained using the Methyl Primer

Express® v1.0 software (Applied Biosystems) from promoter region sequences searched on Genome Browser (chr12:47,841,537-47,905,022) (Figure 2).

DNA amplification by PCR was carried in 20 µl reactions containing 10 µl of Go Taq Hot Start Green Master Mix (1X) (Promega Corporations); 1.4 µl of the

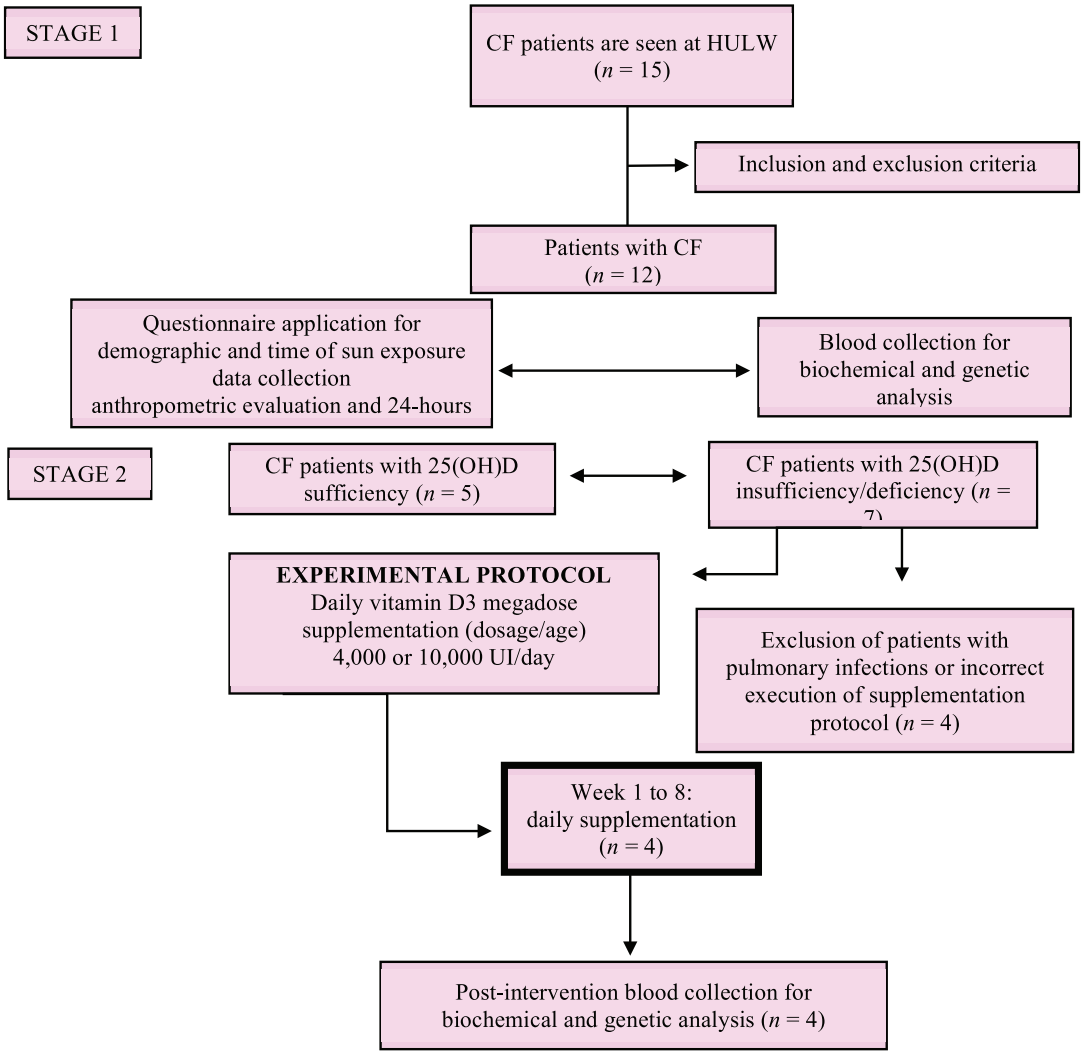

Figure 1. *Study flowchart.*

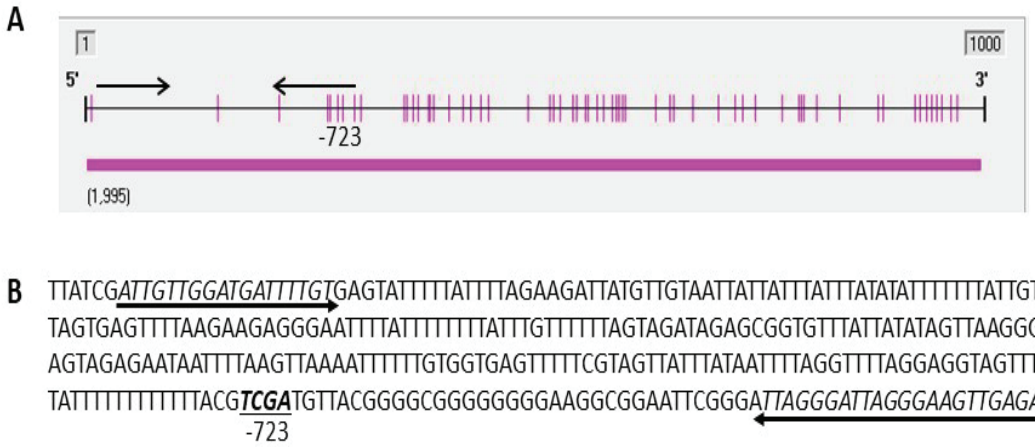

Figure 1. *VDR gene promoter sequence (chr12:47,841,537-47,905,022-Genome Browser) (A) Representation of CpG island inside the gene's promoter region, from -1,000 to -1 bp. (B) CpG island partial sequence (bisulfite-treated sequence with methylated CpG sites). Primers flank a 325 bp region and are indicated by arrows; CpG site-723 is underlined. CpG island-horizontal bar; CpG sites-vertical lines. CpG island was confirmed on Methyl Primer Express® software (Applied Biosystems) according to the following parameters: minimum length of 200 bp; a total of C + G > 50% and CpG observed and expected ratio >0.6.*

Table 1. *Demographic, metabolic, epigenetic, and hematological data of CF patients seen at HULW in João Pessoa-Paraíba, Brazil.*

	Reference values	Patients with sufficient 25(OH) D Serum concentrations	Patients with insufficient/ deficient 25(OH)D serum concentrations**	p^1
Patients	-	41.66% (*n* = 5)	58.33% (*n* = 7)	-
Age (years)	-	12.0 ± 5.24	14.85 ± 1.95	0.21
Time of sun exposure (minute/day)	-	45.00 (IC-3.54-81.04)	30.00 (IC 7.48-96.81)	0.93
PI	-	0	0	-
BMI/age (kg/m²)	-	13.80 ± 1.79	16.03 ± 0.64	0.21
Vitamin D (ng/dl)	**>30**	**34.14 ± 2.63**	**21.91 ± 6.02**	**0.00**
Blood sugar (mg/dl)	**66-99**	**84.50 (IC 78.03-89.46)**	**91.00 (IC 82.63-103.65)**	**0.02**
PTH (pg/ml)	18.5-88.0	18.65 (7.71-30.33)	26.50 (14.89-50.27)	0.06
CRP (mg/l)	<6.0	7.35 (-14.81-42.96)	4.40 (0.65-13.60)	0.80
AGPA (mg/l)	41-121	103.10 (37.93-224.41)	100 (75.43-129.41)	0.29
Creatinine (mg/l)	1 ano a <11 anos: 0.17-0.64 11 a 15 anos: 0.42-0.81 >18 anos: 0.53-1.3	0.53 (0.38-0.72)	0.58 (0.49-0.62)	0.85
Urea (mg/l)	8-36	19.50 (11.24-24.75)	19.00 (16.12-24.72)	0.70
AST (U/l)	10-37	20.50 (13.39-30.10)	26.00 (17.27-31.01)	0.80
ALT (U/l)	**10-37**	**14.50 (4.73-24.26)**	**24.00 (15.97-39.74)**	**0.05**
Uric acid (mg/l)	**Mas: 1.50-6.0 Fem: 0.5-5.0**	**3.74 ± 0.45**	**5.34 ± 0.29)**	**0.02**
Calcium (mg/l)	4.80-5.52	9.8 (9.07-10.97)	9.80 (9.25-10.14)	0.80
Erythrocytes (×10^6/mm³)	4.0-5.2	5.01 (4.44-5.48)	4.60 (4.39-4.83)	0.46
Hematocrit (%)	35-46	27.35 (21.17-34.17)	29.70 (27.47-34.12)	0.29
Hemoglobin (g/100ml)	12-16	13.35 (9.79-17.75)	13.60 (13.07-14.18)	0.32
Platelets (×/mm³)	150.000-450.000	300.00 (169.85-513.19)	337.00 (264.33-399.09)	0.80
		***VDR* methylation profile**		
Methylated		0	0	-
Unmethylated		41.66% (*n* = 5)	58.33% (*n* = 7)	-

Data shown as mean ± standard error, median (confidence interval), or frequency (%).

*25(OH)D > 30 ng/ml and **25(OH)D ≤ ng/ml. Significant values: $p \leq 0.05$ according to *T* test or Mann-Whitney test.

BMI = Body mass index; 25(OH)D = 25-hydroxyvitamin D; PTH = Parathyroid hormone; CRP = C-reactive protein; AGPA = alpha-1-acid glycoprotein; AST = glutamic-oxaloacetic transaminase; ALT = glutamic-pyruvic transaminase.

primer sets (10 µm), 2 µl (~100 ng) of bisulfite-treated DNA; and water. Forty-cycle amplifications occurred in a thermocycler (Applied Biosystems 2720 Thermal Cycler) with an annealing temperature of 55ºC for the 60 seconds. Enzymatic digestion of the amplified DNA was performed using TaqI (Thermo Fisher Scientific), according to manufacturer's recommendations.

The digested products were submitted to vertical electrophoresis in 15% polyacrylamide gels for 3h30. The methylated condition was detected by recognition and cleavage of the 325 bp fragment TCGA site (CpG-723) by TaqI, yielding two fragments of 264 and 59 bp. The unmethylated condition corresponded to the absence of cleavage on the bisulfite-treated TCGA site. Completely methylated and demethylated DNA controls (Cells-to-CpG™ Methylated & Unmethylated gDNA Control Kit, Life Technologies) were used to ensure cleavage specificity on *VDR*.

Statistical analysis

Tests for homogeneity and normality (Levene's and Shapiro-Wilk, respectively) were performed, and variables were classified according to their distribution and asymmetry. Independent *T*-test was used for parametric variables and Mann-Whitney test for the

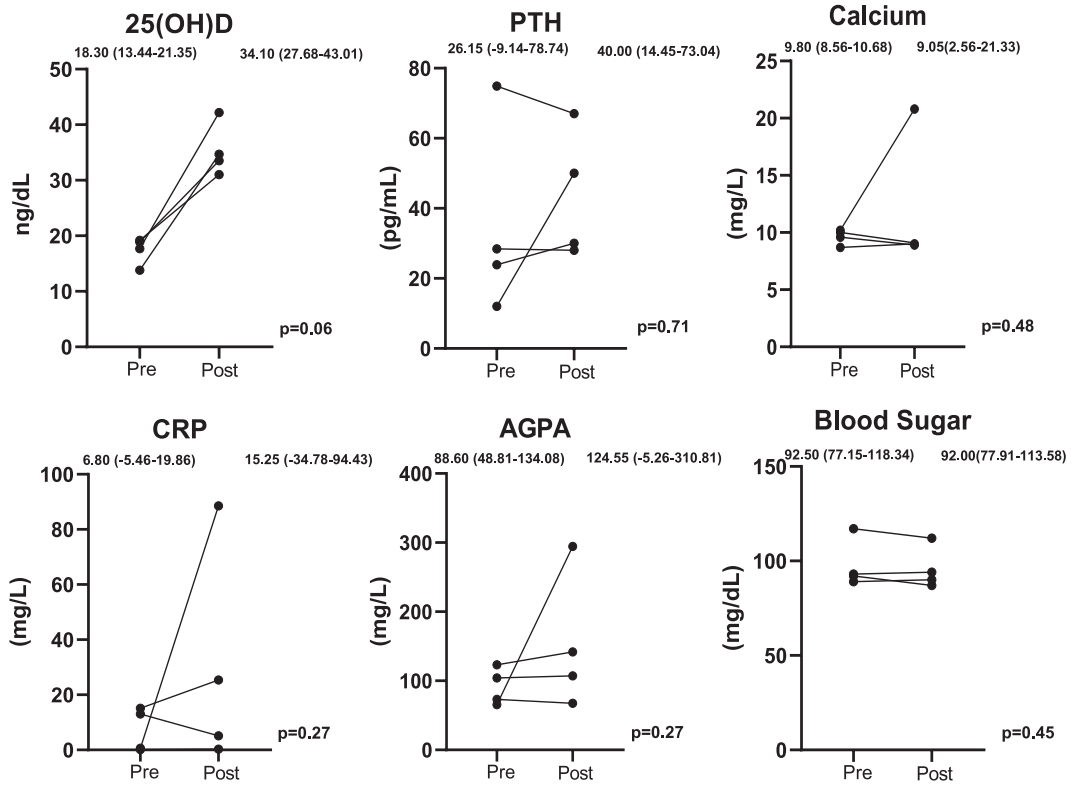

Figure 3. *Pre- and post-intervention single-subject analysis. Values are shown as median (confidence interval). 25(OH)D: 25-hydroxyvitamin D. PTH: parathyroid hormone; CRP: C-reactive protein; AGPA: alpha-1-acid glycoprotein. Significant values: p ≤ 0.05 according to the Wilcoxon test [Reference Values (RV): 25(OH)D: >30 ng/dl; PTH: 18.5-88.0 pg/ml; Calcium: 4.80-5.52 mg/dl; CRP: < 6 mg/l; AGPA: 41-121 mg/l; Blood Sugar:66-99 mg/dl].*

non-parametric ones. Wilcoxon test was used for post-intervention analysis. The resulting data were tabulated into single-subject analysis graphs on Statistical Package for the Social Sciences® Statistics 25.0 software, considering a *p*-value ≤0.05 for significant associations with the outcome.

Results

Demographic data showed that the studied population (*n* = 12) presented an average monthly income ranging from 1 to 5 Brazilian minimum wages. Approximately 58.33% of all participants' guardians completed middle school, while only 41.66% completed high school. No renal or hepatic diseases were observed, only a sole case of chronic disease (Diabetes mellitus). Hypovitaminosis D prevalence among participants reached 58.33% (*n* = 7), comprising two female and five male individuals with ages between 12 and 18 years old, and mean 25(OH)D serum concentration of 21.91 ng/dl (13.8 ng/dl-29.8 ng/dl). Calcium and Vitamin D3 intake values (1,168 mg/day - 5.52 µg/day, respectively) were lower than the Dietary Reference Intakes recommendation for their age. According to the nutritional diagnosis (BMI/age), 57.14% (*n* = 4) of vitamin D insufficiency/deficiency subjects (*n* = 7) had low body weight (BMI = 15.9 ± 1.12) when compared to 40% (*n* = 2) of the sufficiency group (*n* = 5) (BMI = 10.13 ± 0.54). The insufficiency/deficiency group presented the shortest time of sun exposure, 30 minutes per day (CI 7.48-96.81), compared to 45 minutes in the sufficiency group (CI-3.54-81.04). Levels of 25(OH)D were also significantly lower in the individuals with insufficiency/deficiency ($p = 0.00$), as shown in Table 1. Nevertheless, blood sugar, ALT, and uric acid levels were significantly higher in the insufficiency/deficiency group when compared to the sufficiency group individuals ($p = 0.02$, 0.05, and 0.02, respectively). No significant differences were found between groups regarding the DNA methylation profile since all of the participants presented the unmethylated condition for the studied *VDR* gene promoter fragment.

Only four participants from the insufficiency/deficiency group completed the experimental intervention protocol adequately. The supplemented group (*n* = 4) consisted of male individuals only, with ages ranging from 15 to 18 years old and a mean value of 15 years old. Vitamin D supplementation did not influence this group's DNA methylation since their profile remained the same as in the pre-intervention period. This indicates that vitamin D insufficiency/deficiency and its supplementation outcome are associated with the same DNA methylation profile for the site CpG-723 on *VDR*.

Figure 3 shows the biochemical and inflammatory variables before and after the intervention. After vitamin D3 megadose, 25(OH)D levels from all supplemented individuals increased in a good manner, from 18.3 to 34.1 ng/dl, even though no significance was detected with the statistical test ($p = 0.06$) due to the small sample size.

After the intervention, it was possible to observe alterations in the median values for inflammatory markers

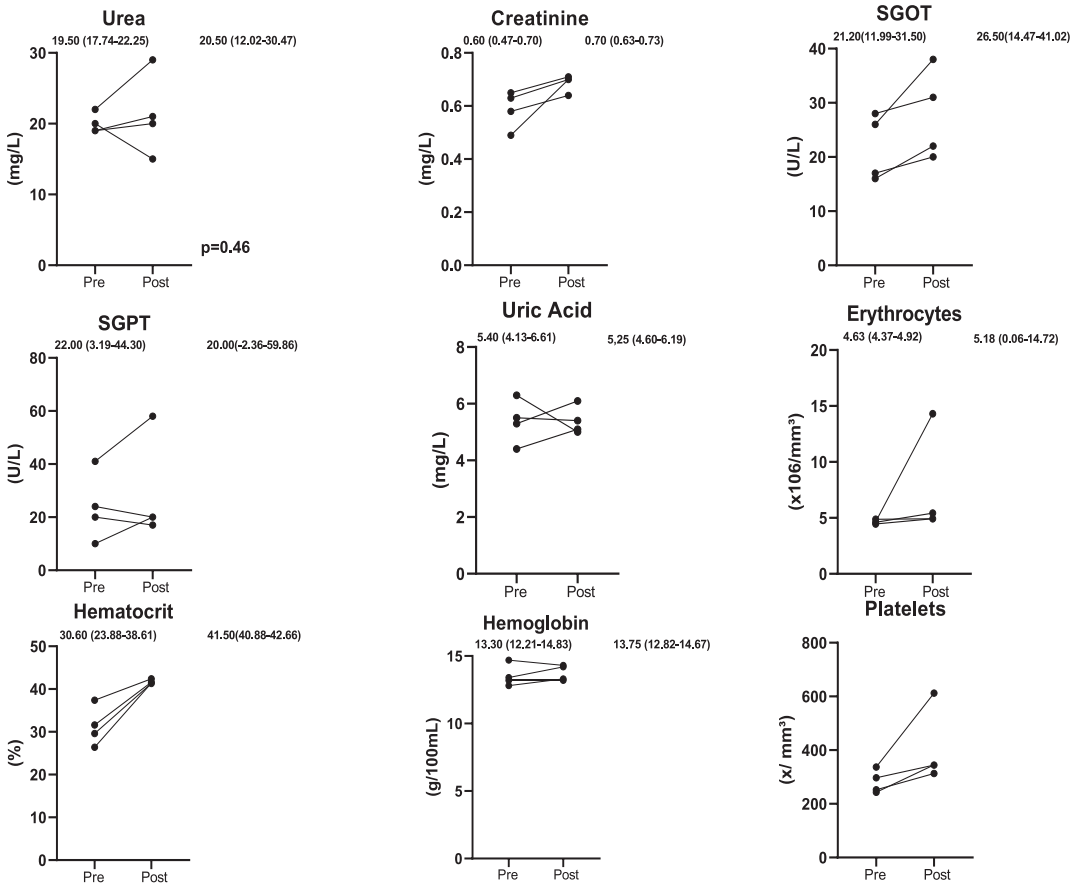

Figure 4. *Pre and post vitamin D3 intervention single-subject analysis. Values are shown as median (confidence interval)-significant values: p ≤ 0.05 according to the Wilcoxon test.*

(CRP and AGPA). Still, these were also non-significant since they refer to a sole patient whose base prognosis worsened during the experimental protocol. No other significant values were observed for the other markers. Apart from discrete modifications (Figure 4), vitamin D megadose supplementation did not lead to any significant differences in the biochemical and hematological parameters, especially for hepatic and renal markers.

Discussion

This is the first study to perform an epigenetic analysis of *VDR*, reporting significant differences between hepatic, renal, and glycemic markers of fibrocystic disease patients with Vitamin D insufficiency/deficiency and patients with sufficient vitamin D levels. This study shows a 58.33% prevalence (n = 7) of hypovitaminosis D in patients with CF, superior to values observed in other current studies such as the review (19) of 148 medical records of children with CF, ranging from 10 months and 12 years old, which reported a prevalence of 43%. They also found that this deficiency was present even in patients without PI. Another example is a study carried out in the Northeast of Brazil (20), which reported a 33.33% prevalence of hypovitaminosis D in children and adolescents with fibrocystic disease. For the first time in the literature, biochemical data from the pre-intervention period showed a significant increase in ALT values in CF patients with insufficient/deficient vitamin D levels compared to those with vitamin D sufficiency. Hepatic enzymes tend to be elevated in CF patients, especially ALT. This marker has been associated with predictive factors of poor prognosis for the nutritional status, in particular for female subjects with this illness (21). Nevertheless, higher ALT levels and AST and GGT (gamma-glutamyl transpeptidase) are considered great non-invasive markers for diagnosing early hepatic impairments caused by CF (22). Another pre-intervention data that varied between groups and had not been reported in the literature yet was the uric acid levels. Those were higher in the insufficiency/deficiency group. Recent studies (23-24) demonstrated that when in elevated concentrations, this marker of renal complications produces direct proinflammatory effects upon macrophages *in vitro*, possibly through the urate transporter 1. They also reported its association with low vitamin D3 levels associated with the higher risk of developing diabetes type II in adult individuals. The insufficiency/deficiency group also presented elevated blood sugar levels, and those underwent no significant changes after the intervention. Health care improvements led to an increase in CF patients' survival, despite the emergence of long-term complications. The most common one is cystic fibrosis-related diabetes (CFRD), which has a prevalence of 33% in some populations of CF patients (25). A multicenter study involving 898 patients with CF showed that 25(OH)D serum concentrations were related to the patients' glycemic profile, increasing the risk of developing CFRD (26). Still, no data on the influence of supplementation and insulin resistance improvements were found.

Vitamin D megadose supplementation effectively increased 25(OH)D levels, even though the statistical tests detected no significance. This variation corroborates with one recently-published intervention study (27) that tested higher cholecalciferol dosages (50,000 UI) in 47 CF patients ranging from 6 to 21 years old, and in which 66% of them reached higher concentrations of 25(OH)D after the intervention. This demonstrates the need for researches involving megadose-supplemented children and adolescents since similar studies with adults are already found in the literature (28-29). In our study, no significant alterations in renal and hepatic markers were observed after the intervention. This data agrees with findings of a previously published review paper (30), which suggests that CF patients need a superior dosage compared to healthy individuals when it comes to hypovitaminosis D. The supplementation was not able to decrease CF patients' inflammatory response. Since this study's sample size was relatively small, it is impossible to confirm the supplementation's lack of effect. Regulating CF patients' inflammatory responses is primordial, and studies with adults (31-32) have reported beneficial effects arising from nutritional supplementation interventions. Hence the necessity for more researches in the area.

Regarding the *VDR* sequence methylation profile, the unmethylated (or hypomethylated) condition was observed in leukocytes, without any association to vitamin D3 serum levels. CpG-723 is located on a CpG island, which suggests participation in gene expression regulation. It is also recognized by a restriction enzyme, a requirement of the COBRA technique. It is important to comment that the 325 bp fragment investigated in the present study can be accessed by other restriction enzymes, meaning that the methylation profile of other CpG sites may be revealed for the exact location. It is known that different CpG sites in the same region may present different methylation profiles in response to environmental factors (33). However, our data suggest that the unmethylated profile of site-723 in leukocytes is independent of the vitamin D status and a common aspect of children and adolescents with CF. Few studies are assessing the *VDR* sequence methylation profile in different pathologies and tissues and intervention studies, and nothing was known about a population with CF to this day. A study in Australia (34) reported a hypomethylated profile in leukocytes for the *VDR* gene promoter region (17 CpG sites) in individuals over 65. Although our study investigated younger individuals, DNA methylation results were similar. On the other hand, in the previously mentioned study, a positive correlation between DNA methylation profile and vitamin D plasma levels was observed. Another study with leukocytes was carried out with colorectal cancer patients, in which a hypomethylated profile was also prevalent in this population compared to the control group. Lower vitamin D plasma levels for the colorectal (35) cancer group were also found. The literature reports an association between DNA hypomethylation and gene expression, but little is known about the *VDR* gene, as well as the epigenetics of vitamin D (36). The present study's limitations include small sample size, which was influenced by the rareness of the disease, and the lack of a control group for epigenetic studies. Nevertheless, its importance lies on the scarcity of studies with children and adolescents, primarily as it reports data on the rarely explored epigenetics of Vitamin D. Our data reinforce the continuity of researches on this population's inflammatory, glycemic, hepatic, renal, and genetic markers, as well as on the methylation profile of other CpG sites in the same *VDR* gene promoter fragment. Epigenetic data may contribute to the implantation of personalized nutritional interventions (37).

Conclusion

This study's results demonstrated significant differences in blood sugar, ALT, and uric acid levels of CF patients with Vitamin D insufficiency/deficiency compared to a group with sufficient levels, along with elevated inflammatory markers. Cholecalciferol megadose increased 25(OH)D serum values even though it did not influence the patients' inflammatory, glycemic, or genetic parameters. Therefore, further studies on these themes are encouraged.

Acknowledgement
The authors thank the faculty members and professionals from the Graduate Program in Nutrition Sciences of the Federal University of Paraíba, as well as the patients and respective guardians that participated in this research, without whom this study would not be possible.

Author contribution
MPP, MCR conceived and designed the study. MPP and ASS performed the statistical analysis. MPP, DJQ, and CCSJ were involved in data collection and writing the manuscript. MPP edited the manuscript. All authors reviewed and approved the final draft of the manuscript.

List of Abbreviations

25(OH)D	25-hydroxyvitamin D
AGPA	Alpha-1-acid glycoprotein
ALT	Glutamic-pyruvic transaminase
AST	Glutamic-oxaloacetic transaminase
CF	Cystic fibrosis
CFRD	Cystic fibrosis-related diabetes
CFTR	Cystic fibrosis transmembrane conductance regulator
CRP	C-reactive protein
HULW	Hospital Universitário Lauro Wanderley
PI	Pancreatic insufficiency
PTH	Parathyroid hormone

Funding
None.

Declaration of conflicting interests
The authors of this article have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Ethical approval
The study protocol was approved by the Hospital Universitário Lauro Wanderley Research Ethics Committee under protocol number 87354018.1.0000.5183.

Consent to participate
Informed consent was obtained from the patients.

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