Revista da Sociedade Brasileira de Medicina Tropical
Journal of the Brazilian Society of Tropical Medicine
Vol.:52:e20190133: 2019
doi: 10.1590/0037-8682-0133-2019

Major Article

Association of vitamin D3, *VDR* gene polymorphisms, and LL-37 with a clinical form of Chagas Disease

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Abstract

Introduction: Chagas disease (CD) is an important public health problem in Brazil and worldwide. Aging and obesity are important matters in patients with CD, as is hypovitaminosis D3, which can decrease the quality of life of these patients. Immunomodulation mediated by vitamin D3, especially the production of antimicrobial peptides such as cathelicidin LL-37, might be related to the severity and symptoms of CD. This study aimed to determine the serum levels of vitamin D and LL-37 and *VDR* gene polymorphisms in patients with chronic CD. **Methods:** This study included male patients with cardiac and indeterminate clinical forms of CD. Clinical, anthropometric, and blood parameters were obtained. Serum levels of 25(OH)D3 and LL-37 were determined by chemiluminescence and enzyme-linked immunosorbent assay respectively. Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236) polymorphisms of the *VDR* gene were investigated by PCR-RFLP. **Results:** Sixty-four patients were included in the study: 18 of the cardiac form and 46 of the indeterminate form. No differences in age, ethnicity, BMI, arterial hypertension, diabetes mellitus, or dyslipidemias were observed between groups. However, the serum levels of 25(OH)D3, but not of LL-37, were lower in the cardiac form group. The association among polymorphisms, vitamin D, and clinical form was not significant. **Conclusions:** Decreased levels of vitamin D suggest an association with the cardiac form of CD. Studies investigating the roles of vitamin D and LL-37 in the immune response and their associations with VDR polymorphisms and disease susceptibility are necessary.

Keyword: Chagas Disease. Vitamin D3. LL-37.

INTRODUCTION

Chagas disease (CD), a neglected disease caused by the protozoan parasite *Trypanosoma cruzi*, is present in 21 Latin American countries. In Brazil, approximately 2 to 3 million people are infected, with 6,000 deaths annually[1]. CD is an emerging public health problem in North America, Europe, and Japan owing to immigration from endemic areas[2]. In addition to its clinical and epidemiological importance, CD affects health services financially because the establishment of early symptoms can lead to the need for long-term treatment and highly complex surgical procedures[3].

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Received 8 May 2019
Accepted 24 July 2019

CD occurs in two distinct phases: an acute and a chronic phase that can progress to symptomatic forms and affect the gastrointestinal tract and/or heart[4]. Cardiac involvement is considered the most important CD manifestation given its high frequency and severity, with signs and symptoms ranging from conduction disturbances to more severe conditions such as cardiomegaly, heart failure, and sudden death[5,6].

Although several studies on CD pathogenesis are available, few have sought to unravel why some patients remain asymptomatic and others develop more severe disease manifestations. Some suggest a relationship with the inflammatory response, the immune mechanisms involved in parasite elimination, and the interaction between the parasite and host[7].

The lack of drugs that completely eliminate the etiological agent of CD causes infected individuals to develop the chronic phase and the parasite persists in host tissues, thus inducing severe lesions. Hence, adjuvant treatments that can prevent or

attenuate these severe cardiovascular complications or those can limit CD progression, with a consequent increase in survival, are important[8]. With this context, control of chronic degenerative comorbidities such as hypertension, diabetes mellitus, dyslipidemias, and aging-associated obesity has become the focus of research on CD. The control of these comorbidities aims to attenuate irreversible damages to the cardiovascular system in patients with CD[9].

Obesity has gained prominence among CD-related comorbidities. Navarro et al.[10] noted that 86% patients with the indeterminate form of CD were overweight/obese, suggesting that these patients should receive the same care as the general population. Thus, identifying these comorbidities in CD may improve the quality of life of these patients, especially those with the cardiac form of the disease[11].

Obesity has been associated with hypovitaminosis D3 and cardiovascular disease risk[12]. This association is important for public health strategies as vitamin D3 supplementation is a safe and inexpensive therapeutic option[13].

In addition to cardiovascular disease risk, vitamin D3 is important in the body's immune response owing to its immunomodulatory activity[14]. Vitamin D3 serum levels directly influence macrophages, increasing oxidative burst—with the activation and production of cytokines, acid phosphatase, hydrogen peroxide, and antimicrobial peptides—and inhibition of some inflammatory cytokines. Moreover, vitamin D stimulates neutrophil motility and phagocytic function, thus reducing local and systemic inflammatory responses[15,16].

Antimicrobial peptides, considered to be endogenous antibiotics, are responsible for the elimination of microorganisms such as bacteria, viruses, fungi, and parasites[17-20]. An antimicrobial peptide that is influenced by vitamin D is cathelicidin (LL-37), which is found in different immune cells. Cathelicidin is involved in immune response activation and control; it increases cytokine and chemokine release[21,22] . In addition to its role in innate immunity, host defense, and inflammation, LL-37 is related to angiogenesis and arteriogenesis[23].

Binding of vitamin D3 to the vitamin D receptor (VDR), belonging to the steroid hormone receptor family located in the cell nucleus, is required for vitamin D3 to exert its physiological effects. In addition to the nucleus, it is believed that VDR is expressed in various tissues and cells, suggesting a paracrine/autocrine effect of vitamin D[24-27].

VDR is encoded by the *VDR* gene located on chromosome 12, position 12q13.11. This gene contains 11 exons and the coding region that expresses the VDR protein, which comprises exons 2 to 9[28]. Genetic variations in restriction enzyme sites of this gene, called single nucleotide polymorphisms (SNPs), may have physiological effects[29]. These SNPs might be related to variations in susceptibility to disease development. Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236) are the most commonly used polymorphisms in genetic association studies[30-33].

As the degree of adiposity or obesity can interfere with vitamin D levels and the reduced level of this vitamin is associated with lower antimicrobial peptide production, more severe symptoms of CD may be developed. Therefore, this study aimed to determine the serum levels of 25(OH)D3 and cathelicidin LL-37 and *VDR* gene polymorphisms in adult male patients with cardiac and indeterminate forms of chronic CD.

METHODS

This study was approved by the Research Ethics Committee of the Botucatu Medical School (Approval No. 1.576.519/2016). All participants received information about the study and signed the informed consent form.

Adult male patients with indeterminate and cardiac forms of chronic CD seen at the outpatient clinic that treats nutritional and metabolic disorders of patients with tropical diseases, University Hospital of the Botucatu Medical School (HCFMB), UNESP, were selected. Patients with chronic digestive and mixed forms of CD and those who refused to be tested for the confirmation of the disease and its clinical form were excluded. Additionally, patients who refused to sign the consent form or who did not attend the scheduled appointments for clinical examination and blood collection were not included.

Patients with the indeterminate form included those with positive serology by at least two methods (chemiluminescence, hemagglutination, or indirect immunofluorescence) and with electrocardiogram (ECG), opaque enema esophagogastric-duodenum without alterations, and absence of clinical symptoms. Patients with the cardiac form included those who, in addition to positive serology, exhibited changes in ECG and chest X-ray and clinical symptoms such as palpitations and arrhythmias (ventricular extrasystole, tachycardia, and different types of heart blockages).

Patients underwent clinical examination such that their anthropometric information such as weight (kg), height (m), BMI (kg/m^2), and waist circumference (cm) could be obtained. Age (years) and the existence of comorbidities such as arterial hypertension, diabetes mellitus, and dyslipidemia were obtained from medical records. The BMI was classified according to the parameters established by the World Health Organization: low weight (BMI < 18.5 kg/m^2), eutrophic (BMI 18.5-24.9 kg/m^2), overweight (BMI 25.0-29.9 kg/m^2), obesity I (BMI 30.0-34.9 kg/m^2), obesity II (BMI 35.0-39.9 kg/m^2), and obesity III (BMI ≥ 40 kg/m^2). Patients with a waist circumference > 102 cm were classified as high cardiovascular disease risk according to the National Cholesterol Education Program - Adult Treatment Panel III (ATPIII).

Determination of Plasma 25(OH)D3 Levels

Peripheral venous blood samples (5 mL) were collected for the determination of serum 25(OH)D3 and cathelicidin LL-37 levels and *VDR* gene polymorphisms. A 1 mL aliquot of whole blood was separated for DNA extraction and the remaining sample was centrifuged to separate the plasm for 25(OH)D3 and cathelicidin LL-37 measurement.

25(OH)D3 serum levels were determined at the Laboratory of Clinical Analysis, University Hospital of Botucatu - SP, using the Abbott 25OHD Kit (Abbott Laboratories, North Chicago,

IL, USA) by Microparticle Chemiluminescent Immunoassay on the Architect i2000SR (Abbott Laboratories, North Chicago, IL, USA), according to manufacturer's instructions. The results were classified according to the Endocrine Society, with values > 30 ng/mL defined as vitamin D sufficiency, between 20 ng/mL and 30 ng/mL as insufficiency, and < 20 ng/mL as deficiency.

Determination of Serum Cathelicidin (LL-37) Levels

Cathelicidin LL-37 was determined by sandwich enzyme-linked immunosorbent assay (ELISA) using the CAMP ELISA Kit, Human OKEH00728 (Aviva Systems Biology, San Diego, CA, USA), according to manufacturer's instructions. A plate containing antibodies to cathelicidin LL-37 was incubated with standards and samples. The standard curve used ranged from 0.125 to 8 ng/mL.

Characterization of Polymorphisms

Genomic DNA Extraction: to investigate the polymorphisms, genomic DNA was extracted from peripheral venous blood leukocytes using the PureLink® Genomic DNA Kit (K182001; Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The extracted DNA was kept in a freezer at −80 °C until analysis. DNA was quantified in a NanoVue Plus spectrophotometer (GE Healthcare, Little Chalfont, Buckinghamshire, UK). The following SNPs in the *VDR* gene were selected from the literature and dbSNP database at National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/projects/SNP): Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236).

Polymerase Chain Reaction Detection of SNPs: SNP amplification by PCR was performed in a 25-μL volume containing 50 ng genomic DNA, Milli-Q water, buffer, 3.0 mM $MgCl_2$, 0.2 mM of each dNTP, 20 pM of each primer, and 1 U Taq DNA Polymerase (Cellco®, São Carlos, Brazil). The nucleotide sequences of each primer and the fragment size are as follows:

***Taq (*T/C) rs731236-** forward/ 5′CAGAGCATGGACAGGGAGCAA3′,

reverse/ 5′GCAACTCCTCATGGCTGAGGTCTC3′ (745 bp);

***Fok (*T/C) rs2228570-** forward/ 5′AGCTGGCCCTGGCACTGACTCTGGCTCTG3′,

reverse/5′ATGGAAACACCTGCTTCTTCTCCCTC3′ (265 bp);

***Bsm (*A/G) rs1544410-** forward/ 5′AGTGTGCAGGCGATTCGTAG3′,

reverse/5′ATAGGCAGAACCATCTCTCAG3′ (191 bp);

***Apa(*T/G) rs7975232-** forward/ 5′CAGAGCATGGACAGGGAGCAA3′,

reverse/ 5′GCAACTCCTCATGGCTGAGGTCTC3′ (745 bp)

For the Apa (rs7975232) and Taq (rs731236) SNPs, the PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 20 s, annealing at 59 °C for 30 s, and extension at 72 °C for 2 min, with a final extension at 72 °C for 10 min. For Fok (rs731236), the PCR conditions were initial denaturation at 95 °C for 5 min, 35 denaturation cycles at 95 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 45 s, with a final extension at 72 °C for 10 min. For Bsm (rs1544410), the conditions were as follows: initial denaturation at 94 °C for 4 min, 35 denaturation cycles at 94 °C for 30 s, annealing at 58.5 °C for 30 s, and extension at 72 °C for 30 s, with a final extension at 72 °C for 5 min.

The PCR products were separated by electrophoresis on 2% agarose gel stained with 0.1% ethidium bromide (4 μL in 50 ml agarose gel) and visualized and photodocumented under an ultraviolet transilluminator (UVP, Upland, CA, USA).

Digestion of Polymerase Chain Reaction - Restriction Fragment Length Polymorphism (PCR-RFLP) Products: after amplification, 10 μL of each PCR product was subjected to RFLP with 1 μL of its corresponding restriction enzyme for digestion, i.e., FokI, ApaI, and BsmI (New England BioLabs, Boston, MA, USA) and TaqI (Jena Bioscience, Munich, Germany), which cuts the product at specific sites, resulting in new fragments of different sizes. The PCR products were incubated with the enzymes at a constant temperature for 1 h according to manufacturer's instructions.

The genotypes and alleles of the *VDR* gene SNPs were established based on the absence (upper case) or presence (lower case) of the restriction site.

Statistical Analysis

Statistical analysis was performed using the SigmaPlot® 12.5 software for Windows® (Systat Software, Inc., San Jose, CA, USA). Qualitative data were analyzed by the chi-square test or Fisher's exact test, depending on the values of the data in the contingency tables. For quantitative data, parametric ANOVA and the student's t-test were used for normally distributed variables, and the Kruskal-Wallis and Mann-Whitney tests were used for variables with an asymmetric distribution. The nonparametric Pearson correlation test was applied to evaluate the correlation among vitamin D levels, cathelicidin, BMI, and waist circumference. A p-value < 0.05 was considered to indicate significant differences between groups.

RESULTS

Sixty-four male patients were included in the study: 46 had the indeterminate form and 18 the cardiac form. The mean age was 60 and 62 years in the two groups, respectively, and no significant difference was found between the groups (p = 0.45). The other patient characteristics included in the study according to clinical form are shown in **Table 1**.

No differences were found between groups for ethnicity, weight, height, waist circumference, BMI, or obesity-associated comorbidities such as hypertension, diabetes mellitus, and dyslipidemias. However, dyslipidemias were present in 62.5% of the patients. BMI evaluation revealed overweight (BMI 25.0 - 29.9 kg/m^2) patients in both groups.

Regarding serum 25(OH)D3 levels, patients with the cardiac form had lower levels than patients with the indeterminate form (**Table 1 and Figure 1**). Despite the reduced levels of 25(OH)D3 in patients with the cardiac form, the serum levels of cathelicidin were similar in the two groups (**Table 2 and Figure 2**).

TABLE 1: Characteristics of patients with Chagas disease.

Variable	Clinical form		*p*
	Indeterminate (n = 46)	Cardiac (n = 18)	
Age (years)[1]	60.3 ± 8.1	62.2 ± 11.0	0.453
Ethnicity[2]			
White	38 (82.6)	11 (61.1)	
Brown	3 (6.5)	5 (27.8)	0.065
Black	5 (10.9)	2 (11.1)	
Waist circumference[1] (cm)	96.3 ± 9.4	97.8 ± 12.5	0.592
Weight[1] (kg)	75.6 ± 11.5	75.0 ± 14.9	0.859
Height[1] (m)	1.68 ± 0.06	1.69 ± 0.06	0.509
BMI[1] (kg/m^2)	26.9 ± 3.8	26.2 ± 4.3	0.506
Arterial hypertension[2]			
Yes	17 (37.0)	11 (61.1)	0.141
No	29 (63.0)	7 (38.9)	
Diabetes mellitus[2]			
Yes	13 (28.3)	7 (38.9)	0.60
No	33 (71.7)	11 (61.1)	
Dyslipidemias[2]			
Yes	32 (69.6)	8 (44.4)	0,114
No	14 (30.4)	10 (55.6)	
25(OH)D3[1] (ng/mL)	29.3 ± 5.8	25.4 ± 7.3	**0.03**
LL-37[3] (ng/mL)	3.98 (0.01 - 33.41)	2.68 (0.03 - 18.76)	0.286

[1]Values expressed as mean ± standard deviation (t-test); [2]values expressed as n (%) (chi-square test); [3]values expressed as mean (range) (Mann-Whitney test.

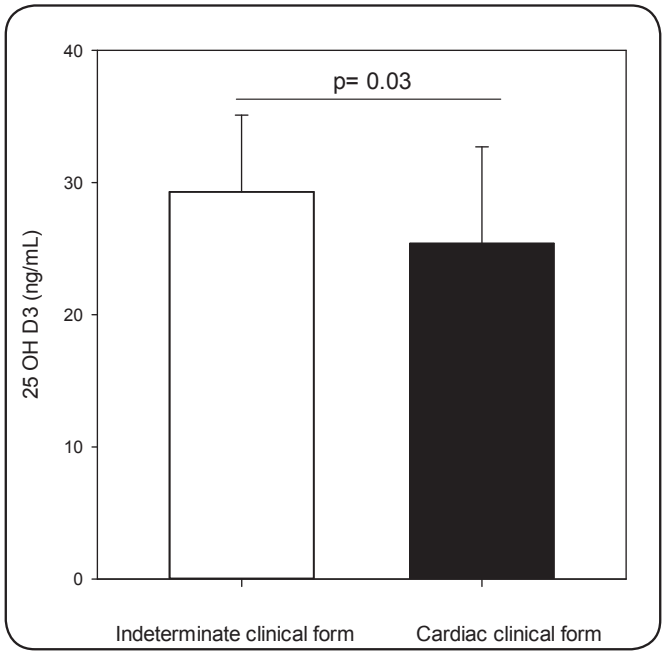

FIGURE 1: Serum levels of 25(OH)D3 according to clinical form of chronic Chagas disease.

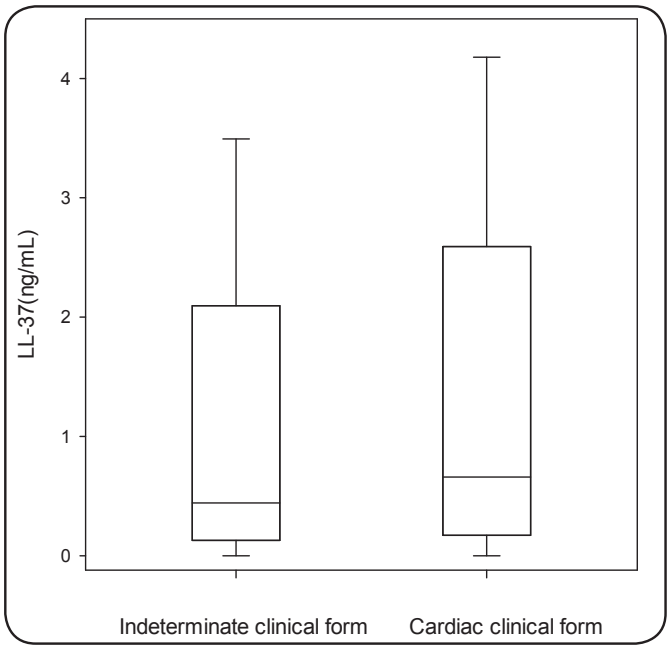

FIGURE 2: Serum levels of LL-37 (cathelecidin) according to clinical form of chronic Chagas disease. Box-plot with percentile p95, p75, p50, p25, and p5.

TABLE 2: Serum levels of 25(OH)D3 and cathelicidin according to clinical form of chronic Chagas disease.

Variable	Clinical form		*p*
	Indeterminate (n = 46)	Cardiac (n = 18)	
Vitamin D[1] (ng/mL)	29.3 ± 5.8	25.4 ± 7.3	**0.03**
Sufficient[2]	19 (41.3)	4 (22.2)	
Insufficient[2]	24 (52.2)	10 (55.6)	0.119
Deficiency[2]	3 (6.5)	4 (22.2)	
LL-37[3] (ng/mL)	3.98 (0.01 - 33.41)	2.68 (0.03 - 18.76)	0.286

[1]Values expressed as mean ± standard deviation (t-test); [2]values expressed as n (%) (chi-square test); [3]values expressed as mean (range) (Mann-Whitney test). ng/mL: nanogram per milliliter. Sufficiency: > 30 ng/mL; insufficiency: 20 to 30 ng/mL; deficiency: < 20 ng/mL.

The correlation of serum 25(OH)D3 levels with cathelicidin, BMI, or waist circumference was not statistically significant in either clinical form (**Figure 3**). The same was observed for the correlation of cathelicidin with BMI and waist circumference.

For the analysis of the *VDR* gene SNPs, six patients were excluded for Taq (rs731236), five for Fok (rs731236), four for Apa (rs7975232), and three for Bsm (rs1544410) because DNA extraction and amplification by PCR-RFLP was impossible or the material for analysis was insufficient. Regarding the distribution of the *VDR* gene SNPs (**Table 3**), the allele and genotype frequencies observed for the Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236) variants did not differ significantly between serum 25(OH)D3 levels. Furthermore, differences in the frequency of the *VDR* gene polymorphisms or association with each clinical form of CD were not found between the clinical forms analyzed, as shown in **Table 4**.

DISCUSSION

Vitamin D3 levels were lower in patients with the cardiac form of chronic CD. The deficiency of this vitamin is observed in approximately 85% of older adults, of which 90% of them were institutionalized, and in 50% of young adults[34]. The percentage of patients with vitamin D3 sufficiency was 36% and that of patients with insufficiency and deficiency was 64%. These values are higher than those reported by Martini et al.[35] for adults and older

TABLE 3: Distribution of genotypes and allele frequency (AF) according to serum 25(OH)D3 levels in patients with chronic Chagas disease.

		Genotype	Deficiency	Insufficient	Sufficient	Total	Allele frequency (AF)		*p*
							Allele	Frequency	
Polymorphisms	***Taq*** (rs731236)	TT (M)	5	16	10	**31**	T	0.74	0.617
		Tt (H)	1	13	10	**24**			
		tt (W)	0	2	1	**3**	t	0.26	
		n	**6**	**31**	**21**	**58**			
	Fok (rs731236)	FF (W)	3	8	7	**18**	F	0.56	0.767
		Ff (H)	2	17	11	**30**			
		ff (M)	2	5	4	**11**	f	0.44	
		n	**7**	**30**	**22**	**59**			
	Apa (rs7975232)	AA (M)	3	17	11	**31**	A	0.69	0.837
		Aa (H)	3	12	6	**21**			
		aa (W)	1	3	4	**8**	a	0.31	
		n	**7**	**32**	**21**	**60**			
	Bsm (rs1544410)	BB (M)	1	8	6	**15**	B	0.60	0.207
		Bb (H)	6	23	14	**43**			
		bb (W)	0	0	3	**3**	b	0.40	
		n	**7**	**31**	**23**	**61**			

M: mutated homozygote; **W:** wild-type homozygote; **H:** heterozygote.

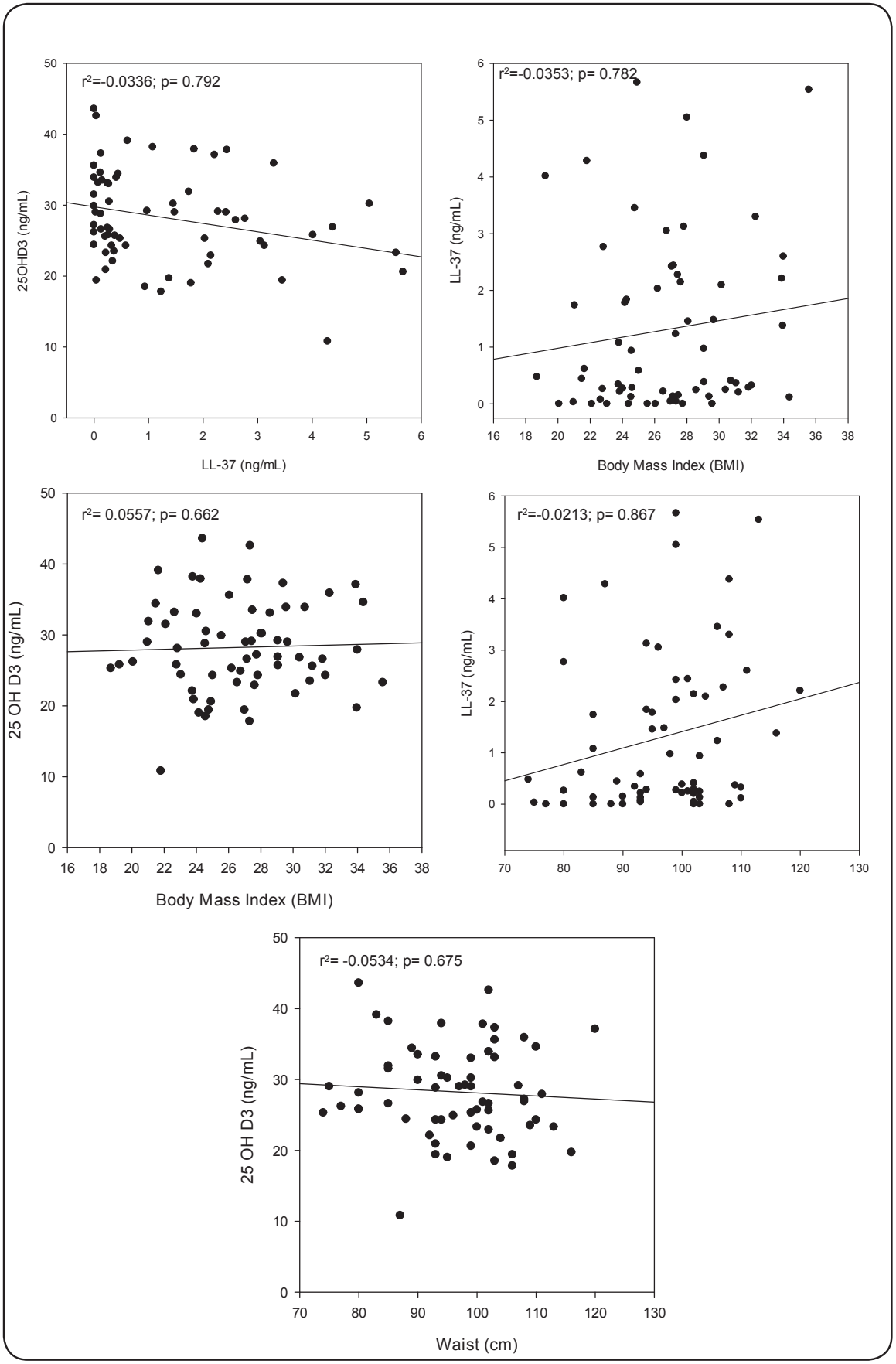

FIGURE 3: Correlation of serum 25(OH)D3 and LL-37 levels with body mass index (BMI) and waist circumference in patients with Chagas disease. Pearson test. Pearson test. 25(OH)D3: 25-hydroxyvitamin D3.

TABLE 4: Distribution of genotypes and allele frequency (AF) according to clinical form of chronic Chagas disease.

			Clinical form			Allele frequency (AF)		*p*
		Genotype	Indeterminate	Cardiac	Total	Allele	Frequency	
Polymorphisms	***Taq*** (rs731236)	TT (M)	21	10	**31**	T	0.74	0.458
		Tt (H)	18	6	**24**			
		tt (W)	3	0	**3**	t	0.26	
		n	**42**	**16**	**58**			
	Fok (rs731236)	FF (W)	11	7	**18**	F	0.56	0.511
		Ff (H)	23	7	**30**			
		ff (M)	8	3	**11**	f	0.44	
		n	**42**	**17**	**59**			
	Apa (rs7975232)	AA (M)	26	5	**31**	A	0.69	0.078
		Aa (H)	13	8	**21**			
		aa (W)	4	4	**8**	a	0.31	
		n	**43**	**17**	**60**			
	Bsm (rs1544410)	BB (M)	12	3	**15**	B	0.6	0.422
		Bb (H)	30	13	**43**			
		bb (W)	3	0	**3**	b	0.4	
		n	**45**	**16**	**61**			

M: mutated homozygote; **W:** wild-type homozygote; **H:** heterozygote.

adults. Factors such as ethnicity, skin color, age, and BMI that could interfere with vitamin D3 levels did not differ between the groups studied, suggesting that the difference in vitamin D3 levels was related to the clinical form of chronic heart disease.

A meta-analysis of 72 Brazilian studies evaluating the spatial distribution of vitamin D status in different age groups demonstrated mean concentrations of 25(OH)D of 67.7 nmol / L (27.1 ng/mL) and prevalence of insufficiency and deficiency of 45.3% and 28.2%, respectively. The highest prevalence of deficiency was from the South and Southeast regions, while the highest prevalence of vitamin D insufficiency were observed in the Southeast and Northeast regions of the country[36]. Representative samples of subjects from the São Paulo city, in Brazil, showed that the highest concentration of 25(OH) D in the autumn (20.7 ng / mL) and the lowest in the summer (12.0 ng / mL)[35].

Patients with the cardiac form generally exhibit several immune imbalances such as systemic and local inflammations, with inflammation of tissues infected with the parasite thus leading to tissue damage. Within this context, hypovitaminosis D3 may be important for the adequate immune response to *T. cruzi* because vitamin D3 deficiency increases systemic and local inflammations[,37,38]; additionally, deficiency or hypovitaminosis results in uncontrolled Th17 response, as observed in autoimmune diseases, as well as tissue damage caused by IL-17[39,40]. This fact is supported by studies demonstrating an increase in IL-10 production by CD4+ and CD25+ regulatory T (Treg) cells after treatment with vitamin D3[41].

Vitamin D3 has been shown to suppress the production of proinflammatory cytokines such as IFN-γ, IL-17, and IL-21, but does not appear to substantially affect the division of CD4+ and CD25+ Treg cells[37-39,42]. Vitamin D3 also plays an anti-inflammatory role[43], inducing a Th2 profile and the production of antimicrobial peptides such as cathelicidins and defensins[21]. Low levels of vitamin D3 may promote inflammation and shift the host immune response to an unbalanced and nonprotective Th1 profile. The formation of CTLA-4+ and FoxP3+Treg cells, which can suppress the immune response significantly, is also stimulated by the presence of vitamin D3, of which a deficiency of the latter can lead to a deleterious immune response[39].

Experimental studies on BALB/c mice have demonstrated that vitamin D3 is related to the maintenance of lung epithelial integrity and suppression of inflammatory cytokines. Supplementation of deficient mice decreased the production of inflammatory cytokines and the number of macrophages and neutrophils in bronchoalveolar lavage[44,45]. Studies with human macrophages infected with Dengue virus showed that vitamin D3 supplementation resulted in decreased expression of receptors for mannose and induces moderate TNF-alpha and IL-1 beta secretion[46]. Vitamin D3 downregulates the accumulation of cholesterol deposits in macrophages, thus reinforcing the need for vitamin supplementation in patients with the cardiac form of chronic CD[47].

Hypovitaminosis D3 may be a consequence of age and overweight/obesity in patients with chronic CD. These conditions can aggravate both CD and age-related comorbidities,

although the groups studied did not differ in age, ethnicity, or BMI. The hypovitaminosis D3 observed was an important finding owing its association with patients with chronic CD, and the decrease was more accentuated in the clinical cardiac form. Vitamin D3 has been shown to mediate the activity of macrophages against *Mycobacterium tuberculosis*, expressing VDR and CYP27B1 via TLR-2/1 stimulation. The interaction between VDR and vitamin D3 stimulates the production of antimicrobial peptides such as cathelicidin[48]. Hypovitaminosis D3 may facilitate the inflammation and destruction of infected tissues through mediation by the absence of suppressive mechanisms such as IL-10 and Treg production. The destruction of infected tissues with no effective mechanisms to eliminate the parasite may aggravate CD and the clinical condition of infected individuals.

Routine measurement of vitamin D3 would facilitate the clinical follow-up of patients. The significant difference in serum 25(OH)D3 levels between patients with the cardiac and indeterminate forms of chronic CD reinforces the need for vitamin D3 supplementation in these patients. However, serum cathelicidin LL-37 levels did not differ between the two clinical forms nor were they correlated with serum vitamin D3 levels or the clinical forms studied. However, this finding does not diminish the importance of this peptide in CD as it can be modulated by cytokines. Cathelicidin is produced by macrophages and may be influenced by cytokines that negatively or positively regulate the response of macrophages, particularly IL-17 and IL-22[49-50].

Cathelicidin acts as an immunoregulatory agent on angiogenesis, cell proliferation, cytokine secretion, chemotaxis, and mast cell degranulation, favoring the phagocytosis and modulation of gene expression[51-53]. Its proinflammatory activity is related to the regulation of specific chemokines (MCP-1 and IL-8) and binding to chemokine receptors such as IL-8RB, CCR, and CXCR-4[54]. Cathelicidin LL-37 can also interact with host cells and generate direct immune responses, as their receptors are found on various cells such as monocytes, mast cells, T-helper cells, and epithelial cells[55,56].

The cathelicidin LL-37 serum levels observed were similar to the plasma levels found in pregnant women, which were 1.74 ng/mL, different from the 27.2 ± 4.9 ng/mL reported by Jeng et al.[57] in healthy control subjects. This fact suggests that the immune response of the individuals evaluated is compromised and reinforces its relationship with serum vitamin D levels as 64.1% of the individuals had hypovitaminosis D[57,58]. According to Jeng et al.[57], serum vitamin D levels below 20 ng/mL interfered with the full expression capacity of cathelicidin LL-37. Thus, the inadequate serum levels of cathelicidin LL-37 in relation to the levels found in healthy individuals were likely caused by D3 hypovitaminosis, although they did not differ between the clinical forms included in the study.

With respect to VDR receptor polymorphisms, frequency difference in the Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236) *VDR* gene polymorphisms was not found when associated with serum 25(OH)D3 levels; consequently, cathelicidin LL-37. Nevertheless, the Fok SNP (rs2228570) is known to be related to a higher transcriptional activity of the VDR receptor and a better response to 1α,25(OH)2D3 because the exchange of a thymine base with a cytosine base (T-C) creates an additional start codon (ATG → ACG) that changes the translation start site and synthesizes a protein that lacks three amino acids[59].

Although the association of *VDR* gene polymorphisms with susceptibility to the development of infectious diseases has been widely discussed, allelic variations may affect the link of the receptor with vitamin D3 and a specific immune response[60]. In tuberculosis, Selvaraj et al.[61] suggested that the Bsm (rs1544410) and Taq (rs731236) SNPs and Bsm/Apa/Taq haplotypes are associated with reduced phagocytic activity of *M. tuberculosis*-infected macrophages. Salimi et al.[62] also found an association of the Fok polymorphism (rs731236) with susceptibility to pulmonary tuberculosis. Neela et al.[63] identified an association between the Fok (rs731236) and Apa (rs7975232) variants and leprosy, in addition to genotypes that may contribute to the risk of developing the disease.

Studies investigating the association of these SNPs with parasitic diseases are scarce as they require a large number of patients. Sortica et al. (2014)[64] observed that *VDR* gene SNPs influenced the immune response in *Plasmodium vivax* infections, suggesting that the TaqIC/BsmIA haplotype may be a marker of susceptibility to intracellular pathogens. In *T. cruzi* infections, only one study investigated the possible relationship between SNPs in the *VDR* gene and susceptibility to and clinical manifestations of CD. The study thereof revealed an association between the Fok SNP (rs2228570) and the risk of developing the cardiac form of CD from data of 1,172 patients[65].

Limitations of this study include the non-consideration of seasonality in sample collection and of women or patients with digestive and mixed forms of CD. This was not a multicenter study and not all patients with the indeterminate and cardiac forms of the disease seen at the Tropical Diseases Outpatient Clinic of HCFMB were included.

Serum 25(OH)D3 levels differed between patients with the cardiac and indeterminate forms of chronic CD, with lower concentrations in the active cardiac CD in comparison with the indeterminate clinical form. Serum levels of 25(OH)D3 were not correlated with serum cathelicidin levels in patients with CD. The serum concentrations of cathelicidin were similar in the clinical forms studied. Association analysis of the Fok (rs731236), Bsm (rs1544410), Apa (rs7975232), and Taq (rs731236) *VDR* gene polymorphisms indicated no difference in genotype or allele frequency according to serum vitamin D3 levels or clinical form of CD. However, the association between SNP and disease susceptibility and the immunomodulatory role of vitamin D3 support the need for more comprehensive studies of these polymorphisms.

ACKNOWLEDGMENTS

The authors thank the nursing team and physicians at the Tropical Diseases Outpatient Clinic of the University Hospital, Botucatu Medical School, and of the Graduate Program in Tropical Diseases, Botucatu Medical School (UNESP). This work was supported by Master's fellowships from CAPES to Luiz Roberto de Oliveira Junior and Thaysa Buss Carvalho.

Conflict of interest

The authors declare no conflict of interest.

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