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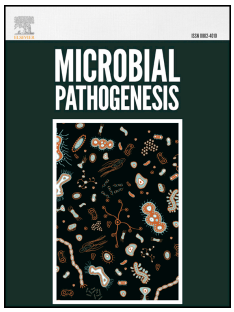

Hypovitaminosis D and reduced cathelicidin are strongly correlated during the multidrug therapy against leprosy

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PII: S0882-4010(20)30739-7

DOI: https://doi.org/10.1016/j.micpath.2020.104373

Reference: YMPAT 104373

To appear in: *Microbial Pathogenesis*

Received Date: 24 April 2020

Revised Date: 25 June 2020

Accepted Date: 1 July 2020

Please cite this article as: Grossi de Oliveira AL, Chaves AT, Cardoso MS, Gomide Pinheiro GR, Parreiras de Jesus AugustoCé, Aparecida de Faria Grossi M, Lyon S, Bueno LL, Otávio da Costa Rocha M, Fujiwara RT, Alves da Silva Menezes C, Hypovitaminosis D and reduced cathelicidin are strongly correlated during the multidrug therapy against leprosy, *Microbial Pathogenesis* (2020), doi: https://doi.org/10.1016/j.micpath.2020.104373.

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Belo Horizonte, June 24th, 2020

Microbial Pathogenesis

Dear Jean-Pierre Gorvel

Editor-in-Chief

We attached the revised version of the manuscript entitled “Hypovitaminosis D and reduced circulating cathelicidin are strongly correlated during the multidrug therapy against leprosy”.

Besides, we are grateful for allowing us to revise our manuscript. We are providing the answers to the reviewers questions and also accepted their suggestions. The modifications were highlightened by line numbers in the original version of the manuscript which we are enclosing with this letter.

Yours sincerely,

Professora Cristiane Alves da Silva Menezes
Departamento de Análises Clínicas e Toxicológicas
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Hypovitaminosis D and reduced cathelicidin are strongly correlated during the multidrug therapy against leprosy

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ABSTRACT

Mycobacterium leprae infection depends on the competence of the host immune defense to induce effective protection against this intracellular pathogen. The present study investigated the serum levels of vitamin D and the antimicrobial peptide cathelicidin, to determine the statistical correlation between them in leprosy patients before and post-six months of multidrug therapy (MDT), household contacts, and healthy individuals. Previous studies associated these molecules with high risks to develop mycobacterial diseases, such as tuberculosis and leprosy. A total of 34 leprosy patients [paucibacillary (n=14), multibacillary (n=20)], and 25 household contacts were recruited. Eighteen healthy adults were selected as a control group. Serum concentrations of vitamin D ($25(OH)VD_3$) and cathelicidin were measured using high-performance liquid chromatography (HPLC), and an enzyme-linked

immunosorbent assay (ELISA) kit, respectively. There were no significant differences in serum levels of 25(OH)VD_3 between all groups, and the overall prevalence rate of vitamin D deficiency was 67.1%. Cathelicidin levels were significantly lower in both untreated and treated patients when compared to controls and household contacts ($p<0.05$). Strong correlations between hypovitaminosis D and reduced cathelicidin in untreated ($r= 0.86$) and post-six months of MDT ($r= 0.79$) leprosy patients were observed. These results suggest that vitamin D status and cathelicidin levels are strongly correlated during multidrug therapy for leprosy and nutritional supplementation from the beginning of treatment could strengthen the immune response against leprosy.

Keywords: Leprosy, *M. leprae*, Vitamin D_3, Cathelicidin, Leprosy drug therapy

1. Introduction

Leprosy remains an important public health problem around the world. According to the World Health Organization (WHO), an estimated 200,000 new cases have been reported each year, and over 11,000 individuals had disabilities at the time of diagnosis [1]. The prevalence and progression of leprosy are previously associated with several factors including socioeconomic situation, food shortage, dietary diversity, household food insecurity [2,3]. In this sense, malnutrition and micronutrient deficiencies, as hypovitaminosis D, constitute crucial risk factors for developing infectious diseases [4–6].

In Leprosy, vitamin D may play antimicrobial and anti-inflammatory functions through modulating immunological pathways to eliminate *Mycobacterium leprae*, the intracellular bacterium that causes Hansen's disease [7–9]. Therefore, vitamin D deficiency would promote susceptibility to leprosy by compromising the innate immune defense, leading to a reduced or inhibited transcription of the antimicrobial peptides genes, as cathelicidins [9–11].

Cathelicidins are extraordinary antimicrobial peptides that are synthesized by humans and animals in response to several pathogen agents. The LL-37 human cathelicidin exhibits deeply spectrum of antimicrobial activity against Gram-positive and Gram-negative bacteria. Besides, its antimicrobial functions, increasing evidence indicates that cathelicidin influences the function of cells involved in adaptive and

innate immune response regulating physiological and pathological processes, as inflammatory responses. This peptide can stimulate pro- and anti-inflammatory mediators, such as cytokines and chemokines, contributing to host homeostasis [12–15].

Little information is available about the close connection between vitamin D and cathelicidin serum concentrations, as well as the general of the correlation before and after leprosy multidrug therapy. Therefore, the present study aimed to determine the vitamin D and cathelicidin serum levels and the statistical correlations between these biomarkers in leprosy patients before and after six-months of MDT, as well as in healthy individuals and household contacts in Brazil.

2. Subjects and Methods

Subjects

Thirty-four leprosy patients, eighteen healthy individuals, and twenty-five household contacts from Brazil were included in this study. Patients were diagnosed by experts on leprosy and enrolled from a Regional Reference Center for Infectious Diseases in Belo Horizonte, the capital of Minas Gerais State, Brazil, from 2014 to 2017. All experiments were carried out at the Universidade Federal de Minas Gerais (UFMG).

Leprosy patients were classified according to the WHO, based on the skin lesions: paucibacillary (PB), when skin lesions were ≤ 5 (n=14), and multibacillary (MB), those with more than 6 lesions (n=20). Clinical forms were confirmed by histopathology, ML-Flow test, and bacilloscopic index (BI). Within the leprosy group both patients, untreated patients (LepT0, n = 15) as well as patients with MDT of 6 months (Lep T6 - MDT group, n = 19) were included (Table 1). The inclusion criteria: men and women older than 18 years with leprosy diagnostic. From these patients, we re-collected blood samples for re-analysis after six months of MDT. Exclusion criteria: Pregnant or nursing women, individuals under the age of 18 years, and patients with comorbidities, co-infections, and inflammatory episodes were excluded, as far as possible, by detailed clinical examination.

Leprosy patients received the standard WHO-MDT for leprosy as described: (1) rifampicin and dapsone for six months to PB patients and (2) rifampicin, dapsone and clofazimine for 12 months to MB or another therapeutic regimen according to medical prescription. After recruited index patients, we invited their households and

enrolled consenting household contacts (n= 25) after clinical examination to rule out the diagnosis of leprosy. Healthy individuals (n= 18) were selected from the non-endemic leprosy area. The ethical statement was obtained by the Bioethical Committee Board of UFMG, and Eduardo de Menezes Hospital, Fundação Hospitalar do Estado de Minas Gerais (FHEMIG), under protocol numbers: #14887414.0.0000.5149 (leprosy patients), and #54988016.0.0000.5149 (household contacts). All individuals were included in this study only after signing the informed consent document.

Methods

Laboratory analyses

Serum samples were stored at -80°C before analysis. Serum 25-hydroxyvitamin D_3 ($25(OH)VD_3$) concentrations were measured by high-performance liquid chromatography (HPLC) following previous studies [16,17], and cutoff values were considered: ≤20 ng/mL and 20–30 ng/mL classified as deficiency and insufficiency, respectively. The normal level was taken to be >30 ng/mL, following to the Sociedade Brasileira de Patologia Clínica/Medicina Laboratorial (SBPC/ML), and the Sociedade Brasileira de Endocrinologia e Metabologia (SBEM) [18].

The cathelicidin serum levels were determined by an enzyme-linked immunosorbent assay (ELISA) using 96 microwell plates (Cloud-Clone Corp., Houston, Texas, USA), exactly according to the manufacturer product manuals. A detection limit of 47.4 ng/mL was observed, and the inter-assay coefficient of variation was <12%. The absorbance was read at 450 nm using the SpectraMax Plus384 microplate reader (Molecular Devices, San Jose, CA, USA) and SoftMax Pro Software to obtain the concentration in ng/mL. Cathelicidin measurements were conducted in duplicate and the concentrations were calculated from a standard curve with a maximum value of 10,000 ng/mL (3x dilutions).

Statistical analyses

Statistical analyses were performed with the statistical package GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA, USA). Shapiro-Wilk test was used to check the dataset normality. Parametric data were analyzed by ANOVA, and Tukey posthoc test to compare means between two or more groups. Non-parametric data were analyzed using the Kruskal-Wallis, and Dunn's test was used to

correct for multiple comparisons. Correlations between vitamin D and cathelicidin serum levels were tested using Pearson's test. Pearson's correlation coefficient r> 0.70 was considered for strong correlations between variables. Statistical significance was defined as $p < 0.05$.

3. Results

General characterization

The general characterization of the PB and MB leprosy patients, healthy control, and household contacts were described in **Table 1**.

Table 1. General characterization of PB/MB leprosy patients, healthy control individuals, and household contacts.

General characteristics	Leprosy patients	Healthy Control	Household Contacts
Age (y)	49.21±12.35	30.3±10.5	32±12.15
Age range [n (%)]			
18 to 60 [68 (88.3)]	27 (79.4)	18 (100.0)	23 (92.0)
> 60 [9 (11.7)]	7 (20.6)	0	2 (8.0)
Sex [n (%)]			
Male [44 (57.1%)]	21 (61.8)	15 (83.3)	8 (32.0)
Female [33 (42.9%)]	13 (38.2)	3 (16.7)	17 (68.0)
Ethnicity [n (%)]			
White [22 (28.6)]	8 (23.5)	6 (33.3)	8 (32.0)
Afro-descendent [54 (70.2)]	25 (73.6)	12 (66.7)	17 (68.0)
Indian [1 (1.3)]	1 (2.9)	0	0
Education [n (%)]			
Unschooled [2 (2.6)]	1 (2.9)	0	1 (4.0)
Elementary [37 (48.0)]	26 (76.5)	2 (11.1)	9 (36.0)
High School [18 (23.4)]	5 (14.8)	4 (22.2)	9 (36.0)
Technician [9 (11.7)]	1 (2.9)	5 (27.8)	3 (12.0)
College [11 (14.3)]	1 (2.9)	7 (38.9)	3 (12.0)
Total (77)	**34**	**18**	**25**

Of the 77 individuals studied, 44 (57%) were male, 68 (88.3%) had 18-60 years, 54 (70.1%) subjects self-declared as Afro-descendant, and 37 (48%) attended at least Elementary education degree **(Table 1)**. Considering the operational classification (WHO), 14 (41.2%) were paucibacillary and 20 (58.8%) multibacillary. Twenty-four (70.6%) showed negative results for ML-Flow test, also zero for BI; 20 (58.8%) had more than six skin lesions; 21 (61.8%) patients showed no neural impairment; 24 (70.6%) had none physical disability; and 16 (47.1%) of patients developed type 1 or type 2 inflammatory immune reactions, throughout the first year of treatment. All leprosy patients were treated following the MDT regimen according to the WHO scheme including rifampicin, dapsone, and clofazimine **(Table 2)**.

Table 2. Clinical characteristics of enrolled leprosy patients.

Leprosy classification (WHO)	**n (%)**
Paucibacillary (PB)	14 (41.2)
Mutibacillary (MB)	20 (58.8)
ML-Flow	**n (%)**
Negative	24 (70.6)
Positive	10 (29.4)
Bacilloscopic Index	**n (%)**
0	24 (70.6)
1 to 2	1 (2.9)
>2 to 3	2 (5.9)
>3 to 4	3 (8.8)
>4	4 (11.8)
Skin lesions	**n (%)**
≤ 5	14 (41.2)
> 5	20 (58.8)
Disabilities degree	**n (%)**
0	24 (70.6)
1	5 (14.7)
2	5 (14.7)
Inflammatory episodes	**n (%)**
No	18 (52.9)
Type 1	13 (38.2)
Type 2	3 (8.8)
Multidrug therapy (MDT)	**n (%)**
Untreated	0
Treated	34 (100%)
Total	**34**

Vitamin D and Cathelicidin serum levels

The median of 25(OH)VD_3 levels was 12.83 ng/mL (Q1=9.27-Q3=54.37 ng/mL) and 15.94 ng/mL (Q1=13.67-Q3=42.74 ng/mL) in the untreated and treated leprosy patient groups, respectively. The quantification of 25(OH)VD_3 levels was not statistically different between leprosy patients and other groups. The overall prevalence rate of vitamin D deficiency (≤20 ng/mL) was 67.1% considering all groups. Healthy control (median: 10.97 ng/mL; Q1=8.40-Q3=19.04 ng/mL) and household contacts (median: 9.65 ng/mL; Q1=6.87-Q3=47.78 ng/mL) presented the lowest vitamin D_3 serum levels than leprosy patients, probably because some patients had taken vitamin D supplementation after leprosy diagnosis. In terms of nutritional aspects, 14 (41.2%) patients were supplemented following the medical protocol: 8 (23.5%) took an association of calcium carbonate plus vitamin D and 6 (17.7%) used ferrous sulfate, vitamin B12 or a non-specified mix of vitamin and minerals. As for sun exposure at work, 11 patients (32.3%) reported activities that included some exposure to the outdoors for at least part of the day **(Table 3)**.

Table 3. 25(OH)D_3 serum levels of included participants and nutritional aspects related to intake and absorption of vitamin D in leprosy patients.

25(OH)VD_3 serum levels	Median (ng/mL) (Q1-Q3)	Deficiency (<20ng/mL)	Insufficient (20-30ng/mL)	Sufficient (>30ng/mL)	Total
Leprosy T0	12.83 (9.27-54.37)	8 (53.3)	-	7 (46.7)	15
Leprosy T6	15.94 (13.67-42.74)	12 (63.1)	1 (5.3)	6 (31.6)	19
Healthy control	10.97 (8.40-19.04)	12 (80.0)	-	3 (20.0)	15
Household contacts	9.65 (6.87-47.78)	15 (71.4)	-	6 (28.6)	21
Total		**47**	**1**	**22**	**70**
Nutritional Supplementation	n (%)	**Supplements**		n (%)	
No	20 (58.8)	None		20 (58.8)	
Yes	14 (41.2)	Calcium carbonate + vitamin D		8 (23.5)	
		Others (ferrous sulfate, vitamin B12, and mix)		6 (17.7)	
Total	**34**			**34**	
Sun exposure (labor)	n (%)				
Non-informed	2 (5.9)				
Yes	11 (32.3)				
No	21 (61.8)				
Total	**34**				

Leprosy T0: untreated leprosy patients; Leprosy T6: post-six months of MDT in leprosy patients.

To characterize 25(OH)VD_3 serum levels, **Figure 1A** presents the median with the interquartile interval for all participants; the majority of them had a vitamin D status lower than 20 ng/mL, considering a total of 70 individuals analyzed. The cathelicidin serum concentration was monitored by ELISA (n=47) and showed reduced values for untreated and post-six months of MDT leprosy patients, compared to healthy control and household contacts **(Figure 1B)**. However, there were no significant differences among leprosy patients. There was a strongly significant correlation between lower 25(OH) VD_3 and lower cathelicidin levels **(Figure 1C)** for both groups, untreated (r=0.86, p=0.0003) and treated patients (r=0.79; p=0.002). These results showed a close connection between these compounds independently of the time of treatment.

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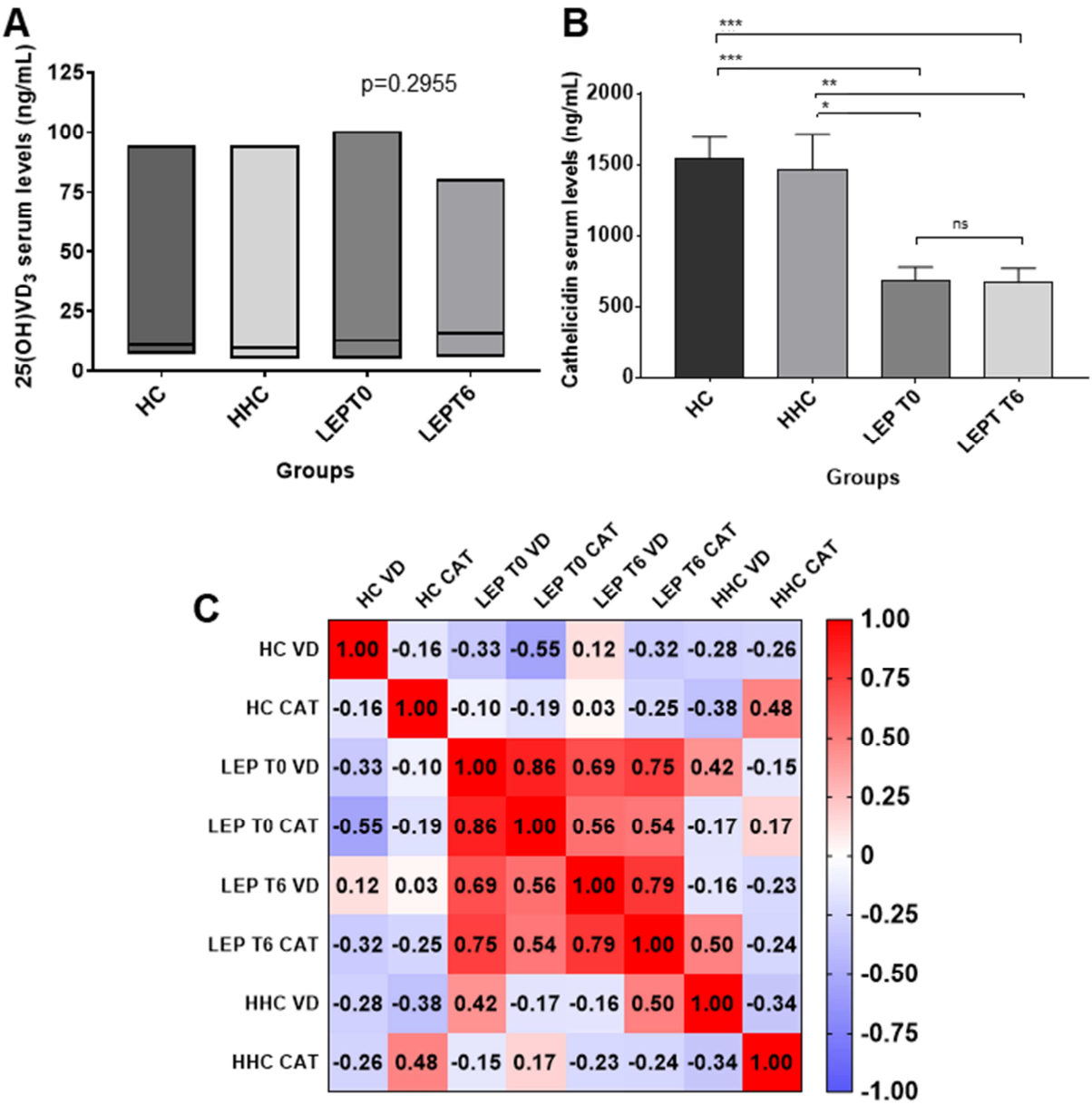

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199 **Fig 1. 25(OH)VD$_3$ and cathelicidin serum levels. (A) 25(OH) Vitamin D$_3$** levels were monitored by
200 HPLC in healthy control (n=15), household contacts (n=21), and untreated leprosy patients (n=15) and
201 post-six months of MDT (n=19). Median with interquartile interval (min and max values) of vitamin D is
202 shown for all groups. No statistically significant differences were observed between studied groups.
203 Non-parametric Kruskal-Wallis test was applied ($p<0.05$). **(B) Serum levels of cathelicidin in** leprosy
204 patients who recently developed the disease (692.5±90.38 ng/mL) and post-six month of treatment
205 (675.2±98.39 ng/mL), healthy individuals (1539±155.2 ng/mL) and household contacts (1462±250.8
206 ng/mL). Data are mean ± SD of n=16, 8, 11, 12 as shown in the graph, respectively. Cathelicidin levels
207 were significantly reduced in both T0 and T6 leprosy patients when compared to healthy controls (T0:
208 p= 0.0008; T6: p= 0.0004) or household contacts (T0: p= 0.0131; T6: p = 0.0092). ANOVA plus Tukey

post-test was performed. Statistically significant differences ($p<0.05$) were represented by * compared between groups in which *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$; ns: statistically not significant. **(C) Correlation between vitamin D and cathelicidin** serum levels in all groups. Vitamin D serum concentrations were strongly correlated with cathelicidin serum levels (red color intensity) in newly diagnosed leprosy patients ($r=0.86$; $p=0.0003$) and either to post-six months of treatment ($r=0.79$; $p=0.002$). The red color indicated strong correlations and blue color demonstrated weak correlations between vitamin D and cathelicidin for all groups. The positive and negative numbers inside the cells are Pearson's correlation coefficient (r). HC: healthy control; HHC: household contacts; LEPT0: untreated leprosy patients; LEPT6: post-six month of MDT in leprosy patients; VD: Vitamin D; CAT: cathelicidin serum levels.

4. Discussion

The induction of vitamin D-dependent antimicrobial pathway in infectious diseases is an important condition for the immune protection against mycobacteria, as *M. leprae* [19–21]. Previous studies postulated that vitamin D is a metabolite that regulates the expression of cathelicidin through its nuclear receptor to activate pathways in the innate and adaptative immune responses against intracellular pathogens [22,23].

Furthermore, vitamin D is correlated to cathelicidin in tuberculosis patients, indicating the existence of a clinical linkage between them and the risk of severity of this disease [24–27]. Besides, low cathelicidin levels have been associated with severe disease in chronic neutropenia [28], strongly induced in human keratinocytes [29], reduced expression in atopic dermatitis [30], and also correlated to oral infections [31,32].

In this study, we provided statistical evidence that there were tight correlations between $25(OH)VD_3$ and cathelicidin serum levels, before and after six-months of MDT, in leprosy patients. It was observed hypovitaminosis D in all studied groups, however, the vitamin D deficiency strongly correlated with low serum cathelicidin in untreated patients ($r= 0.86$), as well as, after six months of MDT ($r= 0.79$). These strong correlations were not observed for household contacts ($r= -0.34$) and healthy control groups ($r= -0.16$).

Besides, it was observed that leprosy patients had a combination of risk factors for vitamin D absorption, such as dark skin, low daily sun exposure, and the absence of nutrient supplementation. It is important to consider that the conversion of

vitamin D is fully influenced by the color of the skin since it is a natural barrier to the UV radiation entry [33,34]. Perhaps, this condition reflects an important point to be considered in leprosy patients, since around 73% of them declared to be Afro-descendants. Together, these elements contribute to a deficient immune response in leprosy patients.

Indeed, previous studies have suggested that low vitamin D levels may be related to leprosy. Firstly, Matzner *et al.*, 2011 [22] observed low serum levels of cathelicidin in untreated and treated Yemen patients. Also, Lyrio *et al.*, 2015 [35] demonstrated a positive cathelicidin modulation on human keratinocytes during the disease. Kim *et al.*, 2018 [9] showed that the activation of the vitamin D pathways during macrophage differentiation increased the antimicrobial responses against *M. leprae* infection. In this study, the reduced cathelicidin actions may have effectively contributed to 47.1% of patients developing leprosy reactions during the first year of treatment.

An effective host immune response against intracellular *M. leprae* requires both innate immune mechanisms as well as cell-mediated immune responses [36]. Our data advert that leprosy is associated with strikingly decreased serum levels of vitamin D and cathelicidin, especially for untreated patients. However, this fact seems to alert to a condition of immediate response in leprosy, since there were no strong correlations between these two biomarkers when analyzing the household contacts and healthy individuals, even under hypovitaminosis D status.

Moreover, the decreased vitamin D might also lead to defective transcription or polymorphism of the vitamin D receptor (VDR), as shown to Fok1 and Taq1 [37], which could lead to a decrease in cathelicidin production, since the human cathelicidin antimicrobial peptide gene is a direct target of VDR [38]. Thereby, a defective VDR signaling could result in impaired immunity against *M. leprae* mediated by this receptor [38,39]. Cathelicidin depends on vitamin D activity, with its binding to the intracellular VDR, explaining the proposed link between hypovitaminosis D and susceptibility to mycobacterial diseases [40].

On the other hand, the weak correlations between reduced serum vitamin D levels and cathelicidin in healthy individuals and household contacts suggest that these immunological mechanisms are truly related to infection by *M. leprae* in leprosy patients, regardless of the time of treatment. Cathelicidin acts as a chemoattractant for several inflammatory and immunological cells, stimulating phagocytosis,

promoting wound healing and angiogenesis, all expected events, and related to the success of treatment in leprosy [41–44].

Thus, understanding the processes that regulate the immune response can be fundamental for the clearance of *M. leprae* infection. Strong correlations between hypovitaminosis D and reduced cathelicidin are an intriguing link for leprosy susceptibility. These data emphasize the importance of maintaining adequate vitamin D serum levels, especially for people exposed to the direct influence of *M. leprae* as household contacts. Therefore, the monitoring of vitamin D deficiency can contribute to managing clinical evolution in leprosy. Initiating appropriate dietary changes and introducing nutritional supplementation from the beginning of treatment could strengthen the immune response to leprosy.

Acknowledgments

We are grateful for the engagement of the healthcare workers and patients of the Hospital Eduardo de Menezes – FHEMIG, healthy volunteers, and household contacts who participated.

Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG) (RTF: #APQ-04035-17; CASM: #APQ-02871-15; MOCR: #APQ-02332-13). ALGO was supported by a doctoral's fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico - CNPq. RTF (#303345/2018-7) and MOCR (#485891/2013-1) are CNPq Research Fellows (Bolsa de Produtividade em Pesquisa).

Conflicts of interest statement: The authors declared that they have no competing interests.

Author contributions

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Highlights

1. Correlation between Vitamin D and cathelicidin levels in leprosy patients was investigated.
2. Vitamin D deficiency was detected in the majority of analyzed individuals.
3. Cathelicidin serum levels were significantly lower in leprosy patients.
4. Hypovitaminosis D and low cathelicidin levels are correlated in leprosy patients.
5. Vitamin D supplementation could strengthen the immune response against leprosy.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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