Association between *Mycobacterium avium* complex lung disease and serum vitamin D status, antimicrobial peptide levels, and bone mineral density

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**Abstract**

Vitamin D maintains calcium balance and has immunomodulatory effects. Only few studies have revealed the relationship between vitamin D and its associated factors in *Mycobacterium avium* complex (MAC) infection. This study aimed to investigate the effects of MAC infection on serum vitamin D, human cationic antimicrobial protein 18, its C-terminal 37 amino acid fragment (hCAP18/LL-37) levels, and bone mineral density (BMD).

We enrolled 58 patients with MAC lung disease and 15 control participants. Serum 25-hydroxyvitamin D and hCAP18/LL-37 levels were measured via enzyme-linked immunosorbent assay. Lastly, computed tomography scan density readings of the BMD of the thoracic and lumbar vertebral bones (Th4, Th7, Th10, and L1) were assessed.

No significant differences in patient characteristics and serum vitamin D levels were observed. Patients with MAC lung disease had significantly low serum hCAP18/LL-37 levels (P = .049). Moreover, low BMD of the mean thoracic and lumbar vertebral bones was observed (mean Th, P = .012; L1, P = .48, respectively). A higher prevalence of scoliosis (P = .031) was observed in the participants with low BMD compared with the control participants. Based on a multivariate analysis, patients with MAC lung disease had significantly lower body mass index [odds ratio (OR), 19.1; 95% confidence interval (CI), 2.0–190.9; P < .01] and vertebral BMD (OR, 12.4; 95% CI, 1.7–160.6; P = .012) than control participants.

Serum hCAP18/LL-37 level and BMD were significantly decreased in patients with MAC lung disease without relation to serum vitamin D level. The vitamin D–independent pathway might affect the waning of antimicrobial peptides and decrease in BMD.

**Abbreviations:** BALF = bronchoalveolar lavage fluid, BMD = bone mineral density, BMI = body mass index, CAMP = cathelicidin antimicrobial peptide, CI = confidence interval, CT = computed tomography, DEXA = dual-energy x-ray absorptiometry, ER = endoplasmic reticulum, hCAP/LL-37 = human cationic antimicrobial protein 18, IFN-γ = interferon-gamma, MAC = *Mycobacterium avium* complex, NTM = nontuberculous mycobacteria, OR = odds ratio, ROI = region of interest, VDR = vitamin D receptor.

**Keywords:** antimicrobial peptide, bone mineral density, cathelicidin, *Mycobacterium avium* complex, nontuberculous mycobacteria, vitamin D

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KF and YI contributed equally to this manuscript.

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1. Introduction

The incidence of Mycobacterium avium complex (MAC) lung disease is increasing worldwide.[^1-3] Not only immunocompromised patients but also middle-aged and older women without predisposing factors acquire this disease. Various host traits are considered risk factors for MAC lung disease.[^4] Numerous studies have shown that vitamin D deficiency is associated with the susceptibility and progression of various infectious diseases.[^5,6] Recently, vitamin D–deficient patients with pulmonary nontuberculous mycobacteria (NTM) disease are highly susceptible to disease progression.[^7]

A previous study revealed that vitamin D regulates human cathelicidin antimicrobial peptide (CAMP) gene expression.[[^8] The only human cathelicidin, human cationic antimicrobial protein (hCAP) 18 and its C-terminal 37 amino acid fragment (LL-37) have multiple functions and play important roles in the innate immune system. hCAP18/LL-37 suppresses mycobacterium growth in macrophages and has antimicrobial effects.[[^9] Vitamin D is now known to have immunomodulatory effects. Because only few reports on the relationship between patients with mycobacterial disease and in vivo hCAP18/LL-37 levels are available, the nature of hCAP18/LL-37 is still unknown.[[^10,11]

Vitamin D is also known to regulate not only antimicrobial peptides but also metabolic factors that affect the bones. Patients with chronic viral infectious disease, such as HIV and hepatitis C virus infection, are also deficient in vitamin D and thus have causal osteoporosis.[[^12-14] MAC lung disease causes chronic inflammation and the deterioration of the bronchi and alveoli. Patients with MAC lung disease may be deficient in vitamin D and have an imbalance of its associated factors, therefore causing osteoporosis. This finding is similar for patients with chronic infectious diseases. However, no report on the relationship between chronic MAC infection and osteoporosis is available.

Here, we conducted a prospective cohort study to investigate the association between MAC lung disease and serum vitamin D status, hCAP18/LL-37 levels, and bone mineral density (BMD) of the thoracic and lumbar vertebrae. Part of this study was presented at the European Respiratory Society International Congress 2013 in Barcelona, Spain.

2. Methods

2.1. Study design, population, and the evaluation of factors

We prospectively enrolled 58 patients with MAC lung disease who matched the 2007 American Thoracic Society diagnostic criteria[[^15] and 15 control participants from January 2011 to January 2014. All participants were postmenopausal women without HIV infection and active endocrine disorder and do not use drugs that affect bone metabolism and any vitamin supplements. Control participants who had no obvious lung involvement were selected from routine medical checkups. We obtained written informed consents from all participants, and the institutional review board (Kyoto University Hospital Ethics Committee) approved this study (Approved number: E1077). Next, we evaluated serum 25-hydroxyvitamin D and vitamin D–associated factors. In the study, hCAP18/LL-37 and BMD are considered as the associated factors of vitamin D. The serum 25-hydroxyvitamin D and hCAP18/LL-37 levels and BMD of the vertebrae were measured.

2.2. Measurement of serum 25-hydroxyvitamin D and hCAP18/LL-37 levels

Peripheral blood samples were collected from all participants. The serum was separated from the blood samples and stored at −80°C. We measured the serum 25-hydroxyvitamin D and hCAP18/LL-37 levels with the enzyme-linked immunosorbent assay (ELISA) technique using serum 25 (OH) vitamin D Xpress kit (Immundiagnostik, Bensheim, Germany) and serum LL-37 HUMAN ELISA kit (Hycult Biotech, Uden, The Netherlands).

2.3. Measurement of BMD

The BMD of the fourth, seventh, and tenth thoracic and the first lumbar vertebrae was measured using computed tomography (CT) scan data in accordance with the previous reports.[[^16-19]] We used 5 sequential chest CT scan slices. First, a midvertebral slice was selected using reconstructed images from CT scan data of 0.5-mm thick slices. Second, the elliptical region of interest (ROI) was encompassed manually as the largest possible area at the anterior portion of each vertebral body on the selected slice. Finally, the mean CT scan density of the ROI was measured. BMD was calculated as the mean CT scan density using the following formula: BMD (mg/mL) = 0.77 × CT scan density (HU) + 1.83. This formula was derived from the calibration phantom (Kyoto Kagaku Co, Ltd, Kyoto, Japan), which contained 8 tubes of known concentrations of hydroxyapatite (0, 50, 100, 150, 200, 250, 300, and 400 mg/mL).

3. Statistical analysis

All statistical analyses were performed using JMP version 8 (SAS Institute Inc, Cary, NC). Results for continuous variables are presented as the mean ± standard deviation. Group comparisons were made using the chi-square, Fisher exact, and Wilcoxon tests. We used the Spearman correlation test to evaluate the degree of association between variables. To identify independent contributing factors to MAC lung disease, variables were included in a stepwise regression analysis if the probability values obtained via a univariate analysis are <.05. Odds ratios (ORs) and their respective 95% confidence intervals (CIs) were computed as estimates of relative risk. A P value of <.05 was considered statistically significant.

4. Results

4.1. Characteristics of the study participants

The characteristics of the participants are shown in Table 1. All participants were postmenopausal women and aged older than 60 years. Patients with MAC lung disease were predominantly infected with the M. avium strain (81%), and a high incidence of scoliosis, a thoracic abnormality, was observed in these patients (27.6% vs 0%, P=.031). Body mass index (BMI) was significantly lower in patients with MAC lung disease than in control participants (18.9±2.4 vs 22.1±4.1, P<.01). No significant differences in the underlying diseases were noted.

Laboratory findings showed that white blood cell, lymphocyte, and platelet counts were significantly lower in patients with MAC lung disease than in control participants.

4.2. 25-Hydroxyvitamin D and hCAP18/LL-37 levels and BMD

Comparisons of vitamin D level and vitamin D–associated factors are shown in Table 2. No significant difference in serum vitamin
Laboratories

| Clinical characteristics | Pulmonary MAC patients (n = 58) | Control subjects (n = 15) | P  |
|--------------------------|---------------------------------|---------------------------|----|
| Age, y                   | 67.1 ± 4.6                      | 64.3 ± 8.4                | .23|
| Body mass index, kg/m²   | 18.9 ± 2.4                      | 22.1 ± 4.1                | <.01|
| Smoking status (never)   | 55 (94.8)                       | 14 (93.3)                 | .99|
| Underlying disease       |                                 |                           |    |
| Severe pneumonia (hospitalization) | 10 (17.2)                     | 0 (0.0)                   | .11|
| COPD                     | 2 (3.4)                         | 0 (0.0)                   | .99|
| Asthma                   | 2 (3.4)                         | 0 (0.0)                   | .99|
| History of tuberculosis  | 2 (3.4)                         | 1 (6.7)                   | .5 |
| History of malignant disease | 6 (10.3)                    | 3 (20.0)                  | .38|
| Diabetes mellitus        | 4 (6.9)                         | 2 (13.3)                  | .6 |
| Autoimmune disease       | 3 (5.2)                         | 2 (13.3)                  | .27|
| Injected MAC strain (M. avium) | 47 (81.0)                    | -                         | -  |
| Duration of MAC disease, y | 6.9 ± 5.8                      | -                         | -  |
| Positive sputum smear    | 10 (17.2)                       | -                         | -  |
| Positive sputum culture  | 32 (55.2)                       | -                         | -  |

| Laboratories |
|--------------|
| WBC, ×10⁹/µL | 5.3 ± 1.7 | 6.6 ± 1.5 | <.01 |
| Neutrophil, ×10⁹/µL | 3.3 ± 1.5 | 3.8 ± 1.1 | .11 |
| Lymphocyte, ×10⁹/µL | 1.5 ± 0.47 | 2.2 ± 0.59 | <.01 |
| RBC, ×10⁶/µL | 4.3 ± 0.30 | 4.5 ± 0.23 | .094 |
| Hemoglobin, g/dL | 12.8 ± 0.97 | 13.2 ± 0.61 | .18 |
| Platelet, ×10⁹/µL | 2.3 ± 0.60 | 2.6 ± 0.44 | .046 |
| Total protein, g/dL | 7.2 ± 0.44 | 7.1 ± 0.31 | .33 |
| Albumin, g/dL | 4.2 ± 0.36 | 4.2 ± 0.24 | .63 |
| ESR, mm/h    | 23.4 ± 21.3 | 20.8 ± 4.2 | .46 |
| CRP, mg/dL   | 0.34 ± 0.54 | 0.36 ± 1.0 | .14 |

| HRCT findings |
|---------------|
| Nodule        | 52 (89.7) | - | - |
| Consolidation | 36 (62.1) | - | - |
| Bronchiectasis| 53 (91.4) | - | - |
| Cavity        | 24 (41.4) | - | - |

| Thoracic abnormality |
|----------------------|
| Scoliosis            | 16 (27.6) | 0 (0.0) | .031 |
| Pectus excavatum      | 10 (17.2) | 1 (6.7) | .44 |

| Location of HRCT findings |
|---------------------------|
| Right/left upper lobe     | 43 (74.1) | - | - |
| Right middle lobe/Influenal | 55 (94.8) | - | - |
| Right/left lower lobe     | 46 (79.3) | - | - |

Data are shown as mean ± standard deviation (SD) or number (%).

Table 3
Correlation between antimicrobial peptide and bone mineral density.

|                      | Pulmonary MAC patients (n = 58) | Control subjects (n = 15) | P   |
|----------------------|---------------------------------|---------------------------|-----|
| hCAP18/LL-37         |                                 |                           |     |
| Serum                |                                 |                           |     |
| Total MAC            | −0.117 ± 0.39                   | 0.07 ± 0.16               | .83 |
| Mean Th BMD          | 0.199 ± 0.16                    | −0.662 ± 0.019           | .019|
| L1 BMD               | 0.189 ± 0.19                    | −0.483 ± 0.11            | .11 |

4.4. Factors associated with MAC lung disease

Table 5 depicts the results of the univariate and multivariate analyses of factors associated with MAC lung disease. We selected variables with a P value of .05 obtained via the univariate analysis of continuous variables for the multivariate analysis. We analyzed each continuous variable after changing the categorical variable. In the multivariable analysis, patients with MAC lung disease had significantly lower BMI (OR, 19.1; 95% CI, 2.0–419.0; P < .01) and lower vertebral BMD (OR, 12.4; 95% CI, 1.7–160.6; P = .012) than control participants.

5. Discussion

In this study, we showed 2 major implications related to vitamin D and its associated factors in patients with MAC lung disease. First, we found that serum hCAP18/LL-37 levels significantly decreased in patients with MAC lung disease, and it was associated with blood cell counts but not with serum 25-hydroxyvitamin D levels. Previously, 2 studies evaluated the relation between serum 25-hydroxyvitamin D level and hCAP18/LL-37 in patients with mycobacterial infections. Yamshchikov...
et al.\textsuperscript{[10]} from the United States, reported that the serum vitamin D level was not correlated with the serum hCAP18/LL-37 level in patients with active tuberculosis. Another study that was conducted by Kim et al.\textsuperscript{[11]} in Korea also revealed no correlation between hCAP18/LL-37 level and vitamin D level in patients with NTM lung disease. Both reports failed to demonstrate any correlation between vitamin D levels and hCAP18/LL-37 levels, and these findings were consistent with previous reports. The regulatory functions of vitamin D are involved in the localized upregulation of hCAP18/LL-37 expression through the stimulation of immune cells by the vitamin D active metabolite.\textsuperscript{[8]} A recent study showed higher concentrations of hCAP18/LL-37 in bronchoalveolar lavage fluids (BALFs) obtained from patients with pulmonary tuberculosis.\textsuperscript{[20]} Our study evaluated the serum concentration of hCAP18/LL-37, which represents the systemic concentration. Thus, the systemic concentration of hCAP18/LL-37 did not correlate with serum vitamin D levels. Another study also depicted that the systemic concentration of hCAP18/LL-37 did not correlate with serum vitamin D levels in healthy participants who received vitamin D supplementation.\textsuperscript{[21]} Therefore, hCAP18/LL-37 may have different dynamics in local and systemic environment. In our study, a significant correlation between blood cells and hCAP18/LL-37 levels was noted. Because neutrophils and monocytes are known as hCAP18/LL-37 sources, a positive correlation with white blood cell counts is possible.\textsuperscript{[22]} On the contrary, the correlation between other blood cells and hCAP18/LL-37 levels is still not clear. Recently, vitamin D–independent pathways have been reported. One is an endoplasm reticulum (ER) stress signaling pathway\textsuperscript{[23]} and the other is curcumin pathway.\textsuperscript{[24]} The former, ER stress increases hCAP/LL-37 expression via nuclear factor-kappa B (NF-\textkappa B)-CCAAT-enhancer-binding proteins (NF-\textkappa B/C/EBP\textalpha) activation, which is independent of vitamin D receptor (VDR) pathway. The latter, curcumin activates hCAP/LL-37 expression by unknown mechanisms, which are independent of both VDR pathway and ER stress pathway. Possibility of involving these mechanisms to our results will be a subject of future investigation.

In the present study, patients with MAC lung disease showed lower hCAP18/LL-37 levels than control participants. Other studies presented that the hCAP18/LL-37 level was not increased in patients with NTM lung disease, whereas a higher concentration of hCAP18/LL-37 was observed in patients with active pulmonary tuberculosis and positive sputum smear.\textsuperscript{[10,11]} Recently, Honda et al.\textsuperscript{[25]} discovered that \textit{M. avium} and \textit{M

### Table 4

Factors correlated with hCAP18/LL-37 and bone mineral density in patients with \textit{Mycobacterium avium} complex lung disease.

| factor                          | Serum hCAP18/LL37| Bone mineral density (mean Th) | Bone mineral density (L1) |
|--------------------------------|------------------|--------------------------------|---------------------------|
|                                | Spearman ρ       | P                              | Spearman ρ               | P                              |
| Age                            | –0.177 .19       | –0.452 <.01                    | –0.484 <.01              |
| BMI                            | 0.186 .18        | –0.173 .22                    | 0.256 .064               |
| hCAP18/LL-37                   | 0.0695 .62       | 0.0695 .62                     | 0.092 <.01               |
| Mean Th BMD                    | 0.0695 .62       | 0.0695 .62                     | 0.092 <.01               |
| L1 BMD                         | –0.069 .63       | 0.92 <.01                      | –                       |
| WBC                            | 0.283 .034       | 0.0124 .03                     | 0.0345 .81               |
| Neutrophil                     | 0.221 .1         | –0.0013 .99                    | 0.072 .61                |
| Lymphocyte                     | 0.268 .046       | 0.0087 .85                     | –0.0625 .66              |
| Hemoglobin                     | 0.315 .018       | 0.03 .83                       | –0.091 .52               |
| Platelet                       | 0.33 .012        | –0.049 .73                     | 0.0029 .98               |
| Total protein                  | 0.165 .23        | 0.179 .2                       | 0.192 .17                |
| Albumin                        | 0.0601 .67       | –0.059 .68                     | –0.155 .28               |
| ESR                            | –0.0924 .53      | 0.145 .34                      | 0.123 .42                |
| CRP                            | 0.122 .37        | –0.0668 .63                    | –0.131 .35               |
| Nodule                         | –0.136 .32       | 0.0065 .85                     | 0.0701 .62               |
| Consolidation                  | 0.205 .13        | 0.0394 .78                     | 0.0407 .77               |
| Bronchiectasis                 | 0.116 .39        | 0.203 .14                      | 0.262 .058               |
| Cavity                         | 0.117 .39        | –0.223 .11                     | –0.0983 .48              |
| No. of involved lobes          | –0.22 .1         | 0.0334 .81                     | 0.129 .36                |
| Scoliosis                      | 0.011 .94        | 0.113 .42                      | 0.101 .47                |
| Pectus excavatum               | 0.127 .35        | 0.095 .62                      | 0.108 .44                |

BMD = bone mineral density, BMI = body mass index, CRP = C-reactive protein, ESR = erythrocyte sedimentation rate, hCAP18/LL-37 = human cationic antimicrobial protein 18 and its C-terminal 37 amino acid fragment, WBC = white blood cell.

### Table 5

Factors associated with \textit{Mycobacterium avium} complex lung disease.

| factor                          | Univariate analysis | Multivariate analysis |
|--------------------------------|---------------------|----------------------|
|                                | OR (95% CI)         | P                    | OR (95% CI)         | P     |
| Low BMI (<20.0), kg/m\textsuperscript{2} | 4.3 (1.3–15.7)      | <.01                 | 19.1 (2.0–419.0)    | <.01  |
| Low lymphocyte (<1.9), \times10\textsuperscript{3}/μL | 7.9 (2.1–39.0)      | <.01                 | 3.6 (0.45–37.4)     | .23   |
| Low platelet (<2.45), \times10\textsuperscript{3}/μL | 3.3 (0.92–13.5)     | .068                 | 4.7 (0.56–67.0)     | .16   |
| Low hCAP18/LL-37 (<38), ng/mL | 4.5 (1.2–22.4)      | .029                 | 4.5 (0.50–62.3)     | .18   |
| Low mean Th BMD (<170), mg/mL | 6.0 (1.8–24.2)      | <.01                 | 12.4 (1.7–160.6)    | .012  |

BMD = bone mineral density, BMI = body mass index, CI = confidence interval, hCAP18/LL-37 = human cationic antimicrobial protein 18 and its C-terminal 37 amino acid fragment, OR = odds ratio.
intracellular} were resistant to hCAP18/LL-37, regardless of the presence of glycopeptidolipids. Moreover, recent study showed that when hCAP18/LL-37 is exposed to NTM, it loses its lost antimicrobial effect. Based on these basic experimental data, the hypothesis that hCAP18/LL-37 in our patients with MAC lung disease may be attenuated by exposing to NTM and decreased serum concentration. On the contrary, we need to think the differences between local and systemic inflammations. Our results showed decreasing serum concentration of hCAP18/LL-37, which showing systemic status. But there is the potential for an increasing concentration of hCAP18/LL-37 in local site, especially alveolar area. To confirm this speculation, we need analysis of BALF or lung biopsy specimens. This will be also subject for a future study.

Second, we found that low BMD was observed in patients with MAC lung disease. However, it is not correlated with serum 25-hydroxyvitamin D level and low BMI. To our knowledge, this is the first study on the quantitative evaluation of BMD in participants with or without MAC lung disease via CT. Although the standard technique used for the evaluation of BMD is a dual-energy x-ray absorptiometry (DEXA), the use of CT has more advantages. CT can evaluate a 3-dimensional BMD, which reflects volume density, and is more precise than DEXA, which reflects area density. In addition, if a calibration phantom is observed, CT scan data, which are intended for the evaluation of MAC lung disease, can be used for evaluating 3-dimensional BMD, and additional x-ray exposure is no longer needed. CT has already been used for evaluating BMD in patients with several pulmonary diseases, such as chronic obstructive pulmonary disease, interstitial pneumonia, and sleep apnea syndrome. No correlation between low BMD and serum 25-hydroxyvitamin D level and low BMI was observed in this study. Furthermore, the vitamin D-independent mechanism might decrease the BMD in patients with MAC lung disease. Because persistent MAC infection causes chronic inflammation, BMD is naturally decreased in patients with MAC lung disease than that of control participants. Numerous studies had revealed that chronic inflammation causes the reduction of BMD and leads to osteoporosis. Mycobacterial infection is characterized by cellular immune reactions. The expression of interferon-gamma (IFN-γ) plays an important role not only in immune reactions but also in bone metabolism. In elderly patients, IFN-γ-mediated inflammations were significantly associated with decreased BMD. Other proinflammatory cytokines, including osteoponint and interleukin (IL)-1, IL-6, IL-11, and IL-17, were also released by antigen-primed T cells and promote bone resorption by mediating the production of the receptor activators of nuclear factor kappa-B ligand, which interact with RANK and enhance osteoclast proliferation and differentiation. Patients with MAC-LD in our study had showed decreased inflammatory cells (Table 1) and this phenomena indicated less inflammation which suggesting paradoxical status of decreased BMD potentially caused by chronic inflammation. Previous articles provided some suggestions in this paradoxical status. Elderly patients with immunosenescence shows the inflammation, which is characterized by chronic low-grade inflammation. In this status, inflammatory cells in blood do not always reflect chronic inflammatory cell infiltration in the local sites. Systemic inflammation status does not always appear in phenotypic manifestations. Although this is our speculation, there may exist the imbalances between intravital inflammation and the number of inflammatory cells in blood samples in this study. Although precise mechanisms are unknown, the results of our study may have been affected by vitamin D-independent mechanisms that are responsible for the development of osteoporosis. In addition, further study is needed to clarify these phenomena in patients with MAC lung disease.

This study has some limitations. First, because our study was cross-sectional and conducted only in a single center with a limited number of patients, a longitudinal study with a larger number of patients in a multicenter will be needed to confirm our results. Second, to remove the sex hormone bias, only postmenopausal women were recruited for this study. Our study results are not directly applicable to the general population. Third, because the expression of mRNAs for VDR, mitochondrial 1α-hydroxylase enzyme (CYP27B1), and CAMP was not evaluated, the relationship between gene expression levels and serum concentrations of vitamin D and hCAP18/LL-37 were not discussed. Recent study showed interesting results that the gene expression level of CAMP significantly increased in patients with MAC lung disease than that of the control participants. However, the serum hCAP18/LL-37 level did not differ between patients and control participants. This expression gap between the gene level and serum level should be further investigated in the future.

6. Conclusions

In conclusion, we found both serum hCAP18/LL-37 level and BMD were significantly decreased in patients with MAC lung disease without relation to serum vitamin D level. The vitamin D-independent pathway might affect the waning of antimicrobial peptides and the decrease in BMD. In addition, a large-scale study should be conducted to present all the mechanisms.

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Author Contributions

KF and YI designed this study. KF, YI, and TH recruited and cared for the patients. KF drafted and revised the manuscript. YI and TH revised the manuscript. TO, TM, AN, and TH supervised this study and revised the manuscript.

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