Mycobacterial infection influences bone biomarker levels in Crohn’s disease

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Mycobacterial infection influences bone biomarker levels in Crohn's disease

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Abstract

Patients with Crohn’s disease have higher risk for osteoporosis following decreased level of osteocalcin. We hypothesize that active inflammation following *Mycobacterium avium subspecies paratuberculosis* (MAP) infection results in elevation of undercarboxylated Osteocalcin (ucOC) and downregulation of active Osteocalcin in CD patients and cow disease-model (Johne’s disease). In this study, we measured ucOC, active osteocalcin and calcium levels in sera from 42 cattle (21 infected with MAP and 21 healthy cattle), 18 CD patients and 20 controls. The level of ucOC in MAP+ bovine samples was higher (318±57.2 nmol/mL vs. 289±95.8 nmol/mL) than MAP- controls, P-value >0.05. Consequently, average calcium level in bovine MAP+ was significantly higher (9.98±0.998 mg/dL vs. 7.65±2.12 mg/dL) than bovine-MAP- samples (P-value<0.05). Also, the level of ucOC was higher (561±23.7 nmol/mL vs. 285±19.6 nmol/mL) in CD-MAP+ compared to CD-MAP- (P-value<0.05). Interestingly, the average osteocalcin level in MAP+ bovine was lower (797±162 pg/mL vs. 1190±43 pg/mL) than in MAP- bovine and it was lower (1.89±0.184 ng/mL vs. 2.19±0.763 ng/mL) in CD with MAP compared to CD without MAP infection (P<0.05). The correlation between MAP infection and elevation of sera ucOC, reduction of active osteocalcin and increased calcium supports MAP infection role in CD and complications with osteoporosis.

Keywords

*Mycobacterium paratuberculosis* (MAP), Crohn’s disease, Osteocalcin, undercarboxylated osteocalcin, osteoporosis, IBD.
Introduction

Crohn’s disease (CD) is a severe relapsing form of inflammatory bowel disease (IBD), which has been associated with increasing number of fracture incidents and osteoporosis prevalence among affected patients (Andreassen et al. 1997; Frei et al. 2006). This might be due to several factors, such as nutrient malabsorption, reduced physical activities, prolonged corticosteroid use and vitamin D deficiency (Vos et al. 1998; Habtezion et al. 2002). However, the main reason behind altered bone metabolism in CD remains unclear. About 50% of IBD patients in general have been diagnosed with low bone mineral density (osteopenia) (Compston et al. 1987; Pigot et al. 1992). Higher rates of bone loss have been detected at the lumbar spine by using quantitative computed tomography or dual-energy X-ray absorptiometry (Motley et al. 1988; Roux et al. 1995). These results indicate that CD patients are required to perform a routine assessment of their bone mineral density (BMD) and biochemical markers of bone turnover such as serum bone alkaline phosphatase, serum type 1 procollagen and total osteocalcin level.

Osteocalcin is a bone specific protein that is secreted specifically by osteoblasts and is encoded by the bone gamma-carboxyglutamic acid-containing protein (BGLAP) gene (Raymond et al. 1999). Its synthesis is dependent on both vitamin K and vitamin D (Lian et al. 1989). Osteocalcin plays a major role in bone mineralization and calcium homeostasis (Lee et al. 2007). In addition to that, osteocalcin works like a hormone by inducing insulin secretion in the pancreatic beta cells and adiponectin release by adipocytes (Lee et al. 2007).
Osteocalcin must be Y-carboxylated before it gets incorporated into bone matrix where it functions in bone calcification (Hauschka et al. 1989). Without this step, there will be higher levels of undercarboxylated osteocalcin (ucOC) circulating in the blood leading to bone turnover and lower BMD (Hauschka et al. 1989). In fact, ucOC as an inactive form of osteocalcin was found to be higher in elderly women, which lead to increasing the risk for hip fractures (Szulc et al. 1993). Consequently, following one year of calcium and vitamin D treatment, ucOC level decreased significantly (Szulc et al. 1993).

CD has been found to be a complex disease involving interaction of several factors including environmental triggers, abnormal immune response, genetics and most importantly microbial agents (Sartor et al. 2006). One of the most extensively studied microorganisms involved in the pathogenesis of CD is *Mycobacterium avium* subspecies *paratuberculosis* (MAP) which has been known to be a causative agent for Johne’s disease (JD), which is a chronic gastrointestinal infection affecting ruminants (Naser et al. 2004; Qasem et al. 2017).

This pathogen has been isolated from intestinal tissues, blood and breast milk of CD patients (Naser et al. 2000; Naser et al. 2010; Qasem et al. 2016). Effective eradication of MAP by antibiotic treatment is undergoing an FDA-approved phase III international clinical trial for moderate to severe cases of CD with an investigational Anti-MAP therapy (RHB-104) (Alcedo et al. 2016; Qasem et al. 2016). The main objective of this study was to assess any potential correlation between MAP infection and bone turnover biomarkers characterized by Y-carboxylated osteocalcin level, ucOC and blood calcium level in both humans and bovine blood samples.
Materials and Methods

Bovine Samples

Sera samples from 21 healthy and 21 Johne’s disease infected cattle were kindly provided by Dr. Michael Collins (University of Wisconsin - Madison). MAP infection was tested by using the IDEXX Mycobacterium paratuberculosis (M. pt.) Antibody Test Kit (IDEXX Laboratories, Westbrook, ME, USA) following manufacturer instructions as described earlier (Qasem et al. 2016).

Human Samples

Sample processing

Clinical samples were collected following the University of Central Florida-Institutional Review Board approval number IRB00001138; all subjects have signed a written consent in order to participate in the study. Human blood samples were collected in two separate sets where each subject provided three 6.0-ml K$_2$-EDTA tubes. A total of 27 human blood samples were collected from CD patients (12 males and 15 females) along with 27 samples (10 males and 17 females) of their healthy biological family members (parents or siblings) at the University of Florida (UF). The age of CD patients ranged between 16 to 56 with an average of 32 years old, while their healthy relatives ranged between 18 to 65 with an average of 45 years old. White blood cells and plasma samples were separated as described earlier [18] and stored at -20°C. Based on MAP infection, 18 CD patients and 20 healthy subjects were selected for this study.

DNA extraction and nested PCR analysis
DNA extraction and PCR analysis was done on purified white blood cells separated from blood samples as described earlier (Qasem et al. 2016). Briefly, diagnosis of MAP infection by using nested PCR (nPCR) was dependent on the MAP-specific IS900 derived oligonucleotide primers. The first round of amplification was performed by using P90 (GTTCGGGGCCGTCGCTTAGG) and P91 (GAGGTCGATCGCCCACGTGA) primers at the following conditions: 95 °C for 5 min, then 34 cycles of 95 °C for 1 min, 58 °C for 1.5 min, 72 °C for 1.5 min. Final extension of 10 min at 72 °C. The product size amplified from this round is 398 bp. This was followed by second round of amplification, which involved using AV1 (ATGTGGTTGCTGTGTTGGATGG) and AV2 (CCGCCGCAATCAACTCCAG) primers at the following conditions: 95 °C for 5 min, then 34 cycles of 95 °C for 1 min, 58 °C for 1.5 min, 72 °C for 1.5 min. Final extension of 10 min at 72 °C. The final product sized after this round was 298 bp.

Undercarboxylated Osteocalcin (ucOC) concentration measurement

Levels of undercarboxylated osteocalcin (ucOC) were measured through ELISA sandwich assay. Bovine samples were determined by using the Antibody Research Corporation Bovine undercarboxylated Osteocalcin ELISA Kit (Antibody Research, St Peters, MO, USA) following manufacturer instructions. Human samples were measured by using the Antibody Research Corporation Human Undercarboxylated Osteocalcin (ucOC) ELISA Kit (Antibody Research, St Peters, MO, USA) following manufacturer instructions. Briefly, all samples and reagents were brought up to room temperature then
5 concentrations of standard solutions were prepared. A volume of 50uL of standards, samples and blanks were added to a pre-coated plate. Immediately, 50uL of biotinylated detection antibody was added to each well, followed by 60 minutes incubation at 37°C. The plate was washed 3 times with washing buffer then 100uL of HRP-streptavidin conjugate reagent was added to each well. After 30 minutes of incubation time at 37°C, the plate was washed 5 times with washing buffer, and then 100uL of TMB substrate solution was added to each well. After 15 minutes of incubation at 37°C, 50uL of stop solution was added to the wells. Finally, absorbance was measured at 450nM wavelength using a plate reader and concentrations were calculated from the standard curve linear regression equation.

**Active Osteocalcin concentration measurement**

Levels of active osteocalcin were measured through ELISA sandwich assay. Bovine samples were measured using LifeSpan Biosciences Bovine Osteocalcin ELISA Kit (LifeSpan Biosciences, Seattle, WA, USA) following manufacturer instructions. Human samples were measured using Life Technologies Osteocalcin Human ELISA Kit (Life Technologies, Carlsbad, CA, USA) following manufacturer instructions. Briefly, all samples and reagents were brought up to room temperature. Six different concentrations of standards were provided and they were added to a pre-coated plate along with samples and controls (25uL of each). A volume of 100uL of working Anti-OST-HRP conjugate was added to each well followed by 2 hours of incubation at room temperature. The plate was washed 3 times then 100uL of chromogen solution was added into each well, followed by 30 minutes of incubation time in the dark at room temperature. Finally,
100μL of stop solution was added into each well and absorbance was read by a plate reader at 450nM wavelength and concentration were calculated from the generated standard curve equation.

**Calcium level measurement**
Calcium levels of bovine samples were measured using the Vitros® 350 Chemistry System Ortho-Clinical Diagnostics machine. Briefly, 10μl of serum was added to multilayered Vitros® Ca slides and evenly distributed by the spreading layer to the underlying reagent layer, where the calcium forms a complex with Arsenazo III dye at pH 5.6, which causes a shift in the maximum absorption. After 5 minutes of incubation at 37°C, the reflection density of the formed colored complex was measured by a spectrophotometric assay, which determined calcium concentration in each sample.

**Statistical analysis**
GraphPad Prism® software was used to analyze data for significance by using unpaired, two-tailed t test. P-values of less than 0.05 were considered statistically significant.

**Results**
Undercarboxylated osteocalcin levels were elevated in MAP infected bovine samples.
A total of 19 bovine sera samples from animals diagnosed with Johne’s disease (MAP-positive) and 17 sera from healthy cattle (MAP-negative) were utilized for the study. All 36 sera were analyzed for undercarboxylated osteocalcin level. The average ucOC level was 289 ± 95.8 nmol/mL in healthy bovine sera control compared to 318 ± 57.2 nmol/mL in cattle infected with MAP [Table 1]. The MAP-positive sera had a higher average ucOC concentration, with a difference in means of 29 nmol/mL [Figure 1A]. However, the data was not found to be statistically significant (P>0.05).

Active osteocalcin levels were decreased in MAP infected bovine samples

A total of 18 sera samples from cattle diagnosed with Johne’s disease (MAP-positive) and 20 healthy cattle (MAP-negative) were selected to determine active osteocalcin levels [Table 2]. The average osteocalcin level in healthy bovine sera was 1190 ± 43 pg/mL, compared to a significantly lower level of 797 ± 162 pg/mL in MAP positive bovine with a P-value <0.05 [Figure 1B].

Calcium levels were increased in MAP infected bovine samples

A total of 42 bovine sera samples (21 MAP-negative and 21 MAP-positive) were analyzed for calcium concentration [Table 3]. The average calcium level in healthy cattle was found to be 7.65 ± 2.12 mg/dL, while levels were 9.98 ± 0.998 mg/dL in MAP infected cattle [Figure 1C]. This significant difference (P-value<0.05) can be explained by the fact that MAP-positive cattle have lower levels of active osteocalcin when compared to healthy cattle.
Undercarboxylated osteocalcin levels were elevated in MAP infected human samples among CD patients and their healthy relatives. There were 10 MAP-positive healthy human samples, 10 MAP-negative healthy subjects, 9 MAP-negative CD human samples and 9 MAP positive CD patients that were selected for this study [Table 1]. The average ucOC level was 562 ± 49.9 nmol/mL in MAP-positive healthy subjects, while in MAP-negative healthy subjects the levels were found to be 533 ± 35.6 nmol/mL [Figure 2A]. Among CD patients there was a significant difference between those infected with MAP and those who were not. The average ucOC level in MAP-positive samples was found to be 561 ± 23.7 nmol/mL, while in MAP-negative patients the average ucOC level was 285 ± 19.6 nmol/mL [Figure 2C] with a difference in means of 276 nmol/mL (P-value <0.05).

Active osteocalcin levels were slightly decreased in MAP negative healthy samples but increased in MAP negative CD samples. The average active osteocalcin concentration in MAP-negative healthy human samples was 3.99 ± 0.36 ng/mL, compared to 4.85 ± 0.39 ng/mL in MAP-positive healthy human samples [Figure 2B]. The average active osteocalcin level was 2.19 ± 0.763 ng/mL in MAP-negative CD patients, while in MAP-positive CD samples the levels were 1.89 ± 0.184 ng/mL [Figure 2D]. In both groups, there was not a significant difference between MAP-negative and MAP-positive samples (P-value >0.05).

Discussion

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Complications of CD and IBD in general are numerous, which are also known as extraintestinal manifestations. More than 30% of IBD patients are suffering from different complications beyond the localized intestinal persistent inflammation such as ophthalmological and mucocutaneous problems, as well as metabolic bone disease including osteoporosis and osteopenia (Ott et al. 2013; Cravo et al. 2010). These extraintestinal complications could be a consequence of an abnormal intestinal activity such as nutrient malabsorption or completely independent from the persistent intestinal inflammation (Ott et al. 2013). In both cases, these complications affects patients quality of life and specific supportive treatment is necessary to avoid further complexity of their underlying medical status.

Lower bone density has been frequently observed in CD patients resulting in higher risk of bone fractures, but the main mechanism of this complication remains under investigation (Ali et al. 2009; Adachi 1999). Among the most commonly implicated factors are persistent intestinal inflammation, malabsorption of key nutrient such as calcium and vitamin D, as well as continuous use of steroids and immunomodulators (Siffledeen et al. 2004; Cravo et al. 2010; Reid et al. 1997). On the other hand, there is a supportive evidence suggesting that chronic inflammation by itself has detrimental consequences on bone mineralization, while suppressing inflammation leads to improvement of BMD (Ali et al. 2009; Bernstein et al. 2005).

We have previously investigated the role of MAP in causing systemic oxidative stress in CD patients (Qasem et al. 2016). It was interesting to figure out if the presence of this chronic infection will influence bone biomarkers such as osteocalcin in its active form...
and undercarboxylated osteocalcin (the inactive form). Therefore, we selected 18 CD patients (9 MAP positive and 9 MAP negative), as well as 20 healthy subjects (10 MAP positive and 10 MAP negative) to compare the level of those bone biomarkers in between these 2 groups according to their MAP infection status. In addition to that, we measured those levels in the blood of 21 MAP-infected and 21 healthy cattle since they represent a very good disease model and MAP infection is very well documented causative agent of Johnes’s disease.

We found that undercarboxylated osteocalcin (ucOC) level was increased in MAP infected human and bovine samples, but only statistically significant (p-value < 0.02) among CD patients. The average level in MAP-negative bovine was 289 ± 95.8 nmol/mL vs. 318 ± 57.2 nmol/mL in MAP-positive bovine (P-value >0.05). Among CD patients, MAP-negative had an average ucOC concentration of 285 ± 196 nmol/mL compared to 561 ± 23.7 nmol/mL in MAP-positive patients (P-value <0.05).

Since MAP infected bovine tend to have higher inactive osteocalcin, they are unable to bind calcium properly and are expected to have elevated free calcium levels. Interestingly, free calcium level was significantly higher (p-value < 0.05) in MAP positive bovine samples (9.98 ± 0.998 mg/dL vs. 7.65 ± 2.12 mg/dL). The higher level of calcium is due to lower level of active osteocalcin, which will increase bone resorption since active osteocalcin is being less incorporated into the bone matrix, resulting in a transfer of calcium from bone tissue to the blood (Teitelbaum et al. 2000).

When we measured active osteocalcin level, we found that it was decreased in both MAP infected bovine samples and in CD patients, but slightly elevated in MAP-positive
healthy control samples (P-value>0.05). However, MAP-positive bovine samples had a significantly lower average of osteocalcin (p-value < 0.005) compared to MAP-negative samples (797 ± 162 pg/mL vs. 1190 ± 43 pg/mL). However, we did not find a significant decrease in osteocalcin level in CD patients according to their MAP infection status (P-value >0.05), even MAP infected patients had a lower average concentration compared to MAP-negative CD patients (1.89 ± 0.184 ng/mL vs. 2.19 ± 0.763 ng/mL).

The pathogenesis of CD involves a complex interaction of immunologic abnormalities, genetics and environmental triggers (Loftus et al. 2004). The microbial component of CD development is a central feature of many recent studies (Shanahan et al. 2005; Feller et al. 2007; Behr et al. 2008). Here we think that the unique progression of MAP infection in CD results in an imbalance between anti-inflammatory and pro-inflammatory cytokines, resulting in a chronic inflammatory response. Previous studies have shown that pro-inflammatory cytokines such as IL-6 and IL-1, may have a crucial role in the osteoclast paracrine stimulation, which influences bone resorption ultimately (Manolagas et al. 1995; Schulte et al. 1999). We believe that dysregulated pro-inflammatory cytokine level caused by MAP infection results in hyperactive osteoclast activity, which will affect bone biomarker levels. To our knowledge, this is the first study that considers MAP infection in CD as a possible etiologic factor in the pathogenesis of this extraintestinal manifestation.

Conclusion
The consistent correlation between MAP infection and bone biomarkers (osteocalcin, calcium and undercarboxylated osteocalcin), proves that the presence of this bacterium dysregulates the immune system resulting in higher bone resorption activity, which on the long-term may cause higher risk for bone demineralization among CD patients.

Acknowledgment

This study was funded in part, by the Florida Legislative grant. Our thanks are due to Dr. Michael Collins (University of Wisconsin - Madison) for providing us with cattle sera. A sincere acknowledgement of all subjects whom clinical samples were included in this study.

Competing interests

The authors declare that they have no competing interests.
References


Tables

Table 1: Average undercarboxylated osteocalcin level and MAP presence in bovine and human blood samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Diagnosis</th>
<th>MAP Status</th>
<th>Number of Samples</th>
<th>Average ucOC Level (nmol/mL) - CV***</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Healthy</td>
<td>Negative</td>
<td>17</td>
<td>289 ± 95.8 – 0.33</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>JD*</td>
<td>Positive</td>
<td>19</td>
<td>318 ± 57.2 – 0.18</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Human</td>
<td>Healthy</td>
<td>Negative</td>
<td>10</td>
<td>533 ± 35.6 – 0.07</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Healthy</td>
<td>Positive</td>
<td>10</td>
<td>562 ± 49.9 – 0.09</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Human</td>
<td>CD**</td>
<td>Negative</td>
<td>9</td>
<td>285 ± 19.6 – 0.07</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>CD**</td>
<td>Positive</td>
<td>9</td>
<td>561 ± 23.7 – 0.04</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* JD: Johne’s disease bovine serum samples.
**CD: Crohn’s disease patients plasma samples.
*** CV: Coefficient of variation.
Table 2: Average osteocalcin level and MAP presence in bovine and human blood samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Diagnosis</th>
<th>MAP Status</th>
<th>Number of Samples</th>
<th>Average Osteocalcin Level (pg/mL) - CV***</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Healthy</td>
<td>Negative</td>
<td>18</td>
<td>1190 ± 43 – 0.04</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>JD*</td>
<td>Positive</td>
<td>20</td>
<td>797 ± 162 – 0.20</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Human</td>
<td>Healthy</td>
<td>Negative</td>
<td>10</td>
<td>3.99 ± 0.36 – 0.09</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Healthy</td>
<td>Positive</td>
<td>10</td>
<td>4.85 ± 0.39 – 0.08</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Human</td>
<td>CD**</td>
<td>Negative</td>
<td>9</td>
<td>2.19 ± 0.76 – 0.35</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>CD**</td>
<td>Positive</td>
<td>9</td>
<td>1.89 ± 0.184 – 0.1</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

* JD: Johne’s disease bovine serum samples.

** CD: Crohn’s disease patients plasma samples.

*** CV: Coefficient of variation.
Table 3: Calcium level and MAP presence in bovine blood samples.

<table>
<thead>
<tr>
<th>Source</th>
<th>Diagnosis</th>
<th>MAP Status</th>
<th>Number of Samples</th>
<th>Average ucOC Level (mg/dL) - CV***</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Healthy</td>
<td>Negative</td>
<td>21</td>
<td>7.65 ± 2.12 – 0.28</td>
<td></td>
</tr>
<tr>
<td>Bovine</td>
<td>JD*</td>
<td>Positive</td>
<td>21</td>
<td>9.98 ± 0.998 – 0.10</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* JD: Johne’s disease bovine serum samples.

*** CV: Coefficient of variation.
**Figures**

**Figure 1: Undercarboxylated osteocalcin (ucOC), active osteocalcin and calcium levels in bovine samples.**

a. *Scatter plot* of undercarboxylated osteocalcin level for MAP negative and MAP positive bovine samples. b. *Scatter plot* of active osteocalcin level for MAP negative and MAP positive bovine samples. c. *Scatter plot* of calcium level for MAP negative and Map positive bovine samples.

* denotes statistical significance (P-value<0.05).

**Figure 2: Undercarboxylated osteocalcin (ucOC) and active osteocalcin in human samples.** a. *Scatter plot* of undercarboxylated osteocalcin levels for MAP negative and MAP positive healthy human samples. b. *Scatter plot* of active osteocalcin levels for MAP negative and MAP positive healthy human samples. c. *Scatter plot* of undercarboxylated osteocalcin levels for MAP negative and MAP positive human Crohn’s disease samples. d. *Scatter plot* of active osteocalcin levels for MAP negative and MAP positive human Crohn’s disease samples.

* denotes statistical significance (P-value<0.05).
Figure 1: Undercarboxylated osteocalcin (ucOC), active osteocalcin and calcium levels in bovine samples.

a. Scatter plot of undercarboxylated osteocalcin level for MAP negative and MAP positive bovine samples. b. Scatter plot of active osteocalcin level for MAP negative and MAP positive bovine samples. c. Scatter plot of calcium level for MAP negative and Map positive bovine samples.

* denotes statistical significance (P-value<0.05).
Figure 2: Undercarboxylated osteocalcin (ucOC) and active osteocalcin in human samples. a. Scatter plot of undercarboxylated osteocalcin levels for MAP negative and MAP positive healthy human samples. b. Scatter plot of active osteocalcin levels for MAP negative and MAP positive healthy human samples. c. Scatter plot of undercarboxylated osteocalcin levels for MAP negative and MAP positive human Crohn’s disease samples. d. Scatter plot of active osteocalcin levels for MAP negative and MAP positive human Crohn’s disease samples. * denotes statistical significance (P-value<0.05).