THERAPEUTIC ADVANCES in
Neurological Disorders

Original Research

Propionic acid beneficially modifies osteoporosis biomarkers in patients with multiple sclerosis

Ther Adv Neurol Disord

2022, Vol. 15: 1–10

DOI: 10.1177/17562864221103935

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Abstract

Background: The impact of the gut and its microbiota are increasingly appreciated in health and disease. Short-chain fatty acids (SCFAs) are among the main metabolites synthesized from bacterial fermentation. Recently, we showed the anti-inflammatory and potentially neuroprotective effect of propionic acid (PA) in multiple sclerosis (MS). Osteoporosis is one of the most common co-morbidities for MS patients with limited therapeutic options available. Osteoporosis is closely linked to an imbalance of cells of the immune system and an immune-mediated impact on bone structure *via* the gut has been shown. Interestingly, intake of SCFA leads to bone mass increase and concomitant reduction of inflammation-induced bone loss in mice.

Objective: To determine the impact of PA supplementation on markers of bone metabolism in MS patients.

Methods: We investigated the influence of 14 days supplementation with PA on bone metabolism in 20 MS patients. To this end, β-CrossLaps and osteocalcin, established markers of bone metabolism, were measured in serum before and after PA intake and correlated with phenotypic and functional immunodata.

Results: Supplementation with PA induced a significant increase in serum levels of osteocalcin, a surrogate marker for bone formation. Levels of β-CrossLaps, a marker for bone resorption, were significantly decreased after therapy. Regulatory T-cell (Treg) numbers and suppressive capacity positively correlated with serum levels of osteocalcin while Th17 cell numbers showed an inverse correlation. Our findings are in line with animal studies showing that SCFA induced increased bone formation and reduced bone resorption.

Conclusion: In addition to its immune regulatory, disease-modifying effect on MS disease course, supplementation with PA beneficially influences serum levels of β-CrossLaps and osteocalcin and may thus also protect against osteoporosis, a common co-morbidity in MS.

Keywords: bone metabolism, gut–brain axis, multiple sclerosis, osteoporosis, propionic acid

Received: 14 June 2021; revised manuscript accepted: 12 May 2022.

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Introduction

Multiple sclerosis (MS) is a chronic, autoimmune demyelinating disease of the central nervous system.[1] Growing evidence support the hypothesis that the gut and its microbiome are involved in MS pathogenesis.[2] The disease is associated with various secondary co-morbidities. Osteoporosis is highly prevalent in various autoimmune-mediated diseases, especially in MS.[3,4] Alongside an increased inflammatory environment, the main risk factors for osteoporosis are smoking, physical inactivity due to disease-related disability or fatigue, and regular glucocorticoid treatment.[5] In recent years, the idea of an autoimmune influence on osteoporosis has gained much interest, hence coining the term osteoimmunology. The impact

of Th1 and Th17 cells on bone resorption and an osteogenic effect of regulatory T cells (Treg) have been demonstrated in an animal study.[6] However, current therapy options are limited and often accompanied by side effects.

Short-chain fatty acids (SCFAs) are the most abundant metabolic by-products of intestinal bacteria.[7] Dysbiosis leads to a reduction in SCFA-producing bacteria, resulting in reduced fecal and serum SCFA levels and a pro-inflammatory intestinal environment.[8] The main SCFAs produced are acetate, butyrate, and propionic acid (PA). Recently, the impact of PA on MS has been investigated. PA supplementation over 2 weeks led to an increase in peripheral Treg numbers and function, while Th1 and Th17 cells decreased.[9,10]

Animal studies of the osteoclastogenic impact of Th1 and Th17 cells as well as the osteogenic effect of Treg provide the groundwork for new therapeutic options in osteoporosis. Treg were observed to suppress osteoclast differentiation by interaction *via* CD80/86− CTLA-4 and IDO induction and thereby potentially halt osteoporosis.[11–14]

Supplementation with SCFA leads to a bone-protective shift in T-cell subsets, which was mainly driven by Treg-induced suppressive effects on osteoclast differentiation.[10,15] A direct impact of SCFAs on cell metabolism in osteoclast precursor cells has also been demonstrated.[16] Besides the direct substitution with SCFA, pre- and probiotics are sufficient sources to increase the levels of SCFA within the gut. Increased bone density and SCFA levels were achieved by substitution with pre- and probiotics in animal experiments. This was linked to an increase of bacterial enzymes needed for the catabolism of fiber-rich food to SCFA.[17]

So far, several randomized, placebo-controlled, human studies have shown a positive effect of probiotics on bone metabolism in postmenopausal women.[18–20] Jarfanejad *et al.*[21] demonstrated a reduction of bone resorption markers under therapy with probiotics. Another study involving 90 osteopenic women treated with *Lactobacillus reuteri* showed a reduced loss of bone density in the treatment group.[22] However, most over-the-counter probiotics produce lactic acid and acetic acid, but not PA.

Here, we set out to evaluate whether PA supplementation has an impact on osteoporosis-associated biomarkers by measuring β-CrossLaps and osteocalcin in MS patients who had received PA in a previous study,[9] and of whom we had serum samples stored.

Methods

Study design

Diagnosis of MS was validated by the 2017 McDonald criteria and oligoclonal bands in the cerebrospinal fluid (CSF). A total of 20 MS patients were recruited according to the known sex-dependent incidence of 3:1 favoring female (for additional information on included individuals, please see Table 1). Participants were instructed to supplement 500 mg of sodium-propionate (PA) capsules twice daily for 14 days (PA; Flexopharm, Herne, Germany) together with pre-existing MS-specific therapies. Neurologic examination was performed by experienced, Neurostatus C-certified neurologists. Clinical data, that is, individual MS therapies and Vitamin D supplementation, was assessed at the Department of Neurology of the Ruhr-University Bochum, at St. Josef-Hospital Bochum.

Blood sample collection was performed at the Department of Neurology of the Ruhr-University Bochum, at the St. Josef-Hospital Bochum, and at the Clinic of Neurology II at EVK Hattingen. For immunophenotyping, blood drawn (in EDTA tubes) was processed immediately after collection. Serum samples taken at the same time points were immediately frozen at −80°C for later analysis.

Immunophenotyping

Whole blood cells of MS patients at baseline and after 14 days of PA supplementation were stained by BD Multitest™ 6-Color TBNK (αCD3-FITC; αCD16-PE+ αCD56-PE; αCD45-PerCP Cy5.5; αCD4-PE Cy7; αCD19-APC; αCD8-APC Cy7; BD). T-cell subsets were identified with αCD196 (CCR6)-PerCP Cy5.5 (11A9; BD), αCD4-FITC (RPA-T4; BD), αCD161-PE (DX-12; BD), and αCD183 (CXCR3)-APC (1 C6/CXCR3; BD) for determination of Th1 and Th17 as previously described.[23] For Treg analysis, cells were stained with CD4-FITC (RPA-T4, eBioscience) and CD25-APC (BC96, eBioscience) and intracellular FoxP3-PE (236A/E7, eBioscience) by using Foxp3/Transcription Factor Staining Buffer Set (eBioscience) according to manufacturer

protocol. Phenotyping was performed on BD FACS Canto2 and analyzed by BD FACS DIVA v6 and BD FACS CANTO2 software.

Quantification of osteocalcin and β-CrossLaps

Cross-Laps CTX-I (IDS, Immunodiagnostic Systems, UK) and osteocalcin (OCN) (Invitrogen, Germany) serum enzyme-linked immunosorbent assay (ELISA) were performed according to the manufacturer's instructions.

Suppression assay

Peripheral blood mononuclear cells (PBMCs) from whole blood of MS patients were isolated by Ficoll Paque PLUS (GE Healthcare) gradient centrifugation and separated *via* MACS™ $CD4^+CD25^+CD127^{dim/-}$ Regulatory T Cell Isolation Kit II human (Miltenyi Biotec) according to manufacturer's protocol. Briefly, $CD127^{dim/-}$ cells were negatively isolated from PBMC and subsequently processed by positive selection of $CD25^+$ for regulatory T cells. $CD127^+$ as well as $CD25^-$ cells were pooled at the end of the procedure and used as control cells ($PBMC^-$); approximately 2 million $PBMCs^-$ were stained with CellTrace™ CFSE Cell Proliferation Kit (Thermo Scientific) to analyze proliferation in a mixed coculture. In total, 50,000 cells per well were seeded in duplicates in serum free x-vivo 15 medium (Biozym) in 96-well plates. Treg, as well as unstained $PBMC^-$, were added in a 1:2 or 1:1 ratio to CFSE-stained $PBMC^-$. As a reference for autologous proliferation of CFSE-stained cells, $PBMC^-$ were seeded separately, sans coculture. All assays were cultured at 37°C with 5% CO_2 for 5 days. Proliferation was analyzed by flow cytometry whereby dead cells were excluded using Fixable Viability Dye eFluor® 780 (eBioscience).

Statistical analysis

Statistical analysis was performed with GraphPad Prism v6. Data were presented as individual data points of each patient before and after treatment as well as mean ± standard error of the mean (SEM; Figure 5) unless stated otherwise. Value of n is always displayed in the figure as individual data points, and more information about absolute n numbers can be found in the figure legends. Values of the same individual were tested for statistical significance by paired t-test and correlated data were tested by log-rank test. All statistical tests used were also indicated in the respective figure legends (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$).

Table 1. Characteristics of individuals included in the study.

Characteristics	MS
	(*n* = 20)
Female sex, *n* (%)	14 (70%)
Age, years, mean ± SD	47.1 (±11.2)
Disease duration, years, mean ± SD	15.05 (±10.4)
Subgroups	
Relapsing-remitting MS	8 (40%)
Secondary progressive MS	12 (60%)
Expanded Disability Status Scale, mean ± SD	3.12 (±0.74)
Therapy	
Dimethyl fumarate	4 (20%)
Fingolimod	2 (10%)
Glatiramer acetate	1 (5%)
Rituximab	2 (10%)
Steroids intrathecal	9 (45%)
w/o therapy	2 (10%)

MS, multiple sclerosis; SD, standard deviation.

Results

Serum samples as well as immunodata were taken from a prospective cohort study previously published by us.[9] Immunophenotyping was performed immediately after collecting blood samples from all recruited patients at baseline and after 14 days of PA supplementation. Analysis of T-cell subsets revealed a significant increase of peripheral anti-inflammatory Treg of approximately 50% during PA supplementation while pro-inflammatory Th17 cells were significantly diminished by around 30% [Figure 1(a); $p < 0.0001$; mean of difference: 2.275; 95% confidence interval (CI): 1.799 to 2.751; paired t-test; Figure 1(b); $p < 0.0001$; mean of difference: −2.570; 95% CI: −3.377 to −1.763; paired t-test] in all participating patients. In addition, functional analysis of Treg showed a significant increase in their ability

to suppress proliferation of PBMC by about 25% (Figure 1(c); $p=0.0276$; mean of difference: 51.36; 95% CI: 6.299 to 96.43; paired *t*-test).

To investigate the impact of PA on bone metabolism, serum levels of osteocalcin and β-CrossLaps were determined before and after 14 days of supplementation. While osteocalcin, a well-established marker for bone building, was significantly increased in serum after 14 days of PA intake (Figure 2(a); $p=0.0003$; mean of difference: 6.526; 95% CI: 3.509 to 9.544; paired *t*-test), β-CrossLaps, the main surrogate marker for bone resorption, was significantly decreased (Figure 2(b); $p=0.0398$; mean of difference: −0.06211; 95% CI: −0.1210 to −0.003237; paired *t*-test).

Analysis of possible associations between PA supplementation-induced changes in immune cell differentiation and bone metabolism-related markers showed a significant positive correlation between osteocalcin levels and the number of peripheral Treg ($p=0.0173$; $R^2=0.2765$; Figure 3(a)). This finding is in line with previous data showing a positive influence of Treg on regulation of bone building. Furthermore, we observed a significant negative correlation of Th17 cells with levels of osteocalcin ($p=0.0151$; $R^2=0.2861$; Figure 3(b)). β-CrossLaps did not show any correlation with either T-helper cell subset (Figure 3(c); $p=0.1537$; $R^2=0.1097$; Figure 3(d); $p=0.3943$; $R^2=0.0401$). $CD8^+$ cytotoxic T cells, natural killer cells, or B cells showed any significant correlation with osteocalcin or β-CrossLaps (data not shown).

In order to query the impact of Treg function on bone metabolism, we correlated their suppressive capacity to serum levels of osteocalcin and β-CrossLaps. A significant positive correlation was detected between osteocalcin and the suppressive capacity of Treg, which may indicate a possible link between Treg functionality and maintenance of bone structure ($p=0.0362$; $R^2=0.2215$; Figure 4(a)). β-CrossLaps showed no significant correlation with the suppressive capacity of Treg ($p=0.2157$; $R^2=0.0838$; Figure 4(b)).

As the proportion of male MS patients with reduced bone mass is high and disproportionate to their age,[24] serum levels of osteocalcin were stratified into male and female subgroups. We detected significantly higher amounts of osteocalcin in female patients independent of PA supplementation (Figure 5(a); $p=0.0108$; mean of difference: 6.945; 95% CI: 1.810 to 12.08). After 14 days of PA treatment, osteocalcin levels increased significantly both in male and female patients, albeit more pronounced in males (Figure 5(a); male $p<0.0001$; mean of difference: 11.68; 95% CI: 9.105 to 14.26; female $p=0.0206$; mean of difference: 5.579; 95% CI: 1.005 to 10.15). In order to determine the effect range in both groups, the ratios of changes in osteocalcin levels after PA supplementation were compared between male and female groups, which showed a significantly stronger alteration in male patients (Figure 5(b); $p=0.0002$; mean of difference: −2.785; 95% CI: −4.025 to −1.544).

Discussion

Here, we show that 14 days of PA supplementation in MS patients leads to a significant increase in serum levels of osteocalcin, whereas β-CrossLaps is decreased. Osteocalcin mainly reflects bone-building processes, while β-CrossLaps is a marker for bone resorption. With lower baseline values of osteocalcin in male MS patients, this increase was even more pronounced in the male subgroup. While SCFA initiate a Treg-mediated process during bone formation, previous studies have found a T-cell-independent mechanism during bone resorption.[15,16] In line with these findings, we detected a positive correlation between the function and amount of Treg and osteocalcin levels in serum.

A positive effect of SCFA on bone formation has been shown in animal models.[15,16] Hence, our observed increase in osteocalcin upon administration of PA in MS patients is consistent with previous data. This may be due to two independent effects of PA: first, indirectly by increasing the amount of peripheral Treg, which are capable of inhibiting the function of osteoclasts *via* CD80/86-CTLA4 and IDO; second, PA alters the metabolic profile of osteoclast precursor cells, which results in a blockage of differentiation to osteoclasts.[13,14,16,25] In addition, a human study of 25 women with postmenopausal osteopenia showed a decrease of bone-specific alkaline phosphatase (BALP) after 6 months therapy with multispecies probiotics.[21] BALP is a marker for osteoblast proliferation and higher bone turnover and indicative of active disease. However, the increase of osteocalcin reported in previous studies did not reach significance.[21,26] This is likely explained by the lower effect of probiotics compared to SCFA.[15] Another human study showed reduced decrease in bone density upon

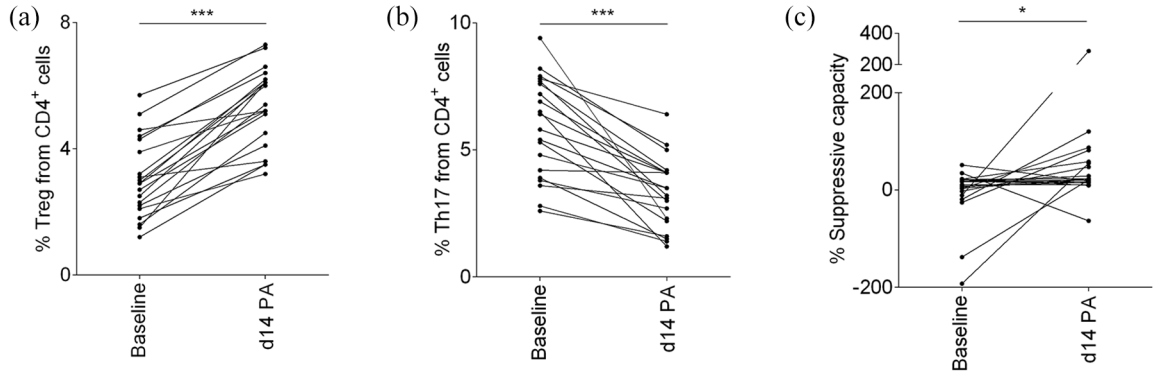

Figure 1. Immunophenotyping displayed a significant increase in peripheral Treg (a; $p < 0.0001$; mean of difference: 2.275; 95% CI: 1.799 to 2.751; paired *t*-test) while pro-inflammatory Th17 were significantly reduced (b; $p < 0.0001$; mean of difference: −2.570; 95% CI: −3.377 to −1.763; paired *t*-test) during PA intake. (c) Analysis of suppression assays also revealed an increase in suppressive capacity of Treg during PA treatment ($p = 0.0276$; mean of difference: 51.36; 95% CI: 6.299 to 96.43; paired *t*-test).

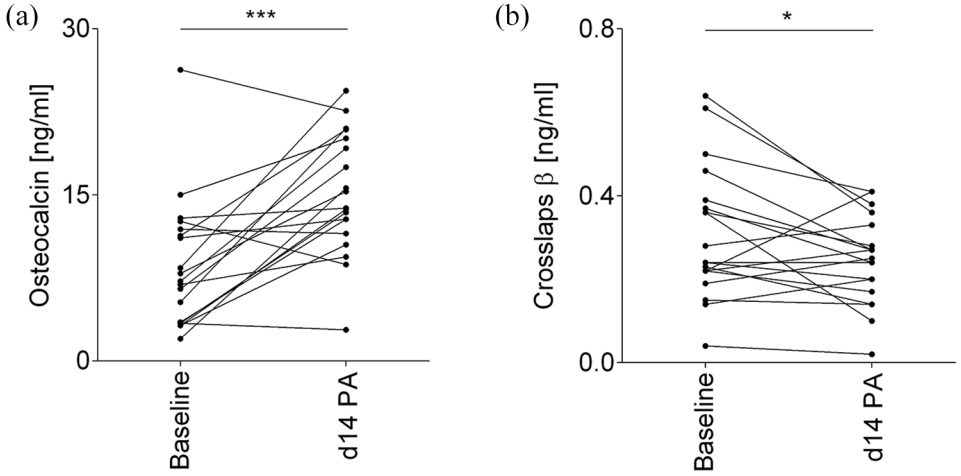

Figure 2. (a) Osteocalcin levels significantly increased in serum of MS patients during PA treatment ($p = 0.0003$; mean of difference: 6.526; 95% CI: 3.509 to 9.544; paired *t*-test). (b) β-CrossLaps concentrations were diminished upon PA administration ($p = 0.0398$; mean of difference: −0.06211; 95% CI: −0.1210 to −0.003237; paired *t*-test).

treatment with a probiotic (*Lactobacillus reuteri*) for 12 months.[22]

Previously, it has been shown in a eugonadic mouse model that the effect of probiotics on bone metabolism is primarily SCFA-mediated. Mechanistically, SCFA lead to a Treg-depended increase in Wnt10b in CD8 T cells, resulting in increased bone density.[15] In order to determine whether a Treg-mediated increase in surrogate markers of bone metabolism depends on cell function, we looked at the suppressive capacity of *in vitro* stimulated Treg. We report here for the first time a correlation between the suppressive capacity of the Treg and serum levels of osteocalcin after PA supplementation in MS patients. This suggests that SCFA might exert their bone-forming effects indirectly *via* increasing the numbers and function of Treg.

β-CrossLaps is a marker for bone resorption. Here, we show that supplementation with PA leads to a significant reduction in serum levels of β-CrossLaps. This is in line with another human

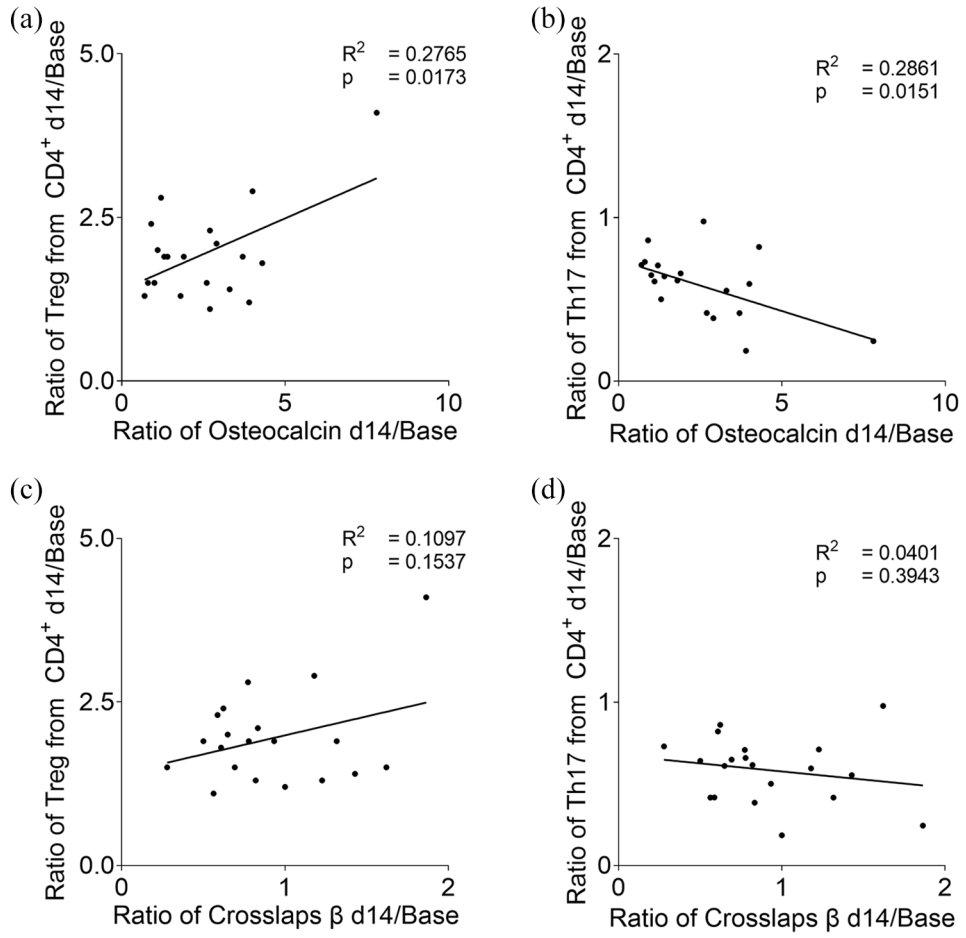

Figure 3. Linear regression analysis of the ratio of T-cell subsets and osteocalcin serum levels displayed a positive correlation between Treg and osteocalcin (a; $p = 0.0173$; $R^2 = 0.2765$), while alterations in Th17 negatively correlated with changes in osteocalcin (b; $p = 0.0151$; $R^2 = 0.2861$). β-CrossLaps did not show any correlation with either T-helper cell subset (c; $p = 0.1537$; $R^2 = 0.1097$; d; $p = 0.3943$; $R^2 = 0.0401$).

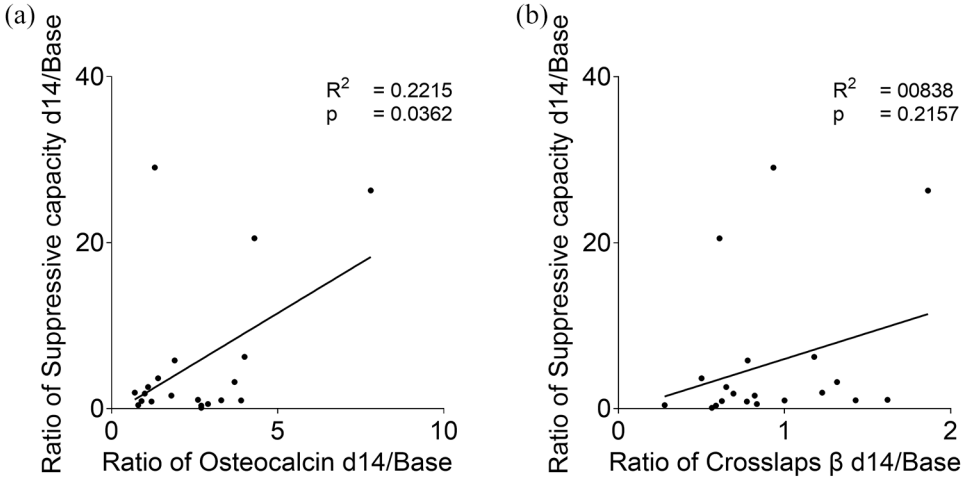

Figure 4. (a) Correlation of changes in bone metabolism markers with suppressive capacity of Treg revealed a positive correlation between alterations in osteocalcin levels and Treg function ($p = 0.0362$; $R^2 = 0.2215$; linear regression analysis). (b) Changes in β-CrossLaps did not correlate with Treg function ($p = 0.2157$; $R^2 = 0.0838$; linear regression analysis).

study where β-CrossLaps was decreased after 6 months therapy with probiotics.[21]

A Treg-independent influence of SCFA and high-fiber diet on bone resorption has been described in an inflammatory arthritis and postmenopausal mouse model.[15] Lucas *et al.* observed a decrease in osteoclasts and β-CrossLaps upon treatment with PA, which was accompanied by reduced bone resorption. SCFA-induced metabolic reprogramming of preosteoclasts appears to be mediated *via* enhanced glycolysis.[11,16,25] It is tempting to speculate that a similar mechanism might decrease bone resorption after supplementation with PA in MS patients, as indicated by reduced serum levels of β-CrossLaps. However, this needs to be addressed in more detail in future studies.

We recently showed a PA-induced reduction of Th17 cells in MS patients.[9] Th17 cells can also reduce bone density,[27] have strong bone-resorbing effects,[28] and have been associated with inflammation-related bone pathologies.[29] Thus, PA-mediated reduction of Th17 cell numbers might also result in a decrease in bone resorption. Interestingly, we also found an inverse correlation between the number of peripheral Th17 cells and osteocalcin levels in serum. Whether this points toward a direct suppressive influence of Th17 cells on bone formation or merely reflects the inverse correlation of Th17 and Treg and their positive contribution to bone formation will have to be addressed in future studies.

In the general population, as well as in patients with MS, there is a gender and age bias, with an increased prevalence of osteoporosis in elderly women.[5] Serum osteocalcin is a valid marker of bone turnover when resorption and formation are coupled and a specific marker of the bone formation when formation and resorption are uncoupled. In a Japanese study with 3250 women aged 15–79 years, higher levels of osteocalcin were observed in osteoporosis patients compared with healthy controls.[30] An increase in osteocalcin can reflect a higher bone metabolism.[31] In our study, we observed elevated baseline serum levels of osteocalcin in women, probably influenced by postmenopausal status.

After 14 days of supplementation with PA, an increase in serum levels of osteocalcin was observed in both sexes. Interestingly, the increase in the male cohort was even more pronounced.

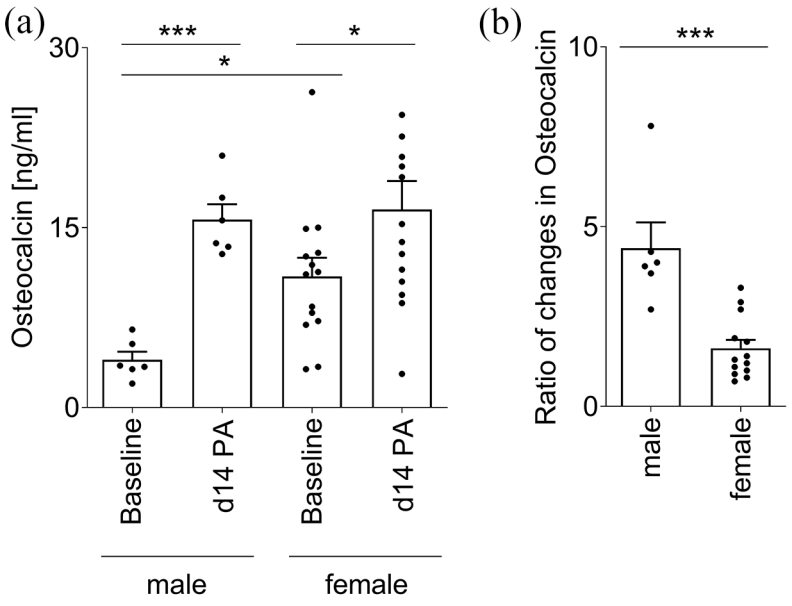

Figure 5. (a) Stratification into male and female patients revealed a significantly higher amount of osteocalcin in female patients independent of PA supplementation ($p = 0.0108$; mean of difference: 6.945; 95% CI: 1.810 to 12.08; unpaired *t*-test). After 14 days of PA intake, osteocalcin levels increased in both groups (male $p < 0.0001$; mean of difference: 11.68; 95% CI: 9.105 to 14.26; female $p = 0.0206$; mean of difference: 5.579; 95% CI: 1.005 to 10.15; paired *t*-test). (b) Overall alterations in osteocalcin levels were more pronounced in male compared with female patients ($p = 0.0002$; mean of difference: −2.785; 95% CI: −4.025 to −1.544; unpaired *t*-test).

Despite the small sample size of 6 male and 14 female subjects, the ratio of osteocalcin values before and after 2 weeks of therapy was different between the two sexes. This gender different effect of PA on osteocalcin levels could be explained by the different baseline values and might be linked to the known gender bias in osteoporosis, resulting in a decreased effect of PA. This in turn would mean that a potential therapeutic use of PA should be made as early as possible during the course of disease. However, due to the limited sample size, these first findings need confirmation in a larger cohort of MS patients.

In sum, we could show that supplementation with PA induced a significant increase in serum levels of osteocalcin in MS patients and that this positively correlated with numbers and function of Treg and negatively correlated with Th17 cells. The main limitation of our study, besides the small sample size, is that we did not measure bone density in our patients and none of them had a confirmed diagnosis of osteoporosis. Instead, we opted for two surrogate markers for bone formation and bone resorption as indicators for bone

metabolism in serum. It would be interesting to observe the variation in bone density, defined as T-score, of MS patients under long time substitution with and without PA in comparison to age-matched controls. Second, some of our patients received intrathecal glucocorticoid therapy. Even though we only enrolled patients at least 6 weeks after the last glucocorticoid dosage, we cannot entirely rule out an influence of glucocorticoid on serum levels of osteocalcin.

Conclusion

To our knowledge, this is the first human study investigating the impact of SCFA supplementation on serum levels of osteocalcin and β-CrossLaps in patients with MS. We could show that PA has a positive influence on surrogate markers of bone formation and resorption, while formation seems to be Treg mediated and resorption Treg independent. Our study suggests that not only the number of Treg but also the increase in function under oral therapy with PA might have a beneficial impact on bone formation. The effect of PA seems to have a larger impact in male patients, which could be due to the often-advanced degree of osteoporosis in female MS patients.

In summary, besides the anti-inflammatory effects of SCFA, supplementation with PA may positively impact on bone metabolism; validation in larger MS cohorts and other autoimmune-mediated diseases associated with osteoporosis is warranted.

Ethics statement

This study was approved by the ethics committee of the Department of Medicine at the Ruhr-University Bochum (registration numbers 15-5351, 4745-13, 17-6235). Prior to study participation, all patients signed informed consent forms. Herein we declare that all authors consent the publication of the presented manuscript.

Author contributions

Alexander Duscha: Conceptualization; Data curation; Formal analysis; Investigation; Writing – original draft; Writing – review & editing.

Tobias Hegelmaier: Conceptualization; Data curation; Formal analysis; Investigation; Writing – original draft; Writing – review & editing.

Kerstin Dürholz: Data curation; Formal analysis; Writing – review & editing.

Christiane Desel: Formal analysis; Writing – review & editing.

Ralf Gold: Conceptualization; Supervision; Writing – review & editing.

Mario M. Zaiss: Conceptualization; Formal analysis; Supervision; Writing – review & editing.

Aiden Haghikia: Conceptualization; Formal analysis; Supervision; Writing – original draft; Writing – review & editing.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: K.D. and M.M.Z. were funded by the German Research Foundation (DFG) SFB1181 – 261193037 project B07. This research received no other specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

The authors declared the following potential conflicts of interest with respect to the research, authorship, and/or publication of this article: R.G. and A.H. have filed a patent on the supportive immunomodulatory effect of C3-C8 aliphatic fatty acids. R.G. is the Editor-in-Chief of this journal; therefore, the the peer review process was managed by alternative members of the Board and the submitting Editor was not involved in the decision-making process. The authors report no other conflicting interests regarding this study.

Availability of data and materials

Further information and requests for resources and reagents should be directed to the corresponding author, Prof. Aiden Haghikia (aiden.haghikia@med.ovgu.de). This study did not generate new reagents.

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