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Research Paper

Multiple Sclerosis

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Increased osteopontin plasma levels in multiple sclerosis patients correlate with bone-specific markers

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Abstract

The pro-inflammatory cytokine osteopontin has been found to be highly expressed in multiple sclerosis lesions and plasma levels are increased during relapses in relapse-onset multiple sclerosis patients.

The objective was to determine the relationship between osteopontin plasma and cerebrospinal fluid levels in relation to the immunoglobulin G index. In addition, osteopontin plasma levels were compared with osteopontin mRNA levels in peripheral blood mononuclear cells and bone-specific markers to analyse whether osteopontin may be peripherally produced.

Osteopontin and bone-specific markers were determined in paired plasma–cerebrospinal fluid samples and serum samples of relapse-onset multiple sclerosis patients ($n = 36$), respectively. Osteopontin mRNA levels were determined by quantitative polymerase chain reaction analysis.

Compared to healthy controls ($n = 20$), plasma osteopontin levels were significantly increased in relapsing-remitting multiple sclerosis patients and correlated ($r = 0.43$, $p = 0.013$) with the immunoglobulin G index. In contrast, cerebrospinal fluid osteopontin levels correlated neither with plasma osteopontin in paired samples nor with the immunoglobulin G index. Since osteopontin mRNA levels in peripheral blood mononuclear cells of relapsing-remitting multiple sclerosis patients did not correlate with osteopontin plasma levels, peripheral blood mononuclear cells might not be the major source for the increased osteopontin plasma levels. Osteopontin plasma levels correlated ($r = 0.42$, $p = 0.035$) with the bone-specific degradation product C-telopeptide of type-1 collagen. In addition, another immunomodulatory molecule involved in bone metabolism, 25-OH vitamin D, correlated negatively ($r = –0.359$, $p = 0.048$) with the immunoglobulin G index.

This study suggests that bone-related molecules like osteopontin and vitamin D with important immunomodulatory functions are related to the immunoglobulin G index in relapsing-remitting multiple sclerosis patients.

Keywords
Multiple sclerosis, osteopontin, bone

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Introduction

Multiple Sclerosis (MS) is an inflammatory demyelinating disease of the central nervous system (CNS) with a prominent role of immune cells and cytokines in degradation of the myelin sheaths.[1] Since the identification of osteopontin (OPN) as the most abundantly expressed cytokine in MS lesions, several studies have confirmed the involvement of osteopontin in MS.[2] OPN levels were found to be increased in the plasma of relapsing-remitting MS (RRMS) patients and a longitudinal study demonstrated that OPN levels were elevated prior to increased disease activity in RRMS patients.[3–5] Recently, OPN has also been found to be increased in the cerebrospinal fluid (CSF) of MS patients, although increased CSF levels were not specific to MS and were also observed in patients with other neurological diseases.[6, 7]

OPN is a member of the SIBLING (small integrin-binding ligand N-linked glycoprotein) family

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of non-collagenous matricellular proteins.[8] These polyanionic SIBLING proteins are believed to play key biological roles in the mineralization of bone.[9] $OPN^{-/-}$ mice have a subtle bone phenotype, with delayed and impaired bone resorption.[10] In the immune system, OPN plays a role in chemotaxis, leading to the migration of macrophages and dendritic cells to sites of inflammation.[11, 12] $OPN^{-/-}$ mice have severely impaired type-1 immunity to viral and bacterial infections.[13] Moreover, cells from draining lymph nodes from these mice demonstrated impaired interleukin-12 (IL-12) and interferon-gamma (IFN-γ) production and increased interleukin-10 (IL-10) production compared to $OPN^{+/+}$ mice.[2]

Further support for the role of OPN in inflammatory diseases of the brain has been provided by work on experimental autoimmune encephalomyelitis (EAE) in mice.[14] In that study, OPN induced relapses and disease progression in EAE through enhanced survival of activated T cells. Moreover, OPN-expressing dendritic cells induced interleukin-17 (IL-17) production by CD4 T cells and anti-OPN antibodies reduced the clinical severity of EAE.[15] Furthermore, OPN-deficient mice were resistant to developing severe EAE.[2]

OPN may be produced by immune and non-immune cells.[11] Among immune cells, OPN is secreted by activated macrophages and T lymphocytes.[16,17] Non-immune sources of osteopontin include bone cells, endothelial cells, epithelial cells, fibroblasts, tumour cells, and brain cells (e.g. astrocytes and neurons).[2, 11] In this study, we determined OPN levels in paired plasma and CSF samples from RRMS patients and evaluated whether the levels correlate with the immunoglobulin G (IgG) index. Furthermore, OPN plasma levels in MS patients were analysed for their association with bone-specific markers and OPN expression levels in peripheral blood mononuclear cells (PBMCs).

Materials and methods

Patient and control characteristics

CSF and plasma samples were obtained from two different libraries stored at the VU Medical Center, Amsterdam and Orbis Medical Center, Sittard-Geleen, The Netherlands. The libraries contain paired CSF and plasma samples from patients having undergone diagnostic lumbar punctures at both hospitals between the years 2001 and 2009. The samples have been stored, coded, and made anonymous in accordance with the MRC (Medical Research Council) guidelines on the ethical use of biological specimen collections in clinical research.

In all RRMS patients ($n = 36$), definite MS was diagnosed according to McDonald criteria by trained neurologists (McDonald et al. 2001)[18]. A trained neurologist scored the symptoms and signs of MS. Age and sex-matched healthy volunteers ($n = 20$) were used as controls for plasma OPN levels. Isoelectric focusing (IEF) with immunoblotting on agarose gels was used for the detection of oligoclonal bands in CSF and serum samples. Albumin and IgG were measured by automated immunoprecipitation nephelometry. The IgG index was calculated according to the standard formula.

Preparation of cell-free cerebrospinal fluid

CSF was sampled in siliconized glass tubes. CSF samples were centrifuged at $1500\times g$ for 10 min at 4°C (to remove cells and other insoluble materials). The cell-free CSF supernatant were recovered in aliquots in polypropylene tubes and stored at –80°C until use.

Osteopontin assay

Quantification of OPN levels in CSF samples was performed using a commercially available enzyme-linked immunosorbent assay kit (Human Osteopontin ELISA, IBL, Hamburg, Germany) according to the manufacturer's protocol. The assay had a minimum OPN concentration detection limit of 5 ng/ml. CSF and plasma samples were diluted to various concentrations to determine the linearity and sensitivity of the assay. The assay was most sensitive at a plasma dilution of 1:10 and a CSF dilution of 1:200, which was used for most samples. All assays were performed in duplicate, and the observer was blinded to the diagnosis.

RNA isolation from peripheral blood mononuclear cells

PBMCs were isolated by Ficoll-Paque (Pharmacia Biotech, Uppsala, Sweden) from 20 ml of citrated blood from 18 MS patients and healthy controls (HCs). After washing in 30 ml of phosphate-buffered saline (PBS) and centrifugation at 100 g for 10 min, the cell pellet was resuspended in 1 ml of PBS. The number of PBMCs was counted in a haemocytometer. After centrifugation, the cell pellet was resuspended in 0.5 ml of guanidinium thiocyanate solution and total RNA was extracted. Before reverse transcriptase polymerase chain reaction (RT-PCR), total RNA was treated with DNase I to remove contaminating genomic DNA. Digestion was conducted at 37°C for 1 h in the presence of 10 units of DNase I (Roche Diagnostics, Mannheim, Germany), 10 mM $MgCl_2$, 0.1 mM DTT, and 50 mM Tris-HCl. After heat inactivation, RNA was extracted using phenol-chloroform and was then ethanol-precipitated.

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Semiquantitative reverse transcriptase polymerase chain reaction of OPN mRNA levels in peripheral blood mononuclear cells

Total RNA (1 mg) was denatured at 65°C for 2 min and annealed with 1 mg of random primers at 37°C for 10 min. RT-PCR was carried out in reaction buffer [50 mM Tris-HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$] with 10 mM DTT, 0.5 mM deoxynucleoside triphosphates, and 0.5 ml of RNase block (Stratagene, La Jolla, California). cDNA was synthesized at 37°C for 1 h using 200 units of Moloney murine leukaemia virus reverse transcriptase (Life Technologies, Inc.). The reaction was stopped at 70°C for 7 min. PCR amplification of OPN cDNA was conducted using the gene-specific primers, 5′-TCACAGCCATGAAGA TATGCTGG-3′ and 5′-TACAGGGAGTTTCCATG AAGCCAC-3′, lying within different exons to give a product of 298 bp. The β-actin mRNA served as an internal control to ensure that an exact amount of high-integrity total RNA was reverse-transcribed to produce cDNA in each assay. PCR was conducted in PCR buffer [20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 2.5 mM $MgCl_2$] added with 0.2 mM deoxynucleoside triphosphates; 30 pmol of sense and antisense primers for OPN or β-actin cDNA; 3 ml of cDNA; and 2.5 units of Taq DNA polymerase (Life Technologies, Inc.). The optimized thermal profile was initiated with a 5-min denaturation at 94°C, followed by 30 cycles of 94°C for 1 min, 61°C (β-actin) or 65°C (OPN) for 1 min and 72°C for 1 min, and a final extension at 72°C for 10 min. Aerosol-resistant pipette tips and separate areas were used for pre-PCR, PCR, and post-PCR procedures. Each sample was analysed in duplicate.

Bone markers assays

Sandwich enzyme immunoassay test kits were used for the quantitative determination of osteocalcin (N-MID™ Osteocalcin One Step ELISA kit, Osteometer BioTech A/S, Herlev, Denmark) and quantification of the bone-specific degradation product carboxy-terminal telopeptide region of type I collagen α1 chain (CrossLaps™ One Step ELISA, Osteometer Biotech A/S, Herlev, Denmark) following the manufacturer's procedure. Quantification of 25-OH vitamin D was performed using the automated method LIAISON® 25-OH Vitamin D TOTAL assay kit (DiaSorin S.p.A., Italy).

Statistical analysis

Differences in OPN levels between the two groups were analysed by the Mann–Whitney test. The association of OPN with other parameters was evaluated by Spearman's correlation analysis. P values lower than 0.05 were considered to indicate statistical significance (confidence interval 95%).

Results

RRMS patients had variable OPN plasma levels ranging from 204 to 663 ng/ml (Figure 1A). Compared to sex- and age-matched controls, OPN plasma levels were significantly increased. Previously, it had been demonstrated that RRMS patients during relapse presented higher OPN levels compared to RRMS patients during remission.[3,4] In this patient cohort, OPN levels significantly correlated with the IgG index (Figure 1B). In contrast, the number of oligoclonal bands did not correlate with OPN plasma levels in RRMS patients (data not shown).

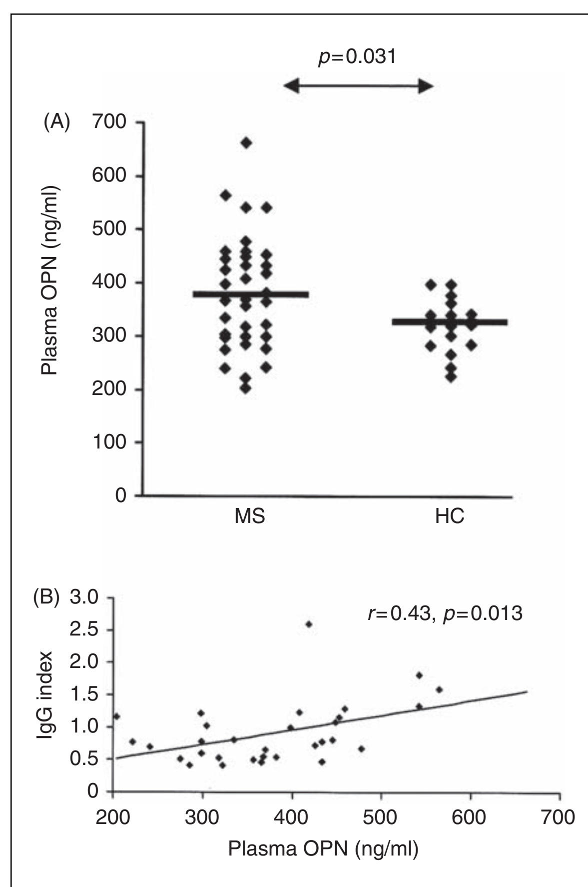

Figure 1. Increased plasma osteopontin (OPN) levels in active relapsing-remitting multiple sclerosis (RRMS) patients. Plasma OPN levels were determined in multiple sclerosis (MS) patients and age- and gender-matched healthy controls (HC) using an OPN-specific enzyme-linked immunosorbent assay (ELISA). Differences between the groups were calculated using Mann–Whitney non-parametric analysis (A). The relationship between OPN plasma levels and the immunoglobulin G (IgG) index was determined by Spearman's correlation analysis (B).

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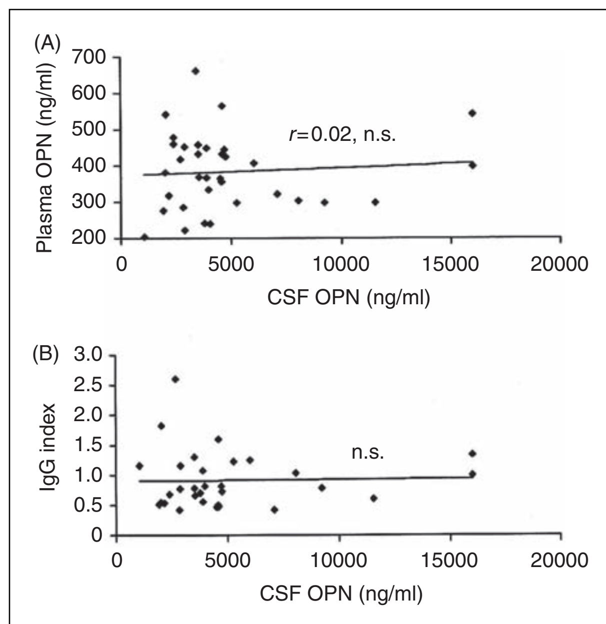

Figure 2. No correlation between cerebrospinal fluid (CSF) osteopontin (OPN) and plasma OPN. OPN levels were determined in paired plasma and CSF samples of multiple sclerosis (MS) patients using an OPN-specific enzyme-linked immunosorbent assay (ELISA). The relationship between OPN levels in CSF and plasma (A) and the immunoglobulin G (IgG) index (B) was determined by Spearman's correlation analysis.

To evaluate whether the increased OPN plasma levels were a reflection of enhanced intrathecal production, OPN levels were determined in paired plasma and CSF samples from RRMS patients. CSF had highly variable OPN levels, ranging from 1046 ng/ml to as high as 16,000 ng/ml (Figure 2A). Remarkably, OPN CSF levels did not correlate with OPN plasma levels or with the IgG index (Figure 2B). Since OPN levels in CSF were on average 10 to 20-fold higher than in plasma, we also evaluated whether OPN plasma levels were associated with albumin levels in CSF, a marker for integrity of the blood–brain barrier. No correlation was found between OPN plasma levels and albumin CSF levels in RRMS patients (data not shown).

OPN plasma levels showed no association with OPN expression level in the PBMCs of RRMS patients (Figure 3). Correction for the total number of PBMCs in each RRMS patient had no effect on the results of the correlation analysis. Two RRMS patients with high OPN mRNA levels in PBMCs had OPN plasma levels below the average level of the patient group. Additionally, OPN mRNA levels in the PBMCs of MS patients were not significantly different from the levels in HCs (data not shown).

Then, the relationship between bone-specific markers in the RRMS patient group was determined (Figure 4). The bone-specific degradation marker

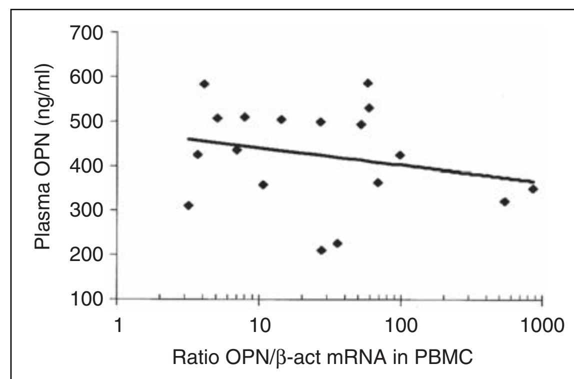

Figure 3. No correlation between plasma osteopontin (OPN) and OPN mRNA levels in peripheral blood mononuclear cells (PBMCs). RNA was extracted from the PBMCs of multiple sclerosis (MS) patients and OPN mRNA levels were determined by semiquantitative polymerase chain reaction (PCR) analysis. The relationship between OPN levels in plasma and the OPN mRNA levels in PBMCs was determined by Spearman's correlation analysis.

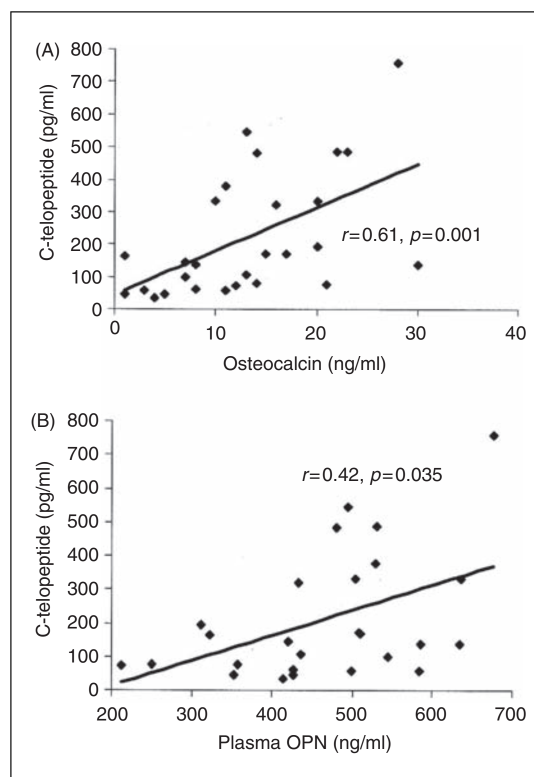

Figure 4. Significant correlation between plasma osteopontin (OPN) and bone-specific degradation product. Plasma OPN, osteocalcin, and the bone-specific degradation product carboxy-terminal telopeptide region of type I collagen α1 chain (C-telopeptide) were determined using specific enzyme-linked immunosorbent assays (ELISAs). The relationship between osteocalcin and C-telopeptide (A) and between OPN and C-telopeptide (B) were determined by Spearman's correlation analysis.

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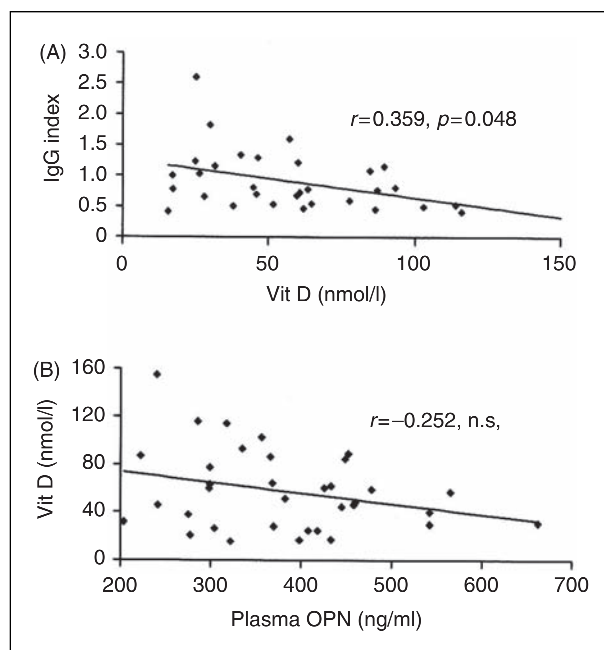

Figure 5. Vitamin D levels negatively correlated with the immunoglobulin G (IgG) index in MS patients. Quantification of 25-OH vitamin D was performed using an automated method. The relationship between vitamin D levels and the IgG index (A) or OPN plasma levels (B) was determined by Spearman's correlation analysis.

C-telopeptide of type I collagen significantly correlated with the bone formation marker osteocalcin in the RRMS patient group. Interestingly, OPN plasma levels were also significantly associated with C-telopeptide of type 1 collagen, whereas no significant correlation was observed between OPN and osteocalcin (data not shown). A significant negative correlation was found between 25-OH vitamin D and the IgG index (Figure 5). RRMS patients with high OPN levels tended to have lower 25(OH)-vitamin D levels than patients with low OPN levels, although correlation analysis revealed no statistically significant relationship. There was no relationship between CSF OPN levels and serum 25-OH vitamin D levels.

Discussion

A limited number of studies demonstrated that OPN levels were increased in plasma of RRMS patients and predominantly in those patients who had an active disease activity state based on the occurrence of a relapse.[3,4] In this study, we showed that OPN plasma levels significantly correlated with the IgG index. The most likely origin of OPN would be intrathecal production, as OPN has been shown to be highly expressed in MS lesions.[2] However, we found no correlation between CSF and plasma OPN levels in paired samples. Moreover, in contrast to OPN plasma levels, no correlation was found between OPN CSF levels and the IgG index.

Although a recent study reported increased OPN CSF levels in MS patients compared to patients with non-inflammatory neurological diseases, OPN CSF levels were not associated with the occurrence of a relapse nor correlated with OPN plasma levels.[7] The absence of correlation with disease activity may be caused by the role of OPN in the CNS repair process. Zhao et al. demonstrated that OPN is extensively expressed by macrophages following CNS demyelination but has a redundant role in remyelination as well.[19] The lack of correlation between OPN CSF and plasma levels may be due to the inability of the acidic 70 kDa glycoprotein OPN to cross the blood–brain barrier. Chowdhury et al. found no significant difference in OPN CSF levels between patients with active and stable disease activation state, although they claim that there was a trend towards higher OPN levels in patients with active disease.[6] The lack of correlation between OPN CSF and plasma levels and the absence of association of CSF OPN with the IgG index indicate that increased OPN plasma levels may be, at least in part, a result of extrathecal production.

Activated PBMCs may express OPN and may contribute to the increased OPN plasma levels in RRMS patients. However, there was no correlation between OPN mRNA expression levels in PBMCs and OPN protein levels in the plasma of RRMS patients. Although the absence of correlation could also be explained by causes like post-transcriptional regulation, the findings are in line with previous microarray data on PBMCs of RRMS patients. In contrast to the high OPN expression levels found in MS lesions, none of the microarray studies performed on the PBMCs of RRMS patients identified OPN as highly expressed mRNA.[20,21]

Several studies demonstrated that MS patients lose bone mass more rapidly than do their healthy, age- and gender-matched controls.[22,23] Since OPN plays an important role in the bone resorption process, we evaluated whether OPN plasma levels in RRMS patients were related to bone-specific markers. Interestingly, OPN levels significantly correlated with the bone-specific degradation product C-telopeptide of type I collagen. Moreover, vitamin D, which stimulates bone growth, correlated negatively with the IgG index and a trend towards a negative correlation with OPN was observed. Other investigators have described the negative relationship between vitamin D and disease activity in RRMS patients.[24,25] The increased OPN levels and decreased vitamin D levels during enhanced disease activity are also in agreement with the proposed immunomodulatory actions of both molecules, stimulating Th1-type immune responses and decreasing regulatory T cell functions.[13,26] Dysregulation of both molecules

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may lead to a disbalanced immune system during relapses in RRMS patients.

Enhanced OPN levels and decreased vitamin D levels may also contribute to osteoporosis in MS patients. Steroid use is another known factor that may increase the bone resorption process. However, studies on bone loss in MS patients showed that bone mineral density in MS patients was associated with ambulatory status and disease duration, whereas no relation could be found with steroid use.[22] These findings suggest that bone homeostasis is impaired during disease progression. Numerous studies have revealed that cells of the immune system like monocytes and lymphocytes present in blood of MS patients show an altered state of activation in relation to disease activity.[27] Increased pro-inflammatory cytokine levels may contribute to the development of osteoporosis.[28] On the other hand, cells in bone tissue produce several proteins, like OPN, that play an important role in inflammation.[29,30]

The awareness that bone cells influence immune cells, and vice versa, have recently lead to the introduction of a novel discipline called osteoimmunology.[31] There is now accumulating evidence that bone not only plays a role in the development of the immune system in the bone marrow, but also influences adaptive immunity. The majority of hormones, cytokines, and cell-signalling pathways regulating bone function also regulate the immune system. For example, vitamin D, parathyroid hormone (PTH), oestrogen, and more recently, leptin, which are well-recognized regulators of bone function, also modulate immune function. They have been found to be dysregulated in MS and may not only contribute to osteoporosis but also to enhanced systemic inflammation and concomitant aggravation of MS disease activity or progression.[25,32,33]

In conclusion, we demonstrated that the bone-regulating and immunomodulating molecules OPN and vitamin D were associated with the IgG index in RRMS patients. The results of this study should be confirmed in larger studies, in which disease activity is monitored by MRI or relapse rate, before firm conclusions could be drawn. Elucidating the mechanisms acting on both bone metabolism and the immune system in RRMS patients may not only lead to novel insights into MS pathology, but may also result in novel markers to monitor disease activity or progression.

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