

BRIEF REPORT

Osteoporosis Associated with Neutralizing Autoantibodies against Osteoprotegerin

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SUMMARY

Autoantibodies against osteoprotegerin, which block the inhibitory effect of osteoprotegerin on signaling by the receptor activator of nuclear factor (NF)- κ B (RANK), were identified in a man with celiac disease who presented with severe osteoporosis and high bone turnover. The osteoporosis did not respond to the treatment of his celiac disease but was completely reversed by bisphosphonate therapy. Autoantibodies against osteoprotegerin were detected in three additional patients with celiac disease. Such autoantibodies may be associated with the development of high-turnover osteoporosis, but whether autoantibodies against osteoprotegerin commonly contribute to the pathogenesis of osteoporosis in patients with celiac disease remains to be determined.

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OSTEOPOROSIS IS A COMMON DISEASE ASSOCIATED WITH REDUCED BONE mass and an increased risk of fracture. Although osteoporosis is a recognized complication of celiac disease,¹ it is generally considered to be secondary to malabsorption and deficiencies of calcium and vitamin D, rather than a consequence of an autoimmune process.¹⁻³ We describe a patient who had celiac disease and autoimmune hypothyroidism and in whom high-turnover osteoporosis developed in association with the presence of neutralizing autoantibodies against osteoprotegerin, an inhibitor of bone resorption produced by osteoblasts and many other types of cells. The antibodies blocked the inhibitory effect of osteoprotegerin on the RANK signaling pathway.

The RANK receptor plays a key role in the regulation of bone remodeling.⁴ When activated by the RANK ligand (RANKL), a signaling cascade is initiated, causing osteoclast differentiation and increased bone resorption. Osteoprotegerin, which acts as a decoy receptor for RANKL, blocks this interaction. The importance of this pathway in bone metabolism is demonstrated by the facts that pharmacologic blockade of RANKL is an effective treatment for osteoporosis,⁵ that an inherited deficiency of RANK or RANKL causes osteopetrosis,⁶ and that loss-of-function osteoprotegerin mutations cause juvenile Paget's disease — an inherited condition characterized by bone deformity, increased bone turnover, and multiple fractures.^{7,8} Genetic variation at the locus encoding osteoprotegerin has been implicated as a risk factor for both osteoporosis and Paget's disease.^{9,10} We investigated whether our patient had an acquired abnormality of osteoprotegerin function that led to bone disease.

CASE REPORT

A 40-year-old man presented with a low-trauma fracture of the left clavicle. He had no history of fractures, despite having taken part in high-impact sports during his

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20s and 30s. Dual-energy x-ray absorptiometry (performed with a Hologic QDR4500 densitometer) showed low bone mineral density at the spine (vertebrae L1 through L4; T score, -6.6) and at the femoral neck (T score, -2.9). Routine tests showed an elevated total alkaline phosphatase level (2610 U per liter; normal range, 25 to 120) and an elevated serum phosphate level (2.36 mmol per liter [7.3 mg per deciliter]; normal range, 0.8 to 1.4 [2.5 to 4.3]) but normal serum calcium, albumin, urea, and electrolyte levels, liver-function tests, and complete blood count. Isoenzyme studies revealed that the increase in the alkaline phosphatase level was of bony origin. The serum parathyroid hormone level was low (8 ng per liter; normal range, 10 to 65), and the serum level of 25-hydroxyvitamin D was 35 nmol per liter (normal range, 25 to 170). The results of a short cosyntropin test were normal. Screening for primary hypothyroidism, performed because the patient had a 12-month history of cold intolerance and lack of energy, revealed a serum free thyroxine level of less than 0.5 pmol per liter (0.04 ng per deciliter; normal range, 10 to 20 [0.8 to 1.6]) and an elevated thyrotropin level (>65 mU per liter; normal range, 2 to 5). Anti-thyroid peroxidase antibodies were detected (243 U per milliliter; normal range, 0 to 82). Tests for thyrotropin receptor-blocking antibodies¹¹ were negative. Serum testosterone and gonadotropin levels were normal.

Treatment with levothyroxine was begun (at a dose of 100 μ g daily), but alkaline phosphatase levels increased further, to 4065 U per liter, and hypercalcemia developed (serum calcium level, 2.8 mmol per liter [11.2 mg per deciliter]; normal range, 2.1 to 2.6 [8.4 to 10.4]). At this point, the serum parathyroid hormone, 25-hydroxyvitamin D, and 1,25-dihydroxyvitamin D levels were undetectable, and the urinary calcium-to-creatinine ratio was elevated (4.78; normal value, <0.5), findings that were consistent with resorptive hypercalcemia.¹² The hypercalcemia resolved with intravenous rehydration. A transiliac bone biopsy, without tetracycline prelabeling, showed high bone turnover and woven bone (Fig. 1A). There was extensive osteoid coverage, but the seams were of normal thickness (Fig. 1B).

The results of investigations to rule out an occult tumor, including measurements of serum parathyroid hormone-related protein, were normal. Radionuclide bone scanning showed a generalized increase in tracer uptake but no focal lesions (Fig. 1C). Levels of transglutaminase IgA

antibodies were elevated (101 U per liter; normal range, 5 to 30), and occult celiac disease was confirmed on small-bowel biopsy.

A gluten-free diet, a regimen of 10,000 units of vitamin D₂ daily, and calcium supplementation (1 g per day) were begun and led to a decrease in the alkaline phosphatase level to about 1000 U per liter over a period of more than 6 months, but the osteoporosis worsened, with an 8% decrease in the T score for bone mineral density at the spine, to -7.1 (Fig. 2A). The patient had a low-trauma fracture of the left humerus and a 6-cm loss in height due to multiple vertebral fractures (Fig. 1D), although repeat duodenal biopsy showed normalization of histologic features of the bowel. The serum 25-hydroxyvitamin D level was normal (68 nmol per liter [27.2 ng per milliliter]). Urinary deoxypyridinoline values were measured at this time and were about 20 times the upper limit of the normal range (93 nmol per millimole of creatinine; normal range, 2.3 to 5.4) (Fig. 2B). Serum osteoprotegerin levels, measured by means of an enzyme-linked immunosorbent assay (ELISA) (Biomedica, Oxford Biosystems) on two occasions, approximately 4 weeks apart; were 0.78 and 0.47 pmol per liter, respectively (normal range, 0.14 to 130). Total serum RANKL levels, as measured with the use of an ELISA (Apotech, Epalinges) at the same time points, were 0.152 and 0.143 nmol per liter, respectively (normal range, 0 to 10).

The patient was given three infusions of 4 mg of zoledronic acid over a 3-month period. After the first infusion, the serum calcium level decreased to 1.91 mmol per liter (7.64 mg per deciliter), accompanied by increases in the parathyroid hormone level to 163 ng per liter and in the 1,25-dihydroxyvitamin D level to 992 pmol per liter (normal range, 20 to 120). No symptoms of hypocalcemia were reported. Subsequently, serum calcium, parathyroid hormone, both 25-hydroxyvitamin D and 1,25-dihydroxyvitamin D, alkaline phosphatase, and urinary deoxypyridinoline levels returned to normal. When the patient was last seen, 42 months after his first presentation, no further clinical fractures had occurred, his height had stabilized, there was no evidence of progression of the vertebral fractures; T scores for bone mineral density at the spine and femoral neck had increased to -1.7 and -0.8 , respectively (Fig. 2A), and the levels of alkaline phosphatase and urinary deoxypyridinoline were normal.

Further studies were performed to determine

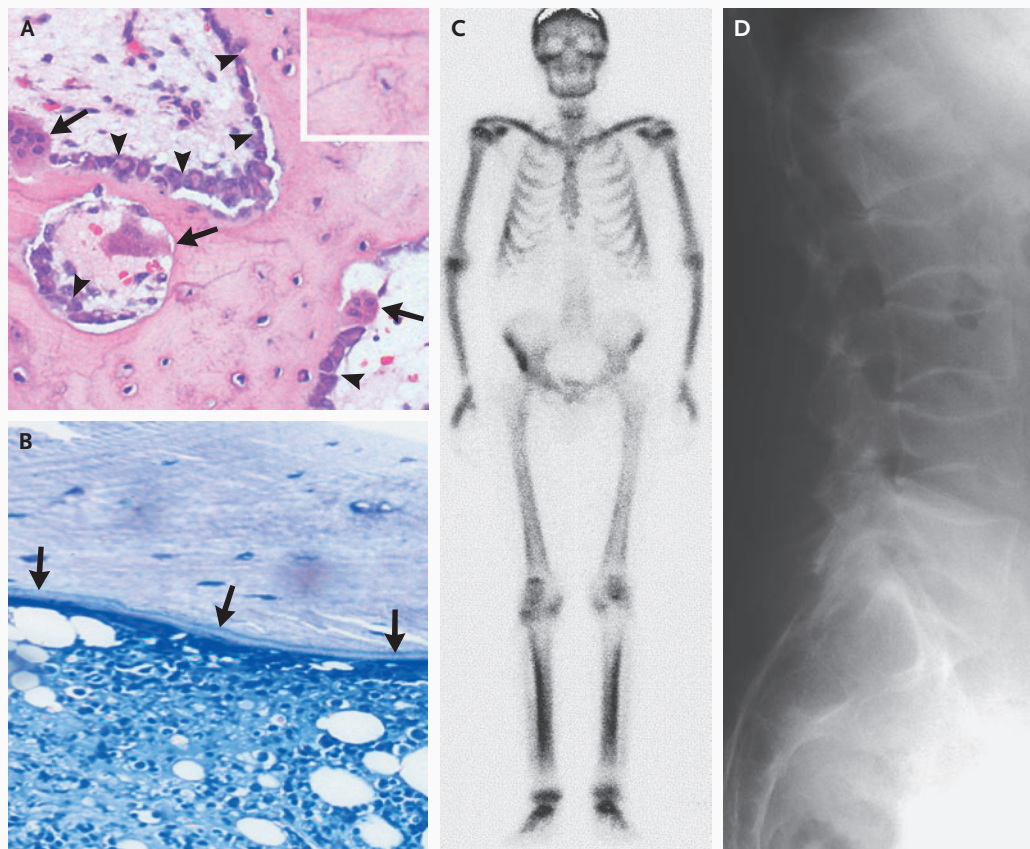


Figure 1. Results of Transiliac Bone Biopsy and Bone Radiography in the Patient.

A photomicrograph of the bone-biopsy specimen (Panel A, hematoxylin and eosin) shows an increase in the numbers of osteoclasts (arrows) and osteoblasts (arrowheads). Woven bone is present throughout the biopsy specimen, as evidenced by the irregular cement lines (higher-power view shown in inset). Staining of the biopsy specimen with toluidine blue (Panel B) shows extensive osteoid coverage, but the seams were of normal thickness (arrows). Radio-nuclide bone scanning (Panel C) showed a generalized increase in tracer uptake but no focal lesions. Radiography of the lumbar spine (Panel D) revealed osteopenia and multiple vertebral compression fractures.

the cause of the increase in bone turnover. This investigation focused on the possibility that neutralizing autoantibodies against osteoprotegerin might have developed, given the high bone turnover and autoimmune diathesis.

METHODS

IMMUNOPRECIPITATION ASSAY FOR OSTEOPROTEGERIN

Nonfasting serum samples were obtained from the patient on several occasions throughout the course of his illness, as well as from 10 age-matched healthy male controls, 15 patients with celiac disease, and 14 patients with autoimmune hypothyroidism. The protein content was mea-

sured with the use of the bicinchoninic acid assay (Pierce). For the immunoprecipitation assay, serum samples were incubated at a 1:100 dilution with 12.5 ng of homodimeric recombinant human osteoprotegerin (R&D Systems) and protein G-coated agarose beads (Calbiochem) that had been preincubated with 5% albumin to reduce nonspecific binding. After incubation for 1 hour at 37°C, the beads were washed five times with phosphate-buffered saline, suspended in 30 μ l of reducing sample buffer, and incubated at 90°C for 5 minutes. After brief centrifugation, the supernatant was loaded onto a 12% polyacrylamide gel (Criterion, Biorad), subjected to electrophoresis at 200 V for 60 minutes, transferred to a membrane, and probed with a mouse mono-

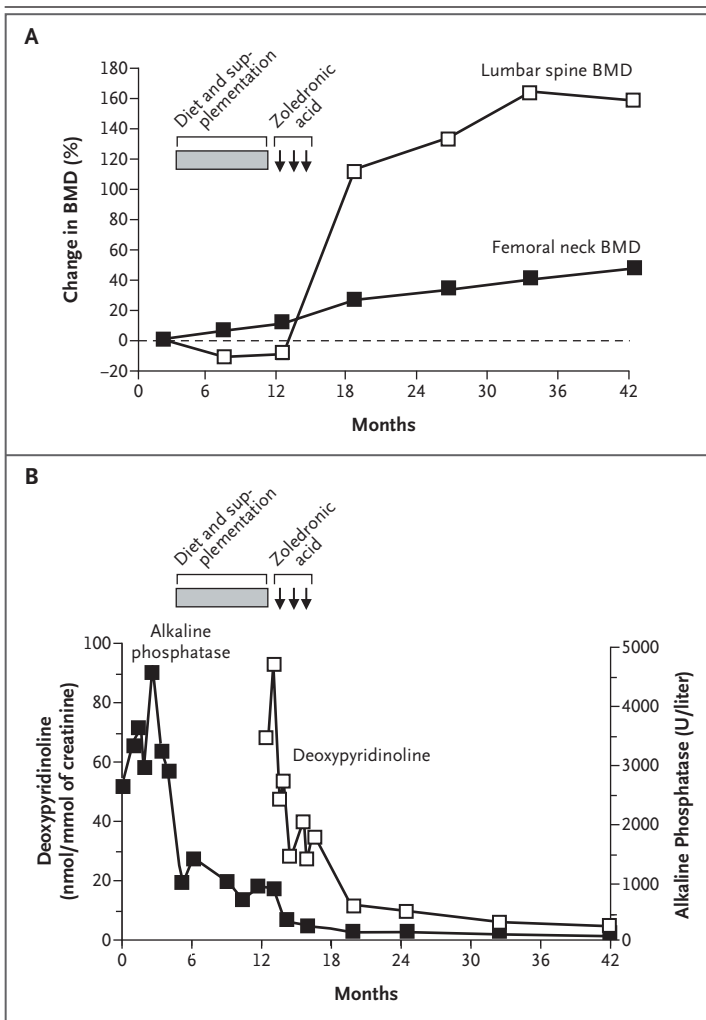


Figure 2. Bone Mineral Density (BMD) and Biochemical Markers of Bone Turnover in the Patient at Presentation and in Response to Treatment.

Treatment included a several-month period of a gluten-free diet and calcium and vitamin D supplementation (gray bars in both panels) and zoledronic acid therapy (arrows in both panels). Panel A shows the BMD values at the lumbar spine and femoral neck. The lumbar spine BMD decreased by about 8% despite the gluten-free diet and calcium and vitamin D supplementation, but both the spine and femoral neck BMD values progressively increased after treatment with zoledronic acid. Panel B shows the levels of urinary deoxypyridinoline (expressed in relation to creatinine) and serum alkaline phosphatase. Alkaline phosphatase levels decreased from more than 4000 U per liter to about 1000 U per liter after a gluten-free diet and calcium and vitamin D supplementation, but deoxypyridinoline levels remained markedly elevated. Treatment with zoledronic acid normalized the levels of both biochemical markers of bone turnover.

clonal antibody against human osteoprotegerin (Abcam). A peroxidase-conjugated donkey anti-mouse antibody (Jackson) at a 1:5000 dilution was used for detection. Loading was assessed by

probing the blot with peroxidase-conjugated goat antihuman antibody (Jackson) at a 1:5000 dilution. Immunolabeled bands were detected with the use of a chemiluminescent substrate (Super-Signal, Pierce) and a chemiluminescence imager (GeneGnome, Syngene).

RANK SIGNALING ASSAY

We studied the effects of immunoglobulins, purified from serum samples obtained from the patient and controls with the use of protein G spin columns (Pierce), on RANKL-induced activation of NF- κ B. For this assay, we used the human embryonic-kidney-cell line HEK293, stably transfected with a NF- κ B-responsive luciferase reporter vector (Cambridge Biosciences). The HEK293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal-calf serum and Hygromycin (Roche) but were serum-starved for 2 hours before stimulation and were maintained thereafter in Dulbecco's modified Eagle's medium with 2% human tissue culture medium supplement (MP Biomedicals) constituted without cytokines. The cells were stimulated with the use of human recombinant RANKL (100 ng per milliliter) for 1 hour at 37°C in the presence of osteoprotegerin (100 to 400 ng per milliliter, R&D Systems) and in the presence or absence of immunoglobulins at a 1:40 dilution. The cells were then lysed, and the lysates were analyzed for luciferase activity with the use of Steady-Glo reagent (Promega) on a microplate reader (Synergy HT, Biotek). All reporter assays were run with five replicates in a 96-well plate containing 5×10^4 cells per well.

RESULTS

Osteoprotegerin immunoprecipitated with the use of the patient's serum was seen in a strong band at 55 kD on Western blotting (Fig. 3A). Serum specimens from the 10 healthy controls, 12 patients with celiac disease, and 14 patients with primary hypothyroidism were negative for the autoantibody (examples shown in lanes 3 through 9), but samples from 3 of 15 patients with celiac disease were positive, albeit at a lower level of intensity than that in the index patient (lanes 10 through 12). Equal loading of the samples was confirmed by probing the same blot for total immunoglobulin (Fig. 3A). The immunoprecipitation assay was

repeated in the presence of gliadin, but this had no effect on the ability of the index patient's serum to immunoprecipitate osteoprotegerin, suggesting that the osteoprotegerin autoantibodies did not cross-react with gliadin. The mean ($\pm$ SE) z score for bone mineral density at the spine was -0.23 ± 0.08 among samples that contained osteoprotegerin autoantibodies, as compared with 0.49 ± 0.08 among samples that did not contain autoantibodies; the z scores for bone mineral density at the hip were -1.00 ± 0.21 and 0.40 ± 0.41 , respectively. These differences were not significant. Serum levels of alkaline phosphatase were normal in all subjects.

The addition of RANKL (100 ng per milliliter) to HEK cells stably transfected with a NF- κ B luciferase reporter construct resulted in robust activation of the reporter-gene expression (Fig. 3B). This activation was inhibited by the addition of human recombinant osteoprotegerin (100 ng per milliliter) in the presence of the immunoglobulin fraction from control serum, but in the presence of immunoglobulins purified from the patient's serum, the inhibitory effect of osteoprotegerin was lost. A higher level of osteoprotegerin (400 ng per milliliter) restored the inhibitory effect in the patient's serum. Finally, the addition of immunoglobulin fractions from either the patient's serum or control serum in the absence of RANKL had no significant effect on reporter-gene expression.

DISCUSSION

Our patient presented in adulthood with severe, high-turnover osteoporosis associated with subclinical celiac disease and autoimmune hypothyroidism. Circulating autoantibodies against osteoprotegerin were present, and immunoglobulins purified from specimens of the patient's serum, but not from control serum, abolished the inhibitory effect of osteoprotegerin on RANKL-induced NF- κ B signaling in vitro, a finding that was consistent with the presence of neutralizing autoantibodies against osteoprotegerin. No evidence of circulating autoantibodies against osteoprotegerin was found in serum samples from 10 healthy controls and 14 patients with autoimmune hypothyroidism, but autoantibodies were detected in samples from 3 of the 15 patients with celiac disease.

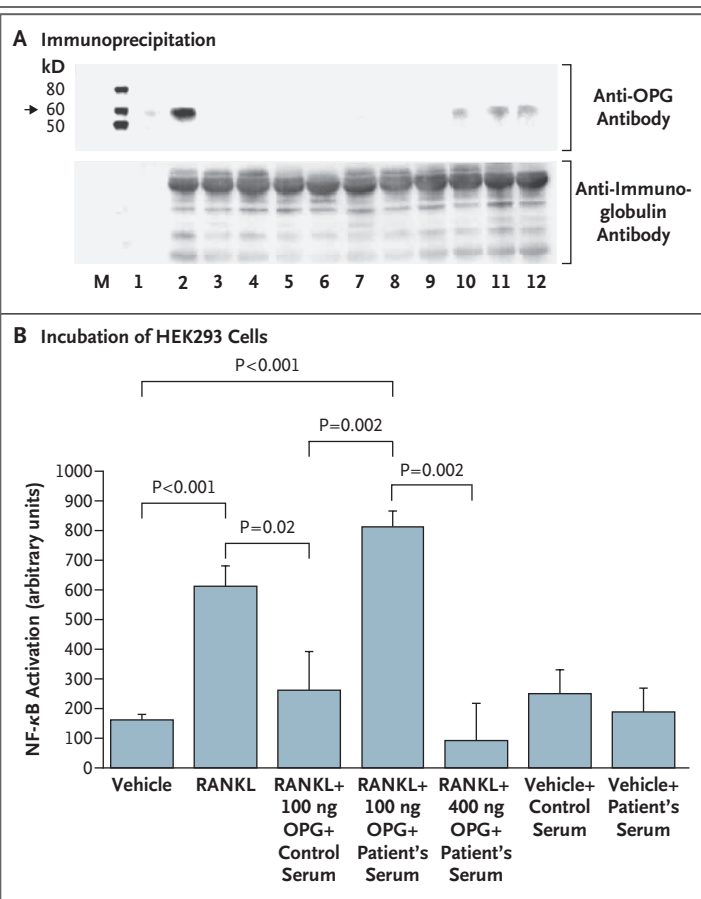


Figure 3. Detection of Serum Autoantibodies against Osteoprotegerin (OPG) and Their Effect on Activation of Nuclear Factor κ B (NF- κ B) by the Receptor Activator of NF- κ B Ligand (RANKL).

Panel A shows the presence of autoantibodies against OPG (top) in a sample of the patient's serum (lane 2), reflected by the detection of a strong band of approximately 55 kD (arrow) on Western blotting with an anti-OPG antibody after immunoprecipitation. Molecular-weight markers of 80, 60, and 50 kD are shown in lane M. Samples from healthy controls (lanes 3 and 4) and patients with primary hypothyroidism (lanes 5 through 8) were negative for the autoantibodies, as were the negative control (lane 1) and 12 of 15 samples from patients with celiac disease (example shown in lane 9). However, 3 of 15 samples from patients with celiac disease (lanes 10 through 12) also showed the 55-kD band indicating the presence of OPG autoantibodies. Western blotting with the addition of staining for immunoglobulin (bottom) confirmed the equal loading of the gel. Panel B shows the effect of OPG on the mean level of RANKL-stimulated NF- κ B activation of the human embryonic-kidney cell line HEK293. Cells were incubated in various combinations of a vehicle (negative control), serum samples from our patient or from controls, RANKL (100 ng per milliliter), and OPG (100 or 400 ng per milliliter). Activation was abrogated by the 100-ng concentration of OPG in control serum but not in the patient's serum. The addition of the higher concentration of OPG (400 ng per milliliter) overcame the inhibition. T bars indicate standard errors. The data in both panels are representative of the three independent experiments performed.

Our patient's bone disease occurred in association with the spontaneous development of autoantibodies against osteoprotegerin. However, one patient in whom treatment with a recombinant Fc-osteoprotegerin construct led to the development of autoantibodies against that fusion protein did not subsequently become ill.¹³ The mechanism by which osteoprotegerin autoantibodies developed in our patient remains unclear, but autoantibodies against other circulating proteins have been reported to develop in patients with autoimmune disease.¹⁴ Presumably, endogenous osteoprotegerin had become the target of an autoimmune response in our patient.

Our patient's high-turnover osteoporosis, elevated alkaline phosphatase level, and fragility fractures are consistent with the phenotype seen both in mice that have targeted inactivation of the gene encoding osteoprotegerin¹⁵ and humans who have juvenile Paget's disease and osteoprotegerin-inactivating mutations.^{7,8} The severity of the bone disease in our patient and the clinical deterioration despite treatment of the celiac disease and supplementation with calcium and vitamin D are notable, as is the remarkable recovery in response to treatment with zoledronic acid, the potent inhibitor of osteoclastic bone resorption. Thus, elevated bone turnover was the primary cause of this patient's osteoporosis, and the response strongly suggests that his acquired illness was due to the development of neutralizing autoantibodies against osteoprotegerin.

We considered other diagnoses. An inherited form of osteoporosis was ruled out on the basis of the late onset of the disease and the fact that the patient had no history of a fracture despite a highly active lifestyle. Osteoporosis and osteomalacia are known complications of celiac disease caused by malabsorption and the effects of calcium and vitamin D deficiency.^{1,2} However, the osteoporosis and elevated bone turnover in our patient were more pronounced than the usual findings in persons with celiac disease, and our

patient did not have a response to a gluten-free diet and calcium and vitamin D supplementation; these differences argue strongly against celiac disease as the primary cause of the bone disease in our patient.¹⁻³ Fibrogenesis imperfecta ossium was also considered, since it is manifested in adulthood as fragility fractures and a mineralization defect.¹⁶ However, the clinical features in our patient were quite distinct from those of fibrogenesis imperfecta ossium, which is characterized by trabecular thickening on radiography, accumulation of thick osteoid on bone biopsy, and a poor response to treatment.¹⁶

We also demonstrated the presence of autoantibodies against osteoprotegerin in 3 of 15 patients with celiac disease. Although the three patients with the autoantibodies had lower bone mineral density values than those without the autoantibodies, the difference was not significant. These observations suggest that osteoprotegerin autoantibodies may contribute to the pathogenesis of osteoporosis in celiac disease more widely, but the observations should be interpreted with caution, since bone mineral density is a complex trait influenced by many genetic and environmental factors. Future studies should determine whether osteoprotegerin autoantibodies are associated with the development or severity of osteoporosis in patients with celiac disease or other autoimmune diseases.

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Drs. Riches, Fraser, and Ralston report having submitted patent applications on the use of osteoprotegerin antibodies as a diagnostic test and therapeutic target in patients with osteoporosis; Dr. McRorie, receiving lecture fees from Wyeth; Dr. Fraser, receiving consulting fees from Procter & Gamble, Merck Sharp & Dohme, Novartis, Sanofi-Aventis, Nycomed, and Roche, lecture fees from Novartis, Roche, and Nycomed, and grant support from Sanofi-Aventis, Lilly, and Roche; and Dr. Ralston, receiving consulting fees from Procter & Gamble, Novartis, and Merck, lecture fees from Novartis and Procter & Gamble, and grant support from Novartis and Wyeth. No other potential conflict of interest relevant to this article was reported.

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