EXTENDED REPORT

Effect of bisphosphonates on cartilage turnover assessed with a newly developed assay for collagen type II degradation products

H J Lehmann, U Mouritzen, S Christgau, P A C Cloos, C Christiansen

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Background: Animal studies of arthritis have suggested that bisphosphonates may have chondroprotective abilities.

Objective: To evaluate the effect of bisphosphonate treatment on cartilage degradation.

Methods: Type II collagen is almost exclusively localised in cartilage, where it is the major structural component of the tissue. Hence fragments derived from this protein should represent a specific index for cartilage degradation. The urinary concentration of collagen type II C-telopeptide degradation products (CTX-II) was measured by a new immunoassay (enzyme linked immunosorbent assay (ELISA)). The serum concentration of collagen type I C-telopeptide degradation products (CTX-I), a marker of bone degradation, was also measured by ELISA.

Participants: Two groups were studied. The alendronate group included 63 healthy postmenopausal women aged 45–54 randomly allocated to receive three years' treatment with 1 mg, 5 mg, 10 mg, or 20 mg alendronate daily or placebo. In the third year the women receiving 20 mg were switched to placebo. The ibandronate group included 119 women at least 10 years after the menopause aged <75 randomly allocated to receive 12 months' treatment with 0.25 mg, 0.5 mg, 1.0 mg, 2.5 mg, or 5 mg ibandronate daily or placebo followed by 12 months without treatment.

Results: 20 mg of alendronate and 2.5 and 5 mg of ibandronate treatment produced significant decreases in urinary CTX-II to about 50% of baseline. The level reached after three months of treatment remained practically constant during the following 12–36 treatment months. When treatment was withdrawn CTX-II values returned towards baseline. Serum CTX-I also decreased rapidly within three months, but to a level of about 30% of baseline.

Conclusions: The urinary excretion of CTX-II, a new marker of cartilage degradation, decreases significantly in response to bisphosphonate. This suggests that bisphosphonates may have chondroprotective effects in humans. By measurement of CTX-II it should be possible to monitor the effects of drugs that potentially inhibit cartilage destruction.

See end of article for authors' affiliations

Correspondence to:
Dr H J Lehmann, Centre for
Clinical and Basic
Research, Ballerup Byvej
222, 2750 Ballerup,
Denmark; hjl@ccbr.dk

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Arthritis is a general term used to describe more than 100 chronic diseases of the joints, bones, and muscles. The most common type of arthritis is osteoarthritis (OA), a chronic and debilitating disease characterised by degradation of the joint cartilage. In the Western world at least 10% of the population have symptoms of OA and 50% of them will require treatment with drugs. Hence, the condition is one of the leading disabilities in the Western world.¹

Despite the high prevalence of OA, current diagnostic tools for assessing structural damage of affected joints are not very sophisticated. The diagnosis and follow up on progression relies on assessing radiographic changes such as narrowing of joint space, osteophytes, and sclerosis of subchondral bone.² Even though significant efforts have been made to standardise scoring methods for assessing these variables, they remain difficult to quantify and extended follow up periods are needed to see significant changes on successive radiographs.²

With the aim of improving the diagnosis and monitoring of diseases affecting the joint cartilage, we recently developed an immunoassay for measurement of the urinary concentration of collagen type II C-telopeptide degradation products (CartiLaps).³ Type II collagen is almost exclusively localised in cartilage, where it is the major structural component of the tissue. Hence fragments derived from this protein should represent a specific index for cartilage degradation.

Osteoporosis and osteoporotic fractures are prevented by treatment with bisphosphonates.^{4–7} Bisphosphonates have also been assessed for other indications, and animal studies of

arthritis have suggested that bisphosphonates may have chondroprotective abilities.^{8–9} Thus a recent study of patients with Paget's disease has in line with this shown that treatment with zoledronate decreases the urinary concentration of CartiLaps.¹⁰

In this paper we present the results of urinary CartiLaps measurements performed in two groups of elderly women treated for two years with various dosages of either alendronate or ibandronate.

MATERIALS AND METHODS

Participants

The present study included CartiLaps measurements in urine samples from two double blind, placebo controlled trials with two different bisphosphonates.^{4,5}

The alendronate study

The original study was designed to investigate the effect of different doses of alendronate on the bone loss and bone turnover in postmenopausal women without osteoporosis.⁴ The inclusion criteria were healthy women aged 45–54 who

Abbreviations: ANOVA, analysis of variance; BMD, bone mineral density; CTX-II (I), collagen type II (I) degradation products; CV, coefficient of variation; ELISA, enzyme linked immunosorbent assay; mAb, monoclonal antibody; OA, osteoarthritis

Table 1 Baseline data for the two study cohorts. Results are given as mean (SEM)

	Study subjects	Age (years)	Height (cm)	Weight (kg)	BMD spine (g/cm ²)	u-CartiLaps (ng/mmol)	s-CrossLaps (pmol/l)
Alendronate	63	52.8 (2.1)*	163.7 (5.2)	61.6 (6.8)	0.93 (0.09)	178.7 (110.6)	4423.0 (1527.1)
Ibandronate	119	64.7 (5.3)*	163.0 (5.4)	68.1 (11.8)	0.86 (0.13)	218.0 (231.0)	4607.6 (1691.2)

*p<0.001.

had undergone a natural menopause six months to three years previously. Women who had undergone hysterectomy could be included if follicle stimulating hormone was in the postmenopausal range. Spinal bone mineral density (BMD) had to be within $\pm 2SD$ of the young mean value. None of the women had diseases or drug treatment that could interfere with the bone metabolism. Seventy nine women volunteered to participate in the study and fulfilled the inclusion criteria. They were randomly allocated to receive two years' treatment with 1 mg, 5 mg, 10 mg, or 20 mg alendronate daily or placebo; 74 women completed the first year, 67 the second year, and 63 the third year of the study. In the third year the group receiving 20 mg was switched to placebo. In the present study only the women who completed all three years are included and they were distributed as 1 mg (n=13), 5 mg (n=13), 10 mg (n=13), or 20 mg (n=11) alendronate daily or placebo (n=13).

The ibandronate study

This study was originally designed to investigate the effect of different doses of ibandronate on the bone loss and bone turnover in elderly postmenopausal women.⁵ The inclusion criteria were healthy women aged <75 and with a natural menopause at least 10 years ago. Forearm BMD had to be below $-1.5SD$ of the young mean value (t score). All women were without diseases or drug treatment that could interfere with the bone metabolism. One hundred and eighty women were randomly allocated to receive one year's treatment with 0.25 mg, 0.5 mg, 1.0 mg, 2.5 mg, or 5 mg ibandronate daily or placebo; 141 women completed the first year. For the second year all treatment was withdrawn and 119 women completed the second year of the study. In the present study only the women who completed both years are included and they were distributed as 0.25 mg (n=22), 0.5 mg (n=21), 1.0 mg (n=19), 2.5 mg (n=20) or 5 mg (n=16) ibandronate daily or placebo (n=21).

Design

In addition to the results on CartiLaps, earlier published^{4, 5, 11} results on serum CrossLaps, a marker of bone resorption, are included in the paper for comparison.

Blood and urine samples were drawn in the morning between 08 00 and 10 00 after overnight fasting. The study was performed according to the Helsinki Declaration, and approved by the appropriate ethics committee. All participants gave their written informed consent.

Analytical methods

Urinary levels of collagen type II degradation fragments (CTX-II)

Urinary CartiLaps was measured by enzyme linked immunosorbent assay (ELISA)³ (Osteometer Biotech A/S, Herlev, Denmark). The monoclonal antibody (mAb) F46 specific for CTX-II was used in a competitive ELISA format developed for measurement of urine samples. Briefly described, the assay was performed as follows: biotinylated collagen type II C-telopeptide derived peptide CT2 (EKGDPD) was diluted in coating buffer (1.5 μ g/l) and 100 μ l pipetted into each well of a streptavidine coated microtitre plate (Micro-Coat, Munich, Germany). The plate was incubated for 30 $\pm$ 5 minutes at 18–22°C. The plates were washed five times with washing

buffer, and 40 μ l of calibrators, controls, or unknown samples were pipetted into the wells. All samples were measured in duplicate. One hundred μ l of primary antibody (mAb F46) diluted in assay buffer to a concentration of 19 μ g/l was pipetted into each well. After incubation overnight, the plates were washed and a peroxidase labelled antimouse antibody was added, followed by visualisation of bound antibody with a chromogenic peroxidase substrate. The concentration of the unknown samples was determined by constructing a standard curve from measurement of the calibrators with known concentrations of CT2 peptide. The concentration of the CartiLaps ELISA (ng/l) was standardised to the total urine creatinine (mmol/l): concentration/creatinine = ng/mmol. The precision of the CartiLaps assay was determined to an intra-assay coefficient of variation (CV) <7.1%, interassay CV below 8.4%, and a dilution recovery averaging 100 (11.7)%.³

Serum levels of bone resorption derived collagen type I fragments (CTX-I)

The Serum CrossLaps One Step ELISA (Osteometer Biotech A/S, Herlev, Denmark) is a sandwich assay using two monoclonal antibodies specific for a β -aspartate form of the epitope EKAHDGGR derived from the carboxy terminal telopeptide region of type I collagen $\alpha 1$ chain.^{12, 13} The assay is based on the application of two highly specific monoclonal antibodies against the amino acid sequence of AHD- β -GGR, where the aspartic acid residue (D) is β -isomerised.¹⁴ In a one step incubation procedure, a biotinylated antibody and a peroxidase conjugated antibody capture the degradation products containing crosslinked di-isomerised EKAHD- β -GGR peptides. The generated complex is then bound to the streptavidine surface through the biotin conjugate. Desalted urinary antigens are used for standardisation, and parallelism is seen with serum samples. Results are obtained in <2.5 hours, and both inter- and intra-assay imprecision are <8% and dilution recoveries average 100 (5)%.

Statistical analysis

The characteristics of the treatment groups at the beginning of the study were compared with non-parametric *t* test (Mann-Whitney) for unpaired data and analysis of variance (ANOVA) (Kruskal-Wallis). Changes in CartiLaps and CrossLaps levels over time were compared with baseline values by Student's *t* test for paired values. Comparisons between groups were made by ANOVA and the Mann-Whitney *t* test. The chosen level of significance was 0.05.

RESULTS

Table 1 shows the age, height, and weight and the baseline values of urinary levels of CTX-II and serum levels of bone resorption derived CTX-I in the alendronate and ibandronate groups. The alendronate group was, as selected, younger and had higher BMD than the total ibandronate group (p<0.001). The alendronate group had lower body weight, lower bone resorption (serum CTX-I) and lower urinary CTX-II levels, but these differences between the cohorts were not statistically significant. The groups that received different doses of alendronate and ibandronate were well randomised with no significant differences between any baseline data for any of

Figure 1 Mean (SEM) percentage values of urinary concentration collagen type II degradation products (CTX-II) as a function of treatment time in the alendronate group (A) and the ibandronate group (B).

the variables reported here.^{4,5} At baseline there was no correlation between urinary levels of CTX-II and either spine BMD or serum CTX-I levels. There was a significant inverse correlation between spine BMD values and serum CTX-I levels ($r=-0.22$, $p<0.05$).

Figure 1 displays the mean percentage values of urinary CTX-II as a function of treatment time in the alendronate group (fig 1A) and the ibandronate group (fig 1B). There was a significant dose dependent relationship in both studies (ANOVA). The alendronate treated groups showed apparent decreases in urinary CTX-II in the 10 mg and 20 mg groups to 78% and 51% after three months of treatment, but only the decrease in the 20 mg group was significantly different from placebo ($p<0.001$). The value reached after three months of treatment remained practically constant during the following two years. At 24 months CTX-II was still depressed to 76% and 59% of baseline in the 10 mg (NS) and 20 mg ($p<0.05$) groups, respectively. At 24 months, the active treatment was stopped in the 20 mg group and the CTX-II levels then returned towards baseline. The 10 mg group, that at all times showed the same trend as the 20 mg group, but with no statistical significance, regressed insignificantly toward baseline between months 24 to 36, although the treatment was continued (random variation).

Figure 2 shows the corresponding serum CTX-I values for comparison. This variable also decreased rapidly within three months, but to a lower level—that is, to about 30% of baseline. The three highest doses (20 mg, 10 mg, and 5 mg) all decreased significantly ($p<0.001$), whereas the group receiving 1 mg and the placebo group had virtually no changes in bone resorption.

In the ibandronate groups (fig 1B) the two highest doses (5 mg and 2.5 mg) significantly decreased urinary CTX-II levels at three months to 45% and 53% of baseline, respectively (both $p<0.001$). At 12 months the corresponding values were 34% and 49% ($p<0.001$). When the treatment was withdrawn urinary CTX-II returned to baseline values within the following 12 months. CTX-II was significantly decreased at three months in the groups receiving 1 mg and 0.5 mg to 72% and 79% of baseline ($p<0.01$ and 0.05 , respectively), but thereafter the mean values were similar to those in the placebo group. The 0.25 mg and placebo groups had unchanged values.

Figure 2 Mean (SEM) percentage values of serum collagen type I degradation products (CTX-I) as a function of treatment time in the alendronate group (A) and the ibandronate group (B).

Figure 2B shows the serum CTX-I levels in the women treated with ibandronate. CTX-I decreased significantly to about 30% of baseline in the groups receiving 5 mg and 2.5 mg. When the treatment was withdrawn at 12 months bone resorption returned to baseline.

There was a significant correlation between the change in CTX-I and CTX-II seen in the two cohorts. After the first three months of the study the change in CTX-I and CTX-II correlated significantly ($r=0.33$, $p<0.001$). An even higher correlation was seen between changes in serum CTX-I levels and the annual change in spine BMD ($r=-0.49$, $p<0.001$), whereas the correlation between change in CTX-II levels and BMD was more poorly correlated ($r=-0.21$, $p<0.01$).

Among the women treated with bisphosphonate doses shown to have a significant effect on CTX-II levels (20 mg alendronate; 1, 2.5, and 5 mg ibandronate), there was a significant correlation between baseline levels of CTX-II and the magnitude of the decrease ($r=0.34$, $p<0.01$).

DISCUSSION

CartiLaps ELISA is a new assay that measures breakdown products from type II collagen. This type of collagen is almost exclusively present in joint cartilage. The antibody used in the CartiLaps immunoassay is raised against a synthetic peptide sequence that is specific for type II collagen. In vitro studies have shown that chondrocyte cell cultures produce CTX-II that are detected by the assay.³ In addition, osteoclast cell cultures grown on bone surface produce nothing that is detected by the CartiLaps assay specific for collagen type II derived protein fragments, but an abundance of breakdown products from collagen type I, the predominant bone collagen.³ In vivo studies have shown that CTX-II levels are increased in patients with OA and rheumatoid arthritis compared with healthy controls.³ In contrast, the CTX-II level is normal in patients with Paget's disease, a condition that only affects bone tissue.³ One study has shown, furthermore, that CTX-II correlates with joint surface area.¹⁵ Thus substantial evidence supports the CartiLaps assay as a measure of cartilage degradation.

This paper is a step on the way towards proving the possible value of a biochemical marker of cartilage degradation. The postmenopausal women who participated in the two studies were not selected because of their OA. However, the prevalence

of OA is higher in women than in men, and the incidence increases dramatically after the menopause.¹⁶ This suggests that the two cohorts represent a highly relevant group for assessment of potential treatments aimed at preventing or decreasing the accelerated cartilage degradation, which results in increased OA incidence/prevalence. However, it must be emphasised that the relatively short follow up periods and lack of arthritis assessment procedures in the two studies hamper a precise conclusion about the long term effects of bisphosphonate treatment on OA incidence, and this must be dealt with in future studies.

The alendronate group was, on average, 53 years old and the ibandronate group 65 years. The ibandronate group had higher CTX-II values than the younger group, but this difference was not significant. The CTX-II levels found at baseline in both cohorts were comparable with values found in a large healthy reference population (Mouritsen *U et al*, unpublished data).

The introduction of bisphosphonates for prevention and treatment of osteoporosis and other bone diseases characterised by increased bone resorption has been extremely important for patients and the scientific field. Numerous *in vitro* and *in vivo* studies have considered the very potent inhibitory effect of bisphosphonates on osteoclast activity. Furthermore, bisphosphonates have also been shown to interact directly with osteoblast and regulate their interactions with the osteoclasts.¹⁷ Chondrocytes originate from the same stem cell lineage as osteoblasts, and thus, possibly, bisphosphonates affect these two cell types by common mechanisms. Another possible mode of action of bisphosphonates on cartilage metabolism might involve the inhibitory action of these compounds on osteoclasts in the subchondral bone. Animal models of OA have also suggested that treatment with bisphosphonates is partially chondroprotective.^{8,9}

The present study demonstrates that bisphosphonates can induce significant reductions in cartilage degradation as measured by the CartiLaps ELISA. Although the results obtained with the CartiLaps assay may appear similar to the previously published effects of bisphosphonates on bone resorption, as assessed by CrossLaps measurements, there are important differences. In the alendronate group CTX-I decreased by approximately 50% in the 5 mg group (the dose approved for bone loss prevention) and by 70% in the 10 mg and 20 mg group (1 mg had no effect). CTX-II levels decreased less—5 mg had virtually no effect on CTX-II. In the ibandronate group 2.5 mg and 5 mg decreased bone resorption to about 70%, whereas CTX-II caused a decrease of about 50%. Hence, the data suggest that slightly higher doses of bisphosphonates are needed to protect the cartilage than those required to inhibit bone loss. This study cannot, however, answer the question: What is the mechanism of the bisphosphonate effect on cartilage break down? It may be a direct effect on the cartilage or an indirect effect through a primary suppression of bone turnover, but further studies will hopefully clarify this issue in the near future.

Overall, this study shows that the urinary excretion of CTX-II decreases significantly in response to bisphosphonate treatment in doses perhaps a little higher than those used for prevention and treatment of osteoporosis. The data suggest that bisphosphonates may have chondroprotective effects in

humans. Measurement of CTX-II by the CartiLaps assay should provide a new means of monitoring the effects of drugs that potentially inhibit cartilage destruction.

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Authors' affiliations

H J Lehmann, U Mouritzen, C Christiansen, Centre for Clinical and Basic Research, Ballerup, Denmark

S Christgau, P A C Cloos, Osteometer Biotech A/S, Herlev, Denmark

REFERENCES

- Elders MJ**. The increasing impact of arthritis on public health. *J Rheumatol Suppl* 2000;60:6–8.
- Boinin S**, Guillemin F. Radiographic scoring methods as outcome measures in rheumatoid arthritis: properties and advantages. *Ann Rheum Dis* 2001;60:817–27.
- Christgau S**, Garnero P, Fledelius C, Moniz C, Ensig M, Gineyts E, *et al*. Collagen type II C-telopeptide fragments as an index of cartilage degradation. *Bone* 2001;29:209–15.
- Ravn P**, Weiss SR, Rodriguez-Portales JA, McClung MR, Wasnich RD, Gilchrist NL, *et al*. Alendronate in early postmenopausal women: effects on bone mass during long-term treatment and after withdrawal. Alendronate Osteoporosis Prevention Study Group. *J Clin Endocrinol Metab* 2000;85:1492–7.
- Ravn P**, Clemmesen B, Riis BJ, Christiansen C. The effect on bone mass and bone markers of different doses of ibandronate: a new bisphosphonate for prevention and treatment of postmenopausal osteoporosis: a 1-year, randomized, double-blind, placebo-controlled dose-finding study. *Bone* 1996;19:527–33.
- Watts NB**, Harris ST, Genant HK, Wasnich RD, Miller PD, Jackson RD, *et al*. Intermittent cyclical etidronate treatment of postmenopausal osteoporosis. *N Engl J Med* 1990;323:73–9.
- Liberman UA**, Weiss SR, Broll J, Minne HW, Quan H, Bell NH, *et al*. Effect of oral alendronate on bone mineral density and the incidence of fractures in postmenopausal osteoporosis. The Alendronate Phase III Osteoporosis Treatment Study Group. *N Engl J Med* 1995;333:1437–43.
- Podworny NV**, Kandel RA, Renlund RC, Grynpas MD. Partial chondroprotective effect of zoledronate in a rabbit model of inflammatory arthritis. *J Rheumatol* 1999;26:1972–82.
- Tanahashi M**, Funaba Y, Itoh M, Kawabe N, Nakadate-Matsushita T. Inhibitory effects of TRK-530 on rat adjuvant arthritis. *Pharmacology* 1998;56:242–51.
- Garnero P**, Christgau S, Delmas PD. The bisphosphonate zoledronate decreases type II collagen breakdown in patients with Paget's disease of bone. *Bone* 2001;28:461–4.
- Christgau S**, Bitsch-Jensen O, Hanover Bjarnason N, Gamwell Henriksen E, Qvist P, Alexandersen P, *et al*. Serum crosslaps for monitoring the response in individuals undergoing antiresorptive therapy. *Bone* 2000;26:505–11.
- Rosenquist C**, Fledelius C, Christgau S, Pedersen BJ, Bonde M, Qvist P, *et al*. Serum Crosslaps One Step ELISA. First application of monoclonal antibodies for measurement in serum of bone-related degradation products from C-terminal telopeptides of type I collagen. *Clin Chem* 1998;44:2281–9.
- Christgau S**, Rosenquist C, Alexandersen P, Bjarnason NH, Ravn P, Fledelius C, *et al*. Clinical evaluation of the Serum Crosslaps One Step ELISA, a new assay measuring the serum concentration of bone-derived degradation products of type I collagen C-telopeptides. *Clin Chem* 1998;44:2290–300.
- Cloos P**, Christgau S. Non enzymatic covalent modification of proteins: mechanisms, physiological consequences and clinical applications. *Matrix Biol* 2002;21:39–52.
- Garnero P**, Piperno M, Gineyts E, Christgau S, Delmas PD, Vignon E. Cross sectional evaluation of biochemical markers of bone, cartilage, and synovial tissue metabolism in patients with knee osteoarthritis: relations with disease activity and joint damage. *Ann Rheum Dis* 2001;60:619–26.
- Felson DT**, Nevitt MC. The effects of estrogen on osteoarthritis. *Curr Opin Rheumatol* 1998;3:269–72.
- Yu X**, Schöller J, Foged NT. Interaction between effects of parathyroid hormone and bisphosphonate on regulation of osteoclast activity by the osteoblast-like cell line UMR-106. *Bone* 1996;4:339–45.