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5 2 **Bisphosphonates alter trabecular bone collagen cross-linking and isomerization**
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7 3 **in beagle dog vertebra**
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28 15 **Running title:** Anti-remodeling agents and collagen
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34 **Changes in organic matrix may contribute to the anti-fracture efficacy of anti-remodeling**
35 **agents. Following one year of treatment in beagle dogs, bisphosphonates alter the organic**
36 **matrix of vertebral trabecular bone while raloxifene had no effect. These results show that**
37 **pharmacological suppression of turnover alters the organic matrix component of bone.**
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1 Abstract

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3 **Introduction:** The collagen matrix contributes significantly to a bone's fracture resistance yet the
4 effects of anti-remodeling agents on collagen properties are unclear. The goal of this study was to
5 assess changes in collagen cross-linking and isomerization following anti-remodeling treatment.

6 **Methods:** Skeletally-mature female beagles were treated for one year with oral doses of vehicle
7 (VEH), risedronate (RIS; 3 doses), alendronate (ALN; 3 doses), or raloxifene (RAL; 2 doses). The
8 middle dose of RIS and ALN, and lower dose of RAL approximate doses used for treatment of post
9 menopausal osteoporosis. Vertebral trabecular bone matrix was assessed for collagen
10 isomerization (ratio of α/β C-telopeptide [CTX]), enzymatic (pyridinoline [PYD]) and
11 deoxypyridinoline [DPD]), and non-enzymatic (pentosidine [PEN]) cross-links. **Results:** All
12 doses of both RIS and ALN increased PEN (+34-58%) and the ratio of PYD/DPD (+14-26%), and
13 decreased the ratio of α/β CTX (-29-56%) compared to VEH. RAL did not alter any collagen
14 parameters. Bone turnover rate was significantly correlated to PEN ($R = -0.664$), α/β CTX ($R =$
15 0.586), and PYD/DPD ($R = -0.470$). **Conclusions:** Bisphosphonate treatment significantly alters
16 properties of bone collagen suggesting a contribution of the organic matrix to the anti-fracture
17 efficacy of this drug class.

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26 **Key words:** alendronate, anti-remodeling, bone markers, pentosidine, raloxifene, risedronate

1 Introduction

2 Bisphosphonates, such as alendronate and risedronate, significantly increase spine BMD and
3 reduce vertebral fractures in post menopausal women (1-3). Raloxifene, a selective estrogen
4 receptor modulator (SERM), also decreases vertebral fracture risk to a similar degree in the spine,
5 despite smaller increases in BMD (4-6). Collectively, these anti-remodeling agents are proposed to
6 reduce fracture predominantly by suppressing bone turnover, slowing the rate of bone loss and
7 increasing the mean degree of tissue mineralization (7, 8).

8 Whether or not a bone fractures is dependent on numerous factors including its mass,
9 geometry, and intrinsic (material) properties (9). Numerous studies have shown anti-remodeling
10 agents maintain bone mass and geometry yet significantly less is known about the effect of these
11 agents on changes to the bone material (e.g. mineral and organic matrix). Anti-remodeling agents
12 increase the amount and homogeneity of mineral within the tissue (10), as well as the structure and
13 homogeneity of mineral crystals themselves (11). The effect of anti-remodeling agents on the
14 organic component of bone is largely unknown.

15 The bone organic matrix is predominantly type I collagen. Following secretion from the
16 cell, collagen undergoes numerous post-translational modifications and is eventually stabilized by
17 intra- and inter-molecular cross-links formed through both enzymatic and non-enzymatic processes
18 (12). Trivalent enzymatic cross-links, such as pyridinoline (PYD) and deoxypyridinoline (DPD),
19 are generally indicative of mature collagen (12). Non-enzymatic cross links (e.g. pentosidine,
20 vesperlysine) exist in skeletal collagen due to spontaneously interaction of collagen proteins and
21 free sugars or via oxidation reactions. Levels of non-enzymatic cross-links are generally higher in
22 bone having a greater mean tissue age. Additionally, as mean tissue age increases collagen
23 undergoes isomerization reactions on the aspartyl acid or asparagine residues, altering the structure
24 of the collagen molecule (12, 13). Quantifying the ratio of native (α) to isomerized (β) collagen

1 provides an index of collagen maturity and has the additional benefit of being able to be measured
2 in urine samples of humans (14).

3 The organic matrix contributes to a bone's fracture resistance (12, 15) although its specific
4 effects are not well understood. We and others have previously reported that anti-remodeling
5 treatment significantly alters mechanical properties of beagle dog vertebral bone (16-20). These
6 changes are only partially explained by treatment-induced changes in bone volume, mineralization,
7 and microdamage suggesting other factors likely contribute to the mechanical alterations (16, 17,
8 21). Therefore, the goals of this study were to determine the effect of anti-remodeling agents
9 (risedronate, alendronate, and raloxifene) on collagen cross-links and isomerization. Given the
10 previously noted differences in turnover suppression between the bisphosphonates and raloxifene
11 (17), we hypothesized that the bisphosphonates (risedronate and alendronate), but not raloxifene,
12 would significantly alter collagen cross-linking and isomerization compared to vehicle-treated
13 animals. We also hypothesized a significant inverse relationship would exist between the rate of
14 bone turnover and both collagen cross-linking and isomerization.

15 16 **Materials and Methods**

17 *Animals*

18 One hundred and eight skeletally mature female beagles (average age 1.3 ± 0.2 years) were
19 purchased from Marshall Farms USA (North Rose, NY). Upon arrival, lateral X-rays of all dogs
20 were obtained to confirm skeletal maturity (closed proximal tibia and lumbar vertebra growth
21 plates). Animals were housed two per cage in environmentally controlled rooms at Indiana
22 University School of Medicine's AALAC accredited facility and provided standard dog chow and
23 water. All procedures were approved prior to the study by the Indiana University School of
24 Medicine Animal Care and Use Committee.

1 *Experimental Design*

2 Specifics regarding the study design are described in more detail elsewhere (16, 17). Briefly,
3 animals were assigned to treatment groups (n=12/group) by matching body weights. All dogs were
4 treated daily for 1-year with oral doses of vehicle (1 ml/kg/day saline), raloxifene (RAL, 0.50 or
5 2.5 mg/kg/day, Lilly Research Labs, Indianapolis, IN), risedronate sodium (RIS, 0.05, 0.10, or 0.50
6 mg/kg/day, Procter and Gamble Pharmaceuticals, Inc) or alendronate sodium (ALN, 0.10, 0.20, or
7 1.00 mg/kg/day, Merck and Co., Inc.). The middle dose of RIS (0.10 mg/kg) and ALN (0.20
8 mg/kg) correspond to treatment doses for post menopausal osteoporosis on a mg/kg basis while the
9 lower dose of RAL (0.50 mg/kg) was chosen to produce serum levels equivalent to those
10 documented in post menopausal women. RIS and ALN were dissolved in saline and RAL was
11 diluted in 10% hydroxypropyl- β -cyclodextrin made with distilled water. Drugs were administered
12 in equivalent volumes (1 ml/kg/day) each morning after an overnight fast and at least 2 hours prior
13 to feeding. Prior to necropsy, animals were injected with calcein (0.20 mL/kg, i.v.) to label active
14 bone turnover sites. Animals were euthanized by intravenous administration of sodium
15 pentobarbital and lumbar vertebrae were dissected and saved for analyses.

16 17 *Bone Turnover*

18 Detailed methods for these variable measurements have been published previously (16, 17).
19 Second lumbar vertebrae were embedded undecalcified in plastic for histological analyses of
20 fluorochrome labels. Measurements were made on a 5 x 5 mm region of trabecular bone using a
21 semiautomatic analysis system (Bioquant OSTEO 7.20.10, Bioquant Image Analysis Co.) attached
22 to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2 microscope, Nikon).
23 Ac.f was calculated (bone formation rate / wall thickness) in accordance with ASBMR
24 recommended standards (22).

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1 *Biochemical analyses of bone collagen*

2 Following mechanical testing, a trabecular bone core from the fourth lumbar vertebrae was isolated
3 and powdered in liquid nitrogen using a freezer mill (Spex Industries, Metuchen, USA). The bone
4 powder was defatted in chloroform methanol (3:1 v/v), extensively washed, and lyophilised. The
5 lyophilised bone powder was separated into two portions for determination of collagen cross-links
6 and collagen isomerization.

7 To determine levels of pyridinoline (PYD), deoxypyridinoline (DPD), and pentosidine
8 (PEN) cross-links, a portion of the lyophilised bone powder was hydrolysed by 6N HCl and pre-
9 treated on SPE columns (Macherey Nagel GmbH & Co.KG, Düren, Germany) to remove
10 interfering fluorophores according to previously published methods with slight modifications (23).
11 Briefly, acetonitril and an internal pyridinium standard (Bio-Rad, Hercules, CA, USA) were diluted
12 in acetic acid and added to the collagen hydrolysates (6-1-1, respectively). Interfering fluorophores
13 were removed by washing the column with 10 mL of a solution containing acetonitril, glacial
14 acetic acid, and water (8-1-1) respectively. Pyridinium cross links and PEN were then eluted with
15 600 μ L of 1% n-heptafluorobutyric acid and then separated using high performance liquid
16 chromatography (HPLC).

17 PYD, DPD and PEN were separated by HPLC on an Alliance 2695 separation module
18 (Waters Corp., Milford, MA, USA) using an Atlantis dC18, 3 μ m, 4.6x100 mm reversed phase
19 column protected by an Atlantis dC18, 3 μ m 4.6 X 20 mm guard cartridge (Waters Corp., Milford,
20 MA, USA) and quantified by fluorescence (2475 multi λ fluorescence detector, Waters Corp.,
21 Milford, MA, USA). Briefly, molecules were separated by using a gradient solution. Solvent A
22 consisted of 0.06 % of HBFA, and solvent B was 50% of solvent A and 50% of acetonitrile. The
23 column was equilibrated with 14% solvent B prior to use. The flow rate was 1.2 ml/min and the
24 column temperature 40°C. PYD and DPD were separated during the first 12 minutes of an isocratic
25 step at 14% of solvent B, and pentosidine was eluted during the following 24 minutes of gradient

1 from 14 to 31% solvent B. PYD and DPD were monitored for fluorescence at an emission of 395
2 nm and an excitation of 297 nm. Pentosidine fluorescence was assessed at an emission of 385 nm
3 and an excitation of 335 nm. Pyridinium cross links were quantified against a supplied calibration
4 standard (Metra Biosystems Ltd). A pentosidine standard was synthesized (24) and calibrated with
5 a standard of pentosidine generously gifted by Dr. Masaaki Takahashi (Hamamatsu University
6 School of Medicine, Shizuoka, Japan). The amount of collagen was determined by hydroxyproline
7 HPLC assay (Biorad, Muchen, Germany).

8 The remaining portion of the lyophilized bone powder was used to assess native (α) and
9 isomerized (β) forms of C-teleopeptide (CTX). Briefly, the bone powder was washed in 2M NaCl
10 solution and then demineralized with 0.5M EDTA Tris buffer, pH 7.4 for 72 h at 4°C with a daily
11 change in the EDTA. Demineralized bone residues were washed extensively with deionised water
12 and then lyophilized. A portion of the demineralized bone residue (10 mg) was digested with
13 collagenase 1A (0.133 mg/ml) overnight at 35°C. The supernatants were removed and the
14 concentration of α CTX and β CTX fragments was measured by the sandwich assays: Urinary
15 ALPHA CrossLaps and Serum CrossLaps ELISA (Nordic Bioscience, Herlev, Denmark),
16 respectively (25). α/β CTX is inversely proportional to collagen maturity with decreases indicative
17 of more mature collagen.

18 *Statistics*

19 All statistical tests were performed using SAS software (SAS Institute, Inc.). One-way ANOVAs
20 were used to compare the drugs to VEH, and to evaluate dose-responses within each drug
21 treatment. For each ANOVA, when significant overall F values ($p < 0.05$) were present,
22 differences between individual group means were tested using Fisher's protected least-significant
23 difference (PLSD) post-hoc test. Dose-equivalents of RIS and ALN were compared using
24 Student's T-tests. A Pearson correlation was used to determine the relationship between PEN and
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1 Ac.f. Because ratios are inherently non-parametric, Spearman correlations were used to determine
2 the relationship of PYD/DPD and α/β CTX to Ac.f. For all tests, $p \leq 0.05$ was considered
3 significant. Data are presented as mean \pm standard error.

4 5 **Results**

6 At all doses, both risedronate- and alendronate-treated animals had significantly higher
7 concentrations of pentosidine (PEN) in the vertebral trabecular bone matrix compared to vehicle-
8 treated animals (Figure 1A). For RIS, levels of pentosidine were +36% (0.05 mg/kg), +50% (0.10
9 mg/kg), and +58% (0.50 mg/kg) higher than VEH (all $p < 0.05$). The highest RIS dose had
10 significantly higher PEN concentrations compared to the lowest RIS dose. For ALN, levels of
11 PEN were +34% (0.10 mg/kg), +37% (0.20 mg/kg), and +52% (1.00 mg/kg) higher than VEH (all
12 $p < 0.05$); the highest dose had significantly higher concentrations of PEN compared to the lowest
13 dose. There was no significant difference in PEN levels between RIS and ALN at any of the three
14 dose-equivalents. There was also no difference between VEH and either of the raloxifene-treated
15 groups.

16 The ratio of pyridinoline (PYD) to deoxypyridinoline (DPD) was significantly higher for
17 all doses of both RIS and ALN compared to VEH (Figure 1B). The ratio of PYD/DPD was +20 to
18 +24% in RIS-treated animals compared to VEH, with no difference among the three doses. ALN-
19 treated animals had a +14 to +26% higher PYD/DPD ratio compared to VEH with no difference
20 among the three doses. There was no difference between RIS and ALN at any of the dose-
21 equivalents. There was also no difference between VEH and either of the RAL-treated groups.
22 Changes in the ratio of PYD/DPD in the RIS- and ALN-treated groups were the result of lower
23 DPD levels (Table 1). All doses of ALN resulted in significantly lower DPD compared to VEH,
24 while RIS showed a trend toward lower DPD levels ($p = 0.057$) compared to VEH. There was no
25 change in PYD between VEH and any of the treatment groups ($p = 0.21$ to 0.79).

1 The ratio of native (α) to isomerized (β) C-teleopeptide (CTX) provides an index of
2 collagen maturity, with a decrease in the ratio indicative of more mature collagen. All doses of
3 both RIS and ALN resulted in a significantly lower α/β CTX ratio compared to VEH (Figure 1C).
4 For RIS, the α/β CTX ratio was -29% (0.05 mg/kg), -46% (0.10 mg/kg) and -56% (0.50 mg/kg)
5 lower compared to VEH (all $p < 0.05$), with significant differences existing between the lowest and
6 highest doses. For ALN, the α/β CTX ratio was significantly lower than VEH (-38% to -45%)
7 compared to VEH with no difference among the three doses. RAL did not significantly change the
8 α/β CTX ratio compared to VEH. These changes in the α/β CTX ratio in RIS- and ALN-treated
9 animals were driven by significantly lower α CTX levels compared to VEH, with no change in β
10 CTX (Table 1).

11 The amount of collagen did not differ for any of the three drug treatment compared to
12 vehicle-treated animals. Collagen content within the demineralized bone residues ranged from
13 3.88 to 4.09 mg per 10 mg of tissue (Table 1).

14 There was a significant relationship between vertebral bone turnover and both collagen
15 cross-links (both enzymatic and non-enzymatic) and collagen isomerization among all animals
16 (Figure 2). Activation frequency (Ac.f) was inversely correlated to PEN ($R = -0.664$, $p < 0.0001$)
17 and PYD/DPD ($R = -0.470$, $p = 0.0005$). A significant positive correlation existed between α/β
18 CTX and Ac.f ($R = 0.586$, $p = 0.0001$), showing that collagen isomerization (and therefore collagen
19 maturity) increases as bone turnover decreases.

20 21 **Discussion**

22 Although well-accepted that the organic matrix contributes to bone's fracture resistance, the effects
23 of anti-remodeling agents on the organic component of bone are largely unknown. Our results
24 document that bisphosphonates, but not raloxifene, have significant effects on collagen cross-
25 linking (both enzymatic and non-enzymatic) and collagen isomerization (an index of collagen

1 maturity). These changes appear to be determined, at least in part, by the degree of turnover
2 suppression in vertebral trabecular bone.

3 At all doses used in the current study, both risedronate and alendronate significantly
4 increased non-enzymatic cross-linking (pentosidine), altered the ratio of enzymatic cross-links
5 (pyridinoline to deoxypyridinoline), and increased collagen isomerization. These doses
6 approximate those used for the treatment of post menopausal osteoporosis (middle dose of each)
7 and for the treatment of Paget's disease (highest dose of each). Changes with the bisphosphonates
8 are contrasted with raloxifene, which had no significant effect on any of these collagen parameters.
9 The most plausible explanation for this class-specific effect is that raloxifene has a smaller effect
10 on turnover suppression compared to the bisphosphonates. In these same dogs, raloxifene
11 suppressed turnover ~ 20% compared to vehicle while the bisphosphonates suppressed turnover
12 between 40 and 80% in vertebral trabecular bone (16, 17). Although RIS and ALN have been
13 shown to produce different levels of turnover suppression in clinical trials (26), the level of
14 turnover suppression was only different at the lowest dose-equivalents in the current study (16).
15 This likely explains the similar changes in organic matrix parameters with both bisphosphonates in
16 the current study.

17 Pyridinoline (PYD) and deoxypyridinoline (DPD) are two trivalent collagen cross-links
18 that are derived from an enzymatic pathway initiated by the enzyme lysyl oxidase. Guenther et al.
19 (27) have shown dichloromethanediphosphonate, a diphosphonate, produced a 20% increase in
20 DHLNL (dihydroxylysyl-norleucine), a 50% reduction in HLNL (lysyl-norleucine), and a 2.2-fold
21 increase in the DHLNL/HLNL ratio in rat tibia. As DHLNL and LHNL are the borohydride
22 reduction forms of in vivo intermediates for PYD and DPD, respectively, these results are
23 consistent with our data. We document bisphosphonate-treatment results in significantly lower
24 levels of DPD with no change in PYD, effectively increasing the ratio of PYD/DPD compared to
25 vehicle-treated animals. Although bisphosphonates significantly alter the PYD/DPD ratio, the total

1 level of pyridinolines is similar among treatments (PYD + DPD ~280-290 mmol/mol collagen). In
2 addition to pyridinolines, bone collagen contains pyrrole cross-links, which are also trivalent
3 enzymatically mediated (28-30). Analyses of a sub-set of samples from the current study showed
4 no difference in pyrrole cross-links (data not shown), further supporting evidence that the total
5 number of enzymatic cross-links is not altered with bisphosphonate treatment, but rather the
6 relative proportion of specific cross-links. Interestingly, studies have consistently showed that the
7 PYD/DPD ratio, but not the individual levels of either PYD or DPD alone, has the greatest
8 association with bone strength and stiffness (29, 31-34).

9 Pentosidine (PEN) is one of several advanced glycation end products (AGEs) that result
10 from a non-enzymatic condensation process of arginine, lysine and free sugars to form
11 characteristic fluorescent cross-links of collagen (35, 36). Pentosidine constitutes a small fraction
12 of non-enzymatically glycated (NEG) cross-links, but is often used as a marker of changes in NEG
13 content. It is possible that the increased non-enzymatic cross-linking of bone collagen resulting
14 from bisphosphonate treatment contributes to the widely reported reduction in bone toughness that
15 underlies this treatment (17-20). Cross-links formed through non-enzymatic processes make the
16 tissue more brittle (37), either preventing the stress relaxation caused by crack initiation, or
17 allowing cracks that are created to grow more easily (38, 39). Increased pentosidine concentration
18 in bone has been shown to reduce the ultimate strain (40) and amount of post-yield deformation
19 (41-43), both traits associated with increased brittleness. Recently, Viguet-Carrin et al. (23) showed
20 that when combined with BMD in a multiple regression, increased pentosidine concentration was
21 negatively associated with work to fracture in human lumbar vertebrae obtained at necropsy ($r^2 =$
22 0.67, $p < 0.0001$). Thus, as the concentration of PEN increased, the work to fracture decreased,
23 consistent with the in vitro results from Vashishth and co-workers (37, 38, 41, 42, 44). Saito et al.
24 (45) showed increased PEN concentration in both high and low mineralized fractions of bone in
25 women with intracapsular hip fractures, compared to non-fracture controls. The increased non-

1 enzymatic cross-linking found in the bisphosphonate-treated groups may help to explain both the
2 increased stiffness and the reduced toughness found in these groups (16, 17). Although the
3 absolute level of pentosidine in the current study is only ~ 0.7 mmol / mol collagen higher than
4 VEH, theoretical analyses suggest small alteration in NEG can have magnified effects on changes
5 to bone toughness (46). Interestingly, both increased cross-linking and decreased toughness (17)
6 were absent in animals treated with raloxifene. Since mechanical properties are dictated by several
7 factors that concomitantly change by remodeling-suppression induced increases in mean tissue age
8 (e.g. increased mineralization, increase microdamage) (21), the independent effect of altered cross-
9 linking on biomechanical properties with anti-remodeling treatments is unclear.

10 Quantifying the ratio of native (α) to isomerized (β) collagen provides an index of collagen
11 maturity (14). Isomerization, the non-enzymatic transfer of the peptide backbone from the aspartyl
12 residue on the α -carboxyl group to the side chain of the β -carboxyl group, occurs in the organic
13 matrix of various tissues. Similar to AGEs, isomerization of collagen occurs over time and
14 therefore is considered an index of mean tissue age. Our results, showing a greater isomerization
15 (a decreased ration of α/β CTX) of trabecular bone collagen with bisphosphonate-treatment are
16 consistent with increases in mean tissue age resulting from reductions in turnover.

17 FTIR, which measures a ratio of PYD to the divalent, reducible cross-link dehydro-
18 dihydroxylysinonorleucine (deH-DHLNL), has been used extensively to examine collagen cross-
19 linking in human biopsies (47-50). Using this technique, Paschalis et al. showed a 40% increase in
20 collagen cross-links ratio (pyridinoline / deH-DHLNL) of iliac crest biopsies from post-
21 menopausal women following two years of hormone replacement therapy (47). As HRT
22 suppresses bone turnover, these data support the findings of the current study linking a suppression
23 of turnover to increased collagen cross-linking, although the specific cross-links measured in these
24 two studies differ. Recently, using FTIR Durchschlag et al. (51) reported no effect on collagen
25 cross-linking at resorbing surfaces in iliac crest biopsies following a 3 or 5 year course of

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3 1 risedronate treatment, and a reduction in cross-linking at forming surfaces following 5 years of
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5 2 treatment compared to baseline values. These results from human bone are not necessarily
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7 3 incompatible with the results reported in the current study, as different parameters were assessed
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9 4 using different techniques. Measurements at forming surfaces would not capture the cross-links in
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11 5 the older, pre-existing bone with greater mean tissue age which would be expected to be more
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13 6 mature and have more cross-links (especially non-enzymatic). Those data simply reflect that a
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15 7 long course of risedronate does not affect collagen of newly forming bone; we did not discriminate
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17 8 between newly formed and pre-existing bone in the current study. Measurements at resorbing
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19 9 surfaces may reflect older more mineralized bone, but the FTIR measurements are very local and
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21 10 may not accurately depict the nature of the collagen cross-links of the older bone deeper within the
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23 11 trabecular core.

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27 12 Increased bone mineral density accounts for only a small portion of vertebral fracture risk
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29 13 reduction, ~4% for raloxifene (52) and 16-28% for bisphosphonates (53-55). Our data suggest
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31 14 changes in the organic matrix may contribute to the fracture risk reduction of anti-remodeling
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33 15 agents. Collagen cross-linking is related to bone strength, stiffness and the amount of energy that
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35 16 can be absorbed by the tissue after yielding, with different kinds of cross-linking having different
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37 17 mechanical effects. As outlined above, increased non-enzymatic cross-linking decreases energy
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39 18 absorption by allowing microdamage formation which may accelerate brittle fracture (37, 38, 41,
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41 19 42, 44). However, increased enzymatic cross-linking has been associated with improved
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43 20 mechanical properties such as strength and stiffness (29). Thus, collagen cross-linking, like other
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45 21 material-level properties of bone such as mineralization, appears to have dichotomous effects on
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47 22 biomechanical integrity of the bone, improving some aspects (e.g. strength and stiffness), while
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49 23 reducing others (toughness) (21). The changes in collagen, specifically with bisphosphonates,
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51 24 likely explain a portion of the discrepancy between changes in BMD and fracture risk.
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1 The data presented should be considered within the context of various limitations.
2 Collagen parameters were only assessed in the trabecular portion of the vertebrae, and may not
3 reflect changes occurring in cortical bone. As cortical turnover is slower than trabecular,
4 alterations in collagen parameters of cortical bone with anti-remodeling treatments may be smaller.
5 Also, based on our analyses technique, it was not possible to determine whether the changes in
6 collagen parameters stem from a focal fraction of bone deposited during the treatment year, or
7 rather from a change to the pre-existing tissue. Finally, the use of intact, non-ovariectomized
8 beagle dogs may limit the translation of these results to how anti-remodeling treatment alters the
9 organic matrix in post menopausal women.

10 In conclusion, our data show that suppression of bone turnover is associated with
11 alterations in collagen cross-linking and isomerization (an index of maturity) of the bone matrix.
12 Bisphosphonates exert more profound changes in the organic matrix, as compared to raloxifene,
13 most likely due to their more potent suppression of turnover. As the organic matrix is known to
14 contribute to biomechanical properties, these data suggest changes to the non-mineral component
15 may contribute to changes in mechanical properties, and therefore fracture risk, with
16 bisphosphonate treatment.

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1 Figure Legends

2 **Figure 1. Changes in collagen cross-linking and isomerization with anti-remodeling agents.**

3 Pentosidine (A), the ratio of PYD/DPD (B) and the ratio of α / β CTX (C) were assessed in
4 trabecular bone from vertebrae of dogs treated for 1 year with vehicle, risedronate, alendronate, or
5 raloxifene. An increase in the PYD/DPD ratio is indicative of increased enzymatic collagen cross-
6 links while a decrease in the α / β CTX ratio indicates increased collagen maturity. Data presented
7 as mean \pm SE. Numbers in bars represent percent difference compared to Vehicle. (a) $p < 0.05$
8 versus vehicle, (b) $p < 0.05$ versus low dose within drug.

10 **Figure 2. Relationship between bone turnover and collagen cross-linking and isomerization.**

11 Significant linear relationships existed between the rate of vertebral bone turnover (activation
12 frequency) and pentosidine (A), enzymatic cross-link ratio (B), and collagen isomerization (C).
13 Vehicle (\bullet), risedronate (\blacksquare), alendronate (\blacktriangle), raloxifene (\blacklozenge).

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For Peer Review

Table I. Differences in the individual components of PYD/DPD and α / β CTX ratios

	PYD mmol / mol collagen	DPD mmol / mol collagen	Alpha CTX ng / mg collagen	Beta CTX ng / mg collagen	Collagen content mg / 10 mg tissue)
Vehicle	232 ± 5	51.0 ± 2.3	352 ± 43	863 ± 59	3.90 ± 0.11
RIS 0.05	240 ± 6	44.5 ± 2.3	232 ± 9 a	969 ± 167	4.00 ± 0.10
RIS 0.1	246 ± 9	43.4 ± 2.0	269 ± 26 a	1247 ± 203	3.99 ± 0.11
RIS 0.5	248 ± 6	45.3 ± 1.1	226 ± 15 a	1225 ± 142	3.88 ± 0.12
ANOVA	<i>0.384</i>	<i>0.057</i>	<i>0.004</i>	<i>0.286</i>	<i>0.810</i>
ALN 0.1	235 ± 6	44.9 ± 1.5 a	240 ± 11 a	1021 ± 143	3.91 ± 0.11
ALN 0.2	237 ± 7	43.0 ± 1.5 a	240 ± 22 a	1072 ± 121	4.09 ± 0.11
ALN 1.0	250 ± 7	43.5 ± 2.0 a	205 ± 15 a	1167 ± 199	4.03 ± 0.10
ANOVA	<i>0.210</i>	<i>0.017</i>	<i>0.001</i>	<i>0.580</i>	<i>0.564</i>
RAL 0.5	233 ± 6	49.5 ± 1.5	361 ± 21	867 ± 72	3.94 ± 0.10
RAL 2.5	237 ± 5	51.6 ± 2.4	333 ± 26	688 ± 63	4.02 ± 0.12
ANOVA	<i>0.792</i>	<i>0.747</i>	<i>0.767</i>	<i>0.105</i>	<i>0.735</i>

Data presented as mean ± SE. (a) $p < 0.05$ vs VEH

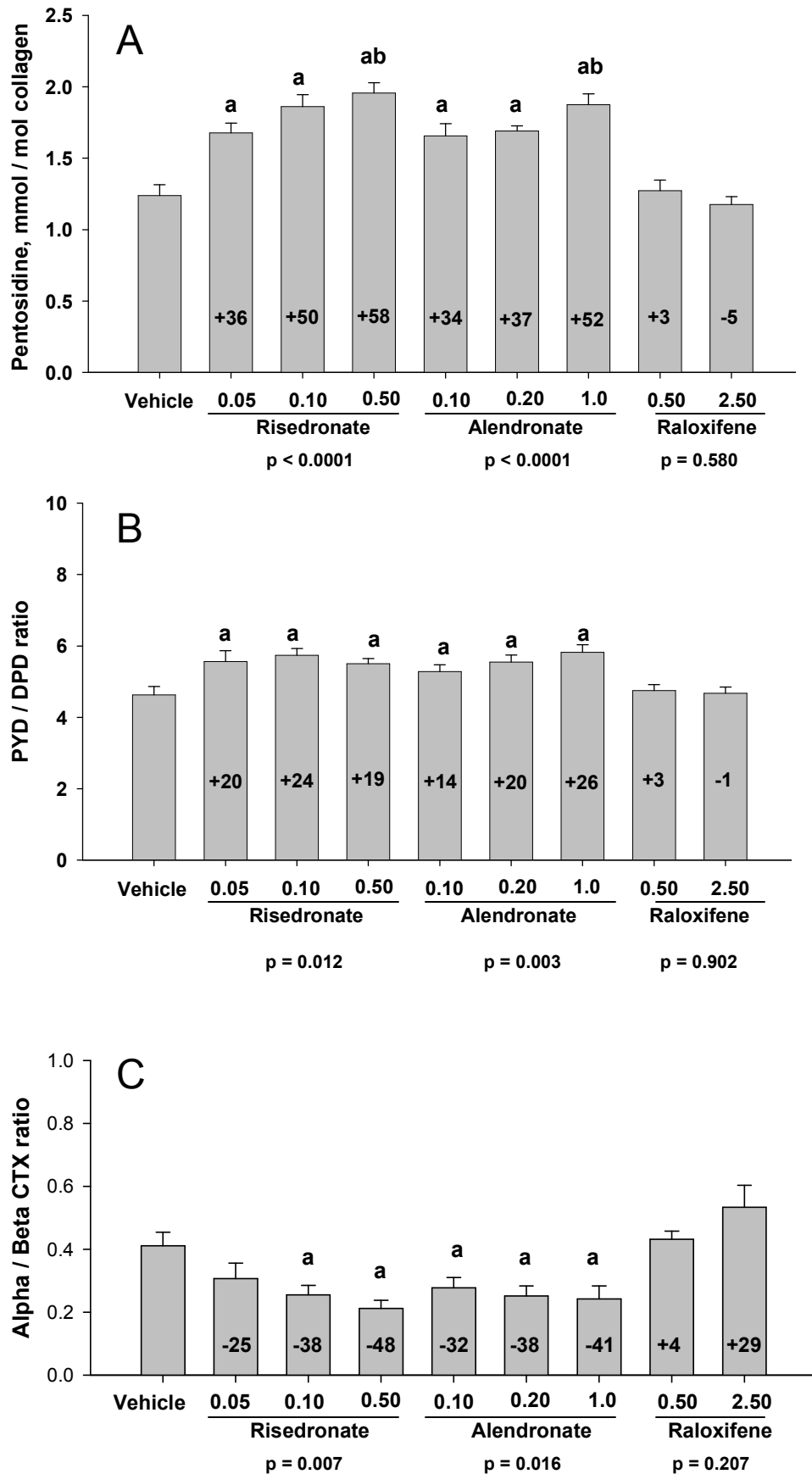


Figure 1

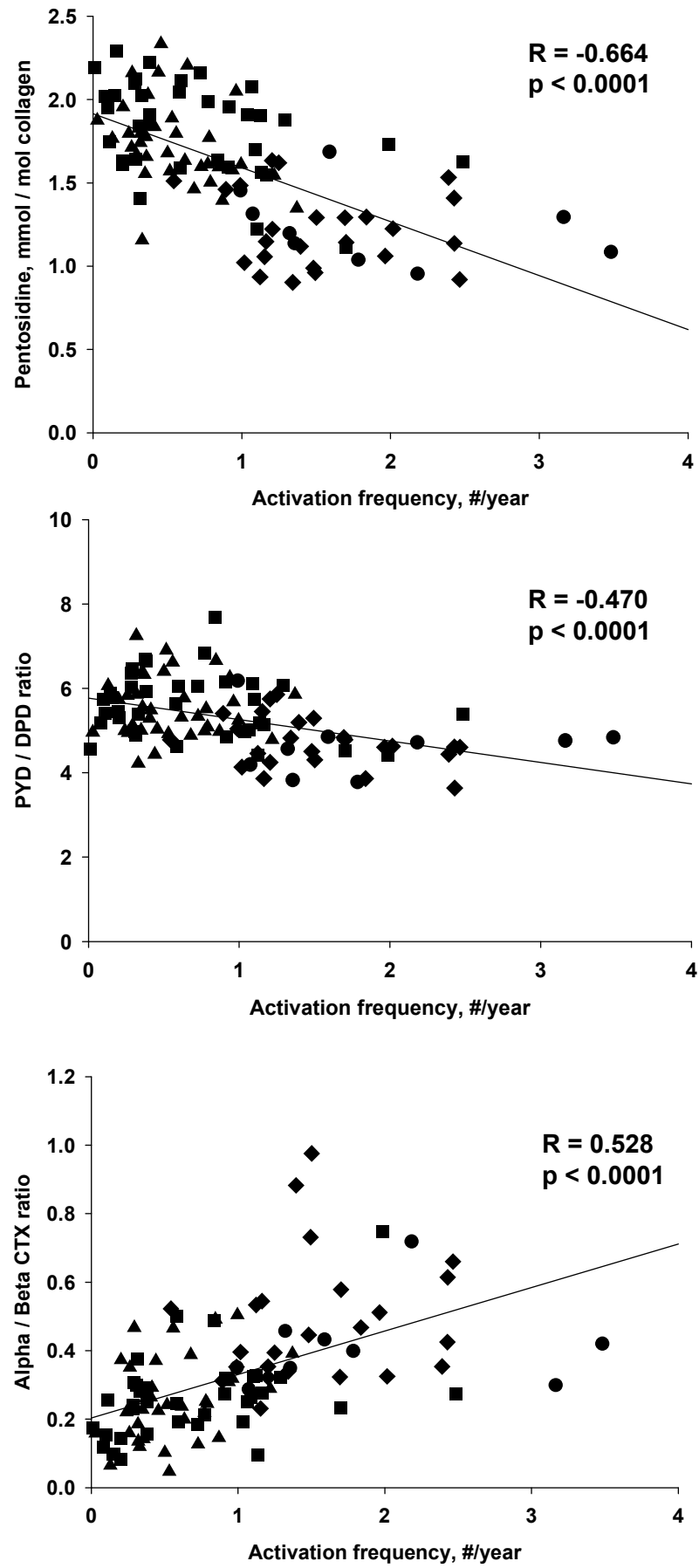


Figure 2