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## Changes in non-enzymatic glycation and its association with altered mechanical properties following 1-year treatment with risedronate or alendronate

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### Abstract

**INTRODUCTION**—Non-enzymatic glycation (NEG) is a post-translational modification of the organic matrix that results in the formation of advanced glycation end-products (AGEs). In bone, the accumulation of AGEs play an important role in determining fracture resistance, and elevated levels of AGEs have been shown to adversely affect the bone's propensity to brittle fracture. It was thus hypothesized that the suppression of tissue turnover in cortical bone due to the administration of bisphosphonates would cause increased accumulation of AGEs and result in a more brittle bone matrix.

**MATERIALS AND METHODS**—Using a canine animal model (n=12), we administered daily doses of a saline vehicle (VEH), alendronate (ALN: 0.20 mg/kg, 1.00 mg/kg), or risedronate (RIS: 0.10mg/kg, 0.50 mg/kg). After a one-year treatment, the mechanical properties, intracortical bone turnover, and the degree of non-enzymatic crosslinking of the organic matrix were measured from the tibial cortical bone tissue of these animals.

**RESULTS**—There was a significant accumulation of AGEs at high treatment doses (+49 to +86%;  $p < 0.001$ ), but not at doses equivalent to those used for the treatment of postmenopausal osteoporosis, compared to vehicle. Likewise, post-yield work-to-fracture of the tissue was significantly reduced at these high doses (−28% to −51%;  $p < 0.001$ ) compared to VEH. AGE accumulation inversely correlated with post-yield work-to-fracture ( $r^2 = 0.45$ ;  $p < 0.001$ ), suggesting increased AGEs may contribute to a more brittle bone matrix.

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**CONCLUSION**—High doses of bisphosphonates result in the accumulation of AGEs and a reduction in energy absorption of cortical bone. The increased accumulation of AGEs in 4 of 28 these tissues may help explain altered bone matrix quality due to the administration of BPs in animal models

### Keywords

Bisphosphonates; alendronate; risedronate; non-enzymatic glycation; advanced glycation end-products; animal models; osteoporosis

## INTRODUCTION

Bisphosphonates, such as alendronate and risedronate, are a class of anti-remodeling drugs that are commonly used for clinical treatment of osteoporosis [1]. Bisphosphonates specifically inhibit osteoclastic activity during resorption by preventing attachment to the bone surface and by inducing early apoptosis to reduce the amount of bone resorbed [2,3]. This suppression of resorption in bone leads to a reduction in remodeling space, increased average tissue mineralization, and altered tissue mineral density distribution [4], which, in turn, decreases fracture risk [5]. Despite the reductions in fracture risk, previous studies have shown that bisphosphonates-mediated alterations adversely affect the energy dissipation capabilities of bone, reducing bone's overall toughness [6–10].

Because bone is a composite consisting of organic and mineral phases, changes to either of its constituents would inevitably alter tissue fracture resistance. Collagen, composing approximately 90% of the organic matrix, is susceptible to the accumulation of advanced glycation end-products (AGEs) created by a series of post-translational modifications, through the process of non-enzymatic glycation (NEG). NEG occurs when reducing sugars spontaneously condensate with free amino groups such as lysine and arginine, resulting in the formation of AGEs. The accumulation of AGEs may be more pronounced in long-lived tissues [11] because AGEs are removed from the extra-cellular matrix when the afflicted tissues are remodeled [12–15]. Thus, the accumulation of AGEs may be a consequence of imbalanced tissue turnover relative to the rate of NEG formation [16,17]. More importantly, the increased accumulation of AGEs has been shown to modify the organic matrix by reducing the energy dissipation mechanisms at the whole bone, apparent, and tissue levels [18–21].

Because bisphosphonates suppress turnover, leading to an overall increase in mean tissue age [4], the reduction in remodeling also may result in the decreased removal of AGEs from the extracellular matrix. Thus, we hypothesized that (1) bisphosphonate-mediated suppression of tissue turnover would result in an increased accumulation of AGEs in cortical bone and (2) this increased accumulation of AGEs would be associated with alterations in material-level mechanical properties. In order to test these hypotheses, intact beagle dogs were treated with daily doses of either vehicle, alendronate (ALN: 0.20mg/kg, 1.00mg/kg), or risedronate (RIS: 0.10mg/kg, 0.50mg/kg) for one year. Mechanical properties and the degree of non-enzymatic crosslinking (e.g. AGEs) were measured from the tibial cortical bone tissue.

## METHODS

### Animals

Detailed methods regarding experimental design have been previously reported [8]. Briefly, sixty skeletally mature female beagles ( $1.3 \pm 0.02$  years old) were assigned to five treatment groups ( $n = 12/\text{group}$ ) by matching body weights. All dogs were treated daily for 1 year with oral doses of vehicle (VEH), risedronate sodium (RIS, 0.10 or 0.50 mg/kg/day; Procter and Gamble Pharmaceuticals, Inc.), or alendronate sodium (ALN, 0.20 or 1.00 mg/kg/day; Merck and Co., Inc). These doses were chosen to approximate, on a mg/kg basis, doses used for the

treatment of post-menopausal osteoporosis (ALN 0.2 and RIS 0.1) or Paget's disease (ALN 1.0 and RIS 0.5). Both risedronate and alendronate were dissolved in saline and administered to the dogs orally with a syringe. Vehicle-treated animals received 1 mL/kg/day of saline. Prior to necropsy, animals were injected with calcein (5 mg/kg, intravenously dosed on a 2-12-2-5 schedule) to allow histological measures of dynamic bone formation. At sacrifice, the distal tibia was processed for histology while the remainder of the cortical shaft was processed for mechanical testing and measurement of AGEs.

## Histology

Using an automatic tissue processor (Shandon/Lipshaw, Pittsburgh, PA), distal tibia specimens were cycled through a graded series of ethanols, cleared using xylene, and infiltrated with methyl methacrylate (MMA; Aldrich, Milwaukee, WI) using routine embedding procedures. Transverse sections (~100µm) were cut using a diamond wire saw (Histosaw; Delaware Diamond Knives, Wilmington, DE). Histological measurements were made on a single cross-section using a semiautomatic analysis system (Bioquant OSTEO 7.20.10; Bioquant Image Analysis, Nashville, TN) attached to a microscope equipped with an ultraviolet light source (Nikon Optiphot 2; Nikon, Tokyo, Japan). Intracortical bone remodeling was assessed by counting the number of labeled osteons (L.On.N). This parameter was used as bisphosphonates have consistently been shown to alter mineralizing surface, of which L.On.N is a corollary for the intra-cortical envelope [8,22].

## Mechanical testing

Cortical bone microbeams were sectioned longitudinally from the mid-diaphysis, near the superior end in the anterior-medial quadrant, (1.5mm<sup>2</sup> × 8mm) using an ISOMET 11–1180 low-speed diamond blade saw (Buehler Corp., Lake Bluff, IL). The microbeams were loaded to failure in 3-point bending (Span: 4.9mm; 0.01mm/s) using a custom-made bending jig and the Enduratec Bose ELF 3200 micromechanical test system (Enduratec Inc, Eden Prairie, MN). Microbeam deflections were independently measured using a high-resolution Canon video camera system (Canon USA, Lake Success, NY), and analyzed using a custom image analysis program written in Matlab (Mathworks Inc, Natick, MA). The following parameters were computed from the force-displacement data and the respective geometries: Bending modulus (E - Eq. 1), ultimate stress (σ<sub>Ult</sub> - Eq. 2), loss of tangent stiffness (Eq. 3 – Fig 1a), the elastic work (defined as the area under the linear region of the force displacement curve; Fig 1a), and the post-yield work-to-fracture (defined as the difference between total work and the elastic work; Fig 1b). The transition from to yielding determined using an automated criteria (executed in Matlab) that uses a 10% deviation from the initial stiffness.

$$E = \frac{(\frac{P}{y})L^3}{48I} [1 + 2.85(\frac{h}{L})^2 - 0.84(\frac{h}{L})^3] \quad (\text{Eq. 1})$$

Where P is the applied load, y is the displacement, L is the length of the lower span, h is the height of the specimen, and I is the bending moment of inertia (defined as

(defined as  $I = \frac{bh^3}{12}$ ).

$$\sigma_{\text{ultimate}} = \frac{(\frac{P_{\text{ult}}}{2})L(\frac{h}{2})}{I} \quad (\text{Eq. 2})$$

Where P<sub>ult</sub> is the maximum load on the force-displacement curve.

$$\text{Loss of tangent stiffness} = 1 - \frac{k_{\text{final}}}{k_{\text{elastic}}} \quad (\text{Eq. 3})$$

Where  $k_{\text{elastic}}$  and  $k_{\text{final}}$  are the slopes between the origin and the end point of the linear region force-displacement curve, and between the origin and ultimate point of the force-displacement curve, respectively. A decrease in the loss of tangent stiffness is associated with a more brittle fracture.

### Measurement of Advanced Glycation End-products (AGEs)

After mechanical testing, the bone tissues were demineralized by incubating the specimens in a 20% formic acid solution for seven days. The solutions were renewed every 24 hours. Demineralization end-point determination assays (Polysciences, Warrington, PA) were used to verify demineralization of each specimen. The demineralized bone tissues were digested with papain collagenase (0.4mg/ml in 0.1 mM sodium acetate buffer, pH 6.0, 16 hours, 65°C). AGEs content was determined using fluorescence readings taken with a Synergy-HT Microplate reader at wavelengths of 370nm/440nm excitation/emission (Biotek USA, Winooski, Vermont) against a quinine sulfate standard [18], and normalized by the collagen content for the sample. The amount of collagen for each cortical bone specimen was approximated based on the amount of hydroxyproline. Collagen is assumed to contain 14% hydroxyproline by mass [23,24], and it was determined using the same plate reader as above that recorded the absorbance of the digested samples against a commercially available hydroxyproline standard (Sigma-Aldrich USA, St. Louis, MO) at the wavelength of 570nm.

### Statistical analyses

ANOVA was used to determine the effects of bisphosphonates treatment on mechanical behavior, AGE composition, and bone remodeling of the cortical bone tissue. Separate ANOVAs were also used for clinical doses vs the VEH, and high doses vs the VEH to determine whether the effects were present due to low- or high- doses. A Kolmogorov-Smirnoff test for normality was performed on all data, and when found to be non-normally distributed, a Kruskal-Wallis non-parametric ANOVA was used instead. Post-hoc comparisons between treatments were done using Fisher's protected-least-significant-difference (pLSD) for parametric data, or Mann-Whitney U tests for nonparametric data, when overall p-values were less than 0.05. The relationships between two measured parameters were evaluated with either linear or nonlinear regressions. Groups were considered statistically significant when the p-value was less than 0.05. All statistical analyses were performed using SigmaSTAT 3.0 (Chicago, IL, SPSS, Chicago, IL). Error bars in the figures represent standard errors of the mean of the respective data.

## RESULTS

At doses equivalent to those used for treatment of post-menopausal osteoporosis, there was no significant effect of either risedronate or alendronate on AGEs accumulation ( $p=0.75$ ; Figure 2) or mechanical properties of the cortical bone tissue at the tibial diaphysis (Table I). However, higher bisphosphonates doses significantly increased AGEs accumulation ( $p<0.001$ ; Figure 2), including an 81% increase for risedronate, and a 49% increase for alendronate compared to vehicle treatment (Figure 2). The high doses of alendronate and risedronate also significantly decreased the post-yield work-to-fracture ( $p<0.001$ ; Figure 3) and loss of tangent stiffness ( $p=0.03$ ; Figure 4) compared to vehicle. No differences were found in the bending modulus ( $p=0.95$ ) and ultimate stress ( $p=0.88$ ) even at these higher doses of bisphosphonates treatment

(Table I). There were no differences between RIS and ALN at equivalent doses for any measured parameters.

The significant increase in AGEs was inversely correlated with post-yield work-to-fracture ( $p < 0.001$ ; Figure 5) and with the loss of tangent stiffness ( $p = 0.004$ ; Figure 6).

Intracortical bone turnover was not significantly different with either the high ( $-51\%$ ,  $p = 0.13$ ) or clinical ( $-8\%$ ,  $p = 0.27$ ) doses compared to VEH (Table 1). Error bars in all figures represent the standard errors of the mean of the respective means.

## DISCUSSION

The data presented here show that advanced glycation end-products (AGEs) do not accumulate in cortical bone of the canine tibia following one year of risedronate or alendronate treatment at doses comparable to those used for the treatment of postmenopausal osteoporosis. They do demonstrate, however, that significant accumulation of AGEs occur with higher doses, similar to those used for the treatment of Paget's disease. This may contribute to the documented observation of reduced tissue toughness, both in this study and others [8–10,25] using higher doses. Our data clearly show that accumulation of AGEs is associated with reduced post-yield energy dissipation of bone tissue prior to fracture. Therefore, any significant accumulation of AGEs could have serious implications for the health of the bone tissue.

Modification of the organic matrix through nonenzymatic glycation (NEG) results in the formation of AGEs that form cross-links such as pentosidine and vesperlysine (among others) that can be detected by fluorescence of the bone tissue [12,26]. NEG occurs when extracellular reactive sugars form Schiff's bases with free amino groups in lysine, hydroxylysine, or arginine residues on the collagen molecule. The subsequent structure then undergoes Amadori rearrangement and ultimately results in a family of molecules known as AGEs [12]. Because there are numerous unstable and stable intermediates during this process, many of which are not fully characterized, the use of fluorescence spectroscopy offers a more generalized means of AGE assessment since multiple products will fluoresce at the characteristic wavelength [27–29]. This technique is well-established and has been validated in a variety of tissues and across anatomical locations [11,30]. AGE-formation occurs over a period of years [14], thus proteins with long half-lives, such as collagen, can accumulate substantial AGEs with age [16,19,21]. Because NEG of collagen occurs in the presence of extracellular sugars, diabetic bone is known to be highly glycated and has been demonstrated to have poor fracture resistance [32–33].

The accumulation of AGEs in the organic matrix may be regulated by bone turnover, which removes the AGEs that are formed as a consequence of NEG [17]. In this study, labeled osteon number (L.On.N.) was used as a metric of bone turnover and displayed a decreasing relationship to AGE accumulation. The accumulation of AGE with reduced turnover is consistent with a previous study using the same group of animals that showed activation frequency, a measure of bone tissue turnover, significantly correlated in an inverse manner with pentosidine at the vertebral body, that explained 36% of the variation in AGE accumulation [34]. The lack of significant difference between bone turnover in this study may be related to the inherently lower rate of turnover in cortical bone than in cancellous bone in the control animals [35]. Taken together, these findings support the idea that increased accumulation of AGEs in the extra-cellular matrix could be mediated by suppression of bone remodeling.

Bone primarily derives its stiffness from its mineral phase, and its post-yield properties for its organic phase [36]. Consequently, because NEG affects only the organic moiety of bone, parameters that account for post-yield aspects of the force-displacement curve are the most

sensitive to changes in the accumulation of AGEs. Increased post-yield work-to-fracture and loss of tangent stiffness are indicators of the energy dissipation mechanisms in bone, and increased quantities are desirable aspects of overall tissue mechanical behavior. It is important to note that although these parameters are both related to the post-yield behavior at the material level, post-yield work-to-fracture and loss of tangent stiffness are independently derived parameters that reflect different aspects of bone behavior ( $p=0.02$ ;  $r^2 = 0.09$ ). Post-yield work-to-fracture is a parallel parameter to post-yield strain energy and represents bone's ability to sustain deformation without catastrophic failure. Thus the decrease in post-yield work-to-fracture observed here is consistent with decreased energy dissipation due to the accumulation of AGEs [18,21]. Loss of tangent stiffness represents the material's ability to resist fracture through toughening mechanisms. Therefore the decreased values of tangent stiffness found here indicate that bisphosphonates treatment is associated with the reduction of bone's toughening mechanism [18,21].

AGEs are inversely correlated to bone toughness [18,21], and specific products such as pentosidine explained up to 35% of the variation in cortical bone toughness in bending [19], while fluorescent AGEs can explain up to 43% of loss of post-yield mechanical behavior [21]. Increased AGE concentration in bone also has been shown to reduce the ultimate strain [37] and post-yield deformation [19,21]. This is because crosslinks formed through non-enzymatic glycation stiffen the organic matrix of bone and subsequently reduce the energy dissipation characteristics of bone [21]. Consistent with previous studies, this increased accumulation of AGEs due to bisphosphonates treatment can explain the 15–20% reduction in bone toughness following one year of treatment [6–8], and the 27% following three years of treatment with alendronate at doses used for the treatment of osteoporosis [10]. Because the administration of bisphosphonates affects may alter fracture resistance in the bone tissue, such tissue mineralization [8–10], microdamage accumulation [8–10], and AGEs, it is thus not possible to isolate the effects of NEG due to BPs administration. Although changes in bone toughness are often associated with increased microdamage accumulation in these animal experiments, recent studies strongly suggest accumulated microdamage is not a key contributor to changes in toughness [10,38]. Changes in mineralization have also been found to have no significant correlation to changes in toughness in these experiments [39]. Furthermore, changes in one aspect of matrix quality may affect other aspects of matrix quality. For example, NEG has been demonstrated to alter microdamage formation in bone [40]. In this study we have examined specific parameters that has been previously demonstrated to be associated with increased AGEs [18,21]. Based on the regressions shown in this study, NEG may directly or indirectly account for 45% of the decrease in tissue-level fracture resistance due to bisphosphonates administration.

The use of non-ovariectomized beagle dogs in this study may limit the translation of these results to rapid turnover and low bone mass conditions such as post-menopausal osteoporosis. Although the higher doses used in the current study are equivalent, on a mg/kg basis, to those used for treatment of Paget's disease, the dosing at these high levels is not for a year. As such, it is not clear to what extent AGEs would accumulate, and how the material properties would be reduced, when high doses are administered for shorter periods of time.

In conclusion, one-year of daily oral bisphosphonates therapy at doses exceeding those used to treat postmenopausal osteoporosis increased the level of advanced glycation end-products and decreased tissue-level fracture resistance. These changes did not occur at dose-levels equivalent to those used to treat postmenopausal osteoporosis. Overall, there was a significant inverse correlation between AGE accumulation and tissue-level fracture resistance

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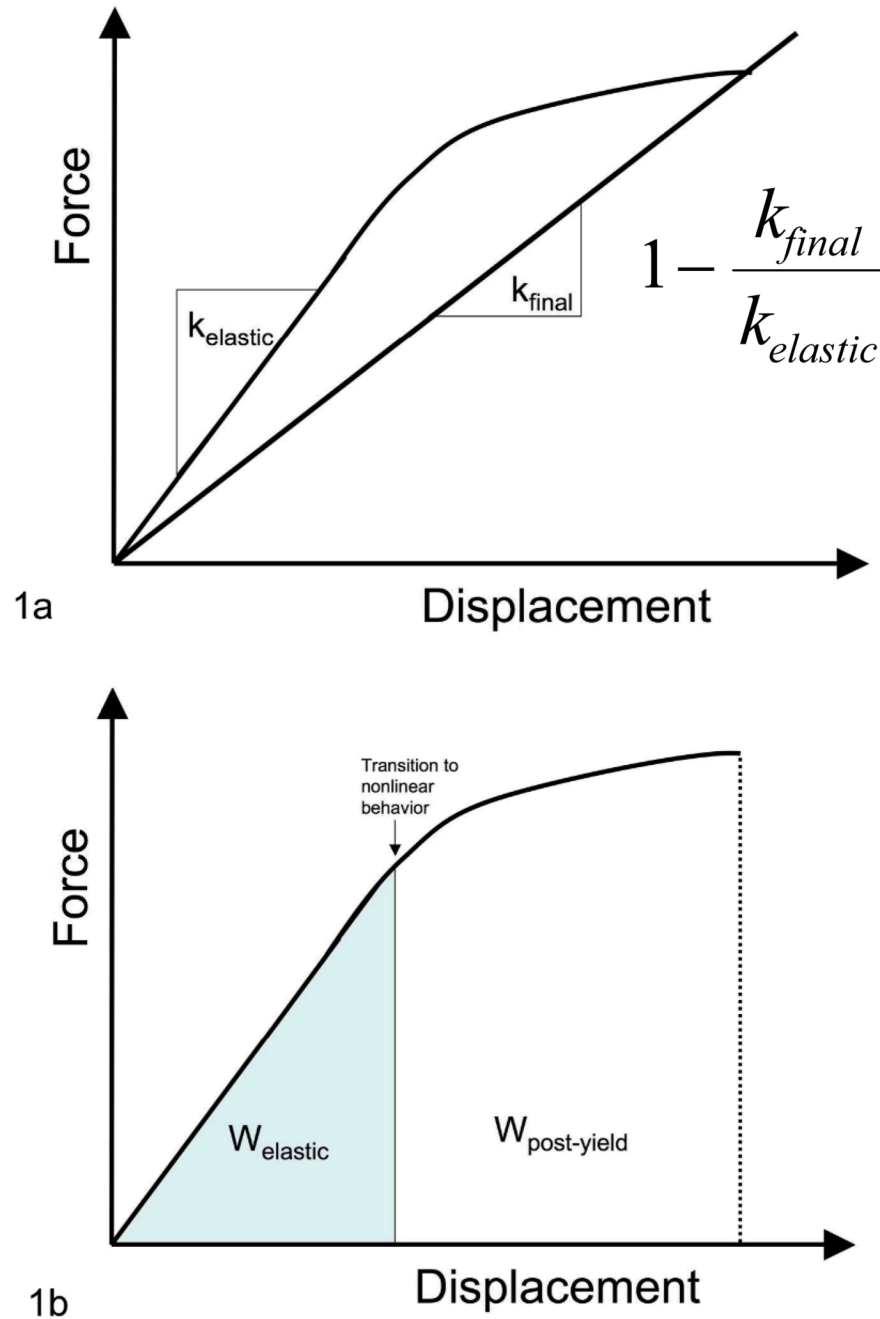
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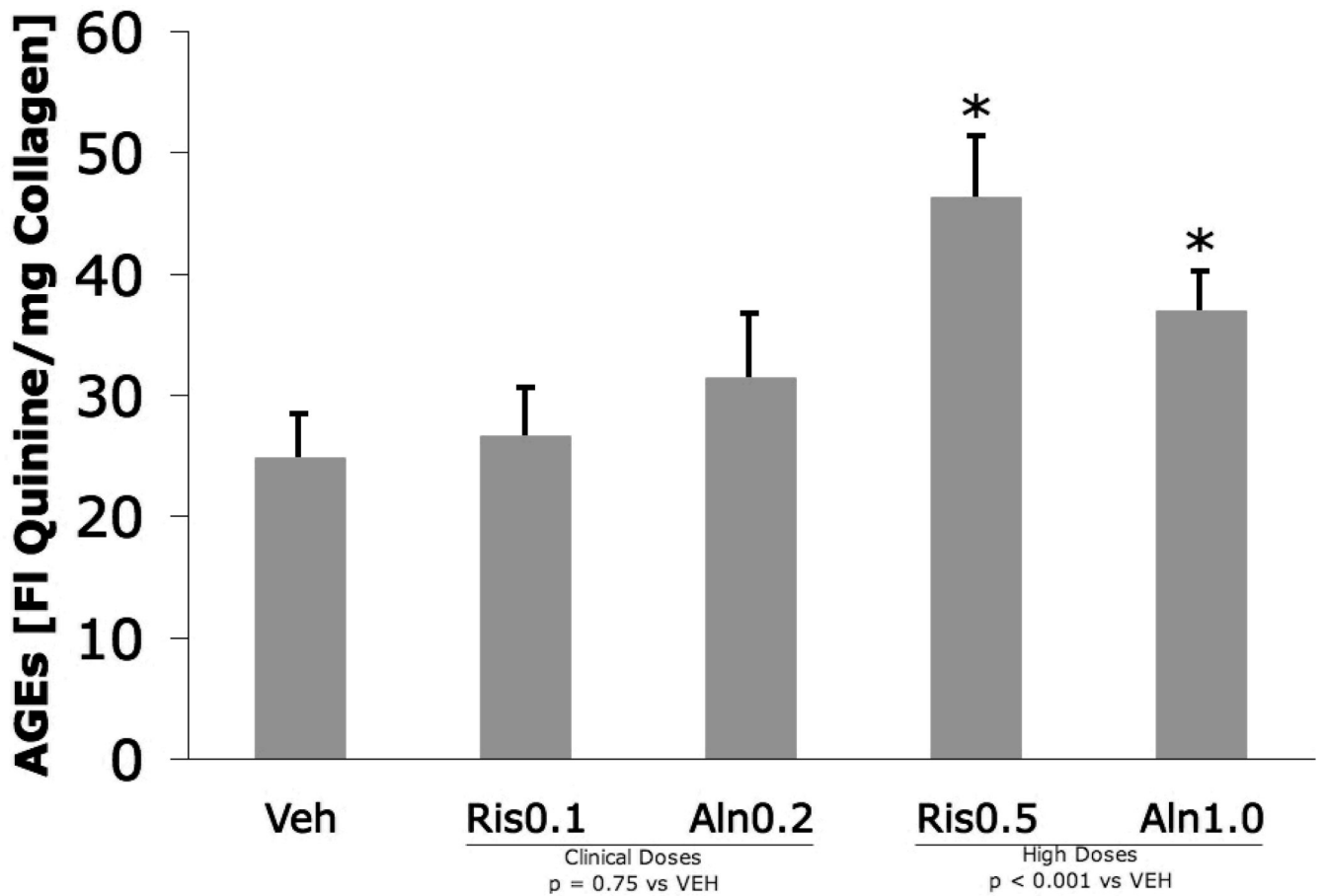
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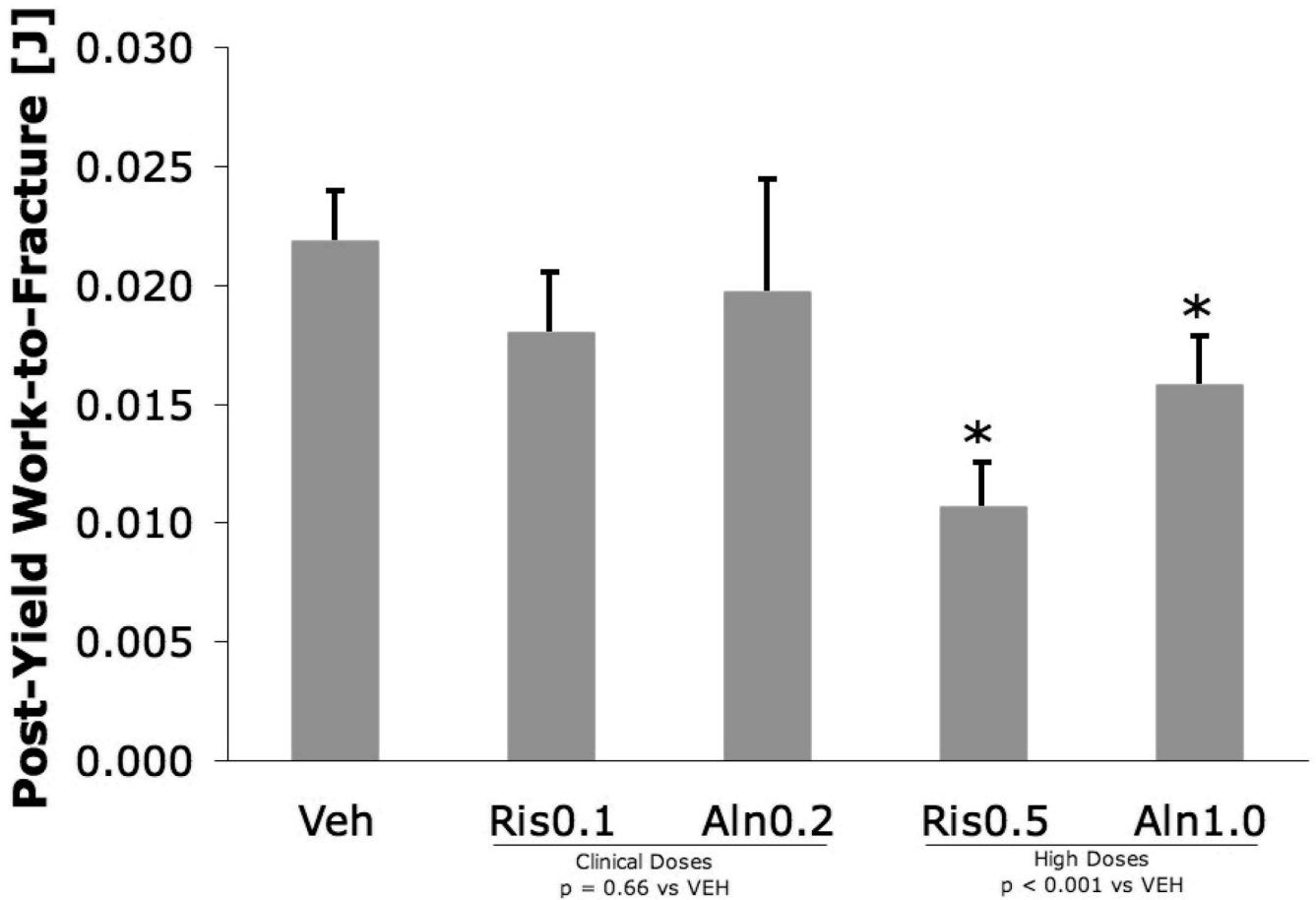


**Fig 1.** Schematics for the calculation of the loss of tangent stiffness (Top -1a); and elastic and post-yield work-to-fracture (Bottom -1b) on the force-displacement curve. Where  $k_{elastic}$  and  $k_{final}$  are the slopes between the origin and the end point of the linear region force-displacement curve, and between the origin and ultimate point of the force-displacement curve, respectively. The elastic work ( $W_{elastic}$ ) is the area under the linear portion of the force displacement curve and the post-yield work-to-fracture ( $W_{post-yield}$ ) is the difference between the total area under the force-displacement and  $W_{elastic}$ . The linear portion of the force displacement curve was determined by an automated criteria (executed in Matlab) that uses a 10% deviation from initial stiffness to calculate the transition to non-linear behavior.



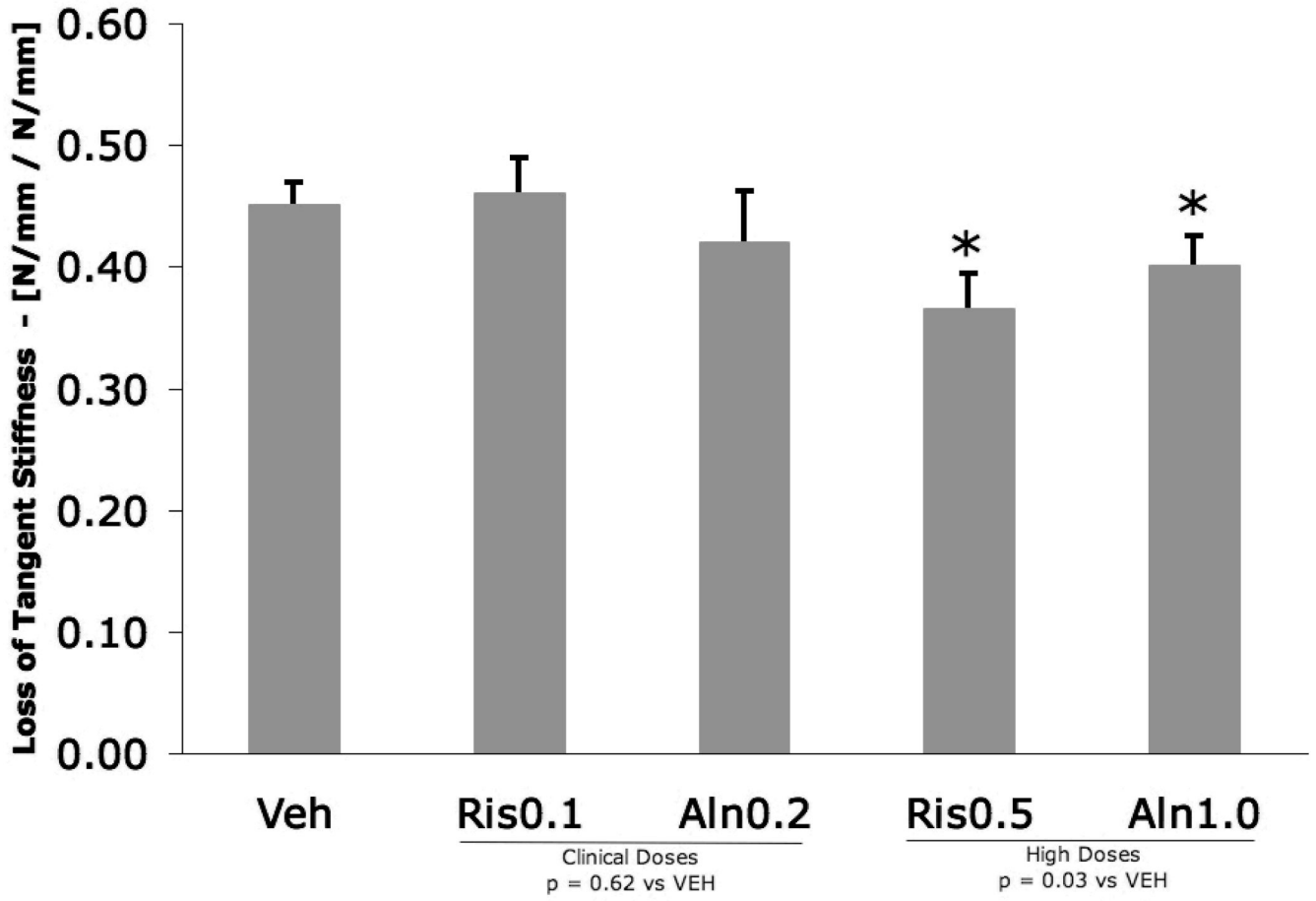
**Fig 2.**

There was no significant effect of bisphosphonates at clinical doses on AGE accumulation. High doses (5x clinical dose) resulted in significant AGE accumulation ( $p < 0.001$ ; ANOVA). ALN and RIS-treated animals showed no significant difference between the drugs at dose-equivalents. Asterisks denote significance when compared to the vehicle.

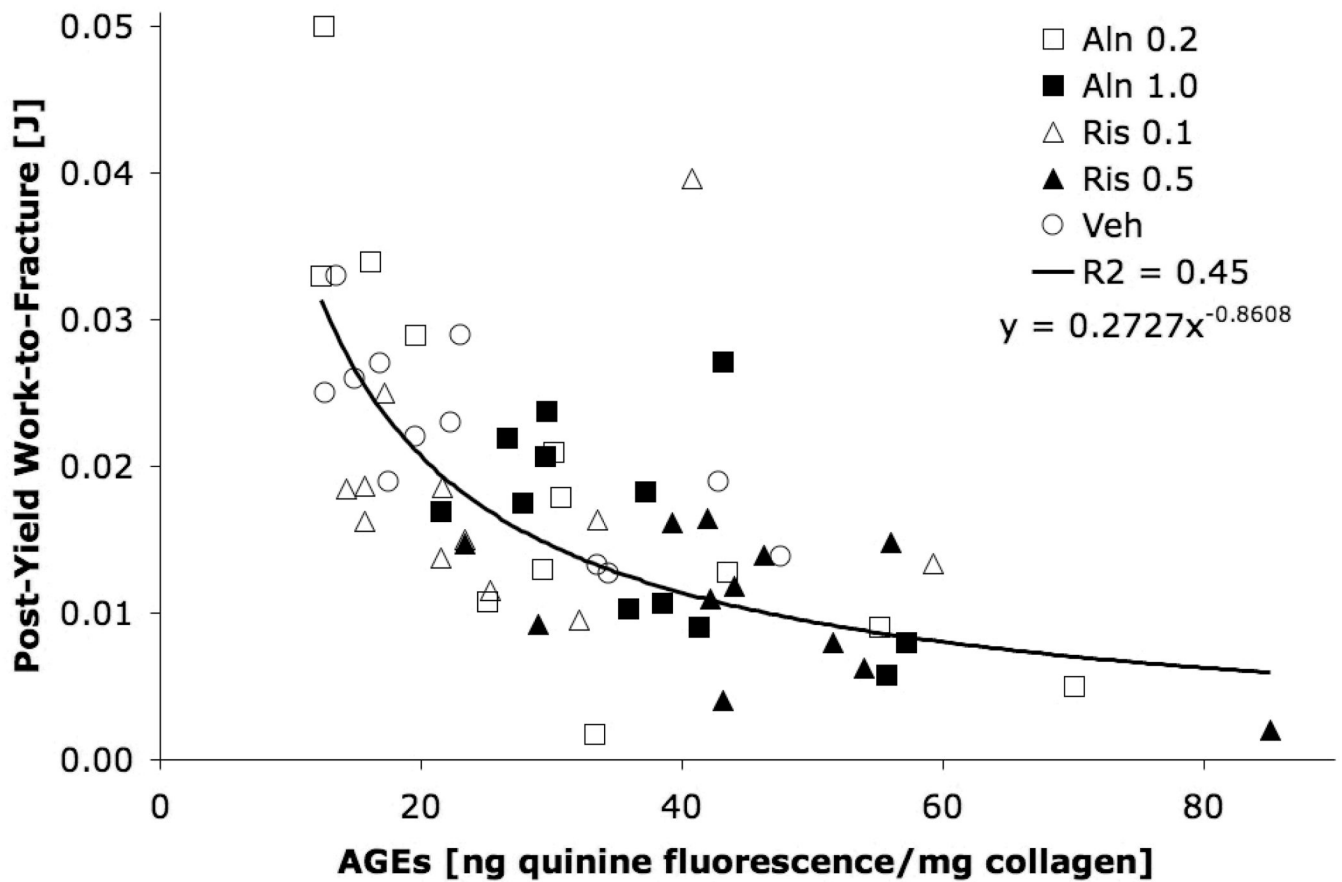


**Fig 3.**

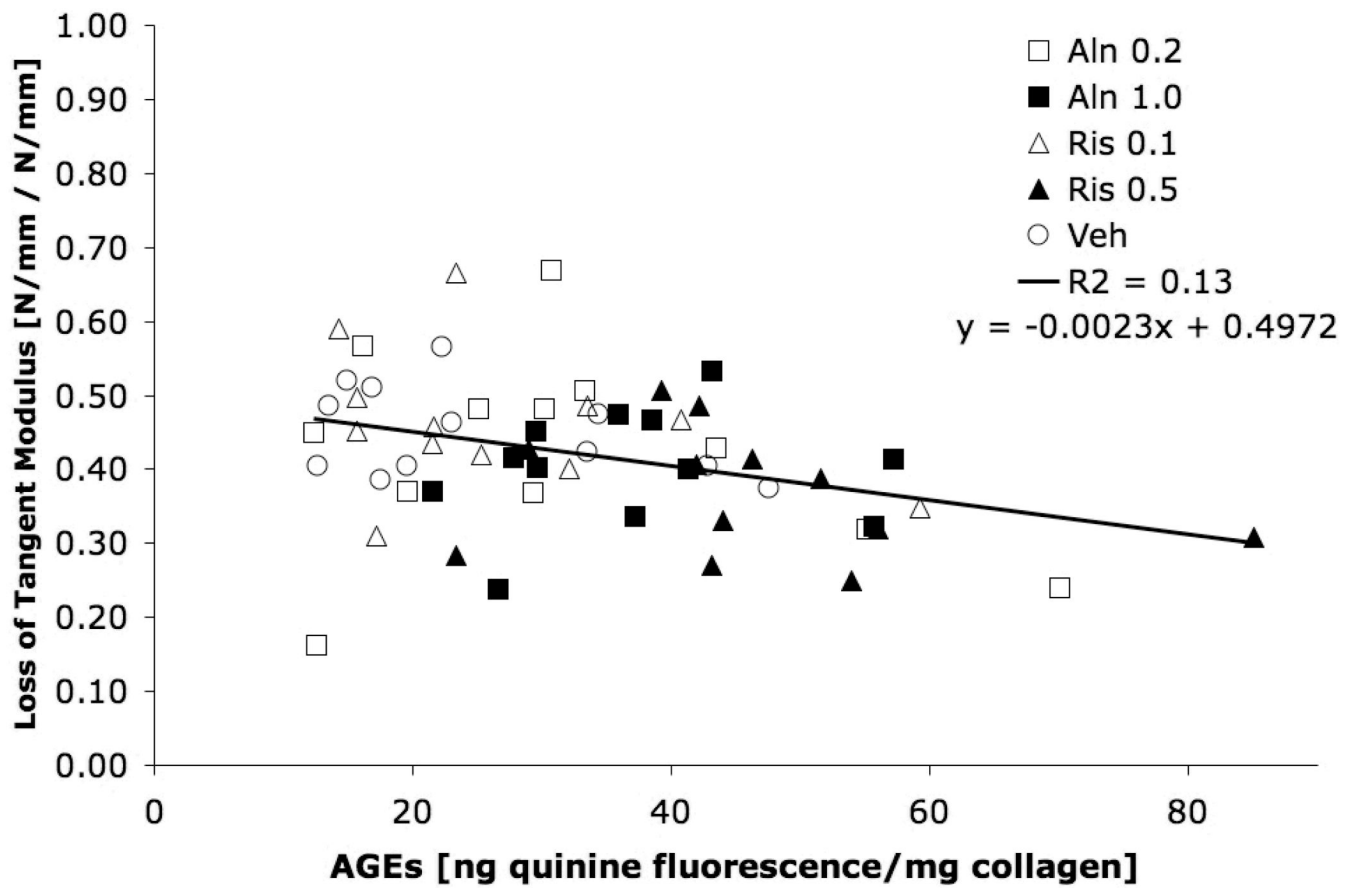
There was no significant effect of bisphosphonates at clinical doses on post-yield work-to-fracture when compared with the vehicle. Higher doses (5x clinical dose) resulted in a significant reduction in the post-yield work-to-fracture ( $p < 0.001$ ) in ALN and RIS-treated animals with no significant difference between the drugs at dose-equivalents. Asterisks denote significance when compared to the vehicle.



**Fig 4.** There was no significant effect of bisphosphonates at clinical doses on the loss of tangent stiffness of the cortical beams compared with the vehicle. Higher doses 5x clinical dose resulted in a significant reduction in the loss of tangent stiffness of the cortical bone tissue in ALN and RIS-treated animals (p=0.03; ANOVA) with no significant difference between the drugs at dose-equivalents. Asterisks denote significance when compared to the vehicle.

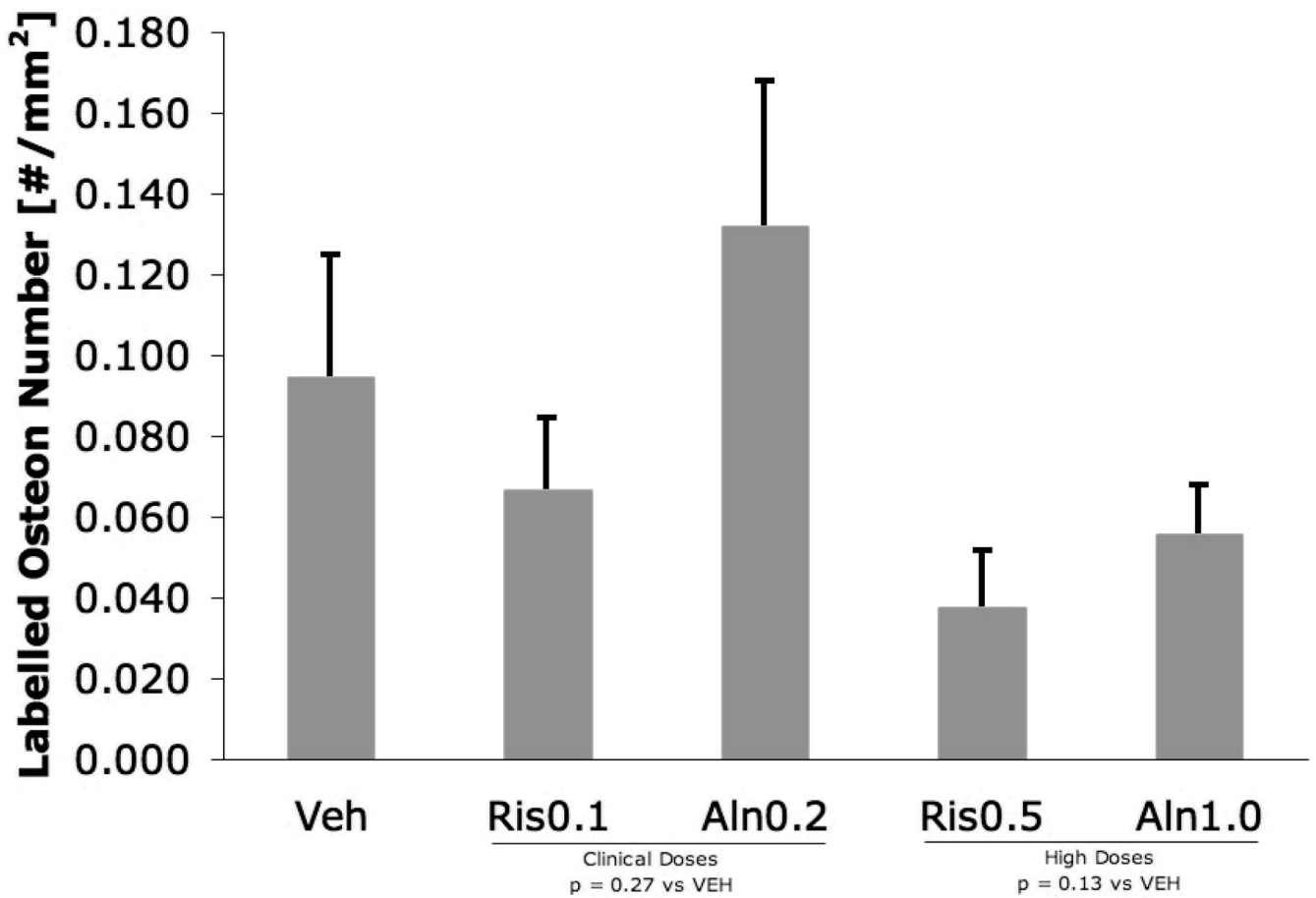


**Fig 5.** Post-yield work-to-fracture in cortical microbeams shows a significant negative correlation with AGEs accumulation ( $p < 0.001$ ; Non-linear regression).



**Fig 6.**

Loss of tangent stiffness in the cortical bone tissue shows a significant negative correlation with AGE accumulation ( $p=0.004$ ; Linear regression).



**Fig 7.** Intracortical remodeling of the tibia, as marked by L.On.N, was suppressed 51% with high-dose bisphosphonates compared to VEH ( $p=0.13$ ) while clinical doses had minimal effect ( $-8\%$  vs VEH;  $p = 0.27$ ) after one year.



Table 1

Average values of measured parameters (standard error of the mean in parentheses). p-values are from either ANOVA. Bending modulus (E), ultimate stress ( $\sigma_{ult}$ ), loss of tangent stiffness ( $k_{loss}$ ), the elastic work ( $W_{elastic}$ ), post-yield work-to-fracture ( $W_{py}$ ), degree of nonenzymatic crosslinking (AGEs), and labeled osteon number (L.On.N) are shown. Asterisks denote significance ( $p < 0.05$ ; ANOVA)

	Vehicle	RIS0.1	ALN0.2	p-values (vs VEH)	RIS0.5	ALN1.0	p-values (vs VEH)
E [GPa]	8.03 (0.60)	7.99 (0.48)	8.01 (0.73)	0.96	8.07 (0.65)	8.37 (0.53)	0.90
$\sigma_{ult}$ [MPa]	36.5 (3.3)	35.3 (2.0)	35.2 (3.0)	0.93	33.0 (2.0)	34.1 (1.8)	0.57
$k_{loss}$ [N/mm / N/mm]	0.451 (0.018)	0.461 (0.029)	0.421 (0.042)	0.62	0.366 (0.028)	0.402 (0.023)	<b>0.03*</b>
$W_{elastic}$ [J]	0.0603 (0.0069)	0.0550 (0.0045)	0.0636 (0.0063)	0.56	0.0590 (0.0047)	0.0607 (0.0042)	0.78
$W_{py}$ [J]	0.0219 (0.0021)	0.0180 (0.0025)	0.0198 (0.0047)	0.66	0.0107 (0.0018)	0.0158 (0.0020)	< <b>0.001*</b>
AGEs [ng quinine fluorescence/mg collagen]	24.9 (3.6)	26.7 (3.9)	31.2 (5.1)	0.75	46.3 (5.3)	37.0 (3.2)	< <b>0.001*</b>
L.On.N [# /mm <sup>2</sup> ]	0.0948 (0.030)	0.0667 (0.018)	0.132 (0.036)	0.27	0.0377 (0.014)	0.0558 (0.012)	0.13