



Published in final edited form as:

*Osteoporos Int.* 2015 March ; 26(3): 977–985. doi:10.1007/s00198-014-2978-9.

## Changes in skeletal collagen crosslinks and matrix hydration in high and low turnover chronic kidney disease

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### Abstract

**Purpose/Introduction**—Clinical data have documented a clear increase in fracture risk associated with chronic kidney disease (CKD). Preclinical studies have shown reductions in bone mechanical properties although the tissue-level mechanisms for these differences remain unclear. The goal of this study was to assess collagen cross-links and matrix hydration, two variables known to affect mechanical properties, in animals with either high or low turnover CKD.

**Methods**—At 35 weeks of age (>75% reduction in kidney function), the femoral diaphysis of male Cy/+ rats with high or low bone turnover rates, along with normal littermate (NL) controls, were assessed for collagen cross-links (pyridinoline (PYD), deoxypyridinoline (DPD), and pentosidine (PE)) using a high performance liquid chromatography (HPLC) assay as well as pore and bound water per volume (pw and bw) using a <sup>1</sup>H nuclear magnetic resonance (NMR) technique. Material-level biomechanical properties were calculated based on previously published whole bone mechanical tests.

**Results**—Cortical bone from animals with high turnover disease had lower Pyd and Dpd crosslink levels (–21% each), lower bw (–10%), higher PE (+71%), and higher pw (+46%), compared to NL. Animals with low turnover had higher Dpd, PE (+71%), and bw (+7%) along with lower pw (–60%) compared to NL. Both high and low turnover animals had reduced material-level bone toughness compared to NL animals as determined by three-point bending.

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#### CONFLICT OF INTEREST

Matthew R. Allen, Christopher L. Newman, Neal Chen, Mathilde Granke, Jeffrey S. Nyman, and Sharon M. Moe declare they have no conflicts of interest related to this work.

**Conclusions**—These data document an increase in skeletal PE with advanced CKD that is independent of bone turnover rate and inversely related to decline in kidney function. Although hydration changes occur in both high and low turnover disease, the data suggest that non-enzymatic collagen crosslinks may be a key factor in compromised mechanical properties of CKD.

### Keywords

advanced glycation end products; pentosidine; bound water; bone material properties; toughness

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## INTRODUCTION

There are clear and consistent data showing that chronic kidney disease (CKD) is associated with an increased fracture risk compared to individuals without CKD [1-5]. More concerning is that individuals with CKD who experience a fracture are at greater risk of mortality compared to people without CKD who fracture [6, 7]. There are numerous challenges to reducing fracture risk in CKD patients because of the complexity in the underlying bone disease. CKD is associated with significant metabolic derangements, including secondary hyperparathyroidism, yet some patients have low levels of parathyroid (PTH) for unclear reasons [1, 8, 9]. At the skeletal level, CKD appears to have a preferential impact on cortical bone, leading to increased porosity, and likely playing a major role in fracture [10-14]. Preclinical studies have documented CKD-associated reductions in biomechanical properties of whole bones, as well as at the micro- and nano-scale levels of the hierarchical organization of bone [14-18]. Properties of the tissue, such as mineral content, have not shown clear differences between normal and diseased bone leaving a cloud of uncertainty as to what properties of the tissue are responsible for this compromised mechanical phenotype associated with CKD [16].

Increased attention to ‘bone quality’ (those aspects other than BMD that account for a bone’s resistance to fracture) has led to a greater understanding of various skeletal properties [19-21]. Collagen plays a vital role in bone with changes in collagen cross-links showing clear influence on bone mechanical properties [22-26]. CKD-induced collagen changes have been noted in serum and several soft-tissues [27] but data in bone are limited [28, 29]. A less often discussed bone quality variable, bone hydration, also affect bone mechanical properties [30-32], but only recently has it become clear that *in vivo* modulation of hydration can play a role in a bone’s fracture resistance capacity [33-35].

While there is an increasing awareness of the importance of bone quality in CKD [20, 21], few studies have explicitly examined material properties in animal models [14-16, 18, 29]. The goal of the present study was to examine two specific properties, collagen crosslinks and matrix hydration (bound and pore water) in a rat model with progressive development of CKD that can be manipulated to have either high or low turnover disease [14, 36]. We hypothesized that CKD adversely affects both collagen cross-links and matrix hydration, that these parameters would be most affected in high turnover disease, and there would be relationships between these properties and bone mechanical outcomes.

## MATERIALS AND METHODS

### Animal Model

The current study utilized a slowly progressive animal model of CKD, the Cy/+ rat, which has been described in detail several times [15, 16, 18, 36-38]. Tissues analyzed in the current study were from select groups of animals that were part of a larger study [14]. Specifically, the animals were in one of three groups: Cy/+ animals that were untreated and thus had high turnover bone disease, Cy/+ given calcium supplementation in their drinking water to suppress PTH starting at 25 weeks of age (low turnover disease), or normal littermate controls (n=8-10/gp). All animals were euthanized at 35 weeks of age and femora (used to study both outcome measures) were saved wrapped in saline soaked gauze at -20C. Prior to the assays reported in this paper, femora were tested in 3 point bending [14]. Blood was also collected at the end of the experiment for biochemical analyses. Dynamic histomorphometry (detailing turnover rates), 3 point bending, and biochemical assays have all been previously reported [14]. All procedures were conducted under the approval of Indiana University School of Medicine Institutional Animal Care and Use Committee protocol # 10479.

### Collagen Cross-Linking

Segments of the femoral diaphysis (~3 mm in length) were processed for collagen cross-links as previously described [16]. Briefly, following demineralized in 20% EDTA (0.68 M, pH 7.4), demineralized bone was hydrolyzed. Each hydrolysate was resuspended in ultrapure water, split into two equal portions, and dried. Half the residue was resuspended in ultrapure water with an internal standard ( $5 \times 10^{-6}$  g/L pyridoxine) and assayed by a high performance liquid chromatography (HPLC) system (Beckman-Coulter System Gold 168). Standards with varying concentrations of pyridinoline (Pyd) (Quidel), deoxypyridinoline (Dpd) (Quidel), pentosidine (PE) (International Maillard Reaction Society), and a constant amount of pyridoxine were also assayed. Using a Waters 2475 fluorescence detector (excitation/emission of 295/400 nm for Pyd and Dpd and 328/378 nm for PE), chromatograms were recorded to determine the amount of each crosslink. These amounts were then normalized by collagen content (hydroxyproline), which was determined from the other half of each hydrolysate by another HPLC assay. The calculated mass of hydroxyproline was then multiplied by 7.5 (assuming 13-14% of type I collagen by mass) and divided by the molecular weight of collagen (30,000 Da), thereby giving crosslink concentration as mol/mol of collagen. For each chromatogram, the area of the peak is calculated and divided by an internal standard. These values are then plotted onto a standard curve that plots the known masses of the standards to their given areas, providing an estimate of the mass of the given molecule in the unknowns (hydroxyproline, pyridinoline, deoxypyridinoline, and pentosidine).

### <sup>1</sup>H Nuclear magnetic resonance spectroscopy (NMR)

A ~5 mm cross-section of the femur mid-shaft was placed into a low proton, loop-gap-style radio-frequency (RF) coil [39] along with a reference microsphere of water ( $T_2 \sim 2$  s). The NMR analysis to separate proton signals within the bone was then performed in a 4.7T horizontal-bore magnet (Varian Medical Systems, Santa Clara, CA) using 90°/180° RF pulses of ~ 9/18  $\mu$ s duration and collecting Carr-Purcell-Meiboom-Gill (CPMG)

measurements with 10,000 echoes at 100  $\mu$ s spacing. To generate the spectrum of transverse relaxation time constants ( $T_2$ ), the echo magnitudes were fitted with multiple exponential decay functions [40]. Upon normalizing the integrated areas of bound water ( $T_2 = 100 \mu\text{s}$ -800  $\mu\text{s}$ ) and pore water ( $T_2 = 800 \mu\text{s} - 600 \text{ms}$ ) to the area of the reference ( $T_2 = 600 \text{ms}$ -10 s) [41], the volume of bound water and the volume of pore water were divided by the specimen volume (calculated from Archimedes' principle) to give bound water (bw) and pore water (pw) volume fractions.

### Material-level biomechanical properties

Whole bone material properties, determined using 3 point bending of the femora, have been previously reported [14]. Material-level properties were estimated using standard beam theory equations [30]. Bone diameters were measured at the mid-diaphysis using digital calipers while cross-sectional moment of inertia was measured using micro computed tomography.

### Statistical Analysis

All analyses were performed using SAS software. Comparisons among the groups were made using student's t-tests as the focus of this work was to independently assess high/lower turnover conditions versus control. Pearson product moment correlation tests were used to assess the relationship between variables across all treatments with the exception of hydration for which Spearman correlations were used, as the data were not normally distributed. *A priori*  $\alpha$ -levels were set at 0.05 to determine significance.

## RESULTS

### High-turnover CKD model

Details about the phenotype of these animals with secondary hyperparathyroidism have been previously published [14]. Briefly, measures of kidney function and PTH were both significantly higher compared to normal (+200 and 2800%, respectively), indicative of active disease. Trabecular bone turnover, measured by dynamic histomorphometry, was more than 4-fold higher in compared normal; cortical porosity was more than doubled. Structural biomechanical properties, ultimate load and stiffness, were both significantly lower than controls with no difference in energy to fracture, a biomechanical property that is not independent of bone structure [14].

All measured forms of collagen cross-links were significantly different compared to normal. The two mature enzymatic cross-links, Pyd and Dpd were both significantly lower (-21%;  $p = 0.004$  and  $0.02$ ) compared to control (**Figure 1A**). There was no significant difference in the Pyd/Dpd ratio. PE, an advanced glycation product, was 71% ( $p = 0.001$ ) higher compared to normal (**Figure 1B**). Pore water volume fraction within the femoral cortex was higher (+46%;  $p = 0.024$ ) while matrix bound water volume fraction was significantly lower (-10%;  $p = 0.04$ ) compared to normal animals (**Figure 1C**).

Ultimate stress was significantly lower than normal animals (-22%;  $p=0.001$ ) with no difference in modulus and a trend toward lower toughness (-21%;  $p = 0.11$ ) (**Table 1**).

### Low-turnover CKD model

Details about the phenotype of these animals, where PTH is suppressed with the administration of calcium in the drinking water, have been previously published [14]. Briefly, measures of kidney function were significantly higher (+150%), while PTH was significantly lower (−75%) compared to normal. Trabecular bone turnover, cortical porosity, and structural properties were not different from controls.

Levels of Pyd in the bone matrix were not different from controls, while Dpd levels were significantly higher (+24%;  $p = 0.002$ ) compared to normal (**Figure 2A**). There was no significant difference in the Pyd/Dpd ratio. PE was 72% higher ( $p = 0.001$  compared to normal (**Figure 2B**). The NMR-derived pore water within the femoral cortex was lower (−60%;  $p = 0.001$ ), and bound water was significantly higher (7%;  $p = 0.04$ ) compared to normal animals (**Figure 2C**).

Toughness was significantly lower than normal animals (−27%;  $p = 0.005$ ) (**Table 1**). Ultimate stress and modulus were similar to normal animals.

### Correlations

To examine relationships between outcome measures, correlations were assessed across the entire data set (controls, low turnover CKD and high turnover CKD). Kidney function (assessed by BUN) was significantly correlated to PE ( $R = 0.68$ ) (**Figure 3A**). PTH was significantly correlated to several outcomes including pore water ( $R = 0.81$ ), bound water ( $R = -0.70$ ), pyd (−0.58), Dpd ( $R = -0.72$ ), and PE ( $R = 0.46$ ). PE levels were negatively correlated to ultimate stress and toughness ( $R = -0.44$  and  $-0.57$ , respectively) (**Figure 3B**).

## DISCUSSION

It is becoming clear that the increased risk of skeletal fracture associated with chronic kidney disease is multi-factorial and occurs due to changes in both bone quantity and quality [12, 20, 21, 42]. We have previously documented in an animal of progressive kidney disease, that compromised biomechanical properties can be quantified at the whole bone level as well as at the micro- and nano-scales [16]. These data illustrate that loss of bone mass, as has been repeatedly documented in both clinical and preclinical studies, is just one piece of the CKD fracture risk puzzle. Given the nano-scale changes in bone mechanical properties, our laboratory has focused on measuring properties of the tissue matrix that contribute to this level of mechanical integrity [16]. The results of the current work, in a preclinical animal model of progressive CKD, point to modification of both collagen crosslinking and bone hydration in late stage CKD. The changes in crosslinks confirm previous preclinical work in a different CKD model of high turnover bone [29], while the hydration data results represent novel data and a potential variable that could be used for non-invasive clinical assessment of bone quality [33, 43].

Collagen crosslinks in bone are either enzymatically-mediated (via lysyl oxidase) or non-enzymatically mediated (in the form of advanced glycation end products (AGEs) such as pentosidine) [22]. The predominant mature enzymatic crosslinks – pyridinoline and deoxypyridinoline (also known as hydroxylslypyridinoline and lyslypyridinoline,

respectively) – have been shown to be associated with changes in bone mechanical properties [22]. Previous work by our lab in the same animal model as the current study showed no difference in Pyd or Dpd in CKD animals at 30 weeks [16]. We have documented that this animal model has rapid disease progression, in high-turnover states, between 30 and 35 weeks of age [14, 15], leading us to hypothesize that major changes in collagen cross-links might manifest with progression to advanced disease. Although this turned out to be the case, the story is more complicated as the enzymatic crosslink changes were dependent of rate turnover; high turnover groups had reduced enzymatic cross-links while low turnover animals had higher than normal levels of Dpd.

The simplistic relationship between bone turnover and collagen crosslinks is an inverse one. High bone turnover leads to lower levels of mature enzymatic crosslinks because the mean tissue age is lower; while low turnover bone typically has high levels. Yet this relationship model assumes that the process of collagen crosslinks formation/maturation is not disrupted. Levels of lysyl oxidase, the key enzyme involved in enzymatic crosslinking, are tissue-specific. Kidney levels of lysyl oxidase have been shown to be higher in CKD and this is associated with increased fibrosis in renal tissue [44]. Serum measures of pyridinoline have been shown to be increased in patients with end stage renal disease, and these levels were higher in patients with high turnover compared to those with low turnover [45]. Yet these serum measures do not provide insight into skeletal levels, as the latter are determined both the rate of formation/breakdown (i.e., bone turnover) and the rate of crosslink maturation (i.e., divalent to trivalent bonded). Skeletal levels of mature crosslinks have been found to be lower in patients with high-turnover end stage renal disease [27], while animal models of low turnover have shown reductions in bone lysyl oxidase levels [28]. Reduction in lysyl oxidase would reduce enzymatic cross-links – although neither the clinical nor preclinical study measured both enzyme and crosslinks. Unfortunately, levels of lysyl oxidase were not assessed in the current work and thus definitive mechanisms underlying changes in mature cross-links cannot be ascertained.

Clinical studies have demonstrated that non-enzymatic cross-links (pentosidine, specifically) are increased in the circulation and soft tissue of patients with CKD [46]. Increased oxidative stress in CKD leads to increased production of AGE, and AGEs worsen oxidative stress creating a vicious cycle that is independent of blood glucose levels [47]. Skeletal levels of AGEs have been shown to be elevated in a small clinical study in patients on dialysis [27]. Although we have recently shown that at 30 weeks of age there is no difference in skeletal AGEs in our progressive CKD model, the current results show that at 35 weeks there is a striking difference (>70% higher than normal). This suggests that, like the enzymatic cross-links, major changes occur between 30 and 35 weeks in this animal model. More interesting is that the levels of AGEs in the bone are independent of bone remodeling rate – both high and low turnover models had similarly higher AGE levels compared to controls (**Figures 1B and 2B**). AGEs are typically linked to mean tissue age – since they accumulate in tissues over time when mean tissue age is higher AGEs are higher. It therefore follows that, in normal situations, bone with higher turnover rates will have lower AGEs compared to bone with lower turnover rates. This does not seem to hold true in the setting of CKD suggesting there is some intrinsic aspect of the disease that results in

more AGE accumulation in bone independent of tissue age, perhaps due to ongoing AGE production in CKD [47] and reduced clearance by the kidney [48]. We hypothesize that elevations in oxidative stress may be underlying the accumulation of AGEs in this model. Thus, the severity of kidney disease may be more important than the level of bone turnover for determination of skeletal AGEs. The importance of the severity of kidney function is supported by a modest positive correlation between serum BUN and skeletal pentosidine ( $R = 0.679$ ) in the current study (**Figure 3A**).

The influence of hydration on bone mechanical properties has long been appreciated as it pertains to *ex vivo* mechanical testing [30, 31]. More recently data have emerged showing modulation of hydration can occur *in vivo* [34], suggesting that it could play a role in disease-related bone mechanical phenotypes. The results of the current study show that in high turnover disease, pore water is significantly higher, while in low turnover disease pore water is lower, than normal animals. Changes in pore water track closely to changes in cortical porosity, which is high in high turnover animals and low in low turnover animals at 35 weeks of age [14]. The changes in bound water, which have been shown to have predominant mechanical effect on bone [34], were unexpected and the mechanisms through which bound water is modulated *in vivo* remain unclear. Bound water tends to be inversely related to mineralization and at least at 30 weeks of age mineralization characteristics (determined by Raman spectroscopy) are not different from controls [16]. It is possible that mineral has changed by week 35 in these animals yet unfortunately tissue is not available for such analyses. Also, as intracortical porosity increases, there is more pore water but less matrix interactions with water molecules. This is confirmed in the current work by the positive correlation between pore water and porosity ( $R = 0.69$ ).

Both AGE levels and bone hydration have been documented as having significant effects on bone mechanical properties. Increasing AGE levels either *in vitro*, or *in vivo*, are inversely related to bone toughness – the ability of the bone tissue to absorb energy before fracture [23, 49]. Reductions in bone toughness reflect a bone that fractures more easily and in a more brittle fashion [50]. Conversely to AGEs, modulating bone hydration (specifically bound water) has been shown to have a positive relationship to bone toughness [34, 51]. Estimation of material-level mechanical properties in the current study shows that both high and low turnover disease lower bone toughness relative to normal animals, with only the lower turnover animals reaching statistical significance (**Table 1**). Interestingly, these two conditions had contrasting effects on pore and bound water, as well as enzymatically-mediated crosslinks. The only common feature of the two conditions was a significant increase in levels of AGEs (pentosidine). Skeletal pentosidine was negatively correlated to bone toughness ( $r = -0.57$ ; **Figure 3B**). While we cannot definitively conclude that the higher AGE levels are fully responsible for the biomechanical phenotype, the results of this work suggest it is an outcome that should be a primary focus in future studies.

In conclusion, using an animal model of progressive chronic kidney disease, we have shown that both collagen crosslinking and skeletal hydration are affected in late-stage renal disease. Although both of these variables are known to play roles in bone mechanical properties, the data suggest that accumulation on non-enzymatic collagen crosslinks may be a key change

that is responsible for altering mechanical properties associated with late-stage disease progression.

## ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health grants AR58005 (SM), DL100093 (CN), AR063157 (JSN), and the Indiana Clinical Translational Science Institute grant TR000162 (CN). The cross-link analysis is the result of work supported with resources and the use of facilities at the VA Tennessee Valley Healthcare System. All authors were involved in the design, conduct and analyses of the study. The authors would like to thank Drew Brown, Shannon Roy, and Kali O'Neill for technical assistance. We would also like to acknowledge the late Dr. Vincent H. Gattone II (1951-2013), who was instrumental in developing this animal model.

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**SUMMARY**

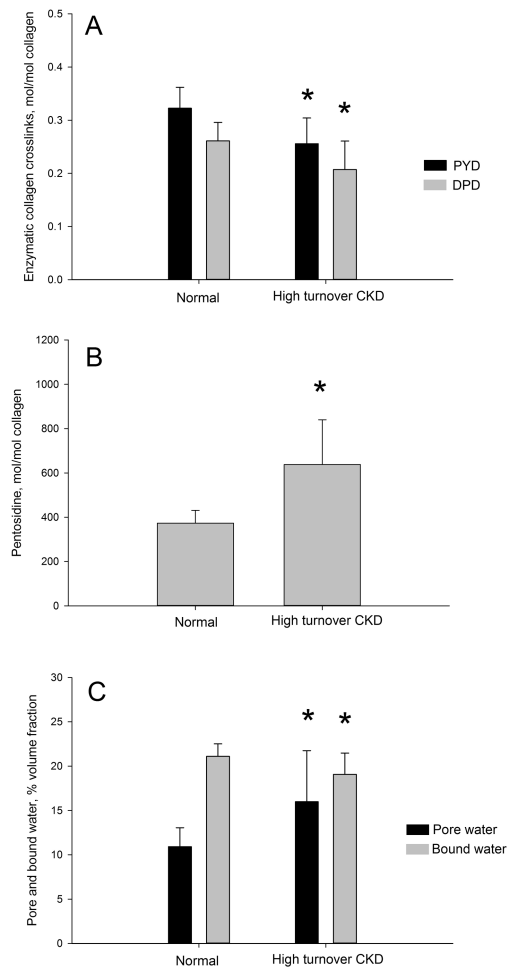
Chronic kidney disease (CKD) increases fracture risk. The results of this work point to changes in bone collagen and bone hydration as playing a role in bone fragility associated with CKD.

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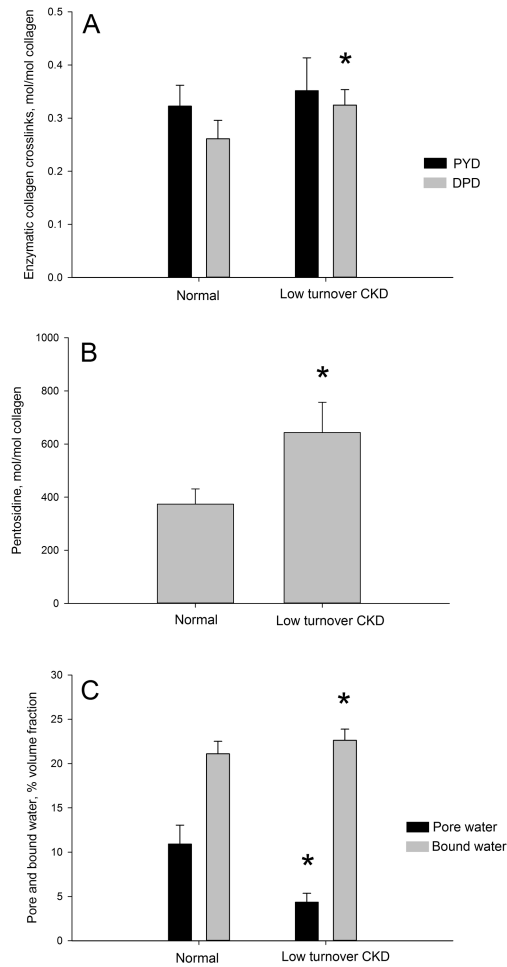
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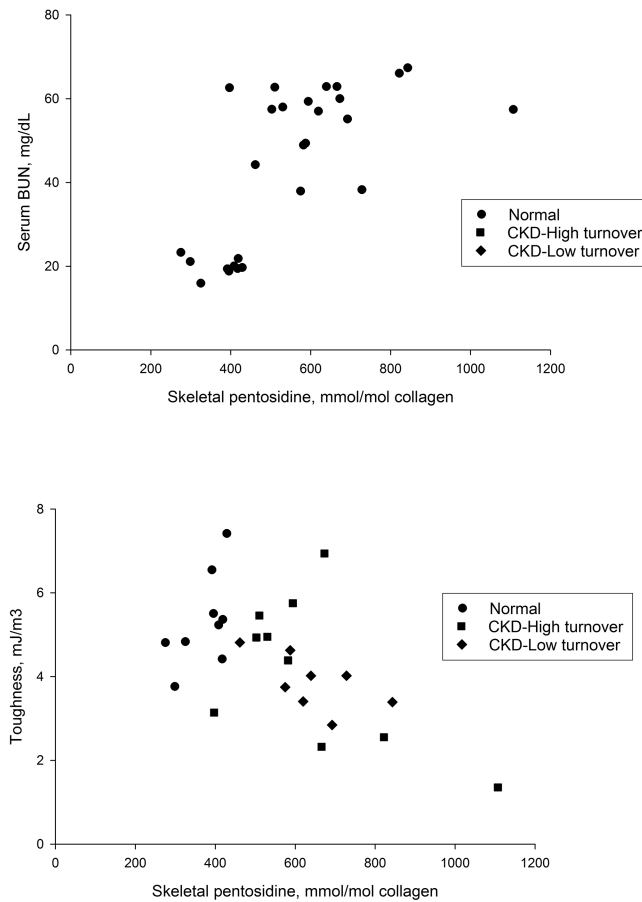
**Figure 1.**

High-turnover CKD associated changes in skeletal collagen crosslinking and tissue hydration. (A) Enzymatic collagen cross-links (pyridinoline, PYD and deoxypyridinoline, DPD) were significantly lower in CKD compared to normal littermates. (B) Pentosidine, an advanced glycation end product, was found in significantly higher concentrations in CKD bone compared to normal. (C) Pore water fraction was significantly higher and bound water fraction significantly lower in CKD animals compared to normal. \*  $p < 0.05$  compared to normal animals.



**Figure 2.**

Low-turnover CKD associated changes in skeletal collagen crosslinking and tissue hydration. (A) The enzymatic collagen cross-link (deoxypyridinoline, DPD) was significantly higher than normal in low-turnover disease; pyridinoline, PYD was unaffected. (B) Pentosidine, an advanced glycation end product, was found in significantly higher concentrations in CKD bone compared to normal. (C) Pore water fraction was significantly lower and bound water fraction significantly higher in CKD animals compared to normal. \*  $p < 0.05$  compared to normal animals.



**Figure 3.**

Correlations between skeletal pentosidine levels and kidney function (A) and mechanical properties (B). As both high and low turnover CKD models were shown to have higher pentosidine levels compared to normal we explored the relationship between kidney function and pentosidine. This relationship was statistically significant ( $r = 0.68$ ,  $p < 0.001$ ). Given the known role of pentosidine in modulation of mechanical properties we also explored the relationship between pentosidine and bone toughness (B). This relationship was statistically significant ( $r = -0.57$ ,  $p = 0.002$ ).

**Table 1**

Femoral diaphysis morphology and calculated material-level biomechanical properties

	Normal	High turnover CKD	Low turnover CKD
Bone diameter, mm	3.7 ± 0.2	3.6 ± 0.2	3.7 ± 0.1
Porosity, %	0.1 ± 0.1	4.1 ± 5.4 *	0.5 ± 0.2 *
Cross-sectional moment of inertia, mm <sup>4</sup>	10.1 ± 1.5	8.56 ± 0.9 *	11.1 ± 1.4
Ultimate stress, MPa	180 ± 17	140 ± 25 *	176 ± 27
Modulus, MPa	6777 ± 831	6609 ± 750	6701 ± 880
Toughness, MJ/m <sup>3</sup>	5.3 ± 1.1	4.2 ± 1.8	3.9 ± 0.7 *

Values are presented as mean ± standard deviation.

\* p < 0.05 versus normal controls.