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Moderate Chronic Kidney Disease Impairs Bone Quality in C57Bl/6J Mice

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Abstract

Chronic kidney disease (CKD) increases bone fracture risk. While the causes of bone fragility in CKD are not clear, the disrupted mineral homeostasis inherent to CKD may cause material quality changes to bone tissue. In this study, 11-week old male C57Bl/6J mice underwent either 5/6th nephrectomy (5/6 Nx) or sham procedures. Mice were fed a normal chow diet and euthanized 11 weeks post-surgery. Moderate CKD with high bone turnover was established in the 5/6 Nx group as determined through serum chemistry and bone gene expression assays. We compared nanoindentation modulus and mineral volume fraction (assessed through quantitative backscattered scanning electron microscopy) at matched sites in arrays placed on the cortical bone of the tibia mid-diaphysis. Trabecular and cortical bone microarchitecture (μ CT) and whole bone strength were also evaluated. We found that moderate CKD minimally affected bone microarchitecture and did not influence whole bone strength. Meanwhile, bone material quality decreased with CKD; a pattern of altered tissue maturation was observed with 5/6 Nx whereby the newest 60 micrometers of bone tissue adjacent to the periosteal surface had lower indentation modulus and mineral volume fraction than more interior, older bone. The variance of modulus and mineral volume fraction were also altered following 5/6 Nx, implying that tissue-scale heterogeneity may be negatively affected by CKD. The observed lower bone material quality may play a role in the decreased fracture resistance that is clinically associated with human CKD.

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Keywords

bone quality; kidney disease; CKD; CKD-MBD; nanoindentation; qBSE

1. Introduction

Chronic kidney disease (CKD) is recognized as a global health problem [Levey *et al.*, 2007]. An estimated 7.2% of adults over age 30 and 23-36% of persons aged 64+ have at least moderate kidney dysfunction [Zhang & Rothenbacher, 2008]. Bone fragility is an important consequence of CKD. In a three-year study, patients with moderate CKD had a 1.6 - 2.4 fold increase in fracture prevalence compared with age-matched controls having mild or no disease. With severe CKD, fracture rate increases by 3.1 – 5.1 fold [Naylor *et al.*, 2014]. Moreover, the presence of moderate CKD increases fracture-related mortality by two fold in patients over 70 years of age [Nitsch *et al.*, 2009]. The mechanisms by which bone fragility develops in CKD are incompletely understood.

Although bone mineral density is used clinically to diagnose fracture risk, bone fragility in CKD cannot be explained by declines in bone mineral density alone [Nitsch *et al.*, 2009]. However, a growing body of work across several models of bone disease demonstrates that bone strength and fracture resistance also depend on bone quality. Bone quality is the comprehensive state of tissue microarchitecture and bone material properties including mechanical response, mineralization, collagen cross-linking, and accumulation of microdamage [Donnelly 2011, Seeman & Delmas, 2006]. CKD is a disease of profoundly dysregulated mineral homeostasis, and thus, changes in bone mineralization and other parameters of bone quality may be expected [Moe *et al.*, 2006, Wolf 2015].

Few previous studies have investigated bone quality in CKD. Some of these prior works employed 5/6th nephrectomy (5/6 Nx), which is well-established to produce moderate CKD with high-turnover osteodystrophy that mimics key aspects of human kidney dysfunction [Fleck *et al.*, 2006, Gava *et al.*, 2012]. Bone quality has been observed to decrease after 5/6 Nx; diminished bone microarchitecture (BV/TV, Tb.N, Conn.D, and increased Tb.Sp) was observed for tibial trabecular bone in Crlj:CD1 mice 16 weeks following 5/6 Nx [Kadokawa *et al.*, 2011]. Microscale cortical bone material properties have been assessed after 5/6 Nx, but results for these studies are in disagreement about whether bone material quality is diminished or preserved. In a complex model of CKD (5/6 Nx with additional thyroparathyroidectomy (TPTx) in rats), bone developed a low-turnover response while Raman Spectroscopy revealed higher mineral to matrix ratio, carbonate to phosphate ratio, collagen maturity, and pentosidine to amide ratio in cortical bone compared with TPTx alone [Iwasaki *et al.*, 2011]. In a subsequent study by the same group, rats were given either 5/6 Nx alone or alongside TPTx. 5/6 Nx produced high-turnover bone response as well as increased mineral to matrix ratio, while 5/6 Nx and TPTx resulted in low-turnover and decreased mineral to matrix ratio. Increased pentosidine to matrix ratio, decreased crystallinity and degree of orientation of the c-axis of bone mineral crystals were general to both uremic models [Iwasaki *et al.*, 2015]. Meanwhile, Kadokawa *et al.* did not observe changes in FTIR-observed mineral to matrix ratio, mineral maturity, collagen maturity, or

indentation modulus in mice after 5/6 Nx compared with sham. Critically, these studies all employed microscale materials characterization techniques (*e.g.*, nanoindentation, FTIR, Raman Spectroscopy) within the middle of the cortical thickness [Iwasaki 2011 *et al.*, Iwasaki *et al.*, 2015, Kadokawa *et al.*, 2011]. Yet new rodent bone is apposed primarily on periosteal and endosteal surfaces [Ferguson *et al.*, 2003, Donnelly *et al.*, 2010]. It is possible that the inconsistent observation of bone material changes after 5/6 Nx results from surveying bone that predominantly existed prior to nephrectomy; that is, material property assessment regions of interest were not necessarily placed on bone actively forming under the influence of CKD.

Bone material quality has not been studied in rodent cortical bone for tissue apposed after nephrectomy. In this study, we evaluate the influence of moderate CKD established *via* 11 weeks of 5/6 Nx in C57Bl/6J mice, a low bone density, well-characterized inbred mouse strain. We seek to characterize how bone microscale material properties may diminish in bone established during CKD, and together with microarchitecture and whole bone strength describe bone quality in a mouse model of moderate CKD with high bone turnover.

2. Materials and Methods

2.1 Specimens

Eight-week-old male C57Bl/6J mice ($n = 26$) were obtained from Jackson Laboratories (Catalog number 000664, Bar Harbor, ME). Mice were maintained on a 12-h light / 12-h dark cycle and housed ($n = 5$ max) in polycarbonate cages with standard bedding. Mice were fed a normal rodent chow diet with water freely available. We randomly assigned mice to sham ($n = 12$) or 5/6 Nx ($n = 14$) groups. The 5/6 Nx group underwent a two-stage nephrectomy procedure; at 10 weeks of age, 2/3 of the right kidney was ablated, followed by complete ablation of the left kidney one week later. The control group received sham operations with the same timeframe. Mice were anesthetized using 1.5% isoflurane during procedures. A post-operative dose of buprenorphine (0.5 mg/kg) was administered after surgeries and prior to recovery and every 12 hours for the following 2 days. Calcein (10 mg/kg) and tetracycline (20 mg/kg) were administered *via* intraperitoneal injection two weeks and two days before euthanasia, respectively. Mice were euthanized at 11 weeks following the second procedure by exsanguination and midline thoracotomy. Following euthanasia, femurs, tibiae, and humeri were harvested. The right femur diaphysis was cleaned of soft tissue and marrow, and then snap-frozen and stored at -80°C for gene expression. Other harvested bones were stored in phosphate buffered saline (PBS) soaked gauze at -20°C until analyses or embedding. All animal procedures were approved by the Institutional Animal Use and Care Committee at the University of Colorado Denver. Investigators were blinded to specimen treatment status for methods described in §2.2-§2.6. For methods described in §2.7 – §2.9, specimens were randomly selected from each treatment group.

2.2 Serum chemistry

Serum biochemistry analyses were performed on blood drawn at sacrifice. Plasma creatinine concentrations were determined using kits from BioAssay System (Hayward, CA). Blood

urea nitrogen (BUN), plasma phosphate and plasma calcium levels were measured with kits from Stanbio Laboratory (Boerne, TX). Intact plasma parathyroid hormone (PTH) level was measured with kits from Immutopics (San Clemente, CA). Phosphate, calcium, and creatinine assays were performed for sixteen samples each (5/6 Nx: n = 8, sham: n = 8). BUN and PTH were assessed for twelve samples (5/6 Nx: n = 6, sham: n = 6), and eleven samples (5/6 Nx: n = 5, sham: n = 6), respectively.

2.3 qPCR

Relative gene expression was performed using quantitative real-time polymerase chain reaction (qPCR). Frozen femurs were powdered with two 30-second pulses 2600 rpms per manufacturer's instructions (Sartorius mikro-dismembrator S). RNA was extracted and cDNA prepared as previously described by King *et al.* [King *et al.*, 2009]. The fast SYBR green qPCR method (Bio-Rad CFX connect real-time system) was used with the primer sequences listed in Table 1. All primers were designed and then individually validated to the MIQE guidelines of amplification efficiency between 90% and 110% [Bustin *et al.*, 2009]. Conditions were set at an initiation temperature of 95° C for 20 seconds, followed by 40 cycles of denaturing at 95° C for 3 seconds and annealing at 60° C for 30 seconds. Following this DNA amplification, a melting temperature sequence was used to determine size of amplicon. Data were analyzed using the C_q method for relative fold change (equations 2.3.1 – 2.3.3) with 18s as the reference gene. For this method, C_q is defined as the number of cycles necessary to reach the threshold cycle of target amplification, and C_q is the difference between the number of cycles to threshold for the gene of interest and the reference gene. Smaller values for C_q indicates the presence of more copies of RNA, because less time is necessary to reach the peak of the reverse transcription reaction. C_q is the difference between C_q for 5/6 Nx and sham, and its value has reverse orientation of the fold change.

$$\Delta C_q = C_{q_{\text{gene of interest}}} - C_{q_{18s}} \quad \text{Equation 2.3.1}$$

$$\Delta\Delta C_q = \Delta C_{q_{5/6Nx}} - \Delta C_{q_{\text{sham}}} \quad \text{Equation 2.3.2}$$

$$\text{Fold change} = 2^{-\Delta\Delta C_q} \quad \text{Equation 2.3.3}$$

2.4 μ CT

Left tibiae were cleaned of non-osseous tissue and stored in 70% ethanol at 4°C. Left femurs were cleaned and fresh frozen in PBS soaked gauze at -20°C. The femurs were defrosted at 4°C overnight before micro-computed tomography imaging (μ CT). Microarchitecture was evaluated *via* μ CT for all 26 study specimens (10 μ m voxel size; MicroCT 80, Scanco Medical AG, Basserdorf, Switzerland). For the tibiae, scans extended in the distal direction from immediately below the proximal growth plate. The scans were acquired using a 70-kVp peak X-ray tube potential, a 0.5 mm Al filter, and an integration time of 800 ms to minimize beam hardening effects and optimize the signal-to-noise ratio. Each sample was

contoured semi-automatically and subjected to Gaussian filtration. Thresholding was site-specific, using values of 450 and 723 mg HA/cm³ for tibial and femoral analyses, respectively. The proximal tibia was evaluated for parameters including trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular spacing (Tb.Sp), trabecular thickness (Tb.Th), and connectivity density (Conn.D). Femurs were scanned at the mid-diaphysis, with μ CT cortical bone parameters including total mineral density (TMD), cortical thickness (Ct.Th), cortical bone area (BA), total area (TA), cortical porosity (Ct.Po), moment of inertia about the anterior-posterior axis ($I_{\max}$), moment of inertia about the medial-lateral axis ($I_{\min}$), and the distance between the centroid and bone surface in the anterior-posterior direction ($C_{\min}$) [Bouxsein *et al.*, 2010]. Contours for cortical bone were fit tightly to the periosteal and endosteal surfaces, without gaps, so as to minimize measurement variance. Contours for trabecular bone were drawn a distance away from the endocortical surface to avoid erroneously including cortical bone.

2.5 Ashing

Humeri from all 26 mice were first oven-dried at 105° C for 24 hours and weighed to obtain dry bone mass. The bones were then oven-dried at 800° C for 24 hours to obtain the bone mineral mass. Percent mineralization was calculated from the ratio of bone mineral mass to dry bone mass.

2.6 Whole Bone Mechanical Testing

Following μ CT, all left femurs underwent three-point bending and femoral neck testing (Insight II Material Testing System, 250 N load cell, MTS Systems Corporation, Eden Prairie, MN). We removed bones from the PBS-soaked gauze and placed them on fixtures for mechanical testing while taking care to maintain their hydration levels. Femurs were first tested to failure in three-point bending using a deflection rate of 5 mm/min on a custom anvil with an 8-mm span. The proximal femur fragment was then placed in a pencil vise fixture mounted in-line with the main compressive direction, with the femoral head pointing up [Jämsä *et al.*, 1998]. The femoral neck was tested to failure using a deflection rate of 5 mm/min.

For both three-point bending and femoral neck tests, load-displacement curves were analyzed for mechanical properties including stiffness, maximum load, displacement at maximum load, energy at maximum load, load at yield, displacement at yield, energy at yield, post-yield displacement, load at fracture, displacement at fracture, and energy at fracture. The yield point was defined as the intersection of a secant line drawn with a 10% reduction in slope from the initial tangent stiffness and the load-displacement curve [Jepsen *et al.*, 1996]. Using $I_{\min}$ and $C_{\min}$ from μ CT, we estimated material properties including bending modulus, maximum stress, and toughness from the three-point bending data using standard beam bending equations as applied to the mouse femur [Turner & Burr, 1993].

2.7 Mineral Apposition Rate

Fluorescent labels administered in this study were too diffuse to allow reliable dynamic histomorphometry, including an estimate of mineral apposition rate (MAR). In order to calculate MAR for the purposes of estimating the amount of bone tissue formed following

nephrectomy, we employed published femoral mid-diaphysis periosteal mineral apposition rates for the same strain, sex, and age range of mice [Ferguson *et al.*, 2003]. In that study, bone formation was characterized for femurs from male C57Bl/6 mice at 18 time points between 4 and 104 weeks of age. Mice killed at 10, 12, 15, 18, and 22 weeks of age and administered tetracycline labels at 6-day intervals permit characterization of MAR relevant to the current study. Periosteal MAR was calculated as the bone formation rate (bone area / time between labels) divided by the linear extent of the mineralizing perimeter. Using this calculated periosteal MAR for the age of mice in the present study, mean linear apposition (calculated from a crescent-shaped area) was estimated to be 80 μm .

2.8 Nanoindentation

Subsequent to μCT analysis, tibiae were histologically dehydrated in ethanol and embedded in poly(methyl)methacrylate (PMMA). Embedded bones were sectioned at 0.8 mm proximal to the tibia-fibula junction using a low speed diamond saw (Isomet, Buehler, Lake Bluff, IL). Specimens were ground using wet silicon carbide paper (600 and 1200 grit), and then polished using aluminum oxide pastes (9, 5, 3, 1, 0.1, 0.05 μm) and Rayon fine clothes (South Bay Technologies, San Clemente, CA) to a final finish of 0.05 μm . Samples were sonicated between each polishing step.

Nanoindentation was performed for twelve randomly selected samples (5/6 Nx: n = 6, sham: n = 6) using a Hysitron TI 950 Triboindenter (Hysitron Inc., Minneapolis, MN) and 5 μm diamond spherical tip. The nanoindentation array was situated on the posterior-lateral quadrant of the cortical tibia mid-diaphysis (Figure 1). Arrays had a minimum of six points in each row, and eight rows such that material properties were evaluated throughout the cortical thickness. The first row was parallel to, and within 20 μm from, a tangent line to the periosteal surface. Spacing in both x and y directions between indents was 20 μm . The load function was depth controlled; indentation rate was 31 nm/s until a max depth of 500 nm. Max depth was maintained for 120 s to allow dissipation of viscoelastic energy. Indentation modulus was calculated using Oliver-Pharr analysis considering the first 45% of the unloading curve [Bushby *et al.*, 2004].

2.9 Quantitative Backscattered Scanning Electron Microscopy

Following nanoindentation, samples were sputter coated with carbon in preparation for scanning electron microscopy using backscattered electron imaging (qBSE, JEOL JASM6490 LV, 20 kV, aperture 60 μm , 14-mm working distance) [Boyde *et al.*, 1999, Campbell *et al.*, 2012]. Images were acquired at 300x to include the nanoindentation array using both backscatter compositional and shadow modes. Raw grey levels of compositional images were calibrated using borosilicate glass reference standards [Campbell *et al.*, 2012]. Custom Matlab code was employed to measure the mean grey level in circular regions of interest (ROI) situated over the nanoindentation indents, and convert mean grey level to corresponding mineral volume fraction (minVf) based on a previously published calibration curve [Campbell *et al.*, 2012]. The ROI radius was selected to be 2.5 times the indent contact radius (R) after performing a sensitivity analysis with ROI ranging from 1-3; minVf was observed to stabilize at and beyond $2.5R$. The indent impression was excluded from the ROI for analysis to reduce error from topographic shadowing. Black pixels were not included in

mean grey level calculation, as post-calibration pixel values of 0 indicate regions without mineral content, such as cracks, lacunae, or PMMA. If an ROI overlapped with a crack, lacuna, or the bone/PMMA interface, the ROI and its corresponding indent were excluded from minVf and indentation modulus datasets, respectively. The distance of indents from the periosteal surface was measured using the shadow-mode images and the ImageJ measurement tool [Rasband 1997]. Endosteal perimeter (Endo.P), periosteal perimeter (Peri.P), endosteal area (Endo.A), periosteal area (Peri.A) and cortical area (Cort.A, the difference between periosteal and endosteal areas) were also calculated from qBSE images using ImageJ.

2.10 Data Analysis

Data are presented as mean $\pm$ standard error of the mean. The average and variance for modulus and minVf were calculated per row (eight rows spaced at 20 μ m, with 6-9 points each). The average modulus and minVf at each row were compared between 5/6 Nx and sham groups using repeated-measures ANOVA. Post-hoc testing was conducted between row means within each treatment group, and for each row between treatments. A modified Bonferroni procedure was employed to control family-wise error at $\alpha = 0.05$. Variance in modulus and minVf was compared between treatment groups at each indentation row for all indents *via* Levene's method (Table 6).

All other measures were compared for 5/6 Nx and sham groups utilizing unpaired t-tests, with significance set *a priori* to $p < 0.05$. Data sets were examined for both normality and equal variance. All statistical analyses were performed with Minitab (v.17).

3. Results

3.1 Kidney Function

Moderate CKD following 11 weeks of 5/6 Nx was confirmed with significantly higher BUN (sham = 7.42 mg/mL, 5/6 Nx = 44.68 mg/mL, $p < 0.001$) and creatinine levels (sham = 0.37 mg/mL, 5/6 Nx = 0.50 mg/mL, $p = 0.042$) in 5/6 Nx than sham [Levey *et al.*, 2003]. Mean PTH was not statistically different between groups (sham = 211.12 pg/mL, 5/6 Nx = 359.27 pg/mL, $p = 0.120$). Serum calcium and phosphate did not change with treatment status.

3.2 Gene Expression

The expression of genes coding for mouse alkaline phosphatase (*Alp*) and mouse bone sialoprotein (*Ibsp*) were significantly higher in mice that received nephrectomy (Table 2). The expression of osteocalcin was nearly 5-fold higher with nephrectomy, but was not significant, potentially due to variation among the control mice (as indicated by a higher standard deviation in C_q data). Type I collagen showed no difference.

3.3 Bone microarchitecture

μ CT of the tibia revealed significantly decreased Tb.N (-5.87% , $p = 0.016$) and increased Tb.Sp (7.74% , $p = 0.008$) with 5/6 Nx. For the femur, TMD was lower after 5/6 Nx (-1.89% , $p < 0.001$) (Table 3). Cortical porosity was higher following 5/6 Nx ($+107.90\%$, $p = 0.139$), but this difference was not significant because of high variance. No other μ CT

measures of the tibia (BV/TV, Tb.Th, vBMD) or femur (pMOI, Bone Area, Total Area, BA/TA) differed between sham and 5/6 Nx. No differences were found in Endo.P, Peri.P, Endo.A, Peri.A, or Cort.A as measured from qBSE images of the tibial midshaft.

3.4 Ashing

No differences were observed between sham and 5/6 Nx for dry mass (sham = 21.22 ± 0.67 ; 5/6 Nx: 21.74 ± 0.34), mineral mass (sham = 12.53 ± 0.37 ; 5/6 Nx: 12.46 ± 0.14), or percent mineral (sham = 59.81 ± 2.85 ; 5/6 Nx: 57.39 ± 0.55).

3.5 Whole Bone Mechanical Properties

Sham and 5/6 Nx groups did not differ in mechanical properties at maximum load, yield, or fracture as measured by three-point bending and femoral neck testing (Tables 4-5). Material properties (bending modulus, maximum stress, toughness) calculated from three-point bending were also unchanged with 5/6 Nx.

3.5 Microscale Mechanical Properties

Repeated measures ANOVA found a significant main effect of row away from the periosteal surface, as well as a significant interaction between treatment and row. Sham mice had the lowest modulus in the first 20 μm from the periosteal surface, but no row means were significantly different for this treatment group (Figure 2). Nephrectomized mice had a larger region of lower modulus tissue than observed in sham. Modulus was significantly lower in rows 1 and 2 (0-40 μm) than in rows 4 – 7 (80-140 μm). Row 2 (20-40 μm) and row 3 (40-60 μm) also did not differ, indicating that the zone of lower modulus tissue from 5/6 Nx extends through the first 60 μm of tissue. Row 8 had a similar modulus to Rows 2 and 3, which may indicate altered material properties in newly apposed endosteal bone. Comparing each row between treatments, modulus was lower in 5/6 Nx than sham for row 1 ($p = 0.028$) and row 2 ($p = 0.042$). These between-group comparisons reveal noteworthy trends, but p-values were higher than the critical α adjusted for family-wise error. Variance was also compared for all points between treatment groups by row using Levene's test. For row 1 (0-20 μm), variability in modulus was significantly lower (-65.96%) in 5/6 Nx than sham, indicating decreased heterogeneity of tissue-scale stiffness with kidney disease. Row 2 (20-40 μm) also had significantly lower variance (-56.81%) in 5/6 Nx than in sham. All other rows had variance that did not significantly differ between treatments.

3.6 Microscale bone mineralization

Sham mice had similar minVf throughout cortical bone; no differences in minVf were seen with distance from the periosteal surface. By contrast, in 5/6 Nx, a periosteal band of undermineralized tissue was clearly visible in bone expected to have apposed post-nephrectomy (Figure 3). In repeated measures ANOVA with factors of row and treatment, the main effect of row was significant as was the interaction between row and treatment. There were no differences in minVf between any rows for sham mice. A markedly different mineralization profile was evident with 5/6 Nx (Figure 3). Row 1 (0-20 μm) had significantly lower minVf than more interior rows 5-7 (80-140 μm). The area of undermineralized tissue with 5/6 Nx was observed to extend through row 4, as the first four

rows (0-80 μ m) did not significantly differ in minVf (Figure 4). Row 8, which on some bones approached the endosteal surface, was similar to rows 2-4; this last row may be influenced by newly apposed endosteal bone. Variance in minVf was also compared between treatment groups for each row. For bone 0-20 μ m from the periosteal surface, variability in minVf was 122.98% higher in 5/6 Nx than sham, but variances for the two groups were not significantly different. Bone 20-40 μ m in distance had significantly higher variability (+100.50%) in mineral for 5/6 Nx than for sham. At 40-60 μ m, variance in minVf also significantly higher (+128.87%) for 5/6 Nx than for sham. All other rows had variance that was not significantly different between treatments.

4. Discussion

The purpose of this investigation was to evaluate bone quality alterations, including tissue microarchitecture and material properties, in a mouse model of high-turnover chronic kidney disease (CKD). Kidney disease was induced at 11 weeks of age *via* 5/6th nephrectomy (5/6 Nx) in male C57Bl/6J mice. The significantly elevated BUN and creatinine were observed without concomitantly increased PTH, and thus without evidence of secondary hyperparathyroidism, suggesting that CKD was moderate in severity [Joy *et al.*, 2007, Levey *et al.*, 2003]. We observed markedly altered maturation of bone material properties with distance from the periosteal surface for 5/6 Nx compared with sham. These deleterious alterations occurred despite only minimal changes to bone microarchitecture and without significant differences in whole bone mechanical response or material properties derived from three-point bending. Our findings of altered microscale mineral volume fraction and modulus may provide new insight into the clinically observed increase in bone fragility that accompanies human CKD.

Our work fills a critical gap by assessing microscale bone material properties in arrays extending through newer and into older bone tissue, including bone close to the periosteal surface that is likely established after 5/6 Nx. While previous studies of rodent bone quality in simple (i.e., 5/6 Nx) and complex (i.e., 5/6 Nx compounded with thyroparathyroidectomy) surgical models of CKD have surveyed cortical bone for material properties, each study evaluated bone material quality at interior cortical bone that likely formed prior to CKD [Iwasaki *et al.*, 2011, Iwasaki *et al.*, 2015, Kadokawa *et al.*, 2011]. In our study, bone material quality was similar in the oldest, most interior bone for CKD and sham groups, indicating that previously established bone was not affected by kidney dysfunction. In contrast to sham, 5/6 Nx was characterized by a 40-60 μ m band of bone with both lower modulus and lower mineral volume fraction compared with more interior, older bone. In other words, older, more mineral-dense cortical bone was preceded by a zone of newer, lower quality bone tissue for 5/6 Nx (Figure 3). These observations underscore the importance of assessing microscale material properties in newly formed as opposed to pre-existing bone in nephrectomy models of CKD.

It was necessary to assess whether the reduced indentation modulus and mineral volume fraction observed in new bone apposed after 5/6 Nx were caused by CKD, or were instead explained by normal bone mineral maturation, *i.e.*, the temporal mineralization of osteoid. Because mice in this study were not skeletally mature at the time of 5/6 Nx, substantial bone

formation and cortical expansion were concurrent with CKD [Ferguson *et al.*, 2003, Glatt *et al.*, 2007]. It is known that newly deposited osteoid increases in mineral content, crystallite size, and stiffness over time until achieving the chemistry, structure, and mechanical response of mature bone [Donnelly 2010]. We therefore compared bone maturation patterns between sham and CKD groups. We observed a normal pattern of bone tissue maturation in new cortical bone for sham mice. This maturation was exhibited by increasing indentation modulus values, measured within micrometer-sized regions of bone, within the first ~20 μm distance from the periosteal surface and then a plateau in modulus values at distances > 20 μm . Sham mice did not evidence differences in mineral volume fraction with distance from the periosteal surface. By contrast, tissue maturation was altered with CKD; the region of less-stiff tissue extended to 40-60 μm from the periosteal surface and was also undermineralized.

While our fluorescent labels confirmed periosteal apposition at the site of our nanoindentation arrays, they were too diffuse to allow calculation of mineral apposition rate or clearly distinguish sites of newly formed bone tissue. Consequently, it was not possible to determine the exact boundary between bone tissue apposed before and after nephrectomy. However, a zone of new, lower-quality bone tissue 60 μm in width is quite possible; from mineral apposition rates measured for the femur for the same strain, sex, and age range of mice, an estimated 80 micrometers of periosteal apposition may be expected [Ferguson *et al.*, 2003]. Because the periosteal and endosteal perimeters (and cortical area) were not different for 5/6 Nx and sham, mineral apposition is comparable between groups. Furthermore, gene expression indicates significantly raised *Alpl* and *Ibsp*, both of which are related to matrix mineralization. While we cannot determine if these changes were due to a change in osteoblast/osteocyte activity or number, because osteocalcin and collagen gene expression were not greater in the CKD group, change in activity as opposed to number seems more likely. These increased markers of matrix mineralization do not necessarily imply a greater content of mineral in new bone tissue; indeed, we observe that mineral volume fraction is decreased in new bone with CKD. Instead, increased expression of *Alpl* and *Ibsp* likely corresponds with high-turnover bone response and the deposition of a substantial quantity of new tissue following 5/6 Nx. The exact boundary of new and pre-existing bone within these 160 μm wide arrays is less important than the observation of altered tissue mineral and modulus maturation with CKD in arrays that include both new tissue and bone established before nephrectomy. Indeed, abnormal mineral maturation kinetics have been observed in other diseases where bone quality is reduced and fracture risk increased, such as osteoporosis and osteogenesis imperfecta [Roschger *et al.*, 2008].

Our observations may also imply a reduction in bone toughness with CKD. The marked increase in mineralization and modulus between new and extant bone potentially introduces a dissimilar material interface with CKD; abnormal interfaces in bone can reduce tissue toughness [Wagermaier *et al.*, 2015]. Microscale bone tissue heterogeneity is altered for bone apposed during CKD, which may also imply a reduction of bone tissue toughness. Specifically, variance was decreased for indentation modulus and increased for mineral volume fraction. Lowered heterogeneity in indentation modulus has not been directly related to fracture risk [Lloyd *et al.*, 2015], yet has been experimentally observed in bones where fracture has occurred [Tjhia *et al.*, 2011]. Cortical bone in atypical bone fracture with

severely suppressed bone turnover had reduced variance in indentation modulus measured at 500 nm, the same depth as assessed in our study, when compared with an age-matched group without fracture [Tjhia *et al.*, 2011]. Variance in indentation modulus may serve to increase tissue-scale toughening mechanisms. Fratzl *et al.* analytically established that periodically varying elastic and inelastic regions should increase toughness [Fratzl *et al.*, 2007]. Additionally, experimentally-measured heterogeneous indentation modulus was seen to promote energy dissipation in a finite element model when compared with homogenous modulus [Tai *et al.*, 2011]. While the Tai *et al.* study assessed heterogeneity in elastic properties from indents at 40 nm of depth, we indented to 500 nm in order to minimize the effects of surface roughness while maintaining the same coefficient of variance as indents as shallow as 100 nm [Paietta *et al.*, 2011]. It is beyond the scope of the present study to evaluate if the energy-dissipation mechanisms proposed by Tai *et al.* extend to this greater length scale. Meanwhile, altered heterogeneity in mineral volume fraction may also contribute to bone fragility. Abnormally high or low variance in mineral volume fraction has been noted in backscatter SEM studies comparing bone samples with and without fragility fractures. The coefficient of variance of bone mineralization was lower in iliac crest biopsies from women with vertebral fractures versus healthy cadaveric controls [Ciarelli *et al.*, 2009]. Yet in another study of bone mineralization at the iliac crest, cadaveric trabeculae from women with osteoporotic vertebral fractures had a wider distribution of bone mineral content than healthy trabeculae [Busse *et al.*, 2009]. Increased heterogeneity in mineralization has also been reported in both trabecular and cortical bone for fracture-prone children [Tamminen *et al.*, 2014]. Thus abnormal variance in mineral volume fraction, whether too high or too low, may be indicative of lower tissue toughness and reduced fracture resistance. Tissue toughness was not directly evaluated in this study; however, the emergence of a materials interface, less variable indentation modulus, and more variable mineral volume fraction are characteristic of less-tough bone. This potential reduction of bone toughness, along with lower stiffness and mineralization, may comprise important factors influencing skeletal fragility in CKD.

The decrease in indentation modulus variance concurrent with increase in mineral volume fraction variance was unexpected. From tissue composite theory, it is expected that the infilling of stiff mineral within a softer matrix should substantially increase the overall bone tissue modulus. However, in considering the dependence of modulus on mineral volume fraction as published in prior works, the variance in mineral volume fraction only poorly explains that of modulus within the range of mineral volume fraction data observed in our study [Currey 1998, Oyen *et al.*, 2008]. An alternate explanation is that lowered mineral volume fraction observed in CKD has a minor influence on tissue stiffness, and that the variance in modulus may be better explained by coincident changes related to mineral deposition or growth, such as mineral crystal size and distribution, mineral chemistry, or modifications to the underlying collagen matrix.

The length-scale of observation is important for identifying bone quality changes in bone-affecting metabolic diseases [Roschger *et al.*, 2008, Karunaratne, *et al.*, 2012], and appears to be particularly crucial in CKD. At the micrometer length scale, bone material quality was worse with CKD. Yet lowered material quality did not impair mechanical or resulting material properties at the whole bone scale. How and to what degree whole bone properties

change with CKD remains unclear. Iwasaki *et al.* observed changes in whole bone storage modulus and tan delta in rats after 16 weeks with 5/6 Nx and a high-phosphate diet [Iwasaki *et al.*, 2015]. Meanwhile, in a Cy/+ genetic CKD model in rats, diseased bones had significantly lower ultimate stress, a trend towards lowered toughness, and no change in modulus as measured from three-point bending [Allen *et al.*, 2014]. In another study with Cy /+ rats, CKD significantly reduced ultimate stress and stiffness, while energy to failure was not changed [Moe *et al.*, 2014]. That whole bone mechanical properties were not compromised in our study could result from a lesser quantity of diseased bone or less severe CKD. To gain insight into the influences of poor bone quality on whole bone properties, we performed a first order approximation of flexural load at the yield point for either sham or diseased cases. The load at yield for a whole long bone can be estimated by calculating whole bone modulus in three point bending [Turner & Burr, 1993]. For this calculation, moments of inertia were taken from microCT, moduli were chosen to be the mean of the three closest nanoindentation rows to the periosteal surface for either sham or 5/6 Nx groups (from Figure 2), mean displacement at yield was 0.15 mm (from Table 4), and span length was maintained at 8-mm. Since load scales linearly with whole bone modulus, the load at yield for bones consisting entirely of diseased tissue was predicted to decrease by -16.6% as compared to healthy bones. However, we observed from qBSE that on average 21% of cortical bone area had apparently diminished minVf following 11 weeks of 5/6 Nx, therefore the expected reduction in load at yield should be substantially less than the aforementioned extreme example of a fully diseased bone. Thus, the 10-20% standard deviation observed around max, yield, and fracture loads exceeds the magnitude of the estimated effect size of 5/6 Nx. While whole bone strength is one important part of the suite of bone quality assessments, the uncertainty inherent in mouse bone flexural testing may limit the ability to resolve important changes in bone quality in moderate CKD [Ferguson *et al.*, 2003, Jämsä *et al.*, 1998]. Instead, the use of microscale techniques such as nanoindentation and qBSE may more reliably reveal changes to bone quality that could contribute towards fracture risk.

Our study had several limitations. The primary limitation was that dynamic bone histomorphometric measurements were not possible because of prohibitively diffuse fluorescent labels. Dynamic histomorphometry would have provided additional insight into how bone formation and resorption are altered with CKD and will be implemented wherever possible in future studies. Also limiting was the single time-point for gene expression assessment. Longitudinal analysis of serum markers of bone would aid in interpretation of bone material changes and should be performed in future analyses. Furthermore, in our study and others, rodents were skeletally immature at the time of nephrectomy [Kadokawa *et al.*, 2011, Iwasaki *et al.*, 2011, Iwasaki *et al.*, 2015]. The tendency of bone microarchitecture and whole bone mechanical properties to diminish with CKD may have been overshadowed by substantial bone growth as mice underwent skeletal maturation. The confounding influences of age and CKD could be clarified with a study of young, middle-aged, and mature mice.

In summary, microscale material properties of the cortical bone within the tibia diaphysis were impaired for mice that experienced surgically-induced CKD concurrent with skeletal maturation. Bone apposed between 11 weeks of age (time of 5/6 Nx) and 22 weeks (euthanasia) possessed lower and less variable indentation modulus, as well as lower and

more variable mineral volume fraction, compared with bone from sham mice. The current work addressed an important inconsistency in whether microscale material properties change with CKD; namely, through placing arrays in a location of actively forming bone, lower bone material quality was readily observed. A preponderance of evidence collected from this study and others now suggests that CKD does affect bone material properties, and that these properties may involve the quantity of mineral or its maturation (*e.g.*, current work, Iwasaki *et al.*, 2011, Iwasaki *et al.*, 2015), or the collagen network supporting mineralization [Allen *et al.*, 2014]. Looking ahead, a site-matched approach employing nanoindentation alongside Raman Spectroscopy may reveal greater detail about how formation and maturation of bone mineral and matrix are specifically altered with CKD, and how these changes contribute to diminished microscale mechanical properties.

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Abbreviations

CKD	Chronic Kidney Disease
5/6 Nx	5/6 th nephrectomy
TPTx	thyroparathyroidectomy
qBSE	quantitative backscattered scanning electron microscopy
BUN	blood urea nitrogen
PTH	parathyroid hormone
BV/TV	trabecular bone volume fraction
Tb.N	trabecular number
Tb.S	trabecular spacing
Th.Th	trabecular thickness
Conn.D	connectivity density
TMD	total mineral density
Ct.Th	cortical thickness
BA	cortical bone area
TA	cortical total area
Ct.Po	cortical porosity
I_{max}	moment of inertia about the anterior-posterior direction
I_{min}	moment of inertia about the medial-lateral axis

C_{min}	distance between the centroid and bone surface in the anterior-posterior direction
Alpl	gene for alkaline phosphatase
Ibsp	gene for bone sialoprotein
Bglap	gene for osteocalcin
Col1a1	gene for type I collagen α 1 chain
MAR	mineral apposition rate
PMMA	poly(methyl)methacrylate
minVf	mineral volume fraction
Endo.P	endosteal perimeter
Peri.P	periosteal perimeter
Endo.A	endosteal area
Peri.A	periosteal area
Cort.A	cortical area

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Research highlights

The role of CKD induced by 5/6th nephrectomy in lowering bone quality was examined in C57Bl/6J male mice.

- Microscale material property assessment arrays were positioned to include bone tissue established before and after 5/6 Nx.
- CKD lowered indentation modulus and mineral volume fraction in recently established cortical bone.
- Variance in mineral volume fraction and indentation modulus were altered with CKD.

Figure 1.

Modulus and minVf were measured at matched micrometer-scale sites in arrays placed on the posterior-lateral mid-diaphysal tibia, with points spaced 20 μm in x & y. Arrays extended through the cortical thickness, sampling bone tissue of varying age, including bone likely to have been established either before or after CKD.

Microscale material property measurements of the cortical tibia.

A **B**

Distance (μm)	Indentation modulus (GPa)	
	Sham	5/6 Nx
0-20	26.25 ± 0.82 ^A	23.71 ± 0.34 ^C
20-40	28.87 ± 0.96 ^A	26.32 ± 0.52 ^{B,C}
40-60	29.48 ± 0.89 ^A	28.05 ± 1.10 ^{B,A}
60-80	28.96 ± 1.00 ^A	29.86 ± 0.88 ^A
80-100	29.31 ± 0.67 ^A	30.77 ± 0.58 ^A
100-120	29.32 ± 0.46 ^A	30.32 ± 0.69 ^A
120-140	29.31 ± 0.56 ^A	30.41 ± 0.97 ^A
140-160	28.44 ± 0.84 ^A	29.51 ± 0.57 ^{B,A}

Figure 2.

A) Indentation modulus increases with distance from the periosteal surface. Modulus increases with distance in sham mice as bone tissue matures until a plateau is reached 20 μm from the periosteal surface. Modulus is initially lower and matures more slowly with 5/6 Nx. B) Mean and standard error for indentation modulus at each row. Within each treatment group, measures that do not share a letter subscript are significantly different. Significance was set at family-wise α of 0.05. Error bars indicate standard error of the mean. Indentation modulus measured at 20 μm increments from the periosteal surface.

Figure 3.

Mineral volume fraction was assessed with qBSE at the cortical mid-diaphysis of the tibia for A) sham and B) 5/6 Nx mice. Images used for analyses were captured at 300× for regions of interest (dotted lines). Mineral volume fraction does not differ with distance from the periosteal surface for C) sham, but is lower in the newest (*i.e.*, most recently apposed) periosteal bone for D) 5/6 Nx.

Quantitative Backscattered SEM images of the cortical tibia for sham and 5/6 Nx.

A **B**

Distance (μm)	minVf (%)	
	Sham	5/6 Nx
0-20	44.93 ± 0.79 ^A	42.15 ± 1.13 ^B
20-40	44.92 ± 0.63 ^A	43.17 ± 0.82 ^{A,B}
40-60	45.15 ± 0.87 ^A	44.22 ± 1.17 ^{A,B}
60-80	44.87 ± 0.82 ^A	46.22 ± 0.99 ^{A,B}
80-100	45.11 ± 0.84 ^A	46.66 ± 0.87 ^A
100-120	44.73 ± 0.85 ^A	46.93 ± 0.68 ^A
120-140	45.41 ± 0.71 ^A	46.38 ± 0.96 ^A
140-160	45.11 ± 0.80 ^A	45.47 ± 0.56 ^{A,B}

Figure 4.

A) Mineral volume fraction (minVf) increases with distance from the periosteal surface for 5/6 Nx, but not for sham. B) Mean and standard error for minVf at each row. Within each treatment group, measures that do not share a letter subscript are significantly different. No row means differ between treatment groups. Significance was set at family-wise α of 0.05. Error bars indicate standard error of the mean

Mineral volume fraction measured at 20 μ m increments from the periosteal surface.

Table 1

Primer sequences used for mRNA analysis.

Gene	Forward Sequence	Reverse Sequence
18s	CGCCGCTAGAGGTGAAATTCT	CGAACCTCCGACTTTCGTCT
<i>Alpl</i>	GAGGGACGAATCTCAGGGTA	TTTCAAGGTCTCTTGGGCTT
<i>Ibsp</i>	CTTACCGAGCTTATGAGGATGAAT	AAATGGTAGCCAGATAAGACAG
<i>Bglap</i>	AGACAAGTCCCACACAGCAG	TTGGACATGAAGGCTTTGTC
<i>Colla1</i>	GCTCCTCTTAGGGGGCACT	CCACGTCTCACCATTGGGG

Alpl = gene for alkaline phosphatase; *Ibsp* = gene for bone sialoprotein; *Bglap* = gene for osteocalcin; *Colla1* = gene for type I collagen $\alpha 1$ chain.

Table 2

Expression of bone matrix and mineralization genes in the femurs of sham and 5/6 Nx treated C57Bl/6J male mice (n = 11 / group).

Gene	Treatment	C _q ± StdError	C _q ± StdError	Fold-change (significance)
<i>Alpl</i>	Sham	19.4 ± 0.5	0 ± 0.5	0
	5/6 Nx	17.5 ± 0.5	-1.9 ± 0.5	6.1 (p = 0.020)
<i>Ibsp</i>	Sham	17.4 ± 0.6	0 ± 0.6	0
	5/6 Nx	15.7 ± 0.5	-1.7 ± 0.5	5.4 (p = 0.031)
<i>Bglap</i>	Sham	16.5 ± 0.6	0 ± 0.6	0
	5/6 Nx	14.5 ± 0.5	-1.5 ± 0.5	4.8 (p = 0.067)
<i>Colla1</i>	Sham	13.5 ± 0.5	0 ± 0.5	0
	Nephrectomy	12.6 ± 0.5	-0.9 ± 0.5	2.3 (p = 0.206)

Alpl = gene for alkaline phosphatase; *Ibsp* = gene for bone sialoprotein; *Bglap* = gene for osteocalcin; *Colla1* = gene for type I collagen α1 chain; C_q = quantification cycle (also known as quantification threshold, C_t).

Table 3Cortical and trabecular microarchitecture assessed by μ CT (mean $\pm$ standard error).

	Cortical bone					Trabecular bone							
	TMD (mg HA / cm ³)	Ct.Th (mm)	BA (mm ²)	TA (mm ²)	CLPo (%)	I _{max} (mm ⁴)	I _{min} (mm ⁴)	C _{min} (mm)	BV/T V (%)	Tb.Th (mm)	Tb.N (mm)	Tb.Sp (mm)	Conn.D
Sham n = 12	1043.70 ± 3.57	0.17 ± 0.00	0.77 ± 0.03	0.77 ± 0.03	0.19 ± 0.04	0.29 ± 0.02	0.13 ± 0.01	0.66 ± 0.01	15.41 ± 0.72	0.05 ± 0.00	4.81 ± 0.08	0.20 ± 0.00	126.32 ± 7.89
5/6	1023.98	0.16 ±	0.71 ±	0.71 ±	0.40 ±	0.26 ±	0.12 ±	0.67 ±	15.35	0.05 ±	4.53 ±	0.22 ±	136.52 ±
Nx	± 3.23 *	0.00	0.01	0.01	0.13	0.01	0.00	0.02	± 0.46	0.00	0.07*	0.00*	7.90
n = 14	(p < 0.001)										(p = 0.016)	(p = 0.008)	

Cortical and trabecular microarchitecture were assessed at the femur mid-shaft and proximal tibia, respectively. Significant differences ($p < 0.05$) between treatment groups are noted with *. TMD = total mineral density; Ct.Th = cortical thickness; BA = bone area; TA = total area; Ct.Po = cortical porosity; I_{max} = moment of inertia about the anterior-posterior axis; I_{min} = moment of inertia about the medial-lateral axis; C_{min} = distance from the centroid to the surface in the anterior-posterior direction; BV/TV = trabecular bone volume fraction; Tb.Th = trabecular thickness; Tb.N = trabecular number; Tb.Sp = trabecular spacing; Conn.D = connectivity density.

Table 4

Femur mechanical properties determined by three-point bending (mean $\pm$ standard error).

Parameter	Sham (n = 12)	5/6 Nx (n = 14)
Stiffness (N/mm)	115.63 $\pm$ 4.29	114.54 $\pm$ 5.39
Load at Yield (N)	15.64 $\pm$ 0.90	15.58 $\pm$ 0.77
Displacement at Yield (mm)	0.15 $\pm$ 0.01	0.15 $\pm$ 0.01
Energy at Yield (mJ)	1.30 $\pm$ 0.11	1.29 $\pm$ 0.10
Post-Yield Displacement (mm)	0.34 $\pm$ 0.03	0.33 $\pm$ 0.06
Max load (N)	18.46 $\pm$ 0.59	17.75 $\pm$ 0.61
Displacement at Max Load (mm)	0.25 $\pm$ 0.018	0.25 $\pm$ 0.020
Energy at Max Load (mJ)	2.99 $\pm$ 0.32	2.97 $\pm$ 0.32
Load at Fracture (N)	15.51 $\pm$ 0.68	15.27 $\pm$ 0.86
Displacement at Fracture (mm)	0.49 $\pm$ 0.02	0.48 $\pm$ 0.06
Energy at Fracture (mJ)	6.99 $\pm$ 0.49	6.48 $\pm$ 0.78
Bending Modulus (GPa)	8.89 $\pm$ 0.99	9.42 $\pm$ 0.81
Maximum stress (MPa)	189.36 $\pm$ 8.22	195.60 $\pm$ 8.16
Toughness (N/mm ²)	8.87 $\pm$ 0.54	8.94 $\pm$ 1.07

No significant differences were observed between sham and 5/6 Nx for any measure.

Table 5

Femur mechanical properties determined by femoral neck fracture test (mean $\pm$ standard error).

Parameter	Sham (n = 12)	5/6 Nx (n = 14)
Stiffness (N/mm)	175.54 $\pm$ 5.58	170.35 $\pm$ 5.25
Max load (N)	21.46 $\pm$ 0.89	22.25 $\pm$ 0.67
Displacement at Max Load (mm)	0.16 $\pm$ 0.01	0.19 $\pm$ 0.02
Energy at Max Load (mJ)	1.97 $\pm$ 0.13	2.53 $\pm$ 0.29
Load at Yield (N)	19.05 $\pm$ 1.01	17.86 $\pm$ 0.94
Displacement at Yield (mm)	0.12 $\pm$ 0.01	0.12 $\pm$ 0.00
Energy at Yield (mJ)	1.26 $\pm$ 0.11	1.14 $\pm$ 0.10
Post-Yield Deflection (mm)	0.07 $\pm$ 0.15	0.09 $\pm$ 0.02
Load at Fracture (N)	18.30 $\pm$ 1.43	20.71 $\pm$ 0.86
Displacement at Fracture (mm)	0.19 $\pm$ 0.01	0.20 $\pm$ 0.02
Energy at Fracture (mJ)	2.57 $\pm$ 0.26	2.84 $\pm$ 0.37

No significant differences were observed between sham and 5/6 Nx for any measure.

Table 6

Variance in microscale material properties calculated at each array row.

Row	Modulus		minVf	
	Sham	5/6 Nx	Sham	5/6 Nx
1	6.58	2.24*	3.23	8.54
2	7.34	3.17*	2.01	4.03*
3	7.52	5.97	3.81	8.72*
4	7.59	5.72	4.26	6.00
5	5.30	2.71	4.20	4.84
6	3.51	4.49	4.09	3.30
7	2.62	6.99	3.03	5.58
8	4.81	2.51	3.30	2.55

Variance calculated from points pooled across specimens within each treatment group per row. Significant differences ($p < 0.05$) between 5/6 Nx and sham are indicated by *.