Supplementary Material
for
Tolerance of the Human Kidney to Isolated Controlled Ischemia

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Complete Methods

Study Design

Between January 2009 and October 2010, 40 subjects were enrolled in this prospective clinical study after approval by the UTHSC San Antonio Institutional Review Board and informed consent. A single surgeon conducted all procedures. A data safety and monitoring committee periodically monitored the study progress. The surgeon was blinded to the study results until study completion.

Study Participants

Any patient with a renal mass undergoing an open partial nephrectomy and at least 18 years old was eligible for inclusion. Exclusion criteria included patients scheduled for a laparoscopic partial nephrectomy, pregnant patients, and patients with a solitary kidney or end stage renal disease.

Surgical Procedure and Renal Biopsies

Study participants underwent an open partial nephrectomy for renal mass excision. The kidney was mobilized and the vascular pedicle was isolated in the standard fashion. After induction of anesthesia and prior to clamping of the hilar vessels all study participants received 25 g of intravenous mannitol. Needle biopsies with an 18 gauge needle biopsy gun (Bard Monopty) were obtained from the kidney at a site remote from the tumor on all study participants at specified time points: one biopsy just prior to renal pedicle clamping, one biopsy every 10 minutes during ischemia up to unclamping of renal vessels and one biopsy at five minutes after reperfusion. The biopsy protocol was modified to focus more on endpoint biopsies after review of the first 18 patients indicated sufficient ischemia time course data. The subsequent 22 participants underwent biopsies at the following time points: two biopsies just prior to renal pedicle clamping, two biopsies at end of maximum duration of ischemia and two biopsies after five minutes of reperfusion. One core was processed for ultrastructure and the other for immunofluorescence. All biopsies were done under direct visualization from non-tumor bearing areas of the kidney.

A Satinsky vascular clamp was used to control the renal hilum *en bloc*. The decision to use warm or cold ischemia was based on the size and complexity of the renal mass with cold ischemia reserved for the most complex lesions. Surface hypothermia for cold ischemia was induced by using ice slush for 10-15 minutes after clamping of the hilum. Renal mass excision with parenchymal reconstruction was performed in a standard fashion. All patients received intravenous normal saline at 125-150 ml/hr in the post-operative 24 hours. All complications were recorded and graded according to the Clavien classification system [1].

Serum and Urine Biomarker Collection and Analysis

Serum and urine were collected at the preoperative visit and at 2, 6 (urine only), and 24 hours postoperatively and then daily until discharge (serum only). Full assays were done on all available samples from the first 24 hours. Serum was analyzed for creatinine, cystatin C, and neutrophil gelatinase-associated lipocalin (NGAL). Urine was analyzed for creatinine, NGAL, N-acetyl-beta-D-glucosaminidase (NAG), interleukin-18 (IL-18), kidney injury marker-1 (KIM-1), liver fatty acid binding protein (LFABP) and albumin. Serum creatinine was measured in the hospital clinical laboratory. The other samples were stored at -80 C until assay. The laboratory investigators were blinded to the sample sources and clinical outcomes. The urine NGAL ELlSA was performed using a commercially available assay (NGAL ELlSA Kit 036; Bioporto, Grusbakken, Denmark) that specifically detects human NGAL [2]. The intra-assay coefficients of variation were 2.1 % (range: 1.3-4.094) and inter-assay variation was 9.1 % (range: 6.8-18.1 %). Urine IL-18 and L-FABP were measured using commercially available ELlSA kits (Medical &Biological Laboratories Co., Nagoya,

Japan and CMlC Co., Tokyo, Japan, respectively) per manufacturer's instructions (12). The urine KIM-1 ELlSA was constructed using commercially available reagents (Duoset DY1750, R & D Systems, Inc., Minneapolis, MN) as described previously. [3]. Urine microalbumin was measured by immunoturbidimetry using MALB flex reagent on a Siemens Dimension Xpand Plus with HM clinical analyzer (Siemens Healthcare USA, New York, NY). Intra- and inter-assay CV values for the assay are 2.3% and 5.9%, respectively. Creatinine values were obtained using a colorimetric creatinine kit based on the Jaffe reaction (Enzo Life Sciences, Plymouth Meeting, PA). lntra- and inter-assay CV's for the creatinine assay are 2.4% and 3.15% respectively. Cystatin C was measured by N-latex nephelometry on a Siemens BNll clinical nephelometer (Siemens Healthcare USA, New York, NY). The inter- and intra-assay coefficient variations were 5% for batched samples analyzed on the same day and <10% for samples measured 6 months apart. The plasma NGAL ELISA was performed using an established and validated assay as previously described [4,5]. Briefly, microtiter plates precoated with a mouse monoclonal antibody raised against human NGAL (#HYB211-05; AntibodyShop, Gentofte, Denmark) were blocked with buffer containing 1% bovine serum albumin, coated with 100 µl of samples (plasma) or standards (NGAL concentrations ranging from 1 to 1,000 ng/ml), and incubated with a biotinylated monoclonal antibody against human NGAL (#HYB211-01 B; AntibodyShop) followed by avidin-conjugated horseradish peroxidase (Dako, Carpinteria, CA). TMB substrate (BD Biosciences, San Jose, CA) was added for color development, which was read after 30 minutes at 450 nm with a microplate reader (Benchmark Plus; Bio-Rad, Hercules, CA).

Renal biopsy specimen processing and analysis

Thick sections for toluidine blue and thin sections for electron microscopy: Needle biopsy cores obtained using 18 gauge Bard Monopty biopsy instruments were immediately ejected into 4% formaldehyde and 1% glutaraldehyde in 0.1M sodium phosphate buffer pH 7.2. After subdivision into multiple pieces, the tissue was washed in cold 0.1M Na cacodylate buffer pH 7.4, fixed over ice in the dark in 1% osmium tetroxide in water for 1 hour, washed in cold water and incubated overnight in the dark in cold, saturated uranyl acetate in water. After thorough washing in cold distilled water, the tissue was dehydrated in ascending concentrations of cold acetone in water. After incubation in absolute acetone with 2 changes, the tissue was transferred to propylene oxide with 3 changes to remove acetone, and embedded in epoxy resin by standard techniques. One micron thick sections of epoxy embedded tissue were cut with diamond knives and stained with toluidine blue. After light microscopic verification of the tissue architecture, the block was trimmed further for microtomy using diamond knives to obtain 600-800 Å thick sections. The sections were mounted on copper grids, stained with uranyl acetate and lead citrate and prepared for electron microscopy. Sections were viewed using a JEOL 1230 electron microscope and images recorded digitally.

Immunostaining and rhodamine phalloidin staining on frozen sections: Biopsies were ejected into periodic acid–lysine–paraformaldehyde (PLP), transported to the laboratory, and stored in PLP overnight (18-24 hours) at 4 degrees C. They were then washed x3 in PBS (phosphate buffered saline) and stored at 4 degrees C in PBS-0.02% NaN3. For sectioning, they were cryoprotected in 10% sucrose-PBS for 1 hour, then 30% sucrose-PBS, prior to embedding in OCT compound, freezing in isopentane, and storage at -80^{o}C. Cryosections of 6 µm thickness were cut on a Reichert-Jung Fridgocut-N 2800 cryostat and placed on glass slides pre-coated with 1% poly-L-lysine, then stored at -80^{o}C until stained. For staining, slides were rinsed in phosphate buffered saline (PBS), then permeabilized with 0.3% Triton X-100 in PBS for 4 min. at room temperature. They were then dip washed for 2 min. in PBS prior to 10 min. room temperature blocking with 10% Donkey Serum (S30, Millipore, Billerica, MA). Following another PBS wash, primary antibodies diluted in 10% donkey serum in PBS were applied for 60 min. at

room temperature in a humidified chamber. Primary antibodies were mouse monoclonal antibodies to phosphotyrosine (4G10, Millipore, 1:50), β1 integrin (MAB1981, Millipore, 1:100), and ICAM-1 (sc-107, Santa Cruz Biotechnology, Santa Cruz, CA, 1:50). After antibody exposure, slides were washed and CF^{TM}488A–conjugated donkey anti-mouse IgG (20014, Biotium, Hayward, CA) was applied at a 1:100 dilution in PBS as the secondary. After another 60 min. slides were rinsed with PBS and then treated with a 1:40 dilution of rhodamine phalloidin (00027, Biotium) followed by postfixing in 1% paraformaldehyde in PBS. Slides were then dried and sections were overlaid with Prolong Gold with DAPI (Invitrogen, Carlsbad, CA), cured overnight at room temperature, sealed with nail polish and stored at -20 C until viewing. Viewing was done on a Leica DM IRB fluorescence microscope (Leica, Bensheim, Germany) using a 40x HCX PL Fluotar lens and L5, N2.1, and A filter sets for CF488, rhodamine phalloidin, and DAPI respectively. Images were captured using an Olympus DP70 camera and DP controller software (Olympus, Center Valley, PA).

Grading criteria for quantitation of thick section and ultrastructure morphology, immunofluorescence, and rhodamine phalloidin staining for actin

Biopsy specimens processed for light microscopy of toluidine blue-stained thick sections and ultrastructure were analyzed by two of the authors (MAV and JMW) who were blinded to the clinical information from the patients during the evaluation.

***Thick section evaluation*:** Single pre-clamp, end-clamp, and post-clamp slides for each case.
0 – Normal
1 – mild focal tubule epithelial swelling, occasional tubules with blebs.
2 – moderate to severe tubule epithelial swelling affecting a minority of tubules. Swollen cells exhibit rounded prominent and discrete organelles. Tubules with blebs, focal. Focally frayed brush borders.
3 – Severe tubule epithelial cell swelling affecting a majority of tubules. More extensive blebbing affecting a larger population of tubules than in (2). Frayed brush borders.
4 – More extensive and severe tubule epithelial cell swelling. Necrosis of epithelium affecting a minority of tubules. Severe blebbing and fraying of brush borders.
5 – Necrosis of a majority of tubules.

Ultrastructure analysis: 10-15 sets of EM photographs for each biopsy totaling >6000 images on 178 biopsies were reviewed.
Parameters assessed:
a) Reversible mitochondrial swelling proximal tubule (PT) vs. distal tubule (DT).
b) Mitochondrial condensation.
c) Mitochondrial permeability transition lethal swelling.
d) Brush border membrane (BBM) clubbing, fragmentation, thinning, discontinuities.
e) Apical membrane blebbing PT vs. DT.
f) Presence of free blebs in lumens.
g) Expansion of intercellular spaces – ± biopsy artifact since seen in pre-clamp biopsies.
h) Pale cytosol with otherwise intact structure – ± biopsy artifact since seen in pre-clamp biopsies.
Composite Scale:
0 - absolutely pristine.
1 - Minimal BBM discontinuity, apical membrane blebbing without shedding , mild mitochondrial swelling limited to DTs. Mild occasional intercellular expansion. Occasional pale cells.
2 - Moderate mitochondrial swelling in PTs, moderate to severe swelling DTs. Mitochondrial condensation. BBM fragmentation, thinning or discontinuities. Occasional lumenal blebs.

3 - BBM thinning, fragmentation. Lumenal bleb casts. Uniform higher amplitude mitochondrial swelling in PTs and DTs, but with preservation of cristae and overall architecture. Changes present in any tubules, but not present in all.
4 - Stage 3 changes seen in every tubule.
5 - Presence of necrotic cells with large amplitude permeability transition-type mitochondrial swelling, plasma membrane disruption.

Quantitation of alterations of apical membrane integrity by rhodamine phalloidin staining for polymerized actin:
Parameters assessed were thinning, irregularity and absence of the brush border and apical membrane blebbing.
0 – No abnormalities in any tubules on the section
1 – <10% of tubules with any abnormalities. These were invariably mild
2 – 10-30% of tubules with abnormalities.
3 – 30-50% of tubules with abnormalities
4 – 50-70% of tubules with abnormalities
5 – >70% with abnormalities and/or substantial numbers of tubules with complete BBM loss, bleb casts

Semiquantitative analysis of immunofluorescence results for phosphotyrosine, β1 integrin, and ICAM-1 and of rhodamine phalloidin staining for actin:
End-Clamp and Post-Clamp sections were compared to the corresponding Pre-Clamp section and were assigned one of three categories: 1) Neither End-clamp nor Post-Clamp different from the corresponding Pre-Clamp, 2) End-Clamp different, 3) Post-Clamp different. Signal locations for each protein in normal tubules (Supplemental Figure 2) and types of changes seen in the End-Clamp and Post-Clamp samples for each parameter assessed as part of this analysis were:
1) Phosphotyrosine – This signal was basally located in most tubules, but was also seen in apical membranes of S3 segments of the proximal tubule. It became more heterogeneous in affected injury samples with loss of signal in some tubules, but intensification in others.
2) β1 integrin – Fluorescence was seen in the glomerular capillaries and basally in all tubule segments in pre-clamp samples. The abnormality seen was decreased signal intensity and was seen in only one of the Post-Clamp samples.
3) ICAM-1 – Fluorescence was seen in the glomeruli and peritubular capillaries in pre-clamp samples. The change in the End-Clamp and Post-Clamp samples was an increase in extent and intensity.
4) Actin – Fluorescence was intense in the apical brush border membrane of proximal tubules and was also present basally in proximal tubules and both basally and apically in distal segments of pre-clamp samples. The main changes in the end-clamp and post-clamp were in the proximal tubule brush border membrane as described above.

Statistical Analyses
For the statistical analyses, underlying data distributions were determined using the Shapiro-Wilk test. Parametric tests (t-test, paired t-test, ANOVA) were used for normally distributed data, non-parametric tests (Wilcoxon Rank Sum, Wilcoxon Signed Rank, Kruskal-Wallis) were used for data with skewed distributions, and categorical data were analyzed using a chi-square test.
To study the relationship of biomarkers to recovery time, vertical scatter plots were used to display the data at various time points post-surgery. Median values were plotted and differences at each time point from preoperative values were determined. Functional biomarkers (creatinine and serum cystatin C) ratios (24 hour to pre-operative) were plotted against ischemia time. To correlate urine and serum

biomarkers with functional changes and ischemia time, the ratios of biomarker peak values to pre-operative values were calculated. These data were plotted with either the creatinine ratio (peak to pre-operative values) or ischemia time on XY graphs. Peak ratios of structural biomarkers were also plotted against peak creatinine ratio. Linear regression analyses were used to fit lines to the data and an F statistic was calculated to determine if the slope of the regression line was significantly different from zero. Power analysis indicated that with a sample size of 40, we have 80% power to detect a moderate correlation of ± 0.43 or greater as compared to the null hypothesis of no correlation (r=0.0; two-sided test, alpha=0.05) and to detect a slope of ± 0.41 or greater as compared to the null hypothesis of slope=0 (two-sided test, alpha=0.05, assume standard deviation for both x and y=1).
To determine differences in morphology scores between the three sampling times, a paired analysis was done comparing pre- to end-clamp, pre- to post-clamp, and end- to post-clamp. A paired t-test was used to test for differences and a Bonferroni correction was used to adjust the p-values for multiple testing. Statistical analyses were performed using SAS ver. 9.2 (SAS Institute, Cary, NC) and GraphPad Prism 5 (GraphPad Software, La Jolla, CA). All tests were two-sided with significance set at $p<0.05$.

Reference List

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3. Chaturvedi S, Farmer T, Kapke GF: Assay validation for KIM-1: human urinary renal dysfunction biomarker. *Int J Biol Sci* 5:128-134, 2009

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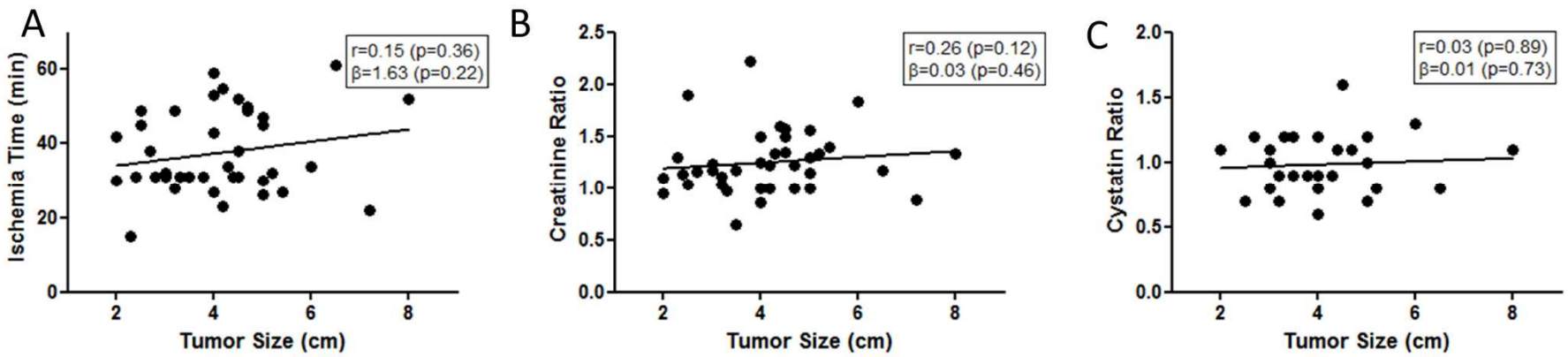
5. Hirsch R, Dent C, Pfriem H, Allen J, Beekman RH, III, Ma Q, Dastrala S, Bennett M, Mitsnefes M, Devarajan P: NGAL is an early predictive biomarker of contrast-induced nephropathy in children. *Pediatr Nephrol* 22:2089-2095, 2007

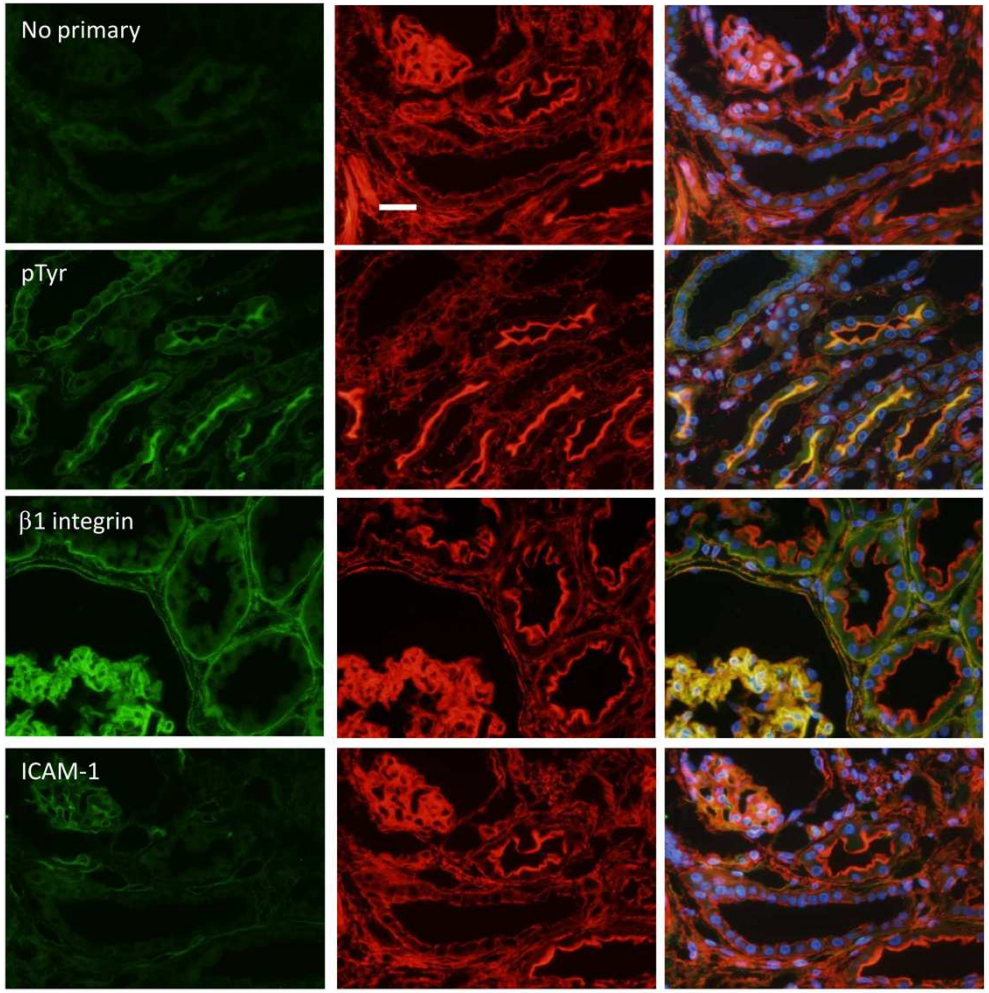
Supplemental Table 1. "One Kidney" AKIN Categorization[a]

Total number of evaluable patients	**38**	
	24 hrs	**≥48 hrs**
Stage 1, >1.25x or 0.15	***19***	**8**
Stage 2, >1.5x	**4**	**1**
Stage 3, >2.0x	**1**	**0**
Total	**24/38 = 63%**	**9/38 = 24%**
Warm Ischemia	**16/26=62%**	**7/26=27%**
Stage 1	**14/16**	**4/7**
Stage 2	**1/16**	**2/7**
Stage 3	**1/16**	**0/7**
Cold ischemia	**8/12=66%**	**2/12=17%**
Stage 1	**5/8**	**2/2**
Stage 2	**3/8**	**0/2**
Stage 3	**0/8**	**0/2**

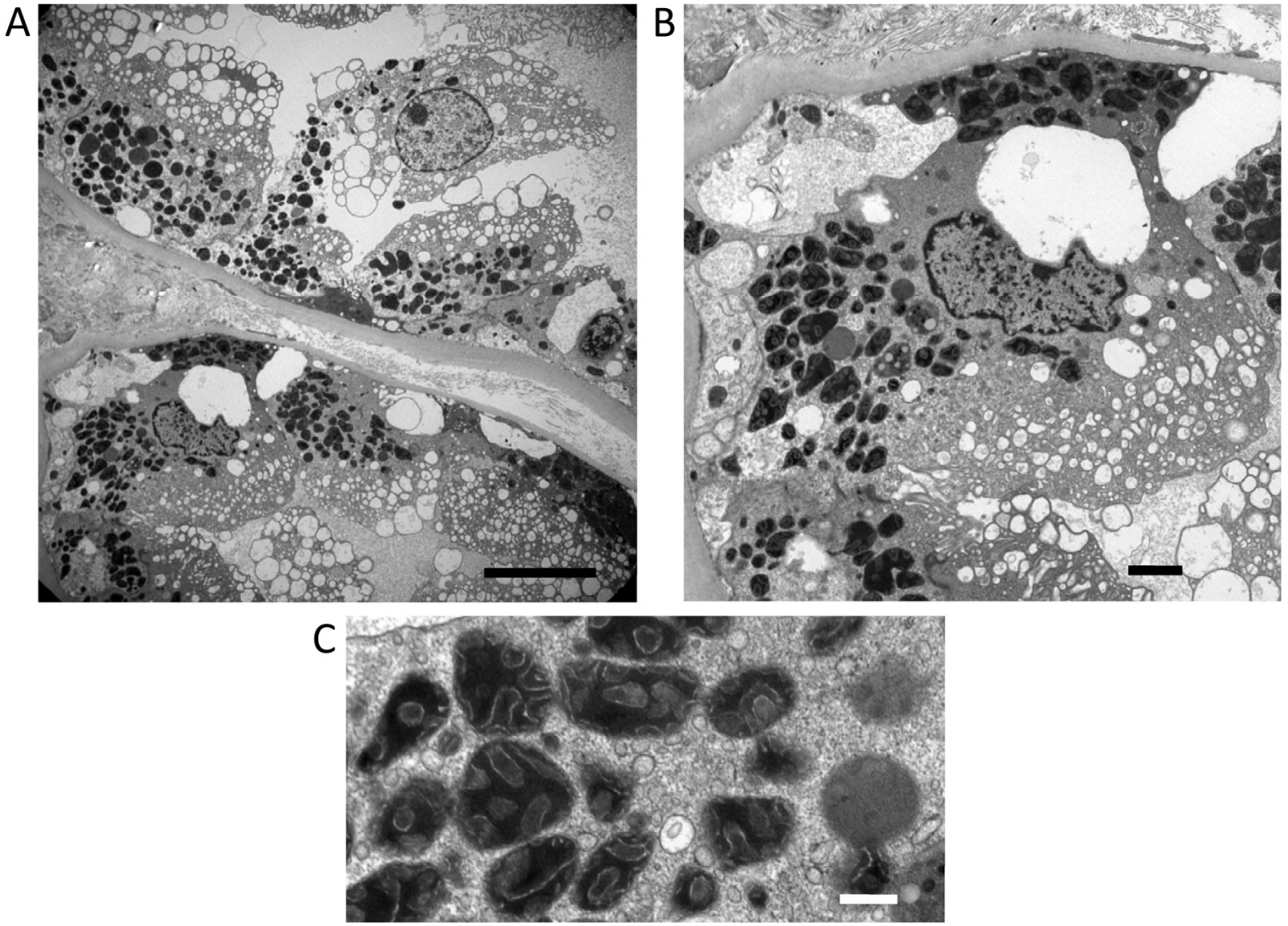
a. Creatinine critera for each stage (fold increase vs preoperative or increment) are as indicated in first column. 2 of the 40 patients did not have sufficient serum creatinine data for this analysis.



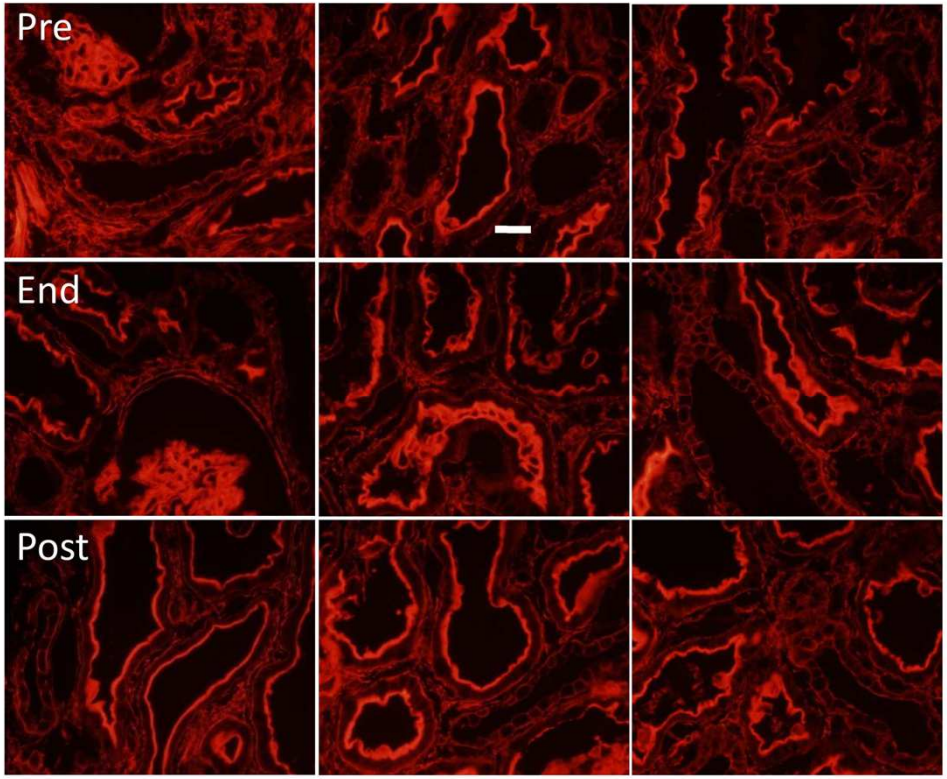
Supplemental Figure 1. Relationships between tumor size, ischemia time and clearance markers. A) tumor size vs. ischemia time, B) tumor size vs. ratio of creatinine at 24 hours to preoperative, C) tumor size vs. 24 hour cystatin C to preoperative.



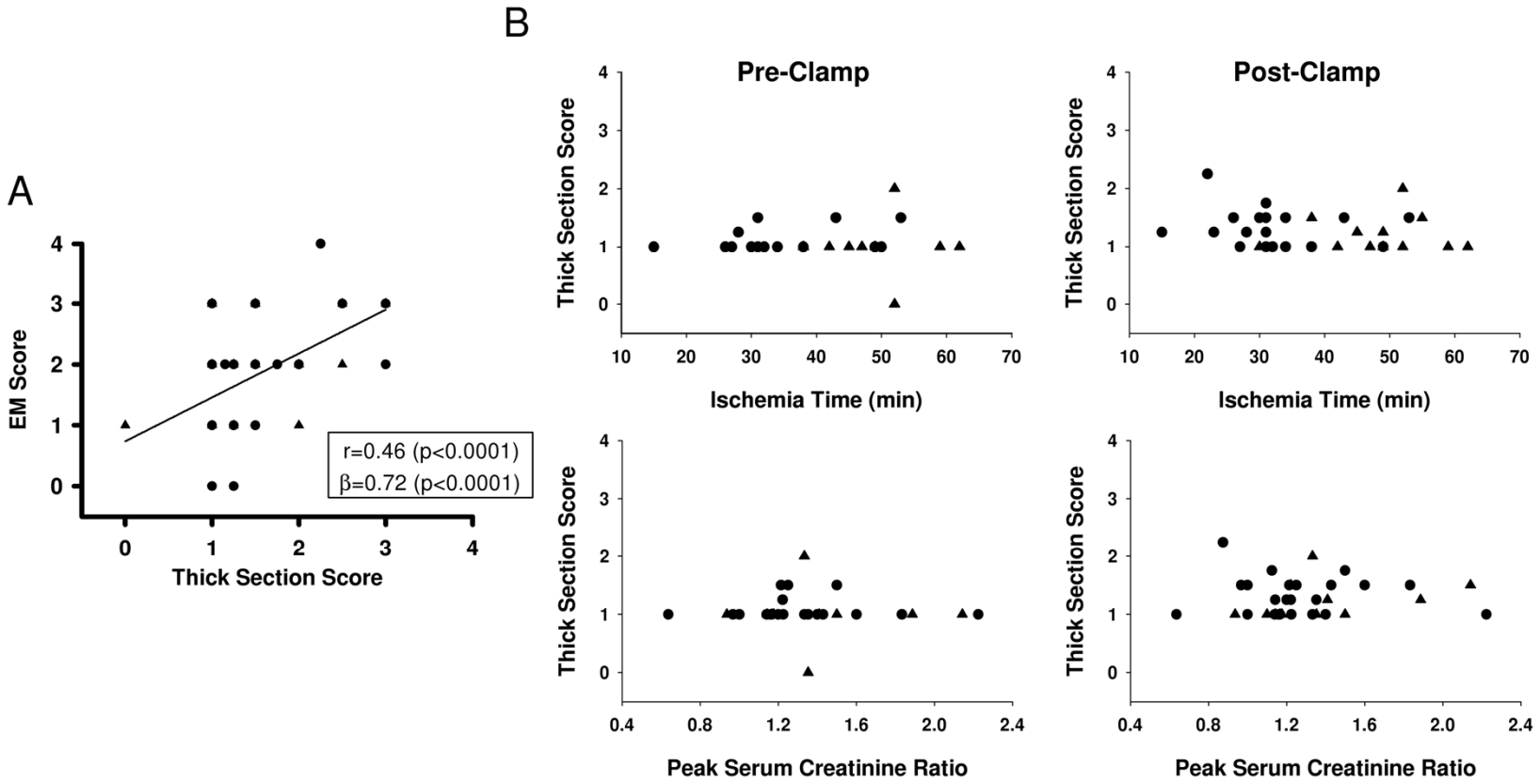
Supplemental Figure 2. Appearance in pre-clamp samples of each of the parameters assessed by immunofluorescence. Left panels show the green fluorescent signals from the primary antibody and the CF^{TM}488A-conjugated secondary, middle panels are the red rhodamine phalloidin signals for polymerized actin, right panels are an overlay that also shows the DAPI-stained nuclei. Size marker 10 microns.



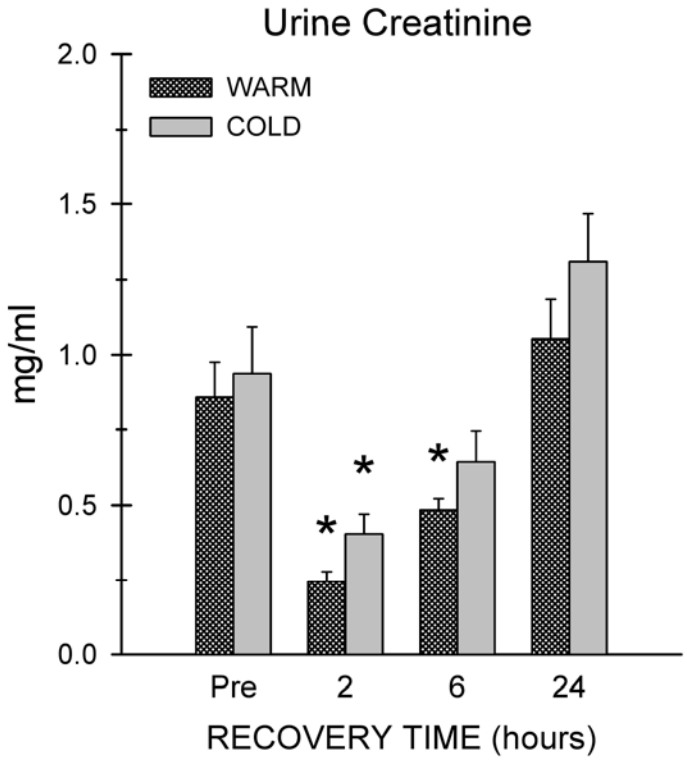
Supplemental Figure 3. Ultrastructure showing severe mitochondrial condensation during reflow. The changes shown here were found in small areas of 2 cases. The images are three magnifications from the same region. Size markers are: A) 10 microns, B) 2 microns, C) 0.5 microns.



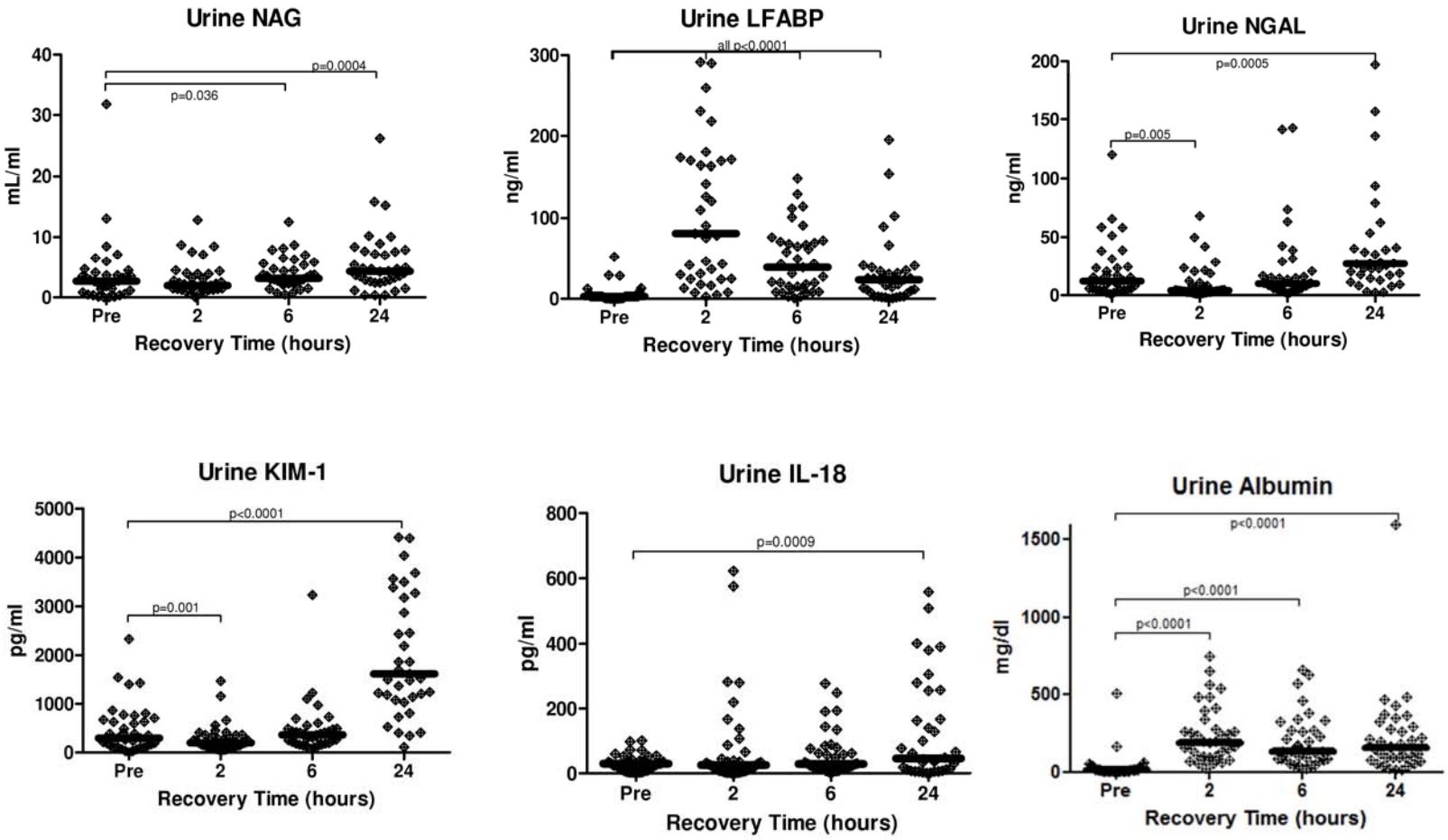
Supplemental Figure 4. Additional examples of rhodamine phalloidin staining. Sets of three images from the pre-clamp, end-clamp, and post-clamp samples from one case. Size marker is 10 microns.



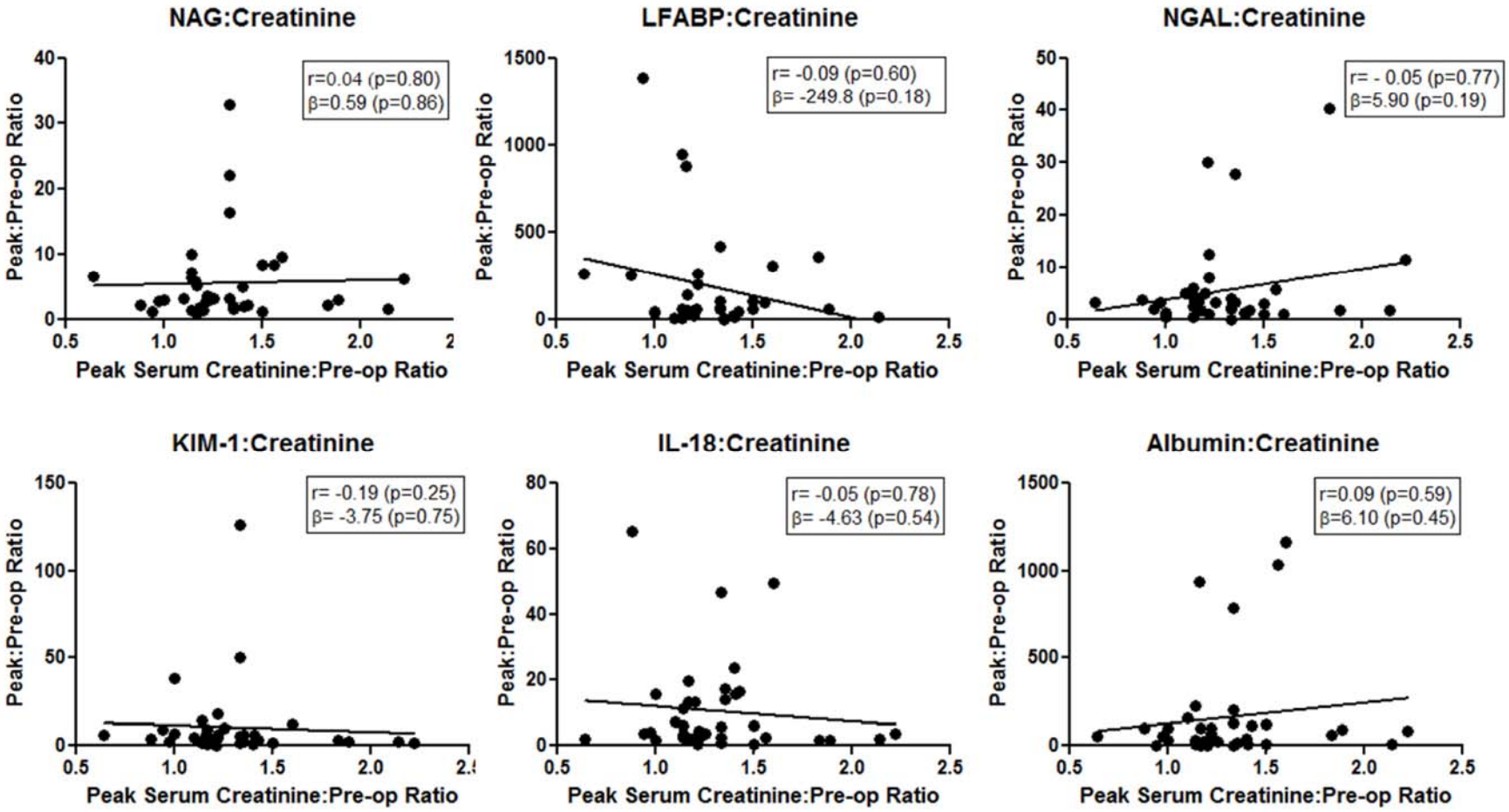
Supplemental Figure 5. Relationships between thick section scores, EM scores, ischemia time, and creatinine changes. A) Correlation between thick section and ultrastructure (EM) scoring including all points sampled. Overlapping points are not separated. 95% confidence intervals were 0.29 to 0.61 for r and 0.47 to 0.97 for β. B) Relationships between ischemia time and ratio of peak serum creatinine to preoperative versus the pre-clamp and post-clamp thick section scores. Circles are warm ischemia. Triangles are cold ischemia.



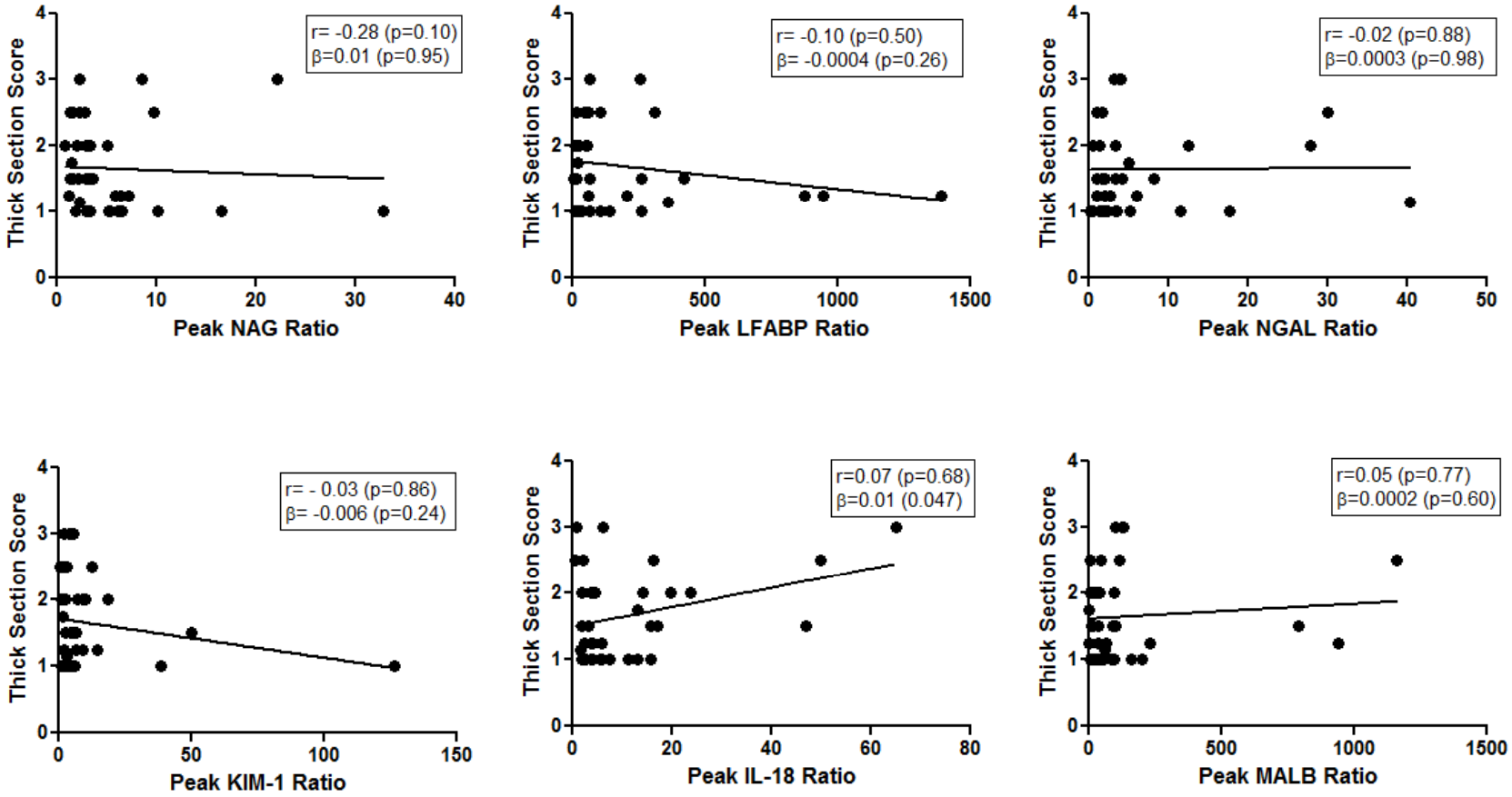
Supplemental Figure 6. Urine creatinine concentrations preoperatively and at 2, 6, and 24 hours of recovery. Values are means±SEM. *P < 0.05 vs. preoperative (Pre) value.



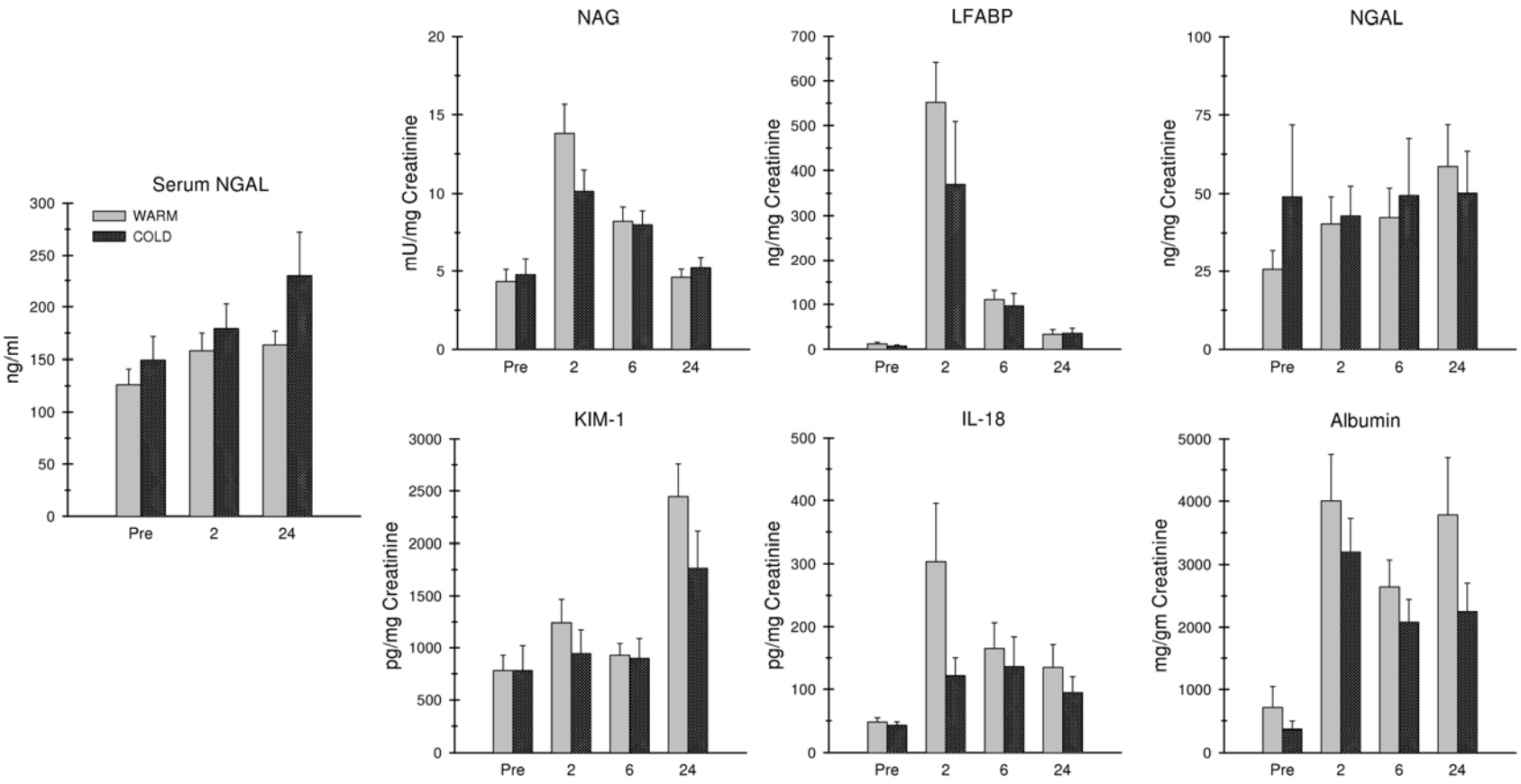
Supplemental Figure 7. Urine biomarkers as absolute concentrations not factored for creatinine. Bars indicate medians. Pre, preoperative.



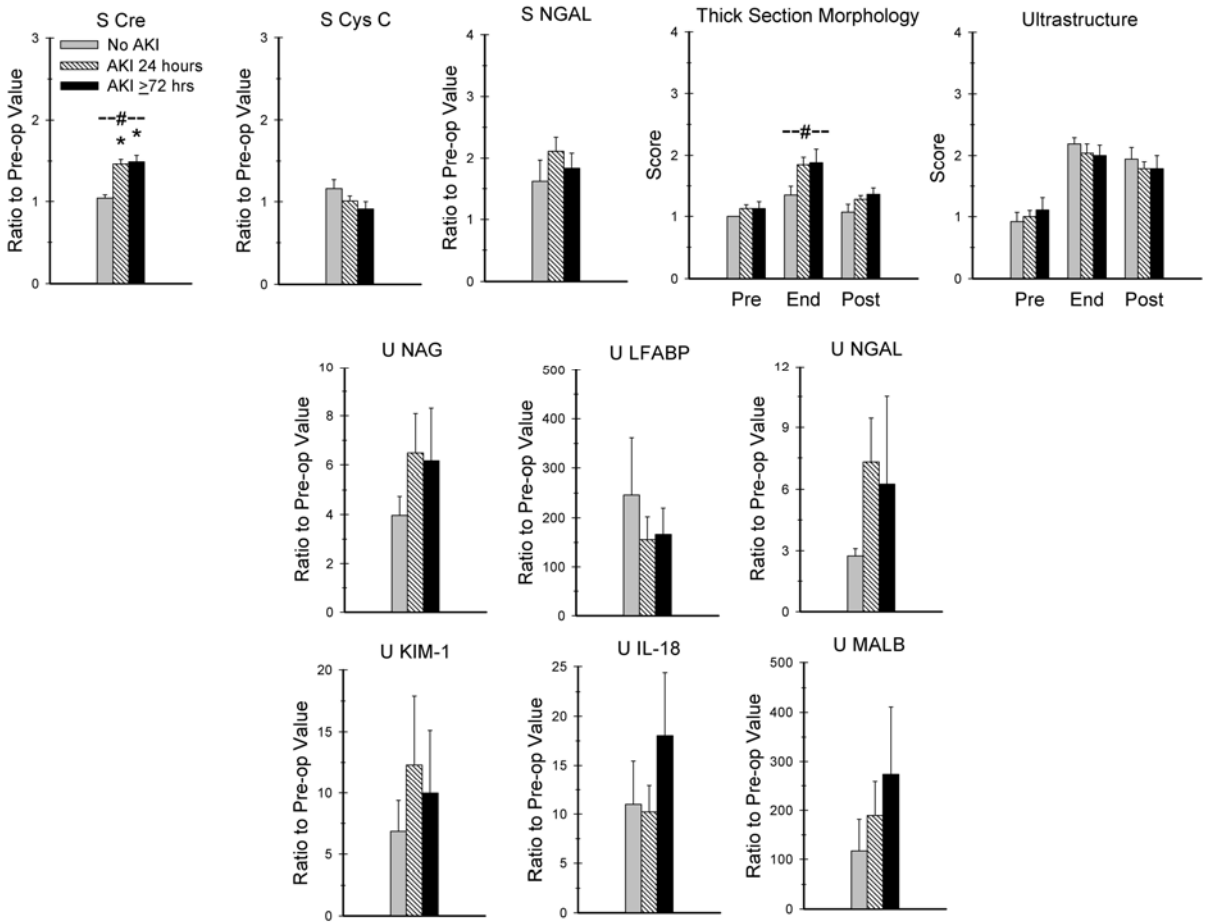
Supplemental Figure 8. Relationships between changes of serum creatinine and biomarkers. Serum creatinines and biomarker are ratios between the peak and preoperative (Pre-op) values.



Supplemental Figure 9. Relationships between creatinine-factored urine biomarkers and end-clamp thick section scores. Biomarker values are the ratios of the peak to preoperative values.



Supplemental Figure 10. Serum NGAL and urine biomarkers categorized according to whether patients were subjected to warm or cold ischemia during the clamping period. Values are means±SEM. None of the cold vs. warm differences were statistically significant.



Supplemental Figure 11. Behavior of biomarkers and structural parameters relative to 'modified AKIN' categorization. The 'modified AKIN' categorization is detailed in Appendix Table 1. Values are means±SEM for each parameter sorted according to whether patients had no AKI or met the 'modified AKIN' criteria at 24 hours or at ≥96 hours. S – serum, U – urine. Other abbreviations are as in the text. Scores for thick section morphology and ultrastructure are as defined in Methods. The other values are all ratios of the peak value measured to preoperative (Pre-op). --#-- indicates overall significant differences among the conditions ($P<0.001$ for creatinine and $P=0.013$ for thick section morphology). * indicates significantly different ($P< 0.05$) from the corresponding "No AKI" group.

High Resolution Main Paper Figures
for
Tolerance of the Human Kidney to Isolated Controlled Ischemia

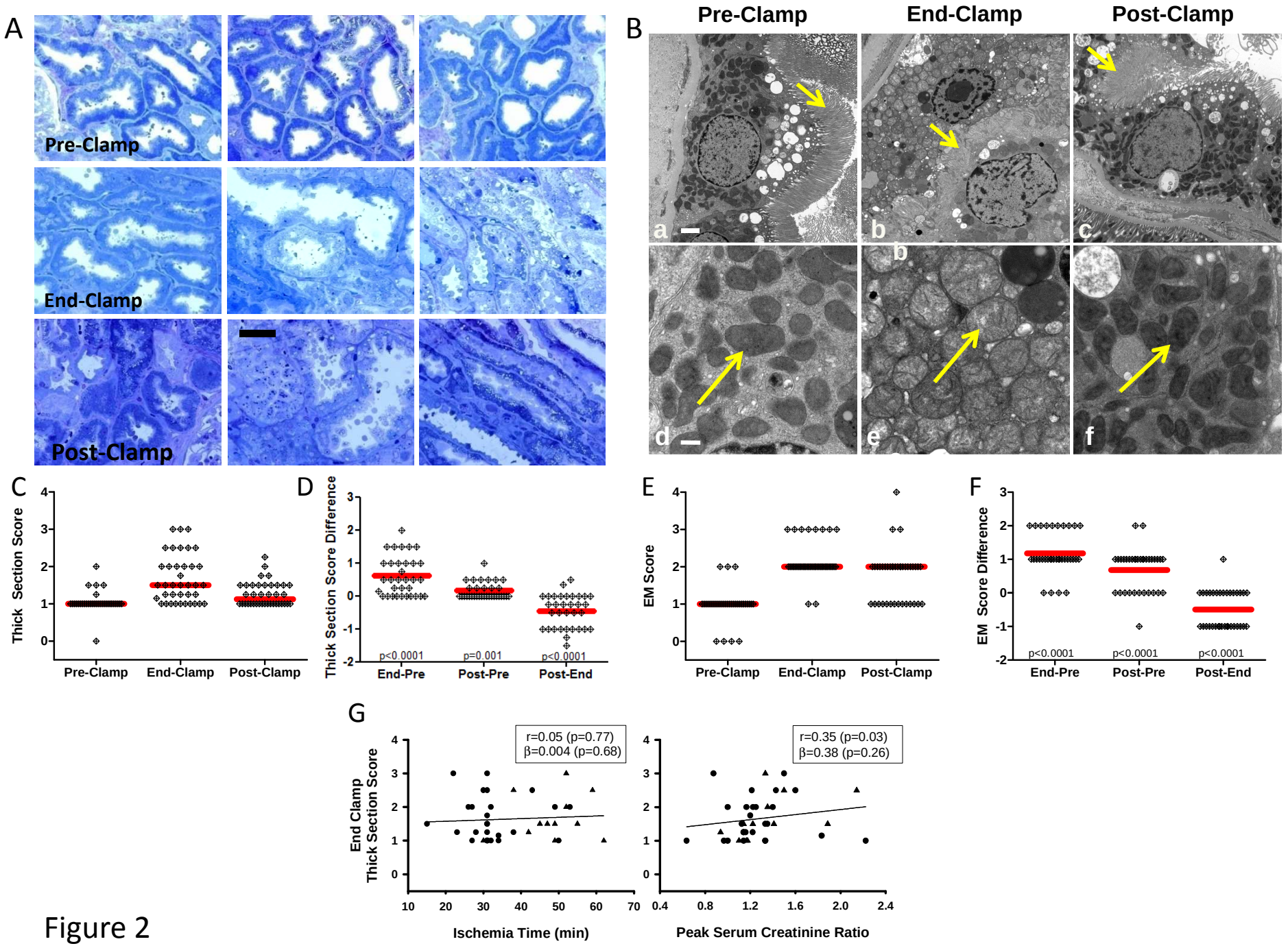
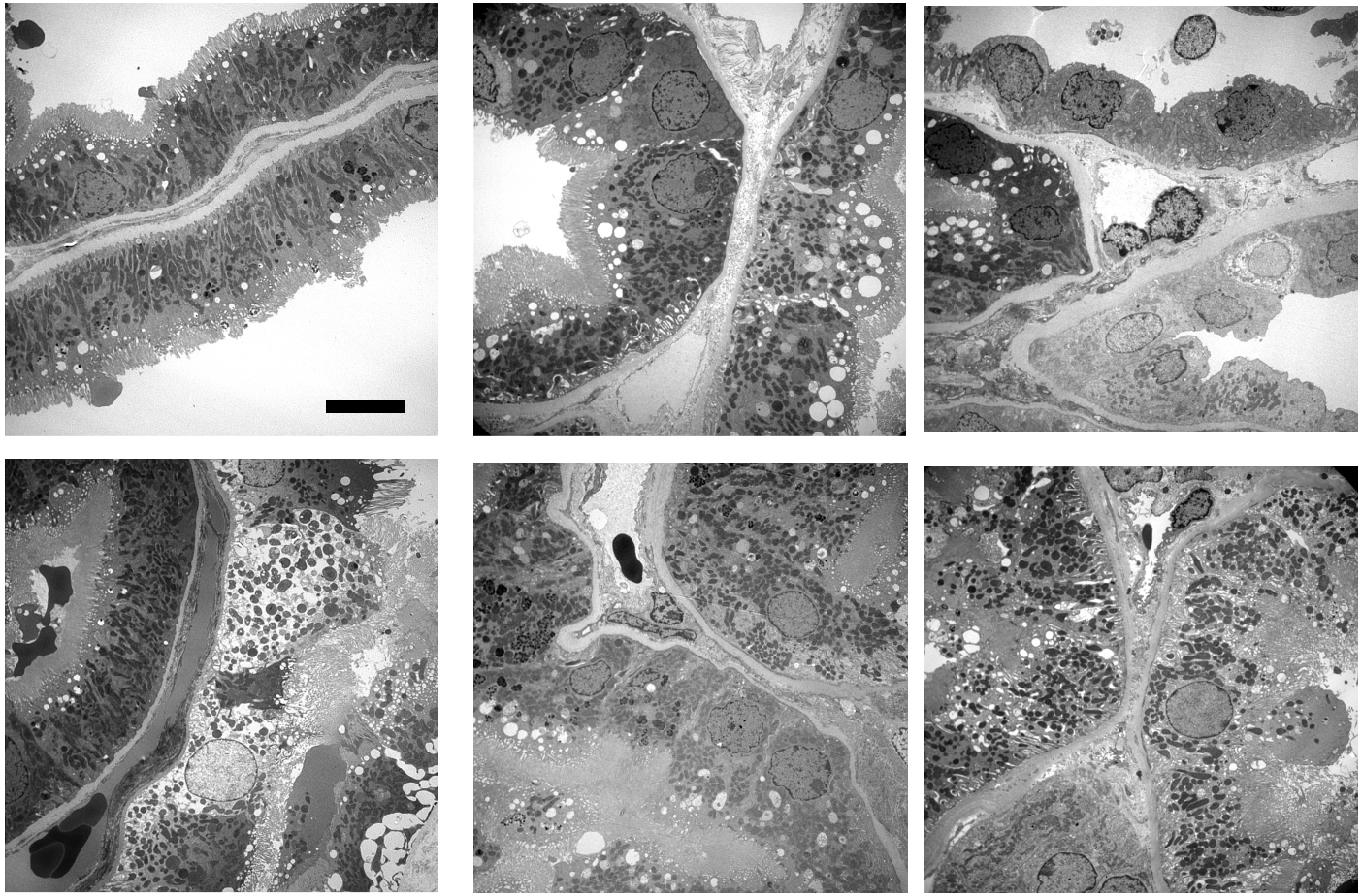
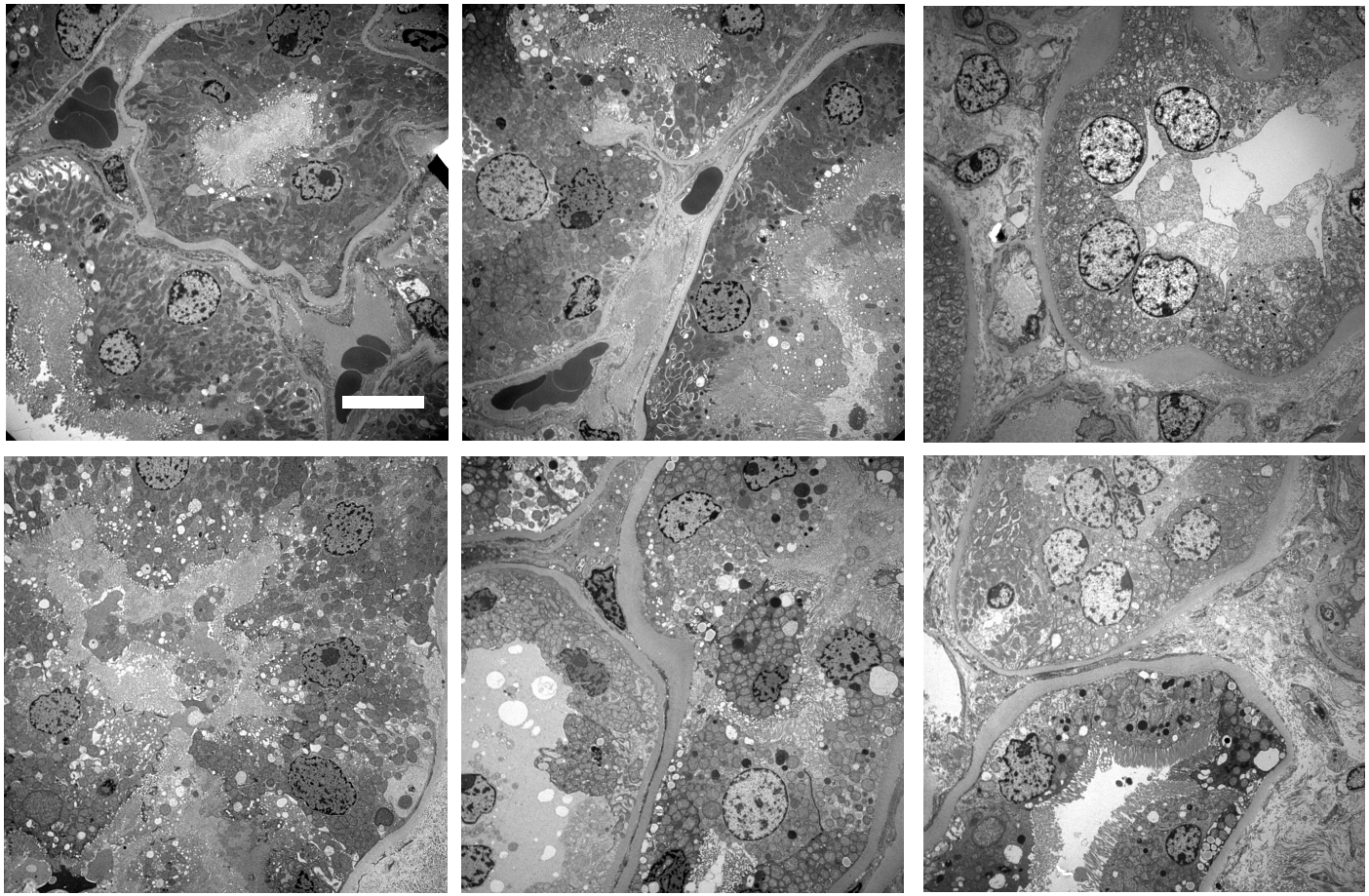

A
Pre-Clamp
End-Clamp
Post-Clamp
B
Pre-Clamp
End-Clamp
Post-Clamp
a
b
c
d
e
f
C
Thick Section Score
Pre-Clamp
End-Clamp
Post-Clamp
D
Thick Section Score Difference
p<0.0001
p=0.001
p<0.0001
End-Pre
Post-Pre
Post-End
E
EM Score
Pre-Clamp
End-Clamp
Post-Clamp
F
EM Score Difference
p<0.0001
p<0.0001
p<0.0001
End-Pre
Post-Pre
Post-End
G
End Clamp
Thick Section Score
r=0.05 (p=0.77)
β=0.004 (p=0.68)
Ischemia Time (min)
r=0.35 (p=0.03)
β=0.38 (p=0.26)
Peak Serum Creatinine Ratio

Figure 2



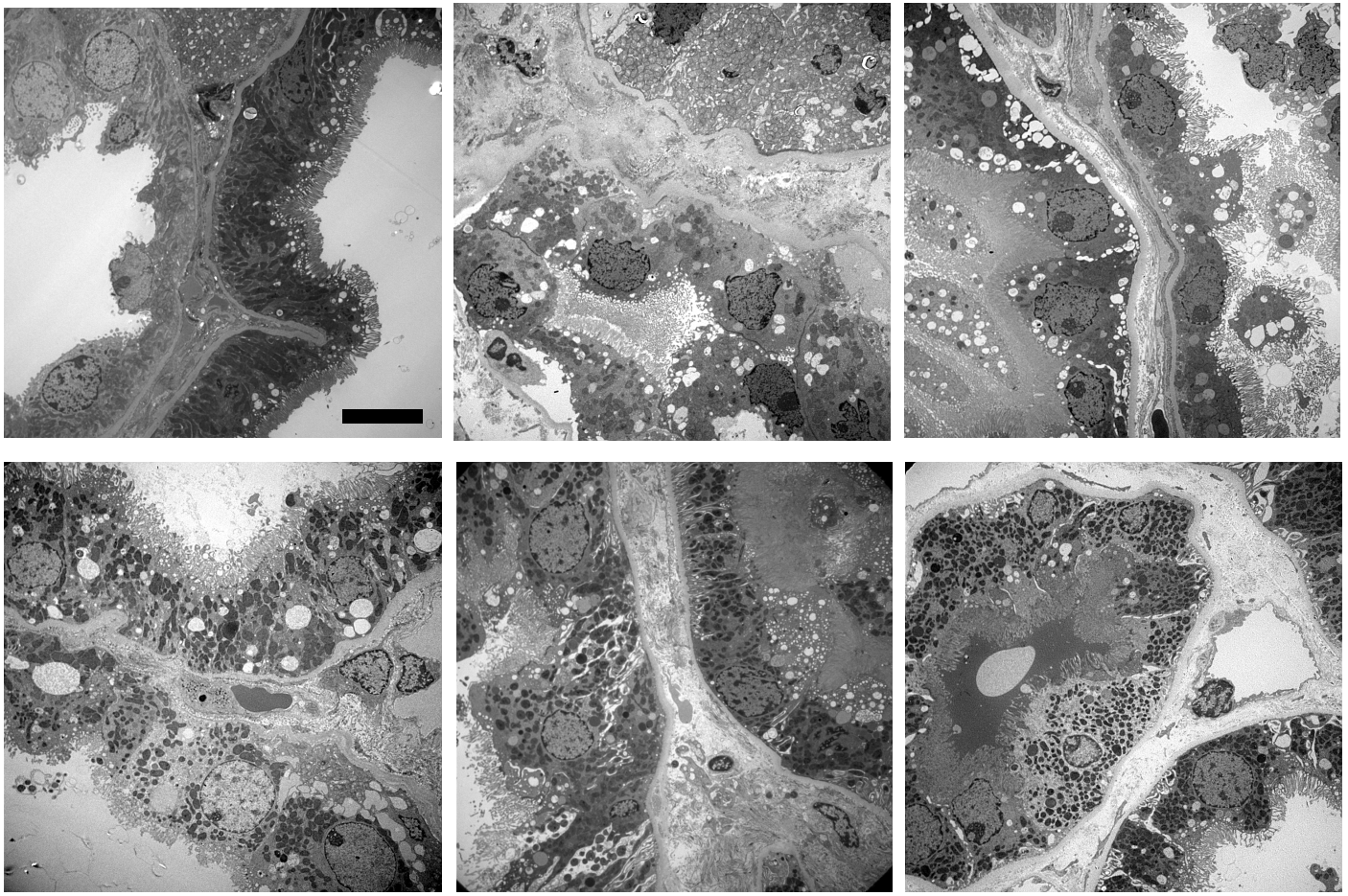
Pre-Clamp

Figure 3A



End-Clamp

Figure 3B



Post-Clamp

Figure 3C

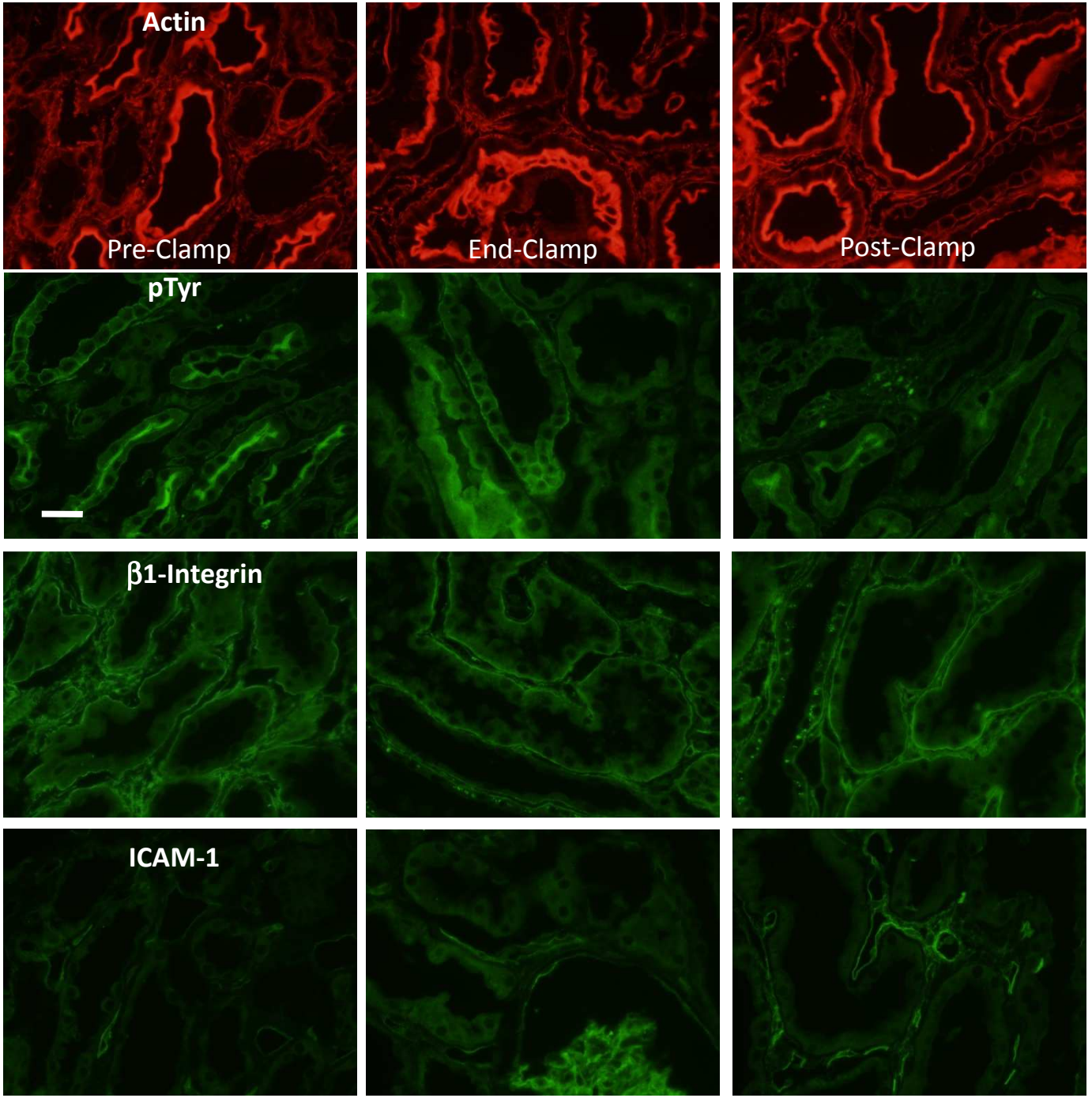


Figure 4

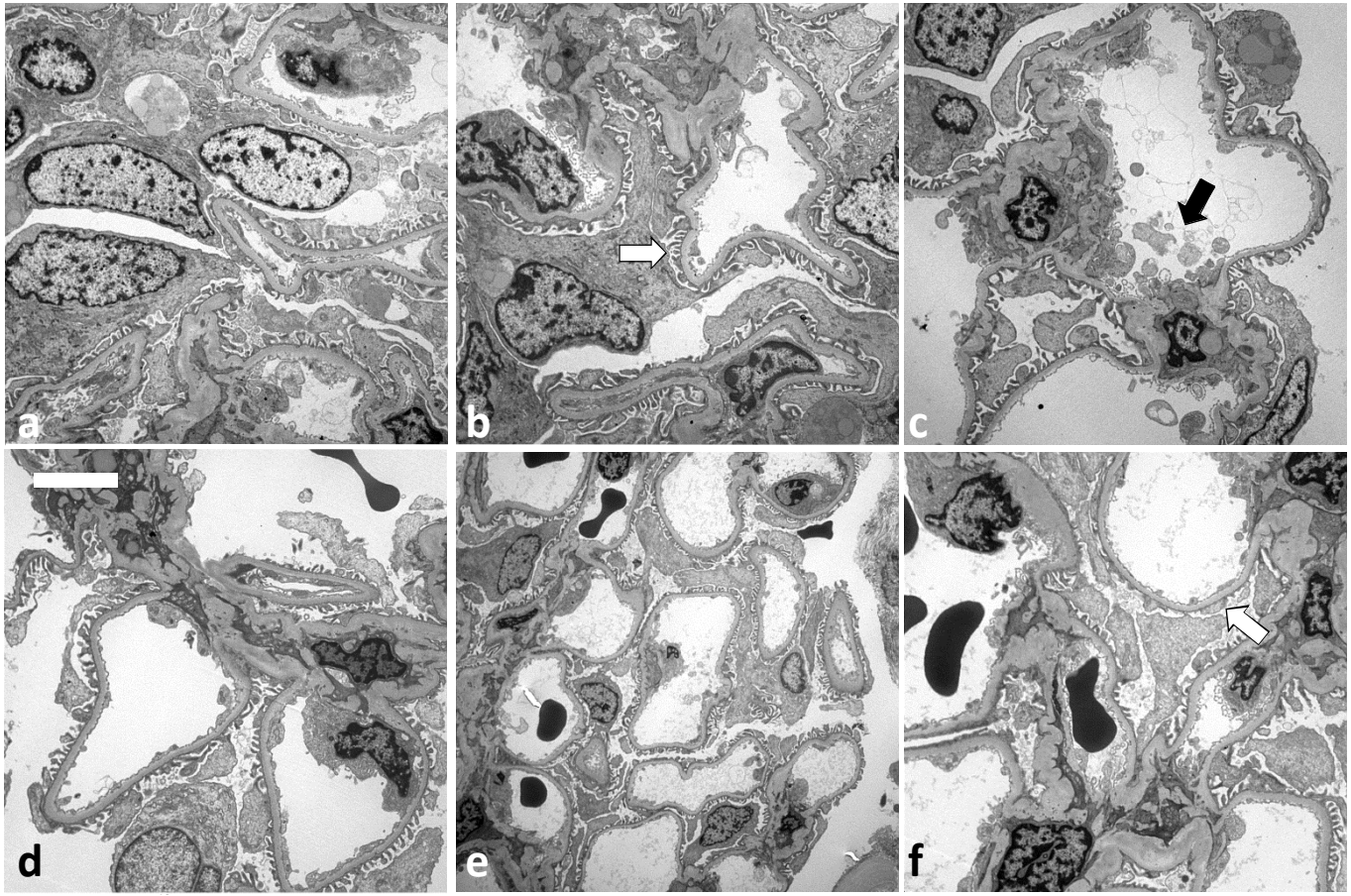


Figure 9