Online Data Supplement

Urinary glycosaminoglycans predict outcomes in septic shock and ARDS

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Supplementary Methods:

Exclusion criteria for prospective study of septic shock and trauma.

Exclusion criteria for the prospective observational cohort of septic shock/trauma patients included anuria, pregnancy, prisoners, absence of a urinary collection device (placed as part of usual care), gross hematuria, or history of a genitourinary malignancy.

Patient enrollment and sample collection.

At enrollment, 5 ml of urine was collected from subjects' urinary collection devices, centrifuged at 2000 RPM for 10 min, and frozen in aliquots. Written, informed consent was obtained from the patient. If the patient lacked decisional capacity during the 24 hour window of enrollment, urine was collected (and documented in the medical record), processed, and stored. Once the patient regained decisional capacity, written informed consent was obtained. If at that time the patient declined to participate in the trial, the stored urine was destroyed. If the patient expired prior to regaining decisional capacity, collected urine and data were kept for analysis, in accordance with Colorado Multiple Institutions Review Board (COMIRB) policies applying to minimal-risk protocols.

Definition of septic shock.

Septic shock was defined by (a) the presence of 2 or more systemic inflammatory response syndrome criteria (temperature < 36 ^{0}C or > 38 ^{0}C; heart rate > 90 beat/min; respiratory rate > 20 breaths/min or $PaCO_2$ < 32 mm Hg; white blood cell count < 4,000/µl or >12,000/µl or > 10% bands); (b) evidence of infection (chest imaging, blood/urine cultures; clinically-apparent soft tissue-infection, or other source per clinician judgement); and (c) vasopressor medications administered for > 4 hours over previous 24 hours of ICU hospitalization (despite initial >30 ml/kg intravenous crystalloid resuscitation).

Mass spectrometry.

Using human urine samples (including a subset of samples from our trauma "control" cohort), we have recently optimized the isolation, purification, and mass spectrometry characterization of urinary GAGs (E1). Briefly, urine supernatants were thawed, filtered, and washed. In the presence of a digestion buffer, urine GAGs were digested into oligosaccharides using a mixture of heparin and chondroitin lyases. Samples were freeze-dried and labeled with 2-aminoacridone (AMAC). Similarly, GAG disaccharide standards (representing 17 different structure and sulfation patterns) were labeled with AMAC. Samples and standards were then analyzed via liquid chromatography (Agilent Poroshell 120 EC-C18) and (via electrospray injection) triple quadrupole mass spectrometry (Thermo Fisher Scientific) using multiple reaction monitoring (E1).

In accordance with other studies of urinary GAGs (E2), we controlled for urinary dilution by normalizing mass spectrometry measurements of GAG content to urine creatinine (E3). Urine creatinine was measured in a blinded fashion by the Colorado Clinical and Translational Sciences Institute (CCTSI, Aurora CO) using a Beckman Coulter AU Analyzer.

Urinary heparanase activity.

We determined heparanase activity in freshly thawed urine via measurement of HS degradation activity (Genway), as previously described (E4). This activity was normalized to urine creatinine to control for urinary dilution, as described above.

Urinary bikunin assay.

Bikunin (Mochida Pharmaceuticals) was prepared at 0.1 mg/ml in saline, serially diluted, and 2 μl aliquots were spotted onto a nitrocellulose membrane. Urine samples (2 μl) were similarly spotted and the membrane was dried, blocked with 5% bovine serum albumin (BSA) in tris-buffered saline containing 0.05% Tween20 (TBS-T) for 1 hour, incubated with rabbit anti-bikunin

(Abcam) diluted in 5% BSA in TBS-T at a dilution of 1:2000 for 30 minutes, and washed three times with TBS-T. Blots were then incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology) at a dilution of 1:5000 for 30 minutes, washed three-times with TBS-T, detected with a chromogenic horseradish peroxidase substrate (Pierce 1-Step Ultra TMB Blotting Solution) and color was quantified using ImageJ software.

DMMB assay.

We used the dimethylmethylene blue (DMMB) colorimetric assay to measure urine sulfated GAG concentrations, as previously described (E1). Briefly, DMMB reagent (0.04 mM) containing glycine, sodium chloride, and acetic acid was used to prepare a standard curve using serial dilutions of a 3-mg/ml CS stock solution. Standard and samples (8 µl each) were transferred to individual wells of a 96-well plate, and DMMB reagent (200 µl) was added to each well. The absorbance at 525 nm was immediately measured, and the concentrations of sulfated GAGs were determined by comparison to the standard curve.

We tested the DMMB assay at pH values of 3.0, 4.0 and 5.0. The color reaction was the most sensitive at pH 4.0. At pH 4.0 and with a standard range from 0-5 µg/mL, test linearity was optimal (Figure E5a in the online supplement). The recovery of chondroitin sulfate at pH 3.0, 4.0 and 5.0 was determined to be 119%, 105%, and 92%, respectively.

We also tested the effect of DNA (100 ng/µl), protein (100 ng/µl), and urine matrix (the solution of urine through 3 kDa spin column) on the DMMB reaction. Proteins or matrix did not affect the results of DMMB assay. At pH 3.0, DNA was not detected by DMMB, but as the pH increased to 4.0 and 5.0, it is detected with increasing sensitivity. Based on these observations, we selected pH 4.0 as this afforded the optimal detection of chondroitin sulfate with minimal interference by DNA. While we cannot fully exclude a confounding effect of urine cell-free DNA in our patient samples, our DMMB assay is relatively insensitive to levels described in the

plasma of severe sepsis patients (< 6 ng/µl, reference E5). The presence and significance of urine cell-free DNA in septic shock or ARDS is unknown.

As noted in Figure E5b, we additionally examined the DMMB assay's ability to detect different GAGs: CS, HS, and heparin (a highly-sulfated HS). At the same concentration of each GAG, the color for (oversulfated) heparin was the darkest, followed by HS and CS. As described above, we selected CS as the standard in the current study. Thus, if the sample contained more highly-sulfated GAGs (e.g. HS) than CS, the result of the DMMB assay would potentially skew higher than the actual concentration of GAGs present in the sample. As such, the absolute values determined by DMMB may be higher than the results of mass spectrometry (Fig 5a).

Statistical analyses

Correlative data are presented as scatter plots and analyzed using Pearson's correlation. Mean concentrations of urinary GAGs and HS degradation activity are presented with standard error of the mean. Normally-distributed bivariate analyses were performed using t-tests; non-normally distributed data were logarithmically transformed. Multiple comparisons were performed via ANOVA, with Bonferroni post-hoc testing. Receiver-operating characteristic (ROC) analysis was performed to measure predictive validity of data. Areas under the receiver operating characteristic curves (AUCs) were constructed and used to evaluate predictive effect of individual GAGs for the acute kidney dysfunction and hospital mortality outcomes. To control for severity of illness, multivariate regression analyses were performed after adjusting for APACHE-II scores with either new onset renal dysfunction or hospital mortality as outcomes variables. Kaplan-Meier survival curves were analyzed via log-rank (Mantel-Cox) testing.

Analyses were performed using Prism (Graphpad, La Jolla, CA); with the exception of multivariate regression modeling (JMP v11, SAS, Cary, NC).

Supplementary References:

E1. Sun X, Li L, Overdier KH, Ammons LA, Douglas IS, Burlew CC, Zhang F, Schmidt EP, Chi L, Linhardt RJ. Analysis of Total Human Urinary Glycosaminoglycan Disaccharides by Liquid Chromatography–Tandem Mass Spectrometry. *Anal Chem* 2015; 87: 6220-6227.

E2. Zarbock A, Meersch M, Van Aken H, Görlich D, Singbartl K. Urinary Hyaluronic Acid as an Early Predictor of Acute Kidney Injury After Cardiac Surgery. *J Am Coll of Cardiol* 2014; 64: 737-738.

E3. Ginsberg JM, Chang BS, Matarese RA, Garella S. Use of Single Voided Urine Samples to Estimate Quantitative Proteinuria. *N Engl J Med* 1983; 309: 1543-1546.

E4. Schmidt EP, Yang Y, Janssen WJ, Gandjeva A, Perez MJ, Barthel L, Zemans RL, Bowman JC, Koyanagi DE, Yunt ZX, Smith LP, Cheng SS, Overdier KH, Thompson KR, Geraci MW, Douglas IS, Pearse DB, Tuder RM. The pulmonary endothelial glycocalyx regulates neutrophil adhesion and lung injury during experimental sepsis. *Nat Med* 2012; 18: 1217-1223.

E5. Dwivedi DJ, Toltl LJ, Swystun LL, Pogue J, Liaw KL, Weitz JI, Cook DJ, Fox-Robichaud AE, Liaw PC. Prognostic utility and characterization of cell-free DNA in patients with severe sepsis. *Crit Care* 2012; 16: R151.

Supplementary Data:

Figure E1. Urine heparan sulfate degradation activity in septic shock and major trauma. **(a)** Urine heparan sulfate (HS) degradation activity was significantly elevated in patients with septic shock as compared to surgical ICU patients with major trauma. **(b)** Urine HS degradation activity was highly associated with urine HS fragmentation. **(c)** Similar to urine HS (Fig 1b of the main manuscript), urine HS degradation activity did not correlate with severity of illness, as measured by APACHE II. * $p < 0.05$

Figure E2. Survival analyses of urine heparan sulfate (a) and hyaluronic acid (b) in septic shock. Test cut-offs chosen as described in Supplementary Tables E3 and E6. Testing performed via Log-rank.

Figure E3. **Urine HS/CS disaccharide sulfation and AKI**. In urine samples collected from ARDS patients at 0, 1, and 3 days after study enrollment, mass spectrometry revealed distinct patterns of urine HS **(a,c,e)** and CS **(b,d,f)** disaccharide sulfation in patients who later developed acute kidney injury (AKI) as compared to those who did not develop AKI. * $p < 0.05$

Figure E4. **Urine HS/CS disaccharide sulfation and hospital mortality**. In urine samples collected from ARDS patients at 0, 1, and 3 days after study enrollment, mass spectrometry revealed distinct patterns of urine HS **(a,c,e)** and CS **(b,d,f)** disaccharide sulfation in patients who later died as compared to those who survived their hospitalization. * $p < 0.05$

Figure E5. DMMB test optimization. **a.** Test validation assays revealed maximum sensitivity and linearity for CS at pH of 4.0. **b.** Highly-sulfated GAGs, such as heparin (HP, a highly-sulfated HS) react more densely with the DMMB assay. As such, when measuring a mixtures of sulfation-enriched GAGs, DMMB measurements of total GAGs may produce higher results than mass spectrometry (as demonstrated in Figure 5a of the main manuscript).

	Septic Shock	Trauma	*p*-value
Number of patients	30	25	
Age, years (mean, SD)	57.0 ± 11.0	42.2 ± 18.7	0.0008
Gender (male)	57%	76%	*NS*
Ethnicity (n, %)			
Hispanic/Latino	12 (40.0%)	8 (32.0%)	*NS*
Black/African American	3 (10.0%)	3 (12.0%)	*NS*
Native American	0 (0%)	1 (4.0%)	*NS*
Asian/Pacific Islander	1 (3.3%)	1 (4.0%)	*NS*
White	26 (86.7%)	20 (80%)	*NS*
BMI (mean, SD)	26.8 ± 9.4	25.0 ± 4.4	*NS*
Infection site (n, %)			
Pulmonary	18 (60%)	n/a	
Extrapulmonary	17 (57%)		
Use of therapeutic heparin (unfractionated, low molecular weight, fondaparinux)	1 (3.3%)	0 (0%)	*NS*
ARDS at study enrollment (n, %)	6 (20%)	n/a	
New ARDS within 72 h of urine collection (n, %)	2 (6.7%)	n/a	
Injury severity scale (mean, SD)	n/a	25.0 ± 10.2	
APACHE-II score (mean, SD)	30.2 ± 11.2	14.0 ± 7.8	2.77×10^{-8}
Serum creatinine at study enrollment, mg/dl (median, IQR)	1.70 (1.20-2.66)	0.82 (0.73-0.99)	0.0001
AKI onset within 72 h of urine collection (n, %)	14 (46.7%)	1 (4.0%)	0.002
Hospital deaths (n, %)	17 (56.7%)	2 (8.0%)	0.0002

Table E1: Baseline characteristics of prospectively-enrolled MICU/SICU patients

	ROC *p*-value	ROC AUC
Heparan sulfate degradation activity (U/mg creatinine)		
	0.01	0.7768
Heparan sulfate (ng/mg creatinine)		
TriS	*NS*	
NS6S	*NS*	
NS2S	0.018	0.7545
NS	0.014	0.7634
2S6S	*NS*	
6S	0.018	0.7545
2S	0.004	0.8125
0S	0.042	0.7188
Total (unadjusted)	0.014	0.7634
Total (adjusted for APACHE-II)	0.02	0.8036
Chondroitin sulfate (ng/mg creatinine)		
TriS	*NS*	
2S4S	*NS*	
4S6S	*NS*	
2S6S	*NS*	
4S	0.001	0.8438
2S	*NS*	
6S	*NS*	
0S	*NS*	
Total (unadjusted)	0.018	0.7545
Total (adjusted for APACHE-II)	0.022	0.7417
Hyaluronic acid (ng/mg creatinine)		
Total (unadjusted)	0.018	0.7545
Total (adjusted for APACHE-II)	0.01	0.7723
Total GAGs (ng/mg creatinine)		
Total (unadjusted)	0.003	0.8170
Total (adjusted for APACHE-II)	0.003	0.8213

Table E2: Urinary predictors of worsening renal function (new AKIN >2 within 72h of enrollment) in patients with septic shock.

Septic Shock Cohort	Cutoff	Sensitivity (%)	Specificity (%)	(+) Likelihood Ratio
Heparan sulfate				
Renal dysfunction	>695.2 ng/mg Cr	92.86	37.5	1.486
	>1503 ng/mg Cr	71.3	75	2.852
	>2608 ng/mg Cr	46.15	93.75	7.385
Mortality	>868.5 ng/mg Cr	94.12	76.92	4.078
	>868.5 ng/mg Cr	94.12*	76.92*	4.078*
	>2008 ng/mg Cr	47.15	92.31	6.118
Chondroitin sulfate				
Renal dysfunction	>9173 ng/mg Cr	85.71	56.25	1.959
	>11328 ng/mg Cr	71.43	75	2.857
	>20605 ng/mg Cr	28.57	93.75	4.571
Mortality	*NS*			
	NS			
	NS			
Hyaluronic acid				
Renal dysfunction	>858.7 ng/mg Cr	92.86	31.25	1.351
	>1283 ng/mg Cr	78.57	68.75	2.514
	>2215 ng/mg Cr	42.86	93.75	6.857
Mortality	>1109 ng/mg Cr	88.24	76.92	3.824
	>1109 ng/mg Cr*	88.24*	76.92*	3.824*
	>1520 ng/mg Cr	64.71	92.31	8.412
Total glycosaminoglycans				
Renal dysfunction	>12.00 µg/mg Cr	92.86	62.5	2.476
	>15.97 µg/mg Cr	71.43	81.25	3.81
	>23.86 µg/mg Cr	42.86	93.75	6.857
Mortality	*NS*			
	NS			
	NS			
DMMB				
Renal dysfunction	>23.50 µg/ml	85.71	68.75	2.743
	>29.00 µg/ml	78.57	87.5	6.286
	>29.00 µg/ml*	78.57*	87.5*	6.286*
Mortality	>7.00 µg/ml	82.35	15.38	0.973
	>44.50 µg/ml*	64.71*	92.31*	8.412*
	>44.50 µg/ml*	64.71*	92.31*	8.412*

Table E3: Predictive Test Characteristics of Urinary Glycosaminoglycans in Patients with Septic Shock. Cutoffs chosen to favor sensitivity (top row), specificity (bottom row), or an intermediate cutoff that maximizes sensitivity and specificity (middle row). In select cases (*), these cutoffs may be identical. *NS*: area under ROC curve not statistically significant.

	ROC *p*-value	ROC AUC
Heparan sulfate degradation activity (U/mg creatinine)		
	0.008	0.7873
Heparan sulfate (ng/mg creatinine)		
TriS	*NS*	
NS6S	*NS*	
NS2S	0.0008	0.8643
NS	0.001	0.8507
2S6S	*NS*	
6S	0.0007	0.8688
2S	0.004	0.8145
0S	0.002	0.8281
Total (unadjusted)	0.0009	0.8597
Total (adjusted for APACHE-II)	0.0003	0.914
Chondroitin sulfate (ng/mg creatinine)		
TriS	*NS*	
2S4S	0.03	0.7376
4S6S	0.01	0.7692
2S6S	*NS*	
4S	*NS*	
2S	*NS*	
6S	*NS*	
0S	0.002	0.8326
Total (unadjusted)	*NS*	
Total (adjusted for APACHE-II)	*NS*	
Hyaluronic acid (ng/mg creatinine)		
Total (unadjusted)	0.0009	0.8597
Total (adjusted for APACHE-II)	*NS*	
Total GAGs (ng/mg creatinine)		
Total (unadjusted)	*NS*	
Total (adjusted for APACHE-II)	0.033	0.7911

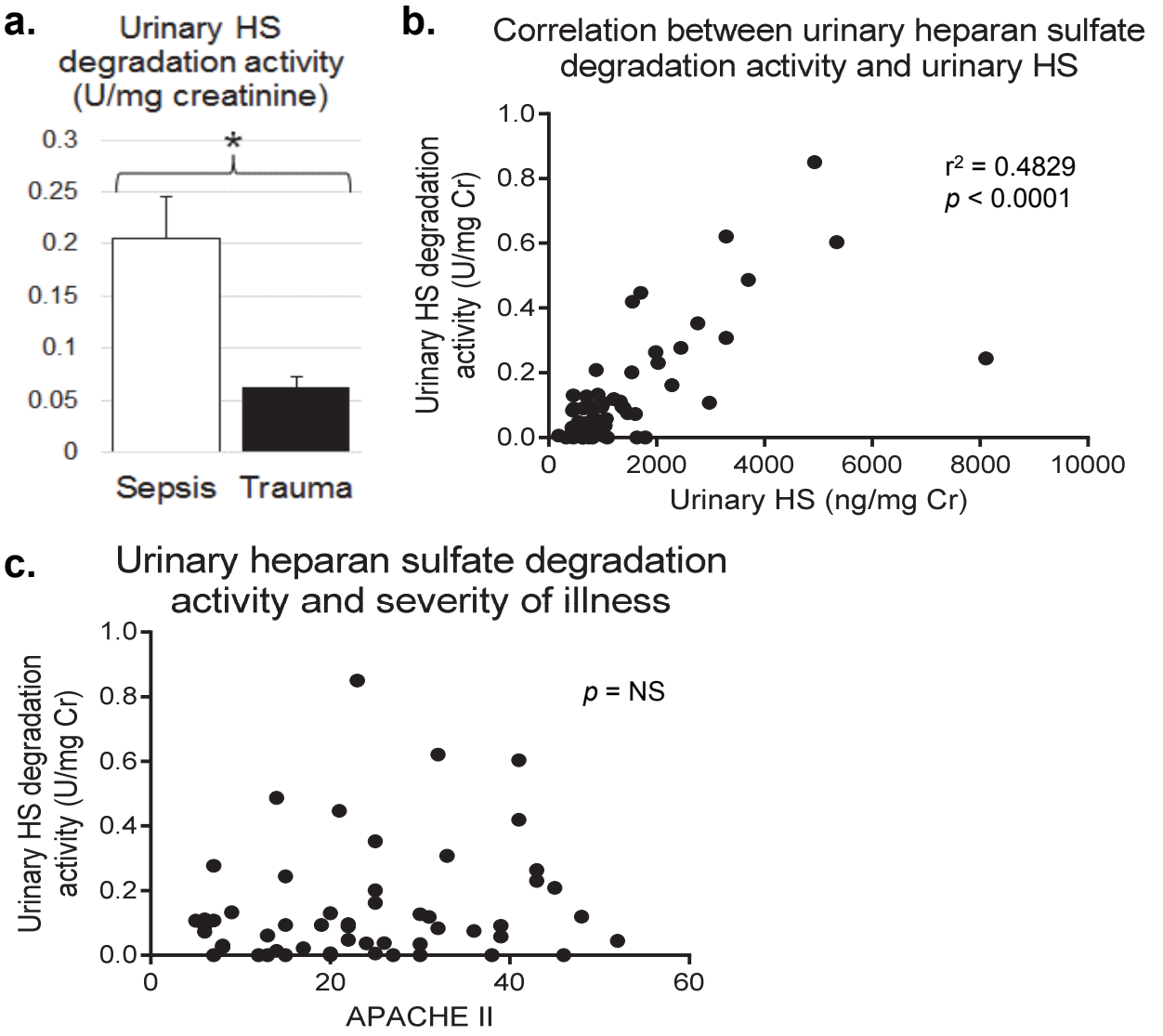
Table E4: Urinary predictors of hospital mortality in patients with septic shock.

	AKI	No AKI	*p*-value
Number of patients	22	48	
Age, years (mean, SD)	50.8 ± 6.39	50.4 ± 1.81	*NS*
Gender (male)	45%	50%	*NS*
Ethnicity (n, %)			
Hispanic/Latino	0 (0%)	4 (8.3%)	*NS*
Black/African American	5 (22.7%)	11 (22.9%)	*NS*
Native American	0 (0%)	1 (2.1%)	*NS*
Asian/Pacific Islander	1 (4.5%)	0 (%)	*NS*
White	16 (72.7%)	32 (66.7%)	*NS*
Cause of ARDS			
Direct (pneumonia)	14 (64%)	38 (62%)	*NS*
Indirect (sepsis)	8 (36%)	18 (38%)	*NS*
Time of AKI onset (days after enrollment)	3.4 ± 0.33	n/a	
Hospital deaths (n, %)	16 (72%)	14 (29%)	0.0003

Table E5: Patient Characteristics in ARDS Validation Cohort

ARDS Cohort (Day 3)	Cutoff	Sensitivity (%)	Specificity (%)	(+) Likelihood Ratio
Heparan sulfate				
Renal dysfunction	*NS*			
	NS			
	NS			
Mortality	*NS*			
	NS			
	NS			
Chondroitin sulfate				
Renal dysfunction	>16438 ng/mg Cr	88.89	52.17	1.859
	>26319 ng/mg Cr	66.67	80.43	3.407
	>28123 ng/mg Cr	61.11	82.61	3.514
Mortality	>16438 ng/mg Cr	85.19	62.16	2.251
	>17003 ng/mg Cr	81.48	64.86	2.319
	>25436 ng/mg Cr	55.56	83.78	3.426
Hyaluronic acid				
Renal dysfunction	*NS*			
	NS			
	NS			
Mortality	>127.5 ng/mg Cr	81.48	64.86	2.319
	>161.5 ng/mg Cr	74.07	72.97	2.741
	>256.5 ng/mg Cr	51.85	83.78	3.198
Total glycosaminoglycans				
Renal dysfunction	>26.54 µg/mg Cr	77.78	54.35	1.704
	>34.13 µg/mg Cr	66.67	69.57	2.190
	>34.13 µg/mg Cr	66.67	69.57	2.190
Mortality	>18.89 µg/mg Cr	88.89	37.84	1.430
	>34.13 µg/mg Cr	62.96	75.65	2.588
	>47.17 µg/mg Cr	40.74	86.49	3.015
DMMB				
Renal dysfunction	*NS*			
	NS			
	NS			
Mortality	>75.00 µg/mg Cr	85.19	45.95	1.576
	>88.50 µg/mg Cr	62.96	59.46	1.553
	>194.0 µg/mg Cr	33.33	89.19	3.083

Table E6: Predictive Test Characteristics of Urinary Glycosaminoglycans in Patients with ARDS (Day 3 samples). Cutoffs chosen to favor sensitivity (top row), specificity (bottom row), or an intermediate cutoff that maximizes sensitivity and specificity (middle row). *NS*: area under ROC curve not statistically significant.



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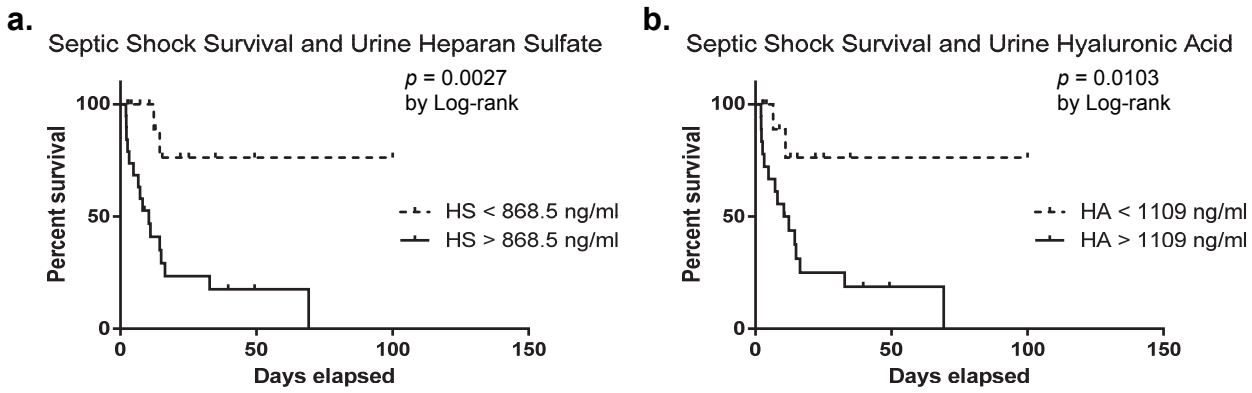

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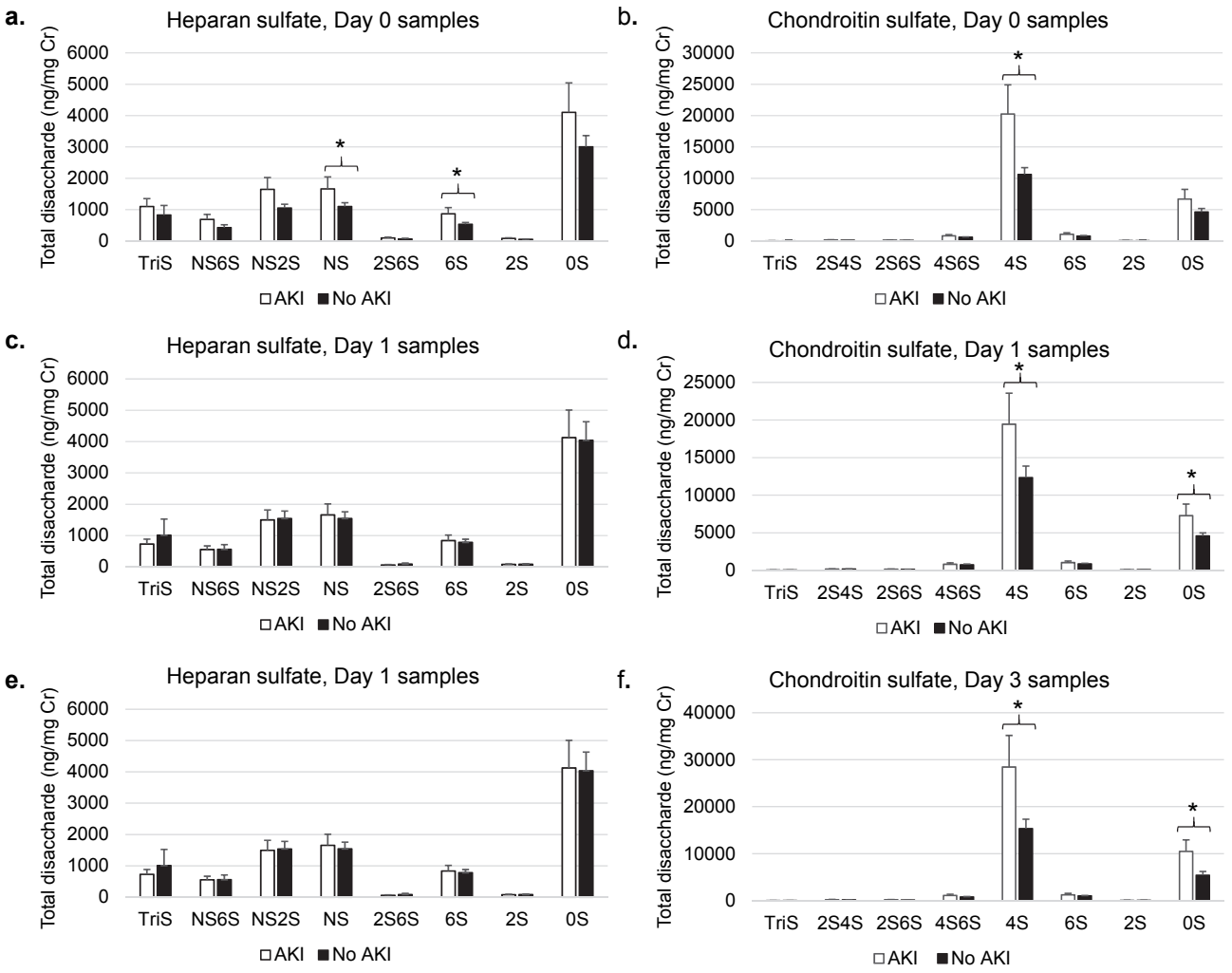

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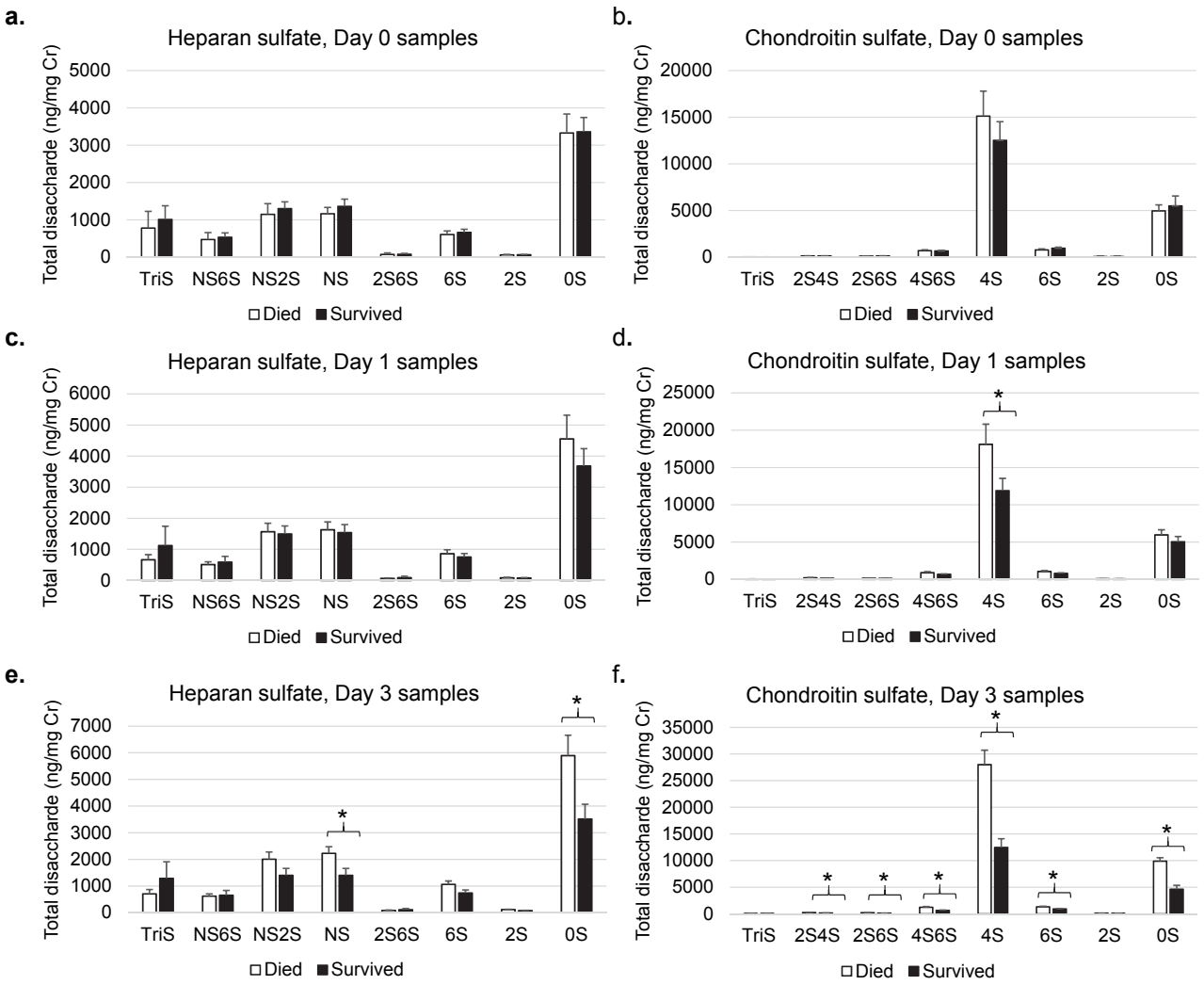

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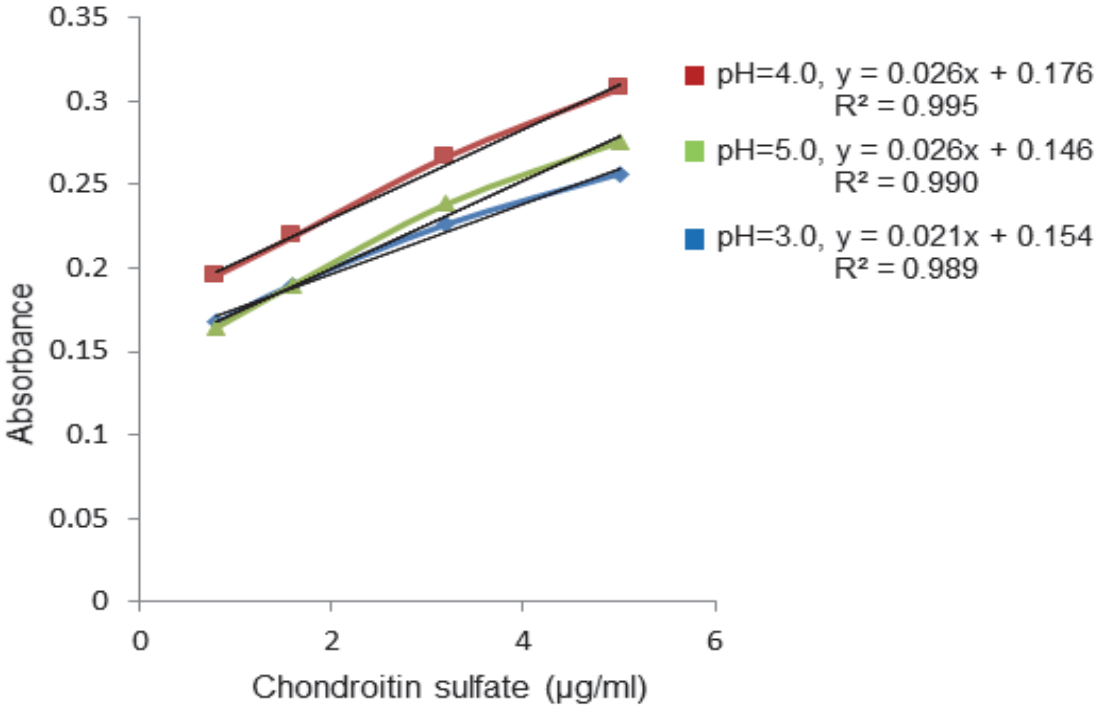
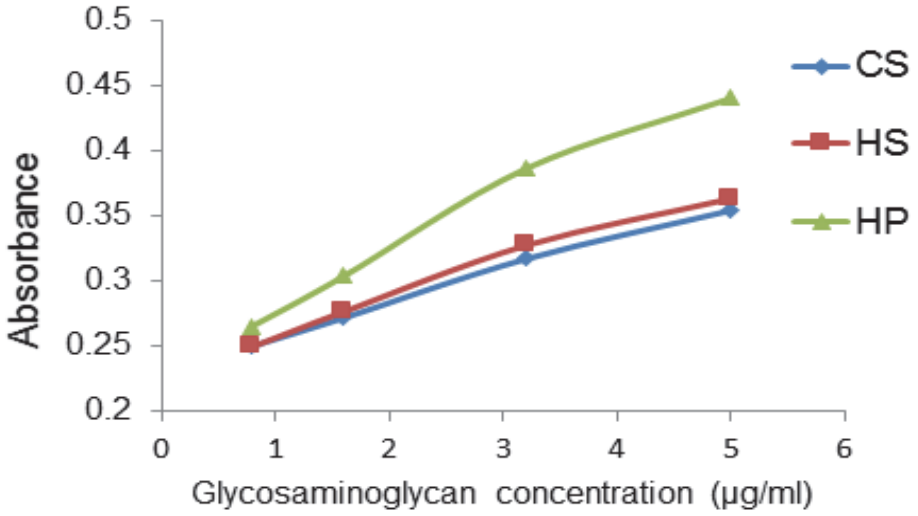
a.



b.



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