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Apoptosis Inhibitor of Macrophage (CD5-Like Antigen) in Healthy Dogs and Dogs With Acute Kidney Injury

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ABSTRACT

Background: Apoptosis inhibitor of macrophage (AIM) is a fundamental mediator of recovery from acute kidney injury (AKI) in mice, but its role in AKI in dogs is unknown.

Hypothesis/Objectives: To detect and quantify AIM in serum and urine from both healthy dogs and dogs with AKI, and to determine if AIM is higher in dogs with AKI compared to healthy controls.

Animals: Eight dogs with International Renal Interest Society (IRIS) Grade II–V AKI and 10 healthy adult dogs.

Methods: Retrospective case–control study. Liquid chromatography-mass spectrometry (LC–MS) based targeted proteomics was used to quantify AIM. AIM peak areas were compared between the AKI and healthy cohorts and correlated with selected markers of renal function.

Results: AIM was able to be quantified in the urine of 5/8 dogs with AKI and 1/10 healthy dogs. AIM was quantified in the serum of all dogs, and there was no difference in peak area between the two groups (AKI: median, 67 840 (range, 9797-98 725); control: median, 79 072 (range, 46 400-160 330); $p = 0.274$). In dogs with AKI, AIM was not correlated with serum creatinine, blood urea nitrogen, phosphorus, or potassium concentrations, urine specific gravity, or IRIS AKI grade.

Conclusions and Clinical Importance: AIM was predominantly detected in the urine of dogs with AKI and not in the urine of healthy dogs.

1 | Introduction

Acute kidney injury (AKI) is characterized by abrupt renal parenchymal injury that might lead to impaired function [1, 2]. Despite the fact that renal recovery can occur, AKI in dogs is associated with prolonged hospitalization, development of chronic kidney disease, and death [3–5]. Consequently, further investigation into potential therapeutic targets that could lead to improved outcomes is needed.

Apoptosis inhibitor of macrophage (AIM), also known as CD5-like antigen (CD5L), is a 40-kilodalton protein and a member of the scavenger receptor cysteine-rich superfamily. It is produced by tissue macrophages and was initially found to aid in

Abbreviations: ABC, ammonium bicarbonate; ACN, acetonitrile; AIM, apoptosis inhibitor of macrophage; AKI, acute kidney injury; CD5L, CD5-like antigen; IgM, immunoglobulin M; IRIS, International Renal Interest Society; KG, kilogram; KIM-1, kidney injury moledule-1; LOD, limit of detection; LOQ, limit of quantification; MRM, multiple reaction monitoring; MS, mass spectrometry.

the survival of macrophages [6]. AIM has a host of different functions in the regulation of inflammation and the pathogenesis of various disease states [7]. AIM is a fundamental mediator of the clearance of renal tubular intraluminal debris by injured epithelial cells, which is essential for the recovery of renal tubular structure and function [8]. AIM is usually bound to immunoglobulin M (IgM) in circulation [9] but can dissociate and be found in the urine of mice and people with AKI [8]. AIM dissociated from IgM undergoes glomerular filtration and facilitates kidney injury molecule-1 (KIM-1) mediated removal of intratubular debris [8, 10]. Mice receiving renal transplants have a reduction in renal tissue damage, tubular obstruction, local and systemic inflammation, and improved renal function with recombinant AIM compared to a placebo [11]. Additionally, mice deficient in AIM have impaired clearance of intratubular debris and recovery from AKI compared to wild-type mice, and the administration of recombinant AIM improves clearance of intratubular debris, recovery from AKI, and survival [8].

The understanding of AIM in health and disease of dogs is limited. The expression of AIM protein was originally thought to be tissue macrophage-specific [6]. Subsequently, AIM was shown to be expressed in healthy dogs in the proximal tubules of the kidneys, glomerular endothelial cells, peripheral monocytes and B lymphocytes, and other tissues [12, 13]. It might play a role in the progression of canine histiocytic sarcoma [14], but its function in the pathophysiology of other disease processes of this species, including AKI, is unknown.

The objectives of this study were to detect and quantify AIM in serum and urine from both healthy dogs and dogs with AKI and to determine if AIM was higher in dogs with AKI compared to healthy controls. We used custom-made light and heavy peptide standards to generate calibration curves and determine the level of AIM across samples. Our hypothesis was that AIM concentrations would be detected in both cohorts and be higher in dogs with AKI in both serum and urine.

2 | Materials and Methods

2.1 | Animals

A retrospective case–control study design was used. Banked serum and urine were utilized from healthy dogs and dogs with AKI that were previously prospectively enrolled in studies at Kansas State University Veterinary Health Center between April 2021 and August 2021 (healthy dogs) as well as March 2022 and October 2022 (dogs with AKI). Dogs in both cohorts were client-owned. Sample collection and storage were performed after informed consent was given by clients, and study protocols were approved by the Institutional Animal Care and Use Committee at Kansas State University.

For the healthy dog cohort, dogs > 1 year of age and weighing > 10 kg were eligible for enrollment. Dogs were deemed healthy based on history, physical examination, CBC, serum biochemistry panel, and urinalysis.

Dogs > 1 year of age and weighing > 5 kg that were presented for evaluation of acute azotemia were eligible for enrollment into the AKI cohort. A diagnosis of AKI was made if the onset of clinical signs was acute (≤ 7 days). Clinical signs considered consistent with AKI included lethargy, inappetence, vomiting, polyuria, oliguria, or anuria. Additionally, dogs had to have new or worsening azotemia. Azotemia was defined as a serum creatinine ≥ 1.7 mg/dL, representing International Renal Interest Society (IRIS) AKI Grade II or higher [1]. Worsening of azotemia was defined as a doubling of serum creatinine within the past 6 months. Oliguria was defined as urine production of < 1 mL/kg/h over 6 h, and anuria was defined as no urine production over 6 h [1]. Dogs were excluded if intravenous fluid therapy was administered before presentation since salt loading might lead to decreased AIM dissociation from IgM [15]. AKI etiology was documented if the information was available, but the diagnostic evaluation to determine the cause of the AKI was at the discretion of the attending veterinarian. Placement of a urinary catheter for quantification of urine output was also at the discretion of the attending veterinarian.

2.2 | Sample Preparation

AIM quantification was performed on serum and urine samples that were frozen at −80°C within 1 h after collection. The protein concentration for each sample was measured using the Bicinchoninic Acid Assay (Pierce). For the urine samples, an equivalent volume of 20 μg was precipitated overnight with 4 volumes of cold acetone (−20°C). The supernatant was then removed, and the remaining pellets were briefly dried before being reconstituted in 20 μL 8 M urea in 50 mM ammonium bicarbonate (ABC). An equivalent volume to 20 μg of serum samples was directly dissolved in 20 μL 8 M urea in 50 mM ABC. Twenty μL of reduction/alkylation reagent mixture (97.5% acetonitrile (ACN), 0.5% triethylphosphine, 2% iodoethanol) was added to each sample, incubated at 37°C for 1 h in a thermomixer [16, 17], and dried in a vacuum centrifuge. Samples were then reconstituted in 142.5 μL of 50 mM ABC containing 1 μg trypsin and 7.5 μL ACN. Proteolysis was carried out in a Barocycler (50°C, 120 cycles consisting of 50 s at 20000 PSI and 10 s at 1 ATM) [16]. Peptides were then desalted using Pierce C18 Spin Tips and dried prior to mass spectrometry (MS) analysis.

2.3 | Development and Analytical Validation Targeted MS Assays/Measurements

The multiple reaction monitoring (MRM) analysis was carried out as described previously [18]. Tryptic peptides were selected based on in silico digestion of the CD5L (A0A8P0PIT0) using the MS-Digest software (University of California, San Francisco). At least 4 proteotypic tryptic peptides (no missed cleavages) were then imported into Skyline (version 22.2, Seattle, WA), and the transition list with collision energy parameters was generated, using y-ions as the product ions for monitoring. Two μL of peptide solution from each sample were then separated in a 25 cm nanoflow Aurora Series ultra-high-performance liquid chromatography packed emitter columns by reversed-phase chromatography in a Dionex UltiMate 3000 RSLC system (Thermo Fisher Scientific) coupled to a TSQ Endura Triple-Stage Quadrupole Mass Spectrometer (Thermo Fisher Scientific). The liquid chromatography consisted of a 60-min gradient run, as follows: samples were injected in 2% B and increased in a linear fashion until 27% B was reached at

40 min, 45% B at 45 min, and 100% B at 50 min. The concentration of B was then held at 100% for min before returning to 2% B and maintained at 2% B until the end of the run. The TSQ was set to SRM mode in positive polarity with a spray voltage of 2400 V, and transfer capillary at a temperature of 275°C. The MRM transitions were acquired with a cycle time of 0.8 s, Q1 resolution of 0.7 (full width at half maximum) and Q3 resolution of 1.2. Cysteine ethanolation was set as a fixed modification. Raw data were then imported to Skyline, and peaks containing at least three measured transitions, with consistent retention time across all samples, were considered for further analysis.

2.4 | MRM Calibration Curve and LOD/LOQ Determination

Standard peptides (both unmodified and modified with Heavy Arg (13C15N)) were custom ordered from Thermo Fisher Scientific, with identical sequence LVGGDSPCAGR, which corresponds to the CD5L peptide identified and monitored in the dog samples. Standard peptides were reconstituted in 3% ACN, and 20 μg of each peptide was aliquoted for alkylation. Cystines were alkylated with 2% iodoethanol and 0.5% triethylphosphine in 50% ACN/50% 25 mM ABC for 1 h at 37°C. Peptides were then dried in a vacuum centrifuge and reconstituted in 3%ACN/0.1% formic acid. Light peptides were diluted into 5 pg/μL, 50 pg/μL, 250 pg/μL, 500 pg/μL, 5 ng/μL, and 50 ng/μL. Heavy peptides were then spiked into each diluted solution at a concentration of 5 ng/μL. For blanks, a 3% ACN/0.1% formic acid solution was used. One μL of each standard mix was then injected into a TSQ Endura Triple-Stage Quadrupole Mass Spectrometer using the parameters described above. Raw spectra were imported into Skyline, and limit of detection (LOD) and limit of quantification (LOQ) were calculated using "Blank plus 2 * SD" and "Max LOQ bias = 20% and Max LOQ CV = 20%", respectively.

TABLE 1 | Selected clinicopathological data of the healthy dog cohort and the AKI cohort.

Variable	Healthy	AKI
Hematocrit (%)	48.5 (43–55)	35 (25–47)
White blood cell count (10^3/μL)	7.65 (4.7–9.6)	14.15 (4.7–19.8)
Neutrophil count (10^3/μL)	4.65 (2.9–6.7)	11.85 (3.5–17.6)
Band neutrophil count (10^3/μL)	0 (0–0)	0.05 (0–1.1)
Creatinine (mg/dL)	1.1 (0.8–1.2)	7 (2.5–15.2)
Blood urea nitrogen (mg/dL)	14 (11–21)	180 (43–353)
Sodium (mmol/L)	146.5 (145–148)	146.5 (142–150)
Potassium (mmol/L)	4.35 (4.1–4.9)	4 (3.4–5.4)
Phosphorus (mg/dL)	3.6 (2.2–4.3)	14.15 (5.9–28)
Albumin (g/dL)	3.85 (3.6–4.3)	3.55 (2.7–4.6)
Urine specific gravity	1.044 (1.030–1.053)	1.013 (1.010–1.024)

Note: Data are presented as median (range).

2.5 | Statistical Analysis

A priori sample size calculation was performed using commercially available software (G*Power version 3.1.9.7, Düsseldorf, Germany). Based on the serum AIM data from a cohort of mice with experimentally induced AKI, 7 dogs in each group would provide 80% power to detect a significant increase of 50% in serum AIM [8].

Data analysis was performed using Prism version 10.0.3, GraphPad Software Inc. San Diego, CA. Normality of the variables was assessed using the D'Agostino-Pearson normality test. Since most data were not normally distributed, numerical data are presented as median and range. Quantification of AIM was compared between dogs with AKI and healthy dogs using the Mann–Whitney *U* test. The Spearman correlation coefficient was used to assess the association between AIM and selected markers of renal function (serum creatinine, blood urea nitrogen, phosphorus, potassium, urine specific gravity, and IRIS AKI grade). $p < 0.05$ was considered statistically significant.

3 | Results

Samples for AIM quantification were available for 8 client-owned dogs with AKI. Three were spayed females and 5 were castrated males. The median age was 9 years (2.5–14). The median bodyweight was 20.7 kg (5–36.1). The following breeds were represented: Pitbull terrier ($n = 2$), Beagle ($n = 1$), Chihuahua

TABLE 2 | Transitions monitored for the peptide LVGGDSPCAGR (538.263 m/z), and the five product ions monitored.

Compound	Polarity	Precursor (m/z)	Product (m/z)	Collision energy (V)
LVGGDSPC[+44.026215]AGR(+2)	Positive	538.263	863.3676	20.6
LVGGDSPC[+44.026215]AGR(+2)	Positive	538.263	806.3461	20.6
LVGGDSPC[+44.026215]AGR(+2)	Positive	538.263	749.3247	20.6
LVGGDSPC[+44.026215]AGR(+2)	Positive	538.263	634.2977	20.6
LVGGDSPC[+44.026215]AGR(+2)	Positive	538.263	547.2657	20.6

Note: Collision energy was set at 20.6 V.
Abbreviations: m/z: mass-to-charge ratio, V: volts.

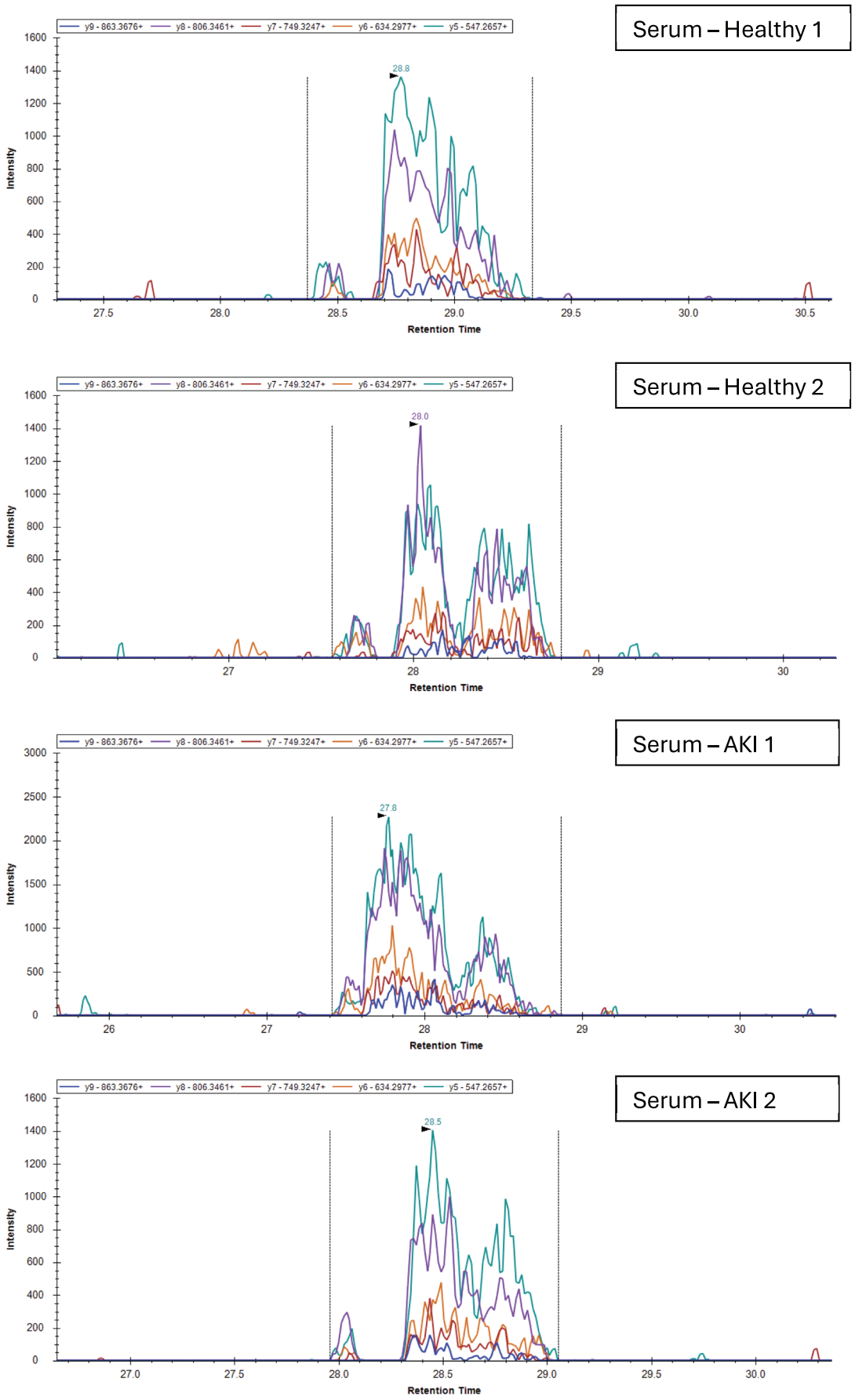

FIGURE 1 | Peaks for all 5 monitored transitions in the multiple reaction monitoring analysis for serum samples. Two representative sample peaks from the healthy and acute kidney injury groups are depicted.

($n=1$), hound mix ($n=1$), Old English Sheepdog ($n=1$), Poodle ($n=1$), and mixed breed dog ($n=1$). The etiological diagnosis of AKI was leptospirosis ($n=3$), pyelonephritis ($n=1$), secondary to hypotension from general anesthesia and/or administration of a non-steroidal anti-inflammatory drug ($n=1$), and undetermined ($n=3$). Upon presentation, 1 dog was categorized as IRIS AKI Grade II, 5 dogs were Grade IV, and 2 dogs were Grade V. The azotemia was newly documented for all dogs, with only 2/8 dogs having pre-AKI renal values available for review. Serum creatinine was normal (<1.4 mg/dL) at 2 months for the IRIS AKI grade II and at 4 months for an IRIS AKI Grade IV dog prior to the AKI. Selected clinicopathological variables of the AKI and healthy cohorts are presented in Table 1. Urine output was quantified during hospitalization for 4/8 dogs, and all were nonoliguric. The median urine output was 4.4 mL/kg/h (1.5–10).

Evaluation of the AKI cohort for comorbidities was not standardized, but abdominal ultrasonography was performed in all dogs, and thoracic radiography was performed in 5/8 dogs. One dog was diagnosed with acute pancreatitis based on ultrasonographic findings of a hypoechoic pancreas with hyperechoic peripancreatic fat. This dog had AKI grade IV (creatinine 6.2 mg/dL) on presentation. Another dog was diagnosed with aspiration pneumonia based on radiographic finding of an alveolar pattern of the right middle lung lobe. This dog had AKI grade V (creatinine 11.8 mg/dL) on presentation. Neoplastic or other systemic disease was not diagnosed in any dog. None of the dogs had a post-renal cause of the azotemia identified on diagnostic imaging. One dog had a single cystolith identified on ultrasonography. Aside from the dog with a clinical diagnosis of pyelonephritis, inflammatory disease of the urinary tract was not apparent based on ultrasonography, urinalysis, and urine culture, including the dog with the cystolith. No dogs had echocardiography performed, but a heart murmur was not heard in any of them.

The healthy control cohort of dogs ($n=10$) consisted of 1 spayed female and 9 castrated males. The median age was 3 years (1–6), and the median bodyweight was 25.3 kg (14.8–31.6). Represented breeds were Border Collie ($n=2$), Goldendoodle ($n=2$), Golden Retriever ($n=1$), Pembroke Welsh Corgi ($n=1$), and mixed breed dog ($n=4$).

To quantify the levels of AIM in dogs from both healthy control and AKI groups, we performed an MRM analysis of a tryptic AIM peptide. We generated a standard curve using serially diluted standard AIM peptides, with concentrations ranging from 50 ng/μL to 5 pg/μL (Figure S1). This calibration curve was then used to calculate both the LOD and LOQ of this method. Additionally, we plotted the serum and urine samples from all dogs onto the standard calibration curve. The total measured peptide amounts for each sample are detailed in Table S1.

AIM was detected and quantified in the serum from all dogs with AKI and all healthy control dogs (Table 2); however, there was no difference in the peak areas between the two groups (AKI: 67840 (9797-98 725); control: 79072 (46400-160 330); $p=0.274$). (Figures 1 and 2) Interestingly, AIM was able to be detected and quantified in the urine of 5/8 dogs with AKI and only 1/10 healthy control dogs. Of the 5 dogs with AKI and AIM detected in urine, 4 of them had a urinary catheter with urine quantification performed. The urine output of one dog averaged 36 mL/kg/day, whereas the other 3 dogs were considered polyuric (> 50 mL/kg/day). A value of 0 was given to those dogs with undetectable AIM in urine. Consequently, urinary AIM peak area was higher in dogs with AKI compared to controls (AKI: 2855 (0–16 200); control: 0 (0–490); $p=0.018$, median difference 2656 (95% CI 0–6228)). (Figures 3 and 4). All but one of the measured AIM peptide peaks fell into the linear range of our calibration curve (Figure S1C,D). This value was below the limit of detection, and the urine sample was from the AKI cohort. Consequently, the sample was assigned a value of 0, and AIM was considered undetectable.

Serum AIM was not correlated to serum creatinine, blood urea nitrogen, phosphorus, potassium, urine specific gravity, and IRIS AKI grade. Modest correlations were found between urinary AIM and serum creatinine ($\rho=0.474$, $p=0.047$), blood urea nitrogen ($\rho=0.516$, $p=0.028$), and urine specific gravity ($\rho=-0.614$, $p=0.01$), and IRIS AKI grade ($\rho=0.595$, $p=0.009$) but not potassium ($\rho=-0.433$, $p=0.073$) or phosphorus ($\rho=0.447$, $p=0.063$). However, the data were reanalyzed with only the AKI cohort, and none of the variables were significantly correlated with urinary AIM peak area.

4 | Discussion

We developed an MRM-based assay to monitor AIM levels across serum and urine samples from a cohort of healthy dogs and dogs with AKI. We identified that AIM detected by MS was present primarily in the urine of dogs with AKI and not in healthy dogs. AIM in serum was found in similar amounts in the AKI and control cohorts. The quantity of AIM in both serum and urine does not appear to be correlated with any of the markers of renal function assessed in this study.

AIM is primarily IgM-bound in health when in circulation but dissociates with AKI [8, 9]. Additionally, AIM found in the urine

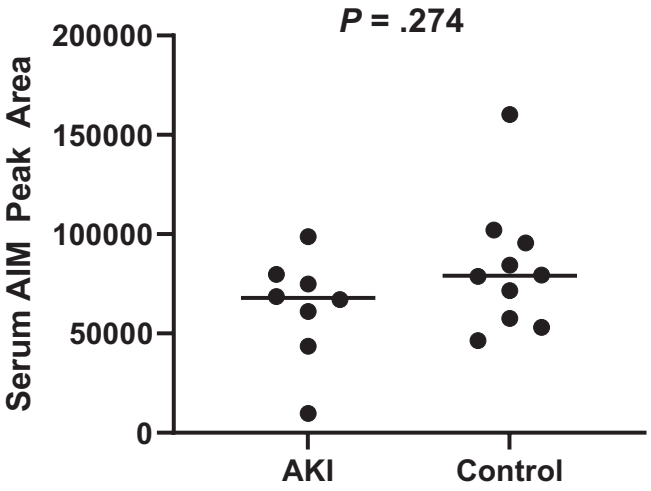

FIGURE 2 | Dot plot of peak areas of apoptosis inhibitor of macrophage measured by liquid chromatography-mass spectrometry in the serum of dogs with acute kidney injury ($n=8$) and healthy control dogs ($n=10$). Individual data points (dots) and group medians (horizontal lines) are represented.

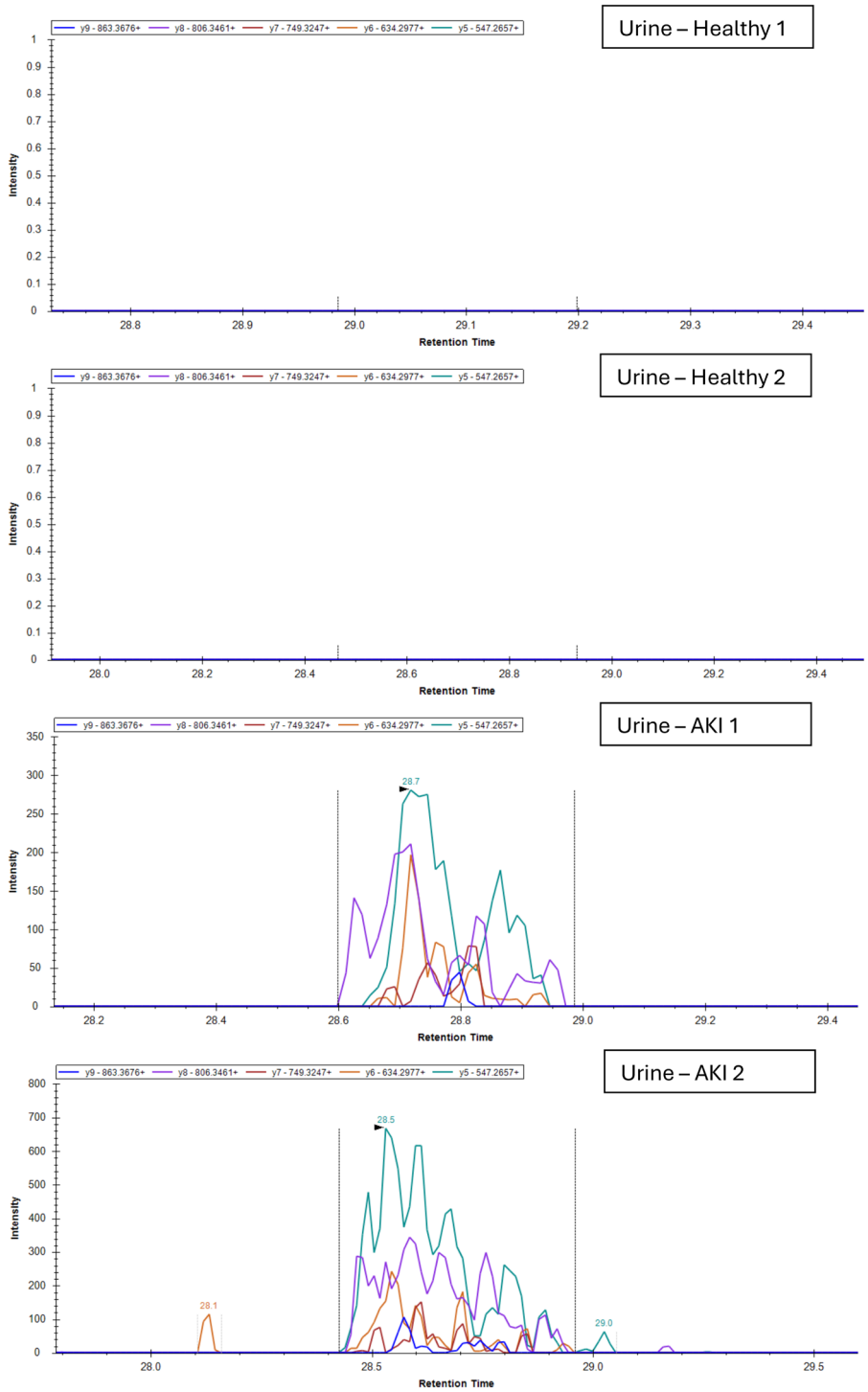

FIGURE 3 | Peaks for all 5 monitored transitions in the multiple reaction monitoring analysis for urine samples. Two representative sample peaks from the healthy and acute kidney injury groups are depicted.

of mice with AKI is in its free form and comes from serum [8]. The mechanism for AIM's dissociation from IgM with AKI is currently unknown. The MRM assay in this study quantified total AIM in serum and urine, and size fractionation to determine the proportion of bound versus free AIM was not performed as a part of this protocol. However, we speculate that this pathophysiology is conserved between dogs and mice since the majority of the urinary AIM in our study was only detected in the AKI cohort. Total AIM concentration in serum remained similar between healthy dogs and dogs with AKI in this study, but it is not yet known if free AIM in circulation increases with AKI.

AIM could not be detected in the urine of 3 dogs with AKI, and clinical differences with these dogs compared to the rest of the AKI cohort were not readily identified. An initial hypothesis was if a deficiency in urinary AIM could lead to a decrease in urine production since renal tubular obstruction is one of the

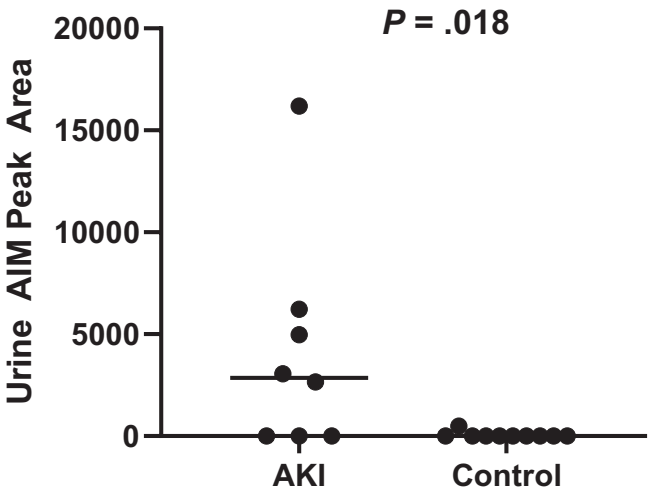

FIGURE 4 | Dot plot of peak areas of apoptosis inhibitor of macrophage measured by liquid chromatography-mass spectrometry in the urine of dogs with acute kidney injury ($n = 8$) and healthy control dogs ($n = 10$). Individual data points (dots) and group medians (horizontal lines) are represented.

contributors to oligoanuria [19, 20]. AKI leads to compromise of the integrity of the renal tubular cytoskeleton resulting in loss of polarity and sloughing of epithelial cells [21]. These cells can combine with proteins and necrotic debris and cause obstruction of the tubules [21, 22]. None of these dogs had their urine output quantified. On presentation, the dogs were classified as IRIS AKI Grades II, IV, and V. Interestingly, the dog with IRIS AKI Grade II (serum creatinine 2.5 mg/dL) represented 5 days after discharge with a serum creatinine of 7.9 mg/dL, and urine output was quantified this time. The azotemia was progressive over the following 2 days prior to euthanasia, and the average urine output was 0.48 mL/kg/h. The other two dogs with IRIS AKI Grades IV and V were euthanized during hospitalization due to a lack of clinical improvement. The reason for the detection of urinary AIM in the single dog in the healthy cohort was not able to be determined since it was deemed to be healthy based on the screening diagnostics performed. It is possible that a subclinical disease was present which led to the presence of AIM in urine.

This study has several limitations, including its retrospective nature and small sample size, which precluded assessment of AIM in dogs with AKI and decreased urine output. Additionally, size fractionation was not performed in order to evaluate the association between AIM and IgM. KIM-1 was also not measured in this study. The interplay between both KIM-1 and AIM is necessary for the enhanced phagocytic activity of macrophages and tubular epithelial cells, but assessment of this association is suspected to be more meaningful in a larger cohort of dogs with AKI with different IRIS subgrades (i.e., oligoanuric vs. nonoliguric). Another limitation of this study is that tissue expression of AIM was not assessed; however, obtaining renal biopsies from client-owned dogs in both the healthy and AKI cohorts would be a clinical challenge due to its invasive nature. Obtaining samples through fine needle aspiration could be considered, but this sampling method poses its own diagnostic challenges. Additionally, comorbidities were identified in several dogs in the AKI cohort, which could influence AIM results since it has a role in many disease states [7].

In conclusion, we developed a targeted proteomic method utilizing an LC–MS-based MRM approach to accurately monitor and quantify the levels of AIM across various biological samples. Our findings revealed that AIM was predominantly present in the urine of dogs diagnosed with AKI, while it was undetectable in the urine of healthy dogs. This observation highlights the potential role of AIM as a biomarker for AKI in dogs.

Disclosure

Authors declare no off-label use of antimicrobials.

Ethics Statement

Institutional Animal Care and Use Committee (IACUC) approval was granted for this study the Kansas State University IACUC. Samples collected as part of protocols 4496 and 4662. Authors declare human ethics approval was not needed.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.