



Influence of clinical and experimental intra-articular inflammation on neutrophil gelatinase-associated lipocalin concentrations in horses

Catina Frydendal DVM | Katrine B. Nielsen DVM | Lise C. Berg DVM, PhD |
Gaby van Galen DVM, MSc, PhD, DECEIM (equine), DECVECC |
Ditte M. T. Adler DVM, PhD  | Stine M. Andreassen DVM, PhD |
Stine Jacobsen DVM, PhD, DECVS-LA 

Department of Veterinary Clinical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence

Stine Jacobsen, University of Copenhagen, Department of Veterinary Clinical Sciences, Section of Medicine and Surgery, Agrovej 8, DK-2630 Taastrup, Denmark.
Email: stj@sund.ku.dk

Funding information

Foreningen KUSTOS af 1881, Grant/Award Number: N/A

Abstract

Objective: To investigate neutrophil gelatinase-associated lipocalin (NGAL) concentrations in serum and synovial fluid (SF) from horses with joint inflammation.

Study design: Experimental studies and retrospective clinical study.

Sample population: Serum and SF samples were available from healthy horses ($n = 19$), clinical cases, and horses with experimental joint inflammation. Clinical cases included horses with ($n = 10$) or without ($n = 10$) septic arthritis. Experimental intra-articular inflammation was induced by lipopolysaccharide (LPS; $n = 7$, severe inflammation), lidocaine ($n = 6$, moderate inflammation), or mepivacaine ($n = 6$, mild inflammation).

Methods: Availability of samples was based on approval from the local ethical committee and from the Danish Animal Experiments Inspectorate. Neutrophil gelatinase-associated lipocalin was measured with a previously validated enzyme-linked immunosorbent assay. Repeated-measurements one- and two-way analysis of variance and correlation analysis were used to analyze NGAL concentrations and white blood cell counts (WBC).

Results: After injection of LPS or lidocaine, SF NGAL concentrations increased 343- ($P = .0035$) and 60-fold ($P = .0038$) relative to baseline, respectively. Serum NGAL also increased in both groups ($P < .05$) but to lower concentrations than in SF. Concentrations were higher after injection of lidocaine SF NGAL than after injection of mepivacaine ($P < .05$) at 6 and 12 hours. Synovial fluid concentrations of NGAL were higher in horses with septic arthritis than in the nonseptic group ($P = .0070$) and in healthy controls ($P = .0071$). Concentrations of NGAL correlated with WBC in SF ($P < .0001$, $R^2 = 0.49$) and in blood ($P = .0051$, $R^2 = 0.27$).

Conclusion: Neutrophil gelatinase-associated lipocalin concentrations increased in SF in response to experimentally induced and naturally occurring

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

© 2021 The Authors. *Veterinary Surgery* published by Wiley Periodicals LLC. on behalf of American College of Veterinary Surgeons.

joint inflammation. Synovial fluid NGAL concentration correlated with WBC and, thus, seems to reflect intensity of joint inflammation.

Clinical significance: Neutrophil gelatinase-associated lipocalin may prove to be a useful biomarker of joint inflammation and infection in horses.

1 | INTRODUCTION

Diagnosing synovial sepsis in horses may be difficult.¹ Bacterial culture is not always reliable, as was shown in a recent study where 48 % of synovial fluid (SF) samples from horses diagnosed with septic synovitis were culture negative.²

Biomarkers in blood and SF are used routinely in horses to indicate presence of joint inflammation and infection,³ to assess degree of inflammation, and to monitor changes in disease activity.⁴ Markers vary in sensitivity and specificity, response pattern, and the degree to which they are influenced by previous interventions in the synovial structure.⁵ It is therefore of great relevance for equine clinicians to have an armamentarium of biomarkers available. Inflammatory markers are essential for early and accurate identification of synovial infection, and they are a prerequisite for prompt intervention.³ Total white blood cell counts (WBC), differential leukocyte counts, and total protein (TP) concentrations are currently the most commonly used SF biomarkers in equine joint disease. Total protein is easily determined by refractometry; however, levels are affected by previous interventions such as arthrocentesis,⁶ arthroscopic surgery,⁷ or intra-articular drug administration,⁸ which renders this marker of limited value for monitoring treatment effects. Total and differential leukocyte counts are performed by manual counting or by using automated cell counters, so leukocyte counts may be time consuming or require skills and/or equipment not commonly available in general practice.³ Within the last 10 to 15 years, serum amyloid A (SAA) has emerged as a useful analyte for identifying and monitoring joint inflammation.^{4,6,9,10} Serum amyloid A has gained widespread use not only because of its desirable diagnostic properties but also because a horse-side test was developed, thus allowing assessment of SAA without the requirement of shipping samples to larger diagnostic laboratories.¹¹ Serum amyloid A can be measured systemically and in SF.⁹ Concentrations in SF increase in response to moderate–severe joint inflammation^{6,10} but remain unaffected by interventions such as repeated arthrocentesis,⁶ intra-articular injection of amikacin,⁸ and joint lavage.^{7,12} One drawback of SAA is that concentrations in SF increase fairly slowly (SAA can be measured 8–12 hours after induction of joint inflammation and concentrations peak at 48 hours).^{9,13}

Neutrophil gelatinase-associated lipocalin (NGAL), also known as *human neutrophil lipocalin*, *lipocalin 2*, or *24p3*, is a protein belonging to the family of lipocalins. In the body, it is present in the form of a monomer, a dimer, or a complex in which it is covalently bound to metalloproteinase 9.¹⁴ In man, NGAL was first described in neutrophil granulocytes,^{15,16} but the protein has since then also been detected in renal tubular cells, hepatocytes, macrophages, epithelial cells, and adipocytes.^{14,17,18}

In man, NGAL has mainly been used as a biomarker of acute kidney injury.¹⁹ It has, however, become increasingly clear that NGAL may also be an inflammatory marker because systemic NGAL levels are elevated in patients with inflammatory or infectious conditions such as sepsis.^{20,21} In a very limited number of studies in man, NGAL has been described as a potential marker of joint inflammation,^{22–25} with the protein being present in SF from people with osteoarthritis²⁴ and rheumatoid arthritis.²⁵ Deirmengian et al²² assessed SF concentrations of NGAL and classified human patients with periprosthetic joint infection (PJI) with 100% sensitivity and specificity.

There are few reports on NGAL in equine medicine. Increased serum NGAL concentrations were found in horses with increased serum creatinine values by researchers investigating NGAL in horses suspected of kidney injury.²⁶ Similarly to in man, NGAL seems to be a marker of inflammation in horses. Serum NGAL concentrations increased after experimental intravenous injection of lipopolysaccharide (LPS) and were also elevated in clinical cases with systemic inflammation.²⁷ There are no published studies on NGAL in equine joint disease.

The objective of the present study was to determine NGAL concentrations in horses with clinical and experimental (induced by LPS or local analgesics [lidocaine, mepivacaine]) joint inflammation. Based on the limited evidence available from human medicine, our hypothesis was that intra-articular inflammation would cause increased serum and SF NGAL concentrations compared with healthy horses.

2 | MATERIALS AND METHODS

2.1 | Animals and samples

For this study, samples were available from two previous studies^{28,29} and from horses admitted to the Large

Animal Teaching Hospital at University of Copenhagen. Blood was drawn from an indwelling jugular venous catheter (in experimental studies) or by puncture of the jugular vein (in clinical cases). Synovial fluid samples were obtained with 19- to 21-gauge needles after aseptic preparation of the arthrocentesis site.

2.2 | Lipopolysaccharide-induced joint inflammation

Serum and SF samples were available from seven horses (four mares and three geldings, 3-15 years old, 425-620 kg) that underwent intra-articular injection of LPS, resulting in severe joint inflammation as described in detail by Andreassen et al.²⁸ In brief, 3 µg LPS (*Escherichia coli* 055: B5, No. L2880; Sigma-Aldrich ApS, Copenhagen, Denmark) was injected into the antebrachio-carpal joint of one front limb. One horse (horse B) erroneously received 12 µg LPS. Before (0 hour) and after (2, 4, 8, 16, 24, 36, 48, 72, and 144 hours) LPS injection, blood and SF were collected and directly transferred to tubes (BD Vacutainer; Becton Dickinson A/S, Albertslund, Denmark) containing no additive (for preparation of serum) and ethylenediaminetetraacetic acid (for assessment of WBC in whole blood and SF and for stabilizing SF for later NGAL measurements). White blood cell count in whole blood was determined by automated cell counting (ADVIA 2120 analyzer; Siemens Healthcare Diagnostics, Deerfield, Illinois) and in SF by manual cell counting with a hemacytometer. Both analyses were performed within 24 hours as described in detail in Andreassen et al.²⁸ Serum tubes and SF samples were centrifuged at 1200 g for 10 minutes and stored at -80°C. Analysis of NGAL concentrations took place 5 years after samples were obtained. The experimental protocol was preapproved by the ethical committee of the Large Animal Teaching Hospital of University of Copenhagen and by the Danish Animal Experiments Inspectorate (permit 2011/561-1996).

2.3 | Joint inflammation induced by intra-articular injection of local analgesics

Serum and SF samples were available from 12 horses (all mares, 5-17 years old, 495-611 kg). Six horses were injected with 200 mg lidocaine (Xylocaine, 20 mg/mL; AstraZeneca, Copenhagen, Denmark), and six horses were injected with 200 mg mepivacaine (carbocaine, 20 mg/mL; AstraZeneca) in one middle carpal joint as described in Adler et al.²⁹ Intra-articular lidocaine injection caused moderate synovial inflammation with peak SF WBC (at 12 hours) of $15.3 \pm 4.6 \times 10^9/L$; intra-articular

mepivacaine injection resulted in mild synovial inflammation with peak SF WBC (at 12 hours) of $1.7 \pm 2.6 \times 10^9/L$ as described in detail in Adler et al.²⁹

Blood and SF were collected before (0 hours) and after (6, 12, 24, 48, and 168 hours) injection of the local analgesics and directly transferred to tubes (BD Vacutainer; Becton Dickinson A/S) containing no additive (for preparation of serum) and ethylenediaminetetraacetic acid (for assessment of WBC in whole blood and SF and for stabilizing SF for later NGAL measurements). Serum tubes and SF samples were centrifuged at 2500 g for 10 minutes at 4°C; all other sample handling and laboratory procedures for determining WBC in whole blood and SF were performed as previously described. Samples were stored at -80°C until analysis of NGAL concentration, which took place 2 years after sample collection. Serum samples from the six horses injected with mepivacaine were not analyzed in the present study. The experimental protocol was preapproved by the ethical committee of the Large Animal Teaching Hospital of University of Copenhagen and by the Danish Animal Experiments Inspectorate (permit 2015-15-0201-00608).

2.4 | Healthy controls

The healthy controls comprised the preinjection serum and SF samples available from the 19 horses involved in the experimental studies. These were the preinjection samples from the seven horses undergoing experimental LPS-induced joint inflammation, the six horses receiving experimental intra-articular lidocaine injection, and the six horses receiving experimental intra-articular mepivacaine injection (no serum was available from this group). Before beginning the experimental studies, horses were determined to be healthy after thorough clinical examination including lameness evaluation and hematology and blood biochemistry analyses.

2.5 | Clinical cases

Serum and SF samples from 20 horses (admitted between 2013 and 2017) were available from the biobank at the Large Animal Teaching Hospital at University of Copenhagen. Blood and SF samples were collected routinely as part of the diagnostic workup of the horses. Serum and SF were obtained and processed, and WBC was determined as previously described. Samples were stored at -20°C until analysis of NGAL concentrations, which took place 1 to 5 years after sampling. Inclusion of horses was based on their final diagnosis, and two distinct groups were assessed. Ten horses had acute septic

synovitis based on anamnesis; clinical examination including assessment of lameness, diagnostic imaging, bacteriology, hematology/blood biochemistry; synoviocentesis/pressure test; SF TP of 30 to 72 g/L (reference value, <25); SF WBC of 60 to 307×10^9 /L (reference value, $< 1.35 \times 10^9$ /L); and SF differential count with 90% to 100% neutrophils. Ten horses had acute septic synovitis based on anamnesis; clinical examination including assessment of lameness; diagnostic imaging; bacteriology; hematology/blood biochemistry; synoviocentesis/pressure test; SF TP of 30 to 72 g/L (reference value, <25); SF WBC of 60 to 307×10^9 /L (reference value, $< 1.35 \times 10^9$ /L); and SF differential count with 90% to 100% neutrophils.

2.6 | Measurements of NGAL

A commercially available, porcine enzyme-linked immunosorbent assay (ELISA; pig NGAL ELISA No. 44; Bio-Porto Diagnostics, Hellerup, Denmark) was used to quantitate NGAL in serum and SF. The ELISA has been validated and found reliable for equine use by our research group.²⁶ Serum samples were diluted 1:1000 to 1:3000, and SF was diluted 1:2000 to 1:20000 to achieve absolute NGAL concentrations. The ELISA was performed according to the manufacturer's instructions. Absorbance was read at 450 nm (reference, 620) in an ELISA reader (Multiskan EX; ThermoFisher Scientific, Hvidovre, Denmark) with Ascent Software version 2.6 to EMS Reader MF (Thermo Labsystems, Philadelphia, Pennsylvania). By using four-parameter logistic regression according to the manufacturer's instructions, absorbances were converted to concentrations (pg/mL) in MyAssays (www.myassays.com). Concentrations were subsequently converted to $\mu\text{g/L}$.

2.7 | Data analysis

Two-way analysis of variance (ANOVA; no assumption of sphericity) with Sidak multiple-comparisons test was used to (1) evaluate concentration changes over time (ie, compare preinjection and postinjection NGAL concentrations) and identify differences between SF and serum NGAL concentrations in the LPS group, (2) evaluate concentration changes over time (ie, compare preinjection and postinjection NGAL concentrations) and identify differences between SF and serum NGAL concentrations in the lidocaine group, and (3) compare SF NGAL concentrations in the lidocaine and mepivacaine groups. Because of differences in sampling time points, NGAL concentrations in the LPS group could not be compared

statistically with concentrations in the local analgesics groups. Assumptions (Gaussian distribution of residuals) were verified on residual and QQ plots. Neutrophil gelatinase-associated lipocalin levels in healthy controls and the two clinical groups (septic and nonseptic) were compared by using one-way ANOVA with Welch's correction (because SD in the groups differed) and Dunnett's multiple-comparison post hoc test. These data were found to be normally distributed according to the D'Agostino & Pearson normality test. Correlation between NGAL concentration and WBC was assessed by using samples from the clinical cases ($n = 20$) and one randomly selected sample from each of the 19 horses that were enrolled in the experimental studies. $P < .05$ was considered significant. Statistical analyses were performed in GraphPad Prism version 8.4.2 (GraphPad Software, La Jolla, California).

3 | RESULTS

3.1 | Neutrophil gelatinase-associated lipocalin concentration in horses with LPS-induced joint inflammation

Intra-articular injection of LPS caused increases in NGAL concentrations in serum and SF compared with baseline (0 hours; $P = .0035$; Figure 1). Synovial fluid NGAL concentrations (mean \pm SD) increased 343-fold from a preinjection concentration of $7.52 \pm 11.30 \mu\text{g/L}$ to a peak concentration of $2582.0 \pm 1713.8 \mu\text{g/L}$ at 8 hours. Serum NGAL concentrations (mean \pm SD) increased 7.4-fold from a preinjection concentration of $22.58 \pm 16.65 \mu\text{g/L}$ to a peak concentration of $165.6 \pm 81.4 \mu\text{g/L}$ at 36 hours (Figure 1). Concentrations of NGAL were higher in SF than in serum between 2 and 24 hours after LPS injection ($P < .05$).

3.2 | Neutrophil gelatinase-associated lipocalin concentration in horses with joint inflammation induced by intra-articular injection of local analgesics

After intra-articular injection of lidocaine, NGAL concentrations in serum and SF increased compared with baseline (0 hours; $P = .0038$; Figure 2). Synovial fluid NGAL concentrations (mean \pm SD) increased 60-fold from a preinjection concentration of $6.22 \pm 1.77 \mu\text{g/L}$ to a peak concentration of $370.3 \pm 155.7 \mu\text{g/L}$ at 12 hours. Serum NGAL concentrations (mean \pm SD) increased 1.8-fold from a preinjection concentration of $21.31 \pm 6.38 \mu\text{g/L}$ to a peak concentration of $37.3 \pm 24.2 \mu\text{g/L}$ at 6 hours

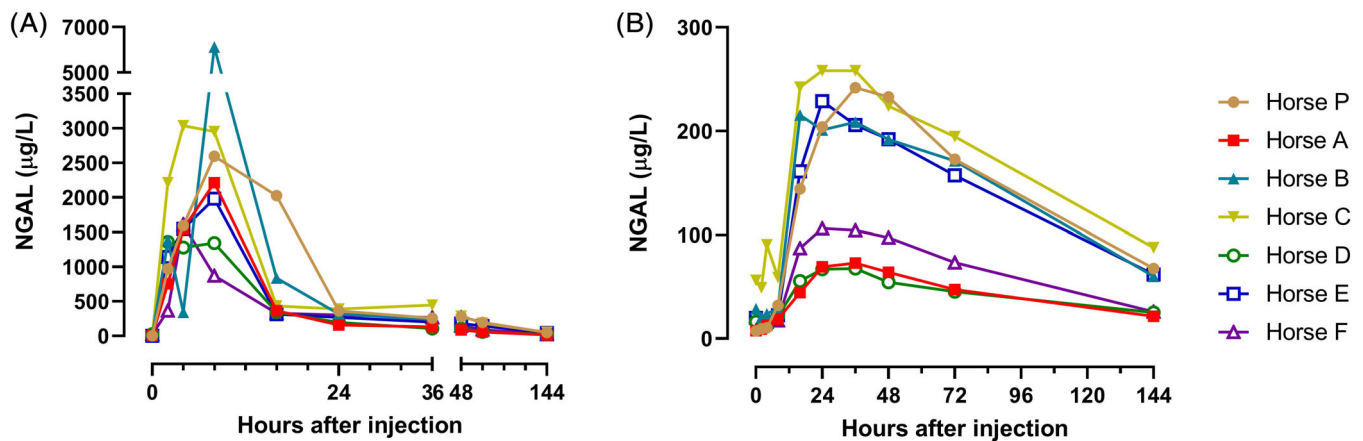


FIGURE 1 Concentrations of NGAL in SF (A) and in serum (B) obtained from seven horses before and 2-144 hours after intra-articular injection of LPS. There are different x- and y-axes in A and B. Concentrations of NGAL were higher in SF than in serum 2-24 hours after LPS injection ($P < .05$). LPS, lipopolysaccharide; NGAL, neutrophil gelatinase-associated lipocalin; SF, synovial fluid

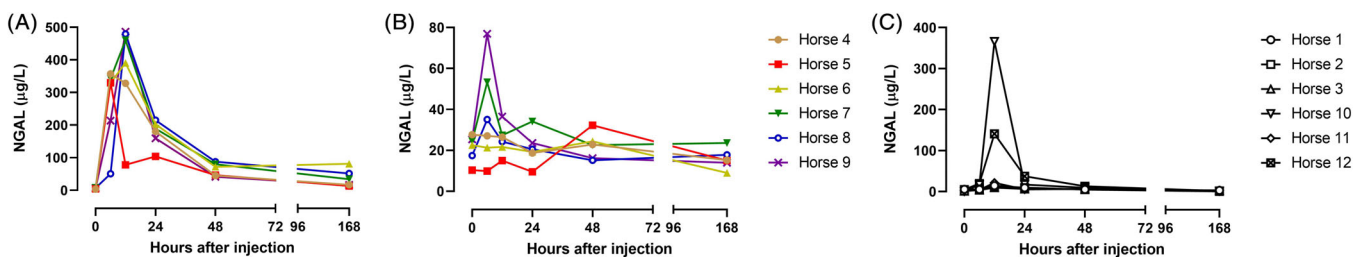


FIGURE 2 Concentrations of NGAL in SF (A) and in serum (B) obtained from six horses before and 6-168 hours after intra-articular injection of lidocaine and in SF (C) obtained from six horses before and 6-168 hours after intra-articular injection of mepivacaine. There are different y-axes in A, B, and C. SF and serum NGAL concentrations after intra-articular injection of lidocaine differed 6-48 hours after injection ($P < .05$). SF concentrations of NGAL were higher in the lidocaine group than in the mepivacaine group at 6 and 12 hours after injection ($P < .05$). NGAL, neutrophil gelatinase-associated lipocalin; SF, synovial fluid

(Figure 2). Concentrations of NGAL were higher in SF than in serum between 6 and 48 hours after injection ($P < .05$).

After intra-articular injection of mepivacaine, NGAL concentrations in SF (mean \pm SD) increased 34-fold from a preinjection concentration of 2.74 ± 2.09 to a peak concentration of 94.2 ± 142.5 $\mu\text{g/L}$ at 12 hours (Figure 2). Synovial fluid concentrations of NGAL were higher in the lidocaine group than in the mepivacaine group at 6 and 12 hours after injection ($P < .05$).

3.3 | Neutrophil gelatinase-associated lipocalin concentration in healthy controls and horses with and without septic synovitis

Synovial fluid NGAL concentrations were higher in horses with septic synovitis (mean [range] = 1966.8 [156.8 - 4684] $\mu\text{g/L}$) than in the nonseptic group (mean [range] = 2.1 [0.0 - 6.1] $\mu\text{g/L}$, $P = .0070$) and in healthy

controls (mean [range] = 5.6 [0.0 - 25.0] $\mu\text{g/L}$, $P = .0071$; Figure 3). Serum NGAL concentrations did not differ between horses with septic synovitis (mean [range] = 188 [11.3 - 778.4] $\mu\text{g/L}$), the nonseptic group (mean [range] = 43.5 [8.4 - 96.4] $\mu\text{g/L}$), and healthy controls (mean [range] = 22 [7.5 - 56.1] $\mu\text{g/L}$; $P > .05$; Figure 3).

3.4 | Correlation between NGAL and WBC in synovial fluid and blood

Total WBC and NGAL concentrations correlated positively in SF ($P < .0001$, $R^2 = 0.49$) and in blood ($P = .0051$, $R^2 = 0.27$; Figure 4).

4 | DISCUSSION

In this study, we introduce NGAL as a marker of joint inflammation in horses and emphasize the diagnostic potential of NGAL concentrations in SF. Synovial fluid

from healthy horses contained very low concentrations of NGAL, and a large dynamic response (>340-fold increase relative to baseline) and a fast rise-and-fall pattern (concentration peaked 8 hours after experimental LPS

injection) were observed during joint inflammation. These features are quite similar to those of SAA, although it seems that SF NGAL concentration peaks even faster than SAA (which has been shown to peak 48 hours after intra-articular LPS injection^{9,13,28}). Similarly to what has been proposed for SAA,⁵ the characteristics of the NGAL response may make this analyte particularly well suited for diagnosing and monitoring joint inflammation. Because of the fast response, NGAL may prove useful for early detection of joint inflammation. In severe synovial inflammation (elicited by intra-articular injection of LPS or observed in the animals with septic arthritis), SF NGAL concentrations were several hundred times higher than in SF from healthy horses. In contrast, measuring NGAL in serum seemed to be less useful. Serum NGAL concentrations did not differ between horses with septic synovitis, the nonseptic group, and healthy controls. During LPS-induced joint inflammation, serum NGAL peak values were only 7.4 times higher compared with preinjection levels. These findings in horses correspond to results from a study in mice, in which experimental induction of arthritis led to only modest increases in serum NGAL concentrations.³⁰

Determining the effects of other diseases or other types of pathology on NGAL concentrations will require further investigation in horses. Neutrophil gelatinase-associated lipocalin levels in plasma and serum have been shown to be influenced by systemic inflammatory conditions (eg, sepsis) as well as other concurrent disease (eg, kidney injury) in man,¹⁴ dogs,³¹ and horses.²⁷ Assessing NGAL in SF may thus have the benefit of reflecting the local inflammatory status in the joint more specifically than systemic concentrations do.

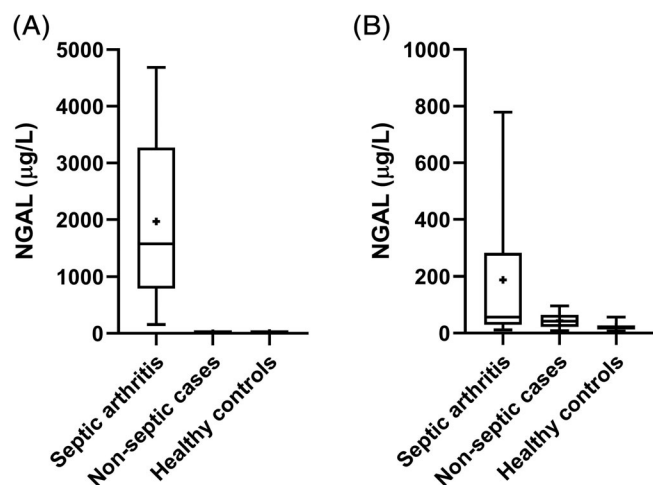


FIGURE 3 Concentrations of NGAL in SF (A) and in serum (B) from horses with septic arthritis ($n = 10$), horses with nonseptic synovial fluid (wound near a synovial structure but no penetration, $n = 10$), and healthy controls ($n = 19$). There are different y-axes in the two panels. SF NGAL concentrations differed between horses with septic arthritis and healthy controls ($P < .0071$) and between horses with septic arthritis and the nonseptic group ($P < .0070$) but not between healthy controls and the nonseptic group ($P > .05$). Serum NGAL concentrations did not differ between groups ($P > .05$). The boxes extend from the 25th to the 75th percentiles, and the whiskers illustrate ranges. Horizontal line, median; dot, mean. NGAL, neutrophil gelatinase-associated lipocalin; SF, synovial fluid

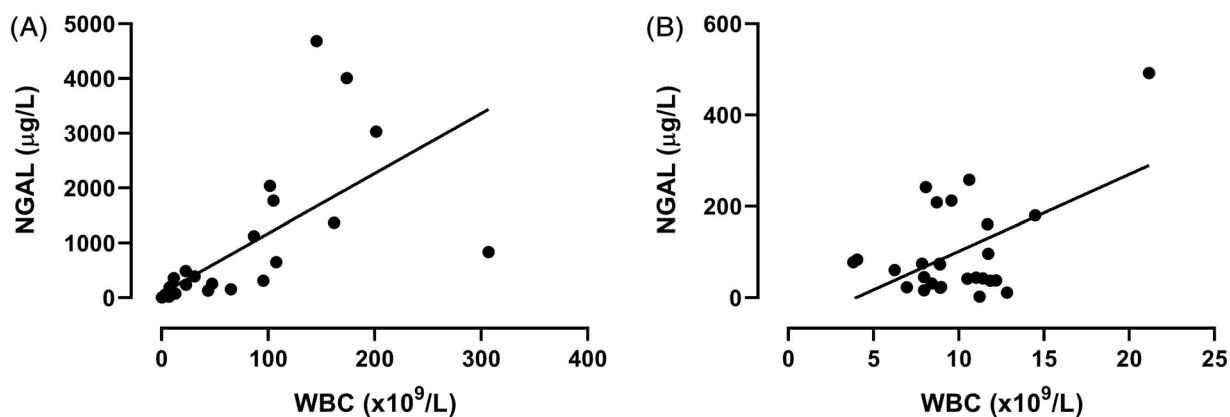


FIGURE 4 Correlation between concentrations of NGAL and WBC in SF (A) and in blood (B) in clinical cases with ($n = 10$) and without (wound near synovial structure but no penetration, $n = 10$) septic arthritis and in samples from horses with experimental joint inflammation induced by intra-articular injection of lipopolysaccharide ($n = 7$), lidocaine ($n = 6$) or mepivacaine ($n = 6$ [SF only because no serum was available from this group]). To obtain independent samples, only one (randomly selected) sample was included from each of the 19 horses from the experimental studies. The x and y-axes are different in A and B. WBC and NGAL concentrations correlated in SF ($P < .0001$, $R^2 = 0.49$) and in blood ($P = .0051$, $R^2 = 0.27$). NGAL, neutrophil gelatinase-associated lipocalin; SF, synovial fluid; WBC, white blood cell count

Few studies in man—and none in animal species—have been focused on investigating NGAL concentrations in SF. In one early study,²³ researchers found very high (mean \pm SD of 1700 ± 1400 $\mu\text{g/L}$) NGAL concentrations in SF from patients with rheumatoid arthritis. Similarly to our findings, concentrations correlated significantly with the neutrophil count in SF, and those researchers concluded that NGAL could be used as a marker of joint inflammation.²³ In two studies, researchers identified NGAL in SF from patients with osteoarthritis^{24,25} but at lower concentrations than in rheumatoid arthritis²⁵ and septic arthritis/PJI.²² Using a cut off value of 2200 $\mu\text{g/L}$, Deimengian et al²² found that NGAL in SF had 100% sensitivity and specificity for septic arthritis/PJI in man, and they concluded that use of NGAL could improve diagnostic accuracy of this condition. To fully understand the diagnostic potential of NGAL in horses, future studies must measure the protein in different types of joint disease and assess its true diagnostic capacity (eg, sensitivity and specificity) in clinically relevant populations.

Concentrations of NGAL in SF from healthy individuals have not previously been reported in the horse or in other species. In our study, concentrations of NGAL in SF from healthy horses were uniformly low (75% percentile was 7.2 $\mu\text{g/L}$). Biomarkers such as NGAL that combine the desirable properties of (1) low levels in healthy individuals and (2) a large-amplitude response to inflammation have a favorable signal-to-noise ratio. This facilitates interpretation of measured concentrations and limits the clinical effect of interindividual variation in baseline concentrations.⁵

A biomarker's ability to reflect severity of inflammation is of importance when the biomarker is used for monitoring changes in inflammatory activity, for example, in response to treatment or during exacerbation/relapse of disease.⁵ Concentrations of NGAL in SF correlated positively with WBC but with moderate effect size ($R^2 = 0.49$), and NGAL may, thus, partially reflect intensity of synovial inflammation. This finding was further corroborated by SF NGAL levels after experimental injection of the two local analgesics. Intra-articular injections of lidocaine and mepivacaine cause moderate and mild inflammation, respectively,²⁹ and this was matched by higher SF NGAL concentrations in the lidocaine group compared with the mepivacaine group. These findings provide evidence that NGAL is a potential new biomarker for assessing severity of/monitoring inflammation similar to what has been demonstrated for SAA.⁴

The source of the NGAL found in SF is not entirely clear. Neutrophil gelatinase-associated lipocalin is released from neutrophil granulocytes,¹⁶ and the significant correlation between NGAL concentrations and WBC in SF found in our study as well as in a study in man²³

provides evidence that infiltrating leukocytes are responsible for the accumulation of NGAL in SF. However, others have found weak correlation between WBC and NGAL in SF from people with septic arthritis/PJI,²² providing evidence that other cell types may contribute to intra-articular NGAL concentrations by local synthesis. The same may be true for horses because the magnitude of correlation between SF WBC and NGAL concentrations was only moderate ($R^2 = 0.49$). Researchers performing *in vitro* studies have found expression of NGAL in cultured murine chondrocytes in response to stimulation with proinflammatory cytokines and glucocorticoids,^{32,33} thus providing corroborating evidence that NGAL may be produced locally by different types of cells in the inflamed joint compartment.

The study presented here has several limitations that must be addressed. The prolonged storage (up to 5 years) of the samples used in the study could be a limitation. However, it has been shown in previous studies that human NGAL is quite stable in biological fluids and that prolonged storage or repeated freeze-thaw cycles have minimal effect on measured concentrations.^{34,35} The main limitations of our study are the assay used and the limited number of clinical cases included. The assay used for this study is an ELISA. Enzyme-linked immunosorbent assays are suited for batch analyses of research samples but are not useful in the clinic, where single test random-access is required.⁵ For NGAL to gain more widespread use in equine arthrology, assays relevant for horse-side assessment of the protein should be developed, similarly to what has been done for other novel biomarkers such as SAA.¹⁰ An additional prerequisite for clinical use of NGAL is that the NGAL response becomes characterized in a variety of different joint conditions and that effects of interventions such as repeated arthrocentesis, joint surgery/lavage, and intra-articular administration of drugs are clarified similarly to what has been done for WBC, TP, and SAA.^{6-8,12,28} Future studies on NGAL response in a variety of joint conditions will further elucidate the usefulness of NGAL for diagnosing and monitoring joint inflammation.

In conclusion, concentrations of NGAL in SF and, to a lesser extent in serum, were elevated in experimental and spontaneous inflammatory joint conditions in horses and appeared to reflect intensity of inflammation. In SF derived from healthy horses, NGAL concentrations were low, and the intra-articular NGAL response to inflammation had a large magnitude. In combination with the fast rise-and-fall pattern found in the experimentally induced joint inflammation, these findings provide evidence that NGAL may serve as a diagnostic marker of joint inflammation and that it possesses the ability to monitor changes in disease activity. These findings warrant exploration of the diagnostic

potential of NGAL in larger, well-defined, and clinically relevant populations of horses with joint disease.

ACKNOWLEDGMENTS

The authors thank the staff of The Large Animal Teaching Hospital of University of Copenhagen for assisting with sampling from horses; and Pia Haubro Andersen and Denis Verwilghen DVM, MSc, PhD, DECVS-LA for their support and supervision, DVM, PhD, DVSci of the experimental studies (LPS, local analgesics) that provided samples for this study; and Claus Ekström, MSc, PhD, University of Copenhagen, for his feedback on the statistical analyses.

The study was funded by the Kustos Foundation of 1881. The foundation had no influence on study design or data interpretation and presentation.

AUTHOR CONTRIBUTIONS

Frydendal C, DVM: Collected the data from clinical cases, performed the NGAL analyses on all samples, assisted with analyses of all data, drafted parts of the manuscript, and approved the final submitted version of the manuscript; Nielsen KB, DVM: Collected the data from clinical cases, performed the NGAL analyses on all samples, assisted with analyses of all data, drafted parts of the manuscript, and approved the final submitted version of the manuscript; Berg LC, DVM, PhD: Assisted with design of the study, assisted with interpretation of the NGAL analysis results, critically revised the manuscript, and approved the final submitted version of the manuscript; van Galen G, DVM, MSc, PhD, DECEIM (equine), DECVS: Assisted with design of the study, assisted with interpretation of the NGAL analysis results, critically revised the manuscript, and approved the final submitted version of the manuscript; Adler DMT, DVM, PhD: Collected and interpreted the data from horses undergoing lidocaine and mepivacaine injection, critically revised the manuscript, and approved the final submitted version of the manuscript; Andreassen SM, DVM, PhD: Collected and interpreted the LPS data, and approved the final submitted version of the manuscript; Jacobsen S, DVM, PhD, DECVS-LA: Conceived and designed the study, assisted with collection of the LPS data, reviewed the medical records of clinical cases, performed all data analyses, interpreted the NGAL analysis results from all groups, and wrote the manuscript.

CONFLICT OF INTEREST

The authors declare no conflicts of interest related to this report.

ORCID

Ditte M. T. Adler  <https://orcid.org/0000-0001-5042-0810>
Stine Jacobsen  <https://orcid.org/0000-0002-2380-4576>

REFERENCES

- Adkins AR. Establishing a diagnosis of septic arthritis/osteomyelitis—a challenging process. *Equine Vet Educ.* 2012; 24:615-617.
- Gilbertie JM, Schnabel LV, Stefanovski D, Kelly DJ, Jacob ME, Schaefer TP. Gram-negative multi-drug resistant bacteria influence survival to discharge for horses with septic synovial structures: 206 Cases (2010-2015). *Vet Microbiol.* 2018;226:64-73.
- Ludwig EK, van Harreveld PD. Equine wounds over synovial structures. *Vet Clin North Am Equine Pract.* 2018;34:575-590.
- Haltmayer E, Schwendenwein I, Licka TF. Course of serum amyloid A (SAA) plasma concentrations in horses undergoing surgery for injuries penetrating synovial structures, an observational clinical study. *BMC Vet Res.* 2017;13:137.
- Kjelgaard-Hansen M, Jacobsen S. Assay validation and diagnostic applications of major acute-phase protein testing in companion animals. *Clin Lab Med.* 2011;31:51-70.
- Jacobsen S, Thomsen MH, Nanni S. Concentrations of serum amyloid A in serum and synovial fluid from healthy horses and horses with joint disease. *Am J Vet Res.* 2006;67:1738-1742.
- Sanchez-Teran AF, Bracamonte JL, Hendrick S, et al. Effect of arthroscopic lavage on systemic and synovial fluid serum amyloid A in healthy horses. *Vet Surg.* 2016;45:223-230.
- Sanchez-Teran AF, Rubio-Martinez LM, Villarino NF, Sanz MG. Effects of repeated intra-articular administration of amikacin on serum amyloid A, total protein and nucleated cell count in synovial fluid from healthy horses. *Equine Vet J Suppl.* 2012;43:12-16.
- Jacobsen S, Niewold TA, Halling-Thomsen M, et al. Serum amyloid A isoforms in serum and synovial fluid in horses with lipopolysaccharide-induced arthritis. *Vet Immunol Immunopathol.* 2006;110:325-330.
- Ludwig EK, Brandon Wiese R, Graham MR, et al. Serum and synovial fluid serum amyloid A response in equine models of synovitis and septic arthritis. *Vet Surg.* 2016;45:859-867.
- Schwartz D, Pusterla N, Jacobsen S, Christopher MM. Analytical validation of a new point-of-care assay for serum amyloid A in horses. *Equine Vet J.* 2018;50:678-683.
- Sanchez-Teran AF, Bracamonte JL, Hendrick S, et al. Effect of repeated through-and-through joint lavage on serum amyloid A in synovial fluid from healthy horses. *Vet J.* 2016;210:30-33.
- Lindgaard C, Glerup KB, Thomsen MH, Martinussen T, Jacobsen S, Andersen PH. Anti-inflammatory effects of intra-articular administration of morphine in horses with experimentally induced synovitis. *Am J Vet Res.* 2010;71:69-75.
- Makris K, Rizos D, Kafkas N, Haliassos A. Neutrophil gelatinase-associated lipocalin as a new biomarker in laboratory medicine. *Clin Chem Lab Med.* 2012;50:1519-1532.
- Kjeldsen L, Bainton DF, Sengelov H, Borregaard N. Identification of neutrophil gelatinase-associated lipocalin as a novel matrix protein of specific granules in human neutrophils. *Blood.* 1994;83:799-807.
- Kjeldsen L, Johnsen AH, Sengelov H, Borregaard N. Isolation and primary structure of NGAL, a novel protein associated with human neutrophil gelatinase. *J Biol Chem.* 1993;268:10425-10432.
- Bolignano D, Donato V, Coppolino G, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a marker of kidney damage. *Am J Kidney Dis.* 2008;52:595-605.

18. Xiao X, Yeoh BS, Vijay-Kumar M. Lipocalin 2: an emerging player in iron homeostasis and inflammation. *Annu Rev Nutr.* 2017;37:103-130.
19. Mishra J, Dent C, Tarabishi R, et al. Neutrophil gelatinase-associated lipocalin (NGAL) as a biomarker for acute renal injury after cardiac surgery. *Lancet.* 2005;365:1231-1238.
20. Saleh NY, Abo El Fotoh WMM, El-Hawy MA. Serum neutrophil gelatinase-associated lipocalin: a diagnostic marker in pediatric sepsis. *Pediatr Crit Care Med.* 2017;18:e245-e252.
21. Martensson J, Bell M, Oldner A, Xu S, Venge P, Martling CR. Neutrophil gelatinase-associated lipocalin in adult septic patients with and without acute kidney injury. *Intensive Care Med.* 2010;36:1333-1340.
22. Deirmengian C, Kardos K, Kilmartin P, Cameron A, Schiller K, Parvizi J. Diagnosing periprosthetic joint infection: has the era of the biomarker arrived? *Clin Orthop Relat Res.* 2014;472:3254-3262.
23. Blaser J, Triebel S, Tschesche H. A sandwich enzyme immunoassay for the determination of neutrophil lipocalin in body fluids. *Clin Chim Acta.* 1995;235:137-145.
24. Gupta K, Shukla M, Cowland JB, Malemud CJ, Haqqi TM. Neutrophil gelatinase-associated lipocalin is expressed in osteoarthritis and forms a complex with matrix metalloproteinase 9. *Arthritis Rheum.* 2007;56:3326-3335.
25. Katano M, Okamoto K, Arito M, et al. Implication of granulocyte-macrophage colony-stimulating factor induced neutrophil gelatinase-associated lipocalin in pathogenesis of rheumatoid arthritis revealed by proteome analysis. *Arthritis Res Ther.* 2009;11:R3.
26. Jacobsen S, Berg LC, Tvermose E, Laurberg MB, van Galen G. Validation of an ELISA for detection of neutrophil gelatinase-associated lipocalin (NGAL) in equine serum. *Vet Clin Pathol.* 2018;47:603-607.
27. Van Galen G, Jacobsen S, Vinther AM, et al. NGAL: a new biomarker in the horse for renal injury and inflammation. *J Vet Intern Med.* 2019;33:1549-1549.
28. Andreassen SM, Vinther AML, Nielsen SS, et al. Changes in concentrations of haemostatic and inflammatory biomarkers in synovial fluid after intra-articular injection of lipopolysaccharide in horses. *BMC Vet Res.* 2017;13:182.
29. Adler DMT, Serteyn D, Franck T, et al. Effects of intra-articular administration of lidocaine, mepivacaine, and the preservative methyl parahydroxybenzoate on synovial fluid biomarkers of horses. *Am J Vet Res.* 2020;81:479-487.
30. Shashidharamurthy R, Machiah D, Aitken JD, et al. Differential role of lipocalin 2 during immune complex-mediated acute and chronic inflammation in mice. *Arthritis Rheum.* 2013;65:1064-1073.
31. Hokamp JA, Nabity MB. Renal biomarkers in domestic species. *Vet Clin Pathol.* 2016;45:28-56.
32. Conde J, Lazzaro V, Scotece M, et al. Corticoids synergize with IL-1 in the induction of LCN2. *Osteoarthritis Cartilage.* 2017;25:1172-1178.
33. Choi WS, Chun JS. Upregulation of lipocalin-2 (LCN2) in osteoarthritic cartilage is not necessary for cartilage destruction in mice. *Osteoarthritis Cartilage.* 2017;25:401-405.
34. Pedersen KR, Ravn HB, Hjørtedal VE, Norregaard R, Povlsen JV. Neutrophil gelatinase-associated lipocalin (NGAL): validation of commercially available ELISA. *Scand J Clin Lab Invest.* 2010;70:374-382.
35. Schuh MP, Nehus E, Ma Q, et al. Long-term stability of urinary biomarkers of acute kidney injury in children. *Am J Kidney Dis.* 2016;67:56-61.

How to cite this article: Frydendal C, Nielsen KB, Berg LC, et al. Influence of clinical and experimental intra-articular inflammation on neutrophil gelatinase-associated lipocalin concentrations in horses. *Veterinary Surgery.* 2021; 50:641–649. <https://doi.org/10.1111/vsu.13582>