ORIGINAL ARTICLE

Veterinary Clinical Pathology
An International Journal of Laboratory Medicine WILEY

Markers of inflammation in free-living African elephants (*Loxodonta africana*): Reference intervals and diagnostic performance of acute phase reactants

Christine Steyrer[1] | Michele Miller[2] | Jennie Hewlett[3] | Peter Buss[3,4] | Emma H. Hooijberg[1] 🆔

[1]Department of Companion Animal Clinical Studies and Centre for Veterinary Wildlife Research, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[2]Department of Science and Innovation-National Research Foundation Centre of Excellence for Biomedical TB Research, South African Medical Research Council Centre for Tuberculosis Research, Division of Molecular Biology and Human Genetics, Faculty of Medicine and Health Sciences, Stellenbosch University, Cape Town, South Africa

[3]Department of Production Animal Studies and Centre for Veterinary Wildlife Research, Faculty of Veterinary Science, University of Pretoria, Pretoria, South Africa

[4]Veterinary Wildlife Services, South African National Parks, South Africa

Correspondence
Emma H. Hooijberg, Department of Companion Animal Clinical Studies, Faculty of Veterinary Science, University of Pretoria, Private Bag X04, Onderstepoort, Pretoria 0110, South Africa.
Email: emma.hooijberg@up.ac.za

Abstract

Introduction: Acute phase reactants (APRs) have not been investigated in free-living African elephants (*Loxodonta africana*), and there is little information about negative APRs albumin and serum iron in elephants.

Objectives: We aimed to generate reference intervals (RIs) for APRs for free-living African elephants, and to determine the diagnostic performance of APRs in apparently healthy elephants and elephants with inflammatory lesions.

Methods: Stored serum samples from 49 apparently healthy and 16 injured free-living elephants were used. The following APRs and methods were included: albumin, bromocresol green; haptoglobin, colorimetric assay; serum amyloid A (SAA), multispecies immunoturbidometric assay, and serum iron with ferrozine method. Reference intervals were generated using the nonparametric method. Indices of diagnostic accuracy were determined by receiver-operator characteristic (ROC) curve analysis.

Results: Reference intervals were: albumin 41–55 g/L, haptoglobin 0.16–3.51 g/L, SAA < 10 mg/L, and serum iron 8.60–16.99 μmol/L. Serum iron and albumin concentrations were lower and haptoglobin and SAA concentrations were higher in the injured group. Serum iron had the best ability to predict health or inflammation, followed by haptoglobin, SAA, and albumin, with the area under the ROC curve ranging from 0.88–0.93.

Conclusions: SAA concentrations were lower in healthy African vs Asian elephants, and species-specific RIs should be used. Serum iron was determined to be a diagnostically useful negative APR which should be added to APR panels for elephants.

KEYWORDS
albumin, clinical decision limit, haptoglobin, serum amyloid A, serum iron

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1 | INTRODUCTION

The acute phase response has been well-studied in veterinary medicine over the past decade, with several publications focusing on zoo and wild animals, including species such as manatees, dolphins, elephant seals, rhinoceros, capybara, and cheetah.[1–6] Acute phase reactants (APRs) are substances that either increase (positive) or decrease (negative) during inflammation and include acute phase proteins, lipoproteins, and non-protein molecules, like serum iron and zinc.[7] Acute phase proteins have been investigated fairly comprehensively in captive Asian elephants (*Elephas maximus*), to a limited extent in captive African elephants (*Loxodonta africana*), and not at all to our knowledge in free-living elephants of either species.[8–12] Studies concerning acute phase reactants (APRs) in captive elephants have focused on serum amyloid A (SAA) and haptoglobin, which are major and moderate APRs, respectively, in these species and increase in conditions common in captive elephants, like elephant endotheliotropic herpesvirus (EEHV) infection, pododermatitis, ulcerative dermatitis, and tusk infections.[8–12]

Few investigations have been carried out to characterize changes in negative APRs, such as albumin and serum iron in elephants. The pathways underlying changes in iron metabolism during inflammation have been well described.[13,14] A major mechanism involves the inflammatory cytokine interleukin-6, which causes increased hepcidin expression. Hepcidin causes degradation of ferroportin, the cellular iron exporter, leading to a reduction in iron transport out of hepatocytes and macrophages, resulting in hypoferremia.[13] Serum iron concentrations decrease rapidly after an inflammatory stimulus; for example, in humans given intravenous lipopolysaccharide, hypoferremia was detectable within 6 hours of the treatment.[15] This nutritional immunity is protective for a host organism as iron is essential for the metabolism of many microbial pathogens.[16] Further details on iron metabolism during inflammation are available in comprehensive reviews on this topic.[13,14] In veterinary medicine, low serum iron has been documented in various animal species with inflammatory diseases, and serum iron has been shown to be a good biomarker for inflammation in horses, cattle, and white rhinoceros.[4,17–21]

African elephants are endangered, with increasing human populations, habitat loss, unsustainable harvesting of ivory and human-elephant conflict all considered significant risks to species survival.[22,23] Optimized diagnostic testing and treatment of diseased or wounded elephants is important as part of a multifaceted approach to the conservation of this species. As mentioned, no information is available concerning APRs or changes in inflammatory markers during disease in free-living elephants. Free-living elephant populations have different social structures, habitats, nutrition, and stressors compared with captive elephants. Diseases reported in free-living African elephants include traumatic injuries as a result of natural causes (inter-species fighting, predators) and human-elephant conflict.[24–26] The latter was reported to be a cause of injury in 67% of free-living elephants requiring veterinary care in Kenya, with injuries resulting from arrows, spears, bullets, and snares.[25] Wire snares around the neck and foot in particular are commonly used to trap other animals for bushmeat, and elephants can be unintentionally trapped or injured.[26,27] Snare injuries can cause deep wounds to the lower limbs and trunks of elephants, and sometimes result in amputation.[25] Wounds, resulting tissue trauma, necrosis, and possible infection, cause acute phase responses.[28,29] As wounds are easily identifiable and are the most common reasons for free-living elephants to be immobilized for veterinary care; we used elephants with wounds from snares and other causes as a model of inflammation in our investigation.

The aim of this study was to investigate the acute phase response in free-living African elephants from the Kruger National Park, South Africa. Specific objectives included (a) generation of reference intervals (RIs) for APRs for this population and (b) assessment of overlap and diagnostic performance of APRs in apparently healthy elephants and elephants with inflammatory lesions. Four APRs were evaluated: albumin, haptoglobin, SAA, and serum iron. We hypothesized that RIs would be similar to those published for captive African elephants, elephants with inflammation would exhibit an acute phase response, and that APRs would have high diagnostic accuracy for diagnosing the presence or the absence of inflammation.

2 | MATERIALS AND METHODS

2.1 | Study population and sample collection

Serum samples used in this study originated from the free-living African elephant population in the Kruger National Park, South Africa. All samples from apparently healthy elephants were collected from animals immobilized for reasons unrelated to this study (ecological, veterinary management purposes, or unrelated research projects), according to the South African National Parks Standard Operating Procedure for the Capture, Transport, and Maintenance in Holding Facilities of Wildlife. Elephants with injuries were immobilized because they required veterinary interventions. The project was approved by the Research Ethics Committee of the Faculty of Veterinary Science and the Animal Ethics Committee, University of Pretoria (certificate number REC132-19).

Reference intervals for serum clinical chemistry measurands were previously generated from these samples; full details of immobilization, sample collection, analyses, and RI generation for albumin specifically, have already been described.[30] Briefly, animals were darted from a helicopter using a combination of etorphine (0.003 mg/kg; Novartis, South Africa), azaperone (0.01 mg/kg; Janssen Pharmaceutical Ltd., South Africa), and hyaluronidase (Kyron Laboratories, South Africa), and immobilization was reversed with naltrexone (Kyron Laboratories, South Africa) at twenty times the etorphine dose in mg. Age was estimated based on molar progression, and weight was estimated by an experienced wildlife veterinarian (PB) based on measured weights of individuals of similar size in this population.[31] Immobilized elephants were positioned into lateral recumbency (the side that they fell onto), and a cursory clinical examination was performed, which

included inspection of the available skin surface and external structures for injuries or abnormalities, examination of the oral cavity, palpation of auricular arteries for pulse rhythm and relative pulse pressure and evaluation of tick load. Elephants without any obvious abnormalities were classified as apparently healthy; if animals had wounds or other abnormalities, these were documented. Demographic and clinical information (sex, estimated age and weight, microchip number, geographical location, and clinical findings) were recorded for each elephant.

Blood was collected from an auricular vein using an 18G needle into two 9 ml serum vacutainer tubes (BD Biosciences, USA). Both tubes were placed upright into a polystyrene cooler box containing cooler bricks; serum was left to clot for at least 30 min. Samples were transported in the cooler box and reached the Veterinary Wildlife Services (VWS) laboratory, Skukuza, Kruger National Park, within 6 hours of collection. After centrifugation (Hermle Z383, Hermle Labortechnik GmbH, Germany; 1300 g, 10 min), serum was aliquoted into cryovials (2 ml, Greiner Bio-One, Lasec S.A., Pty Ltd.) and frozen at −80°C.

2.2 | Analytical methods

For albumin determination, one aliquot of serum (per animal) was thawed and analyzed on-site at the VWS laboratory using a VetScan VS2 (Abaxis, USA) using the Large Animal Rotor (Zoetis, South Africa).[30] This analyzer performs an internal electronic quality control check on each rotor. Between-rotor imprecision for albumin measurement on elephant serum was determined by measuring pooled serum on 20 rotors over 1 day, and the CV was calculated.[30]

When available, a second serum aliquot was transported on dry ice to the Clinical Pathology Laboratory at the Faculty of Veterinary Science, Onderstepoort, South Africa, and stored for a month at −80°C. These samples were batch thawed to room temperature, gently mixed, and evaluated over the course of 1 day. Measurements of haptoglobin, SAA, and serum iron were performed using a wet chemistry analyzer, the Cobas Integra 400 Plus (Roche Products [Pty] Ltd, Basel, Switzerland), as per the manufacturer's instructions. Haptoglobin was measured with a colorimetric peroxidase assay using a modified serum calibrator (species unknown) (PHASE Haptoglobin Assay, Tridelta, Maynooth, Ireland). The limit of the blank for this assay is 0.005 g/L. Serum amyloid A was measured with a multispecies immunoturbidometric assay containing polyclonal and monoclonal anti-human SAA antibodies using a modified human serum calibrator (LZ-SAA Assay, Eiken Chemical Co., Japan). The detection limit for this assay in this laboratory was set at 10 mg/L based on internal validation for other species. Serum iron was determined with the ferrozine zinc method with a modified human serum calibrator (Roche Products [Pty] Ltd, Basel, Switzerland). With this ferrozine method, Fe^{3+} ions are liberated from transferrin and reduced to Fe^{2+} ions, which form a colored complex with ferrozine. The manufacturer's lower detection limit for this assay is 0.9 μmol/L.

For all three methods, assay performance was monitored by daily internal quality control procedures according to laboratory protocols and performance goals published for domestic species.[32,33]

Partial analytical validation was performed for haptoglobin and SAA. Intra-assay imprecision and linearity were determined using recommended protocols.[34] For the linearity experiments, a serum pool with high concentrations of haptoglobin and SAA and an eight-step dilution series performed with triplicate measurements were prepared. Linearity was evaluated using Spearman's correlation coefficient (r) and linear regression analysis. For the intra-assay imprecision study, two pools of sera were created: a low concentration pool from the group of healthy elephants and one from the injured group with expected high concentrations. Analyte concentration was determined from these pools 20 times over the course of 1 day. The performance goals for maximum imprecision were set at 8.5% for haptoglobin and 20% for SAA.[4] An inter-assay imprecision study was not performed as reagents were used up after 2 days.

To assess the potential effect of storage time on APR measurands, the correlation between months in storage and APR concentration was assessed using Pearson's correlation coefficient (rho, ρ), with $p < 0.05$ considered significant.

2.3 | Reference intervals

The RI generation procedure for albumin has been described previously; RIs were generated from 50 samples using the non-parametric method.[30] Results from 49 available samples from apparently healthy elephants were used to generate RIs for haptoglobin, SAA, and serum iron. Statistical analyses were performed using MedCalc software version 19.1.7 (MedCalc Software, Ostend, Belgium) (Shapiro–Wilk test and outlier identification) and Reference Value (RefVal) Advisor version 2.1 (Ecole Nationale Vétérinaire de Toulouse, France) (descriptive statistics and histograms, outlier identification, reference limit [RL] calculations) according to ASVCP guidelines.[35,36] Histograms were visually inspected, and Dixon and Tukey tests were used to identify outliers. If an outlier was detected, then the entire data set from that individual was excluded and the analyses were rerun. This process was repeated until no more outliers were detected. Due to the size of the final reference sample group, a p-value cut-off of >0.2 for the Shapiro–Wilk test was used to increase the specificity of normality testing.[37] If $p > 0.2$, the parametric method was used to determine the RLs; if $p ≤ 0.2$, the nonparametric method was used.[38] The 90% CI (confidence intervals) of the RLs were calculated using a bootstrap method, and the ratio of the upper or lower CI to the RI was calculated by dividing the former by the latter.

2.4 | Overlap performance and diagnostic utility

Acute phase reactant results from the healthy and injured groups were compared using the Mann–Whitney U test; this nonparametric test was used because of the small size of the injured group.

Receiver-operator characteristic (ROC) curve analysis was performed for each measurand that was significantly different between the two groups and the area under the curve (AUC) and diagnostic cut-off value (DCOV; represented by the Youden index, which is the point on the curve where both sensitivity and specificity are optimal) were determined.[39] The sensitivity and specificity at the clinically significant RL (upper RL for positive APRs, lower RL for negative APRs) and the DCOV were determined using MedCalc.[39]

The predictive ability of a combination of inflammatory markers to detect the presence or the absence of inflammation was determined by stepwise logistic regression analysis. The DCOV was used as the cut-off to dichotomize analytes as being negative or positive for the presence of inflammation. Independent variables were only included in the model if the p-value for the regression coefficient was <0.05. Results for SAA < 10 mg/L were set to 10 mg/L for the statistical analyses.

3 | RESULTS

Serum samples from free-living African elephants, collected between October 2014 and August 2019, were included in this study. Samples were analyzed in December 2019. Samples from 77 apparently healthy elephants were available for albumin measurement.[30] Of these, 49 samples contained sufficient material for measurement of the other APRs. Samples from sixteen injured animals were available. Fourteen of these animals had snares present (which were removed), one had a snare injury with no snare present, and one animal had cellulitis of unknown origin (Table 1). The 49 animals in the apparently healthy group included 45 males and 4 females, with 2 calves and 47 adults.

3.1 | Analytical performance

Imprecision for elephant serum albumin, measured on the VetScan VS2, was 1.1% (pool mean 45 g/L).[30] Imprecision for haptoglobin using elephant serum ranged from 0.4% (pool mean 2.33 g/L) to 2.7% (pool mean 0.18 g/L) and for SAA, from 1.3% (pool mean 137 mg/L) to 4.1% (pool mean 11 mg/L).

The haptoglobin assay was found to be linear up to 2.5 g/L (which was also the concentration of the highest calibrator). The SAA assay showed acceptable linearity up to 155 mg/L (highest concentration tested). All samples with initial results greater than the upper linearity limits were diluted 1:2 (haptoglobin; using sample diluent included in the kit) or 1:5 or 1:10 (SAA; using distilled water) to obtain the final results.

There was no significant correlation between months in storage and values obtained for albumin ($\rho = 0.07$), haptoglobin ($\rho = -0.04$) or serum iron ($\rho = -0.12$). No analysis was performed for SAA as many results were < 10 mg/L.

3.2 | Overlap performance

Results from all 49 apparently healthy elephants and from the 16 injured animals were compared (Table 2). Individual results for the injured elephants are shown in Table 1. There was a significant increase in haptoglobin and SAA concentrations and a significant decrease in albumin and serum iron concentrations in the injured compared with the healthy group. Box and whisker plots for measurands with significant changes are presented in Figure 1.

3.3 | Reference intervals

Previously reported reference intervals for albumin are presented in Table 3; 50 samples were included after outlier exclusion.[30] For haptoglobin, SAA, and serum iron, datasets from six of the original 49 reference individuals were excluded after outlier analysis (identified by both MedCalc and RefVal). Four animals had high outliers for SAA, one of which also had a high outlier for haptoglobin. One animal had a high outlier for haptoglobin and one for serum iron. Two of the animals with high outliers for SAA were apparently healthy calves. One of these was captured as it had escaped the reserve with its mother; the other was immobilized to remove a wire snare hanging loosely around its neck, with no skin injury. The remaining four apparently healthy animals were adults, captured for other studies. The final group of 43 elephants used for reference interval generation included 1 female and 42 males, all adults. The haptoglobin and serum iron data were non-Gaussian, and the nonparametric method was used to generate RLs. After outlier elimination, all results for SAA were less than the set laboratory detection limit for this assay (10 mg/L). All results pertaining to RIs are presented in Table 3.

3.4 | Diagnostic utility

The prevalence of inflammation in elephants selected for this study, as represented by the injured animals, was 24.6% (16/65). Based on ROC-curve analysis, serum iron had the highest AUC, followed by haptoglobin, SAA and albumin (Table 4). All these measurands had an AUC that was significantly greater than 0.5, but their AUCs were not significantly different from one another. Using the lower RL (albumin, serum iron) or upper RL (haptoglobin, SAA) as a diagnostic limit for the presence of inflammation generally resulted in high specificity but moderate to low sensitivity (Table 4). The DCOVs were within the RIs for albumin and haptoglobin, and associated with improved sensitivity but decreased specificity compared with the RL for these measurands. The DCOV was below the lower RL for serum iron and above the upper RL for SAA and was associated with improved specificity with no loss of sensitivity, compared with the RL. Positive LRs at the DCOVs for this population ranged from 7.66 (haptoglobin) to 42.87 (serum iron), and negative LRs from 0.07 (haptoglobin) to

TABLE 1 Demographic and clinical information and results for acute phase reactants in 16 free-living African elephants from the Kruger National Park with snare and other injuries, representing animals with inflammation

Elephant Number	Sex	Life Stage	Injury	Albumin (g/L)	Haptoglobin (g/L)	Serum iron (μmol/L)	SAA (mg/L)
1	Female	Prime adult	Snare removal, right hind leg, soft tissue defect, appeared infected	29	6.39	5.1	80
2	Female	Young adult	Snare removal, around thorax, just behind mammary glands, deep tissue cut	38	7.23	5.3	423
3	Female	Young adult	Snare removal (below carpus), tissue appeared infected, severe necrosis	35	3.72	5.7	361
4	Female	Young adult	Snare around right front distal of carpus, wound appeared infected, necrosis, swollen distal limb. Snare probably present for at least 4 months	29	6.09	4.8	386
5	Male	Prime adult	Cellulitis of left front leg, severe nonweight bearing	37	7.41	4.1	301
6	Female	Old	Snare removal right front, poor body condition	41	6.72	4.5	555
7	Male	Young adult	Snare around left carpus, swollen, no skin laceration	45	3.60	7.0	920
8	Male	Young adult	Snare around the trunk, chronic with minimal tissue damage but constricting nares and animal struggling to breath	53	0.39	5.1	<10
9	Female	Not available	Snared	37	5.61	5.6	99
10	Female	Calf	Cable snare around left hind foot	46	9.85	4.1	443
11	Male	Young adult	Snare wound on left hind foot	43	6.03	9.0	129
12	Male	Juvenile (6y)	Cable snare around left hind foot, wounded	30	6.06	3.4	402
13	Male	Prime adult	Cable snare, left hind foot	48	3.33	7.5	30
14	Male	Calf	Snare around the head	38	3.24	19.7	<10
15	Male	Young adult	Snare around right front leg, wounded	30	2.94	5.0	34
16	Male	Sub adult	Snare wound around front leg, wounded	40	6.39	4.5	136

TABLE 2 Acute phase reactant results from free-living apparently healthy and injured African elephants from the Kruger National Park

Measurand	Median (range) healthy elephants	Median (range) injured elephants	Mann–Whitney U p-value
Albumin (g/L)	48 (41–56)	38 (29–53)	<0.0001
Haptoglobin (g/L)	1.28 (0.16–5.37)	6.05 (0.39–9.85)	<0.0001
SAA (mg/L)	10 (10–1093)	218 (10–920)	<0.0001
Serum iron (μmol/L)	11.7 (7.4–21.9)	5.1 (3.4–19.7)	<0.0001

0.26 (albumin). The box and whisker plots in Figure 1 illustrate the results for the two groups with both the relevant RLs and DCOVs. The combined ROC curves for the four measurands are presented in Figure 2.

Of the 16 injured elephants, eight showed changes consistent with an acute phase response for all four APRs, while three more showed typical changes for haptoglobin, serum iron, and SAA concentrations but not for albumin concentrations (Table 1). Three showed changes in two or three of the four APRs in various other combinations. One injured elephant (number 8) showed only hypoferremia, and one (number 14) showed only hypoalbuminemia.

Only serum iron was shown to predict the presence of inflammation and injury significantly, based on stepwise logistic regression analysis (regression coefficient 5.81, $p < 0.001$). The odds that an elephant with a serum iron concentration of ≤7.5 μmol/L would have an inflammatory injury was 336.0 (95% CI 28.4–3985.2). Using a serum iron concentration cut-off value of 7.5 μmol/L, inflammation could be predicted in 95% of cases.
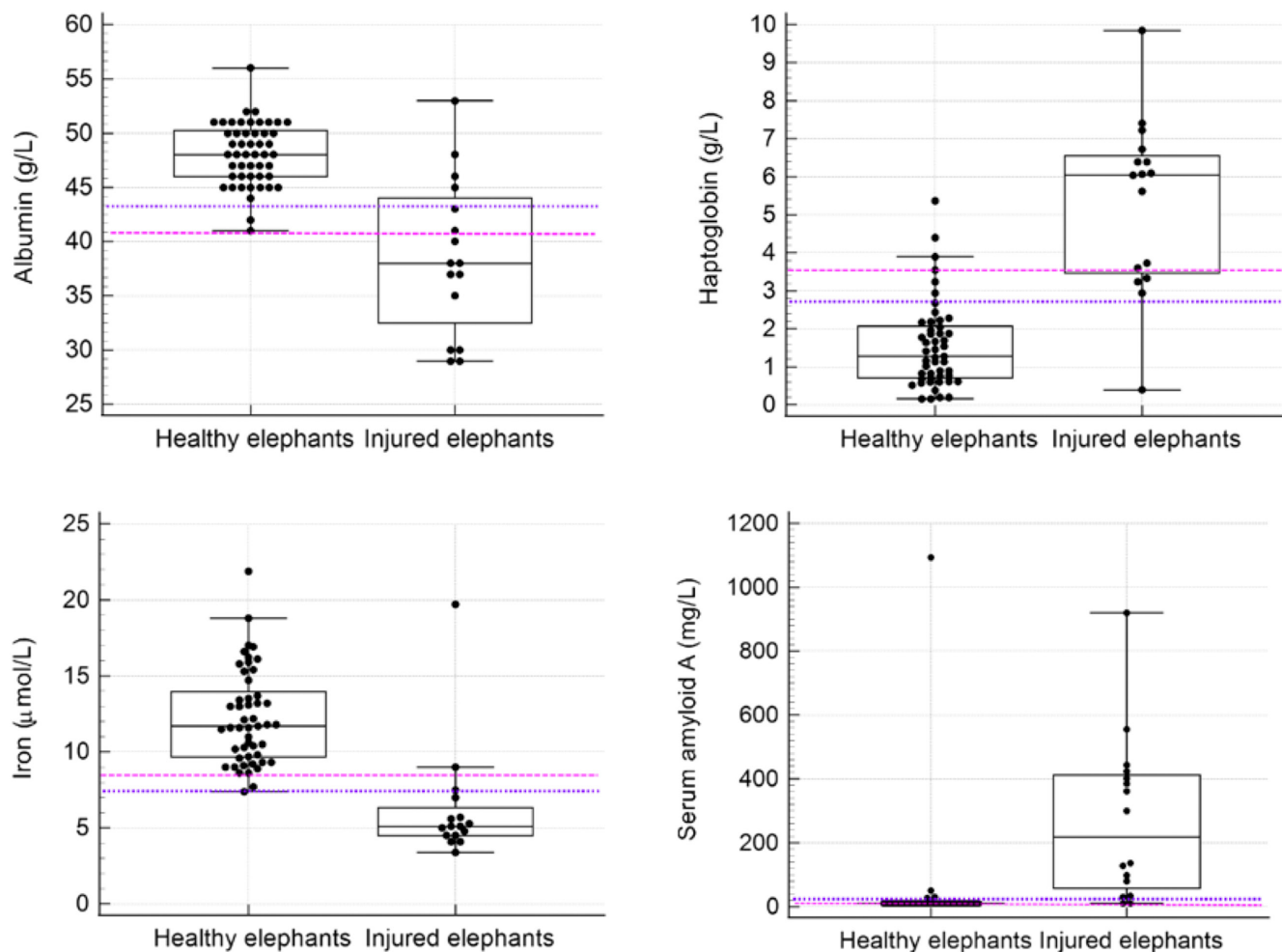
FIGURE 1 Box and whisker-plots comparing acute phase reactants between free-living African elephants (n = 49) and those with inflammatory injuries (n = 16). There was a significant difference ($p < 0.0001$) between groups for all measurands. The black dots represent results from individual animals, the box encompasses the 25th-75th percentiles, the middle horizontal line represents the median, and the outside horizontal lines indicate the minimum and maximum excluding the far-out values. The pink dotted lines indicate the clinically relevant reference limit for each measurand, and the purple dotted lines indicated the diagnostic cut-off value determined by ROC-curve analysis.

4 | DISCUSSION

This study confirmed that SAA and haptoglobin are major and moderate positive APRs in free-living African elephants and showed that serum iron, a negative APR, had strong predictive ability to identify elephants with inflammation.

Although the APRs included in this report have been studied in both captive Asian and African elephants, these measurands have not been investigated in healthy and injured free-living African elephants. The Kruger National Park is 2 million hectares in size, and elephants and other indigenous animals roam freely within the natural savanna ecosystem of the park, with minimal human-animal contact. The environment, free-living status, and nature of the immobilization procedure of the individuals included here mean that obtaining a history and full clinical evaluation for the apparently healthy elephants was not possible, and animals were not screened for internal parasites or infectious diseases. Ticks were present, but the burdens were considered normal for healthy free-living elephants. On the basis of autopsies performed on elephants in the Kruger National Park, this population may have a high prevalence (up to 50%) of hepatic filariasis, causing vasculitis and cholangitis, and bile duct hookworms, also causing cholangitis.[40] Other diseases reported include pulmonary herpesvirus lymphoid nodules, gastric parabronemiasis, lymphadenitis, and ulcerative cystitis.[40] These are all disease processes that could cause systemic inflammation but can only be detected on post-mortem examination, and it is probable that some elephants with pathologies or inflammatory diseases not obvious on clinical examination were falsely included in the apparently healthy group. This misclassification is supported by the outliers detected during RI generation, in particular, the elephant with both high haptoglobin and SAA, with the latter measured as 1093 mg/L (Figure 1). Datasets from these individuals were excluded for RI generation but were included for the evaluation of diagnostic accuracy, as the classification of disease for this analysis was necessarily made based

TABLE 3 Reference intervals for serum acute phase reactants for free-living African elephants in the Kruger National Park, South Africa, using the VetScan VS2 bromocresol green method for albumin, the Tridelta PHASE haptoglobin assay, ferrozine zinc method for serum iron and Eiken LZ-SAA assay on a Roche Cobas Integra 400 Plus chemistry analyzer. The reference sample population for haptoglobin, SAA, and serum iron only contained one female elephant

Measurand (unit)	n	Mean	SD	Median	Min	Max	p-value (Shapiro–Wilk)	Distribution	Method	LRL of RI	URL of RI	CI 90% of LRL	CI 90% of URL
Albumin[30]	50	48	3	48	41	56	0.19	NG	NP	41	55	41–44[a]	52–56[a]
Haptoglobin (g/L)	43	1.34	0.81	1.16	0.16	3.54	0.03	NG	NP	0.16	3.51	0.16–0.22	2.63–3.54[a]
SAA (mg/L)	43	All results <10 mg/L								<10		Not computed	
Serum iron (µmol/L)	43	12.09	2.55	11.70	8.6	17	0.005	NG	NP	8.60	16.99	8.60–8.91	16.42–17.00

Abbreviations: LRL, lower reference limit; n, number of individuals; NG, non-Gaussian; NP, nonparametric; RI, reference interval; SAA, serum amyloid A; SD, standard deviation; URL, upper reference limit.

[a]Width of URL/RI >0.2.

on the presence (inflammation) or the absence (no inflammation) of snare or other injuries.[39]

In terms of the injured group, the wounds present varied in the severity and chronicity of tissue damage, although more detailed information than that presented in Table 1 is not available. The clinical outcome after treatment and snare removal is unknown due to the free-living nature of these elephants. It is well-documented that tissue injury (for example, surgery or wounds) causes an acute phase response.[28,29] The variation in response seen here is likely related to the degree and chronicity of tissue damage. Individual number 8, for example, only had hypoferremia, but injuries were described as mild and chronic, which explains the lack of changes in other APRs. Individual 14 was a calf that only exhibited hypoalbuminemia; detailed information on the nature of the snare injury is not supplied, and it is possible that this animal did not have much tissue damage, given the lack of response for the other APRs. Individuals 1–5, 12, and 16 had severe tissue injury or wounds, infection or necrosis, and exhibited moderate to marked changes in all APRs. Other elephants (individuals 13 and 15) had similar decreases in iron but only mild increases in SAA and no change in haptoglobin. Detailed information on the nature of the snare injuries in these two animals was not recorded, but they may have been more chronic or less severe than in other animals with a more marked acute phase response. Using this type of injury as a model of inflammatory disease does therefore have limitations, as some elephants may not have injuries severe enough to cause systemic inflammation. Because of these factors, the inaccurate discrimination of truly healthy free-living elephants from those with systemic inflammation is an unavoidable limitation of this study.

There was also a sex bias in the groups, in that mostly male elephants were included (54 out 65 animals), and the RIs generated may not be appropriate for female elephants. The sex bias is due to various reasons: bull elephants tend to be solitary and thus easier to immobilize compared with cows who reside in large family groups, bull elephants were prospectively included in protocols for other research studies, and bull elephants require veterinary intervention more commonly.[26] Although only a sporadic feature of human-elephant conflict in the Kruger National Park, crop-raiding by elephants occurs frequently in East Africa as game reserves are unfenced, with male elephants more likely to raid and thus become injured or killed by humans.[26,41]

Samples were stored at −80°C for up to 5 years before analysis. Haptoglobin and serum iron have been shown to be stable in human serum for up to 1 year when stored at −70°C, and serum iron was stable for up to 25 years at −25°C.[42,43] Equine SAA appears to be stable for at least 2.5 years in serum stored at −80°C.[44] There are no studies available detailing the stability of elephant APRs when stored for up to 5 years, and sample stability could be a confounding factor. However, there was no correlation between storage time and APR concentrations, suggesting that time in storage did not significantly affect results.

Although the ASVCP reference interval guidelines recommend the use of the robust method for non-Gaussian data when the

TABLE 4 Diagnostic accuracy (based on receiver-operator characteristic curve analysis) of acute phase reactants for the detection of inflammation in free-living African elephants in the Kruger National Park, South Africa. 95% confidence intervals for sensitivity and specificity and area under the curve are indicated in parentheses

Measurand	AUC	Applicable RL	Se (%) at LRL/URL	Sp (%) at LRL/URL	DCOV	Se (%) at DCOV	Sp (%) at DCOV	−LR at DCOV	+LR at DCOV
Albumin	0.88 (0.78–0.95)	41 g/L (LRL)[30]	68.8 (41.3–89.0)	98.0 (89.1–99.9)	≤43 g/L	75.0 (47.6–92.7)	95.9 (86.0–99.5)	0.26	18.37
Haptoglobin	0.92 (0.82–0.97)	3.51 g/L (URL)	75.0 (47.6–92.7)	93.9 (83.1–98.7)	>2.68 g/L	93.8 (69.8–99.8)	87.8 (75.2–95.4)	0.07	7.66
Serum amyloid A	0.91 (0.81–0.97)	10 mg/L (URL)	87.5 (61.7–98.4)	89.8 (77.8–96.6)	>27 mg/L	87.5 (61.7–98.4)	93.9 (83.1–98.7)	0.13	14.29
Serum iron	0.93 (0.84–0.98)	8.6 µmol/L (LRL)	87.5 (61.7–98.4)	89.8 (77.8–98.6)	≤7.5 µmol/L	87.5 (61.7–98.4)	98.0 (89.1–99.9)	0.13	42.87

Abbreviations: AUC, area under the curve; DCOV, diagnostic cut-off value (Youden index); +LR, positive likelihood ratio; −LR, negative likelihood ratio; LRL, lower reference limit; RL, reference limit; Se, sensitivity; Sp, specificity; URL, upper reference limit.
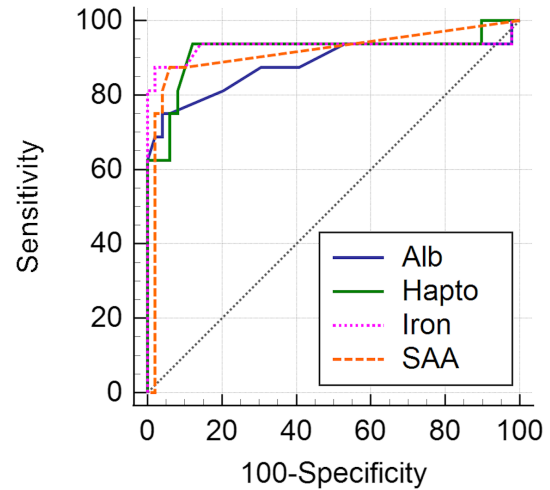

FIGURE 2 Receiver-operator characteristic curves for four acute phase reactants that show significant diagnostic accuracy for detecting inflammation in free-living African elephants in the Kruger National Park, South Africa. The gray line indicates the line of no discrimination. Alb, albumin; Hapto, haptoglobin; Iron, serum iron; SAA, serum amyloid A.

sample number is between 40 and 120, a couple of recent modeling studies have shown that the robust method is the least accurate and precise in this scenario and that the nonparametric method performs best, especially when the data distribution is moderately to highly skewed.[36,38,45] For this reason, the nonparametric method was used on the datasets here.

The role of albumin as a negative APR has not been widely investigated in elephants, although RIs have been reported for both species.[10,11,30] The RIs in our study (41–55 g/L) are higher than those reported previously (captive Asian elephants 29–47 g/L, agarose gel electrophoresis[10]; captive Asian elephants 22–33 g/L, Siemens Dimension Xpand [analytical method not given][11]; captive African elephants 23–35 g/L Siemens Dimension Xpand [analytical method not given]).[11] The method used to measure albumin in our study (VetScan VS2 bromocresol green) has been documented to show variable biases compared with other bromocresol green methods for albumin measurement in other species; with a positive bias demonstrated in pangolins, cats, and owls.[46–50] A positive analytical bias may be a reason for the higher albumin RIs found for free-living African elephants here, and it is important to note that these RIs are method-specific. In terms of changes in disease, in one study that evaluated serum protein electrophoresis changes in Asian elephants, albumin concentration was found to be lower in animals with active clinical disease compared with healthy individuals.[10] In our study, albumin concentration was significantly lower in the injured elephants with high specificity (98.0%) and moderate sensitivity (68.8%), using the lower RL of 41 g/L, but was not found to be a significant predictor of inflammation based on the stepwise logistic regression analysis. It is important to note that albumin can decrease due to a multitude of pathological conditions other than inflammation (i.e., protein-losing nephropathies, enteropathies, and dermatopathies).[51] Of particular relevance is that a significant amount of protein can

be lost through wounds, and protein is needed for wound healing, factors which could lead to hypoalbuminemia in the wounded elephants, independent of the acute phase response.[52,53] Therefore, the true specificity of albumin as a negative acute phase reactant cannot be determined in our study, and the diagnostic indices reported here possibly encompass changes in albumin due to both the primary tissue injury and the secondary inflammation.

The analytical performance of the haptoglobin assay using African elephant serum was acceptable, similar to what has been reported for Asian elephants and other zoo and wildlife species.[4,6,10,12,54,55] Haptoglobin RIs, using the same colorimetric assay as in this study, have been reported for captive Asian (0.1–1.1 g/L[10], 0.24–4.00 g/L[11]) and African (0.21–2.35 g/L[11]) elephants. The RI for free-living African elephants (2.63–3.54 g/L) was higher than that for captive African elephants. This may reflect the presence of chronic, low-grade inflammation related to subclinical infectious diseases and/or internal and external parasites circulating in the free-living population.[56–58] Higher haptoglobin levels have also been described in free-living Florida manatees and Atlantic bottlenose dolphins compared with their captive counterparts.[1,55] The median haptoglobin concentration in the injured elephant cohort was around 4.5 times higher than that of the apparently healthy group, which is consistent with the change expected for a moderate positive APR. Haptoglobin had moderate to high sensitivity (75.0% at the upper RL of 3.51 g/L; 93.8% at the DCOV of 2.68 g/L) and specificity (93.9% at the upper RL; 87.8% at the DCOV), depending on whether the upper RL or DCOV was used, but it was not a significant predictor of inflammation based on the stepwise logistic regression analysis. Although other studies have not investigated the diagnostic accuracy of haptoglobin in elephants, increased haptoglobin concentration was reported in some captive Asian and African elephants with inflammatory conditions including systemic neoplasia, bronchopneumonia, sepsis, pododermatitis, tusk abscesses, and EEHV infection, yet was not increased in other individuals with the same conditions, septic peritonitis, or intra-thoracic abscesses.[9,11,12,59] In our injured group, only one elephant (Table 1, number 8) did not have haptoglobin concentrations above the DCOV of 2.68 g/L (Figure 1). This elephant also showed no changes in the other APRs apart from hypoferremia. The snare injury appeared to be chronic, fibrosed, and mild; thus systemic inflammation was probably not present.

The intra-assay imprecision of the SAA assay using African elephant serum (1.3–4.1%) was acceptable and was lower than the inter-assay imprecision reported using Asian elephant serum in other studies (5.0–10.9%).[10,12] Published SAA RIs using the same immunoturbidimetric assay were higher for captive Asian elephants (0–47.5 mg/L[10]; 0.1–37.6 mg/L[11]) compared with captive African elephants (0.1–6.9 mg/L[11]), and this species difference has been confirmed in our study, with SAA RIs calculated to be <10 mg/L. This finding has been proposed to be due to a higher level of constitutive expression of SAA in Asian compared with African elephants.[10] Differences could also be due to variation in antibody binding in the SAA assay. There was at least a 20-fold increase in the median SAA

concentration of the injured compared with the apparently healthy group; the true increase could not be determined as values below 10 mg/L were not reported due to the laboratory's internal detection limit of 10 mg/L. This finding provides further evidence that SAA is a major APP not only in the Asian but also in the African elephant. Serum amyloid A was found to be increased above the species-specific RI in Asian elephants with muscle trauma, skin wounds, pododermatitis, tusk infection, and some individuals with EEHV viremia, and in African elephants with bronchopneumonia, pododermatitis, ulcerative dermatitis, tusk infections, and septicemia.[9,12,59] Serum amyloid A has also been useful in tracking the clinical progress and viral load in Asian and African elephants infected with EEHV.[8,59] Using the upper RL of 10 mg/L as a cut-off value, SAA had moderate sensitivity (87.5%) and specificity (89.8%); specificity was increased to 93.9% when the DCOV of 27 mg/L was applied, with a positive likelihood ratio of 14.3. However, SAA was not a significant predictor of inflammation based on the stepwise logistic regression analysis. To increase the utility of SAA, we propose that 10–30 mg/L be considered as a gray zone when interpreting SAA in African elephants, with results above 30 mg/L highly specific for the presence of inflammation. As previously mentioned, one elephant in the apparently healthy group had an SAA result of 1093 mg/L, and it is highly likely that this animal had an inflammatory disease process not detected during immobilization.

Serum iron values are available for both healthy Asian and African captive elephants (reference intervals for African, 6.29–21.06 μmol/L; Asian 4.03–16.6 μmol/L; analytical method not specified) as well as for free-living Kruger National Park elephants (range 11.5–25.8 μmol/L, method not specified).[11,60] Disparities between these and the reference intervals published here (8.60–16.99 μmol/L) could be due to discrepancies in the measurement methods and differences in nutrition or the presence of subclinical disease or parasitism. To our knowledge, the use of hypoferremia as a diagnostic marker of inflammatory disease has not been extensively studied in zoo and wildlife species, apart from white rhinoceros, Florida manatees, and rhesus macaques.[4,54,61] In addition, a study evaluating iron regulation in a very small group of captive Asian elephants found that hypoferremia was present in two of three animals with chronic M. tuberculosis infection.[62] Serum iron has been shown to have high diagnostic accuracy for the detection and exclusion of inflammatory disease in horses, cattle, and white rhinoceros, with AUC values ranging from 0.71 to 0.97. It has furthermore been proposed as a substitute marker for SAA and haptoglobin in cattle with respiratory disease.[4,20,21] In white rhinoceros, serum iron had higher diagnostic accuracy than SAA or haptoglobin, and hypoferremia had a specificity of 98% and sensitivity of 60% for identifying animals with inflammation caused by wounds.[4] It is important to note at this point that several nondomestic animal species suffer from serum iron overload disorder (IOD) in captivity. Common species affected include black and Sumatran rhinoceros, tapirs, lemurs, dolphins, and pinnipeds.[63] The pathogenesis of IOD is multifactorial and has not been fully elucidated, but it

is associated with an inflammatory state. Animals with IOD may therefore have increases and not decreases in serum iron concurrent to increases in positive APRs.[3,64] IOD has not been described in elephants. Results from our study showed that using the DCOV of 7.5 μmol/L, serum iron concentration was the best predictor of the presence of inflammation related to injury (sensitivity 87.5%, specificity 98.0%, odds ratio 336.0) and was the only APR that could predict the presence or the absence of inflammation. Assays for serum iron measurement are easily automated and widely available, and serum iron could be more widely used as a marker of inflammation, especially when acute phase protein assays are not available. However, as with albumin, there are other possible reasons for the presence of hypoferremia, apart from inflammation, such as chronic blood loss, nutritional deficiency, or malabsorption. To our knowledge, these conditions were not present in our elephant population but should be considered as alternative etiologies for inflammation if hypoferremia is documented in an animal. Other markers of iron status which assist in differentiating between a true iron deficiency and decreased iron availability due to inflammation, such as transferrin and ferritin, were not measured in this study. Ferritin is a positive APR, which is raised with inflammatory hypoferremia, but decreased with true iron deficiency.[14] Ferritin is measured using species-specific ELISA methods; to our knowledge no ferritin assay has been validated for elephants, and thus ferritin was not measured in our study. The blood iron transport protein, transferrin, is a negative APR and is decreased in both inflammation and true iron deficiency.[14] Transferrin is indirectly measured as the total iron-binding capacity. The latter method requires at least 0.3 ml of serum, which was not available in this study.

5 | CONCLUSIONS

The acute phase response in free-living African elephants is similar to that described for captive elephants. All measurands had good diagnostic accuracy for detecting inflammation in this population. Results outside the relevant RL have high sensitivity; specificity for detecting inflammation can be increased using DCOVs. We have shown that serum iron is a promising biomarker for the detection of inflammation in these animals and should be measured alongside haptoglobin and SAA. The results of our study have also confirmed that species-specific RIs should be used to interpret SAA results in Asian vs African elephants. The direct clinical utility of this study is perhaps limited for the population of free-living elephants in the Kruger National Park due to the large size of the park and elephant numbers, as well as the limited ability to perform ongoing veterinary care for individuals. APR measurements could be useful for the veterinary management of elephants in less extensive reserves or semi-captive situations. The information gained regarding APRs in the Kruger National Park elephant population could, however, provide a basis for population monitoring and comparisons in the future. Future studies should endeavor to include a higher proportion of female animals.

ORCID

Emma H. Hooijberg https://orcid.org/0000-0002-4367-799X

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