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Reference intervals of acute phase proteins in healthy Andalusian donkeys and response to experimentally induced endotoxemia

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Abstract

Background: Assessment of acute phase proteins (APPs) may allow prompt detection of diseases in donkeys, that otherwise may be missed because of the stoic behavior of donkeys. Reference intervals (RIs) of APPs measured using immunoassays and a comparison of the response of these biomarkers to a controlled inflammatory insult are lacking in donkeys.

Objectives: (a) To describe the RIs for APPs in healthy Andalusian donkeys, (b) to study the effects of sex and age on APPs, and (c) to assess the early response of APPs to experimentally induced endotoxemia.

Animals: Seventy-three healthy Andalusian donkeys (67 for RIs and 6 for endotoxemia).

Methods: Serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), ceruloplasmin (Cp), α1-acid glycoprotein (AGP), procalcitonin (PCT), ferritin (Ft), and fibrinogen (Fb) RIs were determined. Endotoxemia was induced and samples for APP determination were obtained at regular intervals for 4 hours.

Results: The RIs in Andalusian donkeys were: SAA (0.1-0.6 mg/L), Hp (75-2261 mg/L), CRP (1.3-7.0 mg/L), Cp (0-745 mg/L), AGP (0-884 mg/L), PCT (0-504 pg/mL), Ft (26.9-31.8 μg/L), and Fb (115-466 mg/dL). Concentrations of SAA were higher ($P < .05$) in jacks. Donkeys <5 years old had higher Cp, AGP, and PCT compared to older donkeys. Concentrations of SAA and Hp were significantly increased in endotoxemic donkeys from 2 hours postinduction.

Abbreviations: γGT, gamma-glutamyl transferase; AGP, α1-acid glycoprotein; APP, acute phase protein; ASVCP, American Society for Veterinary Clinical Pathology; Cp, ceruloplasmin; CRP, C-reactive protein; CV, coefficient of variation; Fb, fibrinogen; Ft, ferritin; Hp, haptoglobin; IL-1, interleukin 1; IL-6, interleukin 6; IQR, interquartile range; LPS, lipopolysaccharide; PCT, procalcitonin; PLI, post-LPS injection; RI, reference interval; SAA, serum amyloid A; SIRS, systemic inflammatory response syndrome; TNFα, tumor necrosis factor-alpha.

Conclusions and Clinical Importance: We illustrated the importance of using species-specific RIs for APPs in donkeys and the effect of age and sex on APP concentrations. Concentrations of SAA and Hp appear to be the most useful biomarkers in donkeys in the early stages of endotoxemia.

KEYWORDS
biomarker, equids, inflammation, sepsis, serum amyloid A

1 | INTRODUCTION

Although donkeys are commonly affected by severe medical disorders such as endotoxemia, pneumonia, and colic[1-3]; their stoic behavior can cause vague signs that preclude early diagnosis and hinder subsequent successful treatment.[4]

Acute phase proteins (APPs) are early, nonspecific biomarkers produced mainly in the liver in response to pro-inflammatory cytokines such as interleukin 1 (IL-1), tumor necrosis factor-alpha (TNFα), and interleukin 6 (IL-6).[5-7] In horses, APP concentrations vary in response to several conditions including bacterial and viral infections, endotoxemia-related systemic inflammatory response syndrome (SIRS), and surgical procedures.[5,8] The APPs can play a useful role in detecting subclinical disease processes and assessing response to treatment.[5,8,9]

In horses, serum amyloid A (SAA), haptoglobin (Hp), C-reactive protein (CRP), fibrinogen (Fb), procalcitonin (PCT), ceruloplasmin (Cp), α1-acid glycoprotein (AGP), and ferritin (Ft) are considered the most important APPs.[6-12] Although several methods have been used to measure APPs (eg, single radial immunodiffusion, sodium dodecyl sulfate polyacrylamide gel electrophoresis), immunoassays allow more sensitive detection of APPs compared to other techniques.[13]

Because species-related differences in APP concentrations and dynamics are well known,[5,6,8,13] both species-specific reference intervals (RIs) and comparative studies on the response of several APPs to a controlled insult are mandatory to properly determine their clinical usefulness. The American Society for Veterinary Clinical Pathology (ASVCP) recently has standardized guidelines for determination of RIs in veterinary medicine according to the Clinical Laboratory and Standards Institute recommendations.[14]

Currently, fibrinogen is the only APP that has a RI specific for donkeys, although it is similar to that of horses.[15] Previous studies using immunoassays in donkeys have evaluated only SAA, Hp, or CRP in a limited number of healthy animals.[16-20] Both comparison of the response of >2 APPs to a controlled inflammatory insult and evaluation of the effect of sex and age on these biomarkers are lacking in donkeys.

Our objectives were to characterize the RIs of the main APPs in healthy adult Andalusian donkeys, evaluate the effect of age and sex on their concentrations and assess their early response to experimentally induced endotoxemia.

2 | MATERIALS AND METHODS

2.1 | Animals

All animals were considered healthy based on normal clinical history, physical examination, and laboratory results (CBC, total protein, albumin, total bilirubin, creatinine and blood urea nitrogen concentrations as well as aspartate transaminase and gamma-glutamyl transferase [γGT] activities). All animals had received regular antihelminthic treatment. No animal had received any treatment for at least 2 months before sampling and no pregnant jennets were included in the study. Sampled animals were not exercised or worked before sampling and had no previous history of SIRS or endotoxemia-inducing diseases.

2.2 | Study 1: RI determination

Blood samples were collected from 67 healthy adult (8.6 ± 5.2 years old) Andalusian and Andalusian crossbred (54 jennets and 13 jacks) donkeys from different farms with similar premises in Southern Spain.

Donkeys were grouped based on age (range, 2-17 years old) in the following groups: Group 1 (<5 years old; n = 15), group 2 (5-10 years old; n = 28), and group 3 (>10 years old; n = 24).

Blood samples were collected by venipuncture into heparinized tubes and plain tubes with clot activator. Samples were centrifugated within 30 minutes of collection at 2000*g* for 10 minutes. Plasma and serum subsequently were stored at −20°C.

2.3 | Study 2: Effect of experimentally induced endotoxemia on APPs

Six healthy adult (7.6 ± 0.8 years old) Andalusian nonpregnant jennets (348 ± 39 kg) housed in the facilities of the Veterinary Teaching Hospital were included. The skin over the left jugular vein was clipped and aseptically prepared. A baseline blood sample was collected 30 minutes before endotoxemia induction and handled as described above.

Endotoxemia was induced following previous protocols described in donkeys.[3,18] A dose of 20 ng/kg of lipopolysaccharide (LPS; *Escherichia coli* O55:B5, Sigma-Aldrich Quimica, Madrid, Spain) diluted in 500 mL of sterile saline was administered over 30 minutes using a polyurethane catheter (Milacath, Mila International Inc, Kentucky). Blood samples then

were collected at: 30, 60, 120, and 240 minutes post-LPS injection (PLI) and handled as described above.

2.3.1 | Acute phase proteins measurements

Samples were analyzed using the following commercially tests: Serum amyloid A Tridelta Phase (Tridelta Development Ltd, Kildare, Ireland), Horse Haptoglobin ELISA, K-Assay (Kamiya Biomedical Company, Washington), Horse CRP ELISA, K-Assay (Kamiya Biomedical Company, Washington), Horse Ceruloplasmin ELISA Kit (MyBiosource.com, California), Horse A1-Acid Glycoprotein ELISA Kit (MyBiosource.com), and Horse Procalcitonin ELISA Kit (MyBiosource.com). These kits previously have been validated in horses.[6,9,12,21,22] Optical densities were read on an automatic plate reader (Multiskan FC Microplate ELISA reader, Thermo Fisher Scientific, Massachusetts). Samples with optical density values above the range of the standard curve were diluted further and reanalyzed. Fibrinogen was measured using the Clauss method in an automated coagulometric analyzer (STart Hemostasis Analyzer, Diagnostica Stago S.A.S., Asnières sur Seine Cedex, France). Ferritin was measured using an immunoturbidimetric method (Ferritin immunoturbidimetry assay 13934. Biosystems S.A., Barcelona, Spain) on an automated chemistry analyzer (A15 analyzer, Biosystems S.A.) as previously described in horses.[23] All samples and standards were measured in duplicate.

2.3.2 | Test validation

Tests were validated for donkeys following a modified protocol previously reported in horses and using published guidelines for immunoassay validation.[24-26] Specific aliquots from the tested donkeys were used for these validations. Precision was evaluated by calculating the intra-assay (same day) and interassay (3 nonconsecutive days) coefficients of variation (CVs) of 5 replicate measurements of 3 donkey plasma pools with different concentrations. Validation of measurements in markedly high ranges was not performed because of a lack of pathological samples fulfilling the required concentrations. Accuracy was investigated by evaluating linearity under dilution (replicate measurements of 5 serial dilutions of a serum pool containing high concentrations of each APP).

2.3.3 | Data analysis

Normality was assessed using the Kolmogorov-Smirnov test. Results were expressed as mean ± SD (endotoxemia data) or as median, interquartile range (IQR; difference between the 75th and 25th quartiles) and 90% confidence intervals (CI) of the median (RI data). The median and IQR were calculated using a Tukey's Hinges test. Following recommendations from the ASVCP, and according to the size and distribution of our population, RIs were obtained using dedicated software (Reference Value Advisor v. 2.1. freeware. Available at: http://www.biostat.envt.fr/reference-value-advisor/, Accessed May 18, 2020).[14] Briefly, the untransformed data were analyzed using a robust method, which utilizes an iterative process to estimate location and spread of data. A bootstrap method included in the software was used to determine and study the limits of the RI.

Mann-Whitney and Kruskal-Wallis tests with the Bonferroni correction were used to study the effect of sex and age on APP concentrations, respectively. The effect of LPS on each APP was analyzed

TABLE 1 Blood concentrations of acute phase proteins in healthy Andalusian donkeys (n = 67)

Acute phase protein	Median (IQR) 90% CI	Reported concentrations in horses[8,22,23,27]
SAA (mg/L)	0.35 (0.31-0.40) 0.33-0.37	0.5-20
Haptoglobin (mg/L)	1167 (822-1460) 950-1243	200-1000
CRP (mg/L)	4.37 (2.90-5.04) 3.42-4.80	7.5
Ceruloplasmin (mg/L)	266 (197-511) 232-392	300-400
AGP (mg/L)	333 (171-591) 237-484	70-90
Procalcitonin (pg/mL)	190 (134-313) 160-254	450
Ferritin (μg/L)	29 (20-36) 25-32	30-100
Fibrinogen (mg/dL)	290 (130-330) 180-310	200-400

Note: Data are expressed as median (IQR; 75th percentile-25th percentile) and below the 90% confidence interval of the median. All proteins were determined in plasma with the exception of ferritin which was measured in serum samples.
Abbreviations: AGP, α1-acid glycoprotein; CI, confidence interval; CRP, C-reactive protein; IQR, interquartile range; SAA, serum amyloid A.

TABLE 2 Proposed reference intervals (RIs) for acute phase proteins in healthy Andalusian donkeys (n = 67)

Acute phase protein	Proposed RIs for donkeys
SAA (mg/L)	<0.6
Haptoglobin (mg/L)	75–2261
CRP (mg/L)	<7.0
Ceruloplasmin (mg/L)	<745
AGP (mg/L)	<884
Procalcitonin (pg/mL)	<504
Ferritin (μg/L)	26.9-31.8
Fibrinogen (mg/dL)	115-466

Note: Proposed RIs were obtained following indications of the American Society for Veterinary Clinical Pathology using a robust method included in a dedicated software for reference intervals.
Abbreviations: AGP, α1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A.

using an analysis of variance followed by a *t* test analysis, both for repeated measures. Correlations between APPs were studied using either the Pearson or Spearman test depending on normality. A *P* value ≤.05 was considered significant.

Linearity under dilution was investigated by linear regression analysis. Runs test was performed to determine whether data deviated significantly from the applied model.

Statistical analysis was performed using commercial statistical software (IBM SPSS Statistics 24, IBM, Illinois).

3 | RESULTS

3.1 | Study 1: RI determination

Results for each APP are shown in Table 1. Proposed RIs for Andalusian donkeys for each APP are presented in Table 2. Differences

TABLE 3 Blood concentrations of acute phase proteins in healthy Andalusian donkeys arranged by sex

Acute phase protein	Jack (n = 13)	Jennet (n = 54)	*P* value
SAA (mg/L)	0.43 (0.31–0.54)	0.35 (0.31-0.39)*	.05
Haptoglobin (mg/L)	1354 (989-1754)	1251 (676-1456)	.17
CRP (mg/L)	4.41 (2.90-5.74)	4.25 (2.89-5.03)	.3
Ceruloplasmin (mg/L)	276 (203-505)	262 (163-511)	.48
AGP (mg/L)	400 (237-660)	306 (155-586)	.18
Procalcitonin (pg/mL)	289 (157-372)	184 (129-309)	.07
Ferritin (μg/L)	21 (18-34)	29 (22-37)	.08
Fibrinogen (mg/dL)	300 (190-380)	260 (170-320)	.5

Note: Data are expressed as median (interquartile range; 75th percentile-25th percentile). All proteins were determined in plasma to exception of ferritin was in serum samples.
Abbreviations: AGP, α1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A.
*$P < .05$ vs Jack.

TABLE 4 Blood concentrations of acute phase proteins in healthy Andalusian donkeys grouped by age

Acute phase protein	Group 1: <5 years old (n = 15)	Group 2: 5-10 years old (n = 28)	Group 3: >10 years old (n = 24)
SAA (mg/L)	0.34 (0.30-0.35)	0.36 (0.34-0.39)	0.37 (0.32-0.44)
Haptoglobin (mg/L)	1350 (1053-2087)	1268 (601-1345)	1290 (651-1762)
CRP (mg/L)	4.41 (3.41-5.46)	4.97 (4.03-5.81)	4.39 (2.50-5.61)
Ceruloplasmin (mg/L)	478 (290-588)	205 (135-512)*	218 (129-270)*
AGP (mg/L)	524 (392-689)	181 (125-528)*	240 (134-400)*
Procalcitonin (pg/mL)	388 (260-509)	159 (112-317)*	159 (114-246)*
Ferritin (μg/L)	32 (20-40)	27 (22-36)	28 (20-32)
Fibrinogen (mg/dL)	310 (200-380)	290 (220-370)	250 (200-290)

Note: Data are expressed as median (interquartile range; 75th percentile-25th percentile). All proteins were determined in plasma to exception of ferritin was in serum samples.
Abbreviations: AGP, α1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A.
*$P < .05$ vs group 1.

TABLE 5 Spearman correlation coefficients (rho) among acute phase proteins in healthy Andalusian donkeys

	Hp	CRP	Cp	AGP	PCT	Ft	Fb
SAA	0.06	0.09	−0.14	−0.11	−0.19	−0.18	−0.07
Hp	–	0.19*	0.03	−0.02	0.06	−0.32*	0.05
CRP	–	–	−0.35*	−0.3*	−0.1	0.07	0.25
Cp	–	–	–	0.93*	0.84*	−0.08	−0.23
AGP	–	–	–	–	0.85*	−0.11	−0.24
PCT	–	–	–	–	–	−0.05	−0.18
Ft	–	–	–	–	–	–	0.02

Abbreviations: AGP, α1-acid glycoprotein; Cp, ceruloplasmin; CRP, C-reactive protein; Fb, fibrinogen; Ft, ferritin; Hp, haptoglobin; PCT, procalcitonin; SAA, serum amyloid A.
*$P < .05$.

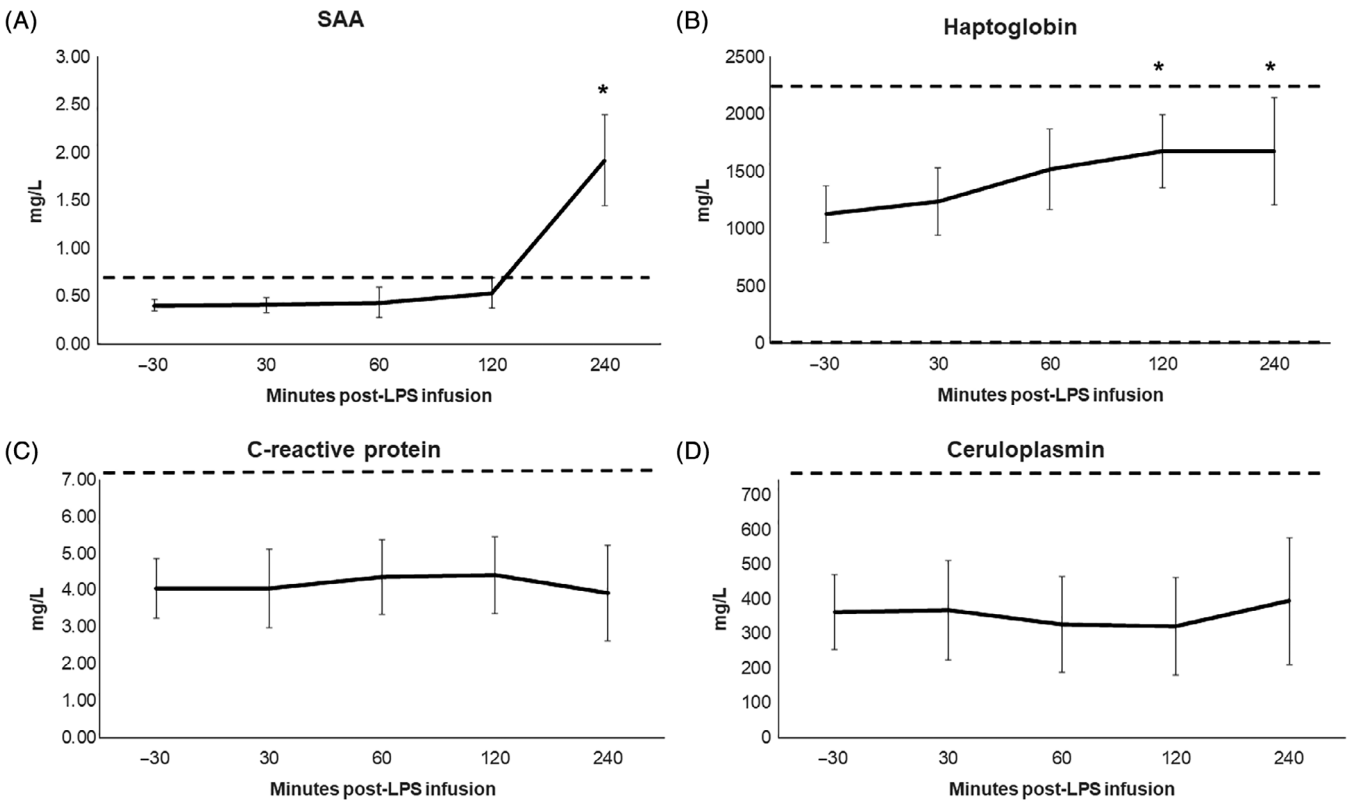

FIGURE 1 Plasma concentrations of serum amyloid A (A), haptoglobin (B), C-reactive protein (C), and ceruloplasmin (D) in Andalusian donkeys with experimentally induced endotoxemia. Data are presented as mean and SD. Dashed lines represent the proposed reference intervals for donkeys. *$P < .05$ vs baseline. SAA, serum amyloid A

between jennets (0.35 [0.31-0.39] mg/L) and jacks (0.43 [0.31-0.54] mg/L) were only significant ($P < .05$) for SAA (Table 3). Younger donkeys (<5 years) had significantly higher concentrations of Cp, AGP, and PCT compared to the other age groups (Table 4). Positive correlations ($P < .05$) were found among Cp, AGP, and PCT in healthy animals (Table 5).

3.2 | Study 2: Effect of experimentally induced endotoxemia on APPs

All of the donkeys developed typical features of SIRS such as tachycardia, fever, leukopenia, and neutropenia (data previously published).[3] The serum SAA concentrations were significantly increased

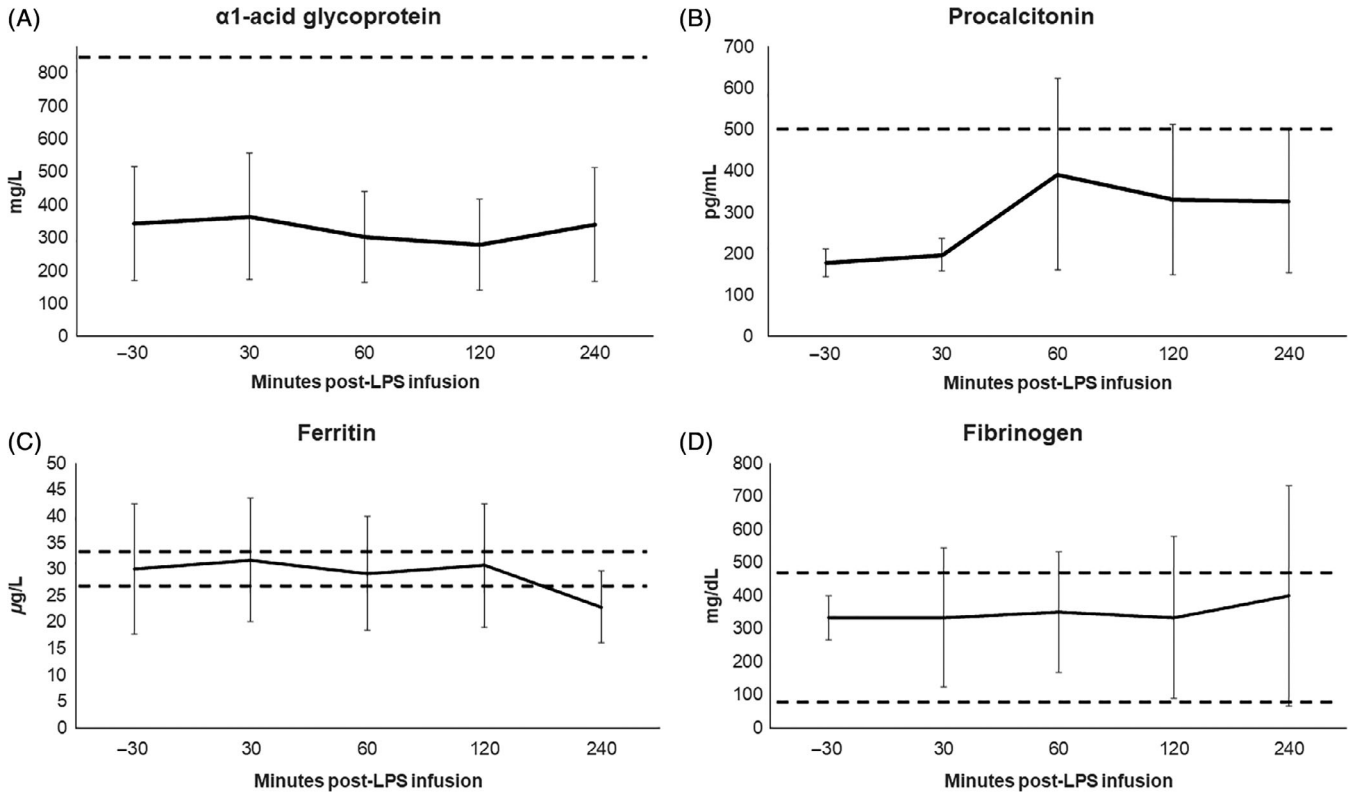

FIGURE 2 Plasma concentrations of α1-acid glycoprotein (A), procalcitonin (B) and fibrinogen (C) and serum concentrations of ferritin (D) in Andalusian donkeys with experimentally induced endotoxemia. Data are presented as mean and SD. Dashed lines represent the proposed reference intervals for donkeys

TABLE 6 Pearson correlation coefficients (*r*) among acute phase proteins in Andalusian donkeys with experimentally induced endotoxemia

	Hp	CRP	Cp	AGP	PCT	Ft	Fb
SAA	0.44*	0.09	0.07	0.07	0.3*	−0.01	−0.36*
Hp	—	0.08	0.06	0.22	0.5*	−0.23	−0.33*
CRP	—	—	0.8*	0.84*	0.12	0.12	−0.1
Cp	—	—	—	0.84*	0.15	−0.12	0.02
AGP	—	—	—	—	0.25	−0.36*	−0.24
PCT	—	—	—	—	—	−0.27	−0.38*
Ft	—	—	—	—	—	—	0.22

Abbreviations: AGP, α1-acid glycoprotein; Cp, ceruloplasmin; CRP, C-reactive protein; Fb, fibrinogen; Ft, ferritin; Hp, haptoglobin; PCT, procalcitonin; SAA, serum amyloid A.
*$P < .05$.

($P < .001$) at 240 minutes PLI compared to basal results (Figure 1). Serum Hp concentration also was significantly ($P < .05$) increased at 120 and 240 minutes PLI (Figure 1). No significant differences were observed in the other APPs in response to LPS infusion (Figures 1 and 2).

Serum Hp concentration showed a moderate positive correlation ($P < .05$) with SAA and PCT whereas CRP was positively correlated ($P < .001$) with AGP and Cp in donkeys with experimentally induced endotoxemia (Table 6).

3.3 | Test validation

Intra- and interassay CVs ranged from 2.5% to 7.9% and 2.8% to 9.7%, respectively. No marked deviations from a slope equal to 1 and a y-

TABLE 7 Validation data from acute phase proteins immunoassays in Andalusian donkeys

	Accuracy					Precision				
Parameter	Concentration range	y-intercept (95% CI)	Slope (95% CI)	*P* (Runs test)	r^2	Mean concentration	Intra-assay CV	Interassay CV	Sensitivity	Detection range
SAA (mg/L)	0.01-0.61	0.01 (0-0.02)	0.98 (0.93-1.02)	.4	0.99	Pool 1 (0.21)	6.9	7.4	0.02	0.05-40
						Pool 2 (0.34)	5.6	5.8		
						Pool 3 (0.51)	2.5	2.8		
Hp (mg/L)	68-2192	86 (−10 to 182)	0.98 (0.88-1.07)	.3	0.99	Pool 1 (208)	3.7	7.1	5	15-3500
						Pool 2 (1004)	5.2	6.1		
						Pool 3 (1953)	2.9	2.9		
CRP (mg/L)	0.21-6.81	0.23 (0.06-0.39)	0.97 (0.92-1.02)	.7	0.99	Pool 1 (1.95)	7.4	8.0	0.05	0.05-50
						Pool 2 (3.81)	4.5	8.1		
						Pool 3 (6.09)	3.7	4.6		
Cp (mg/L)	22-713	29 (5.1-53.5)	0.97 (0.90-1.04)	.4	0.99	Pool 1 (109)	7.9	8.6	0.5	31.2-1000
						Pool 2 (278)	4.0	5.2		
						Pool 3 (689)	6.4	6.7		
AGP (mg/L)	25-815	21 (3.2-38.9)	0.96 (0.91-1.01)	.7	0.99	Pool 1 (114)	4.5	5.4	5	31.2-1000
						Pool 2 (360)	7.3	8.0		
						Pool 3 (764)	2.9	4.0		
PCT (pg/mL)	15-501	26 (−7.1-60.5)	0.97 (0.83-1.12)	.3	0.98	Pool 1 (109)	6.7	6.7	10	50-1600
						Pool 2 (207)	3.9	4.8		
						Pool 3 (415)	5.8	5.8		
Ft (μg/L)	1-30	1 (0.21-1.66)	0.95 (0.90-1.00)	.4	0.99	Pool 1 (20)	5.0	7.2	4	4-500
						Pool 2 (30)	4.7	5.1		
						Pool 3 (37)	5.5	9.7		

Note: Accuracy was investigated by linear regression analysis evaluating replicate measurements of 5 serial dilutions of a plasma/serum pool containing high concentrations of each APP. Precision was calculated on 5 replicate measurements determined in 3 nonconsecutive days. Sensitivity and detection range are displayed as referred by the manufacturers.

Abbreviations: AGP, α1-acid glycoprotein; APP, acute phase protein; CI, confidence interval; Cp, ceruloplasmin; CRP, C-reactive protein; CV, coefficient of variation; Ft, ferritin; Hp, haptoglobin; PCT, procalcitonin; SAA, serum amyloid A.

intercept equal to 0 were observed in the linear regression equation of any APP, and Runs test determined that data agreed with the linear model (Table 7).

4 | DISCUSSION

We determined RIs for APPs specific for donkeys. Our results showed that higher SAA concentrations are present in jacks compared with jennets and higher Cp, AGP, and PCT concentrations were found in animals <5 years of age compared to older donkeys. In addition, we found significant increases in SAA and Hp in response to experimentally induced endotoxemia, which appear as early as 2 hours PLI.

Although RIs for some APPs in donkeys showed wide ranges (Hp, Cp, AGP, and PCT), wide ranges also are observed in horses and other mammals.[7,8,13] When compared with previously reported studies in donkeys using the same technique, our SAA concentrations were lower,[17,18] although interbreed differences partially could be responsible for these differences.[28] Previously reported Hp and CRP concentrations in healthy donkeys using immunoassays would be contained within our proposed RIs.[16,17] Because ours is the first study assessing Cp, AGP, PCT, and Ft using immunoassays in donkeys, there are no comparable values. Nonetheless, Cp and AGP concentrations were higher than those obtained in Pêga donkeys using a sodium dodecyl sulfate polyacrylamide gel electrophoresis technique.[29] Fibrinogen concentrations were within RIs reported in donkeys.[15]

The SAA, Hp, and fibrinogen concentrations in Andalusian donkeys were similar to concentrations in horses using the same techniques.[6,21,27,30] Although CRP, PCT, and ferritin concentrations in donkeys were similar to those obtained in horses using the same methodology by some investigators,[10,21,22] our data showed increased (CRP and PCT) or decreased (Ft) concentrations compared to other studies using horses.[6,12,23] The AGP concentrations in donkeys were higher compared to reports in horses.[8,31] No published data are available on ceruloplasmin in horses using the technique validated in our study. Nonetheless, results from healthy donkeys were within reported concentrations for horses using single radial immunodiffusion methods.[8,32]

To our knowledge, ours is the first report of higher SAA concentrations in jacks compared to jennets. Because age was similar between groups (jennets, 8.9 ± 5.1 years; jacks, 7.5 ± 5.3 years), and donkeys were handled in similar way independently of the sex (without arousal or excitement), these factors could be ruled out as an explanation for our finding. Although most investigators report no sex influence on SAA concentrations in horses,[7,28] testosterone appears to influence in SAA concentrations in other species.[33] Whether this is the case in donkeys and whether this difference could be sufficient to justify sex-specific RIs should be further studied using larger numbers of animals for each sex group. Although significant, this sex-related difference may be of limited clinical interest in sick donkeys, given that SAA often increases several thousand-fold during acute inflammation. We did not observe any other sex effect on APP concentrations, which concurs with findings in horses.

Our findings concerning the effect of age on APPs in donkeys cannot be compared to previous reports in equids because of differences in age distribution. Although differences between foals and adults are described in horses,[5,8] our is the first study identifying lower concentrations in older donkeys compared to animals <5 years of age. Whether this finding is related to a developmental effect, influence of developmental hormones, hepatic metabolism, or other causes should be further investigated.

Experimentally induced endotoxemia caused a significant increase in SAA concentrations at 240 minutes PLI, which was above the upper limit of the RI. A similar early response has been reported previously in donkeys.[18,34] In LPS-challenged horses, using even higher doses, the increase in SAA concentrations was slower.[35,36] Although more rapid, the SAA response in donkeys was milder than reported in horses. Thus, clinicians should closely monitor SAA concentrations in sick donkeys, and even a moderate increase above the species-specific RI could have clinical relevance. The Hp concentrations also significantly increased in endotoxemic donkeys at 120 and 240 minutes PLI, which is an earlier increase compared to previous studies in both donkeys and horses.[34,36] These findings could point to an inherent species-specific rapid response of these APPs. Thus, both biomarkers could be helpful in the early diagnosis of SIRS in donkeys. Ferritin concentrations were below the proposed RI at 240 minutes PLI. Mild hypoferritinemia has been reported previously in horses with colic,[23] which could be in accordance with our finding. Contrary to our results, PCT showed an early increase in horses with experimentally induced endotoxemia,[37] although a higher LPS dose was used in that study. The lack of CRP, Cp, AGP, and Fb change after LPS infusion in our study could be explained by the relative late onset response of these APPs in response to any insult, as previously described in horses.[5,8,11,27] However, additional studies using more animals or higher LPS doses could clarify this apparent idiosyncrasy.

Because several methods are available for measuring APPs, concentrations and cutoffs can easily differ between techniques and results should be compared with caution.[8,13] Species-specific validation of any APP test is mandatory to obtain reliable data.[13] Our validation results show that every immunoassay performed reliably in donkeys, with good repeatability and accuracy (linearity under dilution) in our ranges of measurements, in agreement with requirements for this type of analysis.[24,26]

One limitation of our study is more prolonged sampling post-LPS. Including donkeys with naturally occurring disease could clarify mid- and long-term variations in the concentrations of the APPs studied in this species. Validation studies using samples from sick donkeys with higher APP concentrations also should be carried out in the future to validate these immunoassays for markedly high concentrations.

In conclusion, our results emphasize the importance of using species-specific RIs for APPs in donkeys, and the effect of sex and age on APPs in this species. In addition, SAA and Hp appear to be the most useful biomarkers in donkeys in early stages of endotoxemia.

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CONFLICT OF INTEREST DECLARATION

Authors declare no conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Approval from the Welfare Committee of Animal Experimentation of the University of Cordoba (2015PI/05, approval date: March 19, 2015) and the Rural Development, Fishing and Agriculture Ministry of Junta de Andalucia (19-03-2015-212, approval date: March 19, 2015).

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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