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

Alejandro Perez-Ecija1 | Antonio Buzon-Cuevas1 | Raul Aguilera-Aguilera2 | Carlos Gonzalez-De Cara3 | Francisco J. Mendoza Garcia1

1Department of Animal Medicine and Surgery, University of Cordoba, Cordoba, Spain
2Egabro Veterinary Practice, Cordoba, Spain
3Department of Pathology and Infectious Diseases, University of Surrey, Guildford, United Kingdom

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 colic1-3; 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, endotoxia-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 anthelmintic 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 endotoxia-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 horses8,22,23,27 |
|---------------------|----------------------|------------------------------------------|
| SAA (mg/L)          | 0.35 (0.31-0.40)     | 5.0-20                                   |
| Haptoglobin (mg/L)  | 1167 (822-1460)      | 200-1000                                 |
| CRP (mg/L)          | 4.37 (2.90-5.04)     | 7.5                                      |
| Ceruloplasmin (mg/L)| 266 (197-511)        | 300-400                                  |
| AGP (mg/L)          | 333 (171-591)        | 70-90                                    |
| Procalcitonin (pg/mL)| 190 (134-313)       | 450                                      |
| Ferritin (μg/L)     | 29 (20-36)           | 30-100                                   |
| Fibrinogen (mg/dL)  | 290 (130-330)        | 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.
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 \( \leq .05 \) 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).

### 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 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 (\( \mu \)g/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, \( \alpha \)-1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A.

**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 (\( \mu \)g/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, \( \alpha \)-1-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 (\( \mu \)g/L) | 32 (20-40) | 27 (22-34) | 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, \( \alpha \)-1-acid glycoprotein; CRP, C-reactive protein; SAA, serum amyloid A. \(^*\) \( P < .05 \) vs group 1.
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

### Table 5: Spearman correlation coefficients (rho) among acute phase proteins in healthy Andalusian donkeys

|        | Hp   | CRP  | Cp   | AGP  | PCT  | Ft   | Fb   |
|--------|------|------|------|------|------|------|------|
| SAA    | 0.06 | −     | −0.14| −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
(P < .01) 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

| Parameter | Accuracy | Precision |
|-----------|----------|-----------|
|           | Concentration range | y-intercept (95% CI) | Slope (95% CI) | P (Runs test) | r² | 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) | 0.4 | 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) | 0.3 | 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) | 0.7 | 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) | 0.4 | 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) | 0.7 | 0.99 | Pool 1 (114) | 4.5 | 5.4 | 5 | 31.2-1000 |
|           | Pool 2 (360) | 7.3 | 8.0 |
|           | Pool 3 (764) | 4.9 | 4.0 |
| PCT (pg/mL) | 15-501 | 26 (–7.1-60.5) | 0.97 (0.83-1.12) | 0.3 | 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) | 0.4 | 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.
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, 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. In LPS-challenged horses, using even higher doses, the increase in SAA concentrations was slower. 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. 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 hypoferritemia has been reported previously in horses with colic, which could be in accordance with our finding. Contrary to our results, the SAA response in donkeys was milder than reported in horses. PCT showed an early increase in horses with experimentally induced endotoxemia, 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 low onset response of these APPs in response to any insult, as previously described in horses. Whether, 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. Species-specific validation of any APP test is mandatory to obtain reliable data. 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.

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.
ACKNOWLEDGMENT
Funding provided by the Plan Propio de Investigacion from the University of Cordoba (Spain), by the Programa Operativo de fondos FEDER Andalucia (Spain) and Plan Andaluz de Investigacion (AGR-227 group) from the Consejeria de Salud de la Junta de Andalucia (Spain).

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 (IACUC) OR OTHER APPROVAL DECLARATION INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE
Authors declare no off-label use of antimicrobials.

HUMAN ETHICS APPROVAL DECLARATION
Authors declare human ethics approval was not needed for this study.

REFERENCES
1. Mendoza FJ, Toribio RE, Perez-Ecija A. Donkey internal medicine part II: cardiovascular, respiratory, neurologic, urinary, ophthalmic, dermatology, and musculoskeletal disorders. J Equine Vet Sci. 2018;65:86-97.
2. Thiemann AK, Sullivan RJ. Gastrointestinal disorders of donkeys and mules. Vet Clin North Am Equine Pract. 2019;35(3):419-432.
3. Mendoza FJ, Gonzalez-De-Cara C, Aguilara-Aguilara R, et al. Meloxicam ameliorates the systemic inflammatory response syndrome associated with experimentally induced endotoxemia in adult donkeys. J Vet Intern Med. 2020;34(4):1631-1641.
4. Gonzalez-De-Cara CA, Perez-Ecija A, Aguilara-Aguilara R, et al. Temperament test for donkeys to be used in assisted therapy. Appl Anim Behav Sci. 2017;186:64-71.
5. Petersen HH, Nielsen JP, Heegaard PM. Application of acute phase protein measurements in veterinary clinical chemistry. Vet Res. 2004;35(2):163-187.
6. Cywinska A, Szarska E, Gorecka R, et al. Acute phase protein concentrations after limited distance and long distance endurance rides in horses. Res vet Sci. 2012;93(3):1402-1406.
7. Witkowska-Pilasiewicz OD, Zmigrodzka M, Winnicka A, et al. Serum amyloid A in equine health and disease. Equine vet J. 2019;51(3):293-298.
8. Jacobsen S. Review of equine acute-phase proteins. Proceedings of the 53rd Annual Convention of the American Association of Equine Practitioners. 1st ed. American Association of Equine Practitioners: Lexington, KY: 2007.
9. Cray C, Belgrave RL. Haptoglobin quantitation in serum samples from clinically normal and clinically abnormal horses. J Equine Vet Sci. 2014;34(2):337-340.
10. Hyypaa S, Hoyohtya M, Nevalainen M, et al. Effect of exercise on plasma ferritin concentrations: implications for the measurement of iron status. Equine Vet J Suppl. 2002;34:186-190.
11. Murata H, Shimada N, Yoshioka M. Current research on acute phase proteins in veterinary diagnosis: an overview. Vet J. 2004;168(1):28-40.
12. Bonelli F, Meucci V, Divers TJ, et al. Plasma procollagen concentration in healthy horses and horses affected by systemic inflammatory response syndrome. J Vet Intern Med. 2015;29(6):1689-1691.
13. Cray C, Zaias J, Altman NH. Acute phase response in animals: a review. Comp Med. 2009;59(6):517-526.
14. Friedricks KR, Harr KE, Freeman KP, et al. ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. Vet Clin Pathol. 2012;41(4):441-453.
15. Perez-Ecija A, Mendoza FJ. Characterisation of clotting factors, anti-coagulant protein activities and viscoelastic analysis in healthy donkeys. Equine Vet J. 2017;49(6):734-738.
16. Aziz DM, Hiss-Pesch S, Mielenz B, Sauerwein H. Haptoglobin baseline value in jennies and the effect of ovariectomy on its serum concentration. Anim Reprod Sci. 2012;132(1-2):83-87.
17. El-Ashker M, Abdelhamid F, Risha E, et al. Vitamin C ameliorates gentamicin-induced acute kidney injury in equines: an experimental study. J Equine Vet Sci. 2015;35(3):238-243.
18. El-Ashker MR, El-Sebai MG, Aamer HG. The influence of experimentally-induced endotoxaemia on clinical variables and markers of systemic inflammation in donkeys (Equus asinus). Vet Med. 2017;62(3):117-124.
19. Kay G, Tilgui N, Semmante N, et al. Determining factors and interspecies-specific modeling for serum amyloid A concentrations in working horses, donkeys, and mules. Res Vet Sci. 2019;125:256-265.
20. Jerele S, Davis E, Mapes S, et al. Survey of serum amyloid a and bacterial and viral frequency using qPCR levels in recently captured feral donkeys from Death Valley National Park (California). Animals. 2020;10(6):E1086.
21. Leclere M, Lavoie-Lamoureux A, Lavoie JP. Acute phase proteins in racehorses with inflammatory airway disease. J Vet Intern Med. 2015;29(3):940-945.
22. Zak A, Siwinska N, Elzinga S, et al. Effects of advanced age and pituitary pars intermedia dysfunction on components of the acute phase reaction in horses. Domest Anim Endocrinol. 2020;72:106476.
23. Dondi F, Lukacs RM, Gentilini F, Rinnovati R, Spadari A, Romagnoli N. Serum amyloid A, haptoglobin, and ferritin in horses with colic: association with common clinicopathological variables and short-term outcome. Vet J. 2015;205(1):50-55.
24. Jacobsen S, Vinther AM, Kjelgaard-Hansen M, Nielsen LN. Validation of an equine serum amyloid A assay with an unusually broad working range. BMC Vet Res. 2019;15(1):462.
25. Andreasen U, Perret-Liaudet A, van Waalwijk van Doorn LJ, et al. A practical guide to immunoassay method validation. Front Neurol. 2015;6:179.
26. Jensen AL, Kjelgaard-Hansen M. Diagnostic Test Validation in Schalm’s Veterinary Hematology. 6th ed. Ames, IA: Blackwell; 2010.
27. Crisman MV, Scarratt WK, Zimmerman KL. Blood proteins and inflammation in the horse. Vet Clin North Am Equine Pract. 2008;24(2):285-297.
28. Pihl TH, Andersen PH, Kjelgaard-Hansen M, Mørck NB, Jacobsen S. Serum amyloid A and haptoglobin concentrations in serum and peritoneal fluid of healthy horses and horses with acute abdominal pain. Vet Clin Pathol. 2013;42(2):177-183.
29. Girardi AM, Toledo CZ, Silva PC, Marques LC. The effect of age and sex on serum proteins in the Pega donkey (Equus asinus). Vet Med. 2017;62(1):10-15.
30. Pollock PJ, Prendergast M, Schumacher J, Bellenger CR. Effects of surgery on the acute phase response in clinically normal and diseased horses. Vet Rec. 2005;156(17):538-542.
31. Taira T, Fujinaga T, Tamura K, et al. Isolation and characterization of alpha 1-acid glycoprotein from horses, and its evaluation as an acute-phase reactive protein in horses. *Am J Vet Res*. 1992;53(6):961-965.
32. Okumura M, Fujinaga T, Yamashita K, Tsunoda N, Mizuno S. Isolation, characterization, and quantitative analysis of ceruloplasmin from horses. *Am J Vet Res*. 1991;52(12):1979-1985.
33. Kahl S, Elsasser TH. Exogenous testosterone modulates tumor necrosis factor-alpha and acute phase protein responses to repeated endotoxin challenge in steers. *Domest Anim Endocrinol*. 2006;31(4):301-311.
34. Samimi AS, Samimi K, Karimlaftshar M, Tajik J. Comparative anti-inflammatory effects of insulin and flunixin on acute-phase responses and cardiovascular biomarkers during inflammatory phase in miniature donkeys. *J Equine Vet Sci*. 2019;81:102788.
35. Wearn JG, Suagee JK, Crisman MV, et al. Effects of the insulin sensitizing drug, pioglitazone, and lipopolysaccharide administration on markers of systemic inflammation and clinical parameters in horses. *Vet Immunol Immunopathol*. 2012;145(1-2):42-49.
36. Vinther AM, Heegaard PM, Skovgaard K, et al. Characterization and differentiation of equine experimental local and early systemic inflammation by expression responses of inflammation-related genes in peripheral blood leukocytes. *BMC Vet Res*. 2016;12:83.
37. Bonelli F, Meucci V, Divers TJ, Wagner B, Intorre L, Sgorbini M. Kinetics of plasma procalcitonin, soluble CD14, CCL2 and IL-10 after a sublethal infusion of lipopolysaccharide in horses. *Vet Immunol Immunopathol*. 2017;184:29-35.

How to cite this article: Perez-Ecija A, Buzon-Cuevas A, Aguilera-Aguilera R, Gonzalez-De Cara C, Mendoza Garcia FJ. Reference intervals of acute phase proteins in healthy Andalusian donkeys and response to experimentally induced endotoxemia. *J Vet Intern Med*. 2021;35:580–589. [https://doi.org/10.1111/jvim.16015](https://doi.org/10.1111/jvim.16015)