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ORIGINAL ARTICLE

Peritoneal bile acids concentration in adult horses with hepatic and gastrointestinal disorders

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Summary

Background: Peritoneal bile acids concentration (PBAC) has not been previously reported in horses. A case of liver lobe torsion in which increased PBAC was detected prompted us to study PBAC in horses.

Objectives: (a) To determine a reference range of PBAC in horses; (b) to compare PBAC from horses with either hepatic or gastrointestinal disease and healthy horses and (c) to assess the prognostic and diagnostic values of PBAC.

Study design: Prospective case-control.

Methods: Prospective observational clinical study. Bile acids concentrations were measured in both plasma and peritoneal fluid in selected clinical patients with hepatic or gastrointestinal disease (n = 108) and healthy horses (n = 11). Sixty-eight of 108 patients survived to hospital discharge, and the remaining 40 were nonsurvivors. Additionally, other haematological and biochemistry analyses were performed.

Results: Sick horses were classified according to diagnosis into hepatic (n = 13), gastrointestinal (GI) obstructive (n = 48) and GI ischaemic-inflammatory (n = 47) groups. The hepatic group had significantly higher PBAC (6.8 [2.3-9.4]; median [IQR]) than the control (1.0 [0.6-1.5]) and GI obstructive groups (1.2 [0.8-1.7] μmol/L; $P < .001$). Moreover, the GI ischaemic-inflammatory group (3.3 [1.4-5.5]) also had significantly higher values than the control and GI obstructive groups ($P < .001$). Regarding outcome, the nonsurvivor group (n = 40) had significantly higher median PBAC value than the survivor group (n = 68, 4.1 [1.6-6.5] vs 1.3 [0.8-3]; $P < .001$).

Main limitations: A higher number of horses with abdominal disease is required to confirm the clinical significance of these findings.

Conclusions: PBAC may have a role in the diagnosis of hepatic and gastrointestinal disease and as a prognostic tool in horses with abdominal pain.

KEYWORDS

colic, horse, liver, peritoneal fluid

1 | INTRODUCTION

Bile acids are amphophilic molecules produced by the liver that serve as detergents and make up to 90% of the organic portion of bile. These acids are present in high concentration in the portal circulation, are extracted by the liver with a high level of efficiency and are normally transported away from the liver through the biliary circulation. The enterohepatic circulation normally removes more than 90% of bile acids.[1]

Concentrations of total plasma bile acids between 5 and 28 μmol/L have been reported in normal adult horses.[2] Increased plasma bile acids concentration are highly specific for the presence of liver insufficiency in horses but is not specific for the type of liver disease.[1,3,4]

Certain metabolites and enzymes present in the equine peritoneal fluid have been evaluated as indicators of ischaemia, inflammation or other abdominal disorders.[5-9] Some of these parameters include lactate,[7] pancreatic enzymes (amilase, lipase)[10] and hepatic enzymes (alkaline phosphatase).[11] In addition, peritoneal:serum creatinine ratio is very useful to confirm uroabdomen in neonatal foals.[12] However, peritoneal bile acids concentration (PBAC) has not been reported previously in horses.

Bile peritonitis has been described in human medicine after traumatic gallbladder or bile duct rupture by either a penetrating wound or a closed injury, by leakage following surgical biliary tract procedures, by transudation of bile through the walls of a gangrenous but nonperforated gallbladder, after rupture of a subcapsular cholangitic abscess of the liver or as an idiopathic disorder.[13,14] Bile peritonitis has been described in dogs and cats mainly due to disruption of the biliary tract secondary to trauma or necrotising cholecystitis.[15,16] More recently, liver lobe torsion has been reported in horses as a cause of colic and bile peritonitis.[17,18] Peritoneal bile acids concentration was found to be elevated in an individual case of liver lobe torsion in our hospital while the remainder of the hepatic enzymes and plasma bile acids concentration were not significantly increased. In that case, PBAC was comparable to those found in plasma of horses with severe hepatic insufficiency. However, no previous data of normal PBAC was available for the correct interpretation of that finding.

Therefore, the main objectives of the present study were: (a) to determine a reference range of PBAC in horses; (b) to compare PBAC from horses with either hepatic or gastrointestinal disease and healthy horses and (c) to assess the prognostic and diagnostic validity of PBAC in horses with hepatic or gastrointestinal disease.

2 | MATERIALS AND METHODS

2.1 | Study population

Horses admitted to the Unitat Equina, Fundació Hospital Clínic Veterinari, Universitat Autònoma de Barcelona, Spain for colic or investigation of suspected hepatic disease between June 2010 and June 2014 were considered for enrolment in the study. Foals younger than 12 weeks of age and horses admitted for colic or hepatic disease in which abdominocentesis was not performed during the diagnostic workup were excluded. Additionally, a group of 11 healthy horses from a teaching herd was enrolled as a control group.

All procedures were conducted in compliance with the guidelines established by the institution animal experimentation ethics committee.

2.2 | Classification

Clinical cases were classified according to their final diagnosis into 3 different groups: horses with liver disease (hepatic group), horses with colic due to simple intestinal obstruction or gastric disease (GI obstructive group) and horses with colic due to ischaemic/inflammatory disorders (GI ischaemic-inflammatory group).

The hepatic group included horses with a diagnosis of liver disease based on at least one of the following criteria: (a) plasma hepatic profile suggestive of hepatopathy or hepatic insufficiency, or (b) evidence of significant hepatopathy on histopathologic examination of samples obtained by ultrasound guided percutaneous biopsy or (c) liver samples obtained at necropsy. Horses were not included in the hepatic group solely based on post-mortem findings. Hepatic profile included plasma bile acids, total and direct bilirubin concentrations, gamma-glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH) activities. Horses in this group had at least two of these five parameters above the reference range. Normal plasma bile acids were considered <20 μmol/L.[19] Post-mortem examinations and liver histopathology were performed by an independent board-certified pathologist with knowledge of the clinical findings and diagnostic workup, but unaware of the PBAC results.

Horses in the GI obstructive and GI ischaemic-inflammatory groups were classified based on clinical history, findings of a complete physical examination, rectal palpation and nasogastric intubation, and results of ancillary diagnostic tests (CBC, biochemistry and blood gas analysis, gastroscopy, abdominal ultrasonography and peritoneal fluid analysis). In those horses that underwent abdominal surgery, the diagnosis was confirmed. Liver disease was ruled out in both groups based on either normal plasma hepatic profile and liver ultrasound in surviving horses, or absence of hepatic abnormalities on *post-mortem* examination of nonsurviving cases. The diagnosis of ischaemic, inflammatory, or obstructive gastrointestinal and hepatic disorders was done by the attending clinician based on the aforementioned diagnostic tests, abdominal surgery and *post-mortem* examination where appropriate, and blinded to the results of the PBAC.

The GI obstructive group included horses with nonstrangulating and noninflammatory disorders of the intestinal tract without signs of intestinal devitalisation and horses with gastric ulcers or impaction. The GI ischaemic-inflammatory group included horses with strangulating GI lesions and horses with inflammation of the small intestine, large intestine or peritonitis.

Horses included in the control group were healthy teaching horses whose soundness was assessed by a complete physical examination, peritoneal fluid (PF) analysis (cytology and total protein concentration) and evaluation of plasma hepatic profile (bile acids, total and direct bilirubin concentrations, GGT and GLDH activities).

Horses were also classified according to clinical outcome. The survivor group included all clinical cases that were discharged from the hospital. The nonsurvivor group included those cases that died during hospitalisation or were euthanised due to poor prognosis. Horses from the control group were not included in this classification.

2.3 | Collection and processing of samples

Blood samples were collected from all horses by routine jugular venipuncture into 1mL lithium heparin tubes. Once centrifuged for 15 minutes at 1,000 g, plasma samples were analysed immediately or otherwise frozen at −20°C and analysed within 48 hours. Plasma collected from horses of the hepatic group was used for biochemical analysis (bile acids, total and direct bilirubin concentrations, GGT and GLDH activities) with an automated chemistry analyser (AU400, Olympus).

Peritoneal fluid was obtained aseptically in all horses by abdominocentesis with a sterile blunt teat cannula, ultrasound guided, approximately 2 cm to the right of the midline at the most dependent area of the ventral abdomen. Each PF sample was collected into both 1mL lithium heparin and 1mL K_3-EDTA tubes. Lithium heparin samples were analysed for bile acids concentration using the same enzymatic colorimetric method (Randox) as for plasma determination immediately after centesis or otherwise frozen at −20°C and analysed within 48 hours.

Samples in K_3-EDTA tubes were used for peritoneal total protein concentration, white cell count and cytology. White cell counts were obtained using an automatic cell counter (Advia 120 analyser, Bayer Lab) and total protein concentration was measured using a refractometer.

2.4 | Validation of determination of bile acids concentration in peritoneal fluid

Biochemical measurement of bile acids concentration in horse peritoneal fluid was validated as part of this study following the methodology elsewhere described.[20] For the calculation of intra-assay CV a low concentration sample (7.2 μmol/L) and high concentration sample (187.5 μmol/L) were analysed 10 times. For the calculation of inter-assay CV the same samples were analysed during 5 consecutive days. Mean values for intra-assay CV and inter-assay CV were 0.5% and 0.7% respectively. An individual sample with an initial concentration of 187.5 μmol/L was used to perform serial dilutions up to 1:128 (1.5 μmol/L) of the analyte in order to determine assay linearity. Using linear regression analysis an *r* value of 0.998 was obtained. Finally, the enzymatic colorimetric assay yielded a recovery ranging from 77% to 98% from spiked samples.

2.5 | Data analysis

Results for qualitative variables are described by absolute frequencies and percentages and as median and interquartile range [Percentiles 25th, 75th] for quantitative variables. Differences between diagnostic groups were analysed by means of one-way ANOVA with a nonparametric approach using rank transformation and pair-wise comparison. A Mann-Whitney U test was used to explore the association of PBAC and outcome. Correlation between peritoneal and plasma bile acid concentrations was determined by Spearman correlation coefficient. Statistical analysis was performed using SPSS version 20.0 (SPSS Inc) and a two-sided Type I error of 5% was used in all analyses ($P < .05$).

Additionally, sensitivity, specificity and predictive values of PBAC for the diagnosis of liver or gastrointestinal disease and outcome were calculated.

3 | RESULTS

A total of 119 animals were included in the study, 108 sick horses and 11 healthy horses. Twenty-seven out of 119 were stallions (22.7%), 51/119 were geldings (42.9%) and 41/119 were mares (34.5%). Horses were between 3 months and 30 years old (9.6 ± 6.0, average ±SD). The breed distribution reflected the hospital's referral population, with 37 Andalusian (31.1%), 29 cross-bred (24.4%), 14 Warmblood (11.8%), 13 Arabian (10.9%), 9 ponies (7.6%), 7 Draught-bred horses (5.9%) and 10 horses of other breeds (8.4%). Sick horses were classified according to diagnosis into hepatic (n = 13), GI obstructive (n = 48) and GI ischaemic-inflammatory (n = 47) groups.

The hepatic group included 13 horses with the following diagnosis: liver lobe torsion (2), cholangiohepatitis (4), hepatic lipidosis (2), lymphoma (1), metastatic hepatic melanoma (1), hepatic abscess due to *S equi equi* (1), and uncharacterised hepatopathy and hepatic insufficiency (2). The diagnosis of liver disease was confirmed by histopathology in 9 out of 13 cases (ultrasound guided biopsy n = 4, necropsy n = 5). The GI obstructive group included 48 horses with diagnosis of large colon impaction (22), large colon displacement (18), gastric ulcers (4), small colon obstruction (3) and stomach impaction (1). The "GI ischaemic-inflammatory" group included 47 horses with diagnosis of intestinal volvulus or torsion (21), epiploic foramen entrapment (2), inguinal hernia (4) and caecocolic intussusceptions (3). Horses with enteritis (2), colitis (9) and septic peritonitis (6) were also included in this GI ischaemic-inflammatory group. Considering outcome, a total of 68/108 sick horses survived to discharge and were included in the survivor group. Seventeen out of 68 survivors were in the GI ischaemic-inflammatory group, 44 in the GI obstructive group and 7 in the hepatic group. Forty horses died or were euthanised due to poor prognosis. Thirty of the nonsurviving horses were from the GI ischaemic-inflammatory group, four from the GI obstructive group and six from the hepatic group. Finally, 11 healthy horses were enrolled in the control group.

3.1 | Plasma bile acids concentration

The median value of plasma bile acids concentration in the hepatic group (9.66 μmol/L) was significantly higher than the control (4.16; $P = .04$), GI obstructive (2.64; $P = .003$) and GI ischaemic-inflammatory (2.63; $P = .016$) groups (Figure 1; Table 1). No other statistically

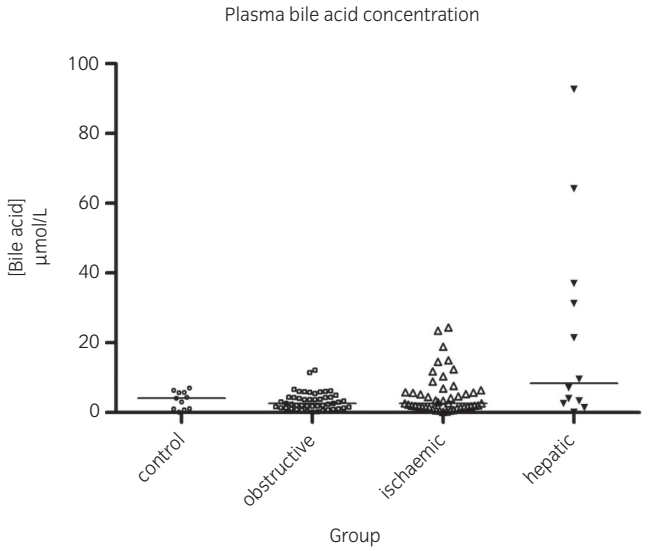

FIGURE 1 Plasma bile acid concentration (µmol/L) of control and sick horses classified into four different groups. The median bile acid concentration is significantly higher in horses with hepatic disease relative to control and gastrointestinal groups

significant differences were observed among diagnostic groups. No differences were observed between survivor and nonsurvivor groups.

3.2 | Peritoneal bile acids concentration

Determination of PBAC was possible in all the samples obtained without technical limitations related to the type of fluid (PF) analysed (ie transudate, modified transudate and exudate).

The median PBAC of the hepatic group was significantly higher than in the control and GI obstructive groups (6.8 vs. 1.0 and 1.2 µmol/L respectively; $P < .001$). Moreover, the GI ischaemic-inflammatory group also had significantly higher median PBAC than the control and GI obstructive groups (3.3 vs. 1.0 and 1.2 µmol/L respectively; $P < .001$). No differences were observed between the GI ischaemic-inflammatory and the hepatic group and between control and GI obstructive groups (Figure 2). Regarding outcome, the nonsurvivor group had significantly higher median PBAC than the survivor group (4.1 vs 1.3 µmol/L respectively; $P < .001$).

Following conventional statistical criteria, we considered as a possible PBAC cut-off value 2.28 µmol/L, that corresponded to 2 standard deviation over the mean of control group (1.04 µmol/L; SD 0.62). Most of the hepatic (9 of 13; 69%) and the GI ischaemic-inflammatory (30 of 47; 64%) horses had a PBAC ≥2.28 µmol/L, whereas only 7 out of 48 horses (15%) with GI obstruction had PBAC ≥2.28 µmol/L. Related to outcome, 68% of nonsurvivor horses had PBAC≥2.28 µmol/L, in contrast to 28% of the survivors.

Correlation of plasma and peritoneal bile acid concentrations was low when all horses were included (rho = 0.44, $P < .001$), and subjectively most of the obstructive colics had normal PBAC and plasma

TABLE 1 Median and IQR of the plasma and peritoneal bile acids concentration obtained in control horses, gastrointestinal disease and hepatic disease groups. Horses are also classified as survivor and nonsurvivor groups

	Hepatic group	GI obstructive group	GI ischaemic-inflammatory group	Control group	Survivor group	Nonsurvivor group	Reference range
Plasma bile acid concentration (µmol/L)	9.66 [3.37-31.22]	2.64 [1.37-4.39][a]	2.63 [1.50-6.40][a]	4.16 [1.00-5.80][a]	2.81 [1.59-5.27]	3.72 [1.31-9.28]	0-20
PBAC (µmol/L)	6.77 [2.27-9.44]	1.20 [0.75-1.66][a,b]	3.29 [1.36-5.5]	1.00 [0.59-1.49][a,b]	1.34 [0.82-3]	4.14 [1.58-6.53][c]	

Abbreviation: PBAC, peritoneal bile acids concentration.

[a]Median significantly different from the hepatic group.

[b]Median significantly different from the GI ischaemic-inflammatory group.

[c]Median significantly different from the survivor group.

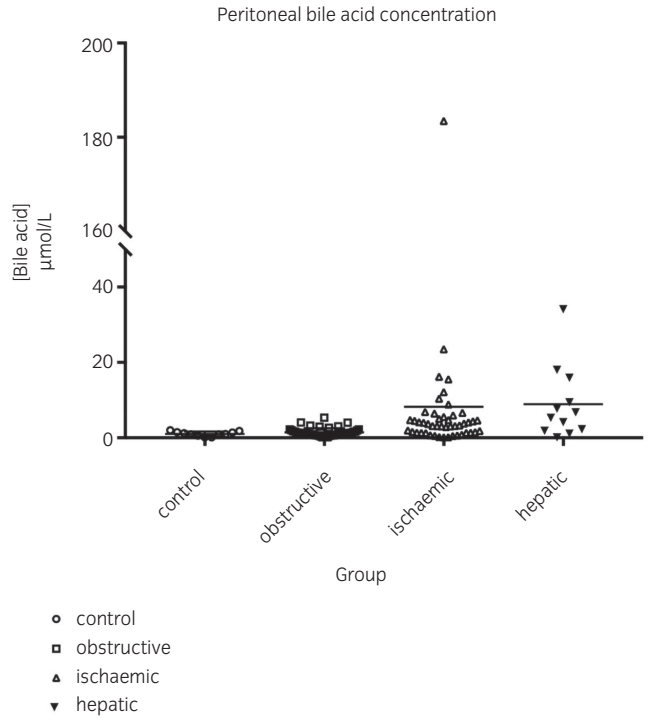

FIGURE 2 Peritoneal bile acid concentration (µmol/L) of control and sick horses classified into four different groups. The median PBAC is significantly higher in horses with hepatic disease relative to control and gastrointestinal obstructive groups. In addition, median PBAC is significantly higher in horses with ischaemic gastrointestinal disease relative to control and obstructive gastrointestinal groups

bile acids, whereas higher increases in PBAC relative to plasma were observed in the ischaemic-inflammatory group (Figure 3).

3.3 | Sensitivity, specificity, positive and negative predictive values of PBAC

Using the population of sick horses (hepatic and nonhepatic groups), the sensitivity, specificity and predictive values of PBAC for the diagnosis of hepatic disease were calculated. Using the cut-off value of 2.28 µmol/L, PBAC showed modest sensitivity (69%, [39%-91%]; [95%CI]) and specificity (61%, [51%-71%]). Although the positive predictive value (PPV) was poor (19.6%, [14%-27%]), the negative predictive value (NPV) was fairly good (94%, [86%-97%]).

Considering that the two groups including horses with more severe abdominal disease (ie hepatic and GI ischaemic-inflammatory groups) had the highest percentage of horses with increased PBAC, we investigated the usefulness of this parameter to diagnose severe abdominal disease. The sensitivity and NPV were moderate (63% [52%-77%] and 72% [63%-78%] respectively) but fair to good values of specificity and PPV (89% [77%-95%] and 84% [73%-92%] respectively) were observed.

In addition, the sensitivity, specificity and predictive values of PBAC for the prognosis of sick horses (hepatic and nonhepatic groups) were calculated. Using the cut-off value of 2.28 µmol/L to predict outcome, PBAC showed modest sensitivity (67% [51%-81%]) and PPV (58% [48%-69%]). Similarly, the specificity (73% [60%-82%]) and NPV (79% [70%-86%]) were moderate.

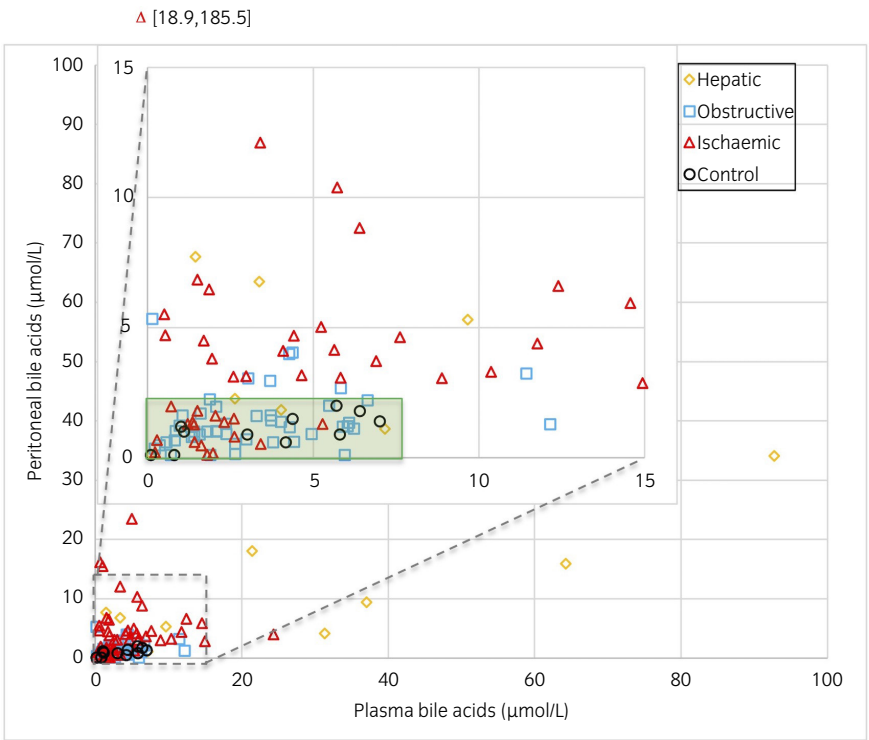

FIGURE 3 Scatterplot of plasma and peritoneal bile acid concentrations. Note that the inset graph is a zoom in to display most of the values in the lower range. An outlier value [X,Y] is depicted outside of the main graph. The green box represents the reference range of peritoneal and plasma bile acid concentration

4 | DISCUSSION

The main findings of this study were: (a) healthy horses have negligible peritoneal bile acids concentrations (three times lower than plasma bile acids), (b) about 70% of horses with ischaemic or inflammatory gastrointestinal disorders or hepatic disease have altered PBAC, and (c) increased PBAC is associated with nonsurvival in horses with colic or hepatic disease.

Peritoneal bile acids concentration has not been established before in horses. One of the main objectives of the study was to determine PBAC in horses and to compare concentrations from horses with either hepatic or gastrointestinal disease and healthy horses. Abdominocentesis and laboratorial analysis of PF are common diagnostic procedures in horses admitted to a referral hospital for gastrointestinal (GI) disorders and other intraabdominal conditions. It is well recognised that colicky horses with a simple obstructive GI lesion (eg pelvic flexure impaction) will usually have normal PF cytology (transudate) and low lactate concentration; in contrast, those with an ischaemic GI lesion (eg small intestinal volvulus) could have abnormal WBC count and TP concentration and/or high lactate concentration relative to plasma.[7] Similarly, determination of creatinine concentration in PF, relative to that in plasma, is of diagnostic value in cases with suspected uroabdomen.[12] The observation of unremarkable plasma liver enzymes but increased peritoneal and plasma bile acids concentration in a horse with severe abdominal pain caused by liver torsion spurred us to investigate the diagnostic and prognostic value of PBAC in horses. At necropsy, complete rupture of the liver lobe capsule secondary to liver lobe torsion was observed and, in the authors' opinion, it could had been the definitive cause of the release of bile acids into the abdomen. Our hypothesis was that determination of PBAC could be useful to diagnose specific gastrointestinal disorders (eg ischaemic lesions) and specific liver diseases (eg liver torsion or cholelithiasis) or could be associated with outcome.

Bile acids, amphophilic derivatives of cholesterol, are synthesised within hepatocytes, conjugated with either glycine or taurine, and are excreted into the bile.[1,21] Given their "detergent-like" properties, bile acids have an important role in digestion and absorption of lipids within the small intestine.[22] Nondegraded bile acids are efficiently absorbed by specific transporters of the ileal mucosa and transported back to the liver via the portal circulation, and about 90% of the bile acid pool is restricted to the enterohepatic circulation.[21,22] Since hepatic metabolism is the only source of bile acids synthesis, and the vast majority of bile acids remain in the enterohepatic circulation, increased plasma bile acids concentration has been described as useful biomarker of liver disease and function in horses.[3,21,23,24] More recently, plasma bile acids and other hepatic biomarkers have been investigated in horses undergoing colic surgery.[25] In this pilot study, horses with markedly increased admission plasma bile acid concentrations and SDH activities did not survive, however, the small number of horses included and the bias towards only surgical cases, does not allow for direct comparison with the present study.

Peritoneal lactate concentration is considered a sensible biomarker of intestinal ischaemia in colicky horses and those with high lactate concentration in PF are less likely to survive.[26-28] Horses with peritoneal lactate concentration over 4 mmol/L after admission to a referral hospital were reported to be three times more likely to suffer a strangulating intestinal lesion.[29] Similarly, as ischaemia and hypoxia progress, intestinal wall devitalisation could result in increased intestinal wall permeability and diffusion of intraluminal substances (ie bile acids) into the peritoneal fluid. Given their biochemical properties, bile acids should not easily diffuse across cell membranes, and increased bile acids concentration in peritoneal fluid could be a marker of bile duct disruption or loss of intestinal wall integrity (ie devitalisation). Therefore, increased bile acids concentration could perhaps be suggestive of more advanced intestinal necrosis and not simply early stages of ischaemia and hypoxia.

The results obtained from healthy horses suggest that in normal physiological conditions, bile acids might be present in small concentration in the peritoneal cavity. On average the ratio of peritoneal to plasma bile acid concentrations in normal horses was 1:3. In contrast, the median PBAC of the hepatic group was significantly higher than the control and GI obstructive groups, and the GI ischaemic-inflammatory group had significantly higher values than the control and GI obstructive groups (Table 1). A large proportion of horses with GI ischaemic-inflammatory disorders had high ratio of peritoneal to plasma bile acids concentration (1:1 and higher). Higher median PBAC in the hepatic and GI ischaemic-inflammatory groups compared to other groups could be related with the compromised liver structure or diffusion of bile acids from the intestinal lumen across a compromised gut wall into the peritoneal cavity.

The NPV of PBAC for the diagnosis of hepatic disease was fairly good (94.5%) meaning that in this population, a horse with a PBAC below 2.28μmol/L has a 94.5% chance of not having hepatic disease. Although the positive predictive value (PPV) was low (19.6%) it was higher than the prevalence of the hepatic disease in this population of hospitalised horses (ie 12%) meaning that this parameter could be valuable as a screening test for the diagnosis of hepatic disease in this type of population.

The specificity and PPV of PBAC to diagnose severe abdominal disease were fair to good (88.7% and 84% respectively). A practical interpretation would be that a horse with a PBAC >2.28μmol/L has 84% possibility of having an inflammatory/ischaemic intestinal disease or hepatic disorder.

Related to prognosis, 67.5% of nonsurvivor horses had PBAC higher than 2.28 μmol/L, in contrast to 27.9% of the survivors. Lactate has been studied as prognostic indicator in colic patients.[7] Considering the results herein described, PBAC could be used in combination with peritoneal lactate concentration in order to predict the chances of survival in colic patients.

Plasma bile acids concentration have been previously determined in horses by the colorimetric method, with values of 5-28 μmol/L considered normal.[2] In another study of horses undergoing colic surgery, normal plasma bile acids concentration was considered <8.6 and mildly high 8.6-20 μmol/L.[25] In the present study, only horses included within the "hepatic" group presented increased plasma bile acids concentration. The reason of admission in five out of 11 horses

of the "hepatic group" was colic signs. Blood analysis and complementary tests allowed us to classify as hepatic disease. In a previous study, 24 out of 26 horses with diagnosis of GI disease in which plasma bile acids were determined resulted within the reference range.[3] This finding is similar to our results in that horses in both colic groups had unaltered plasma bile acids concentration.

The findings of the present study should be interpreted considering several limitations. Firstly, the possibility of misclassification of cases to diagnostic categories, given that a full biochemical hepatic profile was not performed in all horses admitted for gastrointestinal disorders, or histopathologic examination was not performed in 4 out of 13 hepatic cases. Secondly, the preliminary reference range proposed in this study should be validated with an external population, and the 2.28 μmol/L cutoff for PBAC should be interpreted with caution. And finally, these findings in a referral hospital caseload may not be representative of the general population of horses with gastrointestinal or hepatic disorders.

In conclusion, PBAC is not an accurate biomarker of hepatic disease but it could be useful to diagnose horses with ischaemic or inflammatory abdominal disease. In the present study horses with ischaemic or inflammatory gastrointestinal disease had increased PBAC and altered PBAC is associated with nonsurvival. A preliminary reference range of peritoneal bile acids concentration is 0-2.28 μmol/L, which is three times lower than plasma bile acids concentration. A higher number of horses with abdominal disease is required to better understand the clinical significance of these findings in horses with gastrointestinal disease without hepatic involvement.

ETHICAL ANIMAL RESEARCH

Procedures on healthy animals were approved by the Institution's animal experimentation ethics committee.

INFORMED CONSENT

Explicit owner informed consent for inclusion of samples from animals in this study was not sought but owners were given the option to opt out of research.

ACKNOWLEDGEMENTS

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CONFLICT OF INTERESTS

No competing interests have been declared.

AUTHOR CONTRIBUTIONS

ML Rodríguez-Pozo and E. Jose-Cunilleras contributed to study design, data collection and study execution, data integrity, data analysis and interpretation. L. Armengou contributed to study design, and data collection and study execution. J. Viu contributed to data collection and study execution, and data analysis and interpretation. J. Ríos contributed to data integrity, and data analysis and interpretation. All authors contributed to the preparation of the manuscript and gave their final approval.

PEER REVIEW

The peer review history for this article is available at https://publons.com/publon/10.1111/evj.13538.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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