Can prognosis be assessed by a novel biomarker at the time of patient admission for horse colics?

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ABREVIATIONS

APP(s): acute phase protein(s)

APR: acute phase response/reactant

Bpm: beat per minute

$B_r$pm: breath per minute

CLP: calprotectin

CI: confidence interval

CRT: capillary refill time

CV: coefficient of variation (%)

DIC: disseminated intravascular coagulation disorder

EDTA: ethylenediaminetetra – acetic acid

ELISA: enzyme-linked immunoassay

GGT: gamma glutamyl transferase

HMGB1: high mobility group box chromosomal protein 1

HPTG: haptoglobin

HR: heart rate

IQR: interquartile range

ISBER: international society for biological and environmental repositories

LOD: limit of detection

OD450: optical density 450 nm

PF: peritoneal fluid

PVC: packed cell volume

RR: respiratory rate

SAT3: oxidative chromogenic dye
N,N'-Bis(2-hydroxy-3-sulfopropyl)tolidine, disodium salt, tetrahydrate

US: ultrasound

WBC: white blood cells

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1. INTRODUCTION

Colic is a generic term for abdominal pain symptoms. In the equine medicine field, it may range from a short crisis of discomfort to a life-threatening episode, because it can be associated with various etiologies. It is typically detected based on behavioural changes of the animal (SUTTON et al., 2013) and later on confirmed by clinical exam, although the involved pathophysiologic processes can be challenging to identify. The constant interest towards new insights into colic diagnosis proves that struggle still exists with that matter (MAIR, 2009; MAIR and WHITE, 2010).

Colicky horses are an emergency frequently met by practitioners whether they are first opinion or referral veterinarians. WHITE (2005) outlines that over a year in a group of 100 horses, four to 10 will have a colic episode. Eighty to 85% of those are labelled with a non-specific etiology and up to 4% of colic cases require surgical intervention. The diseases causing gastro-intestinal illnesses can be classified in several ways (affected anatomical locations, involved pathophysiological processes, pain level, duration …), yet the most important categorization is linked to the decision tree: surgical versus medical management. The late determination of a surgical case can have major detrimental consequences on survival (WHEAT, 1975).

It is of prime importance to promptly and accurately distinguish the kind of pathology involved in each case. Multiple scanning and laboratory screening tools (FREEMAN, 2002; BECCATI et al., 2011) are now accessible to complement the physical exam. Although from the first identification to the time of decision-making, most of the variables are subjectively interpreted (pain level, ultrasonography and rectal palpation findings). This could potentially lead to cognitive biases (FARRELL et al., 2021). Moreover, management of colic is yet quite problematical besides the latest improvements of treatment plans and drug availability (FREEMAN, 2018). Clearly identifying the real cause of the presented colic, at the early time of admission, would be of assistance to choose the proper treatment strategy. This would lead to less case fatality by reducing erroneous triage choices.

For the past seven years, biomarkers have attracted substantial attention for medical applications (LUDWIG et al., 2023). The validation of biological markers is a long process starting with the identification and selection of good candidates (PERERA et al., 2022). Once their reliability is established and their clinical endpoints are determined, they can be used objectively for decision-making (ATKINSON et al., 2001). For that purpose, acute phase proteins (APPs) have already been widely investigated (PIHL et al., 2013; SOUTO et al., 2019).

So far, preferred biomarkers in colic cases are lactate, creatine kinase (CK), albumin and a few APPs: serum amyloid A (SAA), haptoglobin, fibrinogen and C-reactive protein (CRP). Still, lactate tends to be the only efficient tool as the ratio between peritoneal and blood concentrations indicates bowel ischemia (LUDWIG et al., 2023). A number of other APPs are known to be present in serum or full blood, but not extensively studied yet regarding their potential in equine colic. Among those, calprotectin and HMGB1 are of interest since they are both linked to neutrophils activation. As part of the inflammatory

response, those cells are known to be among the first ones to reach sites of infection, inflammation, or injury (CHAN et al., 2021).

The purpose of this study is to evaluate the relevance of quantifying APPs in serum and in plasma, namely haptoglobin, HMGB1 and calprotectin, as biomarker tools to determine etiology types and prognosis of colic cases.
We hypothesize an increase in HMGB1 and calprotectin concentrations, and a decrease in haptoglobin concentrations in line with colic severity and non-survival of patients.

The specific goals are:

- To assess the differences in concentration of haptoglobin, calprotectin and HMGB1 between serum and plasma.
- To evaluate the prognosis value of each individual protein, haptoglobin, calprotectin and HMGB1, by assessing the correlation of their concentration with the survival of patients.
- To evaluate the diagnostic value of the same individual proteins by measuring the correlation of their concentration with colic types.

1. LITERATURE REVIEW

Considering the limited availability and commercialisation of point-of-care tests, decision-making processes were previously mostly based on variables gathered during physical exams of colic patients. Addition of laboratory parameters to the diagnostic tree helped out define a sharpened image of colic cases. However, because of the poor reliability and definition of cut-off values, the decision process stays empirical and predominantly based on the veterinarian's interpretation.

More than 30 years ago, ORSINI et al. (1988) were already emphasizing that the most useful strategy is to use the values as a combination. They came to the conclusion that combined lactate level in serum and PCV (Packed Cell Volume) value, make a significant tool for prognosticating survival. Later on, CRT (Capillary Refill Time) and blood anion gap are added to the previous parameters by EBERT, R. (1995). Improving strategy to predict outcome, based on routine examination of colic horses, has often been a subject of research. Elevated HR (Heart Rate), elevated PCV, delayed CRT, poor mucous membrane colour and abnormal blood pH, were all assumed to be valuable parameters (REEVES et al., 1989; PARRY et al., 1983).

SANDHOLM et al. (1995) proposed to add a quick on site-testing of D-Dimer at admission time, alongside conventional variables. An increase in concentration of this oligomer gives evidence of ongoing fibrinolytic activity. By doing so, they aim to reduce the non-identified cases of coagulopathies. Once more, the importance of interpreting parameters jointly is raised. D-Dimer is now validated in equine medicine as a sensitive tool. Measured in plasma and peritoneal fluid (PF), it identifies cases of enteritis, peritonitis or onset of disseminated intravascular coagulation (DIC) disorder (DELAGADO et al., 2009; CESARINI et al., 2010). It is also determined in synovial fluid of foals to confirm and monitor septic arthritis (RIBERA et al., 2011).

As more and more studies are carried out on this challenging topic, the use of subjective evaluations from physical exams findings is still matter of discussion. VAN DER LINDEN et al. (2003) analysed the prognosis power of combined white blood cell count (WBC), PCV, blood pH and mucous membrane colour parameters. They came to contradict the previously cited investigations as the combination of clinical parameters of their study is not significantly correlated with the outcome of the horses. Other values like the duration of colic signs, quality of peristalsis and bowel motility, degree of skin tenting, HR, pain level and PF appearance, proved to be useful in identifying survival potential.

Similarly, THOEFNER et al. (2003) questioned the reliability of parameters from the physical exam as their model of decision tree turned out to be non-significant. It was featuring rectal exam findings, PCV, rectal temperature, estimated pain level and PF appearance.

Here again, FARRELL et al. (2021) were able to find relevance in the same base of parameters. The combination of HR, RR (Respiratory Rate), high blood lactate and the identification of abnormal rectal and US (Ultrasound) findings, wears a significantly poor prognosis. On the opposite, increased total calcium concentration is paired with good chances

of survival. Abdominal US is a relatively new tool and the images scanned are, to some extent, subjectively interpreted by clinicians. Nevertheless, BECCATI et al. (2011) proved that abdominal screening can accurately discern pathologies like strangulations, nephrosplenic entrapment, and differentiate small from large bowel involvement. Visualisation of the mesenteric vasculature can also orientate on the type of large colon involvement (MANSO-DIAZ et al., 2020).

The skin tent test and dryness of mucous membrane are assumed to help out assess the percentage of dehydration of patients, together with CRT. Colic horses are often dehydrated and this test is empirically done during the regular exam, fluid therapy being the primary step of procedures. However PRITCHARD et al. (2008) showed that those parameters are not reliable since the duration of the tent and the mucosa tackiness are cancelled by the plasma osmolality of the research subjects.

Keeping in mind the needs to not only get a prognosis tool but also reinforce therapy choices, IHLER et al. (2004) distinguished medical and surgical management. From their results, PCV is the single parameter that orientates towards medical treatment, while the combination of elevated HR and abnormal mucous membrane colour is the cue to surgical procedure. They did not obtain significant results from the incorporation of D-Dimer blood concentration and hypothesise that therapies given before sampling did interfere with their work samples.

SOUTHWOOD and LINDORG (2021) detected that the association of PCV, blood glucose, blood lactate, decreased rectal temperature and first time colic at an advanced age; are parameters associated with strangulating episodes. In a simplified manner, KOS et al. (2022) confirmed that elevated PCV and HR are most common in horses in need of surgical treatment.

1.1. Biomarkers could be the ultimate diagnosis and prognosis tool

Any biological process happening in the body is the result of physiological reactions and activated pathways which require enzymes, proteins and other molecules. This does leave marks whether it occurs locally in a tissue or systemically. Following the changes in concentration of those elements could then give an early warning about processes visible later on only.

Biomarkers associated with intestinal ischemia and injuries have been identified in multiple species, arising from researches in human medicine. In horses, lactate, albumin and creatine kinase are the only ones available so far to foresee ischemic lesions. However just as in humans, their reliability is not sufficient for diagnosis purposes out of the clinical context.
To be efficient, optimum markers need to be strongly sensitive and specific to the related pathology and quantifiable in both local fluid and systemic blood (LUDWIG et al., 2023).

For that purpose, molecules taking part in the Acute Phase Response of the host (proinflammatory cytokines and APPs) are good candidates. They are activated by injured cells in result to inflammation, neoplastic growth, trauma, stress or infection, and circulate in

systemic blood (MURATA et al., 2004; CRISMAN et al., 2008; CRAY and BELGRAVE, 2014). Due to their short life span, cytokines are of limited use. However, elevation of most APPs (glycoproteins) lasts over 48 hours (GRUYS et al., 2005).

APPs are defined by either decreasing (negative) or increasing (positive) concentrations in blood during inflammation. The positive APPs are classified by their dynamic of rise: major, moderate or minor. Within the positive APPs, the major type is defined by proteins sparsely present in the blood of healthy animals that will rise 10 to 1000 fold after trigger. They reach maximum concentration 24 to 48 hours later, before decreasing quickly. Moderate APPs are usually present in blood, multiplying their concentration only five to 10 fold. They rise after major APPs (48 to 72 hours) and slowly decrease. Minors APPs develop gradually and only gain a maximum of a two fold increase (GRUYS et al., 2005; GÜLEÇ et al., 2022).

Besides the differences in concentration variations, all APPs and other reactants are highly species specific. Even though there are all demonstrated in most mammals and birds, they may show different dynamics (AULBACH and AMUZIE, 2017; ECKERSALL and BELL, 2010). In addition, whatever the APP category, some drugs can interfere with the biologic process of the acute phase response. As an example, it was noted that parenteral application of glucocorticosteroids may affect the APRs (GRUYS et al., 2005)

1.2. Selection of recently studied equine APR molecules and their possible implementations

1.2.1. Serum Amyloid A

SAA is a major equine APP, per definition found in elevated amount quickly after injury but rapidly declining in the next days. Those specificities make of serum amyloid a good biomarker for picturing the level of inflammation and assessing the body response to therapy. It does not have the ability to differentiate the types of intestinal injury, nevertheless elevated concentrations are linked with bad prognosis (JACOBSEN, S., 2022; LUDWIG et al., 2023). Many applications are given to SAA, like the identification of systemic infection in neonates (BARR and NIEMAN, 2022) or even at the local level following a limb injury (MÜLLER et al., 2022) and the onset of placentitis as close as 96 hours post induction (COUTINHO et al., 2013).

Considering colic cases, elevated level of SAA is showing thrombophlebitis onset and orienting towards surgical management or humane euthanasia (DANIEL et al., 2016). Thus SAA is a pluripotent yet poorly specific biomarker (WESTERMAN et al., 2016).

1.2.2. Fibrinogen

Fibrinogen appears to be one of the oldest and commonly researched APPs in equine medicine. Even though it is often used to demonstrate systemic inflammation, this protein is not the best biomarker. As a moderate APP, it does not respond in a quickly manner nor is sensitive as it slowly decreases after stimulus (LUDWIG et al., 2023). HULTÉN et al. (2002) identified fibrinogen up to two weeks after the induction of non-infectious arthritis.

1.2.3. C-Reactive Protein

CRP is a major APP in dogs, able to non-specifically pick up infection, inflammation or neoplastic growth (MALIN and 2022). Equine CRP belongs to the group of moderate APPs, starting to increase around three to five days after onset of the disease. In conditions like septicaemia, colic and gastro-intestinal inflammation, blood concentration raises, showing non-specificity in horses (LUDWIG et al., 2023).

1.2.4. Cell Free DNA (cfDNA)

Canine cfDNA is measured significantly in the plasma of dogs during gastric dilatation and volvulus. So far, Equine cfDNA was not proven to be a selective biomarker as it is displayed in horses with colic but also with systemic inflammation (BAYLESS et al., 2021).

1.2.5. Creatine Kinase associated with lactate

LUDWIG et al. (2023) summarize the use of CK as a weakly specific biomarker. Although, used in combination with lactate, it gains potential to identify colic types. When both concentrations are measured in PF, elevated CK is proof of strangulation injury. As it shows to be sensitive but not sufficiently specific, the addition of lactate measurement allows to obtain a sharpen picture.

1.2.6. Matrix-Metalloproteinases (MMP)

Those enzymes are known to degrade the extracellular matrix. Not many studies can be found about equine MMP, besides the ones linked to laminitis and orthopaedic problems (CLUTTERBUCK et al., 2010). BARTON et al. (2021) have highlighted the presence of MMP in serum and PF of colic horses, potentially leading to identification of septicaemia and endotoxemia premises.

1.3. Chosen biomarkers for this study: possibilities and expectations

1.3.1. Haptoglobin

Haptoglobin is a positive moderate APP with the power to bind free haemoglobin. Synthesised in the liver, this protein is massively released into systemic blood during the inflammation response. However in patients with both inflammatory processes and intravascular hemolysis, blood concentration can appear neutral as haemoglobin is increasing and concurrently consumed by haemoglobin binding (GRUYS et al., 2005; CRISMAN et al., 2008).

For this reason, equine haptoglobin is not a reliable biomarker for determination of colic crisis. Serum concentrations are usually lower or the same than in healthy horses. Another reason why it is of limited use might be the short delay between onset of the colic and blood collection, since the haptoglobin response is only identified from 12 or 24 hours after stimulus. Nonetheless, this protein could be of interest to confirm chronic cases of colic (LUDWIG et al., 2023).

There are several contradictory studies on the subject. CRISMAN et al. (2008) reported that haptoglobin is a good marker of equine viral infections, but are no found significant changes in blood samples from colic horses.

In opposition, DA SILVA NOGUEIRA and SANTANA (2011) came up with elevated concentrations of the protein during episodes. They even distinguished horses with obstructed small bowel from the others. Similarly, DONDI et al. (2015) showed that haptoglobin concentration is decreased in horses with strangulating colic compared to non-ischemic ones. They suggested that because of the hemolysis and muscle lesions happening during strangulation, a large amount of haptoglobin is bonded and cannot be taken into account for analysis. Those cases being correlated with poor survival rate, they emphasized the prognosis potential of haptoglobin.

WESTERMANN et al. (2016) and SOUTO et al. (2019) analysed the haptoglobin content of blood from colic horses, based on medical management or surgical cases differentiation. None of the teams obtained significant differences between groups.

PIHL et al. (2013) focused on variations of both serum and PF concentrations. They deducted that PF is a reliable marker of intestinal inflammation since its content in haptoglobin is increased whereas serum concentration is decreased during acute colic. Furthermore, PIHL et al. (2015) explain that in cases with short time interval between onset of the crisis and sampling (less than five hours), haptoglobin significance is low. If the interval is longer than five hours, then both serum and PF contents gradually raise as the episode duration increases. However PF will show changes in concentration before serum and being even higher in ischemia pathologies. They also note a broad reference range in healthy patients. Later on, PIHL et al. (2016) concluded on the good reliability of haptoglobin to identify inflammation cases and differentiate them from pathologies needing surgical intervention. The delay between changes in PF content and serum content should be taken into account as the latter is slower to get altered.

In parallel, haptoglobin has been researched in other processes. Namely, it has been shown that the APP decreases in the serum of endurance horses after racing (MIHELIĆ et al., 2022), and that plasma concentration cannot be used to identify foal septicaemia (ZABRECKY et al., 2015). However, it is present in high levels and remains increased in blood of horses affected with arthritis (HULTÉN et al., 2002) and mares with placentitis (CANISSO et al., 2014).

TAIRA et al. (1992) have explored the variations of haptoglobin along life processes as ageing and pregnancy. They described that healthy neonates to one year old horses have the highest serum concentration compared to adult horses. They evaluated that the protein content in blood is then decreasing with adulthood. However during late pregnancy the haptoglobin level is high reaching its maximum at delivery before reverting to usual concentration.

PAULO et al. (2017) proved the stability of haptoglobin while stored at -20°C. The same study highlighted that ceruloplasmin was better preserved at -80°C than at -20°C. For

haptoglobin samples the comparison was not made. In addition, the drug flunixin meglumine, cyclooxygenase inhibitor, limits the raise of haptoglobin in blood (DI FILIPPOL et al., 2021).

According to previous studies, it is expected that blood concentration in haptoglobin would be a diagnostic biomarker as it usually decreases in ischemic colic. Although, caution is needed to interpret cases if haptoglobin is used alone for diagnosis or prognostication, as parallel pathologic processes could highly interfere.

1.3.2. Calprotectin

Calprotectin, also named S100A8/A9, belongs to the group of positive APPs. It is a pro-inflammatory modulator of interest in both acute and chronic inflammations. It is mostly located in neutrophils (counting for over 5% of cellular proteins) from which it stimulates the chemotaxis properties.

During acute inflammatory reaction, in-situ macrophages and systemic monocytes express calprotectin in small quantity. During chronic inflammation, tissue macrophages do not show the protein expression. By opposition, this APP is highly present in the extracellular fluid during inflammation processes like arthritis and abscesses, mainly released by activated neutrophils. Thus levels of serum calprotectin tend to highlight local processes more than SIRS, unlike CRP (YUI et al., 2003; JUKIC et al., 2021).

Since the protein can be excreted into the intestinal lumen, fecal calprotectin is commonly used in humans. It is recognized as a good differential marker for Inflammatory Bowel Disease (IBD). Blood levels of calprotectin have been tested by MALHAM et al. (2019), concluding that using plasma is better than using serum to identify ulcerative colitis. While researching on children appendicitis, KHARBANDA et al. (2012) obtained the same conclusion as plasma concentration of calprotectin is correlated with the validated disease in patients.

Although serum level of the protein is usually higher than plasma level of the same patient, it appears poorly associated with the studied pathology. NORDAL et al. (2018) hypothesize that handling of serum samples and long or inadequate storage might trigger the delivery of calprotectin in the tube, from activated neutrophils. Additionally they revealed that EDTA (ethylenediaminetetra – acetic acid) plasma samples are significantly stable while frozen at -70°C for more than two years.

Canine calprotectin has been investigated related to septicaemia, SIRS and identified in higher level than the baseline; however a single sample taken at admission time does not allow specific determination of the pathologic process (THAMES et al., 2019). HEILMANN et al. (2012) researched on serum calprotectin to give potential evidence of idiopathic IBD in canine, but they were limited by the concurrent medical treatment. It appears that corticosteroids induce an increase in calprotectin release.

KOSTANJŠAK et al. (2022) used canine serum samples to evaluate the protein stability. They detected that serum, in accordance to studies carried out on human concentrations, is highly affected by storage time at -80°C. Here again, calprotectin content is

increased as soon as eight weeks after handling. It is emphasized that care needs to be taken regarding centrifugation timing of samples, which should not exceed 24 to 48 hours post blood collection.

In horses, calprotectin has been explored related to a few pathologies only. FALEIROS et al. (2009) histologically detected the protein in the hoof tissue of horses with provoked laminitis from black walnut extract. Comparably, CHIAVACCINI et al. (2011) detected a colonic response to the induction. In those, calprotectin is used as the proof of early inflammation response. MIHELIĆ et al. (2022) measured the protein values from serum of competing endurance horses. Calprotectin tends to decrease in blood during exercise, although the exact processes involved are yet to be tested.

Not many studies have been carried out on the subject of colic. GROSCHE et al. (2008) started to seek for neutrophils localisation during induced ischemic and reperfusion episodes. They took biopsy samples of large colon during the experimentation and applied immunostaining process to it, in order to visualise calprotectin. Here, calprotectin filled cells are interpreted as neutrophils. Results indicate the motion of neutrophils throughout the injury. They are quickly gathering in the colonic mucosal veins, reaching their maximal concentration within 30 minutes of reperfusion. After 120 minutes of ischemic condition, the colonic vein content in neutrophils decreases as they are passing into the mucosal tissue. Calprotectin is poorly identified in colonic blood passed 18 hours of reperfusion.

Following the above study, GROSCHE et al. (2011) researched the early characteristics of mucosal lesions by repeating the previous experiment. This time, they limited the ischemia to one hour and sampled the colonic mucosa at several times during reperfusion. Immunohistochemistry staining proved that even after a relatively brief ischemic episode, neutrophils flow towards the injured location follows the same timing than obtained in 2008.
In 2013, GROSCHE et al. compared jugular to colonic blood content in the same experiment settings as previously done. They used an ELISA (Enzyme-Linked Immunoassay) method to measure the calprotectin concentration of both types of serums. Although they did not obtain statistically significant results, they saw a quick decrease in calprotectin content of jugular serum. They hypothesized that neutrophils infiltration to the affected tissue ensues their decrease in the systemic blood. They also assumed that the duration of the ischemia (one hour) was not sufficient to obtain a meaningful inflammatory response. In addition, they raised awareness on the heterogeneity of calprotectin baseline level in jugular blood.

In complement, WEISS and EVANSON (2003) evaluated the content of activated neutrophils in systemic blood collected from colic horses. They determined different ranges of concentration correlated with types of pathologies, showing the diagnosis power of activated neutrophils measurement. In cases with impaction, gas build-up and non-strangulating lesion, no activated neutrophils are found. On the other side, cases with inflammatory bowel injury always have activated neutrophils. For the specific cases of ischemia and strangulation colic, the results are variable and few to none activated neutrophils are seen. Overall, this kind of cell is negatively correlated with survival.

To summarize on calprotectin stability, it is determined that the APP concentration is most reliable when measured in plasma (NORDAL et al., 2018). PEDERSEN et al. (2018)

confirmed it and compared measurements of lithium-heparin plasma and EDTA plasma. They concluded that EDTA plasma is better for dosing calprotectin. Moreover, GAO et al. (2021) tested aliquots from both EDTA plasma and serum samples. They demonstrated a relative stability of calprotectin during up to nine cycles of freezing (-80°C) followed by thawing. Neglect of optimum handling did not significantly affect plasma or serum concentrations. It is important to note that they did not experiment on the time between collection and centrifugation, thus it could still be affecting samples as highlighted by KOSTANJŠAK et al. (2022).

Concerning the samples collected for this study, it is expected that determination of calprotectin concentration is better correlated in plasma than in serum. It is also anticipated that calprotectin values decrease in horses affected by at least two hours of bowel strangulation; and that the longer the duration of the episode before collection, the lower the APP concentration is. On a side note, just after the ischemic onset and for a short period, the protein might be present in higher level in blood. Impaction, non-strangulated and gas colic types should not show evidence of calprotectin in samples. Since the increase of activated neutrophils into general circulation is a proof of early systemic inflammatory reaction, it is assumed that high concentrations will be associated with poor outcome. Calprotectin could be a perfect biomarker for early differentiation and prognostication of horse colic.

1.3.3. High Mobility Group Box 1 (HMGB1)

HMGB1, previously known as amphoterin, is a chromosomal protein widely detected in human and animal tissues (epithelial cells, lungs, joints and smooth muscles). It has several actions and is so far identified as a late phase cytokine. It has a high potential for stimulating macrophages to deliver cytokines, thus activating neutrophils (WANG and ZHANG, 2020). It is considered as a damage-associated analyte, either produced by the liver or leaking from necrotic (ischemic) cells.

HMGB1 appears in blood in higher concentration from eight to 32 hours after onset of the disorder in patients with pneumonia, endotoxemia and septicaemia (HARRIS and ANDERSSON, 2004; AGALAVE and SVENSSON, 2014). Similarly, HUANG et al. (2010) proved the presence of the protein in both serum and tissues during generalized infection, with a much stronger concentration than the baseline of healthy humans and animals. In addition, it has been shown that the drug dexmedetomidine, α_2 agonist, reduces the increase of HMGB1 in blood (NISHIBORI et al., 2020).

In dogs, HMGB1 has been studied in order to identify patients with Systemic Inflammatory Response Syndrome (SIRS). YU et al. (2010) found that the protein is a reliable prognostic factor in affected canines. ISHIDA et al. (2011) compared it with the protein CRP. HMGB1 was not shown to be specific to SIRS, although they confirmed that elevated concentrations are correlated with bad prognosis. Equivalently, LEE et al. (2021) detected high level of serum HMGB1 in dogs with acute pancreatitis and associated poor survival rate. In dogs with gastric dilatation and volvulus, the level of HMGB1 and CRP were studied and

compared. Results showed that the reactive protein is less efficient in detecting the extent of tissue lesions, compared to HMGB1 (UHRIKOVA et al., 2015).

In horses, BAUQUIER et al. (2016) searched for HMGB1 in plasma of colic cases. They attested of a high concentration in colic patients compared to healthy ones. They also highlighted that the concentration in inflammatory colic increases 3.5 fold, whereas in strangulating colic it increases by an average of 5.4 fold. This correlates to the fact that inflammation and strangulation are processes with an increased risk of SIRS development.
HMGB1 is also seen in greater quantities in the synovial fluid of horses with osteochondral joint disease compared to the baseline (BROWN et al., 2009).

Experimentally, HMGB1 has been isolated from heparinized plasma stored at -80°C (LEE et al., 2021), as well as -20°C (ISHIDA et al., 2011). BAUQUIER et al. (2016) analysed their plasma samples that were frozen for two to three years. HMGB1 has also been detected in synovial fluid after storage at -80°C (BROWN et al., 2009).

From those previous studies, it is deducted that HMGB1 is present in plasma and potentially in serum and local tissue of colicky horses, when the onset of the pathologic process was more than eight hours ago. It is expected to obtain at least a reliable prognosis value and perhaps a differentiation biomarker, marking inflammatory and strangulating colic types.

2. MATERIAL AND METHODS

The study population was recruited from three different locations: the clinic of La Nouvetière, Sonzay and Clinique de Conques, Saint-Aubin de Branne, France; and the Veterinary Teaching Hospital of the Faculty of Veterinary Medicine in Zagreb, Croatia. The study was performed over a period of 10 months.

2.1. Ethical commitment

This research was approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, University of Zagreb, approval number: 640-01/22-17/46; 251-61-32-22-01. A written consent was received from the owners.

2.2. Horses

All horses presented with signs of abdominal pain during the study period were considered eligible. Signalment, history and clinical exam at admission were recorded. A note was made about any therapies received before admission or referral to the clinic. After case resolution, the clinical outcome of the horse was added to the record. A sample of the specially designed datasheet is provided in Annex 1.

Patients were further selected based on the availability and reliability of the following inclusion criteria (Figure 1): detailed anamnesis of the present colic episode, clinical examination findings, diagnosis and outcome, correct processing of blood samples. The diagnosis was made by the veterinarian in charge upon clinical exam, laboratory results and intra-surgical findings when available. In cases with several pathological findings, the most influential was used to assign the case to a diagnosis sub-group. The exclusion criteria were: missing main anamnestic data, samples not centrifuged and frozen within 24h.

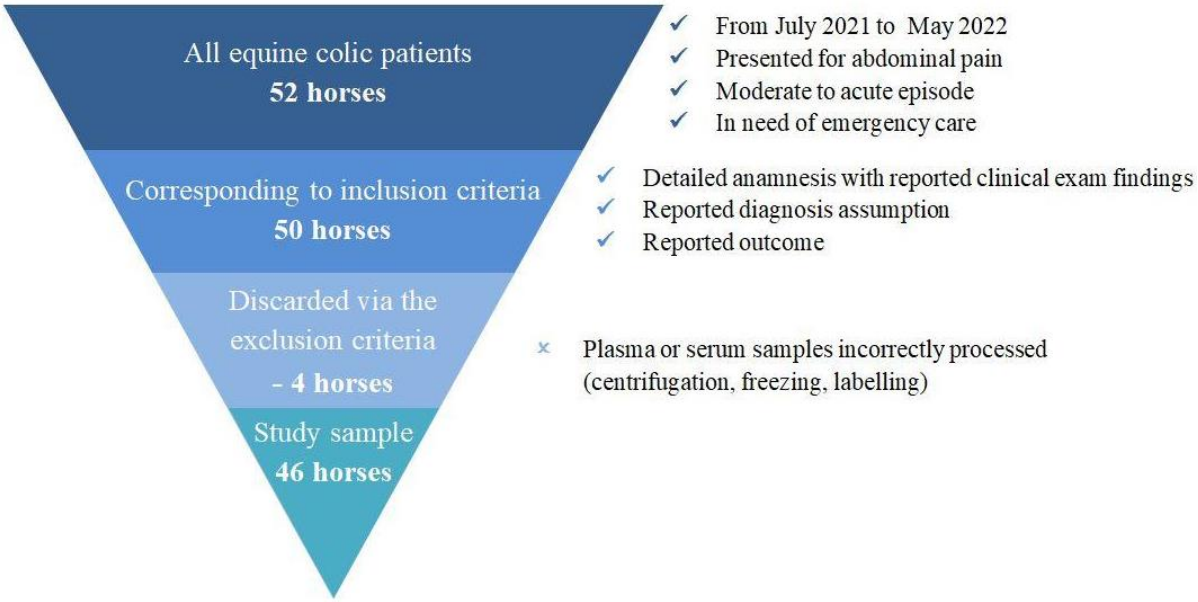

Figure 1: Pyramidal chart of cases selection, from recruitment (52 horses with colic signs) to

the obtained study sample (46 horses with completed reports and correctly processed samples), author's figure.

2.3. Sample collection and processing

At admission, blood was drawn from the jugular vein or the transverse facial venous sinus of horses. The samples were collected in duplicates into four Vacutubes as follows: two Serum Tubes with Gel (5mL) and two Hematology Tubes (6mL) coated with K_2EDTA in order to obtain plasma. All tubes were from LT BURNIK d.o.o., Vodice, Slovenia.
Instructions for handling of samples were provided beforehand (Annex 2). The EDTA tubes were centrifuged between 1300 and 2000g for 10 minutes. The obtained plasma was poured into at least two Eppendorf tubes of 1.5mL. The serum tubes were left to sit for 20 minutes at room temperature then centrifuged within an hour as previously stated. The resulting serum was aliquoted in at least two Eppendorf tubes of 1.5mL. All tubes were frozen at -20°C until analysis.

A few days before analysis, the aliquots obtained in France were shipped to the Veterinary Faculty of Zagreb in an insulated expanded polystyrene box with frozen cooling packs (Koolit® Gel Pad, Cold Chain Technologies, MA, USA), according to the ISBER (2012) best practices.

2.4. Analysis of Acute Phase Proteins and additional parameters

2.4.1. Statistical analysis

Standard curves and conversion of optical densities to concentrations were obtained on the software Microsoft Excel, version 2010 for Windows according to the manufacturer's instructions. All other data analyses were performed using R v4.1.2 (R Core Team (2021): R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL https://www.R-project.org/) in the integrated desktop environment RStudio v. 2023.03.1 (https://posit.co/).

Analyses included exploratory and statistical data analyses. All statistical analyses used alpha error of < 0.05 unless otherwise stated. Exploratory analyses included measures of central tendency (mean and median) and variability (standard deviation, interquartile range, overall range and coefficient of variation) for numerical variables; and counts and relative frequencies for categorical variables. Data included demographic and anamnestic data (age, duration of clinical signs...), clinical data (HR, RR, rectal and US examination findings…), laboratory parameters (hematocrit, WBC, APR concentrations…).

Dependent variables of interest were outcome status (dead *vs* survived) and strangulation status (strangulated *vs* non-strangulated colic types). For these variables, preliminary statistical tests included Shapiro Wilk's test of normality and F test of variance homogeneity in order to select the appropriate two-sample test according to their assumptions. Therefore, evaluation of the differences in numerical variables by horse outcome status and by strangulation status was done as follows. Student t test was used for normally distributed

data with or without Welch correction according to results of the F test, while non-normal data were analysed using Wilcoxon rank sum test.

For exploratory purposes, correlation of variables was analysed using Spearman's correlation test. For more reliable results, Bonferroni correction of *p* values was applied in pairwise correlation tests in order to reduce false positive error rate.

Spearman's *rho* coefficient ranges from -1 to 1 with positive values representing positive correlation between variables and negative values, in turn, negative correlation between variables. The magnitude of the correlation coefficient was judged qualitatively. No correlation was present between 0 and ±0.20, then correlations were considered as poor, moderate, strong and very strong between ±0.20 and ±0.40, between ±0.40 and ±0.60, between ±0.60 and ±80 and between ±80 and ±1, respectively.

For comparison of APP measurements between serum and plasma values, Bland-Altman analyses were performed using absolute values as a standard approach and relative (%) values as recently proposed method by GIAVARINA, D. (2015).

2.4.2. Haptoglobin concentrations

The haptoglobin content of serum and plasma were both determined using a spectrophotometric method adapted from ECKERSALL et al. (1999). This process is based on the innate peroxidase activity of the hemoglobin-haptoglobin complex, which can be quantified at acidic pH with SAT3 as chromogen. The reagent 1 contained 30g/L of hemoglobin prepared using washed and lysed bovine erythrocytes. The reagent 2 was a solution of citric acid (60mM), sodium hydrogen-phosphate (100mM; Na_2HPO_4), Tween 20 (1%), phenol (20 mM), L-cysteine (3.6 mM) and SAT3 (0.32 mM) in citrate buffer at pH 3.8. The reagent 3 was a substrate prepared by addition 100 µL 30% hydrogen peroxide (H_2O_2) in 25 mL of distilled water. The chemicals used for this haptoglobin method were obtained from Sigma-Aldrich (St. Louis, Missouri, USA) and the SAT3 from Dojindo Laboratories (Munich, Germany). The haptoglobin calibration curve was prepared with bovine acute phase serum with known haptoglobin concentration. The analyses were performed on Abbott Architect c4000 Plus (Abbott, Lake County, Illinois, USA). Values below the limit of detection (LOD) were replaced by the associated LOD. The general characteristics of the method are presented in Table 1.

2.4.3. Calprotectin concentrations

The calprotectin content of both serum and plasma were determined using a Horse Calprotectin ELISA Kit E0175HO (Bioassay Technology Laboratory, Zhejiang, China), carrying out a sandwich method for *in vitro* quantitative measurement of equine calprotectin. This kit is registered for our intended use and the assay was done manually up to the incubation step followed by an automated washing (HydroFlex, Tecan, Männedorf, Switzerland), in accordance with the instructions defined by the manufacturer. The optical density was read using a microplate reader (Sunrise, Tecan, Männedorf, Switzerland) set to

450 nm (OD 450). As only one kit was used in this study, the intra-assay CV (coefficient of variation) was produced as a measure of variability. It was estimated from two successive measurements of each standard concentration used in the standard calibration curve (respectively 15, 30, 60, 120 and 240ng/mL). The CV was on average 26% with a range from 14% to 34% across the different concentrations.

The standard curve was interpolated with a linear regression and the sample values were calculated. Negative values of OD450 were assumed to represent very low concentrations in samples and most likely to be under the limits of detection (LOD) or below the linear range of the assay if there was no evidence of invalid result (*e.g.*, haemolytic sample). These samples were replaced with LOD and in the absence of value provided for the kit, the sensitivity value added to the values of 0ng/mL standard concentration was used as an alternative. These results were included in the univariate and correlation analyses and were removed from Bland-Altman analyses of agreement between serum and plasma concentrations. The general characteristics of the method, according to the manufacturer, are presented in Table 1.

2.4.4. High Mobility Group Box 1 (HMGB1) concentrations

The HMGB1 concentrations of both serum and plasma were determined following a similar method to the calprotectin one described previously. Only one Horse HMGB1 ELISA Kit E0124HO (Bioassay Technology Laboratory, Zhejiang, China) was used, based as well on a sandwich method. The calibration curve was built up on standards with, in order, concentrations of 30, 60, 120, 240 and 480ng/mL. The intra-assay precision was calculated as previously and displayed an average of 23%, ranging from 8% to 41%.

In like manner, the standard curve was interpolated with a linear regression, and the sensitivity was used as an LOD replacement. The general characteristics of the method, according to the manufacturer, are presented in Table 1.

Table 1: Characteristics of the methods used for quantification of haptoglobin, calprotectin and HMGB1 in horses of this study. Data concerning ELISA kits is stated by the manufacturer.

Method	Standard curve range	Sensitivity	Limit of detection	Limit of quantification	Intra-assay CV[a]	Inter-assay CV[b]
Haptoglobin adapted from Eckersall et al.[c]	0 – 1g/L	-	0.026g/L	0.053g/L	High concentration: 2.15% Low concentration: 1.55%	High concentration: 3.79% Low concentration: 4.45%
Calprotectin ELISA[d]	3 – 900 ng/mL	1.21ng/mL	-	-	< 8%	< 10%
HMGB1 ELISA[e]	3.75 – 240 ng/mL	1.97ng/mL	-	-	< 8%	< 10%

[a] *Intra-assay CV: coefficient of variation (%), precision within an assay*

[b] *Inter-assay CV: coefficient of variation (%), precision between assays*

[c] *Spectrophotometric method adapted from ECKERSALL et al. (1999)*

[d] *Horse Calprotectin ELISA Kit E0175HO (Bioassay Technology Laboratory, Zhejiang, China)*
[e] *Horse HMGB1 ELISA Kit E0124HO (Bioassay Technology Laboratory, Zhejiang, China)*

2.5. Grouping

Retrospectively, horses were divided into two groups based on their outcome: survivor *vs* non-survivors; where survivors were defined as successfully discharged from the clinic and/or complete resolution of clinical signs without recidivism in the following two weeks. Horses were also grouped based on two types of diagnosis: strangulated bowels or non-strangulated bowels. The non-strangulated cases group was further divided into two sub-groups: identified inflammatory colic or others (without strangulation or inflammation).

3. RESULTS

Out of the 52 identified colic cases during the study period, 46 horses fulfilled the inclusion criteria. Twenty horses were treated by the team of La Nouvetière. Fourteen horses were transported admitted to Conques, and 12 were admitted to the Teaching Hospital of Zagreb.

There were 23 females and 23 males. One mare was seven months pregnant and another one had given birth five days before the colic episode and blood collection. There were 19 warm-blood horses, 10 ponies, 6 Arabians and Anglo-Arabians, 4 Standardbreds, 3 draft horses and 1 Thoroughbred. Three horses had a missing breed data. The average age of the horses was 13.9±8.8 years (from 3 months to 29 years old) (Table 4).

Most of the horses had an occasional activity, defined as up to once a week exercise session (27). Fifteen horses were frequently exercised with more than session per week. The majority were housed in paddocks (23) or turned out. Nineteen patients were receiving regular deworming and 20 had an inadequate frequency (none or occasional).

3.1. Retrospective data of the study population

Prior to admission and sampling, 31 horses had been transported to facilities and 29 had already received therapies. The pre-admission duration of symptoms ranged from one hour to five days with an average of 13.9±20.5 hours (Table 4). It was the first known colic episode of 30 horses while 15 already had shown signs of colic in the past. Final diagnoses were proven in 13% of the horses (6/46) by intra-operative findings or necropsy.

Twenty-six horses (56%) received medical treatment and five (11%) underwent surgery. Twenty-eight out of the 46 patients survived (61%) and were discharged. Eighteen patients (39%) were euthanized including three surgical cases. The details of survival according to diagnosis type and clinical management are presented on Table 2 and Table 3.

Table 2: Survival outcome of horses showing colic signs, according to diagnosis group and clinical management.

Variable	Survivors (n)	Non-survivors (n)	Total (%)
Diagnosis (groups)			
NON-STRANGULATED	27	6	33 (71.7)
STRANGULATED	1	12	13 (28.3)
Clinical management			
MEDICALLY TREATED	26	15	41 (89.1)
SURGICALLY TREATED	2	3	5 (10.9)

n: number of horses

Table 3: Details of the survival outcome of horses with colic signs, according to diagnosis sub-groups (inflammation, strangulation and others).

Variable	Survivors (n)	Non-survivors (n)	Total (%)
Diagnosis (sub-groups)			
OTHERS	23	4	27 (58.7)
STRANGULATED	1	12	13 (28.3)
INFLAMMATION	4	2	6 (13.0)

n: number of horses

The diagnosis sub-group "others" included patients with gastric distension, large colon displacement, nephrosplenic entrapment, colon obstipation, prepubic tendon rupture with associated colic, flexura pelvina impaction/obstipation, gaseous ileus of the colon and non-specific colic findings. The sub-group "strangulated" gathered cases of mesenteric abscess, strangulating lipoma, small intestine torsion, colonic torsion, epiploic foramen hernia, mesenteric laceration with concurrent large colon torsion, and gastric rupture. The sub-group "inflammatory" included colitis, gastric ulcers, and parasitism (*Gasterophilus spp*).

The descriptive values of the anamnestic and clinical parameters are summarized in Table 4 and Table 5.

Table 4: Descriptive values of the numerical parameters of horses with colic signs, from the anamnesis, admission clinical exam and laboratory analyses.

Numerical variables	n	Mean ±SD	CV (%)	Median	IQR	(min – max)
Age (year)	46	13.9 ±8.8	0.6	13.5	16	0.2 – 29
Pre-admission duration (hour)	45	13.9 ±20.5	1.5	6	15	1 – 120
HR (bpm)	45	62.2 ±23.5	0.4	52	24	32 – 120
RR ($b_r$pm)	31	26 ±13.4	0.5	20	19	12 – 64
Temperature (°C)	31	37.5 ±0.7	0	37.4	0.7	36 – 39.5
WBC count (M/mm^3)	39	10.4 ±4.5	0.4	10.9	5	1.2 – 24.3
Neutrophils count (%)	38	71.9 ±12.2	0.2	70.8	17.6	47.5 – 94
Hematocrit (%)	40	41 ±12.7	0.3	36.9	13.3	26.4 – 85.3
Lactate (mmol/L)	32	2.7 ±2.4	0.9	2	1.5	0.8 – 12
Haptoglobin $_{serum}$ (g/L)	46	0.7 ±0.4	0.7	0.6	0.7	0 – 1.7
Haptoglobin $_{plasma}$ (g/L)	38	0.8 ±0.4	0.6	0.8	0.7	0 – 1.8
Calprotectin $_{serum}$ (ng/mL)	46	33.9 ±28.3	0.8	30.9	43.4	2.0 – 102.3
Calprotectin $_{plasma}$ (ng/mL)	38	35.3 ±26.7	0.8	29.9	33	2.0 – 107.3
HMGB1 $_{serum}$ (ng/mL)	46	51 ±32.6	0.6	44.7	41.2	1.2 – 126.5
HMGB1 $_{plasma}$ (ng/mL)	38	53.1 ±26.6	0.5	50.6	31.2	1.2 – 118.4

n: number of horses; SD: standard deviation; CV: coefficient of variation; IQR: interquartile range

Table 5: Descriptive values of the categorical parameters of horses with colic signs, from the admission clinical exam and laboratory analyses.

Categorical variables	**n**	**%**
Mucous membranes colour	*45*	
Normal	33	73.3
Abnormal	12	26.7
Dehydration %	*45*	
None to mild	22	48.9
Mild to moderate	8	17.8
Moderate to severe	15	33.3
Peristalsis (auscultation)	*46*	
Normal	11	23.9
Abnormal	35	76.1
US findings	*34*	
Normal	12	35.3
Abnormal	22	64.7
Rectal palpation findings	*40*	
Loops of small intestine	10	25.0
Tight tenias	9	22.5
Obstipation	9	22.5
Normal palpation	13	32.5

n: number of horses

3.2. Assessment of the differences between serum and plasma values of APP

3.2.1. Haptoglobin

Bland-Altman analysis showed a significant bias between serum and plasma values of haptoglobin. On average, concentration in plasma was 0.11g/L higher than in serum (95% CI= [0.05, 0.17], t= 3.71, dF= 36, p= 0.001). By Giavarina analysis using relative difference from means (%), the bias was also shown to be significant. The average relative bias was 22% (95% CI= [13.09, 30.88], t= 5.01, dF= 36, $p < 0.001$). Results of both analyses are presented in Figure 2.

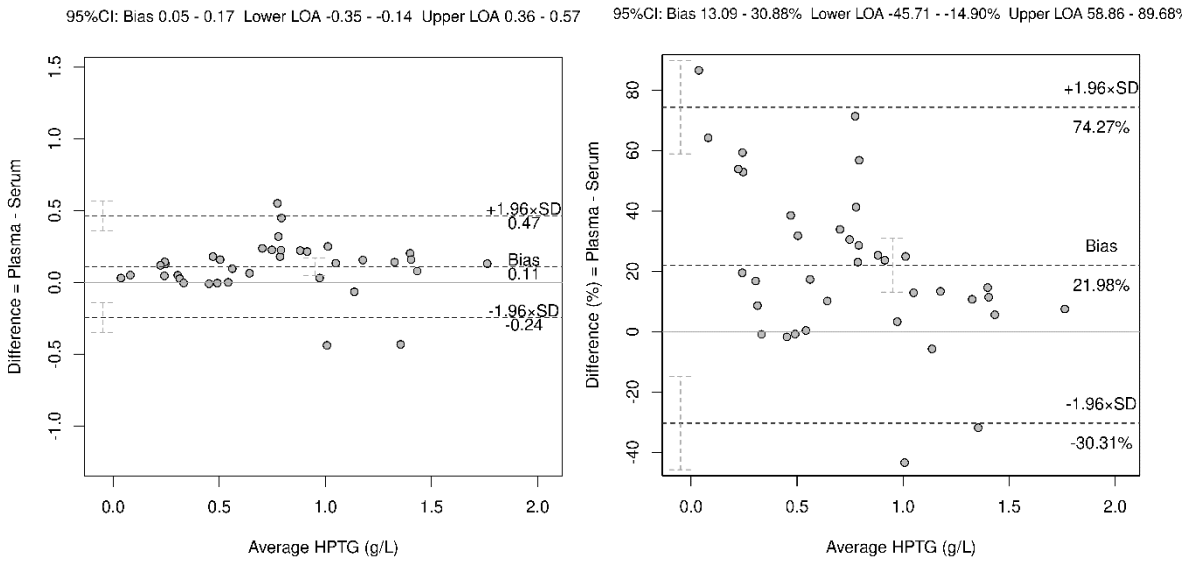

Figure 2: Mean-difference plot for the agreement between serum and plasma concentrations of haptoglobin (HPTG) using Bland-Altman (left) and Giavarina (right) analyses, in horses with colic signs.

Bland-Altman analysis displays absolute bias and Giavarina analysis displays relative bias.
CI: confidence interval; LOA: limits of agreement

3.2.2. Calprotectin

Bland-Altman analysis showed a significant bias between serum and plasma concentrations of calprotectin. Plasma values were on average 5.97ng/mL higher than serum values (95% CI= [0.63, 11.31], t= 2.28, dF= 29, p= 0.030). Conversely, Giavarina analysis highlighted a non-significant relative bias, with on average 23% higher plasma concentrations (95% CI= [-0.33, 46.88], t= 2.02, dF= 29, p= 0.053). However, the model had violations of associated assumptions and the results should be taken with caution. Results of both tests are presented in Figure 3.

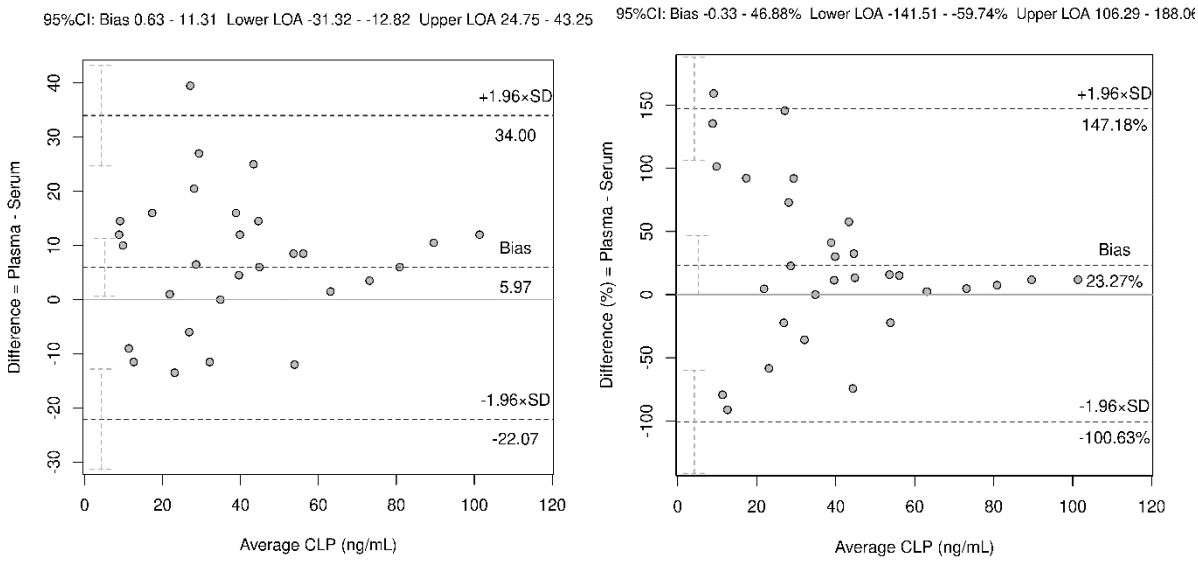

Figure 3: Mean-difference plot for the agreement between serum and plasma concentrations of calprotectin (CLP) using Bland-Altman (left) and Giavarina (right) analyses, in horses with colic signs.
Bland-Altman analysis displays absolute bias and Giavarina analysis displays relative bias.
CI: confidence interval; LOA: limits of agreement; SD: standard deviation

3.2.3. HMGB1

Concerning HMGB1, Bland-Altman analysis indicated a significant bias between serum and plasma concentrations. Plasma values were on average 9.34ng/mL higher than serum (95% CI= [3.1, 15.58], *t*= 3.04, *dF*= 34, *p*= 0.005). Giavarina analysis showed a significant relative bias. On average the relative bias was 23% (95% CI= [5.98, 39.22], *t*= 2.76, *dF*= 34, *p*= 0.009). Plots of both tests are presented in Figure 4.

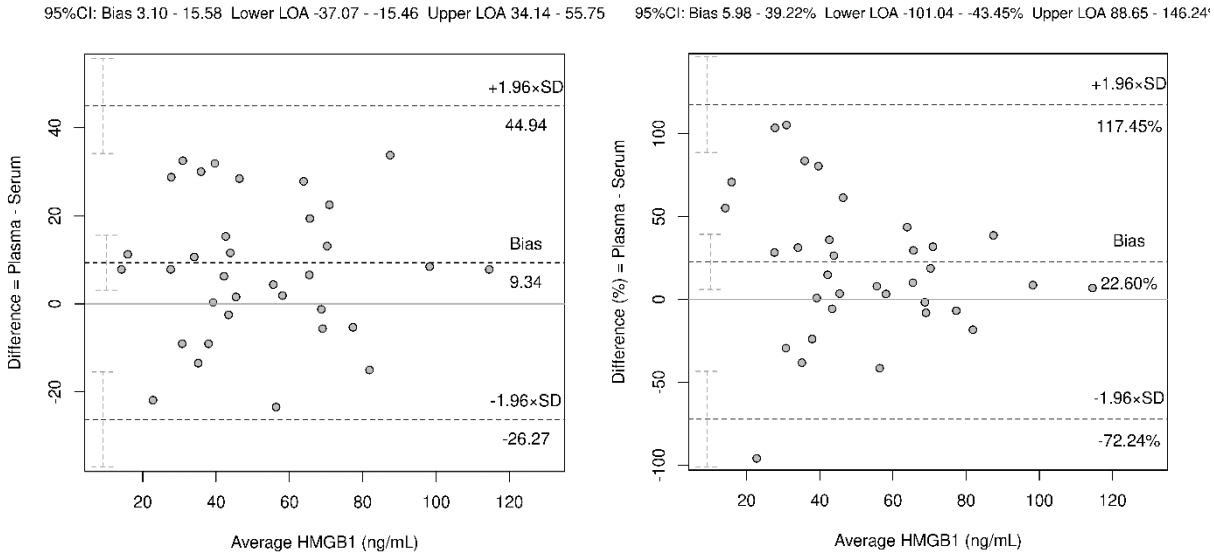

Figure 4: Mean-difference plot for the agreement between serum and plasma concentrations of HMGB1 using Bland-Altman (left) and Giavarina (right) analyses, in horses with colic signs.
Bland-Altman analysis displays absolute bias and Giavarina analysis displays relative bias.
CI: confidence interval; LOA: limits of agreement; SD: standard deviation; SD: standard deviation

As a whole, serum concentrations of the studied APR tend to be significantly lower than plasma concentrations. This is highlighted by both absolute and relative bias, aside from calprotectin relative results.

3.3. Compared values of APR according to survival of horses

A detailed summary of results, tendencies and interpretations is presented in Annex 3.

3.3.1. Haptoglobin

There were no significant differences in haptoglobin concentration between survival status of patients (*t*= 0.52, *dF*= 44, *p*= 0.61 for serum; *t*= 0.42, *dF*= 36, *p*= 0.68 for plasma)

(Figure 5). Concentration in serum and in plasma of survivors were on average respectively 0.07g/L (95% CI= [-0.21, 0.35]) and 0.06g/L (95% CI= [-0.25, 0.37]) lower than for the non-survivors (Table 6).

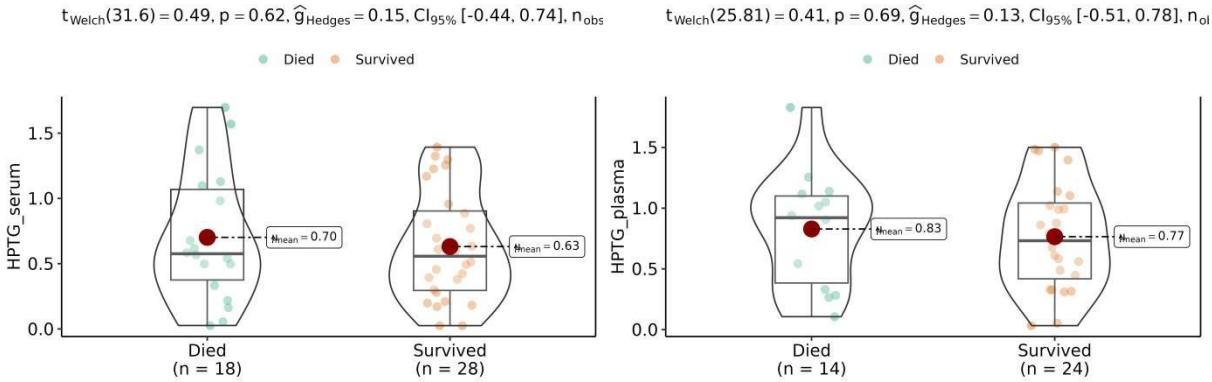

Figure 5: Compared distribution of haptoglobin (HPTG) concentrations between cases of survivors and non-survivors, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

3.3.2. Calprotectin

Serum level of calprotectin was not significantly different between survivors and non-survivors (*W*= 240.5, *p*= 0.8). In plasma, although calprotectin concentration was on average 5.44ng/mL (95% CI= [-23.8, 12.92]) higher in survivors than in non-survivor horses (Table 6), the difference was not significant (*t*= -0.6, *dF*= 36, *p*= 0.55) (Figure 6).

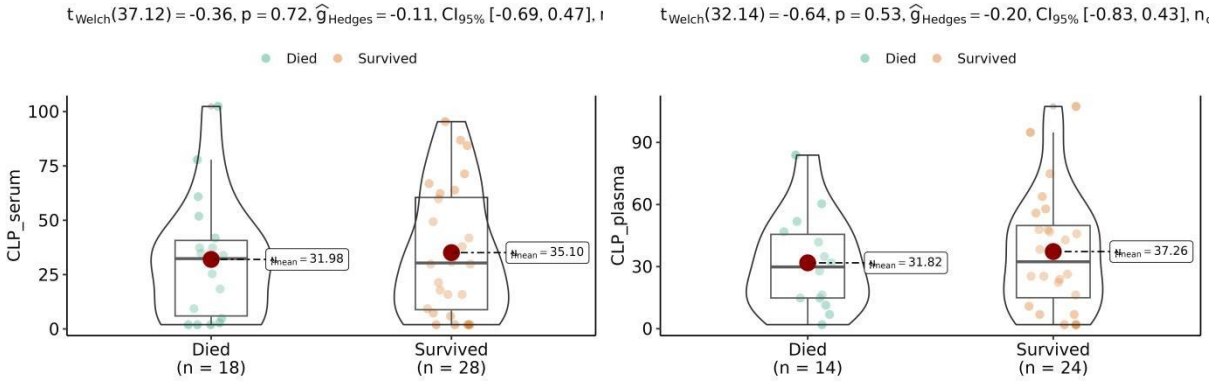

Figure 6: Compared distribution of calprotectin (CLP) concentrations between cases of survivors and non-survivors, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

3.3.3. HMGB1

Concerning HMGB1, the average concentration in serum was 3.93ng/mL (95% CI= [-16.11, 23.97]) lower in survivors compared to the horses that did not survive (Table 6). However, the difference between survival statuses of horses was non-significant (*t*= 0.39, *dF*= 44, *p*= 0.69). Average plasma concentration of HMGB1 was 2.79ng/mL (95% CI= [-

21.16, 15.59]) higher in survivor patients compared to non-survivors (*t*= -0.31, *dF*= 36, *p*= 0.76) (Figure 7).

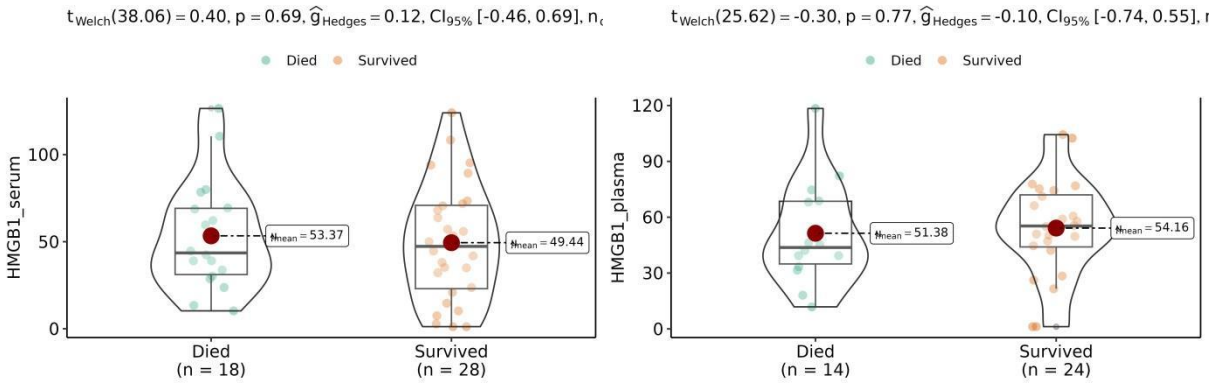

Figure 7: Compared distribution of HMGB1 concentrations between cases of survivors and non-survivors, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

Table 6: Haptoglobin, calprotectin and HMGB1 concentrations in horses with colic signs, according to survival outcome.

Concentration of acute phase proteins	**Survivors (n=28)** Mean ±SD CV (%) Median (IQR, min-max)	**Non-survivors (n=18)** Mean ±SD CV (%) Median (IQR, min-max)
Haptoglobin $_{serum}$ (g/L)	0.6±0.4 0.7 0.6 (0.6, 0 – 1.4)	0.7±0.5 0.7 0.6 (0.7, 0 – 1.7)
Haptoglobin $_{plasma}$ (g/L)	0.8±0.4 0.6 0.7 (0.6, 0 – 1.5)	0.8±0.5 0.6 0.9 (0.7, 0.1 – 1.8)
Calprotectin $_{serum}$ (ng/mL)	35.1±28.9 0.8 30.4 (51.6, 2.0 – 95.3)	32±28.2 0.9 32.4 (34.7, 2.0 – 102.3)
Calprotectin $_{plasma}$ (ng/mL)	37.3±28.8 0.8 32.4 (34.9, 2 .0– 107.3)	31.8±23.2 0.7 29.9 (30.8, 2.0 – 83.8)
HMGB1 $_{serum}$ (ng/mL)	49.4±33.7 0.7 47.3 (47.9, 1.2 – 124)	53.4±31.7 0.6 43.6 (38, 10.3 – 126.5)
HMGB1 $_{plasma}$ (ng/mL)	54.2±26.2 0.5 55.3 (28, 1.2 – 104.3)	51.4±28.3 0.6 43.7 (33.7, 11.8 – 118.4)

n: number of horses; SD: standard deviation; CV: coefficient of variation, IQR: interquartile range

Overall, significant differences of APR concentrations according to the survival outcome of patients were not observed in this study, for both serum and plasma contents.

3.4. Compared values of APR according to colic type (diagnosis groups)

A detailed summary of results, tendencies and interpretations is presented in Annex 3.

3.4.1. Haptoglobin

Concentration of haptoglobin in serum and in plasma of horses affected by non-strangulating colic were on average 0.14g/L (95% CI= [-0.44, 0.16]) and 0.1g/L (95% CI= [-0.45, 0.25]) lower than in strangulating cases, respectively (Table 7). There were no significant differences in concentration between bowel strangulation status (*t*= -0.94, *dF*= 44, *p*= 0.35 for serum; *t*= -0.56, *dF*= 36, *p*= 0.58 for plasma) (Figure 8).

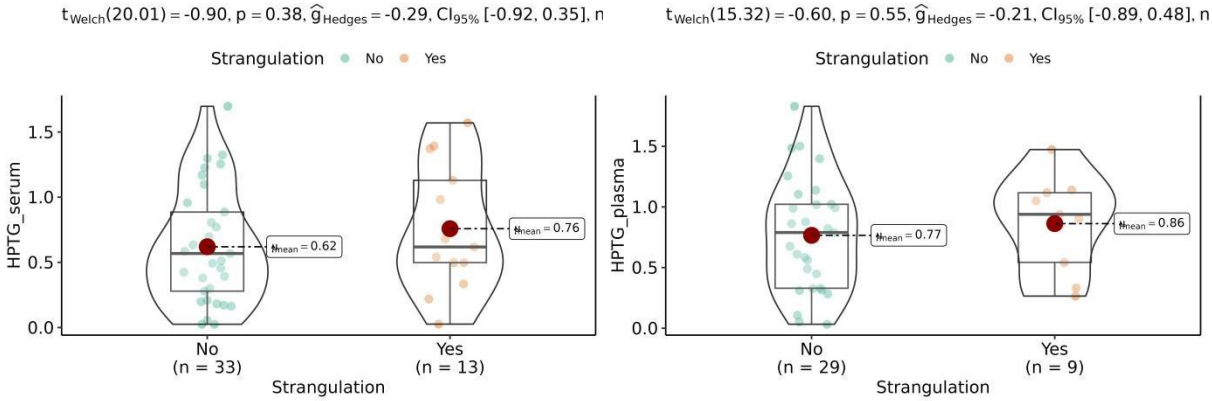

Figure 8: Compared distribution of haptoglobin (HPTG) concentrations between strangulated and non-strangulated bowel cases, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

3.4.2. Calprotectin

Calprotectin in serum was not significantly different between strangulating and non-strangulating colic (*W*= 246, *p*= 0.45). In plasma, although calprotectin concentration of non-strangulating colic was on average 13.26ng/mL (95% CI= [-7.2, 33.71]) higher than during strangulating one (Table 7), there was no significant difference in concentration linked to bowel strangulation status (*t*= 1.31, *dF*= 36, *p*= 0.2) (Figure 9).

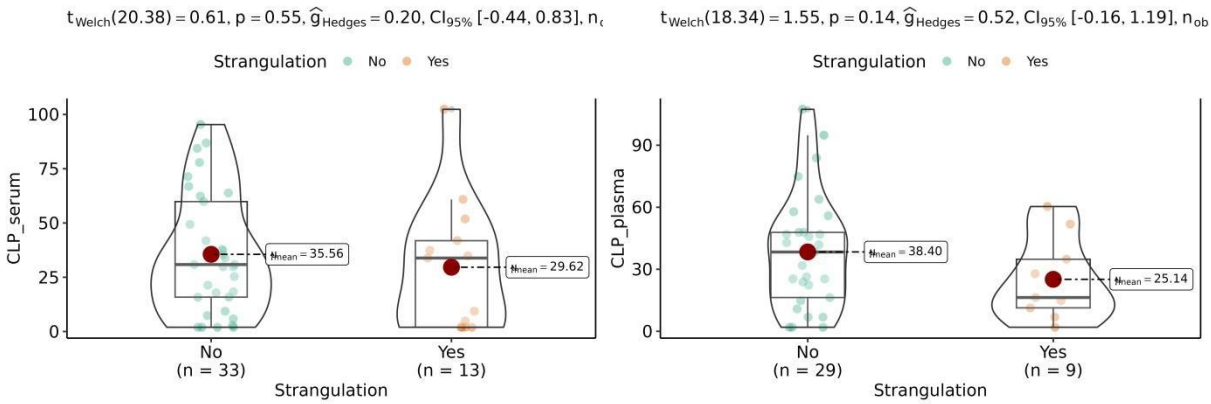

Figure 9: Compared distribution of calprotectin (CLP) concentrations between strangulated and non-strangulated bowel cases, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

3.4.3. HMGB1

Concerning HMGB1, the average concentration in serum and in plasma were 8.55ng/mL (95% CI= [-13.06, 30.16]) and 13.2ng/mL (95% CI= [-7.19, 33.6]) higher in horses with non-strangulated bowels compared to the patients with strangulating colic, respectively (Table 7). However, the difference between strangulated and non-strangulated bowels is shown to be non-significant (t= 0.8, dF= 44, p= 0.43 for serum; t= 1.31, dF= 36, p= 0.2 for plasma) (Figure 10).

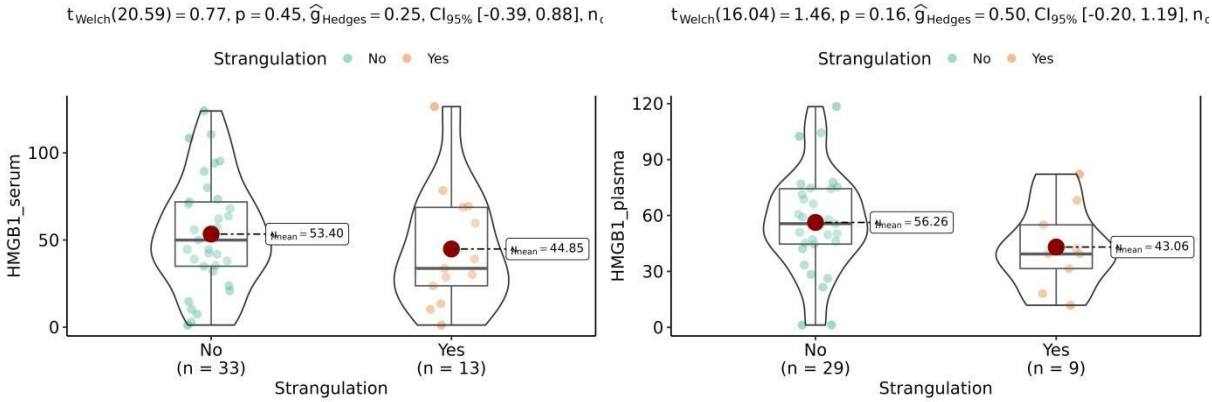

Figure 10: Compared distribution of HMGB1 concentrations between strangulated and non-strangulated bowel cases, using serum (left) and plasma (right), in horses with colic signs.
CI: confidence interval

Table 7: Haptoglobin, calprotectin and HMGB1 concentrations in horses with colic signs, according to strangulation type.

Concentration of acute phase proteins	**Strangulated (n=13)** Mean ±SD CV (%) Median (IQR, min-max)	**Non-strangulated (n=33)** Mean ±SD CV (%) Median (IQR, min-max)
Haptoglobin $_{serum}$ (g/L)	0.8±0.5 0.6 0.6 (0.6, 0 – 1.6)	0.6±0.4 0.7 0.6 (0.6, 0 – 1.7)
Haptoglobin $_{plasma}$ (g/L)	0.9±0.4 0.5 0.9 (0.6, 0.3 – 1.5)	0.9±0.5 0.6 0.8 (0.7, 0 – 1.8)
Calprotectin $_{serum}$ (ng/mL)	29.6±30.4 1 33.9 (39.9, 2 – 102.3)	35.6±27.8 0.8 30.9 (44, 2 – 95.3)
Calprotectin $_{plasma}$ (ng/mL)	25.1±20.3 0.8 16.4 (23.5, 2 – 60.4)	38.4±27.9 0.7 38.4 (31.5, 2 – 107.3)
HMGB1 $_{serum}$ (ng/mL)	44.8±34.6 0.8 33.7 (45, 1.2 – 126.5)	53.4±32 0.6 50 (36.9, 1.2 – 124)
HMGB1 $_{plasma}$ (ng/mL)	43.1±22.5 0.5 39.3 (23.4, 11.8 – 82.2)	56.3±27.3 0.5 55.6 (29.7, 1.2 – 118.4)

n: number of horses; SD: standard deviation; CV: coefficient of variation, IQR: interquartile range

Additionally, descriptive evaluation of the sub-groups Others and Inflammatory (Table 8) were carried out. Since 6 cases are not sufficient to carry out significantly reliable statistical analyses, means and standard deviation were compared. There were no differences in concentrations of APR between the two sub-groups.

Table 8: Haptoglobin, calprotectin and HMGB1 concentrations in horses according to colic sub-group Others and Inflammatory.

Concentration of acute phase proteins	**Others (n=27)** Mean ±SD CV (%) Median (IQR, min-max)	**Inflammatory (n=6)** Mean ±SD CV (%) Median (IQR, min-max)
Haptoglobin $_{serum}$ (g/L)	0.5±0.4 0.7 0.5 (0.5, 0 – 1.3)	1±0.5 0.5 1.2 (0.6, 0.3 – 1.7)
Haptoglobin $_{plasma}$ (g/L)	0.7±0.4 0.6 0.6 (0.7, 0 – 1.5)	1.3±0.6 0.4 1.4 (0.2, 0.3 – 1.8)
Calprotectin $_{serum}$ (ng/mL)	35.2±28.9 0.8 30.9 (42, 2 – 95.3)	37.3±24.3 0.7 33.6 (29.6, 2 – 66.8)
Calprotectin $_{plasma}$ (ng/mL)	39.9±28.9 0.7 40.6 (29.4, 2 – 107.3)	31.2±23.7 0.8 31.9 (25.5, 2 – 63.9)
HMGB1 $_{serum}$ (ng/mL)	53.3±33.6 0.6 50 (35.8, 1.2 – 124)	53.7±26.5 0.5 58.2 (30.5, 10.3 – 80)
HMGB1 $_{plasma}$ (ng/mL)	58.1±28.2 0.5 56.7 (29.5, 1.2 – 118.4)	47.4±23.1 0.5 46.2 (37.8, 21.5 – 74.7)

n: number of horses; SD: standard deviation; CV: coefficient of variation, IQR: interquartile range

There were no significant differences observed in any of the APR concentrations between the different types of colic for both serum and plasma contents. The small number of inflammatory cases in this study did not allow reliable comparisons of concentration distributions in sub-groups.

3.5. Correlation of numerical variables

As presented in Figure 11, Spearman correlation displays different results with potential false positive associations. The addition of Bonferroni correction is shown in Figure 12.
Very strong positive correlations were expressed between:

- calprotectin concentrations in serum and in plasma ($\rho = 0.84$, $S = 1437.4$, $p < 0.001$),
- haptoglobin $_{serum}$ and haptoglobin $_{plasma}$ ($\rho = 0.93$, $S = 598.53$, $p < 0.001$),
- HMGB1 $_{serum}$ and calprotectin $_{serum}$ ($\rho = 0.84$, $S = 2614.2$, $p < 0.001$),
- HMGB1 $_{serum}$ and calprotectin $_{plasma}$ ($\rho = 0.80$, $S = 1835.9$, $p < 0.001$).

Strong positive correlations were demonstrated between:

- HMGB1 concentrations in serum and in plasma ($\rho = 0.68$, $S = 2952.8$, $p < 0.001$),
- HMGB1 $_{plasma}$ and calprotectin $_{plasma}$ ($\rho = 0.79$, $S = 8.0$, $p < 0.001$).

HR and lactate were also strongly positively correlated ($\rho = 0.64$, $S = 1987.3$, $p < 0.001$).

On a side note, pre-admission duration, WBC and neutrophils were not correlated with any of the APP tested. No correlations were noted between haptoglobin, calprotectin and

HMGB1 and the clinical parameters tested in this study. On the other hand, positive correlations were highlighted between calprotectin and HMGB1.

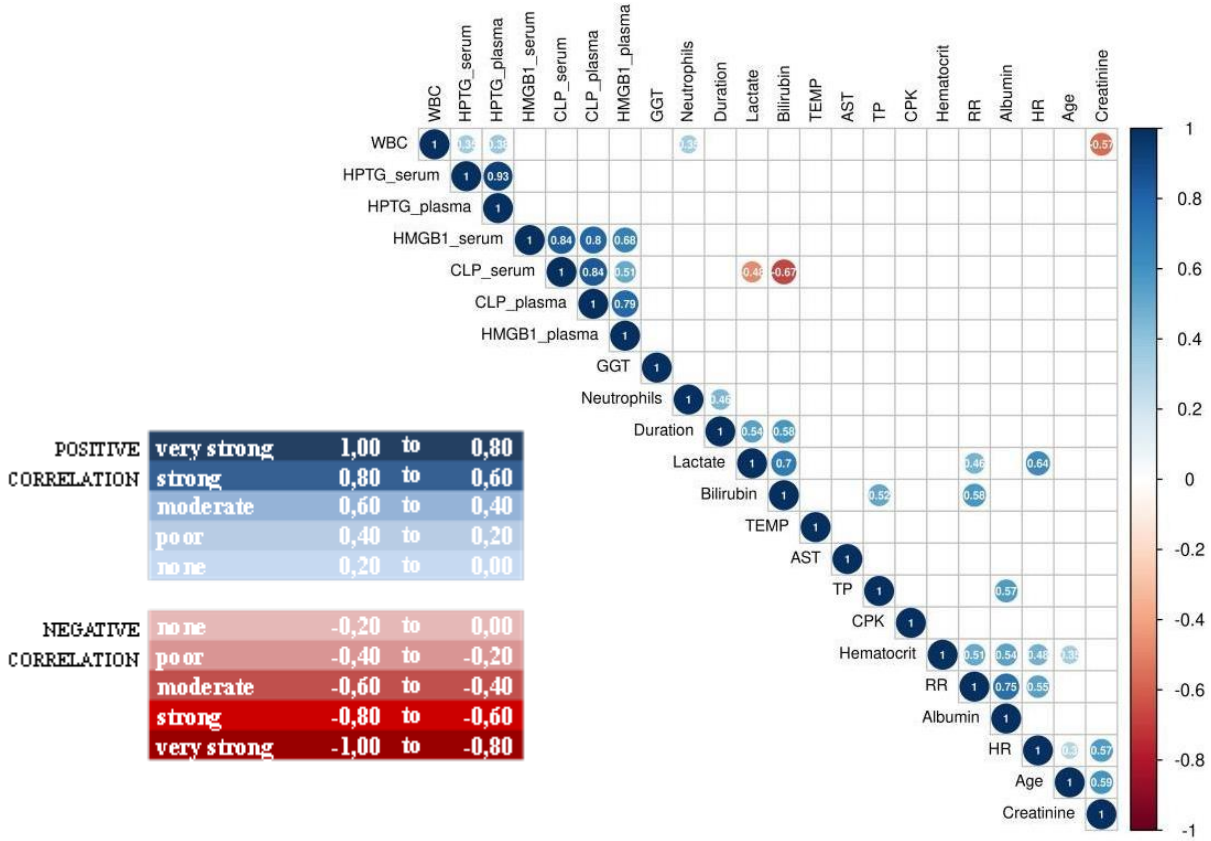

Figure 11: Spearman correlation without correction, exploratory purpose; featuring data from anamnesis, admission clinical exam and laboratory findings of horses with colic signs.
Spearman's rho coefficient is showed in the center of each circle. Positive correlations are displayed in red shades (ρ= 1 to 0) and negative correlations are displayed in blue shades (ρ= 0 to -1).
HPTG: haptoglobin; CLP: calprotectin; Duration: pre-admission duration; TEMP: temperature

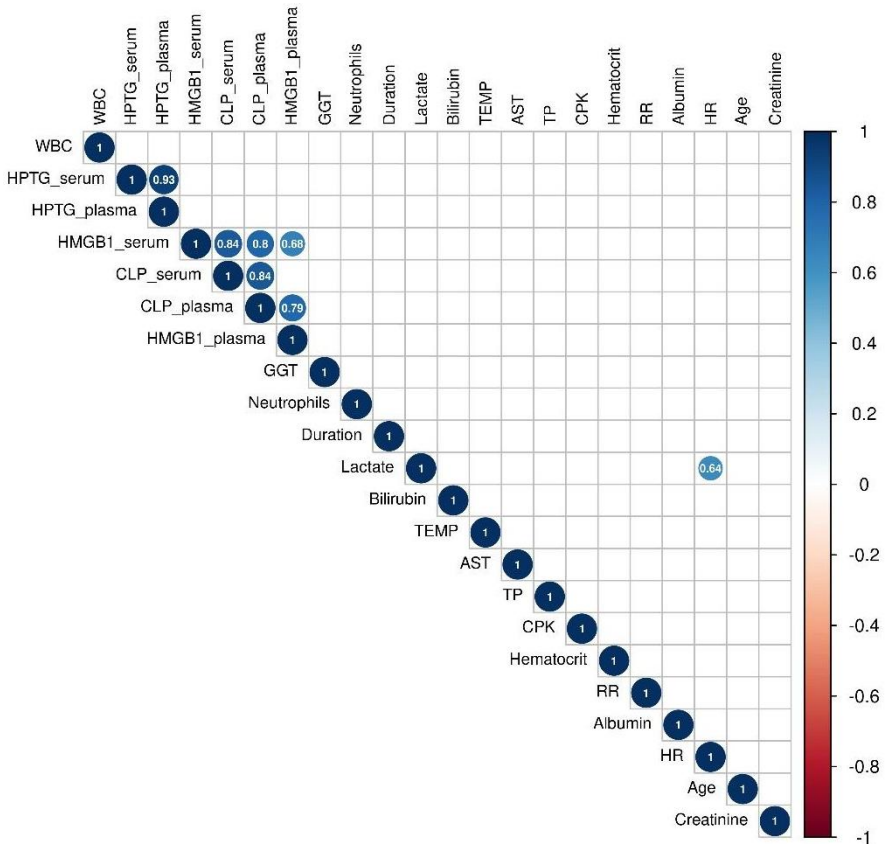

Figure 12: Spearman correlation with Bonferroni correction; featuring data from anamnesis, admission clinical exam and laboratory findings of horses with colic signs.
Spearman's rho coefficient is showed in the center of each circle. Positive correlations are displayed in red shades (ρ= 1 to 0) and negative correlations are displayed in blue shades (ρ= 0 to -1).
HPTG: haptoglobin; CLP: calprotectin; Duration: pre-admission duration; TEMP: temperature

3.6. Compared values of selected additional clinical parameters

There were significant differences in HR between survival and non-survival statuses (*W*= 387.5, *p*= 0.001). The average HR in survivors was 53.1 bpm and 75.9 bpm in non-survivors. According to colic type, HR was also significantly different between cases (*W*= 87.5, *p*= 0.003). It was on average 80.2 bpm in bowel strangulation and 54.9 bpm in non-strangulated cases.

There were significant differences in RR between survival and non-survival of horses (*W*= 169, *p*= 0.005). The average RR in survivors was 22.6 $b_r$pm and 33.2 $b_r$pm in horses that died. However it was not significantly associated with colic type (*W*= 38.5, *p*= 0.06).
Temperature was significantly different between survivors and non-survivors (*W*= 31.5, *p*= 0.003). It was on average 37.7°C in survivors and 37°C in horses that did not survive. On the other hand, temperature was not significantly different between colic types (*W*= 105.5, *p*= 0.13).

Out of the tested laboratory parameters, albumin was on average 6g/L (95% CI= [2.0, 9.9]) lower in survivors, showing a significant difference between survival outcomes (*t*= 3.25, *dF*= 14, *p*= 0.006). The mean albumin concentration was 26±3.3g/L in survivors and

32±1.4g/L in horses that died. Concerning strangulation status, although albumin was on average 5.17g/L (95% CI= [-10.4, 0.1]) higher in strangulated than in non-strangulated cases, it was not significantly different between horses (t= -2.12, dF= 14, p= 0.05). The mean albumin concentration was 32.5±5.6g/L in strangulated bowels and 27.3±3.8g/L in non-strangulated colic.

The GGT concentration was shown to be significantly different between strangulated and non-strangulated bowel cases (W= 39, p= 0.031). It was on average 16.2 U/L in strangulated colic and 67.2 U/L in non-strangulated ones. GGT was not significantly different between survivors and non-survivors (W= 18.5, p= 0.34).

Hematocrit and lactate values were only significantly different between survivors and non-survivors (W= 273.5, p= 0.03 for hematocrit; W= 182, p= 0.04 for lactate). Hematocrit was on average 37% in survivors and 46.8% in non-survivor. The average lactate concentration was 1.9 mmol/L in survivors and 3.7 mmol/L in horses that died. Hematocrit and lactate concentrations were not significantly different between strangulation statuses (W= 116, p= 0.13 for hematocrit; W= 78, p= 0.2 for lactate).

Neutrophil and WBC counts were not significantly associated with outcome (W= 160, p= 0.57 for WBC count; t= 0.99, dF= 36, p= 0.33 for neutrophil count). They were also not significantly associated with strangulation statuses (W= 179, p= 0.62 for WBC count; t= -0.89, dF= 36, p= 0.38 for neutrophil count). Similarly, pre-admission duration was not significantly different within survival groups (W= 298, p= 0.21) and diagnosis groups (W= 174, p= 0.40).

In general, there were few parameters that showed a significant difference associated with outcome. The average values of HR, RR, albumin, hematocrit and lactate were lower in survivors compared to horses that did not survive a colic episode, while the average temperature was higher. Regarding colic types, only HR averages were significantly higher while GGT averages were lower during bowel strangulation. The values of numerical parameters are presented in Annex 3 according to outcome and colic type.

4. DISCUSSION

We aimed to identify one or several novel biomarkers that would assist in determination and decision-making processes related to equine colic cases. To do so, we wanted to measure levels of haptoglobin, calprotectin and HMGB1 in horses with signs of abdominal pain and compare the results with the literature data. The results indicate that, in the settings of this retrospective study, haptoglobin, calprotectin and HMGB1 are not reliable as prognostic biomarkers. Similarly, they are not reliable as diagnostic indicators for differentiation of colic types.

In parallel, we aimed to detect differences between haptoglobin, calprotectin and HMGB1 concentrations measured in serum compared to plasma. Serum concentrations were mainly shown to be significantly lower than plasma concentrations.

As far as haptoglobin and HMGB1 are concerned and to the extent of the author's knowledge, there are no published significant differences of serum concentrations compared to plasma. Nonetheless, calprotectin has been studied on that aspect in humans suffering from rheumatoid arthritis, showing that serum concentration is usually higher than plasma (NORDAL et al., 2018). The current study shows the opposite. As the previous researchers presumed of a release of calprotectin from activated neutrophils to serum during inadequate handling, the present results seem incompatible.

Whether absolute and relative biases between blood substrates are significant or not, the main focus should remain on the determination of clinical significance. Since APR quantification is to be used as a tool to assist the veterinarian in decision making, significant but small variations could be of minor importance for therapy choices. However, the definition of cut-off values, regardless the blood substrate, is far more useful to orientate on best course of action.

APPs measured in this study do not always have known reference ranges. In regards to haptoglobin, many reference values of healthy horses are available in the literature. The mean of serum concentrations of haptoglobin in this study (0.7±0.4g/L) is broadly similar to the mean values described as control group in several studies (KENT and GOODALL, 1991; TAIRA et al., 1992; CRAY and BELGRAVE, 2014; WESTERMAN et al., 2016; MIHELIĆ et al., 2022). However the reference interval announced by CRISMAN et al. (2008) is higher than the interval of this present research.

As far as calprotectin is concerned, there are very few reference intervals of healthy horses known so far. MIHELIĆ et al. (2022) measured a mean of 30.08±7.77ng/mL from an athletic population mainly composed of Arabian breeds. GROSCHE et al. (2013) obtained a median of 3754.9 ng/mL from their control group, which is tremendously higher than this previous mentioned study. Those two values were assessed from serum and in the current research, calprotectin concentration of this substrate (33.9±28.3ng/m) is similar to the results from athletic horses previously named.

Lastly, HMGB1 concentrations in blood of healthy horses are, until now, only described in the plasma of the control group studied by BAUQUIER et al. (2016). They obtained a median of 3.5 ng/mL with a range of 0.1–11.9 ng/mL, which is lower compared to the mean

of our study (53.1±26.6). Nevertheless for all three APPs, it should be considered that values might also depend on the method used for quantification.

Although the difference between survival statuses appeared to be non-significant in the study, just as observed by WESTERMAN et al. (2016) on colicky horses, surprisingly haptoglobin concentration tends to be lower in horses that survived colic.

Considering the approximation that survival rate would decrease with the severity of the colic episode, we would expect from haptoglobin concentration to be lower in cases that died (PIHL et al., 2013). However, DA SILVA NOGUEIRA and SANTANA (2011) obtained an elevated concentration during colic episodes. As highlighted by PIHL et al. (2015), this could be explained by the fact that the inflammatory response is time dependant. Thus, the average pre-admission duration might be insufficient to detect inflammatory reactions. By association, we can also hypothesize that horses that survived the colic episode were the ones quickly taken care of, so damages were still minimal at sampling time.

Moreover, the reference range of heathy horses being quite broad for haptoglobin, the studied population might have a different baseline in healthy settings. As already described, the range values presented in this study are overlapping with ranges of healthy populations shared in other researches (thoroughbreds by KENT and GOODALL, 1991; adult horses by TAIRA et al., 1992; CRAY and BELGRAVE, 2014; pre-race endurance horses by MIHELIĆ et al., 2022).

Fluctuations, in agreement with results from TAIRA et al. (1992), are favoured by the heterogeneity of the sampled horses as two of them were pregnant or had given birth a few days prior, and five of them were less than or around two years old. Additionally, the colic patients that have received therapy before sampling might show reduced concentrations of haptoglobin due to the effects of flunixin meglumin potentially administered, as showed by DI FILIPPOL et al., (2021) after standing castration of horses. Overall, many factors could explain a lower concentration determined in survivors and need further investigation.

Calprotectin concentration was non-significantly higher in horses that survived colic. Those results are the opposite from data on activated neutrophils displayed by WEISS and EVANSON (2003). By extrapolation from their research, calprotectin was expected to appear in higher concentration in non-survivor cases. Nonetheless, WANG et al. (2018) highlighted that calprotectin has both pro and anti-inflammatory properties and is able to stimulate or inhibit leucocytes extravasation. Thus, it is not possible to conclude whether the populations of survivors in the present study had the same regulatory needs compared to the colic horses sampled by WEISS and EVANSON (2003). To date, there are not many published healthy ranges of calprotectin in equine blood but the obtained average in this investigation is close to the pre-endurance race range provided by MIHELIĆ et al. (2022). It appears that the calprotectin range of colicky horses is wider than the one they obtained.

In addition, results from CARDIERO et al. (2022) showed a correlation between calprotectin concentration and neutrophil counts in humans with COVID-19, and conclusions from ÅSBERG et al. (2021) attested of the possibility to measure calprotectin in blood as a substitute to neutrophil count. Considering the lack of correlation between both neutrophil and WBC counts and the concentration of calprotectin obtained in this study, a few storage

mishandling assumptions can be formulated. Among others, sampling-to-centrifugation time as well as sampling-to-freezing time might have an effect on the stability of calprotectin and activated neutrophils.

Results of HMGB1 among groups, even though they are non-significant, are the most unforeseen. Comparably to calprotectin, HMGB1 concentration in plasma was also higher in survivors as opposed to horses that died. Despite the aforementioned tendency, HMGB1 serum concentration was non-significantly decreased in the only case of survivors, as expected considering the known association with poor prognosis in dogs (YU et al., 2010; ISHIDA et al., 2011; LEE et al., 2021). Compared to the average of HMGB1 concentration in heathy horses presented by BAUQUIER et al. (2016), the average and range are considerably higher in the present study. The discrepancy between findings is most probably attributable to samples handling and the choice of blood substrate. In that matter, WENG et al. (2018) concluded that HMGB1 is more stable in plasma than in serum. However, no stability trial has been carried out on equines yet.

More importantly, MORIMOTO et al. (2022) have recently highlighted the regulatory effect of haptoglobin, binding to HMGB1 during brain ischemia. Although no studies have been published so far on bowel ischemia in horses, the potential interaction between haptoglobin and HMGB1 pointed out by YANG et al., (2016) needs to be considered. The formation of haptoglobin-HGMB1 complexes could interfere with ELISA methods and distort the results.

Comparably to the results concerning prognosis potential, the results of diagnosis potential were in disagreement with most expectations. Although the difference between groups was proven to be non-significant, haptoglobin concentration tends to be higher in cases with strangulated lesions. This finding is refuting previous publications on equine colic as the one from DA SILVA NOGUEIRA and SANTANA (2011), who obtained significant elevations of haptoglobin concentration in colic cases (mainly with small intestine obstruction). As well, the publication from DONDI et al. (2015) demonstrates a decreased concentration in strangulating cases compared to non-ischemic ones. As haptoglobin is described by PIHL et al. (2016) to be reliable in identification of inflammation, we suppose that the small number of inflammatory cases enclosed in this study did not allow a proper quantification of the APR potential. Furthermore, haptoglobin is known to react to a large variety of pathologic processes, thus results might need to be interpreted in combination with other parameters in order to be reliable.

Regarding calprotectin concentrations, results were discording from the ones expected according to the study by WEISS and EVANSON (2003) on equine colic. Since they showed different concentrations in inflammatory cases compared to non-strangulated cases, the grouping method and the sample size of our research might not be suited for the corresponding evaluation.

It is important to highlight that calprotectin in strangulated cases reacted according to results shared by GROSCHE et al. (2013). They reported a non-significant decrease in serum concentration during induced bowel ischemia in horses. Besides, calprotectin appears to be

targeting the intestinal mucosa and passes relatively quickly to the interstitium. As a result, assessment of PF and fecal contents when present, could be more relevant in order to identify types of colic.

Concerning HMGB1, concentration was also surprisingly and non-significantly lower in cases with bowel strangulation. Based on research on dogs' volvulus (UHRIKOVA et al., 2015) and on colic horses (BAUQUIER et al., 2016), it was presumed to dramatically increase during bowel strangulation. However the population of our study contains less strangulation cases than the population analysed by BAUQUIER et al. (2016). Correlation findings indicate a positive association with calprotectin, in agreement with the ability of HMGB1 to indirectly activate neutrophils. Thus, as it is linked to calprotectin, comparison of results with PF concentration would be of interest.

In reference to the fact that nowadays, lactate, albumin and creatine kinase are the only ones available to foresee ischemic lesions (LUDWIG et al., 2023), a few other clinical parameters were quantified to assess their diagnosis potential. In this research, HR was showed to be significantly higher in cases of bowel strangulation. This result is in agreement with the studies of IHLER et al. (2004) and KOS et al. (2022). They both determined a significant elevation of HR in colic cases needing surgery. Although they analysed the parameter in combination with hematocrit values, we can highlight again that, in the colic context, HR could be used alone to differentiate diagnosis types.

In the present study, albumin did not show significant differences between concentrations in strangulated and in non-strangulated colic cases. This could be explained because albumin tends to shift to ischemia modified albumin under hypoxic conditions (GUNDUZ et al., 2008).

Also, GGT concentration was proven to be different between colic types, being significantly decreased in strangulated cases. This result was expected, considering the conclusions obtained by GARDNER et al. (2005). They determined that horses with large colon dorsal displaced to the right had higher blood concentrations than in cases of dorsal displacement to the left. They explain it by the potential anatomic compression of the bile duct in cases with colonic displacement to the right, causing occlusion of the channel and followed by elevation of GGT in blood. In our study, whether horses suffer from left or right displacement, we can extrapolate that a shift leading to the compression of the bile duct would be mostly related to strangulation as time passes. Thus it appears rational that GGT could differentiate colic cases, although it still has to be tested.

Interestingly, calprotectin and HMGB1 were shown to be positively correlated between all blood substrate. Concentrations of HMGB1 and calprotectin were correlated with regards to all substrates except plasma HMGB1 to serum calprotectin. The first assumption in connection with those correlations is that calprotectin and HMGB1 might be able to differentiate colicky horses from healthy ones. However, since we did not compare those correlation results with other populations of horses (healthy or with other diseases), we cannot test this assumption. The second assumption is according to the fact that calprotectin and HMGB1 are reacting in the same manner within the investigated colic population. They might

point out towards the same survival outcome or the same diagnosis type, either redundantly or synergistically. CHEN et al. (2020) demonstrated that both HMGB1 and calprotectin were correlated in COVID-19 patients and associated with detrimental cytokine storm and high mortality. The sample size and the cases distribution of our study may not have allowed assessing whether colic horses with deleterious inflammation could be identified at admission. Further research is needed on that specific subject.

Regarding the correlations assessed, it is peculiar that pre-admission duration, WBC count and neutrophil count did not seem associated with any of the APP tested. The pre-admission duration would be expected to correlate positively with the APR, at least in the first set of hours before they decrease. Both WBC and neutrophil counts were expected to correlate with calprotectin, as the APP is secreted by activated neutrophils (YUI et al., 2003). Although since we were not able to identify significant differences concerning APPs between groups, those lacking correlations may be associated to it.

Much variability between expected and achieved results could be explained by many other factors. With regards to the material and methods used, a few points can be mentioned.

Firstly, the exclusion criteria might have been too flexible as the population studied is from two different countries, with many breeds and a large age range. The inclusion of pregnant mares implies that both rectal findings and APR concentration findings might be affected. As horses that already received therapy during the colic episode were also included in this study, laboratory and anamnestic data are affected and might not reflect the right colic type. Moreover, even though the datasheet included a transportation question, the duration of transport was not marked. Transportation was not included in the analysis because CARVALHO FILHO et al. (2022) have proven that length less than 300 km was not significantly changing the values of APPs.

Since the samples were taken in three different locations, they were also processed by different teams and with different centrifugation machines. Although a protocol was provided, one cannot exclude the variability coming from the collection and handling of samples until freezing. Additionally, venepuncture sites were not always the same as some samples were taken from the jugular vein and some other from the transverse facial venous sinus. Nevertheless, studies from HUNTER et al. (2013), DAHAN et al. (2015) and LASCOLA et al. (2017) attested that sites can be used alternatively with no significant differences in laboratory findings.

Because of the reality of field conditions and owners' compliance, horses that had to be humanely euthanized did not get systematically necropsied. Consequently, proven diagnoses were not available in cases that were not treated surgically, which are most of the horses from this study. Additionally, the fact that a horse survived or not a colic episode could be affected by several biases. Even though veterinarians in charge of the cases were experienced, there is a possibility of cognitive biases influencing clinical evaluations (FARRELL et al., 2021). Moreover, the final decision belongs to the owner and there are possible financial restrictions that could influence this choice.

With respect to both calprotectin and HMGB1 ELISA analysis, the obtained intra-assay CVs were too high compared to the values given by the manufacturer. This implies an existing operator issue. However, we performed the calculation on standard duplicates while it was determined by the manufacturer with triplicates of unspecified concentrations. Also, as per instructions reading should be done within 10 minutes after addition of the stop solution. With three consecutive readings made during this period, we have noticed optical density variations for a same sample. Since the exact time of reading what not mentioned in the making of CV calculations, we can only bring attention to the fact that 10 minutes might be an inadequately broad time period.

On a side note, the temperature used to store the samples may not be optimal. To the author's knowledge, there is no published comparison of haptoglobin, calprotectin or HGMB1 stability between storage temperatures concerning freezing. Nonetheless PAULO et al. (2017) demonstrated that the storage of ceruloplasmin was best at -80°C compared to -20°C. By analogy, comparing the stability of haptoglobin, calprotectin and HGMB1 between those two common freezing temperatures would benefit future studies.

Based on the number of horses that were sampled, for most of the analysed parameters the sample size was sufficient for a pilot study. However albumin and GGT were assessed on a smaller number of horses (respectively 16 and 15 cases out of 46) and results need to be considered accordingly. Additional limitations, besides the small sample size and the heterogeneity of the cases, are mostly related to the fact that those APR are not widely studied in the equine field. It appears that concentration ranges might depend on populations, breed, age, training and other factors affecting physiologic processes. Without baselines concerning each APR, it is difficult to identify the modifications made by colic episodes as many unknown are still present. Further research is needed and the comparison between systemic blood and PF concentrations of APPs could be a beneficial investigation for the next step.

5. CONCLUSION

- Haptoglobin, calprotectin and HMGB1 were not reliable as prognostic biomarkers for cases of colic concerning the horse patient.

- Haptoglobin, calprotectin and HMGB1 were not reliable as differentiation tools between strangulated and non-strangulated types of colic.

- Serum concentrations of haptoglobin, calprotectin and HMGB1 were mainly lower than plasma concentrations in equine colic cases.

- Calprotectin and HMGB1 were shown to be positively correlated in horses suffering from colic, and might be increased compared to reference ranges in literature.

- Further research is needed on the topic, with focus on the identification of factors affecting haptoglobin, calprotectin and HMGB1 in healthy horses.

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7. ANNEXES

Annex 1: Datasheet model to report colic cases.

NAME OF THE HORSE: ..... DOCTOR IN CHARGE: .....
BREED: ..... AGE: .....
SEX: ◯ F (◯ PREGNANT (due .....) ◯ M ◯ GELDING
BCS (from 1 to 9): .....
USE: ◯ NONE ◯ LEISURE (once a week or less) ◯ ACTIVE (several times a week) ◯ COMPETING
THE HORSE LIVES IN: ◯ BOX ◯ PADDOCK ◯ BOX/PADDOCK
DEWORMING: ◯ NONE ◯ OCCASIONAL ◯ REGULAR
HAS THE HORSE BEEN TRANSPORTED IN THE LAST 12h ? ◯ YES ◯ NO

START OF SYMPTOMS: Date and time ..... OR ..... hours ago
1ST TIME COLIC: ◯ YES ◯ NO
IF RECURRENT EPISODES: ◯ 1/YEAR ◯ 2 /YEAR ◯ EVERY 3 MONTHS ◯ MORE OFTEN

TRIAS: HEART RATE ..... -MAXIMUM: ..... RESPIRATORY RATE ..... TEMP: .....
MUCOUS MEMBRANE COLOR: ◯ GREY ◯ PALE ◯ PINK ◯ RED ◯ YELLOW
CRT: ◯ <1 ◯ 1.5 to 2 ◯ ≥ 3s MOUTH MUCOSA: ◯ DRY ◯ WET
DEHYDRATION: ◯ DIARRHEA SKIN FOLD: ◯ 1s to 3s ◯ ≥ 4s
PERISTALTISM: ◯ NORMAL ◯ DECREASED ◯ INCREASED
MENTAL STATUS: ◯ APATIC ◯ NORMAL ◯ AGITATED
REFLUX: ◯ SPONTANEOUS APPROXIMATE AMOUNT: ..... pH : .....

PREVIOUS CONDITIONS: ◯ SURGERY (◯ COLIC) ◯ RESPIRATORY DISEASE
◯ OTHER .....
RECTAL FINDINGS: ◯ NOTHING ◯ OBSTIPATION ◯ TIGHT TENIAS
◯ LOOPS OF SMALL INTESTINE ◯ Other.....
US: ◯ INCREASED PERITONEAL FLUID ◯ INCREASED WALL THICKNESS
◯ HYPOMOTILITY-DISTENDED LOOPS ◯ Other.....
OUTCOME: ◯ MEDICAL RESOLUTION ◯ SURGERY
◯ EUTHANIZED (circle: STATUS / COST) ◯ DEAD

SUSPECTED DIAGNOSIS AND FINDINGS: (pick on the left ➔ adjust on the right, and circle details when appropriate)

◯ Stomach
◯ Small intestine (Jejunum / Ileum)
◯ Mesenteric rent
◯ Pedunculated lipoma
◯ Cecum
◯ Large colon (Right / Left – Dorsal / Ventral - Flexura pelvina)
◯ Small colon
◯ Peritonitis ◯ Uterus
Other .....

◯ Entrapment (gastro-splenic / epiploic foramen)
◯ Herniation (scrotal / inguinal / umbilical)
◯ Impaction (ingesta /sand / parasites)
◯ Colitis ◯ Displacement
◯ Enterolithiasis ◯ Intussusception
◯ Rupture ◯ Infarction
◯ Strangulation ◯ Tympany
◯ Torsion – volvulus ◯ Ulcers .....
.....

SAMPLES: Number on the label**: ..... DATE + TIME OF SAMPLING: .....

DID THE HORSE RECEIVE ANY THERAPY BEFORE SAMPLING?
◯ NO (name ..... dose ..... time .....)
(name ..... dose ..... time .....)
(name ..... dose ..... time .....)

REMINDER : **include CBC at sampling time*
*** label the samples with the first letter of your practice + the number of the patient + content of the tube (ex: H-5s for serum or H-5p for plasma taken with EDTA)*

REMARKS (cribbing, necropsy findings, additional info, ...)
.....
.....
.....

Annex 2: Instructions for handling of samples from collection to freezing.

HANDLING OF SAMPLES

- Fill out 2 **dry tubes (serum)** and 2 **EDTA tubes** with blood from the colic horse (at the same time than your CBC tubes)

1 Eppendorf tube = 1.5mL, and I need at least 2mL of serum + 2mL of plasma by colic case

DRY TUBE -serum	EDTA TUBE -plasma
1. Let sit for 20 minutes 2. **Centrifuge IN THE HOUR** AFTER SAMPLING (if this delay has been passed, please mention it on the paper attached to the case) *1300 to 2000g for 10 min* 3. Take the serum and put it into Eppendorf tubes (2 or more) 4. Write the label on the lid of the Eppendorfs, as instructed: first letter of your practice + number of the patient + "s" Example: H-5s	1. Centrifuge *1300 to 2000g for 10 min* 2. Gently aspirate the plasma ONLY and pour it into Eppendorf tubes (2 or more) 3. Write the label on the lid of the Eppendorfs, as instructed: first letter of your practice + number of the patient + "**p**" Example: H-5p

- Freeze immediately at **-20°C**
  Pay attention to sit the Eppendorf vertically, with all the liquid at the bottom

- **Fill out the paper** attached to the case

THANK YOU !

Annex 3: Summary of statistical results, tendencies and interpretations concerning outcome and diagnosis.

p value is expressed as level of significance (is below 0.05, ** is below 0.01 and *** is below or equal to 0.001 and no marks when non-significant)*

Numerical variable	**Overall** Mean ±SD Median (IQR, min-max)	**According to outcome**				**According to colic type**			
		Survivors Mean ±SD Median (IQR, min-max)	**Non-survivors** Mean ±SD Median (IQR, min-max)	**Tendency in survivors**	**Tests details**	**Strangulated** Mean ±SD Median (IQR, min-max)	**Non-strangulated** Mean ±SD Median (IQR, min-max)	**Tendency in strangulated**	**Tests details**
Age (year)	13.9±8.8 13.5 (0.2 – 29) 16	12.1±8.3 10 (0.5 – 29) 13.5	16.8±9.1 16 (0.2 – 28) 13.8	4.7 years younger 95% CI= [-0.5, 9.9]	*t*= 1.81, *dF*= 44 *p*= 0.077	16.6±9.6 16 (0.2 – 28) 13	12.9±8.4 12 (0.5 – 29) 14	3.66 years older 95% CI= [-9.4, 2.1]	*t*= -1.28, *dF*= 44 *p*= 0.208
Pre-admission duration (hour)	13.9±20.5 6 (15, 1 – 120)	13.8±23.9 5 (14, 1 – 120)	14.1±14.6 7.5 (16, 2 – 48)	-	*W*= 298 *p*= 0.205	20.5±32.7 7 (18, 2 – 120)	11.2±12.5 5.5 (13.8, 1 – 48)	-	*W*= 174 *p*= 0.400
HR (bpm)	62.2±23.5 52 (24, 32 – 120)	53.1±16.8 48 (17, 32 – 100)	75.9±25.9 71 (33.5, 40 – 120)	***	*W*= 387.5 *p*= 0.001	80.2±28.1 80 (40, 40 – 120)	54.9±17 49 (16, 32 – 105)	**	*W*= 87.5 *p*= 0.003
RR ($b_r$pm)	26±13.4 20 (19, 12 – 64)	22.6±13.2 16 (7, 12 – 64)	33.2±11.2 30 (16.5, 16 – 50)	**	*W*= 169 *p*= 0.005	33.7±12.6 35 (16, 16 – 50)	24.2±13.1 16 (12, 12 – 64)	-	*W*= 38.5 *p*= 0.061
Temperature (°C)	37.5±0.7 37.4 (0.7, 36 – 39.5)	37.7±0.5 37.7 (0.6, 36.7 – 38.5)	37±1 36.8 (0.7, 36 – 39.5)	**	*W*= 31.5 *p*= 0.003	37.3±1.1 37 (0.5, 36.4 – 39.5)	37.5±0.6 37.5 (0.7, 36 – 38.5)	-	*W*= 105.5 *p*= 0.132
Albumin (g/L)	28.6±4.7 28.5 (5.5, 20- 38)	26±3.3 26 (4, 20 – 31)	32±1.4 32 (3.5, 25 – 38)	6g/L lower 95% CI= [2.0, 9.9] **	*t*= 3.25, *dF*= 14 *p*= 0.006	32.5±5.6 33.5 (5.5, 25 – 38)	27.3±3.8 27.5 (4.8, 20 – 33)	5.17g/L higher 95%CI= [-10.4, 0.1]	t= -2.12, dF= 14 p= 0.052
GGT (U/L)	53.6±58.3 24 (56.5, 11 – 172)	60.7±60.8 24 (91, 15 – 172)	43±58.1 21 (20.2, 11 – 160)	-	W= 18.5 *p*= 0.344	16.2±9.2 12 (4.8, 11 – 30)	67.2±63 33 (100.5, 15 – 172)	*	*W*= 39 *p*= 0.031
Hematocrit (%)	41±12.7 36.9 (13.3, 26.4 – 85.3)	37±9.5 35.1 (9.4, 26.4 – 60.1)	46.8±14.9 41.5 (22.1, 32.3 – 85.3)	*	*W*= 273.5 *p*= 0.025	43.9±11.2 41.5 (18.1, 32.3 – 63)	39.7±13.3 36.1 (10.5, 26.4 – 85.3)	-	*W*= 116 *p*= 0.128

Lactate (mmol/L)	2.7±2.4 2 (1.5, 0.8 – 12)	1.9±1.3 1.6 (1.3, 0.8 – 6.2)	3.7±3 2.7 (3.3, 1 – 12)	*	*W*= 182 *p*= 0.041	3.1±1.9 2.6 (2.6, 1 – 6.5)	2.6±2.6 1.7 (1.6, 0.8 – 12)	-	*W*= 78 *p*= 0.199
Neutrophils count (%)	71.9±12.2 70.8 (17.6, 47.5 – 94)	70.4±10.6 68.8 (12.1, 47.5 – 90.2)	74.4±14.7 79 (21.3, 51 – 94)	4.07 % lower 95% CI= [-4.3, 12.4]	*t*= 0.99, *dF*= 36 *p*= 0.329	74.6±15 79.2 (23, 51 – 94)	70.7±11 68.8 (12.6, 47.5 – 91.2)	3.98 % higher 95% CI= [-12.8, 5]	*t*= -0.89, *dF*= 36 *p*= 0.381
WBC count (M/mm^3)	10.4±4.5 10.9 (5, 1.2 – 24.3)	11.1±4.5 10.5 (4.9, 5.7 – 24.3)	9.3±4.5 10.9 (4.7, 1.2 – 15.6)	-	*W*= 160 *p*= 0.573	9.3±4.5 10.7 (4.5, 1.2 – 15.6)	108±4.6 10.9 (5, 2.6 – 24.3)	-	*W*= 179 *p*= 0.616
Haptoglobin $_{serum}$ (g/L)	0.7±0.4 0.6 (0.7, 0 – 1.7)	0.6±0.4 0.6 (0.6, 0 – 1.4)	0.7±0.5 0.6 (0.7, 0 – 1.7)	0.07g/L lower 95% CI= [-0.2, 0.4]	*t*= 0.52, *dF*= 44 *p*= 0.609	0.8±0.5 0.6 (0.6, 0 – 1.6)	0.6±0.4 0.6 (0.6, 0 – 1.7)	0.14g/L higher 95% CI= [-0.4, 0.2]	*t*= -0.94, *dF*= 44 *p*= 0.351
Haptoglobin $_{plasma}$ (g/L)	0.8±0.4 0.8 (0.7, 0 – 1.8)	0.8±0.4 0.7 (0.6, 0 – 1.5)	0.8±0.5 0.9 (0.7, 0.1 – 1.8)	0.06g/L lower 95% CI= [-0.2, 0.4]	*t*= 0.42, *dF*= 36 *p*= 0.681	0.9±0.4 0.9 (0.6, 0.3 – 1.5)	0.9±0.5 0.8 (0.7, 0 – 1.8)	0.1g/L higher 95% CI= [-0.4, 0.2]	*t*= -0.56, *dF*= 36 *p*= 0.581
Calprotectin $_{serum}$ (ng/mL)	33.9±28.3 30.9 (43.4, 2.0 – 102.3)	35.1±28.9 30.4 (51.6, 2.0 – 95.3)	32±28.2 32.4 (34.7, 2.0 – 102.3)	-	*W*= 240.5 *p*= 0.804	29.6±30.4 33.9 (39.9, 2 – 102.3)	35.6±27.8 30.9 (44, 2 – 95.3)	-	*W*= 246 *p*= 0.448
Calprotectin $_{plasma}$ (ng/mL)	35.3±26.7 29.9 (33, 2.0 – 107.3)	37.3±28.8 32.4 (34.9, 2 .0– 107.3)	31.8±23.2 29.9 (30.8, 2.0 – 83.8)	5.44ng/mL higher 95% CI= [-23.8, 12.9]	*t*= -0.6, *dF*= 36 *p*= 0.552	25.1±20.3 16.4 (23.5, 2 – 60.4)	38.4±27.9 38.4 (31.5, 2 – 107.3)	13.26ng/mL lower 95% CI= [-7.2, 33.8]	*t*= 1.31, *dF*= 36 *p*= 0.197
HMGB1 $_{serum}$ (ng/mL)	51±32.6 44.7 (41.2, 1.2 – 126.5)	49.4±33.7 47.3 (47.9, 1.2 – 124)	53.4±31.7 43.6 (38, 10.3 – 126.5)	3.93ng/mL lower 95% CI= [-16.1, 24]	*t*= 0.39, *dF*= 44 *p*= 0.695	44.8±34.6 33.7 (45, 1.2 – 126.5)	53.4±32 50 (36.9, 1.2 – 124)	8.55ng/mL lower 95% CI= [-13.1, 30.2]	*t*= 0.8, *dF*= 44 *p*= 0.430
HMGB1 $_{plasma}$ (ng/mL)	53.1±26.6 50.6 (31.2, 1.2 – 118.4)	54.2±26.2 55.3 (28, 1.2 – 104.3)	51.4±28.3 43.7 (33.7, 11.8 – 118.4)	2.79ng/mL higher 95% CI= [-21.2, 15.6]	*t*= -0.31, *dF*= 36 *p*= 0.760	43.1±22.5 39.3 (23.4, 11.8 – 82.2)	56.3±27.3 55.6 (29.7, 1.2 – 118.4)	13.2ng/mL lower 95% CI= [-7.2, 33.6]	*t*= 1.31, *dF*= 36 *p*= 0.198

8. ABSTRACT

Juliette, Alexandra Magoga

Can prognosis be assessed by a novel biomarker at the time of patient admission for horse colics?

Equine colic is an emergency frequently met by veterinarians. Causing pathological processes might be challenging to identify and their late determination be fatal. Biomarkers of intestinal lesions could be a valuable tool to assist veterinarians in decision-making, and Acute Phase Proteins (APPs) in particular. This retrospective study aimed to quantify haptoglobin, High Mobility Group Box 1 (HMGB1) and calprotectin in blood of horses hospitalised for colic pain, to assist identifying etiologies and determining cases prognosis. We hypothesised an increase in HMGB1 and calprotectin concentrations, and a decrease in haptoglobin concentration in more severe colics.

Serum and EDTA plasma were collected from 46 colicky horses at admission. History and clinical exam data were recorded. ELISA kits were used to quantify HMGB1 and calprotectin, and haptoglobin was measured using a spectrophotometric method. Horses were grouped by outcome (dead/survived) and colic type (strangulated/non-strangulated). Statistical analyses were based on demographic and anamnestic data, and clinical and laboratory findings. Associations with groups were tested with two-sample tests. Correlations of variables were analysed using Spearman's test with Bonferroni correction. Bland-Altman analysis was used to compare differences between serum and plasma values.

There were no significant differences between groups regarding concentrations of haptoglobin (serum 0.7±0.4g/L; plasma 0.8±0.4g/L), calprotectin (serum 33.9±28.3ng/mL; plasma 35.3±26.7ng/mL) and HMGB1 (serum 51±32.6ng/mL; plasma 53.1±26.6ng/mL). Overall, serum concentrations tend to be significantly lower than plasma concentrations; except for calprotectin for which relative bias was not conclusive. Calprotectin and HMGB1 were positively correlated ($p<0.01$).

The tested APPs were not reliable as prognostic or diagnostic biomarkers. However, calprotectin and HMGB1 might be increased in colic compared to literature reference ranges. Limitations of the study, besides the small sample size and cases heterogeneity, are mostly related to the lack of knowledge on equine APPs. They might depend on breeds, training and other unknown factors.

Key words: equine colic, prognosis, diagnosis, haptoglobin, calprotectin, HMGB1, blood.

9. SAŽETAK

Juliette, Alexandra Magoga

Može li se prognoza konja s kolikom procijeniti pomoću novog biomarkera u trenutku prijema?

Kolike konja su hitni slučajevi s kojim se veterinari često susreću. Uzroke patološke procese može biti teško identificirati, a njihovo kasno utvrđiavenj može biti fatalno. Biomarkeri intestinalnih lezija mogli bi biti vrijedan alat za pomoć veterinarima u donošenju odluka, a posebice proteini akutne faze (APP). Ova retrospektivna studija imala je za cilj kvantificirati haptoglobin, High Mobility Group Box 1 (HMGB1) i kalprotektin u krvi konja hospitaliziranih zbog kolika, kako bi se pomoglo u identifikaciji etiologije i određivanju prognoze slučajeva. Hipoteza istraživanja bila je da će kolike uzrokovati povećanje koncentracije HMGB1 i kalprotektina, te smanjenje koncentracije haptoglobina kod težih kolika.

Serum i EDTA plazma prikupljeni su od 46 konja s kolikama pri prijemu. Zabilježeni su povijest bolesti i podaci kliničkog pregleda. ELISA setovi korišteni su za kvantificiranje HMGB1 i kalprotektina, a haptoglobin je mjeren spektrofotometrijskom metodom. Konji su grupirani prema konačnom ishodu (mrtvi/preživjeli) i vrsti kolika (strangulacijska/nestrangulacijska). Statističke analize temeljene su na demografskim i anamnestičkim podacima te kliničkim i laboratorijskim nalazima. Povezanosti sa skupinama testirane su testovima s dva uzorka. Korelacije varijabli analizirane su Spearmanovim testom s Bonferronijevom korekcijom. Bland-Altmanova analiza korištena je za usporedbu razlika između vrijednosti u serumu i plazmi.

Nije bilo značajnih razlika između skupina u pogledu koncentracija haptoglobina (serum 0,7±0,4g/L; plazma 0,8±0,4g/L), kalprotektina (serum 33,9±28,3ng/mL; plazma 35,3±26,7ng/mL) i HMGB1 (serum 51±32,6 ng/mL; plazma 53,1±26,6 ng/mL). Koncentracije u serumu imaju tendenciju biti značajno niže od koncentracija u plazmi; osim za kalprotektin za koji relativna pristranost nije bila uvjerljiva. Kalprotektin i HMGB1 bili su u pozitivnoj korelaciji ($p<0,01$).

Testirani APP nisu bili pouzdani kao prognostički ili dijagnostički biomarkeri. Međutim, kalprotektin i HMGB1 mogu biti povećani kod kolika u usporedbi s literaturnim referentnim rasponima. Ograničenja studije, osim male veličine uzorka i heterogenosti slučajeva, uglavnom su povezana s nedostatkom znanja o APP kod konja. Oni mogu ovisiti o pasminama, vrsti trening i drugim nepoznatim čimbenicima.

Ključne riječi: kolike konja, prognoza, dijagnoza, haptoglobin, kalprotektin, HMGB1, krv.

10. BIOGRAPHY

Juliette's education started in the south of France, where she took the long path to becoming the vet of her dreams. After finishing a scientific high school (Biology and Ecology), she obtained a Laboratory Technician's degree (biotechnologies, and biologic and agricultural analyses). Later on, she spent one year in an Agricultural Engineering school before coming to Zagreb in 2016 were she was part of the first generation of the English program.

Being a persevering person and a passionate learner, she has been lucky to annually receive the Award of Best Academic Achievement for her generation of English students. However, conscious that good academic results are insufficient to gain competencies, she is always searching for new skills and build herself more every day. Thanks to her previous diploma, she has acquired some experience in the field of research. She has, as well, been in touch with the equine ambulatory practice through the clinic of her relatives, alternatively employed and volunteering since a young age.

During veterinary education, she had externships at small animal and mixed clinics, equine hospital and practices, and a sheep farm. Besides the work experiences, she attended several seminars, summer schools and conferences abroad (Ethics, Proteomics, Epidemiology, One health, Anatomy and Histology, Mountain animal breeding, Biology of Marine Mammals and Entrepreneurial mindset). Wishing to share acquired experiences with her colleagues, she held two presentations with the guidance of her professors, and wrote a few popularisation articles for the faculty journal. By being a Demonstrator in the courses of Histology, Physiology, and Obstetrics, she was able to reinforce her own knowledge and pass it on to younger students, while also improving her soft skills.

Moreover, she has been involved in Faculty life since the first year of her studies, starting with representing her fellow classmates. Thereafter, she joined the IVSA and USVM associations, and in 2018 she integrated the Editorial board of Veterinar. To meet the needs of the students of the English program, she co-founded The Vet Society–University of Zagreb where she was Head of communications until it got established enough to be passed on to the younger generation. She then became Student Coordinator of the newly formed team of Equine volunteers. In 2020 she also joined the team of volunteers of the Internal Medicine clinic.

She frequently answers calls for projects and events (Night of Museums, the 100 years of the Faculty, Plavi Projekt, etc.). Furthermore, she co-created the poster "*Renovation of the Anatomy Students Room*", under the supervision of Professors S. Kužir and M. Đuras. Together with her colleague Irisz Koutis, she presented it at the 10th YGVA meeting – Romania 2019. They devoted themselves to this project during their 3rd year of studies. The same year, she contributed to the article "*False friends in Veterinary terminology: is infection always infection?*" presented by Professor Ž. Klječanin Franić at the 8th Veterinary Science and Profession congress. She also worked on the host-parasite interactions of the Red Deer, with Professor D. Konjević and her dear colleague and friend Irisz Koutis.

While writing her thesis, she was an occasional helper at the clinic of her relatives during foal season, and manager of the online part of recruitment for the next generation of equine practitioners.