Serum Histones in Dogs with Septic Peritonitis as a Prognostic Biomarker

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ABSTRACT

Septic peritonitis (SP) is a life-threating condition. Determining prognosis for dogs suffering from SP remains challenging. Extracellular histones exert cytotoxic, prothrombotic and proinflammatory effects. Our objective was to investigate serum general histone concentrations (sHs) as biomarkers of disease severity and outcome in dogs with naturally occurring SP. Blood samples were collected upon admission and 24 hours post admission from 21 dogs with SP and from 7 healthy controls. Serum general histone concentrations (median; IQR) upon admission were higher in dogs with SP compared to controls (34.2 ng/ml; 39.1 ng/ml vs. 7.3 ng/ml; 1.7 ng/ml; *P*=0.001) and sHs significantly decreased 24 hours post admission in dogs with SP (34.4 ng/ml; 39.3 ng/ml vs. 24.2 ng/ml; 11 ng/ml; *P*=0.018). Serum histones were higher among survivors compared to non-survivors (45.5 ng/ml; 37 ng/ml vs. 24.0 ng/ml; 12 ng/ml; *P*=0.03). This data demonstrates that sHs concentrations significantly increase in dogs with SP and decrease after hospitalization. Future studies are warranted to investigate the reverse relationship between outcome and sHs.

Keywords: Canine; Coagulation; Inflammation; Prognosis; Sepsis.

INTRODUCTION

Septic peritonitis (SP) is an important etiology of sepsis in dogs, and is often associated with leakage of gastrointestinal contents (1,2).

Locally, SP is associated with release of vasoactive substances (e.g., histamine, serotonin, cellular proteases and microbial endotoxins) which lead to increased capillary permeability and vasodilatation (3). As disease progresses, inflammatory cytokines and tissue factors lead to Systemic Inflammatory Response Syndrome (SIRS), activation of the coagulation cascade with diminished platelet aggregation and ultimately disseminated intravascular coagulation (DIC) (4). Consequently, if left undiagnosed and untreated, SP might result in multi-organ failure and death. (1,2,5) Rapid and accurate diagnosis is essential to initiate timely medical and surgical therapy (6).

Histones are highly conserved alkaline, positively-charged proteins, serving as the basic structural block unit of chromatin. Recent studies indicate that in addition to their nuclear function, histones are released from damaged and activated immune cells into the extracellular space (e.g., neutrophils and mast cells) and by neutrophil extracellular traps (NETs) exhibiting toxic, pro-inflammatory and pro-thrombotic properties in a process called Netosis

(5,7,3). Neutrophil extra-cellular traps are networks of extracellular fibers composed of neutrophil chromatin components (including histones) and other antimicrobial factors, which capture and degrade invading microorganisms (5, 8).

Serum histones (sHs) have been considered potential mediators of systemic inflammatory diseases including infections (e.g., sepsis and bacterial peritonitis) and sterile inflammation (e.g., pancreatitis, drug-induced tissue toxicity and heatstroke) (3, 5, 8). Moreover, serum histones are associated with endothelial cytotoxicity as seen both in animal models and humans with sepsis (Xu *et al.* 2009; Ekaney *et al.* 2014).

Determining prognosis for individual dogs suffering from SP remains challenging (6) Since SP is associated with SIRS and sepsis, owing to the proinflammatory and procoagulant properties of histones, we hypothesized that sHs blood concentration would be increased in dogs with SP, and would be associated with disease severity, morbidity and mortality.

MATERIALS AND METHODS

Study design

The current study is an analytical, observational prospective cohort study. Twenty-one dogs diagnosed with SP, admitted to the Hebrew University Veterinary Teaching Hospital (HUVTH) between the years 2016-2018 were included in the study. Seven staff-owned healthy dogs served as control group. Dogs were diagnosed with SP based on the presence of intracellular bacteria confirmed by cytology of the abdominal fluid, and/or the presence of gross contamination of the abdominal cavity with intestinal content during surgery. All dogs included in the study were treated according to the HUVTH SP treatment protocol (supplementary data 1). All dogs went through surgery within the first 24 hrs.

Blood collection

Cephalic or jugular venous blood samples were collected and analyzed upon admission and 24 hours post presentation. For complete blood count (CBC) (Advia 2120i, Siemens, Erlangen, Germany), blood was collected in potassium-EDTA tubes. Blood smears were prepared and stained with modified Wright's staining solution. Samples for serum biochemical analysis (Cobas 6000, Roche, Mannheim, Germany), sHs concentration measurements, were collected in tubes containing no anticoagulant, with gel-separators, allowed to clot and then centrifuged. Biochemical analysis was completed within 60 minutes from collection. Surplus sera were then immediately stored at -80˚C for histone concentration analysis with a maximum storage duration of 12 months. Lipemic and hemolytic sera were removed prior to analysis (10). Additional tests included venous blood gas analysis (Cobas B221, Roche, Mannheim, Germany), total CO_2 (Cobas 6000, Roche, Mannheim, Germany) and blood lactate (Cobas 6000, Roche, Mannheim, Germany). Samples taken between 10 pm to 6 am were carried out on Accutrend lactate analyzer (Roche, Mannheim, Germany). Whole blood samples for hemostatic tests were placed into 3.2% trisodium-citrate tubes (1part citrate: 9 parts blood). For citrated plasma-based tests, the tubes were centrifuged, and plasma was harvested within 30 minutes from collection and analyzed immediately. Global hemostasis was assessed by measurement of the prothrombin time (PT), activated partial thromboplastin time (aPTT), D-dimer, antithrombin (AT) activity and fibrinogen (ACL 9000, IT, Milano, Italy and from 2018-ACL Top 300, IT, Milano, Italy).

Serum Histones analysis

Commercially available sHs ELISA kits were used according to the manufacturer's instructions (Canine Histone Elisa kit, Catalog No. MBS746638, My BioSource©, San Diego, CA, USA) as presented in previous publications (11, 12). The Kit measuring range was between 1ng/ml to 1000ng/ml. Briefly, each sample was divided into 2 aliquots, and analysis was carried out in duplicate. Samples were placed into wells containing canine specific, validated, monoclonal antibodies against sHs and incubated for 2 hours at room temperature. The wells were then washed with a tris-buffered solution (washing buffer), leaving only plate-bound sHs. Specific canine polyclonal antibodies against sHs were added and incubated for 1 hour at room temperature, washed and stained with horseradish peroxide catalyzed reaction for 30 min. The optical density (OD) of the dyed solution was read using a spectrophotometer at 450 nm. The amount of signal read by the spectrophotometer was directly proportional to the sample sHs concentrations. The mean concentration of each duplicate was calculated and used for statistical analysis.

Statistical methods

The sHs concentration results were logarithmically transformed, and their distributions were subsequently deemed normal. The statistical analysis is based on 80% power and α of 5%. Assuming a difference of 8 ng/ml between outcome groups based on our previous study in canine heatstroke (11) a total of 20 dogs (10 dogs in each group) would be sufficient to show statistical differences. Comparison of all normally distributed, continuous variables between the survivors and the non-survivors was performed by using the Student's t-test, while non-normally distributed variables were compared by using the non-parametric Mann-Whitney *U*-test. Spearman correlation test was used to assess the correlation between two quantitative variables. Receiver operator characteristic curve (ROC) analysis was applied for determining an optimal cut off point to distinguish sHs concentrations between groups. All tests were two-tailed, and $P \leq 0.05$ was considered significant. Statistical analyses were performed using a statistical software package (SPSS 22.0 for Windows, SPSS Inc, Chicago, IL, USA).

RESULTS

The study included 21 dogs suffering from SP (11 females and 10 males), of which 12 (57%) survived to discharge and 9 died after a minimum 24 hrs of hospitalization (43%). Median age of all SP dogs was 6.5 years (range, 3 months -14 years), while nonsurvivors were significantly older compared to survivors (median 11 years, range, 4-14 vs. median 4.5 years, range 3 months – 11 years; P=0.003,). Median body weight was 23 kg (range, 8-24 kg) with no significant difference between outcome groups (P=0.8). Seven healthy young (median 3.5 years, range; 2-5 years old), large breed dogs (median body weight 27 kg, range; 20-35 kg), 4 males and 3 females were selected as controls. The median hospitalization time of three days was identical in both groups (survivors and non-survivors). Etiologies of SP included gastrointestinal foreign bodies (6 dogs; 28.5%), gastrointestinal ulcers, pyometra and idiopathic SP with no distinguished etiology (3 dogs each; 14.2%), surgical wound dehiscence (2 dogs; 9.5%), and septic uro-abdomen and intussusception (1 dog each; 4.7%). Median white blood cell count (WBC) was significantly higher among survivors compared to non-survivors, (median 12.4 $10^3/mm^3$, range: 1.7-78 $10^3/mm^3$ vs. 6.3 $10^3/mm^3$, range; 2.9-32.1 $10^3/mm^3$, respectively; P=0.003). The ROC analysis area under the curve (AUC) for WBC as a predictor of survival was 0.81 (95% confidence interval (CI) 0.68-1), with a cut-off point of 7.5 $10^3/mm^3$ corresponding to a sensitivity and specificity of 84% and 72%, respectively (Figure 1). However, no correlation was found between WBC and histone concentration (P=0.3). Additional selected hematologic parameters, although not significant, are presented in Table 1. Moreover, there was no correlation between platelet or red blood cell count and sHs.

Table 1: Selected laboratory analyte results in 21 dogs with septic peritonitis at presentation to the hospital.

Laboratory analyte	Survivors (n=12) Median (range)	Non-survivors (n=9) Median (range)	Reference Interval
Packed cell volume (%)	44 (12.0-68)	45 (23.0-54.0)	37.0-54.0
Platelets ($x10^3/mm^3$)	82.5 (123.0 -437.0)	243.0 (118.0-457.0)	39.0-72.1
Red blood cell ($x10^6/mm^3$)	5.99 (2.27-9.8)	6.32 (4.18-8.81)	5.4-7.8
Glucose (mg/dL)	85.0 (35.0-120.0)	89.0 (53.0-244.0)	31.9-65.7
Albumin (g/dL)	2.44 (1.3-3.8)	2.05 (1.4-3.02)	3.0-4.4
Creatinine (mg/dL)	1.17 (0.03-1.7)	0.75 (0.7-2.25)	0.5-1.8
Prothrombin time (sec)	7.5 (6.4-24.9)	8.3 (7.35-9.9)	6-8.4
aPTT[1] (sec)	14.1 (11.5-53.5)	13.4 (10.4-29.6)	11-17.4
PvO_2[2] (mmHg)	38.8 (27.8-75.7)	39.15 (25.1-89.2)	35-40
$PvCO_2$[3] (mmHg)	28.5 (18.8-47.9)	32.3 (20.6-72.1)	42-52
pH	7.29 (7.2-7.4)	7.34 (6.9-7.5)	7.32-7.41
HCO_3^- (mEq/L)	16.9 (9.4-26.6)	16 (3.9-60.8)	24-28

1. aPTT-activated prothrombin time; 2. Partial venous oxygen pressure; 3. Partial venous dioxide pressure.

Figure 1. ROC analysis of WBC as a predictor of outcome

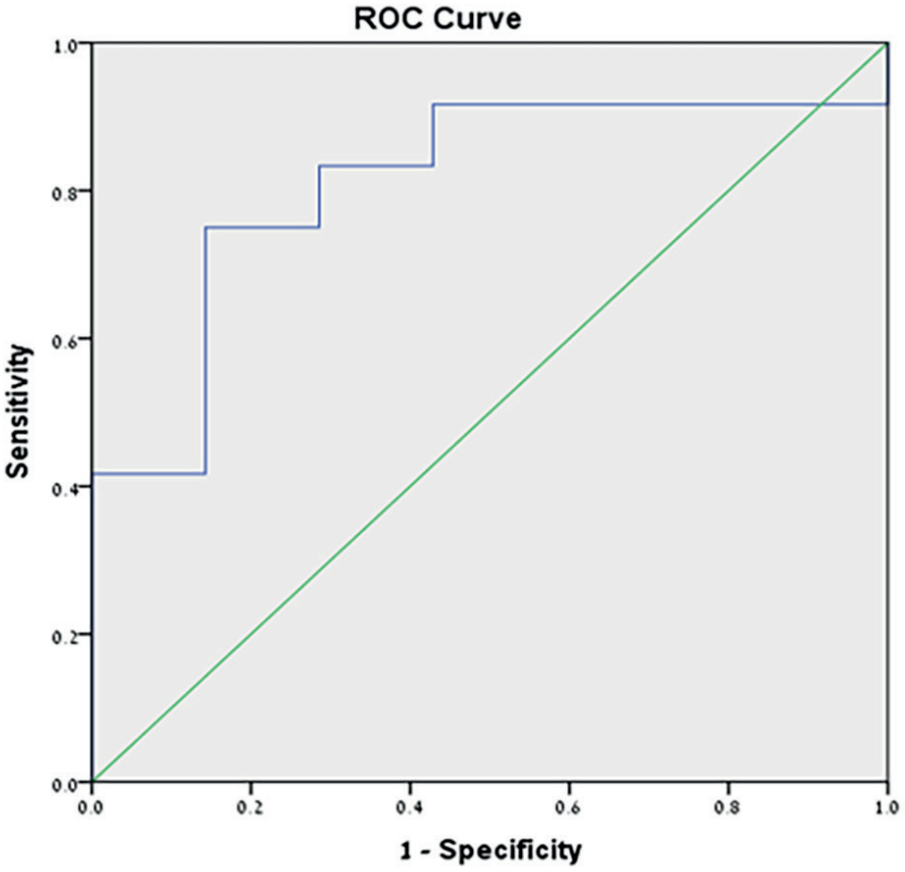

Figure 1: Receiver operator characteristic (ROC) analysis of the leukocytie count as a predictor of outcome in 21 dogs with septic peritonitis. Area under curve (AUC) of 0.81 (95%CI 0.69 100) The optimal cut-off point of was $7.5x10^3/mm^3$ corresponding to sensitivity and specificity of 84% and 72%, respectively.

General serum histones (sHs) were measured in duplicate for each sample. The agreement between duplicates was good with a standard deviation (SD) of 0.57 and intra-assay coefficient of variation of 3.8%. sHs concentration upon admission (median; IQR) was significantly higher in all dogs compared to healthy controls (34.2 ng/ml; 39.1 ng/ml vs. 7.3 ng/ml; 1.7 ng/ml, respectively; *P*=0.001; Figure 2a). Median sHs was significantly higher among survivors compared to non-survivors (45.5 ng/ml; 37 ng/ml vs. 24.0 ng/ml; 12 ng/ml, respectively; *P*=0.03; Figure 2b).

General serum histones (sHs) concentration decreased significantly 24 h after admission in all SP dogs (median 34.4 ng/ml; 39.3 ng/ml vs. 24.2 ng/ml; 11 ng/ml, respectively; *P*=0.018; Figure 3a). However, when each group was analyzed separately (survivors/non-survivors), the decrease was significant only in the survivor group (*P*=0.032; Figure 3b).

The area under the ROC curve for sHs concentration at presentation to the hospital as predictor of survival was 0.88 (95% CI 0.58- 0.99), using a cut-off point of 24.2 ng/ml corresponding to a sensitivity and specificity of 91% and 75%, respectively. Moreover, with the same cut-off point, the positive predictive value (PPV) for survival was 73%, and the negative predictive value (NPV) was 83% (Fig 4).

DISCUSSION

In the present study, sHs concentration was significantly higher in dogs with SP compared to healthy controls, in corroboration with our primary hypothesis and previous results in canine heatstroke (11, 12). However, among the SP dogs, the non-surviving group demonstrated significantly lower sHs level than the survivors. sHs decreased over time in dogs

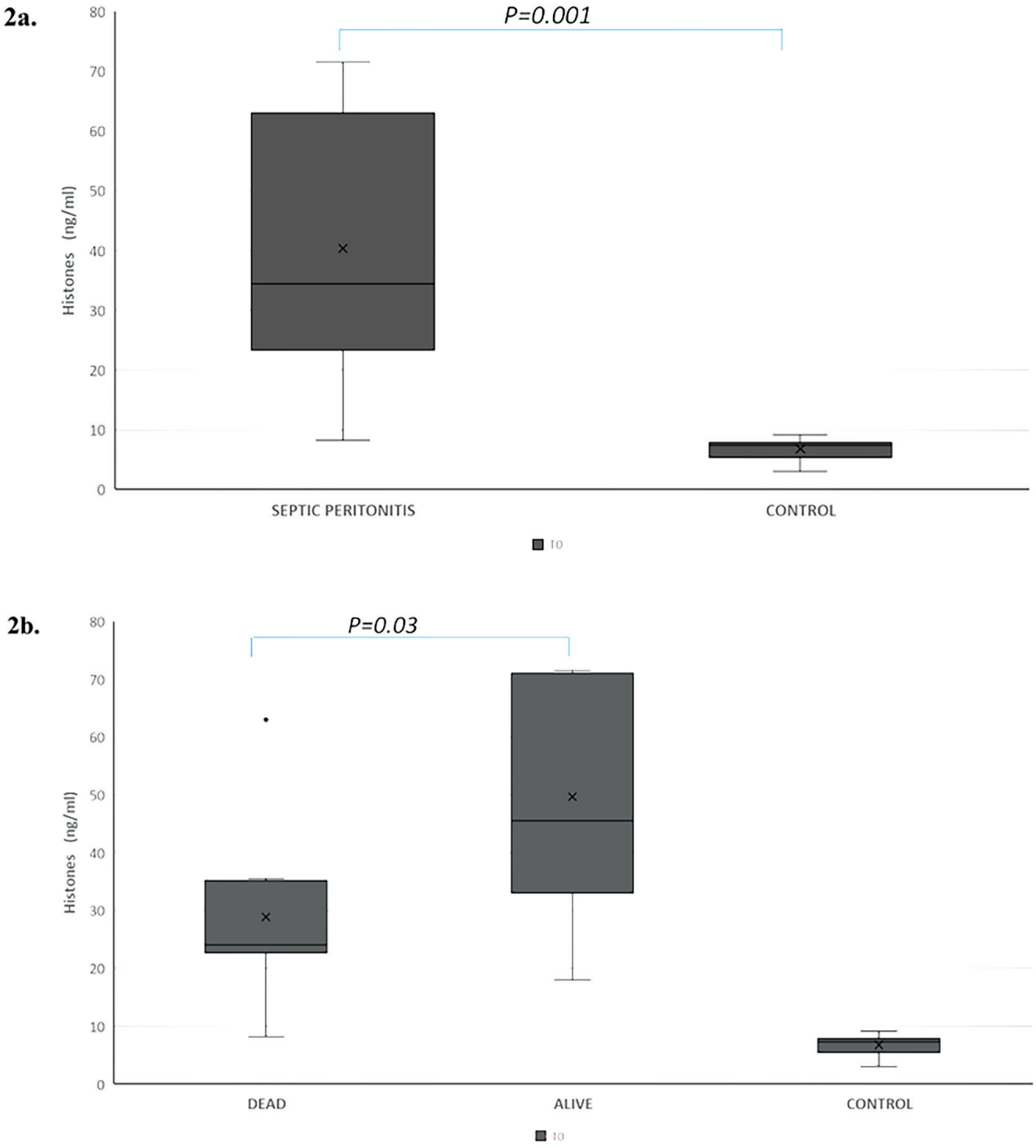

Figure 2: Box and whiskers plots of total serum histone concentration (ng/mL) upon admission to the hospital (T0) in (xx) dogs with septic peritonitis (SP) compared to (xx) controls.

Figure 3a: sHs upon admission and 24 hrs after hospitalization

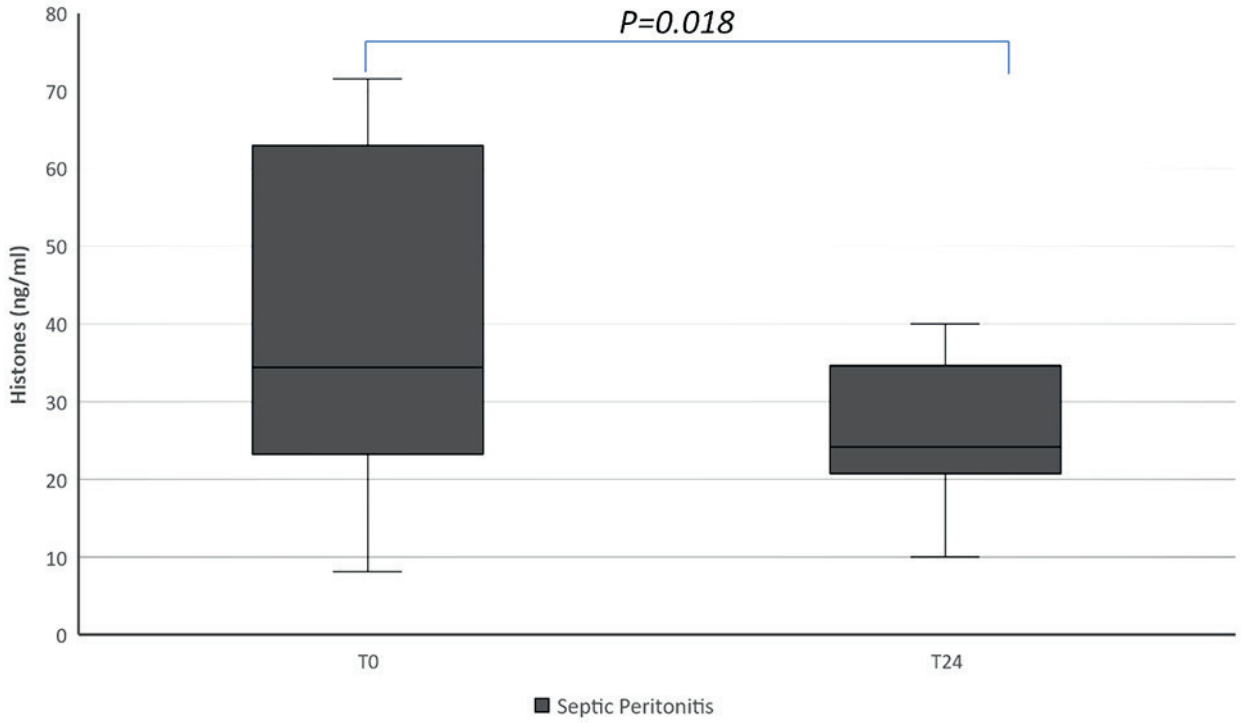

Fig 3b: sHs uppon admision and 24 hrs after hospitalization in survisors and nonsurvivors.

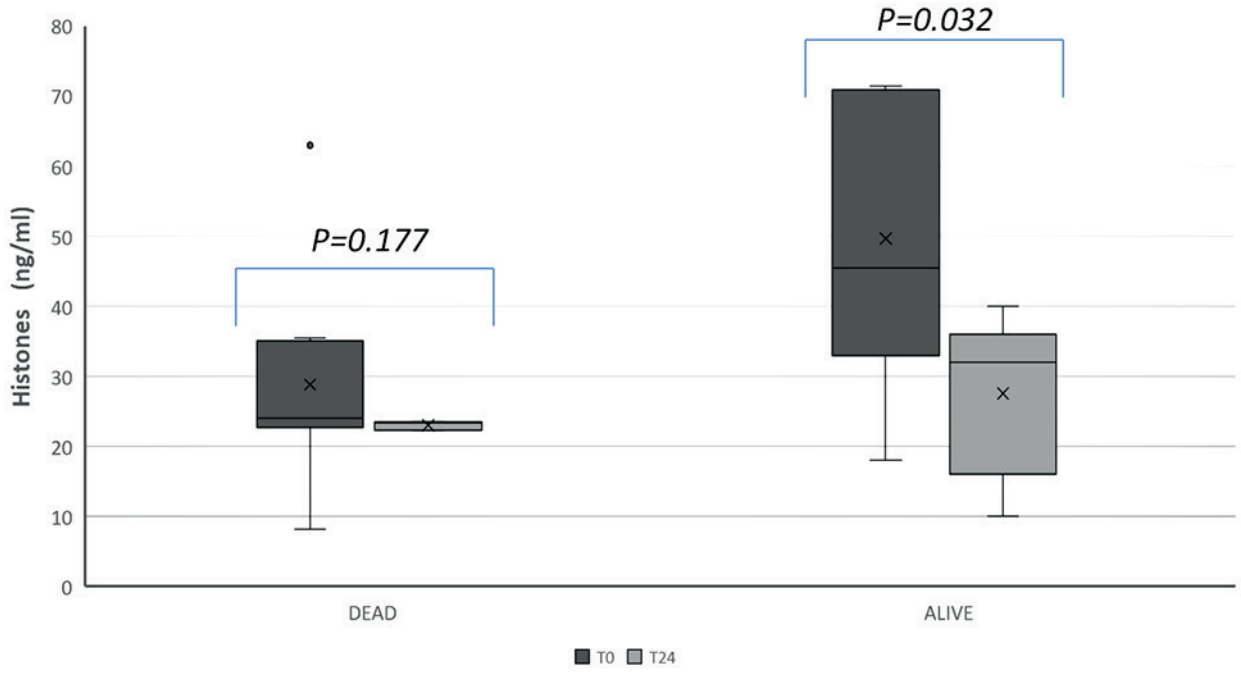

Figure 3: Box and whiskers plots of total serum histone (sHs) concentration (ng/ml) in dogs with septic peritonitis (SP) upon admission (T0)) and at twenty-four hours (T24) post-presentation to the hospital.

Figure 4. ROC analysis of sHs as a predictor of outcome in 21 dogs with septic peritonitis upon admission.

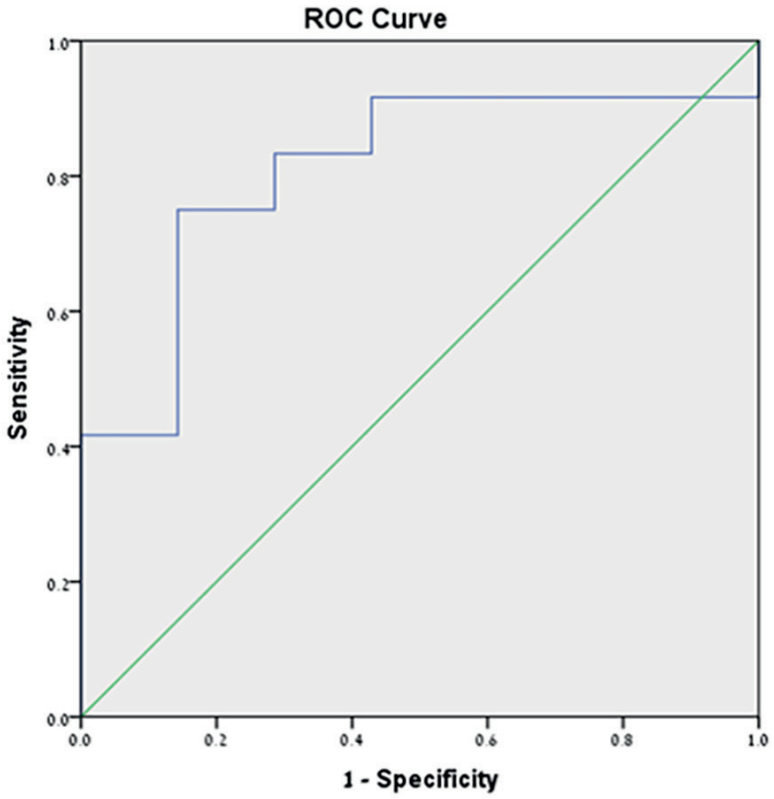

Figure 4: Receiver operator characteristic (ROC) analysis of serum histone concentration (sHs); as a predictor of outcome in 21 dogs with septic peritonitis. Area under curve (AUC) of sHs concentration at presentation to the hospital for predicting survival was 0.88 (95%CI 0.58,0.99); corresponding to a sensitivity and specificity of 91% and 75%, respectively.

with SP herein, possibly supporting an association between sHs and the inflammatory response, in line with previous *in-vitro* and *in-vivo* studies on animal models as well as in humans with SP and dogs with heatstroke (7, 8, 9, 11).

sHs concentrations were higher in survivors compared to non-survivors and decreased during hospitalization. These results are in contrast to previous results in canine heatstroke (Bruchim et al. 2017) (11) and in a naturally occurring acute pancreatitis cases where sHs were lower in survivors compared to non survivors (12). The importance of measuring sHs compared to other inflammatory proteins (e.g. C-reactive protein) is the future therapeutic effect possible as seen in previous research (14, 15, 16).

Higher sHs in survivors might be explained by the antimicrobial activity of histones along with different pathophysiological mechanisms underlying SP and heatstroke, and infective and inflammatory, non-infectious processes (17, 18, 19). In the 1950s, Hirsch *et. al* presented a bactericidal effect of sHs on several bacterial types such as *Escherichia coli, Salmonella* and others (13, 17, 19). This characteristic is more conspicuous with arginine-rich histones (e.g. H3, H4) (18), which constitute an integral part of the innate immune system, referred to as danger/damage-associated molecular patterns (8, 19). These studies might partly account for the present findings, considering the antibacterial effects sHs might have in cases of SP.

The 43% mortality rate from SP demonstrated in this cohort of animals was found to be like previous research (1,2,5,6) and aligns with the overall mortality rate from sepsis in general (2,5,6). Both sHs and WBC were higher in the survivors, however lack of correlation between WBC and sHs might be attributed to the small sample size, variability in the course of the disease and different half-life (8). Additionally, median WBC was significantly higher among survivors compared to non-survivors, but overall was a moderate predictor of outcome in the present study (Fig 1.). The low WBC in

the non-survivors could be explained by over consumption of leukocytes in face of inadequate leukocyte recruitment from the bone marrow, in the severe inflammatory cases, as demonstrated in previous research (20, 21). Furthermore, the higher WBC in the survivors could be interpreted as an indicator of a more robust host defense mechanism, compared to non-survivors, in the face of weaker inflammatory stimuli, leading to less coagulation abnormalities, excess cytokine release and death (8, 21). Since the release of WBCs from the bone marrow to the blood circulation fluctuates (22), the time the blood sampling was done in accordance to the progression of the disease might have been a cofounder. The fluctuations in WBCs during different systemic situations and over time, raises the question whether histones might be a dependent variable as well, as seen in the large variability in sHs concentration in dogs suffering from heat stroke (11).

The current study has several limitations. Firstly, cohort size was limited, thereby decreasing the power of several statistical analyses. Secondly, our cohort study included dogs with SP of various causes, different age, medical background, and was therefore heterogeneous, which might have affected possible associations between laboratory results and outcomes with sHs concentrations. The effect of the serum storage duration (at -80˚C) on sHs is unknown. Moreover, our control group was not necessarily fully comparable with the study group due to changes in breed and age.

In conclusion, sHs concentrations significantly higher in dogs with SP compared to healthy control dogs and decreased over time during hospitalization and after surgery. Contrary to previous studies of SP in humans and animal models, sHs concentration was significantly higher in the survivors. Future prospective studies are warranted to corroborate the present findings and investigate histones' prognostic significance, compare their concentrations with specific WBC subtypes (e.g. lymphocytes and neutrophils), the effect on pro-thrombotic events (e.g DIC) and if the potential therapeutic effects in dogs suffering from naturally occurring SP are reasonable.

Declarations:

Funding – This study was supported by Scil Animal Care Research Grant of EVECCS 2018.

Conflicts of interest/Competing interests – We declare no conflict of interest in this study.

Availability of data and material – All data and material are available contacting corresponding author.

Ethics approval – All dogs in this research were enrolled with their owner's signed consent, and after the study had been approved by the HUVTH's ethical committee.

Consent to participate – Not. Applicable. The data reported here have been retrieved from the scientific literature.

Consent for publication – Not. Applicable. The data reported here have been retrieved from the scientific literature.

Authors contributions – K.H collected serum and stored it in -80 degrees, later conducted the research itself and finalized the results including figures and tables. K.H was the main writer of the manuscript.
R.N helped with writing the manuscript text.
I.G. supervised over the research.
Y.A. helped with conducting the ELISA in the lab and helped with laboratory preparations.
M.H helped with overviewing the writing of the manuscript. Provided the lab for the research itself and supervised the research.
Y.B. was responsible for statistical analysis, helped with collecting the data (serum from dogs) and overviewed the writing of the manuscript.
All authors reviewed and approved the manuscript.

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