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BIOMARKERS OF SEPSIS AND MULTIORGAN DYSFUNCTION SYNDROME IN CRITICALLY ILL DOGS AND CATS

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Abstract

Background

Multiorgan dysfunction syndrome (MODS), *the progressive dysfunction of organ systems following an acute threat to systemic homeostasis*, is a common complication of systemic inflammatory response syndrome (SIRS) and sepsis in the intensive care unit (ICU). Bloodstream biomarkers for MODS prediction and prognostication have received growing attention in human medicine. Literature concerning MODS occurrence and significance is scant in dogs and absent in cats. Criteria for the systematic evaluation of organ dysfunction are lacking, and the use of diagnostic and prognostic biomarkers is limited in veterinary critical care medicine.

Aims

The aim of the proposed research is to investigate novel biomarkers for the prediction of illness severity, organ dysfunction and prognosis in critical dogs and cats hospitalized in the ICU of a veterinary university hospital (VUH).

Materials and Methods

Critically ill dogs and cats hospitalized in the ICU of the VUH of Bologna during the PhD (2014-2017) and diagnosed with SIRS and sepsis have been selected for the studies. The first part of the thesis has been focused on biomarkers evaluating the *host response*, with diagnostic and prognostic purposes. The following studies are presented:

- "Prognostic significance of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in canine SIRS: a prospective observational study"
- "Serum amyloid A in the diagnosis of feline sepsis"

- "Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs"
- "Canine procalcitonin in dogs with sepsis and gastric dilatation-volvulus"

The second part of the thesis has been addressed to specifically evaluate the relevance of *organ dysfunction* and MODS during critical illness, leading to the following investigations:

- "Acute kidney injury in critically ill dogs"
- "Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome"
- "Multiorgan dysfunction syndrome in feline sepsis"

Description of the studies

Prognostic significance of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in canine SIRS: a prospective observational study

The study investigated the prognostic relevance of the $APPLE_{fast}$ score, a validated index of disease severity in critically ill dogs, and other clinicopathologic markers of systemic inflammation in dogs with SIRS. Thirty-three dogs with SIRS were prospectively included and compared to 35 healthy controls; a further comparison between dogs with non-infectious SIRS and sepsis was performed. The study highlighted the usefulness of an extensive panel of traditional and novel blood and urinary biomarkers of canine SIRS. The utility of acute phase proteins to early identify SIRS was confirmed, while laboratory variables including blood lactate, base excess, serum albumin, serum creatinine, urinary protein to creatinine ratio and plasma antithrombin activity were moderately accurate for outcome prediction. Higher values of $APPLE_{fast}$ score were significantly related to increased odds for mortality, confirming the prognostic role of this score of illness severity in a specific clinical setting, like SIRS and sepsis.

Serum amyloid A in the diagnosis of feline sepsis

Cats show unique responses to systemic inflammation, making diagnosis of sepsis more challenging. The study aimed to evaluate the clinical value of serum amyloid A (SAA), the major feline acute phase protein, for sepsis diagnosis and prognostication in critically ill cats. A panel of hematological and chemical variables including SAA was retrospectively compared between 27 cats with trauma as a model of non-infectious SIRS, 29 cats with sepsis and 18 healthy controls. SAA concentrations were significantly higher in sick cats compared to controls. Septic cats had greater SAA concentrations compared to cats with trauma, but the best SAA cut-off detected (>81 mg/l) had only moderate performances to diagnose sepsis. Higher serum bilirubin concentration and toxic neutrophil changes at the blood smear evaluation were further documented in patients with sepsis. The results of the present study significantly enhance the limited literature on feline sepsis and support the role of a complete clinicopathologic evaluation and SAA measurement as valuable tools to facilitate sepsis diagnosis in cats.

Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs

Immature granulocytes and toxic neutrophil changes at the blood smear evaluation are markers of illness severity and infection in human and veterinary medicine. The delta neutrophil index (DNI) is automatically calculated by the ADVIA-series hematological analysers, and provides an estimate of circulating immature granulocytes in people. Despite many clinicians are still unfamiliar with this biomarker, there is a growing number of studies supporting its value for sepsis diagnosis and prognostication in humans. Our retrospective study evaluated the reference interval of the DNI in healthy dogs, and its diagnostic and prognostic utility in dogs with sepsis and immune-mediated hemolytic anemia. The preliminary results of the study support a possible role for the DNI as an aid for sepsis diagnosis and for prediction of sepsis severity: higher DNI values were detected in dogs with sepsis compared to dogs with immune-mediated hemolitic anemia and controls. Moreover,

dogs with septic shock had greater DNI values compared to dogs having sepsis without circulatory failure.

Procalcitonin in dogs with sepsis and gastric dilatation-volvulus

Procalcitonin (PCT), the most promising biomarker for sepsis diagnosis and prognostication in people, has received limited attention in dogs. Validation of a commercially available ELISA kit for quantification of canine PCT was performed. Then, plasma PCT value was tested in two clinical studies involving dogs with sepsis and gastric dilatation-volvulus (GDV). In the first investigation, baseline and serial plasma PCT were measured in 53 dogs with sepsis aiming to evaluate its association with sepsis severity, MODS occurrence and outcome. Baseline PCT concentration was related to sepsis severity, being greater in dogs with septic shock. Baseline PCT was also correlated with MODS occurrence, but not with outcome. Early declining PCT concentrations (during the first 48h of hospital stay) were significantly associated with survival in this population of septic dogs.

The second study evaluated the prognostic significance of plasma PCT, cell-free DNA and high-mobility group box 1 in a population of dogs with gastric dilatation-volvulus syndrome (GDV) undergoing surgery. Citrated plasma samples collected upon admission from 29 GDV dogs and 24 healthy controls were analysed. Presenting lactate concentrations, outcome and evidence of gastric necrosis were recorded. Dogs with GDV had significantly higher biomarkers concentrations compared to healthy controls. A potential prognostic role for plasma PCT and blood lactate concentrations emerged from the results of the study: increased PCT concentrations were detected in non-survivors, while increased lactate concentrations were measured in dogs with evidence of gastric necrosis. A moderate, positive correlation was documented between PCT and lactate concentrations.

Acute kidney injury in critically ill dogs

Acute kidney injury (AKI) is a common clinical condition of the critically ill patient. The aim of this prospective observational study was to evaluate the role of urinary chemistry and fractional excretion (FE) of electrolytes to characterize and prognosticate AKI in critically ill dogs. A total of 135 dogs with AKI were included and graded according the International Renal Interest Society guidelines. Dogs were grouped based on AKI features (volume-responsive vs. intrinsic) and outcome (survivors vs. non-survivors). Blood and urinary variables were measured at the time of AKI diagnosis. The results of the study confirmed the diagnostic and prognostic role of FE of electrolytes in canine AKI as indicators of the severity of renal impairment, as they were greater in dogs with intrinsic AKI and in non-survivors. FE can be considered feasible and cost-effective biomarker able to early differentiate between volume-responsive and intrinsic AKI and aid in outcome prediction.

Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome

Non-thyroidal illness (NTI) occurs in critical illness and seems associated with disease severity and outcome. The purpose of the study was to characterize NTI in dogs with SIRS and sepsis and identify its prognostic significance. Serum total T_3, free T_3, reverse T_3 and total T_4 were retrospectively measured at the time of admission in 10 dogs with pancreatitis (non-infectious SIRS), 31 dogs with sepsis (22 parvovirosis, 9 septic peritonitis) and 15 healthy controls. The $APPLE_{fast}$ score was calculated to assess illness severity. SIRS dogs had several thyroid hormones changes indicating NTI. Lower total T_3 and T_4 were documented in dogs with sepsis and were associated with the $APPLE_{fast}$ score, suggesting NTI occurrence as a marker of higher disease severity.

Multiorgan dysfunction syndrome in feline sepsis

The aim of this prospective observational study was to evaluate the clinical presentation and organ dysfunction in cats with sepsis. Fourty-three cats admitted at the ICU for highly suspected or confirmed sepsis were enrolled and grouped according to final outcome (survivors, non-survivors). Criteria to define selected organ dysfunction were adapted from the available canine literature; MODS was defined as presence of at least two dysfunctional organs simultaneously. Results of the current study significantly enhance the limited literature on feline sepsis, and propose criteria to detect organ dysfunction in this species. Presence of MODS was a common finding in the study population both at the time of admission and during ICU stay. Presence of renal dysfunction, cardiocirculatory dysfunction and MODS was significantly associated with increased odds for death.

Conclusion

The role of biomarkers is becoming crucial in critical care medicine, as they can assist in patient management and predict early and late complications of critical illness. The present thesis contributes to characterize SIRS and sepsis in dogs and cats, and gives novel insights on biomarkers of disease severity and organ dysfunction. A systematic screening for MODS has been proposed in the performed studies, highlighting the need to early recognize this condition at the time of ICU admission and during hospital stay. The prognostic impact of selected organ dysfunction and MODS development has been observed. The presented results improve our understanding of the host response to inflammation and infection, and are the basis for an on-going process to characterize MODS and its sequelae in critical care veterinary medicine.

Riassunto

Stato dell'arte

La Sindrome da Disfunzione Multiorganica (MODS), definita come *la progressiva disfunzione degli organi a seguito di un insulto acuto che compromette l'omeostasi corporea*, rappresenta una complicazione comune della sindrome della risposta infiammatoria sistemica (SIRS) e della sepsi nei reparti di terapia intensiva. L'utilizzo di biomarcatori circolanti che predicano lo sviluppo di MODS e abbiano un ruolo prognostico è sempre più frequente in medicina umana. La letteratura inerente alla MODS è estremamente ridotta in medicina veterinaria, ed è rappresentata da pochi studi soltanto nella specie canina. Negli animali d'affezione, infatti, non sono ad oggi stabiliti dei criteri standardizzati per la valutazione sistematica della disfunzione d'organo, e l'applicazione routinaria di biomarker diagnostici e prognostici risulta piuttosto limitata.

Obiettivi

La presente tesi si propone di valutare il valore clinico di biomarker innovativi nel paziente veterinario ricoverato in terapia intensiva, con l'obiettivo di predirre la gravità della patologia critica, lo sviluppo di disfunzione d'organo e la prognosi.

Materiali e metodi

Le popolazioni di studio considerate sono rappresentate da cani e gatti ospedalizzati in terapia intensiva presso l'Ospedale Veterinario Universitario dell'Università di Bologna nel periodo del dottorato di ricerca (2014-2017), e affetti da SIRS e sepsi. La prima parte della tesi è stata focalizzata su biomarker indicativi della *risposta dell'ospite*, e ha portato alla finalizzazione dei seguenti studi:

- "Prognostic significance of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in canine SIRS: a prospective observational study"

- "Serum amyloid A in the diagnosis of feline sepsis"
- "Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs"
- "Procalcitonin in dogs with sepsis and gastric dilatation-volvulus"

La seconda parte della tesi è stata incentrata sul significato prognostico della disfunzione d'organo e della MODS in corso di malattia critica, ed è stata sviluppata attraverso i seguenti lavori scientifici:

- "Acute kidney injury in critically ill dogs"
- "Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome"
- "Multiorgan dysfunction syndrome in feline sepsis"

Descrizione degli studi

Prognostic significance of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in canine SIRS: a prospective observational study

Lo studio ha valutato il significato prognostico dell'$APPLE_{fast}$ score, uno score di gravità clinica validato nel cane critico, e di un profilo esteso di variabili clinicopatologiche in una popolazione di cani in corso di SIRS. La popolazione di studio era rappresentata da 33 cani con SIRS confrontati con 35 soggetti sani; i cani affetti da SIRS sono stati ulteriormente suddivisi in due gruppi di comparazione in relazione all'origine della flogosi sistemica (SIRS non-infettiva vs. sepsi). Lo studio ha evidenziato il valore di numerosi biomarker sierici ed urinari nel corso di questa sindrome: è stato confermato il ruolo delle proteine di fase acuta come sensibili indicatori di SIRS nel cane, ed è emerso il possibile ruolo prognostico di variabili quali base excess, lattati, albumina sierica, creatinina sierica, rapporto proteine urinarie/creatinina urinaria e attività plasmatica dell'antitrombina. Infine è stata confermata la capacità predittiva di outcome dell' $APPLE_{fast}$ score in questa popolazione, in quanto all'aumentare di tale indice si riscontrava un aumento significativo del rischio di morte.

Serum amyloid A in the diagnosis of feline sepsis

Le manifestazioni cliniche della sepsi sono uniche nella specie felina, e tali per cui la diagnosi precoce di questa sindrome nel gatto è particolarmente complessa. L'obiettivo dello studio è stato quello di valutare il significato diagnostico e prognostico della siero amiloide A (SAA), la principale proteina di fase acuta maggiore nella specie felina, in una popolazione di gatti ospedalizzati in terapia intensiva. Si è proceduto con la comparazione retrospettiva di variabili ematologiche e chimiche, oltre che della SAA sierica, tra 27 gatti con trauma (modello di SIRS non-infettiva), 29 gatti con sepsi e 18 soggetti sani di controllo. Sono state documentate concentrazioni di SAA sieriche significativamente maggiori nei gatti critici rispetto ai soggetti sani, e significativamente maggiori nei gatti con trauma rispetto a quelli con sepsi. In particolare, una SAA >81mg/l ha presentato una capacità moderata per la diagnosi di sepsi all'interno della popolazione ogetto di studio. In aggiunta, i gatti con sepsi erano caratterizzati da concentrazioni più elevate di bilirubina sierica e più frequenti segni di tossicità neutrofilica evidenziate alla lettura dello striscio ematico. Tali risultati apportano utili informazioni alla limitata letteratura disponibile sulla sepsi felina, e supportano il ruolo di una completa valutazione laboratoristica e della misurazione della SAA sierica per facilitare la diagnosi di sepsi nel gatto.

Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs

La presenza di granulociti immaturi alla lettura manuale dello striscio ematico è un marker di gravità della malattia e di possibile sepsi sia in medicina umana che veterinaria. Il Delta Neutrophil Index (DNI) è un parametro calcolato in modo automatizzato dagli analizzatori ematologici della serie ADVIA, e rappresenta una stima dei granulociti immaturi circolanti nell'uomo. Nonostante il DNI sia un biomarker di recentissima introduzione in medicina umana e la sua misurazione non risulti ancora validata in modo sistematico, vi sono numerosi studi a supporto del suo significato diagnostico e prognostico in corso di sepsi. Lo scopo di questo studio retrospettivo è stato quello di

stabilire l'intervallo di riferimento del DNI nel cane sano, e di valutarne il ruolo diagnostico e prognostico in cani affetti da sepsi e da anemia emolitica immunomediata primaria. I risultati preliminari ottenuti supportano il ruolo del DNI come biomarker di sepsi nel cane: in particolare nella popolazione di studio, il DNI è risultato più elevato nei soggetti con sepsi (n=118) rispetto ai cani con anemia emolitica immunomediata (n=20) e ai soggetti sani (n=99). Inoltre, nell'ambito dei soggetti settici, il DNI è risultato più elevato nei cani in shock settico rispetto ai soggetti affetti da sepsi non complicata da disfunzione cardiocircolatoria, a supporto di una possibile correlazione con la gravità della sepsi.

Canine procalcitonin in dogs with sepsis and gastric dilatation-volvulus

La procalcitonina (PCT) rappresenta il biomarker più promettente per la diagnosi, il monitoraggio e la formulazione di una prognosi in corso di sepsi nell'uomo. L'attenzione che la PCT ha ricevuto nel cane è, tuttavia, limitata. All'interno di questo progetto di dottorato ho partecipato ad un primo studio di validazione di un kit ELISA disponibile in commercio per la misurazione della PCT plasmatica nel cane, e ho portato avanti due studi clinici preliminari volti alla quantificazione della PCT nel cane affetto da sepsi e sindrome da dilatazione-torsione gastrica (GDV). Nel primo lavoro clinico si è proceduto alla misurazione retrospettiva della PCT plasmatica all'ammissione in terapia intensiva e in modo seriale (a 24h e 48h di ospedalizzazione) in 53 cani con sepsi, al fine di valutarne l'associazione con la gravità della sepsi, lo sviluppo di MODS e l'outcome. La concentrazione plasmatica di PCT all'ammissione è stata associata alla gravità della sepsi (maggiore nei soggetti in shock settico) e allo sviluppo di MODS, ma non all'outcome. La valutazione seriale della PCT plasmatica ha mostrato un potenziale ruolo prognostico di tale biomarker, con concentrazioni decrescenti dall'ammissione alle 48h nei soggetti sopravvissuti, e differenze non significative nei tempi di monitoraggio nei cani non sopravvissuti.

Nel secondo studio è stato valutato il significato prognostico della PCT plasmatica, del cell-free DNA e dell' high-mobility group box 1 in una popolazione di cani con GDV. Le misurazioni sono

state effettuate su campioni di plasma citrato prelevati all'ammissione da 29 cani con GDV sottoposti a successiva chirurgia, e su campioni di 24 cani sani di controllo. Sono state registrate ai fini dello studio numerose variabili cliniche, di laboratorio (lattati ematici) e di outcome (sopravvivenza alla dimissione, presenza di necrosi gastrica evidenziata in chirurgia). Le concentrazioni plasmatiche dei biomarker oggetto di studio si presentavano tutte significativamente più elevate nei soggetti con GDV rispetto ai cani sani. È inoltre emerso un possibile significato prognostico di PCT e lattati ematici: le concentrazioni di PCT risultavano maggiori nei non-sopravvissuti, mentre quelle dei lattati più elevate nei cani con evidenza di necrosi gastrica. Si evidenziava inoltre una correlazione positiva, moderata, tra PCT plasmatica e lattatemia.

Acute kidney injury in critically ill dogs

Il danno renale acuto (AKI) rappresenta una complicazione frequente del paziente intensivo. È stato condotto uno studio prospettico osservazionale con lo scopo di valutare il ruolo della chimica urinaria in cani con AKI, per caratterizzare il danno renale acuto e individuare biomarker prognostici. Si è proceduto all'inclusione di 135 cani con AKI stadiati in accordo alle linee guida della International Renal Interest Society, e suddivisi in relazione al tipo di AKI (volume-responsivo vs. intrinseco) e all'outcome (sopravvissuti vs. non sopravvissuti). Numerose variabili chimiche ed urinarie sono state confrontate nei gruppi di studio, con particolare attenzione alle frazioni di escrezione degli elettroliti urinari. Queste, in particolare, si presentavano significativamente maggiori nei cani con AKI intrinseca, e più elevate nei soggetti non sopravvissuti. I risultati dello studio confermano le potenzialità delle frazioni di escrezione degli elettroliti urinari come indicatori di facile e rapida applicabilità clinica nella pratica veterinaria per caratterizzare la gravità del danno renale acuto e aiutare nella formulazione della prognosi nel corso di tale sindrome.

Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome

L'euthyroid-sick syndrome (o non-thyroidal illness, NTI) è una transitoria disfunzione tiroidea che si manifesta in soggetti precedentemente eutiroidei nel corso della malattia critica. Lo sviluppo di tale sindrome è associato alla gravità della patologia sottostante e alla prognosi. Lo studio effettuato ha avuto l'obiettivo di caratterizzare la NTI in cani con SIRS e sepsi, e di valutarne il significato prognostico. Sono stati inclusi in modo retrospettivo 10 cani con pancreatite (modello di SIRS non-infettiva), 22 cani con parvovirosi e 9 cani con peritonite settica (modelli di sepsi), confrontati con 15 soggetti sani per la misurazione delle concentrazioni sieriche di T_3 totale, T_3 libero, T_4 totale e reverse T_3 al momento della presentazione in ospedale. È stato calcolato l'$APPLE_{fast}$ score come indice di gravità clinica. I cani con SIRS presentavano numerose alterazioni nelle concentrazioni degli ormoni tiroidei suggestive di NTI. Si è riscontrato un calo più significativo di T_3 e T_4 nei cani con sepsi rispetto ai soggetti con SIRS non-infettiva, e una correlazione tra questi ormoni e l'$APPLE_{fast}$ score, a supporto della presenza di NTI come marker di gravità della malattia critica.

Multiorgan dysfunction syndrome in feline sepsis

È stato condotto uno studio prospettico osservazionale sulla disfunzione d'organo nel gatto con sepsi. Sono stati inclusi 43 gatti ammessi in terapia intensiva con un forte sospetto clinico e/o diagnosi confermata di sepsi, e successivamente suddivisi in relazione all'outcome (sopravvissuti, non sopravvissuti). I criteri per la definizione delle singole disfunzioni d'organo sono stati adattati dalla letteratura presente nel cane; la MODS è stata definita come la presenza concomitante di almeno due disfunzioni d'organo. I risultati dello studio contribuiscono in modo significativo, seppur preliminare, alle limitate informazioni disponibili nella letteratura scientifica sulla sepsi nella specie felina. Nello specifico, la presenza di MODS è stata diagnosticata frequentemente nella popolazione oggetto di studio, sia al momento della presentazione in clinica che durante l'ospedalizzazione in terapia intensiva. La presenza e lo sviluppo di disfunzione renale,

cardiocircolatoria e di MODS erano associate in modo significativo all'aumento del rischio di morte.

Conclusioni

L'utilizzo di biomarker è di crescente importanza nella gestione del paziente intensivo, con le molteplici finalità di assistere nella diagnosi e nel monitoraggio clinico, e favorire il riconoscimento precoce delle complicazioni della malattia critica, in particolare della disfunzione multiorganica. La tesi oggetto di questo dottorato di ricerca arricchisce la letteratura veterinaria in merito alla caratterizzazione della SIRS e della sepsi nel cane e nel gatto, e indaga l'utilità di biomarker innovativi di gravità della malattia critica e disfunzione d'organo. Gli studi presentati propongono un approccio nuovo nel panorama veterinario, incentrato sulla valutazione sistematica della MODS per il notevole impatto prognostico che questa presenta sul paziente sia al momento dell'ingresso in ospedale che durante il ricovero in terapia intensiva. I risultati presentati confermano il ruolo prognostico della MODS in diversi modelli di patologia critica spontanea nel cane e nel gatto, e migliorano le conoscenze mediche in merito alla risposta dell'ospite in corso di infiammazione sistemica e sepsi. Gli studi presentati in questa tesi, pertanto, costituiscono dei tasselli iniziali nel contesto di un filone di ricerca in costante e continuo sviluppo, volto ad anticipare le manifestazioni della disfunzione d'organo e a caratterizzarne le conseguenze, al fine di migliorare in modo complessivo la gestione del paziente critico veterinario.

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1. Introduction

Multiorgan dysfunction syndrome (MODS), *the progressive dysfunction of organ systems following an acute threat to systemic homeostasis*, is a common complication of critical illness, and represents a leading cause of mortality in human intensive care unit (ICU) regardless of the inciting injury which can be infectious (sepsis) or not (Mongardon et al. 2010). The higher the number of failed organs, the higher the mortality risk (Vincent et al. 2006).

Multi-organ dysfunction syndrome represents a state of cellular "metabolic shut-down": mythocondrial dysfunction, alteration in oxygen delivery and utilization, and cellular reprogramming are some of the features explaining the functional -rather than structural- organ failure. Hence, the degree of cellular necrosis documented during hystopathological studies is usually scarce or even absent, and organ recovery frequently occurs in surviving patients without pre-existing organ dysfunction. These findings might suggest the concept of MODS as an adaptive hypometabolic state to survive condition of extreme severe illness, rather than a major irrespective ICU-killer (Abraham and Singer 2007; Mondardon et al. 2010).

Recent years have seen advances in the understanding of the patophysiologic mechanisms underlying MODS in human sepsis: specifically, changes in the concentrations of metabolites, hormones and inflammatory mediators have been investigated as potential bloodstream biomarkers for MODS prediction and characterization, and as aids for individual prognostication (Abraham and Singer 2007; Visser et al. 2008).

Very little is known regarding MODS in veterinary medicine. The association between development of organ dysfunction and decreased survival has emerged in few canine studies (Simpson et al. 2009; Kenney et al. 2010; Ripanti et al. 2012; Ateca et al. 2014). However, standardized criteria to identify dysfunctional organs are lacking, and veterinary clinical practice is far behind the routinary use of biomarkers for MODS prediction and patients stratification.

2. Epidemiology

The history of MODS paralels the advances in medical knowledge and technology. Individual forms of organ dysfunctions were firstly reported during World War II and the Vietnam War, as soldiers surviving the initial battelfield injuries died later on from renal or respiratory failure (Cheadle et al. 2005). In the late 60s and 70s, deaths from multiorgan involvement following systemic diseases were reported with increasing frequency in people: a "lethal and unsolved problem" following initial injury was described, characterized by jaundice, respiratory distress and hypotension (Skillman et al. 1969; Tilney et al. 1973; Baue 1975). Analyses of retrospective clinical studies found that the main threat to survival was not the initial underlying illness, but rather a process of progressive physiologic failure of several interdependant organ systems. Sophystication of life-support technologies, as well as the application of these technologies to an increasingly high-risk patient population, made the occurrence of systemic organ dysfunction more and more common. A new class of ICU patients was created, representing the chronically critically ill with long-term sequelae. Hence, MODS is the complex disease of the latters (Seely and Christou 2000).

In 1991 the American College of Chest Physicians and the Society of Critical Care Medicine held a Consensus Conference to address significant clinical syndromes definitions. The syndrome of multiple organ dysfunction and failure that had been described over the previous 20 years was officially termed "MODS", and defined as the *progressive dysfunction of two or more organ systems following an acute threat to systemic homeostasis*. The term multiorgan failure (MOF) was deemed less appropriate because it implied a static dicothomus event (absence or presence of organ impairment), a more pessimistic outcome, and did not reflect the continuum of physiologic functional derangements observed in the critically ill patient (Bone et al. 1992). The syndrome was further divided into primary MODS, which represents the organ dysfunction resulting from the initial local insult itself (e.g. acute respiratory insufficiency as the immediate result of pneumonia), and secondary MODS, which is the presence of organ dysfunction in distant organ due to systemic

inflammation and the host response (e.g. acute kidney injury and coagulopathy secondary to pneumonia) (Bone et al. 1992; Seely et al. 2000; Johnson et al. 2004).

MODS usually manifests after a lag time (days to weeks) from the major initial insult (e.g. trauma, shock, sepsis, pancreatitis), progresses with the involvement of several organ systems and, ultimately, worsen prognosis. Current therapeutic approaches are based on vital organ systems support, untill they spontaneously recover, without speficic cellular or organ "cure". This generates significant costs from prolonged hospitalization, and poses ethical dilemma regarding withdrawal of therapies in potential survivors versus unnecessarily prolonged life-supports in fatally non-survivors (Mongardon and Singer 2009; Seely and Christou 2000). Sepsis is the main leading cause of MODS in people compared to other forms of critical illness. Mortality rates in people developing MODS is extremely high, ranging between 30-100%, regardless of the initial insult; the higher the numbers of failing organs, the greater the mortality (Seely et al. 2000; Vincent et al. 2006). Moreover, long-term quality of life in people surviving MODS could be scant, due to residual clinical symptoms like asthenia and depression. Interestingly, persistence of residual sequele and long-term symptoms has been related to MODS severity and lenght of ICU stay (Rodriguez-Villar et al 2017).

Recognition of MODS in veterinary medicine has paralleled the human experience, firstly mentioning multiple organ failure in the Veterinary Clinic of North America in 1989 (Hackett 2015). Since that, occurrence of MODS has been occasionally reported in veterinary critical care medicine, and attempts to its monitoring and therapeutics discussed. The attention on veterinary MODS is, however, only recent, and standardized criteria for its definition and extensive investigations are lacking (Hackett 2011; Ripanti et al. 2015; Kenney et al. 2010).

Reported incidence of MODS varies from 4% to 50% in dogs, with sepsis and trauma being the major inciting causes (Kenney et al. 2010; Osteburn et al. 2014; Ateca et al. 2014). MODS development in canine diseases has been associated with poor outcomes. In a large cohort of dogs with blunt trauma and retrospectively analyzed, presence of respiratory, cardiovascular and hemostatic dysfunctions were independently associated with a worse prognosis. Specifically, none

of the dogs developing MODS, defined as dysfunction of at least two organ systems, survived at discharge (Simpson et al. 2009). Similarly, in a retrospective case study including dogs with severe bite wound trauma, MODS occurrence greatly increased the odds ratio for death: dogs with dysfunction of 4 or more organs showed ad an overall mortality of 67% (Ateca et al. 2014). In a prospective study including dogs surgically treated for septic peritonitis, MODS incidence was 50%, and incidence of at least one dysfunctional organ was as high as 78%. Patients with MODS had a significantly lower survival rate (25%) compared to dogs without the syndrome (70%), and mortality increased with the number of dysfunctional organs. Multivariate analysis indicated that respiratory, cardiovascular, renal or coagulation system dysfunction significantly increased the odds of death, independent of other factors (Kenney et al. 2010). Similarly, higher mortality rates were reported in another study including dogs with septic peritonitis when MODS was identified (Craft & Powell 2012). Finally, in a recent canine study, the applicability of the SOFA (Sequential Organ Failure Assessment) score was evaluated in critically ill dogs with systemic inflammation and sepsis. Highest SOFA values were associated with reduced survival, thus supporting that both MODS presence and severity affect outcome (Ripanti et al. 2012). No veterinary report describing MODS in cats is currently available.

3. MODS: a complex non-linear system of adaptation to critical illness?

MODS is a complex clinical syndrome, recognizing a complex pathophysiology. Several theories have been proposed to explain its occurrence in the ICU. It was initially believed that MODS originated from an overwhelming and uncontrolled infection able to precipitate organ failure. However, MODS is frequently encountered in non-infectious inflammatory diseases, where a septic focus is not clinically identifiable (Mongardon and Singer 2009; Seely and Christou 2000). Then, in 1991 the American College of Chest Physicians estabilished the criteria defining the systemic inflammatory response syndrome (SIRS), and defined MODS as the presence of altered organ function in an acutely ill patient such that homeostasis cannot be mantained without intervention". As such, MODS has been indicated as a major SIRS-related complication, arising from the imbalance between the pro- and the anti-inflammatory forces (Bone et al. 1992; Osteburn et al. 2014; Sapan et al. 2016). However, clinical trials attempting to target inflammation in MODS (e.g. attenuating the exaggerated pro-inflammatory host response through cytokines inhibition) have been disappointing overall, as they failed to demonstrate a real benefit, or even showed deleterious effects (Cui et al. 2017).

Modern concepts in physiology and pathology of critical illness propose to consider MODS as a complex, non-linear system, representing the host response to a chronic, potentially lethal, overwhelming systemic injury. In this regard MODS could potentially be an adaptive and protective mechanism, aiming to increase the chances of survival of cells and organs (Mongardon and Singer 2009; Seely and Christou 2000).

A complex non-linear system is a system with interaction between variables are constantly altered and can change as a result of changes in other variables (there is marked connectivity and interdependence between variables). Thus, interconnections and relationships between variables are far more important to be understood rather than the single variables themselves (Seely and Christou 2000). In such systems, homeostasis arises not from the constancy of conditions, but from the complexity of interactions among its dynamic agents. This principle is of utmost importance in

critical illness: complexity belongs to healthy states, while loss of complexity (e.g. loss of specific organ function during MODS) challenges and jeopardizes homeostasis. Recovery from critical illness is, infact, characterized by restoration of complexity and variability in physical parameters (Papathanassoglou et al. 2008). Several biological systems, including animal physiological systems (e.g. immune system, coagulation cascade) as well as their responses to insults (e.g. shock, trauma, infection) are complex non-linear systems by nature, and can be seen as "chaotic systems regulated by emergent orders". Understanding MODS as a complex non-linear syndrome involving endocrine and metabolic processes, explains the lack of success or the unpredictable results in trials investigating specific antimediators therapy, inhibiting or enhancing specific pathways of the inflammatory cascade, or supplementing deficient hormones or metabolites in sepsis (e.g. thyroidal hormones, ionized calcium) (Seely and Christou 2000; Papathanassoglou et al. 2008).

As stated before, MODS carries a negative connotation: it is the most severe threat to body homeostasis, and the major complication of critical illness. On the other hand, MODS represents a transient state: when survival occurs, complete recovery of organ functions is fairly common, especially in the absence of pre-existing organ disease. This is a remarkable event, considering that costituents of organs like liver or kidneys have poor regenerative capacities (Singer et al. 2004). Even in patients who die as a consequence of MODS, hystopathological evidence of cell death or damage in the affected organs is minimal (Mongardon and Singer 2009). Hence, organ failure in the context of MODS is *functional*, rather than structural. As an example, sepsis-associated acute kidney injury (AKI) is usually characterized by preserved or increased renal blood flow, reversible decrease in glomerular filtration rate (GFR) and tubular dysfunction. Acute tubular necrosis is marginal in hystopathological studies, corroborating the idea of MODS leading to cell dysfunction over cell death (Dellepiane et al. 2016). Signs of programmed cell suicide (e.g. apoptosis, necroptosis) can be prevalent over necrosis in several parenchimal tissues (Hattori et al. 2017). Finally, tissue content of oxygen is often normal in septic patients dying of MODS in face of severe cellular hypoxia and reduced oxygen consumption (Kreymann et al. 1993). These findings might

support the idea of MODS as an *energy-saving state*, a condition of metabolic shutdown orchestrated by organisms coping with overwhelming and potentially lethal injuries, aiming to survive. In this novel scenario, MODS could be viewed as an attempt to pursue cell survival during prolonged critical illness towards complex endocrine and metabolic changes, with affected cells experiencing a dormant state analogous to hybernation (Singer et al. 2004; Mongardon and Singer 2009). Mitochondrial dysfunction, indeed, appears to be a major feature of critical illness-related MODS, being the hallmark of reduced cellular metabolism (Singer et al. 2004).

The theory of MODS as an adaptive/protective state of critical illness might also give new insights into its therapeutical approach. People with MODS usually die as a result of iatrogenic interventions rather than their primary disease. Numerous medical procedures are invasive and have risks, and in some cases the harms outweight the benefits. Similarly, excess of supportive cares (e.g. liberal transfusion strategies, aggressive fluid resuscitation, aggressive ventilatory settings, hormone supplementation) have been associated with adverse outcomes in the late phase of diseases. In this regard, major advances in patient prognosis have been reached through a reduction of iatrogenic harm (e.g. conservative fluidtherapy, protective ventilatory strategies) and through the paradigm of "less is more" accepted in critical care (Vincent & Creteur 2015). A better understand of MODS pathophysiology and MODS-related biomarkers might enable us to anticipate complications, ameliorate patient management and avoid counter-adaptive treatments, keeping in mind that reversibility and organ function recovery are possible.

4. Pathophysiology and mechanisms of MODS

MODS is a disease of the cells involving multiple organs at the same time (Papathanassoglou et al. 2008). The pathophysiology of MODS is complex and not entirely understood. According to the historical overview of MODS, three theories have been proposed to explain its development. The "one-hit theory" considers MODS as the early sequela of a massive, systemic insults (massive insult ➔ severe SIRS ➔ early MODS). The alternative scenario, the "two-hit model", views MODS as the consequence of multiple sequential insults: following a severe inflammatory insult, patients enter a less intense SIRS state but remain vulnerable to secondary inflammatory insults (e.g. surgery, or secondary infection) that can amplify SIRS and precipitate late MODS (moderate insult ➔ moderate SIRS ➔ second insult ➔ late MODS). Finally, the "sustained-hit model" postulates that a continuous insult (e.g. drug-resistant infection) can both cause and sustain MODS (Moore & Moore 1995; Hackett 2011).

Regardless of the models behind MODS occurrence, a plethora of mechanisms have been proposed, involving several mediators (cytokines, reactive oxygen species, nitric oxide), different processes (e.g. cellular hypoxia, bacterial translocation, immune dsysregulation, mithocondrial dysfunction) and selected organ dysfunctions (e.g. endothelial dysfunction, coagulation abnormalities) promoting systemic decompensation through organ cross-talk (Johnson et al. 2004; Abraham et al. 2007; Osteburn et al. 2014). In the following chapters the major proposed mechanisms behind MODS in the critically ill will be discussed, with a focus on the causes of specific organ dysfunctions and the pertinent literature available in veterinary medicine.

4.1 MODS effectors

Cytokines

Cytokines synthesis and release characterize the host's innate immune response to a systemic insult, which could be infectious or non-infectious in nature. The rate and the type of cytokines release depend on the severity of the underlying injury (Visser et al. 2008). Tumor necrosis factor alpha

(TNFα), interleukin (IL) 1, IL6, IL10, IL8, high-mobility group box 1 (HMGB1) are some of the cytokines occupying important role in the pathogenesis of MODS during systemic illnesses. Cytokines patterns have been described in several human and veterinary studies during different inflammatory conditions, aiming to understanding the temporal phases of the disease and finding novel pharmacological strategies to modulate inflammation (Visser et al. 2008; DeClue et al. 2009; DeClue et al. 2012). The link between circulating cytokines levels and MODS development has arised from the results of several human studies. In people with trauma, increasing concentrations of selected cytokines (e.g. TNFα, IL6, IL10) are significantly associated with late organ failure and death (Maier et al. 2007). Similarly, in human patients with postoperative sepsis, high TNFα producers tend to develop MODS to a greater extent compared to low TNFα producers (Stuber et al. 1996). Enhanced IL6 and IL8 concentrations have been correlated with the severity of injury and the incidence of organ failure and sepsis in human patients with severe trauma (Visser et al. 2008; Maier et al. 2007). The study of cytokine patterns have received attention in veterinary critically ill patients, too. Increased concentrations of various cytokines (e.g. IL6, monocyte chemoattractant protein-1, IL7, IL15) are well-demonstrated in canine and feline inflammatory diseases (Duffy et al 2010; Schuttler & Neumann 2015; Karlsson et al. 2012; DeClue et al. 2012; DeClue et al. 2009), although their prognostic significance remains unclear (DeClue et al. 2012; Schuttler & Neumann 2015). A biphasic pattern of cytokines release has been proposed in early human and experimental studies, wherein a first hyper-inflammatory phase develops acutely after injury and is characterized by the rise of circulating pro-inflammatory cytokines (e.g. TNF, IL6, IL8), and a subsequent late hypo-inflammatory period is reported after the 24h postinjury. The latter is mainly mediated by other anti-inflammatory cytokines (e.g. IL10), and follows to down-regulate the inflammatory host response to injury. However, this state is usually associated with immunoparalysis and immune dysfunction (Visser et al. 2008; Maier et al. 2007). The imbalance between proinflammatory cytokines and their anti-inflammatory counterpart has been considered as a mechanism for MODS development in human and veterinary critically ill patients (Maier et al 2007; Hackett 2011).

Cytokines play significant roles as systemic inflammatory mediators. As a result, indiscriminate injury concurs to MODS development through a variety of mechanisms, including induction of cellular apoptosis, influence on leukocyte function, enhance of endothelial permeability and activation of coagulation cascade (Johnson & Mayer 2001). Therapeutic approaches aiming to inhibit or modulate the actions of specific inflammatory cytokines have been attempted in experimental trials, and potential beneficial effects have been demonstrated in experimental model of diseases. However, when the same pharmacological strategies were applied to clinical trials, the outcome on clinically meaningful variables remained unclear or even detrimental (Abraham et al. 2007). Pharmacological interventions targeting specific cytokines are likely to be unsuccessful if carried out in spontaneous illness, without the knowledge of the host response phase (hyper vs. hypo-inflammatory response) and the multitude of the modulating effects in which cytokines themselves are involved. However, novel treatments based on continuous cytokines hemoadsorption appear to be beneficial in animal models of sepsis (Kim et al. 2015). Similarly, the use of cytokine adsorbing columns seems promising in preliminary studies in critically ill human patiens, as they contribute to reduce duration of hypotension and vasopressor supports in people with septic shock, preventing additional organ failures (Houschyar et al. 2017).

Nitric oxide and Reactive oxygen species

Nitric oxide synthase (NOS) is enhanced during systemic conditions like SIRS and sepsis, leading to an increase in circulating nitric oxide (NO) concentrations. Circulating NO mainly affects smooth muscles relaxation, thus being major effectors for SIRS- and sepsis-induced hypotension and cardiocirculatory dysfunction (Abraham et al. 2007). Derangement of calcium and NO production are the main mechanisms underlying myocardial dysfunction, as NO is a major circulating myocardial depressant factor. Moreover, NO promotes pro-inflammatory pathways and modulates cytokines release. In addition, NO promotes the synthesis of hypoxia-inducible factors (HIF) and favors the pathways of hypoxia-induced inflammation and organ failure even under non-hypoxic

conditions (Hirota 2015). For instance, NO expressed within the brain favors apoptosis in neurons and microglial cells, while modulates activity of Na/K-adenosine triphosphatase (ATP) membrane pumps in liver cells (Abraham et al. 2007). Finally, NO and its congeners act as potent mitochondrial inhibitors, promoting cytopathic hypoxia and consequent variations in cells bioenergetic status during critical illness (Brealey et al. 2002).

Reactive oxygen species (ROS) are unstable highly reactive molecules characterized by one or more unpaired electrons in their outer orbitals. They are toxic by-product deriving from hypoperfused tissues once blood flow and oxygen supply are restored, hence being considered markers of ischemia-reperfusion injury. However ROS production is not limited to ischemia-reperfusion injury, but can follow non-infectious SIRS (e.g. trauma, pancreatitis) and sepsis (Teng et al. 2017; Gaykwad et al. 2017). Because their high electrical activity, ROS can promote a severe degree of tissue injury, which can be even worse than hypoperfusion alone, through a variety of ways ultimately leading to oxidative stress and cell damage/death. For instance, ROS enhance systemic inflammation acting as secondary mediators in the inflammatory cascade. Additionally they exert direct cytotoxic effect, are involved in the production of reactive nitrogen and ferric oxidant species, and have been described as significant effectors for the development of specific organ dysfunction (e.g. lung dysfunction, endothelial dysfunction) (Johnson & Mayer 2001; Abraham et al. 2007). Uncontrolled ROS production is a common finding in human patients dying from sepsis and trauma, as ROS release leads to cell death, shock and organ failure (Leliefeld et al. 2016; Teng et al. 2017). The subsequent depletion in anti-oxidant molecules results in oxidant/antioxidant imbalance, further worsening the damage inflicted by ROS. In this regard, plasma concentrations of ROS-associated biomarkers have been associated with survival in sepsis, and anti-oxidant therapies are potentially advocated in critically ills at risk of developing MODS (Bime et al. 2016; Johnson & Mayer 2001; Gaykwad et al. 2017). Even if current bundle of care do not address the need of specfic antioxidan therapy, the rational of giving anti-oxidant and ROS scavenging treatments to critically ills does exist. Recent double-blinded clinical trials in people

showed that ascorbic acid (vitamin C) administration was associated with a reduction in organ dysfunction scores, inflammatory biomarkers and vasopressor requirements (Teng et al. 2017). A recent preliminary study showed potential beneficial results linked to the administration of N-acetyl cystein, the precursor of gluthatione and one of the major anti-oxidant molecule of the body, in puppies with parvoviral enteritis (Gaykwad et al. 2017).

Leukocytes

A great body of evidence supports the pivotal role of specific leukocytes patterns and their dysfunctions into MODS developing (Cabrera et al. 2017; Liliefeld et al. 2016). Neutrophils are considered the first-line effectors of innate immunity against both microbial and non-microbial insults. In response to tissue injury, functional neutrophils are capable of chemotaxis, phagocytosis and ROS production, and degranulation of antimicrobials substances into the bloodstream (Leliefeld et al. 2016). Uncontrolled neutrophils activation is, however, responsible of indiscriminate tissue injury and organ failure. Primed neutrophils contribute to ischemia-reperfusion injury through excessive ROS elaboration and release, and marginate to end organs causing direct cytotoxic effects. Once adherent to the endothelium, primed neutrophils release massive amounts of granules containing elastase and proteases that further promote endothelial injury and cellular disruption (Moore & Moore 1995; Dewar et al. 2009; Leliefeld et al. 2016). The process of extracellular killing through formation of neutrophil-extracellular traps (NETs) has received much interest in recent years, and has been implicated in MODS pathogenesis (Leliefeld et al. 2016; Nakazawa et al. 2017). NETs are constituted of DNA fibrils, chromatin and proteins derived from a peculiar mechanism of neutrophils death called NETosis. NETosis occurs in response to a variety of inflammatory stimuli, and NETs possess important antimicrobial functions that help in infections control. However, their excessive and/or inappropriate production has been associated with local and distant organ damage. NETs have cytotoxic components able to induce ongoing inflammation, local cellular necrosis and apoptosis. In turn, necrotic cells further promote neutrophils to undergo

NETosis. Finally, injured organs release NETs and their cytotoxic components into the bloodstream, promoting also systemic inflammation and distant organ injury (Nakazawa et al. 2017).

Other than causing overwhelming and uncontrolled inflammation leading to local and remote tissue damage, leukocytes can also express dysfunctional phenotypes. Disruption of immunity can affect neutrophils in critically ills by means of a variety of mechanisms (e.g. inefficient chemotaxis; downregulation of signalling pathways necessary for pathogen recognition; defective phagocytosis; suppression of the physiological adaptive immunity), and incompetent neutrophils contribute to immunoparalysis and its sequele (e.g. nosocomial infections) (Leliefeld et al. 2016; Hotchkiss et al. 2013). Local conditions like oxygen-tension and tissue levels of antioxidants affect the cellular immune response, thus reducing the proliferation and the respiratory burst of peripheral blood lymphocytes and monocytes, respectively. Experimental data in septic mice reveal specific gene expression profiles of immune cells, that can either be organ specific or common to more than one organ, suggesting the phenomenom of leukocyte reprogramming as the inciting event toward anergy and immunoparesis (Abraham & Singer 2007).

Persistent severe inflammation might also lead to bone marrow exhaustion, with the subsequent marked release of immature neutrophils into the circulation. Few experimental and human studies demonstrated that immature neutrophils have impaired microbicidal functions. Other than reduced phagocytic capacity, immature neutrophils possess abnormal rheological properties, hence they can accumulate in specific microvasculature sites promoting blood sludging, microcirculatory impairment and local organ dysfunction (Leliefeld et al. 2016; Poschl et al. 2005). The number of immature granulocytes has been associated with disease severity and worse outcomes in people with sepsis (Mare et al. 2015). Although no veterinary study has addressed the role of leukocyte patterns and functions to MODS occurrence, there is preliminary evidence suggesting that leukocyte phenotypes influence illness progression and final outcome. Presence of a degenerative left shift (supranormal number of immature granulocytes exceeding the number of mature

neutrophils) has been associated with worse outcomes in dogs and cats regardless of the underlying disease process (Burton et al. 2013; Burton et al. 2014), and functional defects in leukocytes have been demonstrated in sick dogs (Klenner et al. 2010; LeBlanc et al. 2013).

4.2 Effects: major drivers for MODS

There is evidence that a combination of different processes originates MODS in critical illness. Cellular hypoxia, bacterial translocation, immune dysregulation and mitochondrial dysfunctions are recognized as the main pathophysiological mechanisms underlying organ-specific dysfunction. Emerging evidence, however, suggests that immune system dysregulation and mitochondrial dysfunctions have major roles in MODS development (Osteburn et al. 2014).

Cellular hypoxia

Multiple organ injury and shock usually coexist in experimental and clinical models of MODS, pointing out the role of sustained cellular hypoxia as a driver force promoting organ failure (Moore & Moore 1995). The balance between oxygen distribution (DO_2) and oxygen consumption (VO_2) characterizes cells in the healthy. Systemic inflammation and sepsis are characterized by the uncoupling between DO_2 and VO_2 promoting tissue hypoxia and organ dysfunction (Moore and Moore 1995).

There is a large body of evidence postulating hypoxia as both the consequence and the cause of systemic inflammation and organ failure. Hypoxia in the context of SIRS and sepsis recognizes several causes, including microvascular dysfunction, decreased blood and oxygen supply, impaired mithocondrial function and failure for tissues to extract and use oxygen properly (Hirota 2015) (Figure 1). However, tissue hypoxia during inflammation is not a simple bystander process, but it is actively involved in enhancing or attenuating inflammation itself and favoring organ dysfunction. Cells adapt to sustained tissue hypoxia by the transcription of hypoxia-inducible factors (HIF). In

addition, and as stated before, HIF induction can be promoted also under non-hypoxic conditions through several inflammatory mediators including NO, ROS and cytokines (Hirota 2015).

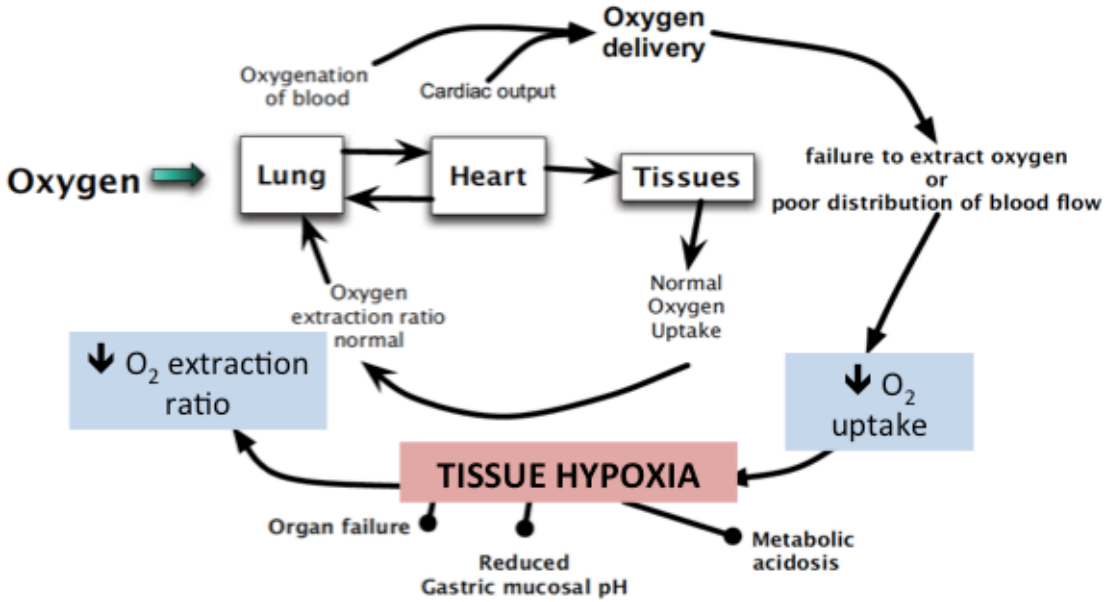

Figure 1. In SIRS and sepsis regulation of oxygen delivery is abnormal. Blood supply and oxygen distribution can be altered, and tissues and organs fail to use oxygen. Responses to hypoxia induce dysregulation in organ function (modified from Hirota 2015).

HIF are key regulator of hypoxia-induced inflammation: they modulate leukocyte patterns and function, and crosstalk with tissue factors (e.g. NF-kB) to modulate inflammation and oxygen homeostasis at tissue levels (Hirota 2015).

Hypoxia and its mediators mainly contribute to MODS development through endothelial cells activation: because endothelial cells line the vascular lumen, they are the first to experience the effects of blood composition alterations, including reduction in oxygen content and blood supply. However, compared to the cells constituting other body systems, the endothelial ones are particularly capable of adaptation during hypoxic states. Indeed, the endothelial physiologic response to hypoxia is called *endothelial activation*, and is characterized by endothelial constrinction (thus systemic vasoconstrinction), development of procoagulant tendencies and further enhance in the inflammatory pathway by means of cytokines release and leukocytes activation. Although protective at the cellular level, systemic endothelial activation triggered by

hypoxia is dramatically deleterious, and plays a significant role in organ failure and death, and hence be maladaptive (Ten and Pinsky 2002).

Bacterial translocation

Initially MODS was thought to be the result of uncontrolled systemic infection (Mongardon and Singer 2009; Seely and Christou 2000). However, infection as the unique etiology for MODS was not in accordance with the various non-infectious causes of organ failure (e.g. trauma, pancreatitis, burns) and the lack of identifiable microrganisms in the affected patients (Mongardon and Singer 2009; Seely and Christou 2000; Johnson & Mayer 2001). The unifying theory of uncontrolled infection recognized the gut as a potential source of circulating infectious agents and/or bacterial products. The gut is normally colonized by an overwhelming number of aerobic and anaerobic bacteria, and bacterial translocation occurs in a variety of physiological and pathological conditions. The liver, which receives a third of its blood supply from the portal circulation, is mainly involved in the processes of microbes and toxins clearance. This explains why bacterial translocation is well-described in the healthy, and usually has limited prognostic implication in conditions of mild/short-term disease (Sertaridou et al. 2015; Unterer et al. 2015; Dahilinger et al. 1997). As examples, bacterial translocation has been described in canine diseases such as acute hemorragic diharrea syndrome and gastric dilatation/volvulus, but no association with illness severity and outcome was documented (Unterer et al. 2015; Winkler et al. 2003). However, in the presence of dysregulated immune function and/or immunoparalysis, liver hypo-function and altered gut environment, bacterial translocation could be the inciting insult for MODS (Sertaridou et al. 2015; Klingensmith & Coopersmith 2016). Several factors sustain the hypothesis of the gut as a driver for MODS: decreased gut perfusion, damage and disruption of the intestinal barrier, gut cells apoptosis, abnormal bowel movements and gut hypomotility, gut microbiome alterations, might favor translocation of bacteria and their toxic products into the systemic circulation, promoting a "second hit" that augments the initial injury (Johnson & Mayer 2001; Sertaridou et al. 2015; Klingensmith &

Coopersmith 2016). The lack of a repeatable isolation of bacteria into the mesenteric lymphnodes (thus of a consistently demonstrable bacterial translocation) (Sertaridou et al. 2015; Moore et al. 1991) arose the theory of "gut-derived sepsis": microbial and *non-microbial* bacterial products are able to translocate from the intestinal tract, reach the lymphatic flow and be motors of sustained SIRS and distant organ damage (Sertaridou et al. 2015; Klingensmith & Coopersmith 2016). Several investigations support the toxic role of these non-bacterial proinflammatory mediators (mainly proteins and lipid factors) in promoting sepsis and MODS during critical illness: in experimental models of critical disease, ligation of the mesenteric lymphatic duct abrogates lung injury and prevents mortality. Interesting novel lines of therapies promoting the restoration of normal gut environment have been proposed in the ICU. Use of prebiotics and probiotics, early enteral nutrition, fecal transplant and selective gut decontamination through the use of short-course antimicrobial treatments showed promising results in large human trials and metanalyses, even if they have not yet modified the standard of care (Sertaridou et al. 2015; Klingensmith & Coopersmith 2016).

Endothelial activation

Impairment in endothelial functions occurs during systemic inflammation and sepsis, and is thought to be a key factor in MODS development. The endothelial cell lining (ECL) constitutes the interface between blood and parenchymal cells, and is essential for the regulation of coagulation, vasomotor tone, osmotic balance, solute transport, immunological functions and trans- and intra-cellular signaling. Thus, failure of the ECL is critical for the progression to MODS (Ince et al. 2016). The endothelial cytoskeleton and the glycocalyx, a gel-like layer lining the luminal membrane of the ECL, mediate the vascular barrier function, leukocyte adhesion, hemostasis control and transmission of shear stress. Cytokines, ROS and different inflammatory mediators instigate glycocalix shedding and disruption, and contribute to failure in endothelial functions. For instance, loss of vascular barrier favors tissue edema, which reduces microvascular perfusion and damages

the glycocalyx layer itself. Additionally, endothelial activation results in a procoagulant state leading to disseminated microvascular thrombosis and local organ ischemia. Glycocalyx shedding exposes adhesion molecules for platelets and leukocytes, which can transmigrate into the parenchyma and participate in loss of tissue function. Finally, endothelial disruption causes loss of miogenic responses at the vascular level, promoting vasoplegia and hypotension (Abraham & Singer 2007; Mikacenic et al. 2015; Ince et al. 2017). Collectively, these changes likely evolved as an adaptive host response to sterile insults and/or extravascular pathogens, allowing for increased blood supply and leukocyte and protein efflux to the affected area. This state may be considered dysfunctional, when an overactive endothelium impairs homeostasis instead of restoring it (Shapiro et al. 2010).

Circulating biomarkers of endothelial activation are currently available for prognostic purpose in human patients with systemic inflammation. In a recent study involving a large cohort of people with SIRS of both infectious and non-infectious causes, markers of inappropriate endothelial activation/dysregulation were independently linked to ongoing inflammation, development of shock and MODS, pointing to their contribution to SIRS-related organ dysfunction and death (Mikacenic et al. 2015).

An activated endothelial phenotype has been demonstrated in animal models of sepsis, as well as in spontaneous diseases, further supporting the role of endothelial dysregulation in SIRS and sepsis patophysiology (Shapiro et al. 2009; Shapiro et al. 2010; Kules et al. 2017). An increase in Von Willebrand factor antigen concentration, a possible marker of endothelial activation, was documented in dogs with sepsis (Rogers & Rozanski 2010). Similarly, circulating markers of endothelial activation were reported in systemic canine diseases like babesiosis and gastric dilation volvulus, and were associated with disease severity and survival (Uhrikova et al. 2015; Kules et al. 2017).

Mitochondrial dysfunction

Mitochondrial respiration through oxidative phosphorylation represents the main cellular process for energy production in form of adenosine triphosphate (ATP) molecules. ATP availability, in turn, is the major rate-limiting step of cellular metabolism (Brealey et al. 2002; Singer et al. 2004). While oxidative phosphorylation is increased in the acute phases of critical illness, it is reported to fall dramatically in presence of ongoing inflammation (>12-16 hours) (Singer et al. 2004). The fall in mitochondrial respiration usually occurs in the presence of normal arterial oxygen content and oxygen delivery, stating the inability for the cells to use oxygen properly (inappropriate VO_2 despite normal DO_2) (Papathanassoglou et al. 2008). These changes in mitochondrial function are prominent for MODS development, and happen under specific circumstances due to the effects of inflammatory, endocrine and metabolic abnormalities (Singer et al. 2004). Various experimental animal studies demonstrate mitochondrial alterations in response to several inflammatory mediators, including NO, ROS and cytokines. In other studies severe hypoxia, rather than inflammation, acts as the main driver for mitochondrial dysfunction through microcirculatory impairment and shock (Brealey et al. 2002; Singer et al. 2004; Kozlov et al. 2017). The influence of hormones on mitochondrial function has recently been recognized: low thyroid hormones reduce ATP synthesis and slow cellular metabolism, thus the non-thyroidal illness syndrome of the critically ill might be associated with a decreased, albeit more efficient, mitochondrial respiration. Similarly, hypercortisolemia associated with chronic or intermittent illness is reported to cause mitochondrial dysfunction (Singer et al. 2004).

The process of mitochondrial shut-down is triggered by induction of ultra-structural mitochondrial changes such as swelling, hyperthrophy and inhibition of mitochondria respiratory enzymes (Singer et al. 2004; Kozlov et al. 2017). Moreover, designated pathways of mitochondrial suicide are activated, leading to the opening of holes into the inner mitochondrial membrane and the subsequent release of mitochondrial DNA (mDNA) (Papathanassoglou et al. 2008). Even in those studies where electron microscopic examination of mitochondria is normal, signs of cellular

damage (e.g. intracellular vacuoles) indirectly linked to mitochondrial dysfunction are present. However, species-specific differences are reported, wherein cats and pigs seem to be particularly predisposed to mitochondrial dysfunction after endotoxin inoculation, while rodents are reported to be more resistant (Kozlov et al. 2017).

The issue of mitochondrial dysfunction as a causative mechanism for MODS or a simple epiphenomenal process is still unsolved. The fall in oxidative phosphorylation reduces ATP availability and profoundly modifies the bioenergetic status of the cells. In this regard, experimental and clinical studies in both humans and animals consistently show a reduction in tissue ATP supply that is associated with organ failures and worse outcomes (Brealey et al. 2002; Kozlov et al. 2017). Mitochondria are also a significant source of ROS, and mitochondrial ROS have been implicated in the genesis of acute liver failure during critical illness (Kozlov et al. 2017). To further support these statements, desensitization of the mitochondrial permeability pore prevents cellular apoptosis and liver damage induced by TNFα administration (Soriano et al. 2004; Kozlov et al. 2017).

Interestingly, mitochondrial dysfunction is a reversible process. Hence the hypothesis that cellular metabolic shut-down and changes in ATP turnover might be seen as attempts by the body to cope with severe critical illness and ensure cell survival (Singer et al. 2004).

4.3 Organ cross-talk in MODS developement

One of the first uses of the term MODS in human medicine came in the late 70s to describe the progressive failure of several organ systems following an acute overwhelming systemic insult. At that time it was already emphasized that *failure of one organ system could cause dysfunction of others* (Baue 1975). The intricate cross-talk provided by temporal changes in inflammatory mediators, hormones, metabolities and oxygen delivery and utilization is behind the interaction between different organ systems (Abraham et al. 2007).

The brain plays an essential role in MODS development through the field of organ cross-talk, as direct or differed damage of the central nervous system has detrimental effects on remote organ functions. In this regard, significant brain injury in people is related with development of respiratory failure, acute kidney injury, hemodynamic instability and sepsis (Junior & da Silva 2014). Brain injury-triggered sympathetic activation and release in neurokinins and neuropeptides are thought to mediate brain-induced MODS by favoring systemic vasoconstrinction and hypertension, endothelial damage and leukocyte adhesion (Quìlez et al., 2012) (Figure 2). On the other hand, distant organ damage could enhance neurologic complications (e.g. uremic and hepatic encephalopathy in the context of AKI and hepatic failure, respectively) and worsen the prognosis of primary neurologic injuries (Doi & Rabb 2016, Junior & da Silva 2014)

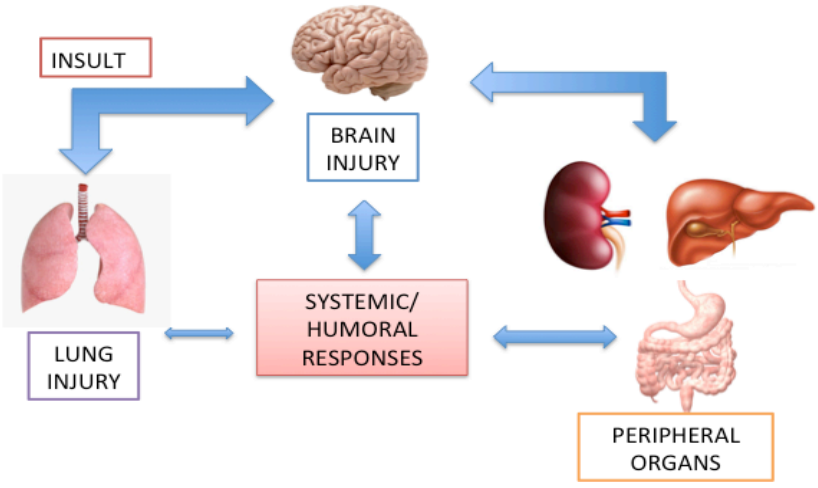

Figure 2. Schematic rappresentation of organ crosstalk during acute lung injury and mechanical ventilation (modified from Quìlez et al. 2012).

Multiorgan failure could also be analyzed from the "heart perspective", since cardiovascular function is the major driver of cardiac output (CO) and hence DO_2 at tissue level. The occurrence of both systolic and diastolic cardiac dysfunction increases mortality in several critical care human and veterinary settings (Donati et al. 2016; Hamacher et al. 2015; Kokaturk et al. 2012), and evidence of myocardial damage has been associated with development of new organ failure and worse outcomes in ICU (Wu et al. 2004; Hamacher et al. 2015; Langhorn et al. 2013). Cardiac dysfunction promotes distant organ failure through different mechanisms resulting in hypoperfusion and inadequate oxygen supply. The cardiocirculatory and the pulmonary systems are tightly interconnected: in this regard, left cardiac dysfunction favors congestive pulmonary edema, while presence of pulmonary hypertension in the context of respiratory dysfunction and ARDS reduces cardiac performances and causes acute *cor pulmonare* and right cardiac failure (Donati et al. 2016).

The terms cardio-renal and cardio-hepatic syndromes fully underline the bidirectional interactions between the heart and distant organ systems. For instance, acute kidney injury can occur secondary to acute cardiac diseases when cardiac output and renal blood supply is abruptly reduced (e.g. hypovolemic shock). Similarly, increased venous pressures during cardiac overload are recognized as a risk factor for AKI in critically ill people (Legrand et al. 2013; Donati et al. 2016; Doi & Rabb 2016; Orvalho & Cowgill 2017). AKI in the context of hemodynamic instability has been occasionally described in small animals (Vaden et al. 1997, Cowgill & Langstone 2011; Ross 2011; Buckley et al. 2017). On the other hand, cardiac arrhythmias and myocardial injuries commonly occur in canine AKI (Keller et al. 2016; Orvalho & Cowgill 2017).

Conditions of both decreased visceral blood supply (low cardiac output) and venous congestion (elevated right-filling pressures) can affect splanchnic perfusion and induce liver and gut impairment (Donati et al. 2016).

Results from experimental studies and significant epidemiologic data link kidney-lung interaction in critically ill patients. For instance, AKI is extremely common in people with ARDS, where increased circulation in inflammatory cytokines and proapoptotic factors might be responsible for

AKI and MODS occurrence. The systemic effects of the *biotrauma* induced by mechanical ventilation further contribute to hormonal and hemodynamic patterns modifications promoting renal damage (Qulez et al. 2012). Presence of renal failure, in turn, promotes lung injury through a variety of mechanisms, including the increase in lung capillaries permeability, leukocyte adherence and infiltration, inflammatory cytokines and chemokines expressions (Doi & Rabb 2016). The occurrence of AKI in the course of systemic diseases and distant organ failures is still not well documented in veterinary patients. A recent retrospective study reported a common incidence of pulmonary abnormalities and respiratory dysfunction in dogs with acute azotemia (Le Boedec et al. 2012), and the overall attention on kidney injury and concomitant MODS has been arised in small animal practice (Hoareau et al. 2017; Keir & Kellum 2015).

The spleen too has been recently recognized as a player into the complex framework of organ cross-talk: specifically, the spleen is part of the reticuloendothelial system responsible for host defense. Potential protective roles of the spleen emerged in the context of experimentally-induced ischemic AKI, as splenectomy before ischemic AKI induction was associated with higher renal damage. Additionally, vagal nerve activation through the splanchnic cholinergic pathway ameliorated inflammation and reduced organ damage in experimental models of sepsis, and novel ultrasound technologies targeting the cholinergic anti-inflammatory pathways might have a role in modulating inflammation and MODS development (Doi & Rabb 2016; Gigliotti & Okusa 2014).

The interplay between coagulation abnormalities and microcirculatory dysfunction has been highlighted from experimental and human data, and development of disseminated intravascular coagulation is thought to have a dominant role in the pathogenesis of organ failure. Microvascular thrombosis occurring during the hypercoagulable phase of disseminated intravascular coagulation contributes to reduce tissue perfusion, while thrombocytopenia and coagulation factors consumption put critically ill patients at risk of bleeding episodes. Additionally, many anticoagulant proteins such as antithrombin and protein C modulate inflammation and influence leukocytes functions. The cross-talk between inflammation and coagulation is actively mediated by endothelial

cells activation: up-regulation of tissue factor leads to local thrombin generation and fibrin deposition in the liver, intestine, kidney and lungs, ultimately contributing to local organ dysfunction (Levi et al. 2012).

Liver is particularly susceptible to inflammation, and is one of the first organ to experience damage and dysfunction in the setting of SIRS. Liver dysfunction, in turn, is associated with encephalopathy and cerebral edema, coagulopathy, renal and respiratory failure and cardiovascular instability, supporting the hypothesis of liver-induced multiorgan failure in the context of critical illness (Kozlov et al. 2017). For example, both acute and chronic liver failure is associated with AKI development (hepato-renal syndrome) by splanchnic vasodilation and vasoconstriction of the renal vascular bed, hence promoting renal hypoperfusion (Doi & Rabb 2016).

Gut functions and gut-microbiome have a tremendous impact on the well-being of other organ systems. Gut-derived bacterial and non-bacterial factors could incite SIRS and MODS in predisposed patients, as already discussed in the chapter above. Alteration in gut-microbiome (e.g. dysbiosis) have been linked with the bloodstream release of uremic toxins contributing to progression of kidney injury, as well as with development of nephropathies with an immune-mediated pathogenesis (e.g. IgA nephropaty, lupus nephritis) (Khodor & Shatat 2017). Occurrence of endotoxemia has been demonstrated during canine parvovirus infection (Isogai et al. 1989; Turk et al. 1990; Otto et al. 1997), and histological evidence of pulmonary alveolitis was documented in a significant percentage of puppies affected by the disease (Turk et al. 1990).

Even iatrogenic interventions supporting the function of one organ system might cause significant distant-organ impairment. Mechanical ventilation with high-PEEP setting, which has long been advocated to support pulmonary function during ARDS, has noticeable hemodynamic effects, as the positive intrathoracic pressures lower CO and reduce myocardial performances, thus negatively affecting preload, afterload and contractility (Donati et al. 2016, Quìlez et al. 2012). Moreover the biotrauma caused by mechanical ventilation has been deemed responsible for the systemic bloodstream release of inflammatory mediators and proapoptotic factors inciting distant organ

failure (Quìlez et al. 2012). Similarly, despite catecholamines are cornestore therapies in the treatment of refractory hypotension in septic shock patients, they have negative non-hemodynamic biologic effects on immune status, mitochondrial function and tissue oxygen requirements. Particularly, catecholamines aggravate hypermetabolism resulting in hyperglicemia and hyperlactatemia, increase oxygen tissue demand, promote immunosuppression, reduce splanchinc perfusion and gastrointestinal motility (Hartmann et al. 2017).

5. Specific organ dysfunction

The most commonly affected organ systems during MODS are the respiratory, cardiocirculatory, renal, coagulation system, central nervous, hepatic and gastrointestinal systems. In some instances, other organ systems including the musculoskeletal and the endocrine systems may be involved (Johnson et al. 2004). An in-detail description of specific organ system dysfunction during systemic diseases is given below, with significant reminds to the available veterinary literature.

Respiratory dysfunction

Lung damage and dysfunction in the course of systemic disease is documented in people and animals and described using the term "ARDS" (Acute Respiratory Distress Syndrome). Specifically, ARDS is defined as acute lung insufficiency characterized by diffuse bilateral alveolar infiltrate, decreased pulmonary compliance and moderate to severe hypoxemia (PaO_2/FiO_2 ratio < 300) in the absence of left-sided heart failure or circulatory overload (Ranieri et al. 2012; Confalonieri et al. 2017). The hallmarks of ARDS are neutrophil infiltration of the lung, damage of alveolar-capillary barrier and vascular leakage. Subsequent accumulation of rich-protein edema into the alveoli and the systemic release of pro-inflammatory mediators contribute to the exudative phase, impair the gas exchange and enhance pneumocytes disruption. The proliferative phase follows after several days through progressive fibrotic tissue formation into the fluid-filled alveoli. Pulmonary thromboembolism and transient pulmonary hypertension might develop. Mechanical ventilation represents the cornerstone of ARDS treatment, aiming to improve the degree of hypoxemia, recruit atelectic lung and reduce the work of breathing. Protocols based on protective ventilation strategies are currently employed to minimize the trauma associated with positive pressure ventilation and the development of ventilator-associated acute lung injury in humans. These include the use of low tidal volumes, higher positive-end expiratory pressures and permissive hypercapnia. Non-invasive ventilation (NIV) and continuous positive airway pressure (CPAP) have been contemplated for the early management of patients with ARDS, but their routine use is

controversial. Protective ventilation has significantly improved outcomes of people suffering from ARDS. The mortality rate reported, however, is still high, reaching an average of 60% in the elderly patients. Nonetheless, many human patients recovering from ARDS do not exhibit signs of long-term pulmonary compromise (Brower et al. 2000; Rubenfeld et al. 2005; Confalonieri et al. 2017).

Data regarding prevalence and prognosis of veterinary patients with ARDS are scant. According to the results of a necropsy-based study in dogs, ARDS was associated with both pulmonary and extra-pulmonary diseases like pulmonary contusions, bacterial pneumonia, sepsis, gastric dilatation volvulus and shock. Clinical correlates of human ARDS were identified in some of the included dogs (Parent et al. 1996). In 2007, the first clinically based veterinary consensus definitions on ARDS and ALI (Acute Lung Injury) were published (Wilkins et al. 2007) (Table 1). The clinical application of such criteria was the topic of a recent retrospective study conducted in dogs and cats presenting for or developing peracute onset of severe hypoxemia. A plethora of risk factors for ALI or ARDS were identified in this setting including SIRS, pneumonia, multiple transfusions, trauma and adverse drug reactions. In mechanically ventilated patients, the median tidal volumes used were generally higher than the ones deemed to be lung-protective in people. An overall survival rate of 10% was documented in the study, with euthanasia due to financial constraint and poor prognosis accounting for the majority of the deaths in both species (Balakrishnan et al. 2017). The elevated costs associated with mechanical ventilation and the need for high-level intensive care support account for the lack of ARDS systematic description in veterinary medicine, and hamper the accurate evaluation of its prognosis. Additionally, no study addressing the benefits of protective ventilator strategies has been published in small animals.

First 4 criteria required	
acute onset of dyspnea (<72h)	
known risk factors	SIRS, sepsis, trauma, multiple transfusion, adverse drug reaction…
pulmonary capillary leak without increased pulmonary capillary pressure	bilateral/diffuse infiltrate on rx proteinaceous fluid within the airways increased extravascular lung water
inefficient gas exchange	PaO/FiO < 300mmHg (ALI) or <200mmHg (ARDS) increased Aa gradient
evidence of pulmonary inflammation	transtracheal wash/bronchoalveolar lavage with neutrophilia or inflammatory biomarkers

Table 1. Diagnostic criteria for ALI and ARDS in small animals (modified from Wilkins et al. 2007).

Cardiocirculatory dysfunction

Cardiovascular dysfunction is a severe complication of MODS in people, and is generally evidenced by hypotension despite adequate fluid resuscitation, requiring vasopressors or inotropic treatment. When fluid-refractory hypotension occurs during sepsis this condition is referred to as septic shock (Vincent et al. 1998; Kakihana et al. 2016; Silverstein & Beer 2015). The hallmarks of cardiovascular dysfunction during MODS are myocardial depression and reduction in systemic vascular resistance (SVR). Cardiac contractility could be altered in the course of SIRS and sepsis, leading to ventricular dilatation, reduced left ventricular ejection fraction, and ultimately reduced cardiac output and hypotension. Several inflammatory mediators including TNF-α, NO, platelet activating factor, oxygen free radicals and arachidonic acid metabolites have been implicated in myocardial depression. The fall in SVR is primarily due to the effects of vasodilating mediators such as NO, histamine, prostaglandins and various cytokines, and is clinically apparent with clinical signs of distributive shock (Vincent et al. 1998). However, pathophysiology of septic shock comprises both warm and cold types. The early phase of septic shock is called hyperdynamic, being characterized by high cardiac output, low SVR and warm extremities. In the late phase hypotension

occurs, followed by a reduction in cardiac output, poor peripheral perfusion, cold extremities and finally death. Notably, ejection fraction is lower and end-diastolic volume is higher in survivors, compared to non-survivors of shock, suggesting that ventricular dilation might be compensatory in order to maintain adequate cardiac output and protect against myocardial depression (Kakihana et al. 2016). Cardiocirculatory dysfunction carries a poor prognosis in people with sepsis and MODS, as it is associated with a mortality rate of 70-90% compared to septic patients without cardiovascular impairment (Kakihana et al. 2016).

Microcirculatory alterations further contribute to the impaired cellular oxygen metabolism occurring in the context of cardiovascular dysfunction. The first imbalance between oxygen distribution (DO_2) and oxygen demand (VO_2) is due to increased oxygen demand and reduced supply. In this first stage, however, oxygen extraction is increased at the tissue level, and DO_2 and VO_2 remain independent. As DO_2 falls further (e.g. with a reduction in cardiac output) and a critical DO_2 is reached, VO_2 becomes DO_2 dependent, and blood lactate levels rise reflecting tissue hypoperfusion and anaerobic metabolism (Vincent et al. 1998). Despite hypotension is generally included in the diagnosis of septic/distributive shock, preserved blood pressure can be associated with markers of tissue hypoperfusion and microcirculatory abnormalities like increased lactate levels and reduced central venous oxygen saturation ($ScvO_2$). Hence, shock has been re-defined as a life-threatening generalized form of acute circulatory failure characterized by decreased oxygen utilization by the cells (Cecconi et al. 2014).

Once appropriate volume resuscitation has been performed, hemodynamic stabilization in the context of septic shock is usually addressed with inotropes and vasopressors administration (Cecconi et al. 2014). In agreement with the 2012 Surviving Sepsis guidelines, norepinephrine is the first-choice vasopressor followed by epinephrine and low-dose vasopressin (Dellinger et al. 2012). A definitive consensus regarding the role of novel inotropic agents (e.g. levosimendan), beta-blockers and vasodilator therapy during septic shock is not currently available in people (Kakihana et al. 2016; Correa et al. 2017).

Myocardial dysfunction and shock have been accepted as one of the most important manifestations of sepsis in small animals, too (Bulmer et al. 2011; Osteburn et al. 2014). Critical-illness induced left ventricular dysfunction has been described in dogs without primary heart disease, mainly affected by sepsis and cancer, and is reported to be reversible (Nelson et al. 2006; Dickinson et al. 2007). Evidence of myocardial injury reflected by increased cardiac troponins has been described in dogs with pyometra, as well as in dogs with various causes of systemic inflammation, and predicts poor prognosis (Hagman et al. 2007; Langhorn et al. 2013). Similarly, a study including 43 puppies diagnosed with parvoviral enteritis documented a more significant reduction in diastolic and systolic function and increased cardiac biomarkers in non-survivors compared to survivors (Kocaturk et al. 2012).

Reported mortality rate of septic dogs suffering from cardiocirculatory dysfunction is as high 80-90%, with increased mortality being associated with a greater number of vasopressors (Kenney et al. 2010; Conti-Patara et al. 2012; Osteburn et al. 2014; Ateca et al. 2014; Gravelyn & Guillaumin 2016). Literature regarding cardiovascular dysfunction is lacking in cats. Relative bradycardia seems to be a peculiar manifestation of shock in cats, but its prognostic significance is controversial (Osteburn et al. 2014; Klainbart et al. 2017). There is still insufficient data to provide conclusions regarding vasopressor choices in animals with naturally occurring septic shock (Silverstein & Beer 2015).

Hepatic dysfunction

Hepatic dysfunction is frequently detected in the context of MODS and is usually defined as progressive hyperbilirubinemia associated with increased liver enzymes (Johnson et al. 2004; Nesseler et al. 2012). Assessing liver dysfunction during critical illness, however, is challenging, as neither static nor dynamic tests can be considered as gold standard. Development of liver dysfunction has several negative consequences during critical illness: aminoacid synthesis and glucose release are impaired; additionally, coagulopathy may be profound and become clinically

significant. Besides its metabolic functions, the liver is also involved in the immune system activation and contributes to host defense and tissue repair during SIRS and sepsis. Thus, bacteria scavenging and detoxification are compromised leading to bacteremia, infections and immunosuppression. In septic people with MODS, liver dysfunction might present with two different clinical features: hypoxic hepatitis as a consequence of hepatocellular hypoperfusion, and sepsis-induced cholestasis (Nesseler et al. 2012; Osteburn et al. 2014). Similarly, in experimental models of canine endotoxemia, liver hystopathology suggested ischemia as the trigger for hepatic injury (Manson et al. 1981). Despite criteria to define hepatic dysfunction differ between studies, the occurrence of liver dysfunction is reported in veterinary patients with MODS. Its overall incidence ranges between 30 and 70% in canine sepsis, with a controversial prognostic significance (Kenney et al. 2010; Osteburn et al. 2014; Bush et al. 2016). Hyperbilirubinemia is also a common finding in cats with sepsis, and has been associated with worse outcomes during this syndrome (Brady et al. 2000; Klainbart et al. 2017; Troìa et al. 2017).

No specific recommendation is currently available to treat hepatic dysfunction during critical illness and sepsis in people and animals. Avoidance of drugs inducing cholestasis or hepatocellular damage, early enteral feeding and therapy with ursodeoxycholic acid, which has choleretic, cytoprotective and immunomodulatory properties, could be used to treat sepsis-induced cholestasis (Nesseler et al. 2012).

Gastrointestinal dysfunction

The gastrointestinal system is a target organ for injury during critical illness, and gastrointestinal motility is frequently affected by systemic diseases. Commonly reported gastrointestinal motility disorders in critically ill humans and animals include esophageal dysmotility, delayed gastric emptying, ileus and colonic motility abnormalities. Despite a reported incidence ranging between 50 and 60% in humans, the exact frequency of overall gastrointestinal dysfunction in people is difficult to gauge compared to other forms of organ dysfunction, due to lack of clear definition and

diagnostic criteria. In humans, gastrointestinal dysfunction predisposes to hyporexia or anorexia, intolerance to enteral feeding, diarrhea, increased gut permeability and bacterial translocation, contributing to prolonged ICU stay and mortality (Chapman et al. 2007; Whiteheat et al. 2016). Additional reported consequences of gastrointestinal dysmotility include esophageal reflux and aspiration, development of gastric ulceration, bacterial overgrowth, fluid sequestration into the gastrointestinal tract, electrolyte abnormalities and delay of nutritional delivery (Chapman et al. 2007; Osteburn et al. 2014; Whitehead et al.2016).

The prevalence of gastrointestinal dysfunction in critically ill dogs and cats has not been reported. Its occurrence, however, is thought to be common in both experimental and spontaneous models of systemic diseases (Johnson et al. 2004; Ostburn et al. 2014). In addition, the gastrointestinal tract is considered to be one of the shock organs in dogs (Hackett 2011). Plasma citrulline concentration has been proposed as a reliable marker of global enterocyte mass in people. A recent canine study reported decreased plasma citrulline concentration in dogs with parvoviral enteritis compared to control dogs, suggesting severe gastrointestinal damage associated with the disease (Dossin et al. 2011). Similarly, markers of increased gut permeability have been reported in critically ill dogs with trauma, parvoviral enteritis and necrotizing pancreatitis (Streeter et al. 2002; Mohr et al. 2003; Chen et al. 2004). The prognostic implication of gastrointestinal damage and dysfunction in critically ill veterinary patients, however, remains controversial (Dossin et al. 2011).

Treatment for gastrointestinal dysfunction is mainly supportive, and include early enteral feeding, use of prokinetics and multi-modal pain control (Mohr et al. 2003; Chen et al. 2004; Whitehead et al. 2016).

Renal dysfunction

Acute kidney injury (AKI) is a severe complication of sepsis and MODS in critically ill people. AKI occurrence in the ICU is extremely common in humans, with septic AKI accounting for approximately the 50% of all AKI diagnosed in the ICU (Bellomo et al. 2017). Diagnosis of AKI in

people relies on clinical assessment, measurement of urinary output and relative or absolute changes in serum creatinine (Kellum et al. 2013). Pathophysiology of AKI in the context of MODS is poorly defined, due to the lack of consistent findings in experimental and clinical studies, and the paucity of renal biopsies performed during the acute setting. Renal ischemia and acute tubular necrosis are documented only in the minority of cases; on the other hand, renal blood flow is usually normal or increased during systemic inflammation and sepsis. Apoptosis of renal tubular cells, local microcirculatory dysfunction, inflammatory and immunological factors concur to incite and promote AKI in the course of critical illness. Moreover nephrotoxic drugs, overzealous fluid therapy, hypoalbuminemia, electrolyte abnormalities and distant organ injuries further predispose the critically ill patient to AKI development (Lunn et al. 2011; Antoniotti et al. 2016). Despite being associated with a significant increase in morbidity and mortality, AKI is usually completely reversible in people surviving sepsis and MODS (Kellum et al. 2013; Antoniotti et al. 2016).

There is limited information on AKI as a component of MODS in veterinary patients: AKI has been mainly investigated as single-organ dysfunction/failure rather than as part of a multisystemic impairment during critical illness; in addition, universally accepted diagnostic criteria are lacking (Johnson et al. 2004; Osteburn et al. 2014; Keir & Kellum 2015). In a study including dogs undergoing surgery for septic peritonitis, AKI was defined as a 0.5 mg/dl increase in post-surgical serum creatinine. An AKI prevalence of 12.3% was documented, and an independent association between development of renal dysfunction and mortality was identified (Kenney et al. 2010). Similarly, in a study including dogs with infectious and non-infectious SIRS, 15% of patients were azotemic; greater serum creatinine concentration and quantitative proteinuria were documented in non-survivors (Giunti et al. 2015).

The overall survival rate of dogs and cats with AKI is variable and strongly related to the underlying disease. Although information regarding the prognostic impact of AKI during MODS are not available in veterinary medicine, developing of acute azotemia is recognized as a strong predictor for mortality in hospitalized dogs and cats (Harison et al. 2012).

Treatment of MODS-related AKI is mainly supportive. Adequate fluid resuscitation, normalization of electrolytes and acid base imbalances and correction of anuria or oliguria are advocated. Renal replacement therapy should be considered in cases of severe azotemia with life-threatening complications, oligo/anuria and fluid overload (Lunn et al. 2011).

Recently, a canine AKI grading system proposed by Cowgill (2010) and accepted by the IRIS (International Renal Interest Society) group has been increasingly applied in clinical settings (De Loor et al. 2013; Segev et al. 2015; Sigrist et al. 2015) (Table 2). Other than classyfing AKI severity based on glomerular filtration rate impairment (hence serum creatinine increase), the above-mentioned criteria point attention on non-azotemic AKI and volume-responsive AKI, whose prevalence and clinical implications are unknown in veterinary medicine.

AKI grade	serum creatinine	clinical description
grade I	<1.6 mg/dl	non-azotemic AKI: • progressive non-azotemic 0.3mg/dl increase in sCr within 48h and/or • measured oliguria
grade II	1.7-2.5 mg/dl	• progressive azotemic increase in sCr and/or • measured oliguria
grade III	2.6-5.0 mg/dl	• increasing severity of azotemia and renal failure
grade IV	5.1-10.0 mg/dl	
grade V	>10 mg/dl	

Table 2. IRIS AKI grading criteria (modified from www.iris-kidney.com) Cowgill 2010, Proceeding of the ACVIM Forum. Measured oliguria: urinari output <1ml/kg/h.

Coagulation dysfunction

The interactions between inflammation and coagulation are well documented in critically ill humans. Coagulation abnormalities can predispose intensive care patients to thrombotic or

hemorrhagic events, and act as independent risk factors for mortality. The mechanisms behind coagulation dysfunction in critical illness are numerous, and involve anticoagulant factor consumption, hyperfibrinolisis, thrombocytopenia, thrombocytopathia and coagulation factors deficiencies (Fourrier et al. 1992; Davies et al. 2014). The link between coagulation and SIRS has been repeatedly demonstrated during sepsis and trauma in people, with progression toward hypocoagulability being generally associated with increased transfusion requirements and worse outcomes (Johansson et al. 2011; Davies et al. 2014). The utilization of more accurate methods for in vitro assessment of coagulation status (e.g. rotational thromboelastometry, thrombin generation assay, platelet aggregometry) has increased the availability of information concerning hemostatic imbalances in veterinary critical care too. Coagulation dysfunction defined as reduced platelet count and increased coagulation times was reported in a population of dogs with septic peritonitis, and significantly increased the odds of death (Kenney et al. 2010). In a different study, a complete coagulation profile and thromboelastography were combined to characterize hemostasis in dogs with septic peritonitis. Decreased activity of endogenous anticoagulants and hypercoagulability were commonly detected, with survivors being more hypercoagulable than non-survivors (Bentley et al. 2013). Similar data were documented in the course of canine leptospirosis, where presence of thromboelastometric evidence of hypocoagulability was related with hemorrhagic complications and increased mortality (Barthèlemy et al. 2017). A recent canine study investigating platelet function using multiple electrode platelet aggregometry, documented decreased platelet function in the course of septic peritonitis. Interestingly, and similarly to human data, collagen-activated aggregometry was significantly reduced in non-survivors, suggesting its role in assessing illness severity (Li & Chan 2016).

Neurologic dysfunction

Neurologic dysfunction is common in the critical care patient, as evidenced by deterioration in mental status, cognitive deficits, changes in awareness and behaviour. In this regard, sepsis-

associated encephalopathy is well described in people hospitalized in the ICU, and is related with a worse prognosis and long-term neurologic complications. Due to its frequent occurrence and its prognostic role in human sepsis, presence of neurologic impairment is also included in the quick Sequential Organ Failure Assessment (qSOFA) score aiming to rapidly identify high-risk patients with suspected infection outside the ICU (Rhodes et al. 2016). Several causes including brake-down of the blood-brain barrier, inflammatory mediators, microcirculatory and coagulation dysfunction contribute to neurologic dysfunction pathogenesis, leading to cerebral edema, thrombosis, infarcts and neuronal cell death (Sharshar et al. 2004).

Incidence and long-term outcomes of neurologic dysfunction in critically ill dogs and cats are not known. However, mental status is usually abnormal in veterinary patients with sepsis and MODS, and its evaluation is included as a required criteria in scores evaluating disease severity and prognosis (Hayes et al. 2010; Hayes et al. 2011). Similarly, the Glasgow Coma Scale, a scoring system created to recognize and grade neurologic impairment in patients with head trauma, has been comprised in the canine SOFA score to assess presence and severity of neurologic dysfunction during critical illness (Ripanti et al. 2015).

Endocrine dysfunction

Acute illness is responsible of dramatic changes in endocrine function, and specifically impairs the hypothalamic-pituitary-thyroid axis and the hypothalamic-pituitary-adrenal axis (Boonen & Van den Berghe 2014).

The term euthyroid-sick syndrome (ESS) specifically refers to the occurrence of low plasma concentration of T_3, increased concentration of reverse T_3 and low to normal TSH and T_4 concentrations in the critically ill patient, in the absence of pre-existing thyroidal disease. Fasting, cytokines, hypoalbuminemia and hypoxia are some of the possible factors involved into ESS pathogenesis. Several human studies reveal an inverse association between the magnitude of

thyroidal hormones alterations and survival, as decreased thyroid function predicts worse outcomes in patients with sepsis and septic shock (Boonen & Van den Berghe 2014; Angelousi et al. 2011).

ESS occurrence has been demonstrated in critically ill dogs with various diseases: specifically, low T_3 and T_4 concentrations were detected in a wide population of dogs with non-thyroidal illness, as well as in dogs with SIRS and sepsis of different etiologies (Mooney et al. 2008; Schoeman JP & Herrtage ME 2008; Pashmakova et al. 2014; Giunti et al. 2017). Although not consistent, low thyroid hormones concentrations seem to paralel disease severity and prognosis in dogs (Mooney et al. 2008; Schoeman JP & Herrtage ME 2008; Giunti et al. 2017).

The clinical implications of ESS are still not completely known. If ESS development is thought to represent an adaptive response to reduce body energy expenditure in the acute phase, it may be maladaptive during prolonged critical illness. That being said, supplementation of thyroidal hormones is not recommended in the acute phase of critical illness, while replacement therapy during persistent disease remains controversial (Boonen & Van den Berghe 2014).

The effects of critical illness on the hypothalamic-pituitary-adrenal axis are even more apparent. High cortisol levels during critical illness likely contribute to provide support to vital organs by influencing glucose metabolism, enhancing inotropic and vasopressor responses to endogenous cathecolamines and modulating inflammation. The initial rise in cortisol concentrations is thought to be dissociated from ACTH stimulus, suggesting tissue-specific glucocorticoid regulation. Despite higher basal levels of serum cortisol, however, tissue resistance to glucocorticoids occurs in the critically ill. The concept of "relative adrenal insufficiency" or "critical illness-related corticosteroid insufficiency" (CIRCI) have emerged in people in the last decade, and refers to the condition in which cortisol production is insufficient despite a maximally ACTH-activated adrenal cortex. Presence of CIRCI is usually defined by low basal cortisol levels and/or a low cortisol response to an ACTH injection, regardless of the initial basal cortisol concentration, indicating the inhability of the body to cope with the stress of illness. Due to inconsistent findings among studies, CIRCI in people is currently diagnosed by evaluating response to treatment with low-dose hydrocortisone,

which is recommended to treat pressor-resistant septic shock (Boonen & Van den Berghe 2014; Annane et al. 2017).

CIRCI has been described in small animals as a reversible, transient adrenal dysfunction causing inappropriately low glucocorticoid concentrations during acute disease. Its overall incidence among veterinary patients is unclear. Earlier studies failed to identify adrenal insufficiency in sick dogs; however populations were heterogeneous and disease severity not always elevated (Prittie et al. 2002; Martin et al. 2011). More recent investigations diagnosed relative adrenal gland insufficiency in dogs with SIRS and sepsis, and noticed that lower delta cortisol concentrations after ACTH stimulation test were related to hypotension and increased mortality (Burkit et al. 2007; Burkitt Creedon 2015). Although alterations in cortisol concentrations and ACTH responses have been reported in critically ill cats, abnormalities were similar between survivors and non-survivors (Prittie et al. 2003; Burkitt Creedon 2015). There is little evidence supported by case reports and expert opinions stating succesfull management of refractory septic shock with low dose glucocorticoid therapy in dogs, cats and foals, but no definitive recomendation is currently available (Burkitt Creedon 2015).

6. Introduction to our experiments: MODS, sepsis and the role of biomarkers

MODS is the leading cause of death in human ICU (Seely et al. 2000; Vincent et al. 2006; Mongardon et al. 2009; Pierrakos & Vincent 2010). Many critical patients with organ dysfunction and failure usually have a concomitant SIRS, which can be infectious or non-infectious in origin. Years of research have shown that it is the host immunological response to this state of systemic inflammation, rather than the inciting insult itself, mainly responsible for organ dysfunction progression, therapeutical success or failure and outcome. Because of the high mortality associated with established MODS, prevention and early recognition of risk factors for organ dysfunction significantly improve patient management and survival (Cheadle & Turina 2005).

Sepsis, *the life-threatening organ dysfunction caused by a dysregulated host response to infection*, is the major inciting condition for MODS (Seely et al. 2000; Gotts & Matthay 2016). Previous approaches considered the spectrum of systemic inflammation and sepsis syndromes (SIRS, sepsis, septic shock) proceeding along a single dimension. Novel approaches, on the other hand, emphasize a continuum of acute inflammation and organ dysfunction. Sepsis definitions have changed, and sequential organ failure scores have been incorporated into its classification (Gotts & Matthay 2016).

Much is known about how sepsis promotes organ injury. As sepsis progresses from a localized infection to SIRS and shock, major perturbations of the cardiocirculatory system develop, leading to tissue hypoperfusion and altered oxygen delivery. Significant alterations to the endothelium occurs, including activation of coagulation, leukocyte adhesion, vasodilation and tissue edema. The loss of endothelial barrier function and the aberrant inflammatory response of the host are the main underlying mechanisms for acute lung injury, acute kidney injury, bacterial translocation from the gut and widespread lethal organ dysfunction. Furthermore, septic organ dysfunction often perpetuates critical illness through self-reinforcing processes, like immune system dysfunction and the potential for iatrogenic arm (Gotts & Matthay 2016).

Due to the wide array of syndromic presentation of sepsis and the variable therapeutical patient responses, studies characterizing the phenotype of the critical patient and the immune systems have emerged (Howell et al. 2011). The PIRO (*Predisposition, Infection, Response, Organ dysfunction*) model describes septic patients across four domains, on the basis of their predisposing conditions, the nature and extent of the insult, the nature and the magnitude of the host response, and the degree of organ dysfunction (Howell et al. 2011; Granja et al. 2013; Gotts & Matthay 2016).

The use of circulating biomarkers of the *host response* and *organ dysfunction* has recently experienced an exponential growth in human medicine, aiming to assess the individual phenotype of the critical patient, classify the degree of disease severity and predict early and late complications of systemic inflammation and sepsis. In this latter regard, biomarkers could be used to anticipate MODS development, moving clinician perspectives from reactive (e.g. following disease progression) to proactive (starting monitoring and treatments before the patient deteriorates) (Vincent & Creteur 2015). Although organ dysfunction is a dynamic complication of critical illness with non-predictable evolution, many physiological and biological biomarkers have been individually shown to be strongly predictive of outcome, even on the first day of ICU admission (Mongardon et al. 2009). Thus, role of early biomarkers seems crucial, as it may be possible that the early response of the immune system plays a pivotal role for healthy or adverse outcomes (Cabrera et al. 2017).

The major challenge of the current research is to approach SIRS and sepsis in dogs and cats in light of the PIRO system, with a main focus on the *host response* and development of *organ dysfunction*.

The primary aim of the project is to investigate the host response to inflammation and infection, testing novel and promising diagnostic and prognostic biomarkers of SIRS and sepsis. The following studies have been carried on:

- "Prognostic significance of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in canine SIRS: a prospective observational study" (J Vet Emerg Crit Care 2015; 25: 226-233)

- "Serum amyloid A in the diagnosis of feline sepsis" (J Vet Diagn Invest 2017; 29: 856-859)
- "Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs" (Vet J 2017; 230: 13-19)
- "Procalcitonin in dogs with sepsis and gastric dilatation-volvulus" (two manuscripts currently under revision).

The second purpose of the present research is to in-depth analyze occurrence and prognostic implication of MODS and selected organ dysfunctions in critically ill dogs and cats. Most investigations have focused on acute kidney injury in critically ill dogs, and non-thyroidal illness in dogs with non-infectious SIRS and sepsis. A preliminary study regarding MODS in septic cats has been performed, too. Results have been finalized with the following studies:

- "Acute kidney injury in critically ill dogs" (manuscript currently under revision)
- "Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome" (J Vet Sci 2017; 4)
- "Multiorgan dysfunction syndrome in feline sepsis" (manuscript to be finalized)

Extended manuscripts are given below. A short summary concerning available human and veterinary data is provided for canine delta neutrophil index and procalcitonin. Abstracts and a summary of major results are given for the manuscripts currently under revision or yet to be finalized.

7. Biomarkers of the host response

7.1

Original Study

Journal of Veterinary Emergency and Critical Care **25**(2) 2015, pp 226–233
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Prospective evaluation of the acute patient physiologic and laboratory evaluation score and an extended clinicopathological profile in dogs with systemic inflammatory response syndrome

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Abstract

Objective – To investigate the prognostic value of the acute patient physiologic and laborartory evaluation (APPLE) score and relevant clinicopathological markers in dogs with systemic inflammatory response syndrome (SIRS).
Design – Prospective observational cohort study.
Setting – Veterinary teaching hospital.
Animals – Thirty-three dogs with SIRS admitted to the intensive care unit (ICU) were compared to 35 healthy control dogs. Dogs with SIRS were divided into septic ($n = 20$) and nonseptic ($n = 13$) etiologies and as survivors (alive to discharge, $n = 22$) and nonsurvivors ($n = 11$: died, $n = 6$, or humanely euthanized, $n = 5$).
Measurements and Main Results – For all dogs, physiological and laboratory parameters were prospectively collected for the calculation of the $APPLE_{fast}$ score. No difference between septic and nonseptic SIRS dogs was detected for any parameter evaluated. Survivors had significantly higher total protein, albumin concentrations, antithrombin activity (ATA), and base excess (BE), as well as significantly lower lactate, urea, creatinine concentrations, urinary protein to creatinine ratio and $APPLE_{fast}$ score compared to nonsurvivors. Higher values of creatinine, lactate, anion gap, alanine transaminase (ALT), and $APPLE_{fast}$ score were significantly associated with an increased risk of death in SIRS dogs, while higher values of total protein, albumin, ATA, and BE were associated with a significantly reduced risk of mortality. When a multivariate binary logistic regression analysis was performed, the $APPLE_{fast}$ score was the only significant parameter retained.
Conclusions – The determination of the $APPLE_{fast}$ score in clinical setting, as well as the measurement of APP, ATA, lactate, BE, anion gap, ALT, urinary proteins, and electrolytes may be beneficial for a better assessment of dogs with SIRS. Identified parameters were significantly related with the presence of SIRS and their evaluation should be considered for the assessment of disease severity, and guidance of the decision-making process in critically ill dogs.

(J Vet Emerg Crit Care 2015; 25(2): 226–233) doi: 10.1111/vec.12257

Keywords: biomarkers, canine, illness severity, urine, sepsis

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Introduction

Systemic inflammatory response syndrome (SIRS) and sepsis are frequently observed conditions in critically ill human patients.[1] Despite the lack of data on the prevalence of SIRS in veterinary patients, this syndrome has gained increasing attention and interest in recent years.[2–12] SIRS is characterized by activation of the acute phase response, hemostatic derangements, impaired tissue perfusion and oxygenation, and can ultimately progress to multiple organ dysfunction syndrome and

Abbreviations

APP	acute phase proteins
APPLE	acute patient physiologic and laboratory evaluation
ATA	antithrombin activity
AUC	area under the curve
BE	base excess
CRP	C-reactive protein
FECa	fractional excretion of calcium
FENa	fractional excretion of sodium
FEP	fractional excretion of phosphorus
iCa	ionized calcium
iHCa	ionized hypocalcemia
ROC	receiver operating characteristic
SIRS	systemic inflammatory response syndrome
TIBC	total iron-binding capacity
TP	total protein
UAC	urinary albumin to creatinine ratio
UPC	urinary protein to creatinine ratio
uUA:C	urinary uric acid to creatinine ratio
OR	odds ratio
CI	confidence interval

death.[2–10] Despite advances in supportive care, SIRS and sepsis remain leading causes of mortality in the ICU setting, with overall mortality rates ranging from 27% to 64% in dogs.[2–4,6–12] Thus, the prompt diagnosis and assessment of disease severity still remain primary goals to improve the therapeutic decision making and the outcome in septic patients. Furthermore, the development of appropriate methods to precisely stratify critical patients according to disease severity, would better assist clinical researchers in the design of clinical trials.

A user-friendly scoring system, acute patient physiologic and laboratory evaluation (APPLE) score, has been recently validated to stratify mortality risk in hospitalized dogs, independent of the underlying disease, by illness severity.[13] The scoring system includes a 10-variable and a 5-variable model ($APPLE_{fast}$) that enable a rapid cage-side calculation based on simple and objective clinical data. Receiver operating characteristic (ROC) curve analysis showed that the area under the curve (AUC) of the APPLE score had a robust value to predict death in ICU patients in both models (AUC 0.91 and 0.85, respectively) and supported their use as prognostic indicators for research purposes in dogs with SIRS.[13]

The number of studies aimed to identify predictive biomarkers for SIRS/sepsis in dogs has grown dramatically in the last decade and several clinicopathological parameters have been evaluated,[2,14,15] including acute phase proteins (APP), antithrombin activity (ATA), ionized calcium (iCa), and urine protein.[3–8,11,16–24]

The aim of this study was to examine the prognostic value of the $APPLE_{fast}$ score (5-variable model) and to evaluate the predictive power of an extended panel of routinely measured clinicopathological markers in dogs with SIRS. In addition, all parameters evaluated were compared between dogs with SIRS and a population of healthy control dogs.

Materials and Methods

A prospective observational study was carried out at the University of Bologna's Veterinary Teaching Hospital between December 2010 and December 2011.

Dogs admitted to the intensive care unit (ICU) were included in the study as nonseptic SIRS if they exhibited 2 or more of the following criteria at admission to the hospital: body temperature <38.1°C or >39.2°C; heart rate >120/min; respiratory rate >20/min; WBC count $< 6.0 \times 10^9$/L [6,000/μL] or $>16.0 \times 10^9$/L [16,000/μL], percentage of bands >3% of the total WBC count.[25] Septic SIRS dogs were identified as patients meeting the above criteria (nonseptic SIRS) in addition to identification of a concurrent septic focus documented by means of cytology or positive culture. Dogs with SIRS were also classified as survivors (alive to discharge) or nonsurvivors (died despite medical treatment or humanely euthanized by the clinical investigators because of moribund conditions or end-stage disease). Dogs that were euthanized for financial reasons were excluded from the study. For all dogs, the length of hospitalization in ICU was also recorded.

Dogs that were younger than 1 year of age, that were diagnosed with chronic kidney disease or parathyroid gland disease, based on history, clinical and clinicopatholgical findings, and imaging results, or that received drugs (eg, steroids, diuretics, vitamin D, phosphate enemas) known to alter calcium metabolism before the admission to the ICU were excluded from the study.

Thirty-five control dogs (client owned and hospital staff-owned dogs) were considered healthy based on history, physical examination, and clinicopathological data, including concentrations of creatinine, glucose, urea, total protein (TP), total bilirubin, cholesterol, phosphorus, total calcium, ionized calcium (iCa), total iron, albumin, Na, K, Cl, Mg, total iron-binding capacity (TIBC), C-reactive protein (CRP), fibrinogen as well as activities of alkaline phosphatase, alanine transaminase (ALT), aspartate transaminase, and γ-glutamyltransferase. Urinalyses including quantitative protein and albumin concentration on urine samples collected by cystocentesis ($n = 35$) were also performed.

Blood and urine from all dogs were collected and analyzed within one hour of ICU admission. Blood sampling was performed by venipuncture with vacutainer system[a] according to standard operating procedures. Urine was collected via cystocentesis or urinary catheterization. The following analyses were performed: venous blood gas with iCa, CBC, serum biochemistry (albumin, TP, glucose, ALT, aspartate transaminase, alkaline phosphatase, total bilirubin, γ-glutamyltransferase, cholesterol, total calcium, phosphorus, total iron) lactate, ATA, APP including CRP, fibrinogen, transferrin (as TIBC), and urinalysis including urinary protein to creatinine ratio (UPC), urinary albumin to creatinine ratio (UAC), urinary electrolytes, and urinary uric acid to creatinine ratio (uUA:C). A previously described standardized method[11] was used to collect venous blood samples to measure iCa and blood gas. Venous blood gas analysis also included pH, base excess (BE), HCO_3, monovalent electrolytes, $PvCO_2$, PvO_2, and anion gap measurements. All parameters were also measured in healthy control dogs.

Ionized hypocalcemia (iHCa) was defined as values lower than the lowest value of iCa measured in healthy control dogs (iCa < 1.21 mmol/L). The presence of an acute phase response was defined by an increased concentration above the reference interval of our lab for CRP (0 – 0.5 mg/dL) or fibrinogen (1.45 – 3.85 g/L). For each dog included in the study an $APPLE_{fast}$ score (5-variable model: glucose, albumin, mentation score, platelet count, and lactate) was calculated. In dogs with SIRS, the score was determined using data collected upon admission to ICU. The study protocol was approved by the local Scientific Ethical Committee for Animal Testing.

Laboratory methods

CBC was determined with an automated cell counter.[b] Lactate concentrations were measured using a portable lactate analyzer.[c] CRP[d] and urine albumin[e] were measured using immunoturbidimetric assays previously validated in our group for dog samples.[26,f] TIBC[g] and ATA[h] were measured using colorimetric methods. Ionized calcium and venous blood gas analysis were obtained using a blood gas analyzer.[i] UPC and UAC were calculated. Fractional excretion of calcium (FECa), sodium (FENa), and phosphorus (FEP) were calculated according to the following equation: fractional excretion = (UX/PX)/(UC/PC), where UX and PX were the concentrations of a specific analyte in urine and plasma, respectively, while UC and PC were creatinine concentrations in urine and plasma, respectively.[27] Urinary uric acid concentrations were measured using a colorimetric method[j] and normalized to urinary creatinine (uUA:C). All analyses were performed with an automated chemistry analyzer.[k]

Statistical Analysis

All data are described using standard descriptive statistics and reproted as median and range or mean ± standard deviation for nonnormal and normal distributions, respectively. Normality was assessed using D'Agostino–Pearson test. Mann–Whitney *U* test and Student's *t*-test were used to evaluate differences between groups. Results were considered statistically significant with P value < 0.05. In the population of dogs with SIRS, univariate logistic regression was used to assess the association between clinical and clinicopathological parameters and outcome. Parameters associated with outcome in the univariate analyses were entered into a multivariable model (backward selection, removing factors with P value > 0.1). Binary logistic regression results were presented as odds ratio (OR) and 95% confidence interval (CI). Overall model fit was assessed by the percentage of outcome correctly classified by the ROC curves analysis and by a significant Hosmer–Lemeshow test ($P > 0.05$). ROC curves were used to find optimal cut-off values for parameters predicting prognosis and to calculate the AUC. Correlations between parameters were assessed using Pearson or Spearman's rank correlation coefficients. All analyses were performed using an online available statistical software.[l]

Results

Sixty-two dogs (age >1 year) with signs of SIRS were admitted to ICU during the study period. Of these cases, 11 were excluded due to the presence of chronic kidney disease, and 10 were not enrolled due to investigators not being notified, causing incomplete blood and urine sampling upon admission. Eight dogs were euthanized for financial reasons and were ineligible for study inclusion.

A total of 33 dogs with SIRS were included in the study. Median age was 7.8 years (range: 1.3–15 y) and median body weight was 25.6 kg (range: 5.1–42 kg). Twenty-two dogs (67%) survived to hospital discharge (survivors), while 11 (33%) died ($n = 6$) or were humanely euthanized ($n = 5$) (nonsurvivors). Of the nonsurvivors, 6 septic SIRS dogs died despite treatments for refractory hypotension and multiple organ failure (4 dogs with septic peritonitis in the postoperative period, 2 dogs with severe urosepsis), 5 were euthanized due to the concomitant presence of multiple organ failure and a diagnosis of malignancy (Table 1). Rectal temperature <38.1°C or >39.2°C was identified in 2 and 26 dogs, respectively. Twenty-four dogs had tachycardia (HR>120/min) and 30 had tachypnea (RR > 20/min), as previously defined.[25] Twenty-six dogs had leukocytosis (WBC count > 16.0×10^9/L [16,000/μL]) and 3 had leukopenia (WBC count < 6.0×10^9/L [6,000/μL]).

Table 1: Diseases affecting 33 dogs with systemic inflammatory response syndrome (SIRS) stratified by outcome

Outcome	Septic SIRS group (20)	Nonseptic SIRS group (13)
Survivors ($n = 22$)	Septic pleuritis (4)	Osteomyelitis (2)
	Bite wound infection (4)	Eosinophilic pneumonia (2)
	Septic peritonitis (2)	Neoplasia (1)
	Pyometra (1)	diskospondylitis (1)
	Pyoderma (1)	Polyarthritis (1)
	Pyelonephritis/urosepsis (2)	Pneumonia (1)
Nonsurvivors ($n = 11$)	Septic peritonitis (4)	Neoplasia (5)
	Urinary tract infection/urosepsis (2)	

SIRS, systemic inflammatory response syndrome.
Number of affected patients in parentheses. To be classified as septic, diagnosis had to be confirmed cytologically or via positive bacterial culture result.

Thirteen dogs (39%) were classified as nonseptic, and 20 (61%) classified as septic. The most common cause of nonseptic SIRS was neoplasia ($n = 6$), while for septic SIRS was septic peritonitis ($n = 6$) (Table 1). For all the performed analyses, none of the tested parameters was significantly different between septic and nonseptic SIRS dogs.

Parameters that were significantly different between control and SIRS dogs, upon admission, are reported in Table 2. Positive (CRP and fibrinogen) and negative (TIBC, albumin, and antithrombin) APP values were all significantly different from control dogs (Table 2). The 22 survivors had significant higher values of albumin and ATA compared to the 11 nonsurvivors, while no differences were detected for the other APP (Table 3).

Urine specimens were collected in all patients; however, samples from 4 dogs were excluded from UPC and UAC analyses because a urinary tract infection with active sediment was observed. UPC and UAC were significantly higher in SIRS compared to the control dogs. UPC was significantly lower in survivors compared to nonsurvivors, while UAC did not vary (Table 3). FENa values were significantly correlated with both UAC and UPC (Table 4).

Survivors also had significantly higher values of serum TP and BE, as well as lower values of anion gap, $APPLE_{fast}$ score, lactate, urea, creatinine, and ALT concentrations compared to nonsurvivors (Table 2).

The $APPLE_{fast}$ score was significantly correlated with TP, urea, BE, total bilirubin, UPC, TIBC, and ATA (Table 4). Length of ICU stay was not significantly correlated to any of the investigated parameters.

Table 5 shows the parameters that were significantly associated with outcome using the univariate binary logistic regression analysis. There were positive associations between odds of mortality and values of creatinine, lactate and $APPLE_{fast}$ score, respectively. Higher values of albumin, TP, ATA, and BE were associated with a significant mortality risk reduction. When the multivariate binary logistic regression analysis was performed, the $APPLE_{fast}$ score was the only parameter retained by the model (Table 5).

Discussion

SIRS and sepsis are important syndromes in critically ill dog.[2–12] It is widely acknowledged that clinical criteria alone fail to identify and stratify these patients adequately. In our study, the potential prognostic significance of a wide panel of clinical and clinicopathological parameters that could be routinely measured in hospitalized dogs was investigated.

The APPLE score has been recently validated to stratify illness severity by mortality risk in hospitalized dogs.[13] In order to obtain a simple and practical calculation of the score, we applied the 5-variable $APPLE_{fast}$ model to a population of critically ill dogs. This score was able to discriminate between survivors and nonsurvivors upon admission with the highest predictive power. Thus, our results support the application of the $APPLE_{fast}$ score in the clinical setting to identify and properly manage high-risk ICU patients. However, its use as an exclusive tool to guide therapeutic decisions needs to be addressed in further clinical trials.

The quantification of APP, particularly C-reactive protein (CRP), allows an early identification of inflammatory processes, and represents an objective monitoring tool to evaluate the response of the patient to selected therapies.[5,16,28] However, the prognostic value of APP still remains controversial.[3–5,20,24] A panel of APP was investigated in the present study, including positive (CRP and fibrinogen) and negative (albumin and transferrin-TIBC) acute phase proteins. The activation of an acute phase response,[29] defined by a concentration above the reference interval of the measured positive APP (CRP, fibrinogen), was noted in all the dogs with SIRS. However, the prognostic role of positive APP is questionable, as their concentrations were not related with outcome, disease severity, or duration of hospital stay. In contrast, dogs with higher albumin concentrations

Table 2: Clinical and clinicopathological parameters in cohort of critically ill dogs with systemic inflammatory response syndrome (SIRS) and healthy controls on admission

Parameter	Units	Reference interval	Control dogs	*n*	SIRS dogs	*n*	*P* value
iCa	mmol/L	1.21–1.35	1.30 ± 0.03	35	1.22 ± 0.08	33	< 0.0001
Total calcium	mmol/L	2.25–2.95	2.6 [2.4–2.8]	35	2.3 [1.8–3.2]	33	<0.0001
Total calcium	mg/dL	9.0–11.8	10.2 [9.7–11.2]	35	9.3 [7.2–12.9]	33	< 0.0001
Anion gap	mmol/L	9–22	20 ± 3	35	23 [20–31]	33	0.008
BE	mmol/L	−2.0 to 2.0	0 [−1 to 3]	35	−2 [−10 to 5]	33	0.0001
Phosphorus	mmol/L	0.84–1.6	1.3 [0.65–1.6]	35	1.8 ± 0.8	33	0.001
Phosphous	mg/dL	2.6–4.9	4.1 [2.0–4.9]	35	5.5 ± 2.5	33	0.001
Creatinine	μmol/L	57.5–119.3	98.1 ± 14.1	35	77.8[38.8–556.0]	33	0.005
Creatinine	mg/dL	0.65–1.35	1.11 ± 0.16	35	0.88 [0.45–6.29]	33	0.005
UPC		0–0.4	0.09 [0.05–0.20]	35	0.80 [0.10–5.80]	29	< 0.0001
UAC		0–0.024	0 [0–0.020]	35	0.100 [0–3.100]	29	< 0.0001
FECa	%	0–0.5	0.09 [0.03–0.68]	35	0.23 [0.06–1.86]	33	0.0007
FEP	%	3–45	11.1 [0.9–48.1]	35	7.3 [0–61]	33	0.009
uUA:C			0.05 [0.03–0.15]	35	0.19 [0.01–0.62]	33	< 0.0001
$Apple_{fast}$ score			13 [7–15]	35	24 [14–39]	33	< 0.0001
CRP	mg/dL	0–0.5	0.28 [0.01–0.6]	35	7.9 [0.20–31.1]	33	< 0.0001
Fibrinogen	g/L	1.45–3.85	2.70 ± 0.70	35	3.25 [1.64–9.60]	33	< 0.0001
ATA	%	105–166	130 ± 14	35	86 ± 18	33	< 0.0001
Albumin	g/L	28.0–37.0	32.9 ± 3.1	35	24.6 ± 7.0	33	<0.0001
Albumin	g/dL	2.80–3.70	3.29 ± 0.31	35	2.46 ± 0.70	33	< 0.0001
TIBC	μmol/L	50.1–84.1	66.6 [51.9–86.6]	35	46.0 ± 15.4	33	<0.0001
TIBC	μg/dL	280–470	372 [290–484]	35	257 ± 86	33	< 0.0001
Total iron	μmol/L	13.4–50.1	28.5 ± 7.5	35	12.5 [5.2–37.9]	33	< 0.0001
Total iron	μg/dL	75–280	159 ± 42	35	70 [29–212]	33	< 0.0001

iCa, ionized calcium; BE, base excess; UPC, urinary protein to creatinine ratio; UAC, urinary albumin to creatinine ratio; FECa, fractional excretion of calcium; FEP, fractional excretion of phosphorus; uUA:C, urinary uric acid to creatinine ratio; CRP, C-reactive protein; ATA, antithrombin activity; TIBC, total iron-binding capacity.
Results are expressed as median and [range] or mean ± standard deviation based on data distribution.

and higher ATA at admission were less likely to die, confirming their prognostic significance in dogs with SIRS and critical illness, as previously reported.[6,7,12,17]

Ionized hypocalcemia has been associated with mortality and longer duration of hospital stay in critically ill dogs.[11,18,19] The pathophysiology of iHCa associated with critical illness remains unclear but could involve increased renal excretion of calcium.[30] No significant association between iCa and mortality, duration of ICU stay or severity of disease was identified in our population. A significant correlation between FECa and iCa in our population was not found, suggesting that calcium excretion should not have a major influence on blood iCa.

An increased loss of urinary proteins, particularly albumin, has been reported in dogs with SIRS and in a variety of ICU settings.[2,21] Presence of albuminuria may be a risk factor for death in critically ill veterinary patients.[20,22] Our findings support that proteinuria and albuminuria are common features during SIRS and SIRS-associated kidney injury.[21] UPC (but not UAC) was significantly different in survivors versus nonsurvivors in our population, suggesting that nonglomerular proteinuria could play a prognostic role in dogs during SIRS.[21] The possibility of a preexisting proteinuria could not be completely excluded and should be considered when interpreting these results.

Although the evaluation of FENa is influenced by numerous parameters, a value above 1% in human patients with acute kidney injury usually indicates intrinsic renal injury, while levels < 1% support prerenal azotemia.[31–33] FENa above 1% was reported in 5 of the 33 SIRS dogs, of whom 3 were azotemic. The significant correlation between FENa and both UAC and UPC might represent an additional relevant index of SIRS-associated kidney injury.

Urinary uric acid is a marker of oxidative stress in people and animals.[34] In normal dogs, 98–100% of glomerular filtrated uric acid is reabsorbed into the proximal tubule and metabolized by the liver.[35] The significant increase in uUA:C noted in dogs with SIRS (Table 2) might have resulted from tissue hypoperfusion or renal damage caused by the underlying condition, since none of the dogs included in our study had a known breed predisposition to hyperuricosuria. The relevance of this finding warrants further evaluation.

A number of limitations of the current study should be considered when interpreting the data presented.

Table 3: Comparison between survivors and nonsurvivors in respect to selected initial clinical and clinicopathological parameters in cohort of critically ill dogs with systemic inflammatory response syndrome (SIRS)

Parameter	Units	Reference interval	Survivors	*n*	Nonsurvivors	*n*	*P* value
Albumin	g/L	28.0–37.0	26.8 ± 5.9	22	20.4 ± 7.3	11	0.011
Albumin	g/dL	2.80–3.70	2.68 ± 0.59	22	2.04 ± 0.73	11	0.011
TP	g/L	56.0–79.0	69.3 ± 12.3	22	54.9 ± 15.8	11	0.007
TP	g/dL	5.60–7.90	6.93 ± 1.23	22	5.49 ± 1.58	11	0.007
ATA	%	105–166	94 ± 15	22	72 ± 15	11	0.0004
BE	mmol/L	−2.0 to 2.0	−1 ± 2	22	−4 [−10 to 5]	11	0.004
Anion gap	mmol/L	9–22	22 [20–30]	22	25 ± 3	11	0.025
Lactate	mmol/L	0–2	1.8 [0.5–7.5]	22	3.8 [2.5–8.6]	11	0.005
Creatinine	μmol/L	57.5–119.34	66.3 [39.8–245.8]	22	88.4 [65.4–556.0]	11	0.009
Creatinine	mg/dL	0.65–1.35	0.75 [0.45–2.78]	22	1.00 [0.74–6.29]	11	0.009
Urea	mmol/L	6.4–19.6	8.2 [4.2–79.3]	22	17.5 [8.6–106.7]	11	0.0005
Urea	mg/dL	18–55	23 [12–222]	22	49 [24–299]	11	0.0005
ALT	U/L	20–55	29 [11–163]	22	195 ± 213	11	0.016
UPC		0–0.4	0.83 ± 0.63	20	1.60 [0.20–5.80]	9	0.04
$APPLE_{fast}$score			22 ± 4	22	31 ± 4	11	<0.0001
iCa	mmol/L	1.21–1.35	1.20 ± 0.09	22	1.23 ± 0.07	11	0.80
CRP	mg/dL	0–0.5	8.2 [2.3–31.1]	22	7.3 [0.2–9.8]	11	0.44
Fibrinogen	g/L	1.45–3.85	3.02 [1.96–9.60]	22	4.65 ± 2.47	11	0.48
TIBC	μmol/L	50.1–84.1	48.3 ± 15.4	22	40.9 ± 15.0	11	0.22
TIBC	μg/dL	280–470	270 ± 86	22	229 ± 84	11	0.22

TP, total protein; ATA, antithrombin activity; BE, base excess; ALT, alanine transaminase; UPC, urinary protein to creatinine ratio; iCa, ionized calcium; CRP, C-reactive protein; TIBC, total iron-binding capacity.
Results are expressed as median and [range] or mean ± standard deviation based on data distribution.

Table 4: Correlations between the acute patient physiologic laboratory evaluation (APPLE) score, base excess, urinary: albumin:creatinine ratio, urinary protein:creatinine ratio and select laboratory parameters in a cohort of 33 dogs with systemic inflammatory response syndrome

APPLE score			Base excess			Urinary albumin: creatinine ratio			Urinary protein: creatinine ratio		
Parameter	***r***	***P* value**	**Parameter**	***r***	***P* value**	**Parameter**	***r***	***P* value**	**Parameter**	***r***	***P* value**
TP	−0.3	0.02	FECa	−0.4	0.03	FECa	0.42	0.02	FECa	0.57	0.001
Urea	0.4	0.004				FENa	0.48	0.008	FENa	0.5	0.004
BE	−0.4	0.02									
TBil.	0.4	0.01									
TIBC	−0.4	0.02									
ATA	−0.4	0.01									
UPC	0.3	0.03									

BE, base excess; UAC, urinary albumin to creatinine ratio; UPC, urinary protein to creatinine ratio; TP, total proteins; TBil, total bilirubin; TIBC, total iron-binding capacity; ATA, antithrombin activity; FECa, fractional excretion of calcium; FENa, fractional excretion of sodium.

The relatively small sample size may have resulted in insufficient statistical power for some of the investigated parameters (eg, iCa, UAC). Furthermore, the intrinsic limitations of the SIRS criteria may have resulted in the inclusion of a very heterogeneous population of patients in terms of disease processes and with varying severity. The low specificity of SIRS criteria[25] utilized may have allowed the inclusion of false-positive SIRS dogs in our population; however, measured APP were highly suggestive that dogs evaluated had systemic inflammatory processes. Four dogs with suspected infection (2 osteomyelitis, 1 dyskospondylitis, 1 pneumonia), but that did not have a focus of infection confirmed by cytology or bacterial culture results, were classified as nonseptic SIRS. The diagnostic challenges of identifying septic patients in the clinical setting could have biased the comparison between septic and nonseptic SIRS dogs in our study. Since we defined survival as an outcome, the inclusion among the nonsurvivors that died naturally and those that were humanely euthanized could have influenced the analysis of the data. However, the exclusion of dogs euthanized for financial constraints may have partly limited this bias.

Table 5: Univariate and multivariate binary logistic regression and receiver operator curve (ROC) analysis results of clinical and clinicopathological parameters measured at admission that were associated with the outcome (survivors/nonsurvivors) in 33 dogs with systemic inflammatory response syndrome (SIRS)

		Univariate binary logistic regression				ROC curve analysis		
Parameter	**Units**	**Regression coefficient**	**SE**	**Odds ratio [95% CI]**	***P* value**	**AUC [95% CI]**	**SE**	***P* value**
Albumin	g/dL	−1.799	0.809	0.166 [0.034–0.809]	0.026	0.785 [0.608–0.908]	0.104	0.006
TP	g/dL	−0.940	0.405	0.391 [0.177–0.864]	0.020	0.777 [0.599–0.903]	0.098	0.005
Creatinine	mg/dL	1.390	62.259	4.016 [1.185–13.607]	0.025	0.783 [0.606–0.907]	0.080	0.0006
Lactate	mmol/L	0.706	0.315	2.027 [1.092–3.761]	0.025	0.861 [0.693–0.957]	0.065	< 0.0001
BE	mmol/L	−0.424	0.199	0.655 [0.443–0.967]	0.033	0.826 [0.641–0.941]	0.103	0.002
Anion gap	mmol/L	0.325	0.164	1.384 [1.003–1.910]	0.048	0.795 [0.601–0.923]	0.085	0.0005
ATA	(%)	−0.114	0.043	0.893 [0.820–0.972]	0.009	0.855 [0.686–0.954]	0.066	< 0.0001
$APPLE_{fast}$ score		0.511	0.175	1.667 [1.183–2.346]	0.003	0.942 [0.798–0.994]	0.038	< 0.0001
		Multivariate binary logistic regression				**ROC curve analysis**		
$APPLE_{fast}$ score		0.560	0.213	1.751 [1.154–2.658]	0.008	**0.95∗ [0.793–0.998]**	**0.0468**	**< 0.0001**

SE, standard error; CI, confidence interval; TP, total protein; BE, base excess; ATA, antithrombin activity.
∗Sensitivity 80% and specificity 90.4% for $APPLE_{fast}$ score >27.
Only parameters with $P < 0.05$ are presented.

In conclusion, the present study underlines the usefulness of performing an extensive evaluation of traditional and newer blood and urinary biomarkers in a population of dogs with SIRS for prognostic purposes. The routine measurement of positive APP could improve the sensitivity and specificity of the criteria commonly used to detect SIRS in dogs.[25] Clinicopathological parameters, including lactate, BE, albumin, creatinine, UPC, and ATA were moderately accurate in predicting outcome in this study population. The $APPLE_{fast}$ score was highly accurate in predicting mortality in dogs with SIRS in the present study, hence its use in the clinical setting is recommended for the early assessment of critically ill dogs. Based on our data, screening of canine patients with SIRS for early renal injury is recommended. The potential role of using illness severity scores in guiding therapeutic decisions should also be further evaluated.

Footnotes

[a] S-Monovette, Sarstedt, Germany.
[b] Advia 2120 Hematology System, Siemens Healthcare Diagnostics, Tarrytown, NY.
[c] Lactate Scout Analyzer, Senslab, Leipzig, Germany.
[d] CRP OSR6147, Olympus/Beckman Coulter, Munich, Germany.
[e] Microalbumin OSR6167, Olympus/Beckman Coulter.
[f] Gentilini F, Mancini D, Dondi F, et al. Validation of a human immunoturbidimetric assay for measuring canine C-reactive protein. Vet Clin Path 2005; 34(suppl):318.
[g] UIBC OSR61205, Olympus/Beckman Coulter.
[h] Antithrombin III, Roche/Hitachi, Mannheim, Germany.
[i] IDEXX VetStat, IDEXX Laboratories, Westbrook, ME.
[j] Uric acid OSR6098 Olympus/Beckman Coulter.
[k] Olympus AU 400, Olympus/Beckman Coulter.
[l] MedCalc Statistical Software 9.5.2.0.

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Brief Communication

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Serum amyloid A in the diagnosis of feline sepsis

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Abstract. Systemic inflammatory response syndrome (SIRS) and sepsis can be challenging to diagnose in cats. Retrospectively, we investigated the diagnostic and prognostic potential of serum amyloid A (SAA), a major feline acute-phase protein (APP), in a population of critically ill cats with SIRS related to trauma or sepsis. A total of 56 SIRS cats (trauma n = 27; sepsis n = 29) were included and compared with healthy controls (n = 18). SAA concentration was significantly increased in SIRS cats compared to controls, confirming its potential for the detection of systemic inflammation in this species. Significantly higher values of SAA were detected in cats belonging to the sepsis group; however, according to the results of the receiver operating characteristic curve analysis, the value of using SAA (>81 mg/L) to discriminate septic cats was only moderate (AUC = 0.76). Additionally, cats with sepsis had significantly higher serum bilirubin concentrations and toxic neutrophil changes compared to the trauma group. Overall, 38 of 56 cats were survivors; 18 of 56 were non-survivors, with 83% of the non-survivors (15 of 18) belonging to the sepsis group. Serum bilirubin concentration, but not SAA, was able to predict outcome. Prospective studies are needed to assess the potential of SAA in the diagnosis of feline sepsis and outcome prediction.

Key words: Acute-phase protein; biomarker; feline; sepsis; serum amyloid A protein; trauma.

The systemic inflammatory response syndrome (SIRS) and sepsis are complex clinical syndromes in critically ill cats, and are associated with substantial disease effect and mortality.[3] Clinical response to sepsis is less predictable and specific in feline patients, potentially delaying early diagnosis and prompt treatment.[17]

Serum amyloid A (SAA), a major feline acute-phase protein (APP), is reported to increase early during inflammation and in cases of tissue damage.[11,12,18] Although its biological role is still unclear, SAA appears to have immunomodulatory activities and protective properties.[16,18] SAA has been shown to be a significant prognostic marker in sick cats with various diseases[20]; however, its potential to discriminate between a septic and a non-septic origin of SIRS is not well defined.[16]

Diagnosis of feline sepsis is still challenging, given that veterinary reports that have investigated the diagnostic performance of selected clinicopathologic variables are limited.[3] A higher percentage of circulating band neutrophils and more severe hypoalbuminemia were associated with the presence of sepsis in a small prospective case-control study of cats with SIRS.[3] The presence of toxic neutrophils was shown to be a common finding in feline diseases of inflammatory and/or infectious origin.[19]

We investigated the potential diagnostic utility of serum SAA concentrations and selected clinicopathologic variables measured at the time of hospital admission for discriminating between SIRS of infectious (sepsis) and non-infectious (trauma) origin in a population of critically ill cats. In addition, SAA data were also assessed for outcome prediction.

Critically ill cats with a clinical condition of SIRS related to trauma or sepsis, hospitalized at the ICU of the University of Bologna Veterinary Teaching Hospital between March 2012 and March 2014, were included retrospectively. The presence of SIRS was defined according to published criteria.[2] Additional inclusion criteria were hospitalization in ICU, complete medical records, and SAA evaluated upon admission, or presence of at least an aliquot of serum collected upon ICU admission and stored frozen at −80°C. The overall population of SIRS cats was divided into 2 subgroups according to the infectious or non-infectious SIRS origin (sepsis vs. trauma). Specifically, the trauma group included cats with blunt trauma associated with motor vehicle accident or high-rise syndrome; the sepsis group included cats with cytologic or bacteriologic evidence of bacterial infection. SIRS cats were also classified as survivors (alive to discharge) or non-survivors (died despite medical treatment or euthanized because of moribund conditions or end-stage disease). Cats euthanized for financial reasons or discharged against medical advice were excluded from

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Table 1. Clinicopathologic results of controls and cats with systemic inflammatory response syndrome (sepsis and trauma groups) analyzed with the Kruskal–Wallis analysis of variance test for comparisons. Only variables with $p < 0.05$ are presented.

Variable	Controls (n = 18)	Sepsis (n = 29)	Trauma (n = 27)
SAA (mg/L)	4 (1–9)	173[a] (1–265)	28[ab] (1–258)
ALT (U/L)	51 (35–80)	49 (15–3,600)	292[ab] (70–3,300)
AST (U/L)	24 (15–36)	130[a] (20–4,000)	310[ab] (43–2,500)
Total bilirubin (μmol/L)	1.7 (1.2–5.8)	6.8[a] (1.7–114)	3.4[ab] (1.7–32)
Albumin (g/L)	37 (33–41)	34[a] (10–41)	28[ab] (15–38)
Total protein (g/L)	81 (72–88)	67[a] (44–107)	59[ab] (29–83)
A/G	0.87 (0.63–1.2)	0.51[a] (0.25–0.94)	0.92[a] (0.51–1.3)
Creatinine (μmol/L)	131 (113–169)	98[a] (45–1,630)	101[ab] (70–597)
Glucose (mmol/L)	5.6 (1.8–8.2)	5.8 (0.3–15.0)	9.3[ab] (3.1–19.1)
Leukocytes (cells × 10^9/L)	8.4 (2.7–14.9)	27.0[a] (0.5–63.6)	13.8[a] (4.3–37.9)
Hematocrit (L/L)	0.41 (0.35–0.47)	0.33[a] (0.18–0.50)	0.31[a] (0.06–0.42)

Values for each analyte are presented as median and range (in parentheses). A/G = albumin-to-globulin ratio; ALT = alanine transaminase; AST = aspartate transaminase; SAA = serum amyloid A. Superscript letters indicate significant differences: from controls ([a]); from sepsis group ([b]).

the study. Controls were client-owned blood donor cats (n = 18) considered healthy according to history, physical examination, and clinicopathologic data.

Hematologic and biochemistry profiles performed upon hospital admission were reviewed in all of the cats enrolled. When SAA values were not available, frozen serum samples were used for analysis. Blood count was determined by an automated hematology system (ADVIA 2120 hematology system, Siemens Healthcare Diagnostics, Tarrytown, NY). May-Grünwald/Giemsa–stained (Merck, Darmstadt, Germany) blood smears were examined, and neutrophil morphology and toxic changes were recorded. The occurrence of Döhle bodies, cytoplasmic basophilia, vacuolation or foaminess, and toxic granulation of neutrophils were recorded.[19] SAA concentrations were measured on serum samples using a commercial immunoturbidimetric assay designed for human SAA (LZ Test Eiken SAA, Eiken Chemical, Tokyo, Japan), as validated previously for cats in our laboratory.[7] All biochemical analyses were performed with an automated chemistry analyzer (AU 400, Olympus/Beckman Coulter, Munich, Germany). All of the investigated variables were also measured in healthy controls.

Nonparametric statistics with post hoc analysis were used to compare variables between the different groups. Data were expressed by standard descriptive statistics and presented as median and range. Categorical variables were compared using Fisher exact test. Univariate logistic regression was used to assess the association between clinical and clinicopathologic variables at the time of hospital admission and the diagnosis of sepsis. Variables associated with a diagnosis of sepsis in the univariate analyses were entered into a multivariable model (stepwise selection). Binary logistic results were presented as odds ratio (OR) and 95% confidence interval (CI). Overall model fit was assessed by the percentage of outcome correctly classified by the receiver operating characteristic (ROC) curve analysis and by a significant Hosmer–Lemeshow test ($p > 0.05$). ROC curve analysis was used to find optimal cutoff values for variables predicting sepsis and to calculate the area under the ROC curve (AUC). Correlation between variables was assessed using the Spearman rank correlation coefficient. A p value <0.05 was considered significant. All analyses were performed using MedCalc statistical software (v.15.6.1, MedCalc Software, Ostend, Belgium).

A total of 56 cats with SIRS satisfied the inclusion criteria and were included in the study. The median age was 4.8 y (0.5–21), and the sex distribution was as follows: 18 of 56 male neutered, 15 of 56 spayed females, 13 of 56 intact males, and 10 of 56 intact females. Overall, 27 of 56 cats were included in the trauma group; 29 of 56 cats were included in the sepsis group. The origin of sepsis was related to pyothorax (10 of 29), septic peritonitis (7 of 29), pyelonephritis (4 of 29), septic arthritis (1 of 29), systemic toxoplasmosis (1 of 29), septic cholangitis (2 of 29), and suppurative cellulitis or abscesses (4 of 29). Data regarding lifestyle were available for 29 of 56 patients; among them, only 3 of 29 lived indoors exclusively; the others were outdoor or indoor and outdoor cats. Frequency of an outdoor lifestyle was not significantly different between the trauma (15 of 27) and the sepsis group (11 of 29). Overall, 38 of 56 (68%) cats were survivors; 18 of 56 (32%) were non-survivors; diagnosis of sepsis was significantly associated with a higher mortality rate (15 of 18, 83%).

Cats in the sepsis group had significantly increased serum SAA and total bilirubin concentration, and a significantly higher frequency of toxic neutrophil changes in blood smears compared to both the trauma group and controls (Table 1). SAA, total bilirubin concentrations, and the presence of toxic neutrophil changes were significantly associated with a diagnosis of sepsis (Table 2). When the multivariate logistic regression was performed, the only variables retained in the model were SAA concentration (OR = 1.01, CI = 1.0–1.02)

Table 2. Univariate and multivariate binary logistic regression of clinicopathologic variables associated with sepsis diagnosis at the time of hospital admission in cats with systemic inflammatory response syndrome. Only variables with $p < 0.05$ are presented.

Variable	Regression coefficient	Standard error	Odds ratio
Univariate binary logistic regression			
Total bilirubin (μmol/L)	0.10	0.048	1.10 (1.00–1.21)
SAA (mg/L)	0.01	0.003	1.01 (1.0–1.01)
Toxic changes (y/n)	2.20	0.67	9.02 (2.4–33.86)
Multivariate binary logistic regression			
SAA (mg/L)	0.01	0.004	1.01 (1.0–1.02)
Toxic changes (y/n)	2.34	0.79	10.4 (2.18–49.79)

Numbers in parentheses are 95% confidence intervals. SAA = serum amyloid A; y/n = presence/absence.

and neutrophil toxic changes (OR = 10.4, CI = 2.2–49.8). However, according to the ROC curve analysis, no significant difference to correctly predict a diagnosis of sepsis was found among serum SAA, serum bilirubin, and neutrophil toxic changes: serum SAA >81 mg/L had a 79.3% sensitivity and a 77.7% specificity (AUC = 0.76); serum bilirubin >3.8 μmol/L had a 72.4% sensitivity and a 74% specificity (AUC = 0.79). The value of AUC for the neutrophil toxic changes was 0.74. No significant correlation was noticed between SAA concentration and the other investigated variables. No difference between survivors and non-survivors was detected for serum SAA concentration and neutrophil toxic changes upon admission. Conversely, serum bilirubin >3.6 μmol/L had a 94.4% sensitivity and a 68.4% specificity (AUC = 0.85) to predict outcome in cats included with a diagnosis of sepsis.

Despite the limited value of the SIRS criteria in humans and dogs,[8,10] diagnosis of sepsis still relies on the evaluation of these criteria in clinical settings. Similar criteria in cats derive from a retrospective autopsy-based study, and their specificity and sensitivity for the detection of feline sepsis have not been reported and evaluated extensively. Diagnosis of feline sepsis is additionally challenging because of the peculiar clinical manifestations of SIRS and the poor diagnostic value of leukocyte counts in cats.[2,16]

In our study, significantly higher SAA concentrations were documented in cats with SIRS of infectious and non-infectious origin compared to healthy controls. This result confirms the role of this APP for the detection of systemic inflammation in cats, and suggests its potential value as a biomarker of feline sepsis. The lack of statistical correlation between SAA concentration and leukocyte count in our population raises a concern regarding the traditional methods used to detect systemic inflammation in cats. Furthermore, significantly higher concentrations of SAA were documented in cats with sepsis compared to cats with trauma. Although higher values of SAA were previously reported in cats with inflammatory and/or infectious diseases compared to other sick cats,[18] we evaluated the potential of SAA in discriminating between septic and non-septic SIRS. However, according to the results of the ROC curve analysis, the performance of a SAA arbitrary cutoff (>81 mg/L) to correctly predict a diagnosis of sepsis in this cohort of animals was only moderate.

Cats included in the sepsis group had significantly increased serum bilirubin concentrations compared to those in the trauma group. Furthermore, this variable was able to predict outcome in the former group, although the accuracy was only moderate. Hyperbilirubinemia is a common finding in critically ill septic humans, being potentially related to sepsis-induced cholestasis or hepatic damage or dysfunction.[9] Increased bilirubin concentration and icterus have been reported previously as common abnormalities in cats with sepsis,[2] potentially indicating the presence of cholestasis in cats with this condition. Further studies evaluating the incidence of cholestasis and its prognostic significance in feline sepsis are warranted.

Leukocytosis with left shift has been reported to be more common in septic cats compared to cats with non-infectious SIRS,[3] and the presence of toxic neutrophils has been associated with various infectious feline diseases.[19] According to our findings, toxic neutrophil changes associated with elevated SAA values may aid clinicians in the diagnosis of feline sepsis.

No association between SAA concentration measured at the time of hospital admission and final outcome was identified in our population of SIRS cats. In humans, elevated SAA concentrations predicted outcome in people with neoplastic or immune-mediated diseases,[5,15] but failed to reach this purpose in other conditions.[1] Similar controversies with respect to prognosis have been reported for SAA in horses,[4,21] and for other APPs in veterinary medicine,[6,14] suggesting that serial monitoring or combined APP profiles rather than single APP values may better predict outcomes. In a retrospective study including 175 cats with various diseases (neoplastic, inflammatory, and other diseases), cats with a SAA concentration above the reported reference interval at the time of the first evaluation had a significantly shorter median survival time compared to cats with non-elevated SAA concentration, regardless of the final diagnosis.[20] However, diagnostic criteria of these diseases were not uniform in the latter study and different treatments before and after diagnosis among the cats might have affected the prognosis.[20] Furthermore, mortality data of the cats with neoplasia were more abundant compared with the cats with other diseases. This might have led to a biased result.

The overall mortality rate for cats with SIRS was 32% in our study, with 83% of the non-survivors belonging to the sepsis group; the mortality rate in the latter group was 52%.

These data are consistent with other reports of mortality of septic cats.[3,13]

Several limitations exist in our study. We included in the sepsis group only SIRS cats with bacteriologic or microbiologic confirmation of sepsis, being confident of their final diagnosis. However, we cannot exclude that some of the cats included in the trauma group may have indeed developed septic complications upon admission or during hospitalization. We included a relatively small number of patients, and this may have limited the statistical evaluation or the prognostic power of the investigated variables. Additionally, because the study was retrospective, we were not able to evaluate a clinical scoring system to stratify patients according to the severity of the clinical condition, preventing further statistical comparisons. Finally, blood sampling was performed at the time of hospital admission only; monitoring over time could have improved the prognostic value of the investigated variables.

Our results support the value of SAA as a potential diagnostic marker of feline SIRS and sepsis. The power of serum SAA in predicting the occurrence of sepsis was only moderate in this population; however, association with the measurement of serum bilirubin concentration and evaluation of the blood smear for the presence of neutrophil toxic changes may further aid the diagnosis of sepsis in cats. The prognostic potential of serum bilirubin in feline sepsis seems promising. Finally, further prospective large-cohort studies are needed to assess the prognostic significance of SAA, serum bilirubin, and neutrophil toxic changes in cats with SIRS, and to assess their value for the early detection of feline sepsis and the prompt recognition of critically ill patients.

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7.3 The Delta Neutrophil Index: background

Granulocytes are key effectors of the host response to inflammation and infection. During severe SIRS and in presence of infection, less mature granulocytes forms enter circulation, including an increased number of bands, metamyelocytes or myelocytes. Presence of immature granulocytes (IG) is still used as a marker of infection or sepsis in clinical practice. Similarly, morphologic changes of granulocytes including toxic granulation, Dohle bodies and cytoplasmatic vacuoles have good sensitivity in predicting infection in humans (Seebach et al. 1997; Park et al. 2011; Mare et al. 2015). Increased IG have been related with disease severity, systemic complications and worse prognosis in septic people (Mare et al. 2015). Whether IG reflect sepsis severity or actively contribute to organ dysfunction is still a matter of debate: interestingly, IG are usually characterized by altered rheological properties compared to mature cells, and might promote obstruction of small vessels, endothelial injury and impaired microcirculation during severe sepsis and septic shock. Additionally, few experimental and human studies indicated that IG have impaired microbicidal functions, and might concurr to immunoparalysis and MODS development (Poschl et al. 2005; Leliefeld et al. 2016). The main limitation to IG assessment and enumeration is related to the manual blood smear examination, which is operator-dependant, non-repeatible and time-consuming (Park et al. 2011).

The Delta Neutrophil Index (DNI) is a relatively new biomarker of sepsis in people. Despite the increasing number of studies evaluating its value in sepsis diagnosis and prognostication, DNI is still unfamiliar to many clinicians and has not been studied world-wide (Park et al. 2017). The DNI can be calculated by hematological analyzers of the ADVIA series (ADVIA 120, Siemens, inc.) as the difference in leukocyte subfractions measured by a cytochemical myeloperoxidase reaction and by a nuclear lobularity assay. Previous human studies demonstrated a high correlation between DNI value and manual IG count, pointing its role in representing the circulating fraction of IG (Nahm et al. 2008). Several investigators documented the usefulness of the DNI to predict early diagnosis, disease severity and outcome in patients with sepsis (Cha et al. 2016). Greater DNI values have

been demonstrated in critically ill patients with sepsis compared to sterile inflammatory conditions mimicking infection, including superimposed pneumonia in heart-failure patients, acute graft pyelonephritis, spontaneous bacterial peritonitis and bacteremia (Park et al. 2011; Lim et al. 2014; Shin et al. 2015; Cha et al. 2016). A recent meta-analysis including 12 articles on the performances of DNI in sepsis reported a pooled sensitivity of 0.67 and specificity of 0.94 (area under the curve 0.89) for the DNI as a predictive factor for infection. Similar data (sensitivity 0.70, specificity 0.78, area under the curve 0.84) were documented for the DNI as a prognostic factor for death. Hence, the DNI seems to have a moderate specificity for sepsis diagnosis, being applicable as a confirmative diagnostic tool, and a moderate prognostic role comparable to other well-studied biomarkers as procalcitonin (Park et al. 2017).

Recent investigations focused on IG as diagnostic and prognostic tools in dogs and cats (Segev et al. 2006; deClue et al. 2011; Burton et al. 2013; Burton et al. 2014). The ADVIA-series hematological analyzers have been used in veterinary medicine to support the manual evaluation of the blood smear. Novel ADVIA parameters able to reflect changes in neutrophil maturity and toxicty have been assessed in dogs. For istance, lower myeloperoxidase index indicating acquired myeloperoxidase deficiency has been demonstrated during localized and systemic canine infections (Klenner et al. 2010). Similarly, changes in myeloperoxidase index and lobularity index suggested myeoperoxidase deficiency and left-shift, respectively, in experimental and spontaneous canine ehrlichiosis (Gianopoulos et al. 2017). Being based on human calculations, the validity of such markers in veterinary patients is still uncertain. However, confirming their significance in future studies might be beneficial, and will emphasize the value of the routinary hematological profile without additional costs or requirements.

As a part of the current PhD research (2014-2016), we conducted a study to retrospectively evaluate the diagnostic and prognostic significance on the DNI in dogs with sepsis and non-infectious SIRS. Preliminary results were presented at the 2016 EVECCS Congress as an oral presentation; the full paper is provided below.

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Original Article

Evaluation of the delta neutrophil index from an automated blood cell analyser in septic dogs

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ABSTRACT

Immature granulocytes (IG) are a marker of severe inflammatory states in human beings and animals, and have been linked to a diagnosis of sepsis and poor prognosis. The delta neutrophil index (DNI), automatically calculated by a haematological analyser, provides an estimate of circulating IG. In particular, an increased DNI value has been associated with the severity of sepsis, and mortality, in critically ill human beings. The aims of this study were to determine the DNI reference interval (RI) in healthy dogs, and to evaluate its diagnostic and prognostic significance in dogs with sepsis. A total of 118 dogs with sepsis undergoing a complete blood cell count (CBC) at the time of hospital admission were included retrospectively. Dogs with sepsis were compared to 20 dogs with primary immune-mediated haemolytic anaemia (IMHA) and 99 healthy controls. The DNI RI was set from 0 to 9.2%. The DNI was significantly higher in dogs with sepsis compared to dogs with IMHA and healthy dogs ($P < 0.001$), and significantly higher in dogs with septic shock compared to septic dogs without circulatory failure ($P < 0.03$). No differences were detected between survivors (78/118) and non-survivors (40/118). Septic dogs with a DNI above the RI had significantly higher frequencies of IG and toxic neutrophil changes on manual blood smear evaluation ($P = 0.03$ and $P < 0.001$, respectively). The DNI had a fair performance in identifying dogs with sepsis in this population and predicted septic shock. Larger prospective studies are needed to validate DNI measurement in dogs and to test its clinical utility.

Introduction

Sepsis is a common disease recognised in the intensive care unit (ICU) and results in high morbidity and mortality in human and veterinary patients (Silverstein, 2012; Gotts and Matthay, 2016). Despite a reduction of in-hospital mortality in the last 40 years, the incidence of sepsis seems to have increased in human beings (Gotts and Matthay, 2016) and the early diagnosis of this syndrome remains a major goal in order to implement prompt and effective treatment (Silverstein, 2012; Hayden et al., 2016).

Neutrophil precursors, including band neutrophils, metamyelocytes and myelocytes, are defined as immature granulocytes (IGs) (Stockham and Scott, 2008; Mare et al., 2015). They are released into the circulation in response to severe inflammation and are included in the diagnostic criteria for systemic inflammatory response syndrome (SIRS) in human beings and animals (Hauptman et al., 1997; Nierhaus et al., 2013; Mare et al., 2015). Recent evidence suggests an association between increased blood concentrations of IGs and a diagnosis of sepsis in human beings (Nierhaus et al., 2013; Mare et al., 2015). In addition, increased concentrations of IGs have been associated with increased disease severity, progression and poor prognosis in different settings of human sepsis (Mare et al., 2015). IGs have altered rheological properties compared to mature cells (Poschl et al., 2005) and, due to poor cell membrane deformability, they may accumulate in specific microvasculature sites, promoting endothelial injury, microcirculatory impairment and local organ dysfunction (van Eden et al., 1997; Poschl et al., 2005). Obstruction of small vessels from activated immature leukocytes has been demonstrated in animal models of low perfusion pressure conditions, such as shock and local ischaemia (Hansell et al., 1993), and in children with Gram negative septicaemia (Poschl et al., 2005).

The potential diagnostic and prognostic value of circulating IGs has been recognised in a number of veterinary studies (Segev et al., 2006; Burton et al., 2013, 2014). The presence of a leukocyte degenerative left shift, defined as the number of IGs exceeding the number of mature neutrophils in circulation, has been associated with an increased risk of death or euthanasia in dogs (Burton et al., 2013). Although the definition of a degenerative left shift is still questionable in respect of the overall number of mature

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neutrophils to consider (Stockham and Scott, 2008), these data suggest that the presence of IGs may have clinical relevance in diseased animals.

The presence and magnitude of a degenerative left shift may be associated with worse outcomes in cats (Burton et al., 2014). In a prospective study, cats with sepsis were more likely to have severe left shift compared to cats with non-infectious inflammation, although no association between IGs and outcome was identified (DeClue et al., 2011). However, although assessment of circulating IGs by manual evaluation of blood smears currently represents the gold standard for leukocyte classification in veterinary medicine, it can be operator dependent and time consuming (Stockham and Scott, 2008; Park et al., 2011).

The delta neutrophil index (DNI) represents the fraction of circulating IGs in the peripheral blood; it is automatically calculated by the ADVIA-series haematological analysers as the leukocyte difference between the myeloperoxidase channel and the nuclear lobularity channel count (Nahm et al., 2008). The DNI has been associated with positive blood cultures, disseminated intravascular coagulation scores, progression of disease and mortality in septic adult and neonatal human patients (Nahm et al., 2008; Park et al., 2011; Lee et al., 2013; Kim et al., 2014). The DNI also appears to be helpful in identifying human patients with an impending risk of organ dysfunction and septic shock (Park et al., 2011).

No data regarding the diagnostic performance of the DNI have been reported in veterinary medicine. The primary aim of the current study was to evaluate the diagnostic and prognostic significance of the DNI in canine sepsis. A specific preliminary aim was to determine the DNI reference interval (RI) in a population of healthy control dogs.

Materials and methods

Study design

This was a retrospective study conducted at the Veterinary Teaching Hospital of the University of Bologna. The study included three groups of dogs: (1) dogs with sepsis; (2) dogs with primary immune-mediated haemolytic anaemia (IMHA); and (3) healthy control dogs. Dogs with sepsis comprised critically ill septic dogs hospitalised in the ICU from January 2014 to January 2016 with a complete blood count (CBC) performed by a standard operative procedure (ADVIA 2120, Siemens Healthcare Diagnostics) at the time of hospital admission. Medical records including outcome information were reviewed. Bacterial sepsis was defined as the presence of SIRS (Hauptman et al., 1997) plus evidence of a septic focus by means of cytology or microbiology. Molecular and serological analyses were used for the diagnosis of specific diseases. Parvoviral enteritis was confirmed by a positive real-time PCR on faecal samples (Battilani et al., 2011). Leptospirosis was diagnosed by combining suggestive clinical and clinicopathological abnormalities with a positive leptospiral microagglutination test (MAT) on serum samples (single titre $\geq$ 1:800 for non-vaccinal serogroups and/or a four-fold increase in titre in paired sera; Mastrorilli et al., 2007).

A diagnosis of primary IMHA was based on the presence of anaemia (packed cell volume $<$ 37%) associated with a positive saline agglutination test and/or a positive Coombs' test and/or evidence of moderate to marked spherocytosis on examination of blood smears (Goggs et al., 2015). Dogs that had received immunosuppressive treatment for IMHA and dogs with evidence of predisposing disease were excluded.

Haematological data of blood donor dogs considered to be healthy according to complete clinical and clinicopathological data were selected in order to determine the DNI RI and for comparative purposes.

Classification of dogs with sepsis

Dogs with sepsis were categorised according to severity of severity (sepsis; severe sepsis; septic shock) based on the following criteria present upon admission or during hospitalisation: (1) severe sepsis was defined by evidence of dysfunction in one of more organs (Kenney et al., 2010); and (2) septic shock was defined as persistent hypotension (systolic blood pressure $<$ 90 mmHg) despite adequate fluid resuscitation (Silverstein and Beer, 2015). Dogs with sepsis were further divided into survivors (alive at hospital discharge) and non-survivors (died despite medical therapy or humanely euthanased because of moribund conditions or end-stage disease).

Haematological analysis

Blood was collected by peripheral vein or jugular venipuncture using a vacuum system and K_3 ethylene diamine tetra-acetic acid (EDTA) tubes (Vacutest Kima) and analysed on a routine basis within 4 h in all dogs. In addition to the CBC, the DNI was calculated automatically by the system and expressed as a percentage according to the following formula (Park et al., 2011):

DNI (%) = (the neutrophil and eosinophil sub-fractions assayed in the myeloperoxidase channel by cytochemical reaction) − (the polymorphonucleated sub-fraction counted in the nuclear lobularity channel by the reflected light beam).

Cellular morphology and toxic changes were assessed by microscopic examination of blood smears stained by the May–Grünwald–Giemsa technique (Merck KGaA, 64271 Darmstadt). Chemistry profiles including C-reactive protein concentrations (CRP) (CRP OSR6147, Olympus/Beckman Coulter) performed at the time of hospital admission were reviewed retrospectively. Analyses were performed using an automated chemistry analyser (AU 400, Olympus/Beckman Coulter). The Acute Patient Physiological and Laboratory Evaluation (APPLE) fast score was retrospectively calculated in the sepsis, IMHA and healthy control groups (Hayes et al., 2010).

Statistical analysis

Normality was tested graphically and using the D'Agostino Pearson test. In view of the non-normal distribution of most variables, non-parametric testing was adopted for all analyses. Data were evaluated using standard descriptive statistics and are reported as median (range). The DNI RI of healthy dogs was determined using the robust method, and 90% confidence intervals (CIs) were specified. Differences between groups were evaluated using the Mann–Whitney U test and the Kruskal–Wallis one-way analysis of variance. If the Kruskal–Wallis test result was significant, a post-hoc analysis for pairwise comparison of subgroups was performed. Receiver operating characteristic (ROC) curve analysis was used to find the optimal cut-off value of the DNI for prediction of sepsis and septic shock, and the presence of IGs and toxic neutrophils on evaluation of blood smears. The areas under the ROC curves (AUC) were compared according to Hanley and McNeil (1983). Correlations between variables were assessed using Spearman's rank correlation coefficients. Categorical results were compared using Fisher's exact test. Statistical analyses were performed using MedCalc Statistical Software version 13.3.1 bvba and GraphPad Prism version 7.02 for Windows. A P value of $<$0.05 was considered to be significant.

Results

A total of 118 dogs with sepsis were considered to be eligible according to the inclusion criteria and were enrolled in the study. Thirty-six of 118 (31%) dogs were intact males, 7/118 (6%) were castrated males, 58/118 (49%) were intact females and 17/118 (14%) were spayed females. The median age was 8 years (range 2 months to 16 years), and the median body weight was 20 kg (range 2–68 kg). Causes of sepsis included pyometra (n = 47), leptospirosis (n = 15), septic peritonitis (n = 16), parvoviral enteritis (n = 13) and miscellaneous causes (urosepsis, n = 6; pneumonia, n = 5; septic cholangitis, n = 4; bite wounds, n = 3; deep pyoderma, n = 2; septic arthritis, n = 2; endocarditis, n = 2; pyothorax, n = 2; septic pericarditis, n = 1). Fifty-two of 118 (44%) dogs were diagnosed with severe sepsis, 12 dogs (10%) were diagnosed with septic shock and 54/118 (46%) had sepsis without evidence of organ dysfunction. Seventy-eight of 118 (66%) dogs were survivors, while 40/118 (34%) were non-survivors; 20 dogs died and 20 were euthanased because of moribund conditions. All dogs with septic shock (n = 12) did not survive to hospital discharge.

Twenty dogs with IMHA were included in the study. Nine of 20 (45%) were intact males, 1/20 (5%) was a castrated male, 4/20 (20%) were intact females and 6/20 (30%) were spayed females. The median age was 7.5 years (range 8 months to 13 years) and the median body weight was 14.6 kg (range 4.3–29.4 kg).

Ninety-nine blood donor dogs were enrolled as healthy controls. Forty-six of 99 (47%) were intact males, 4/99 (4%) were castrated males, 26/99 (26%) were intact females and 23/99 (23%) were spayed females. The median age was 3 years (range 1–9 years) and the median body weight was 27.7 kg (3–70 range).

Table 1
Reference intervals (RIs) and medians (ranges) of APPLE fast scores, delta neutrophil index (DNI) values, C-reactive protein (CRP) concentrations and complete blood counts in dogs with sepsis ($n = 118$), immune-mediated haemolytic anaemia (IMHA; $n = 99$) and healthy control dogs ($n = 20$).

	RI	Sepsis	IMHA	Control group
APPLE fast score	0–50	22 (7–43)	28 (17–39)	11 (7–15)[a,b]
DNI %	0–9.2	4.6 (0.0–86.4)[b,c]	0.6 (0.0–44.1)	2.6 (0.0–14.6)
CRP mg/dL	0–0.5	9.01 (0.34–41.40)	9.44 (5.56–33.84)	0.22 (0.01–0.5)[a,b]
Albumin g/dL	2.8–3.7	2.49 (1.02–4.11)	2.51 (1.76–3.42)	3.32 (2.56–3.89)
Lactate mmol/L	0.2–1.5	1.7 (0.6–16.0)	1.85 (0.70–10.80)	1.2 (0.5–1.8)[a,b]
Glucose mg/dL	70–125	94 (13–725)	98 (49–192)	87 (70–110)
HCT %	37.0–55.0	41.95 (8.4–68.3)	12.75 (4.80–28.00)	48.15 (43.50–57.60)
Hb g/dL	12–18	14.4 (2.5–21.1)	4.3 (2.0–8.9)	16.3 (14.7–16.5)
MCH pg	19.5–24.5	22.7 (19.0–73.1)	24.4 (22.2–60.3)	23.7 (20.9–25.2)
MCV fL	60.0–77.0	66.0 (25.5–81.9)	73.5 (59.1–111.6)	67.5 (62.1–72.4)
MCHC g/dL	32.0–38.0	34.2 (29.9–39.2)	33.75 (26.50–72.40)	34.8 (33.7–35.9)
RDW %	13.0–15.7	12.9 (11.3–20.2)	20.0 (12.7–39.5)	12.6 (11.8–20.2)
RBCs $\times 10^6$/mm^3	5.5–8.5	6.37 (1.23–9.63)	1.67 (5.70–3.69)	7.17 (6.53–8.65)
WBC $\times 10^3$/mm^3	6–17	19.1 (0.3–101.9)[b,c]	25.5 (7.6–68.7)[c]	9.5 (5.4–20.4)
Neutrophils/mm^3	3000–12,000	15,010 (50–80,320)	19400 (6130–62,426)	6505 (4060–10,320)
Band neutrophils/mm^3	0–300	0.0 (0.0–5981.0)[b]	0.5 (0.0–7546.0)[a]	0.0 (0.0–0.0)[a,b]
Lymphocytes/mm^3	1000–4800	1670 (50–37,920)	2445 (920–8980)	2205 (1710–4270)
Monocytes/mm^3	100–1400	1380 (7–10,830)	1640 (310–3018)	500 (150–910)
Eosinophils/mm^3	0–750	110 (0–2380)	140 (0–740)	620 (100–1710)
Basophils/mm^3	0–180	80 (0–1480)	40 (0–650)	55 (20–100)
Platelets $\times 10^3$/mm^3	160–500	266 (16–3800)	147.5 (4.1–465.0)	269 (184–453)
MPV fL	6.6–10.9	12.85 (7.70–30.80)	14.4 (10.8–35.7)	11.3 (9.5–14.2)

HCT, haematocrit; Hb, haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell; RDW, red blood cell distribution width; WBC, white blood cell; MPV, mean platelet volume.
[a] Significantly different from sepsis ($P < 0.05$).
[b] Significantly different from IMHA ($P < 0.05$).
[c] Significantly different from control group ($P < 0.05$).

The RI for the DNI was set 0–9.2% (90% CI 7.8–10.5%). Results of the comparison among groups, including descriptive DNI values, CRP concentrations and the APPLE fast score in septic, IMHA and healthy dogs, are reported in Table 1. Dogs with sepsis had significantly higher leukocyte counts and DNI values than controls, and significantly lower leukocyte counts and higher DNI values compared with dogs with IMHA ($P < 0.05$) (Table 1,Fig. 1a). According to the results of the ROC curve analysis, a DNI > 2.3% had a sensitivity of 67% and a specificity of 75% to correctly predict the diagnosis of sepsis (AUC = 0.71; $P < 0.001$). Dogs with septic shock had significantly higher DNI and APPLE fast score results, and significantly lower leukocyte counts, than dogs with sepsis without circulatory failure ($P = 0.03$, $P < 0.001$ and $P = 0.01$, respectively) (Table 2; Fig. 1b). According to the results of the ROC curve analysis, a DNI > 14.3% had a sensitivity of 58.3% and a specificity of 89.9% to correctly predict septic shock (AUC = 0.69; $P = 0.02$). Comparing ROC curves, no significant differences were found in the prediction of septic shock between DNI, APPLE fast score and leukocyte count (data not shown). When results were compared according to the cause of the septic disease, significantly higher DNI values were documented in dogs with septic peritonitis, pyometra and miscellaneous causes of sepsis, while the APPLE fast score was significantly higher in dogs with septic peritonitis, parvoviral enteritis and miscellaneous causes (Table 3).

Non-survivors had a significantly higher APPLE fast score than survivors; values > 23 had a sensitivity of 81% and a specificity of 67% to correctly predict outcome (AUC = 0.77; $P < 0.0001$). Conversely, serum CRP concentrations, leukocyte count and DNI values were not different according to the final outcome (Table 4).

In dogs with sepsis and IMHA, the frequencies of IGs were 20/118 (17%) and 10/20 (50%), respectively, while the frequencies of toxic neutrophils were 35/118 (30%) and 1/20 (5%), respectively. Both proportions were significantly different between the two groups ($P < 0.05$). Septic dogs with a DNI value above the reported RI (>9.2%) had significantly higher frequencies of IGs and toxic neutrophil changes at the manual blood smear evaluation compared to septic dogs with a DNI value within the RI (42% vs. 6%, $P < 0.01$; 61% vs. 28%, $P = 0.004$, respectively) (Figs. 2a and b).

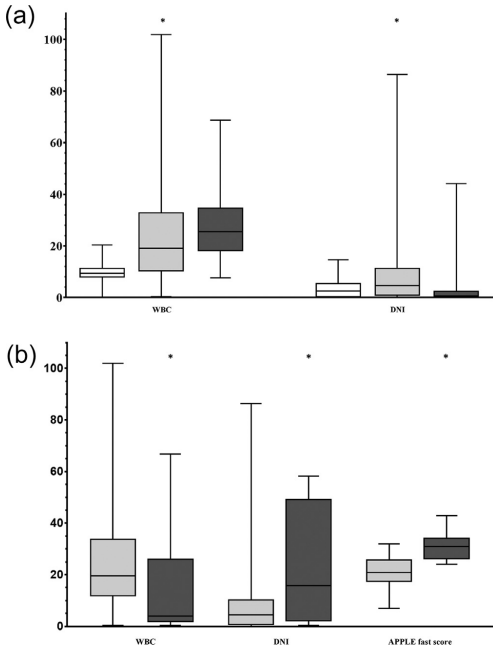

Fig. 1. (a) Box and whisker plots of white blood cells (WBCs, cells $\times 10^3$/mm^3) and delta neutrophil index (DNI, %) values in healthy control dogs (white, $n = 99$), dogs with sepsis (light grey, $n = 118$) and dogs with immune-mediated haemolytic anaemia (IMHA) (dark grey, $n = 20$). *Significant difference from healthy controls and dogs with IMHA ($P < 0.05$). (b) Box and whisker plots of WBCs, DNI and APPLE fast scores among dogs with sepsis (light grey, $n = 106$) and dogs with septic shock (dark grey, $n = 12$). *Significant difference from dogs with sepsis ($P < 0.05$). Boxes represent interquartile ranges, solid horizontal lines through boxes represent medians and whiskers represent minimum and maximum.

Table 2
Reference intervals (RIs) and medians (ranges) of APPLE fast scores, delta neutrophil index (DNI) values, C-reactive protein (CRP) concentrations and complete blood counts in dogs with sepsis ($n = 106$) and septic shock ($n = 12$).

Variable	RI	Sepsis	Septic shock	*P*
APPLE fast score	0–50	21 (7–32)	31 (24–43)	<0.001
DNI %	0–9.2	4.45 (0.0–86.40)	15.85 (0.40–58.30)	0.03
CRP mg/dL	0–0.5	8.98 (0.34–41.40)	9.74 (6.84–40.46)	NS
Albumin g/dL	2.8–3.7	2.35 (1.02–4.11)	2.265 (1.260–3.060)	NS
Lactate mmol/L	0.2–1.5	1.6 (0.6–16)	4.6 (0.9–10.9)	0.01
Glucose mg/dL	70–125	93 (13–733)	87 (16–220)	NS
HCT %	37.0–55.0	41.75 (8.40–68.30)	45.0 (30.6–56.9)	NS
Hb g/dL	12–18	14.35 (2.50–21.10)	15.4 (9.2–19)	NS
MCH pg	19.5–24.5	22.7 (19.0–73.1)	22.8 (19.2–73.1)	NS
MCV fL	60.0–77.0	66.0 (58.2–78.7)	66.05 (25.50–87.90)	NS
MCHC g/dL	32.0–38.0	34.4 (30.1–39.2)	33.45 (29.90–39.10)	0.03
RDW %	13.0–15.7	12.85 (11.30–20.20)	12.95 (11.60–15.50)	NS
RBCs $\times 10^6$/mm^3	5.5–8.5	6.34 (1.23–9.63)	6.67 (4.03–8.44)	NS
WBC $\times 10^3$/mm^3	6–17	19.5 (0.3–101.9)	4.0 (0.4–66.8)	0.01
Neutrophils/mm^3	3000–12,000	15350 (100–80,320)	3020 (50–58,180)	0.01
Band neutrophils/mm^3	0–300	0.0 (0.0–5981.0)	0.0 (0.0–2983.0)	NS
Lymphocytes/mm^3	1000–4800	1940 (50–37,920)	665 (240–8250)	0.04
Monocytes/mm^3	100–1400	1470 (7–10,830)	260 (30–2650)	0.006
Eosinophils/mm^3	0–750	120 (0–2380)	50 (0–1050)	0.02
Basophils/mm^3	0–180	80 (0–1480)	50 (20–410)	NS
Platelets $\times 10^3$/mm^3	160–500	266 (16–3800)	221 (33–584)	NS
MPV fL	6.6–10.9	12.7 (7.7–24.80)	13.7 (9.0–30.8)	NS

HCT, haematocrit; Hb, haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell; RDW, red blood cell distribution width; WBC, white blood cell; MPV, mean platelet volume.
NS, not significant.

Table 3
Reference intervals (RIs) and medians (ranges) of APPLE fast scores, delta neutrophil index (DNI) values, C-reactive protein (CRP) concentrations and white blood cell (WBC) counts in healthy control dogs ($n = 99$) and dogs with sepsis, including dogs with pyometra ($n = 47$), septic peritonitis ($n = 16$), parvovirus ($n = 13$), leptospirosis ($n = 15$) and miscellaneous causes ($n = 27$).

			Causes of sepsis				
	RI	Control dogs	Pyometra	Septic peritonitis	Parvovirus	Leptospirosis	Miscellaneous causes
APPLE fast score	0–50	11 (7–15)	18 (7–30)[a,c,d,f]	28 (16–43)[a]	25 (18–32)[a]	21 (13–29)[a,c,f]	26 (18–33)[a]
DNI %	0–9.2	2.6 (0.0–14.6)	4.6 (0.0–77.7)[a]	9.5 (0.0–86.4)[a]	7.3 (0.0–53.6)	0.7 (0.0–10.4)[b,c,d]	6.6 (0.0–75.1)[a]
CRP mg/dL	0–0.5	0.22 (0.01–0.5)	9.05 (0.34–41.40)[a]	9.39 (1.04–40.46)[a]	9.44 (6.54–30.55)[a]	7.81 (1.85–33.64)[a]	9.64 (0.97–38.85)[a]
WBC $\times 10^3$/mm^3	6–17	9.5 (5.4–20.4)	21.8 (3.3–79.7)[a]	16.0 (1.6–76.2)	2.4 (0.3–29.7)[a,b,c,e,f]	18.8 (10.1–47.1)[a]	20.5 (3.2–101.9)[a]

[a] Significant different from control group ($P < 0.05$).
[b] Significant different from pyometra ($P < 0.05$).
[c] Significant different from septic peritonitis ($P < 0.05$).
[d] Indicates significant different from parvovirus ($P < 0.05$).
[e] Indicates significantly different from leptospirosis ($P < 0.05$).
[f] Indicates significantly different from miscellanea ($P < 0.05$).

Table 4
Reference intervals and medians (means) for APPLE fast scores, delta neutrophil index (DNI) values, C-reactive protein (CRP) concentrations and complete blood counts in survivors ($n = 78$) and non-survivors ($n = 40$).

Variable	RI	Survivors	Non-survivors	*P*
APPLE fast score	0–50	21 (7–32)	26 (16–43)	<0.001
DNI %	0–9.2	5.9 (0.0–77.7)	3.2 (0.0–86.4)	NS
CRP mg/dL	0–0.5	9.05 (0.34–41.40)	8.58 (1.04–40.46)	NS
Albumin g/dL	2.8–3.7	2.56 (1.28–4.11)	2.325 (1.020–3.660)	NS
Lactate mmol/L	0.2–1.5	1.6 (0.6–9.0)	2.0 (0.7–16.0)	0.008
Glucose mg/dL	70–125	93 (13–725)	97.5 (16–733)	NS
HCT %	37.0–55.0	41.9 (21.2–68.30)	42.1 (8.4–60.7)	NS
Hb g/dL	12–18	14.5 (7.0–21.1)	14.4 (2.5–21.1)	NS
MCH pg	19.5–24.5	22.75 (19.00–25.20)	22.75 (19.20–73.10)	NS
MCV fL	60.0–77.0	65.8 (58.4–78.7)	67.3 (25.5–81.9)	NS
MCHC g/dL	32.0–38.0	34.6 (30.2–39.2)	33.8 (29.9–39.1)	0.01
RDW %	13.0–15.7	12.8 (11.3–20.2)	13.0 (11.60–18.70)	NS
RBCs $\times 10^6$/mm^3	5.5–8.5	6.38 (3.08–9.06)	6.40 (1.23–9.63)	NS
WBC $\times 10^3$/mm^3	6–17	19.4 (0.3–79.7)	17.4 (0.4–101.9)	NS
Neutrophils/mm^3	3000–12,000	15,220 (100–67,770)	14,455 (50–80,320)	NS
Band Neutrophils/mm^3	0–300	0 (0–5981)	0 (0–2983)	NS
Lymphocytes/mm^3	1000–4800	1945 (50–5840)	1260 (174–37,920)	NS
Monocytes/mm^3	100–1400	1520 (7–7860)	1220 (30–10,830)	NS
Eosinophils/mm^3	0–750	130 (0–2380)	60 (0–1050)	<0.001
Basophils/mm^3	0–180	80 (0–1350)	90 (20–1480)	NS
Platelets $\times 10^3$/mm^3	160–500	272 (16–3800)	237 (29–1274)	NS
MPV fL	6.6–10.9	12.9 (7.7–24.80)	12.35 (8.80–30.80)	NS

HCT, haematocrit value; Hb, haemoglobin concentration; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular haemoglobin concentration; RBC, red blood cell; RDW, red blood cell distribution width; WBC, white blood cell; MPV, mean platelet volume.
NS, not significant.

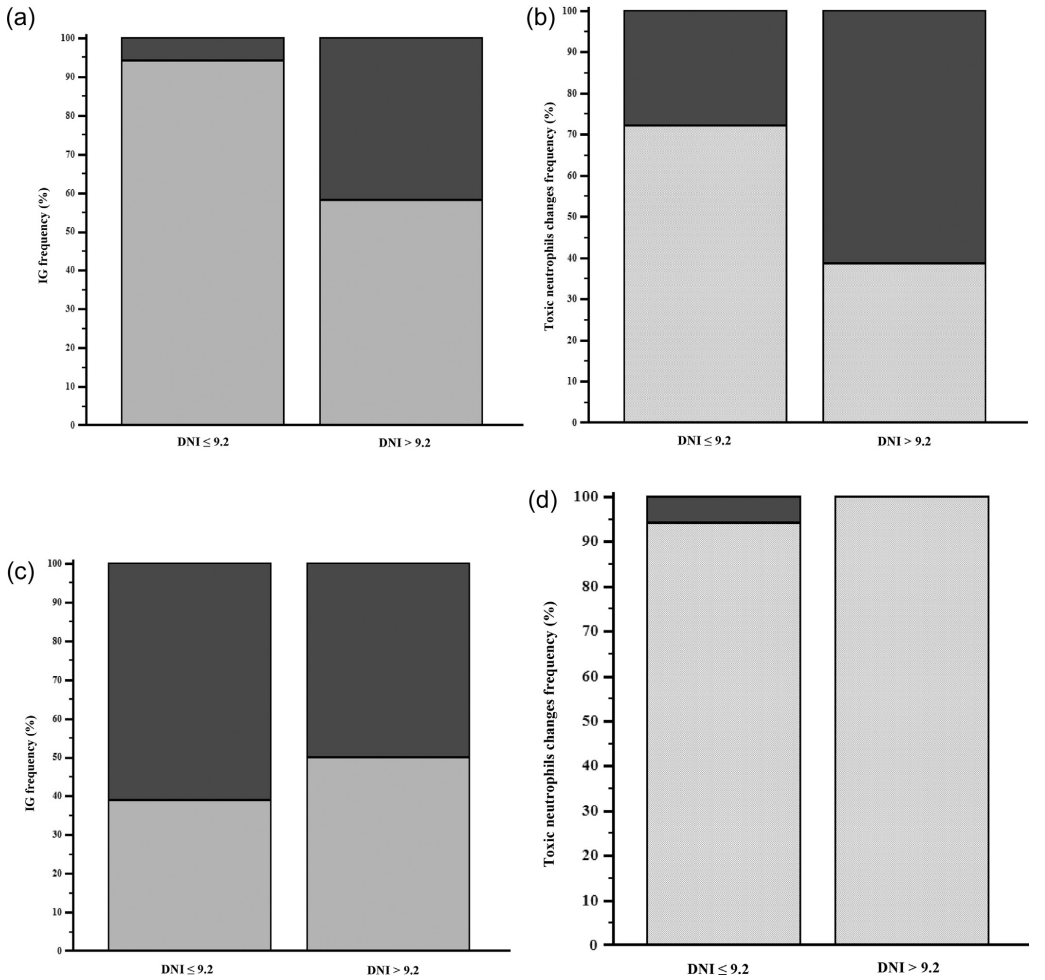

Fig. 2. (a) Frequency bar charts with 100% stacked columns in the presence (dark grey) or absence (light grey) of immature granulocytes (IGs) on blood smear evaluation in dogs with sepsis. Frequencies were significantly different between groups ($P < 0.01$). (b) Frequency bar charts with 100% stacked columns in the presence (dark grey) or absence (light grey) of toxic neutrophil changes detected on blood smear evaluation in dogs with sepsis. Frequencies were significantly different among groups ($P = 0.004$). (c) Frequency bar charts with 100% stacked columns in the presence (dark grey) or absence (light grey) of IGs on blood smear evaluation in dogs with immune-mediated haemolytic anaemia (IMHA). No significant differences were detected ($P = 1.0$). (d) Frequency bar charts with 100% stacked columns in the presence (dark grey) or absence (light grey) of toxic neutrophil changes on blood smear evaluation in dogs with immune-mediated haemolytic anaemia (IMHA). Cases are divided by delta neutrophil index (DNI) cut-off, set to the upper limit of the reference interval (RI, %). Left columns, dogs with DNI ≤ 9.2%; right columns, dogs with DNI > 9.2%.

Moreover, according to the ROC curve analysis, a DNI value > 7.3% had a sensitivity of 87% and a specificity of 71% to correctly predict the presence of IG (AUC = 0.87; $P < 0.0001$), while a value > 10.9% had a sensitivity of 52% and a specificity of 87% to correctly predict the presence of toxic neutrophils (AUC = 0.74; $P < 0.0001$). DNI values were significantly correlated with both IG ($r = 0.5$; $P < 0.0001$) and toxic neutrophil changes ($r = 0.4$; $P = 0.0002$) in dogs with sepsis. On the contrary, the DNI was not able to predict the presence of both IG and toxic neutrophils in IMHA dogs (Figs. 2c and d).

Discussion

An early and accurate diagnosis of sepsis is still a major challenge in order to implement appropriate treatments and interventions, and to reduce sepsis related mortality (Silverstein, 2012; Gotts and Matthay, 2016). Various inflammatory markers have been considered to aid clinicians in the diagnosis of sepsis in human beings and animals; however, they often lack specificity (Karlsson et al., 2013; Du et al., 2016; Pradhan et al., 2016). Circulating IGs have potential diagnostic and prognostic value in

human patients with systemic infection (Nierhaus et al., 2013; Mare et al., 2015). A degenerative left shift in dogs has been associated with severe inflammatory and septic conditions, and with higher risks of death or euthanasia, depending on the underlying disease (Burton et al., 2013). Similar results have been reported in cats (Burton et al., 2014); in this species, leukocytosis with left shift has been linked to sepsis and the presence of toxic neutrophils has been associated with infectious diseases (Segev et al., 2006; DeClue et al., 2011).

The DNI has been used early in the disease course to successfully differentiate septic conditions from non-septic inflammatory states in human beings (Lee et al., 2013; Shin et al., 2015). The DNI is superior to leukocyte counts and CRP concentrations for predicting mortality, as well as the occurrence of severe sepsis and septic shock (Nahm et al., 2008; Park et al., 2011). The results of our preliminary study are partially in line with these human studies. Specifically, serum CRP concentrations were elevated in the overall population of sick dogs, making it impossible to discriminate patients with sepsis from those with non-infectious SIRS (exemplified in our study by IMHA). Conversely, the DNI could be used to identify septic patients with relative accuracy. Furthermore, the DNI in septic dogs had good to fair accuracy in predicting the presence of circulating IGs and toxic neutrophils, respectively, confirming the potential diagnostic value of this automatically calculated variable in canine sepsis. A partial overlap was evident between the DNI values of septic dogs and controls (Table 1). This result is not completely expected and requires further clarification, since the relevance of the DNI may vary according to clinical context, and thus DNI results should be interpreted carefully. Our study enrolled a heterogeneous population of dogs with various causes and severities of sepsis. These factors may have reduced the diagnostic accuracy of the DNI, which appears to be more relevant in specific and more critical septic conditions.

When DNI values were compared according to the category of sepsis severity, no difference was noted between sepsis without organ dysfunction and severe sepsis (data not shown); however, at the time of hospital admission, significantly higher DNI values were found in dogs presenting with or developing septic shock during hospitalisation. This observation is consistent with human data supporting the value of the DNI for prediction of septic shock (Park et al., 2011), although the overall number of dogs with septic shock in our study was low (12/118, 10%). Thus, the prognostic relevance of the DNI needs to be confirmed by further studies in a wider population of dogs with severe sepsis or septic shock.

The APPLE fast score and the leukocyte count showed similar performances in the prediction of septic shock in our study population. The former is a validated score of disease severity, whose prognostic significance has been already documented in critically ill dogs with SIRS (Giunti et al., 2015), while the prognostic role of the leukocyte count could have been influenced by the relative frequency of dogs with septic shock due to parvoviral enteritis (5/12, 42%; data not shown).

The analysis of the DNI values according to the final diagnosis of sepsis showed significantly increased values in dogs with septic peritonitis and pyometra. Although a significant difference was documented between the DNI of these sepsis subgroups and control dogs, only dogs with septic peritonitis had a median DNI above the reported RI. On the other hand, the DNI of dogs with parvoviral enteritis and leptospirosis was not different from the healthy controls. According to the APPLE fast score, dogs with parvoviral enteritis were among the most critical patients in our study population; however, the younger age of the patients and virus-mediated myelosuppression may have had an impact on the DNI results, since IG synthesis and circulation may be different in immunosuppressive states. Limitations of the DNI in assessing bacteraemia have been reported in immunocompromised children (Ahn et al., 2014). These findings suggest that the clinical relevance of the DNI may vary according to the underlying disease or patient characteristics.

When dogs with sepsis were compared on the basis of the final outcome, the APPLE fast score was the only variable that was significantly different between survivors and non-survivors, confirming its prognostic significance in critically ill dogs. No significant association with outcome was found for the DNI. The timing of DNI evaluation for prediction of mortality in the septic human patient is controversial, since DNI values at the time of hospital admission are not necessarily associated with a stronger predictive power (Hwang et al., 2015). The increment of the DNI during hospitalisation has been independently associated with early mortality in human beings with Gram negative bacteraemia (Kim et al., 2014), suggesting that serial DNI assessment may be of value.

The inclusion of a group of dogs with a diagnosis of IMHA was based on the frequent association of IMHA with a strong inflammatory response, representing a well-recognised model of non-infectious SIRS (Goggs et al., 2015). Furthermore, leukocytosis with a left shift is frequently reported in the course of IMHA (Piek, 2011). In the present study, the frequency of IGs on blood smear evaluation was significantly higher in dogs with IMHA than in septic dogs; however, the median DNI in IMHA dogs was not different from that in healthy controls, and the DNI was not able to predict the presence of circulating IGs in dogs with IMHA. These results are not entirely expected and require additional investigations in larger canine populations. If confirmed, they may indicate that interpretation of the DNI in diseased dogs is complex. These findings highlight the potential of the DNI as a marker of sepsis in dogs, although its diagnostic performance in differentiating sepsis and non-septic inflammatory conditions needs to be clarified in further studies.

In this study, 15/118 (12.7%) septic dogs had evidence of toxic neutrophils without a left shift. A lack of correlation between toxic changes and left shift has been reported previously in an experimental canine model of acute inflammation (Gosset et al., 1985) and in cats with spontaneous inflammatory and infectious diseases (Segev et al., 2006). These observations, partially corroborated by our results, suggest that toxic neutrophil changes in the peripheral blood may precede changes in the leukogram.

There are some limitations to consider when interpreting our results. The relatively small number of dogs in some groups, particularly dogs with septic shock and dogs with IMHA, may have reduced the statistical power of the analysis. Dogs with sepsis had a range of diseases and grades of severity of sepsis. In future studies, the inclusion of dogs with non-septic SIRS other than IMHA (e.g. pancreatitis, trauma) may provide more information on the diagnostic performance of the DNI in dogs with systemic inflammation. Due to the retrospective nature of the study, only partial data regarding IGs and toxic neutrophil changes at the blood smear evaluation were available. A prospective study may permit more detailed characterisation of IGs and severity of toxic changes, and clarify their impact on the DNI. Finally, although the DNI has been identified as a promising marker of sepsis in several human case series, there is a need for validation studies of the stability and repeatability of this index in human beings and animals.

Conclusions

This study evaluated the performance of the DNI, measured at the time of hospital admission, as a diagnostic and prognostic variable in canine sepsis. The DNI had fair diagnostic accuracy in identifying dogs with sepsis and had a better performance in

predicting septic shock that current inflammatory biomarkers. Although DNI analysis is limited by the use of a specific haematological analyser requiring myeloperoxidase and lobularity based methods for counting leukocytes, it is performed routinely and thus does not require additional time or costs in the clinical setting. No additional benefit in terms of mortality prediction was evident in our study population. Larger prospective studies are required to determine the diagnostic and prognostic validity of DNI measurement in canine sepsis.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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7.4 Canine procalcitonin: background

Procalcitonin (PCT), the pre-hormone of calcitonin in C cells of the thyroid, is a reliable diagnostic and prognostic biomarker for sepsis in humans. Plasma PCT concentration is low in heatlhy states, while rises early after the exposure to an infectious stimulus. Several studies highlighted its role to early identify bacterial sepsis in people, as well as to assess prognosis and mirror the severity of the septic process (Schuetz et al. 2017). Plasma PCT concentrations have also been proposed as an early predictor of MODS (Zurek & Vavrina 2015). Circulating PCT concentrations are down-regulated during the recovery phase of sepsis; several studies revealed a relationship between persistently elevated PCT concentrations during the first days of ICU stay and increased odds for mortality. Thus, PCT is serially evaluated to guide antimicrobial therapy in selected systemic infections in humans (Schuetz et al. 2017).

Data regarding PCT assessment in dogs are limited. Canine PCT was firstly sequenced in the early 90s (Mol et al. 1991). Increased procalcitonin mRNA expression was then demonstrated in sick dogs compared to healthy ones, and its role as an acute phase protein was suggested (Kuzi et al. 2008). A subsequent study documented the extrathyroidal transcription of PCT gene in dogs with SIRS and sepsis (Giunti et al. 2010). Although increased concentrations of circulating PCT were detected in canine SIRS and sepsis, the lack of reliable species-specific methods limited the value of the results (Giunti et al. 2006; Yilmaz et al. 2008; Floras et al. 2014).

7.4.1 Procalcitonin in dogs with sepsis and gastric dilatation-volvulus

Preliminary studies have been conducted as part of the current PhD thesis to investigate procalcitonin in critically ill dog involving two Veterinary Teaching Hospitals (University of Bologna, Italy, and Cornell University, Ithaca, NY).

A commercially available ELISA assay (Biovendor LLC, Asheville, NC) was used to measure plasma canine PCT. The ability of the assay to identify canine PCT, in addition to assay imprecision and lower limit of detection, were established from our group of research (manuscript currently under revision). Then, two clinical studies were retrospectively performed in order to

evaluate the prognostic significance of canine PCT in dogs with sepsis and gastric dilatation-volvulus (GDV), respectively.

For the first clinical investigation, citrated plasma samples from 53 dogs with sepsis (≥2/4 SIRS criteria plus evidence of infection) were used to measure PCT at the time of ICU admission (T0, n=53) and at 24h (T1, n=35) and 48h (T2, n=30) of ICU stay. Twelve healthy dogs were used as controls. PCT was analyzed using the afore-mentioned commercial canine PCT ELISA. Clinical, clinicopathologic and outcome data were collected, patients were classified as sepsis, severe sepsis or septic shock, and the Acute Patient Physiologic and Laboratory Evaluation (APPLE) score was calculated. Non-parametric statistics was used, and alpha was set at 0.05.

Of the 53 dogs, 18 had sepsis without organ dysfunction, 24 had severe sepsis and 11 had septic shock. Thirty-eight dogs survived to hospital discharge (mortality rate 28.3%). PCT concentrations were significantly higher in dogs with sepsis versus healthy controls and significantly higher in dogs with septic shock versus dogs with sepsis. Declining PCT concentrations were documented in survivors at T1 and T2 compared to PCT at T0. PCT concentrations were mildly positively correlated with the number of dysfunctional organs at the time of admission and duration of hospital stay, and mildly negatively correlated with leukocyte count.

According to these preliminary results, plasma PCT concentrations are increased in dogs with sepsis and are able to early predict occurrence of MODS and septic shock. Serial PCT monitoring seems to be promising in canine sepsis, with early declining in PCT concentrations being associated with survival.

The second study was performed to assess the prognostic significance of procalcitonin in dogs with GDV syndrome, in association with relevant biomarkers previously assessed during the disease. Concentrations of cell-free DNA (cfDNA; Quant-iT High Sensitivity DNA assay Kit, Life Technologies, Grand Island, NY), high-mobility group box 1 (HMGB1, IBL-International, Hamburg, Germany) and PCT (Biovendor LLC, Asheville, NC) were assessed in citrated plasma samples collected from 29 dogs with GDV at the time of hospital admission. Only dogs undergoing

surgery were included. A group of 24 healthy dogs were enrolled as controls. Baseline lactate concentrations, $APPLE_{fast}$ score, evidence of gastric necrosis, post-surgical complications and outcome were recorded. Non-parametric statistics was used, and alpha was set at 0.05.

Dogs with GDV had significantly higher concentrations of cfDNA, HMGB1 and PCT compared to controls, potentially indicating systemic inflammation, tissue hypoperfusion and ischemia/reperfusion injury. PCT concentrations were significantly greater in non-survivors compared to survivors, while lactate concentrations resulted significantly higher in dogs with gastric necrosis compared to the ones without gastric necrosis.

These results, overall, confirm the role of canine PCT as an acute phase reactant able to detect systemic inflammation and sepsis. Baseline and serial PCT measurement might be used as a prognostic tool in critically ill dogs. Both the afore-mentioned studies are currently under revision.

8. MODS and selected organ dysfunctions

8.1 Acute kidney Injury in critically ill dogs

A significant part of this research has been focused on acute kidney injury in dogs, in terms of prevalence, characteristics and overall prognosis.

Acute kidney injury is defined as an abrupt damage or dysfunction of the kidney, frequently associated with electrolytes and acid base disturbances, reduction in glomerular filtration rate and decrease in urine production. AKI diagnosis in humans is based on relative or absolute changes in serum creatinine concentration and urine output (Kellum et al. 2013). Despite AKI occurrence is recognized in dogs and cats, standardized diagnostic criteria are lacking (Harison et al. 2012; Keir & Kellum 2015; Brown et al. 2015).

More recently, the concepts of volume-responsive and volume-unresponsive AKI have emerged in humans. According to this new approach, AKI is characterized by a continuum of volume responsivness/unresponsivness stating different severity of injury and prognosis. The term volume-responsive AKI has replaced the historical "prerenal azotemia", and describes a transient, functional impairment of the kidney that can be usually restored with adequate fluid administration. In most circumstances, a volume-responsive kidney will occur in a volume-responsive patient (e.g. during hypovolemia). However, a patient can be volume-responsive (and experience an increase in cardiac output after fluid administration) whereas kidney function is not. On the other hand, the term volume unresponsive or intrinsic AKI refers to structural damage to the renal parenchyma, usually implying a more severe reduction in kidney function, greater need of assistance and worse outcomes (Himmelfarb et al. 2008; Makris & Spanou 2016).

FE of electrolytes have been proposed as a tool to early differentiate between volume-responsive and volume-unresponsive AKI and aid in its prognostication in humans Specifically, volume-responsive AKI has been characterized by low (<1%) FE of sodium and increased (>35%) FE of urea; this diagnostic paradigm, however, has been questioned in clinical practice, due to the impact of different confounders (Makris & Spanou 2016). The prognostic value of FE of electrolytes has

emerged in a recent study on canine AKI, as sequential reduction in FE of sodium were associated to recovery of kidney function and survival (Brown et al. 2015).

In the course of the PhD (2014-2016), a prospective investigation on critically ill dogs with AKI requiring hospitalization at the Veterinary University Hospital of Bologna has been performed. The main purpose of the study was to evaluate the performance of the fractional excretion (FE) of electrolytes and urea, to early differentiate between volume-responsive and volume-unresponsive AKI and to aid in AKI prognostication. Dogs were diagnosed with AKI according to the IRIS AKI grading system proposed by Cowgill (2010) and previously applied in similar clinical settings (De Loor et al. 2013; Segev et al. 2015; Sigrist et al. 2015). Complete clinical and laboratory data including IRIS AKI grade, the $APPLE_{fast}$ score, complete blood cell count, chemistry profile and complete urinalysis were performed at the time of AKI diagnosis (T0), at 24 h (T1), 48h (T2), 72 h (T3), and 7 days (T7) of hospitalization. Complete urinalysis included measurement of proteinuria (urinary protein to creatinine ratio), albuminuria (urinary albumin to creatinine ratio), FE of electrolytes (sodium, potassium, chloride, magnesium, calcium) and urea, and urinary uric acid to creatinine ratio. FE were calculated according to the following equation:

FEX = uX sCr/uCr sX (based on spot urine sample)

where uX and sX were the concentrations of a specific analyte in urine and serum, respectively.

Non-parametric statistics was used, and alpha was set at 0.05.

According to our results, dogs with volume-unresponsive AKI (n=69) had significantly greater alterations in conventional variables indicating kidney function (greater serum creatinine concentration, IRIS AKI grade and proteinuria) and higher FE of electrolytes compared to dogs with volume-responsive AKI (n=52). Dogs with volume-responsive AKI had significantly higher blood lactate concentration and urinary uric acid to creatinine ratio compared to dogs with volume-unresponsive AKI.

Overall mortality was 41% in the current study population, with volume-unresponsive AKI dogs showing significantly higher frequencies of death compared to the volume-responsive ones.

Variables of renal damage/dysfunction including FE of electrolytes were significantly higher in non-survivors (n=55) compared to survivors (n=80).

The reported results suggest that FE of electrolytes can be routinely applied in the clinical setting with both relevant diagnostic and prognostic implications. Additionally, a role for urinary uric acid as a marker tissue hypoxia/hypoperfusion could be suggested.

The complete study in currently under revision. Preliminary data were presented as an oral presentation at the 2015 EVECCS Congress and published in form of abstract.

A sub-group of this population including dogs with AKI and systemic inflammation was used to specifically evaluate urinary uric acid performances as a marker of volume-responsive AKI and tissue hypoperfusion, and to investigate its relationship with variables of hypoxia and shock. Data were presented as a poster at the 2016 EVECCS Congress and published in form of abstract.

APPLE FAST SCORE AND FRACTIONAL EXCRETION OF ELECTROLYTES IN DOGS WITH ACUTE KIDNEY INJURY

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Introduction: aim of the study was to investigate the prognostic role of Apple Fast Score (AFS) and fractional excretion of electrolytes and urea (FE) in dogs with acute kidney injury (AKI).

Methods: dogs hospitalized at a veterinary teaching hospital (February 2014-January 2015) with AKI graded according to IRIS guidelines were prospectively included. AFS was calculated upon admission (T0) as previously reported. Laboratory analytes including FE (sodium, potassium, calcium, phosphorus, and magnesium and urea) and urinary output (UO) were measured at T0 and after 24 hours (T1). Dogs were divided according to outcome (survivors/non-survivors) and compared to healthy controls (n=56). Non-parametric statistics were used for comparisons. Cox proportional regression analysis was performed to evaluate short term survival (14 days) and hazard ratios (HR) calculated. The significance level was set at $p<0.05$.

Results: 53 AKI dogs were enrolled. FE resulted significantly increased in AKI dogs compared to controls. Non-survivors (n=22) had significantly increased AFS (median 26; range 16-34) and FE (e.g. FE of sodium; median 8.2%; range 0.04-68.9) compared to survivors (n=31) at T0. Non-survivors (n=15) had significantly increased serum creatinine (median 9.0 mg/dL; range 0.98-20.41) and FE (e.g. FE of sodium; median 9.5%; range 0.5-203.2) and decreased UO (median 0.8 mL/kg/h; range 0-2.6) than survivors (n=31) at T1. Survival was significanlty associated with AFS and FE at T0 and with FE, IRIS grade and UO at T1.

Conclusion: AFS and FE have a prognostic value in this cohort suggesting more severe renal tubular dysfunction in non-survivors. Larger prospective studies are needed to confirm these data.

URINARY URIC ACID EXCRETION IN DOGS WITH ACUTE KIDNEY INJURY AND SYSTEMIC INFLAMMATION

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Poster at the 2016 EVECCS Congress, Ljubljana, Slovenia.

Introduction: Acute kidney injury (AKI) can be frequently associated with systemic inflammation in human and veterinary patients and tissue perfusion can be severely compromised during these conditions. Urinary Uric Acid (uUA) is reported to increase in conditions of acute and chronic systemic hypoxia in humans. In normal dogs, 98-100% of glomerular filtrated uUA is reabsorbed into the proximal tubule; however, no data regarding its role as a marker of tubular damage or systemic hypoxia is reported in canine AKI.

Methods: dogs hospitalized at a veterinary teaching hospital (February 2014-December 2015) with a diagnosis of AKI and with evidence of systemic inflammation, were prospectively included. According to the IRIS guidelines, AKI dogs were sub-grouped in volume-responsive and volume-unresponsive (intrinsic AKI). Systemic inflammation was defined according to the presence of at least 2/4 of the systemic inflammatory response syndrome (SIRS) criteria for dogs (Hauptman *et al*., 1997) and/or an increased serum C-reactive protein concentration. Blood donor dogs (n=81) were included as controls. Clinical data, recorded upon admission included the evaluation of the Shock Index (heart rate/systolic blood pressure). Urinalysis, including uUA to urinary creatinine ratio (uUA/C), and blood gas analysis, including blood lactate, were performed. The Mann Whitney U Test and Kruskall Wallis Test were used to compare variables between different groups. A p value of $<.05$ was considered significant. Correlations between variables were assessed using the Spearman rank correlation coefficient.

Results: volume-responsive AKI dogs had a significant increase in Shock Index and blood lactate concentrations compared to dogs with intrinsic AKI and a significant increase in uUA/C compared to dogs with intrinsic AKI and to healthy dogs. Differences in BE and blood pH were not significant between groups.

Conclusion: increased uUA seems to be associated with volume-responsive AKI in this population of dogs with naturally occurring acute kidney disease and systemic inflammation, suggesting its potential as an indirect marker of cellular hypoxia. The presence of intrinsic acute kidney injury did not seem to impact uUA in this population. Further studies investigating the role of uUA as a marker of cellular hypoxia and oxidative stress in different conditions of systemic hypoperfusion are warranted.

Original Article
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JVS

Retrospective evaluation of circulating thyroid hormones in critically ill dogs with systemic inflammatory response syndrome

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Critical illness can be associated with transient alterations in circulating thyroid hormone concentrations, indicating the presence of non-thyroidal illness (NTI). NTI is well described in humans, but there are few reports on its occurrence and prognostic significance in dogs. This retrospective study assessed the occurrence of NTI in a population of dogs with systemic inflammatory response syndrome (SIRS) and investigated its association with disease severity ($APPLE_{fast}$ scores). A total of 41 SIRS dogs were included and were divided by SIRS origin (non-septic SIRS, n = 10; septic SIRS, n = 41) and final outcome (survivors, n = 37; non-survivors, n = 4). Healthy, age-matched dogs (n = 15) were included as controls. Serum thyroid hormone levels including total T3, free T3, total T4, and reverse T3 were measured upon admission. Compared to controls, there were significant changes in serum thyroid hormone concentrations in SIRS dogs, suggesting the presence of NTI. Septic SIRS dogs had higher $APPLE_{fast}$ scores and lower serum thyroid hormones concentrations than those in non-septic SIRS and control dogs. In conclusion, NTI was frequent in dogs with SIRS and may be associated with the presence of sepsis or high illness severity.

Keywords: canine, euthyroid sick syndrome, systemic inflammatory response syndrome, thyroid hormones

Introduction

Critical illness can be associated with dysfunction in multiple organs and remarkable endocrine and metabolic changes [6,25]. Alterations in the circulating levels of thyroid hormones have been widely documented in human medicine and may affect 60% to 70% of critically ill patients with various diseases [6]. This condition is typically characterized by a reduction in the concentration of serum total triiodothyronine (TT3) and a concurrent rise of serum reverse-T3 (rT3) levels; as well, low serum total thyroxine (TT4), free thyroxine (fT4), and, occasionally, thyrotropin (TSH) concentrations are reported with severe and protracted illness [1,4,27]. These are usually transient abnormalities in otherwise euthyroid patients and are commonly recognized under the name of non-thyroidal illness (NTI) [4,27]. The pathogenesis of NTI seems to be multifactorial and mainly attributed to a reduced peripheral deiodination of TT4 to TT3, increased deiodination of TT3 to diiodothyronine, reduced binding of thyroid hormones to transport proteins and nuclear receptors, and impaired intracellular uptake; behind these mechanisms the roles of protracted fasting, hypoxia, ischemia-reperfusion injury, and inflammatory cytokines have been investigated [4].

Thyroid hormones are important for homeostasis and adaptation to stress and pathological conditions, and several studies in critically ill human patients have linked the presence of NTI with poor outcomes and disease severity [1,4]. There is evidence that an acute fall in circulating thyroid hormone concentrations during acute critical illness could represent an adaptive response to reduce energy expenditure and protein breakdown; in contrast, low TT3 and TT4 serum levels during a prolonged or chronic phase of critical illness could be maladaptive [4]. Consensus on therapeutic implications of the above-mentioned abnormalities is currently lacking [4].

There are few reports regarding NTI in veterinary critical care. The syndrome has been documented in some acute conditions in dogs, but its prognostic significance remains unclear [20,24,25]. In a population of puppies with parvoviral enteritis, non-survivors had significantly lower concentrations of serum TT4 during hospitalization [24]. In addition, in a group

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of dogs with naturally occurring infection by *Babesia canis rossi*, lower values of serum TT4 and fT4 were documented in non-survivors [26]. Alterations in TT4, fT4, and TSH concentrations were demonstrated upon admission in dogs with systemic inflammatory response syndrome (SIRS) and sepsis, but no relationship to outcome was identified [20]. Derangement of the thyroid axis was documented in chronic inflammatory conditions and during heterogeneous non-thyroidal diseases [13,15,23]. Finally, significant abnormalities in thyroid function test results have been reported in healthy euthyroid dogs during anesthesia or surgical procedures [28].

The aim of the present retrospective study was to assess the prognostic significance of serum thyroid hormones, including free T3 (fT3), TT3, rT3, and TT4, in a population of dogs with SIRS. We hypothesized that lower serum thyroid hormones concentrations were associated with disease severity ($APPLE_{fast}$ scores) and mortality (survival at hospital discharge).

Materials and Methods

This study involved a retrospective analysis of a population of dogs affected by SIRS associated with acute pancreatitis, parvoviral enteritis, or septic peritonitis that was prospectively enrolled in a previous study performed at our Veterinary Teaching Hospital (VTH) between February 2012 and January 2014. The study was approved by the local Scientific Ethical Committee for Animal Testing (ID 22/79/2014).

Dogs were included in the study if they exhibited two or more of the following criteria: body temperature $< 38.1^{o}C$ or $> 39.2^{o}C$; heart rate $>$ 120/min; respiratory rate $>$ 20/min; WBC count $<$ 6,000/μL or $>$ 16,000/μL, percentage of band cells $>$ 3% of the total WBC count, or a serum C-reactive protein (CRP) concentration $>$1.68 mg/dL [5,10]. At least one aliquot of serum collected at the time of hospital admission and stored frozen at $-80^{o}C$ was obtained from each dog. Dogs were excluded if thyroid hormones or drugs capable of suppressing the thyroid axis (*e.g.*, glucocorticoids, anti-inflammatory drugs, anticonvulsants, and sulphonamides) had been administered in the month prior to hospital admission. Age-matched dogs (n = 15), presented at the VTH for routine screening and prophylaxis, were included as healthy controls based on their anamnestic, physical, and clinicopathological data.

The study population of SIRS dogs was divided in groups according to the origin of SIRS. Specifically, the non-septic SIRS group included dogs affected by acute pancreatitis, while the septic SIRS group included dogs with parvoviral enteritis and septic peritonitis.

Acute pancreatitis was diagnosed by the presence of consistent clinical signs, characteristic ultrasonographic findings (*i.e.*, hypoechoic and/or enlarged pancreas, hyperechoic mesentery, peritoneal effusion), and a positive canine pancreatic lipase immunoreactivity (cPLI) test result (Canine SNAP cPL; IDEXX Laboratories, USA) [14,22]. Clinical diagnosis of parvoviral enteritis was confirmed by a positive real-time polymerase chain reaction for a fecal sample. Sequencing of the VP2 gene was performed to identify antigenic variants of canine parvovirus (CPV) and evaluate their potential associations with disease severity [2]. Septic peritonitis was diagnosed based on cytological or bacteriological evidence of bacterial abdominal infection. The $APPLE_{fast}$ score [11], calculated at the time of hospital admission in order to assess disease severity, and the length of hospital stay were recorded and included as analysis variables. SIRS dogs were also classified as survivors (survived to hospital discharge) or non-survivors (died despite medical treatment or humanely euthanized by the clinical investigators due to moribund conditions or end-stage disease). Dogs that were euthanized for financial reasons were excluded from the study.

Hematological and chemistry profiles, including CRP and albumin concentrations, obtained upon hospital admission were reviewed in all enrolled dogs. Complete blood count was determined by an automated cell counter (ADVIA 2120 Hematology System; Siemens Healthcare Diagnostics, USA). CRP (CRP OSR6147; Beckman Coulter, Germany) level was measured by using an immunoturbidimetric assay that had been previously validated by our group for dog serum samples [8]. All analyses were performed by using an automated chemistry analyzer (OYMPUS AU 400, Olympus Optical, Germany). Serum thyroid hormone levels were measured at the end of the study period in a single batch assay of serum collected upon admission and stored frozen at $-80^{o}C$. The TT3 and TT4 levels were measured by performing radioimmunoassays (RIA) as previously described [17,19]. For analytical purposes, RIA results below the detection limit of the assay ($<$ 0.4 nmol/L for TT3 and $<$ 3.0 nmol/L for TT4) were considered equal to 0.2 nmol/L and 1.5 nmol/L, respectively. The fT3 and rT3 levels were assayed by performing ultraperformance liquid chromatography coupled to tandem mass spectrometry operating in multiple reaction monitoring mode and electrospray ionization positive mode. All analytes were directly determined without the need of derivatization. The linearity of the analytical method was assessed over a wide range of concentrations (0.01−50 ng/mL). The recovery of both fT3 and rT3 was $>$ 82%, with a coefficient of variation $<$ 7%. The within-day and between-day precision ranges were 1.82% to 7.81% and 2.29% to 15.62%, respectively. All investigated variables were also measured in healthy control animals.

Statistical analysis

Normality was checked graphically and by applying the Kolmogorov-Smirnov test. Because of the presence of non-normal distributions for most variables, nonparametric testing was adopted for all analyses. Data were expressed by using standard descriptive statistics and are presented as median and range.

The Mann–Whitney *U* test was used to evaluate differences between the overall population of SIRS and control dogs and for comparisons between survivor and non-survivor SIRS dogs, while a Kruskal–Wallis test was used to compare variables between different groups (controls, septic SIRS, and non-septic SIRS). If the Kruskal–Wallis test result was positive, a Conover test *post hoc* analysis for pairwise comparison of subgroups was performed. Test result p values < 0.05 were considered statistically significant. Correlation between variables was assessed by using Spearman's Rank correlation coefficient. All analyses were performed by using statistical software (MedCalc Software, Belgium).

Results

Forty-one patients met the inclusion criteria and were classified as SIRS dogs. Among the SIRS dogs, median age (8 months, range 2 months to 15 years) and median body weight (17.3 kg, range 3.9–40.2 kg) were not significantly different from those of the control dogs (median age 4.8 years, range 2 months to 8 years; median body weight 20.6 kg, range 4.7–38.0 kg). Overall, 20 dogs were male and 21 were female. Breed distribution of the study population was as follows: mixed breed dogs (18), Spanish Greyhound (4), Labrador Retriever (3), Spanish Mastiff (3), Standard Poodle (3), American Staffordshire Terrier (2), English Bulldog (2), Rottweiler (1), Weimaraner (1), American Pitbull Terrier (1), Manchester Terrier (1), Great Dane (1), and Bernese Mountain Dog (1). Breed distribution of the control dogs was German Shepard Dog (3), mixed breed dogs (3), Flat Coated Retriever (2), Argentine Mastiff (2), Labrador Retriever (1), Whippet (1), Cocker Spaniel (1), Dogue de Bordeaux (1), and Great Dane (1). Thirty-seven of the 41 dogs were survivors, while 4 were non-survivors. All non-survivors were in the septic SIRS group and had a diagnosis of septic peritonitis. Median duration of hospitalization in SIRS dogs was 7 days (range 1–13 days). SIRS dogs had significantly higher $APPLE_{fast}$ score and serum CRP concentration and significantly lower TT3, TT4, and albumin levels compared to those in control dogs (Table 1).

The overall population was divided in two groups according to the origin of SIRS and final diagnosis. Specifically, the non-septic SIRS group consisted of dogs diagnosed with acute pancreatitis (n = 10), while the septic SIRS group included dogs with septic peritonitis (n = 9) and parvoviral enteritis (n = 22). The CPV variants identified by sequencing of the VP2gene were CPV-2c (19/22) and CPV-2b (3/22).

Significantly different clinical and clinicopathological results between septic SIRS and non-septic SIRS dogs are summarized

Table 1. $APPLE_{fast}$ score, acute phase proteins, and thyroid hormone concentrations in dogs with systemic inflammatory response syndrome (SIRS) and control dogs

Variable	SIRS dogs (range) (n = 41)	Control dogs (range) (n = 15)	Reference interval (range)	*p* value
$APPLE_{fast}$ score	25 (12–38)	14 (8–22)	–	< 0.0001
Albumin (g/L)	25.6 (11.3–42.9)	33.6 (27.2–37.1)	(28.0–37.0)	0.0004
CRP (mg/dL)	6.80 (1.80–31.84)	0.01 (0.01–0.3)	(0.01–0.5)	< 0.0001
fT3 (pmol/L)	0.79 (0.41–2.42)	1.0 (0.71–1.71)	(1.41–5.34)	0.05
TT3 (nmol/L)	0.2 (0.2–1.4)	1.1 (0.4–1.6)	(0.8–2.1)	< 0.0001
rT3 (pmol/L)	1.0 (0.29–3.18)	1.23 (0.78–2.21)	(1.23–5.13)	0.09
TT4 (nmol/L)	13.0 (1.50–66.0)	30.0 (15.0–57.0)	(11.0–60.0)	0.001

CRP, C-reactive protein; fT3, free triiodothyronine; TT3, total triiodothyronine; rT3, reverse triiodothyronine; TT4, total thyroxine.

Table 2. Variables with statistically different results between non-septic systemic inflammatory response syndrome (SIRS; pancreatitis, n = 10), septic SIRS (parvoviral enteritis, n = 22; septic peritonitis, n = 9) and control (n = 15) dogs

Variable	Non-septic SIRS (range) (n = 10)	Septic SIRS (range) (n = 31)	Controls (range) (n = 15)	*p* value
APPLEfast score	20 (12–23)*	25 (18–38)*,†	14 (8–22)	< 0.0001
Albumin (g/L)	29.8 (18.6–42.9)	23.7 (11.3–34.6)*,†	33.6 (27.2–37.1)	< 0.0001
TT3 (nmol/L)	0.5 (0.2–1.3)*	0.2 (0.2–1.4)*,†	1.1 (0.4–1.6)	< 0.0001
TT4 (nmol/L)	22.5 (6.0–66.0)	10.0 (1.5–39.0)*,†	30.0 (15.0–57.0)	0.0001

TT3, total triiodothyronine; TT4, total thyroxine; rT3, reverse triiodothyronine. *Difference from controls; †Difference from non-septic SIRS.

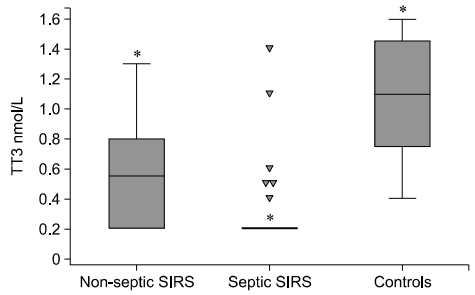

Fig. 1. Box plots of serum total triiodothyronine (TT3) concentrations among dogs with non-septic systemic inflammatory response syndrome (SIRS), dogs with septic SIRS, and control dogs; the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The vertical line extends from the minimum to the maximum value, excluding outside and far out values which are displayed as down-pointing triangles. Asterisk indicates significant ($p < 0.05$) differences among groups.

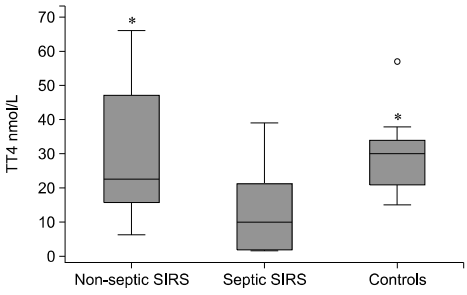

Fig. 2. Box plots of serum total thyroxine (TT4) concentrations among dogs with non-septic systemic inflammatory response syndrome (SIRS), dogs with septic SIRS, and control dogs; the central box represents the values from the lower to upper quartile (25 to 75 percentile). The middle line represents the median. The vertical line extends from the minimum to the maximum value, excluding outside and far out values which are displayed as open circles. Asterisk indicates significant ($p < 0.05$) difference from septic SIRS.

in Table 2. The septic SIRS dogs had a significantly higher $APPLE_{fast}$ score and significantly lower concentrations of serum albumin, TT3, and TT4 than those in non-septic SIRS and control dogs (Figs. 1 and 2). Non-survivors (n = 4) had significantly lower serum albumin (median 15.6 g/L, range 11.3−23.0 g/L) and TT4 concentrations (median 1.5 nmol/L, range 1.5−6.0 nmol/L) compared to survivors (n = 37; median 26.2 g/L, range 18.1−42.9 g/L; median 16 nmol/L, range 1.5−66.0 nmol/L, respectively). Among the survivors, there were no significant correlations between duration of hospital stay and serum thyroid hormone concentrations. Both serum TT4 and TT3 concentrations were negatively correlated with $APPLE_{fast}$ scores ($r = -0.4$, $p < 0.01$ and $r = -0.3$; $p < 0.05$, respectively). Serum TT4 and albumin concentrations were positively correlated ($r = 0.56$; $p = 0.0001$), while the correlation between TT3 and albumin was not significant ($r = 0.3$; $p = 0.05$).

Discussion

The presence of NTI has been documented in different human and veterinary critical conditions including systemic inflammation [1,20]. In the current study, a panel of serum thyroid hormones was assayed in specific canine diseases: acute pancreatitis, parvoviral enteritis, and septic peritonitis. These diseases were included as they are representative and homogeneous spontaneous models of canine infectious and non-infectious SIRS. The high serum CRP concentration in the SIRS dogs confirmed the presence of systemic inflammation in our study population [5]. In order to stratify SIRS patients according to disease severity and mortality risk, as has been previously done [9,20], the dogs' $APPLE_{fast}$ scores were calculated. There was a significantly higher value $APPLE_{fast}$ score in the septic SIRS group than in the non-septic SIRS and control dogs. The reduction in serum TT4 value in the septic SIRS group is in agreement with previous veterinary reports investigating NTI in similar settings [20,24,25], and is a common finding in clinical studies on canine NTI [13,15].

Changes in serum TT3, fT3, and rT3 concentrations have been widely documented in critically ill human patients, but are less reported in veterinary literature [7,13,15,18]. Low levels of TT3 are the most common finding and had the strongest correlation with outcome in a retrospective evaluation of thyroid hormones among heterogeneous non-thyroidal diseases in dogs [15]. Similar results were obtained in a retrospective evaluation of thyroid hormones in critically ill dogs requiring intensive care therapy; low TT3 levels were frequently detected and associated with mortality [7]. In our study, a significant reduction in serum TT3 concentration in canine SIRS was observed, indicating its potential as a sensitive marker of NTI, as has been described in humans [27].

An increase in serum rT3 has been reported during NTI in humans [27]. A similar increase has been reported in healthy euthyroid dogs during general anesthesia and surgery [28], and in a small population of healthy dogs following endotoxin administration [18]; however, no similar results in canine species during spontaneous SIRS have been reported. In the present study, the median concentration of rT3 was not significantly different between SIRS and control dogs. This may indicate that rT3 variations may be less susceptible to NTI in spontaneous severe canine disease, at least with respect to

TT3 abnormalities. It is also possible that rT3 variations are somehow influenced by the onset of the disease, and that serial monitoring of that hormone may reveal different changes in its concentrations. The relevance of the measurement of serum rT3 during canine NTI was apparently limited in this population of SIRS dogs, and that limited role needs to be examined in further studies.

Regarding serum fT3 concentrations, the difference between SIRS and control dogs was not significant, although lower values were detected in the SIRS group. This may indicate that, as observed in humans [27], serum fT3 values do not parallel changes in serum TT3 concentrations and are little affected by the presence of NTI in dogs, at least under acute inflammatory conditions. However, the performance and accuracy of the assay used in this study should be considered when interpreting our result.

The pathogenesis of NTI is incompletely described but is assumed to be multifactorial. The binding of thyroid hormones to circulating proteins and their metabolism at the tissue level are possibly involved. Circulating thyroid hormones are tightly bound to thyroid-binding proteins, including albumin. Such molecules are negative acute phase proteins and may decrease in acute critical illness. The high prevalence of hypoalbuminemia in SIRS dogs, particularly in septic SIRS, may account for the decreased thyroid hormones concentrations observed in our study population. This observation is partially supported by the moderate correlation between TT4 and albumin concentrations. However, other mechanisms in the fall of serum thyroid hormones, particularly for TT3, should be considered. However, such investigations were beyond the scope of the present study.

Dogs with septic SIRS had significantly lower serum thyroid hormones (TT3 and TT4) and higher $APPLE_{fast}$ scores than those in non-septic SIRS and control dogs. These results may suggest that the prevalence and the degree of NTI is strictly related to severity of illness. The negative correlation observed between thyroid hormones (TT3 and TT4) and the $APPLE_{fast}$ score may further support this statement. Derangement in serum thyroid hormone levels have been previously demonstrated in a cohort of dogs with SIRS; however, no relationship with survival or with SIRS origin (infectious *versus* non-infectious) was reported [20]. Different analytical methods, case series compositions, and disease categories may have accounted for the different results observed in our study. The presence of NTI has been associated with a negative outcome in different canine diseases [15,24,25,26], and low TT3 levels were correlated with mortality in critically ill dogs and in canine heterogeneous non-thyroidal diseases [7,15]. In addition, low TT4 concentrations were significantly associated with a negative outcome in puppies with parvoviral enteritis at 24 and 48 hours after admission [24].

In our study, significantly lower TT4 values were found in non-survivor dogs with SIRS. In contrast, there was no difference detected between survivors and non-survivors among the other serum thyroid hormones assessed in this study. However, our survival analysis was limited by the low number of non-survivors in our population; the prognostic significance of thyroid hormones in terms of outcome prediction in canine SIRS should be addressed by further studies.

There are some limitations to be considered before interpreting the results of the current study. The retrospective nature of the study limited the measurement of thyroid hormones in multiple standardized time points, and partially restricted the availability of serum samples for evaluation of a more extended thyroid panel (*e.g.*, to also include fT4 and TSH). However, only dogs diagnosed with selected causes of SIRS and with complete clinical and clinicopathological data were included in the study, allowing improved completeness of data available for analysis upon admission. Concerning the method of subgrouping our patients, we decided to include both dogs with parvoviral enteritis and septic peritonitis in the septic SIRS category. Despite both diseases being considered reproducible models of abdominal sepsis [3,16], a potential age-related difference in clinical and clinicopathological variables among disease groups, including controls, could be a major concern. Specifically, younger dogs with parvoviral enteritis may have partially influenced concentrations of some of the variables investigated (*e.g.*, serum albumin). However, statistical tests performed to compare the different groups divided according to final diagnosis (acute pancreatitis, parvoviral enteritis, septic peritonitis, and controls) produced similar results without adding any other significant information (data not shown). The predominance of variant CPV-2c in our population did not allow comparative analysis of variants in dogs affected by CPV. In addition, breed and sex-related differences have been reported to affect thyroid hormone concentrations in healthy dogs [12,21]. Although sex distribution was homogeneous in our population, and only medium-large breed dogs were included, no breed- or sex-matched controls were considered, which may have partially biased the results. It is theoretically possible that some of the SIRS dogs included in the study may have had concurrent hypothyroidism despite the low prevalence of this disease and the lack of historical and clinical features consistent with its presence. Although the authors consider the occurrence of hypothyroidism unlikely in this population, the additional measurement of TSH and fT4 would have better ruled out this hypothesis and completed the thyroidal evaluation in our SIRS dogs. Finally, the data generated from the current study refer to specific categories of canine SIRS and should not be overinterpreted or extended to different diseases or more chronic situations.

In conclusion, our study confirms a wide frequency of serum thyroid hormones alterations can indicate the presence of NTI in a cohort of dogs with SIRS. Serum concentrations of TT3 and TT4 might be considered useful and reproducible markers of NTI during acute inflammatory states in dogs. Thyroid hormones

abnormalities were more severe in septic than in non-septic SIRS dogs, and they were positively correlated with $APPLE_{fast}$ scores. The results suggest the presence of extensive thyroid axis impairment in SIRS dogs with severe illness. Whether the presence of NTI should be considered as an adaptive response to a critical disease or the consequence of endocrine system dysfunction and failure remains a topic of debate; as well, there is uncertainty about the need for therapeutic strategies with hormone supplementation. Further prospective, large-scale studies investigating the pathogenesis and the prognostic role of NTI in canine SIRS are warranted.

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Conflict of Interest

There is no conflicts of interest.

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8.3 Multiorgan dysfunction syndrome in feline sepsis

There are limited data regarding SIRS, sepsis and organ dysfunction in cats (Brady & Otto 2000; DeClue et al. 2011). Increased bilirubin concentration has been reported in septic cats, potentially reflecting hepatic dysfunction and sepsis-induced cholestasis (Brady & Otto 2000). Evidence of coagulopathy has been described in feline inflammatory conditions including neoplasia, pancreatitis and sepsis (Estrin et al. 2006). Similarly, acute kidney injury and respiratory dysfunction have been reported in the context of systemic diseases (Harison et al. 2012; Balakrishan et al. 2017). However, diagnostic criteria for MODS have not been proposed in this species, and a systematic investigation focused on multiple organ system dysfunction in feline critical care patients is lacking.

A significant part of the present PhD project has been focused on feline sepsis, aiming to evaluate organ dysfunction in the course of this syndrome. We hypothesize that MODS is a frequent complication of feline sepsis, and its development is associated with increased sepsis severity and worse outcomes.

For the purposes of the study, critically ill cats with sepsis presented to the Veterinary Teaching Hospital of the University of Bologna (October 2015-September 2017) were prospectively included. Sepsis was diagnosed as presence of SIRS plus infection, as previously reported (Brady & Otto 2000). Cats were classified as having sepsis and septic shock, and criteria adapted from the available canine literature (Kenney 2010; Ripanti et al. 2012) were used to define organ dysfunction (hepatic, renal, cardiovascular, respiratory, hemostatic). MODS was defined as the simultaneous presence of at least two dysfunctional organs.

A total of 43 cats with sepsis of different origins (thoracic, abdominal, related to feline panleukopenia and miscellaneous diseases) were included in the study. Frequency of organ dysfunction was high in the current population, reaching 58% at the time of hospital admission and 86% during hospital stay. Specifically, all cats developed at least one organ dysfunction during ICU stay. Non-survivors (n=17) had a greater number of dysfunctional organs compared to survivors (n=26) both at the time of admission and during hospitalization. Presence of renal dysfunction,

septic shock and MODS were significantly and independently associated with increased odds for mortality both at the time of presentation and during hospital stay.

Preliminary data of the current study were presented at the LXXI Sisvet Conference.

MULTIORGAN DYSFUNCTION IN FELINE SEPSIS:

PRELIMINARY STUDY ON 37 CATS

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Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Feline sepsis is associated with substantial morbidity and mortality, however is scarcely documented in veterinary literature. The aim of this prospective observational study was to evaluate the clinical response and the presence of multiorgan dysfunction syndrome (MODS) in relation to outcome in feline sepsis. Cats admitted to the Veterinary University Hospital of Bologna (October 2015 - February 2017) with a diagnosis of sepsis defined by the presence of systemic inflammation associated with cytological or microbiological evidence of infection were included [1]. History, comorbidities, clinical and clinicopathological data including the Feline Acute Patient and Laboratory Evaluation (APPLE) score and serum amyloid A (SAA), origin of infection, treatments and exitus were recorded. Major systems dysfunctions (respiratory, cardiovascular, renal, hepatic and hemostatic) were reported at the time of admission and during hospitalization. Non-parametric statistics with post hoc analysis were used to compare variables between the different groups; $P<0.05$ was considered significant. Thirty-seven cats were included in the study: 20 males (14/20 castrated), 17 females (9/17 spayed). Median age was 6 years (0,2-15). Origin of sepsis was categorized as: thoracic (13/37, 34%), abdominal (8/37, 22%), feline panleukopenia (8/37, 22%) and miscellanea (8/37, 22%). Comorbidities were reported in 26/37 (70%) cats. Clinical presentation was characterized by depressed mental status, hypothermia and hypotension associated with hypovolemic and/or distributive shock in the majority of the subjects. Upon admission, 26/37

(76%) cats had MODS (≥2 organs involved) with an increment up to 86% (32/37) during hospitalization. Mortality rate in the study population was 38% (14/37). Non survivors had significantly lower body temperature, systolic blood pressure, white blood cells count and higher APPLE score and coagulation times at the time of hospital admission, compared with survivors. Frequencies of death were significantly higher in cats with septic shock, acute kidney injury and MODS. By univariate logistic regression analysis, variables independently associated with a poor outcome were: APPLE score, body temperature, septic shock, acute kidney injury and the number of affected organs upon admission. The latter was the only variable retained in the multivariate analysis. No association with outcome was reported for SAA and the presence of comorbidities. The present study contributes to describe the clinical features of sepsis in cats, which is mainly characterized by signs of hypodynamic shock. Septic shock and acute kidney injury are critical sequelae of feline sepsis with prognostic implications. MODS is a common complication of feline sepsis, and seems to significantly increase the odd of death, as reported in dogs [2]. Further studies in a wider population are needed to better characterize MODS in feline sepsis.

Conclusion

Multiorgan dysfunction syndrome is a relatively new concept in critical care medicine, since its occurrence has paralleled the advances in scientific knowledge and patient management. Today, MODS represents the unique manifestation of critical illness, greatly contributing to ICU deaths in humans and animals. A better understanding of SIRS, sepsis and MODS pathogenesis is mandatory in order to provide the best possible care for all critically ill patients. Advances in critical care medicine have also modified the way ICU patients are approached. Critical illness is not seen as a disease in itself, but rather as part of a continuing disease trajectory: identifying the early signs of patient decline is of major importance in order to maximize close monitoring, provide adequate supportive care and predict complications of critical illness. The intensivist's perspective has become less reactive and more proactive, anticipating complications and starting treatments before a patient deteriorates too much. Biomarkers have been incorporated in the clinical assessment of patients as objective and reproducible indicators of immune function, disease severity and risk of organ dysfunction and treatment failure.

Valuable insights on emergent biomarkers of disease severity and organ dysfunction are provided through the presented studies. Some of the investigated biomarkers are feasible and cost-effective, and might find a place in the routine monitoring of critically ill veterinary patients. In feline medicine, for istance, the clinical identification of sepsis and systemic inflammation might be particularly ambiguous. The measurement of serum amyloid A along with a complete laboratory evaluation could be easily used to better recognize SIRS. Additionally, elevated SAA concentrations coupled with other potential biomarkers of sepsis, like hyperbilirubinemia and presence of neutrophil toxic changes, could significantly increase the clinical suspect of infection, leading to prioritize diagnostic and therapeutic choices.

Similar positive conclusions could be drown for the use of urinalysis in the contex of acute kidney injury. Several points of controversy have challenged the role of urine biochemistry and fractional excretions of eletrolytes in humans; nonetheless, urine biochemistry is still a major tool in AKI

management. The performed investigation tests biomarkers to early evaluate AKI severity and aid in prognostic assessment. Urine biochemistry and fractional excretions of electrolytes, in particular, emerged as suitable and affordable markers of AKI type, severity and prognosis early at the time of diagnosis. This result corroborates recent veterinary findings supporting the routine use of fractional excretions of electrolytes for the assessment of canine acute kidney injury.

On the other hand, measurement of other biomarkers considered for the present thesis is still restricted to research purposes due to the lack of point-of-care tests available in clinical practice or the need of specific analyzers. Canine procalcitonin, for example, shows some potentials as an acute phase protein in dogs, able to recognize systemic inflammation and sepsis, and as a possible prognostic tool being associated with disease severity, MODS development and outcome in the presented studies. Plasma procalcitonin evaluation, however, is still limited by the method of analysis, as the ELISA test available is not easily performed in clinical practice on an individual basis. Similarly, our preliminary data investigates the delta neutrophil index as a hematological marker of sepsis in dogs. No additional costs and blood requirements are needed for its calculation once the complete blood count is performed; however, its evaluation is intrinsically related to the presence of specific hematological analyzers. Despite these limitations, our data might positively affect the current analytical tecniques and instruments available for biomarkers assessment in veterinary practice. If the validity and reliability of such biomarkers will be confirmed in future studies, user-friendly and practical methods might become available for their analyses.

The present research strongly supports the systematic assessment of organ dysfunction during canine and feline SIRS and sepsis. The results reported here establish that selected organ dysfunction and MODS can be identified in critically ill dogs and cats. Specifically, our investigation proposes a novel approach to characterize canine AKI as a volume-responsive or intrinsic disease. This classification, that parallels the human ones, might aid in the assessment and the prediction of complications associated with the disease in dogs. Concerning dogs with SIRS and sepsis, the occurrence of non-thyroidal illness has been confirmed, and insights of its prognostic

potentials have emerged. Finally, interesting results have been documented in cats with sepsis, which may be particularly valuable due to the lack of scientific information in this species. MODS occurrence has been demonstrated as a frequent complication of feline sepsis, with development of septic shock and acute kidney injury acting as negative prognostic factors.

In conclusion, the presented preliminary results significantly enrich the limited veterinary data regarding MODS and sepsis, and represent the basis for future in-depth analyses on organ dysfunction and outcome prediction in critically ill dogs and cats. Dysfunction of potentially any organ system documented in the ICU should be considered as a negative outcome predictor both in canine and feline species. Future research is warranted in order to define specific consensus criteria to define MODS and improve its early recognition and management in high-risk animals.

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