

UNIVERSITA' DEGLI STUDI DI MILANO

PhD Course in Veterinary and Animal Science

Class XXXI



OXIDATIVE-ANTIOXIDATIVE COMPOUNDS AS POTENTIAL
DIAGNOSTIC AND PROGNOSTIC MARKERS IN ANIMALS WITH
SIRS

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ABSTRACT.....	3
Abstract (italian version)	4
List of Publications.....	5
SCIENTIFIC BACKGROUND	6
Inflammation.....	7
The acute phase response.....	8
SEPSIS and Systemic Inflammatory Response Syndrome (SIRS).....	12
Sepsis and Oxidation.....	15
Early diagnosis: how and why it could make the difference.....	17
Paraoxonase-1.....	18
Protein carbonylation.....	20
Method validation and reference intervals.....	22
AIMS.....	23
Aims of the thesis	24
Specific aims.....	24
DESCRIPTION OF STUDIES I-IV.....	26
I. Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses.....	27
Materials and methods.....	27
Results.....	31
Discussion.....	35
II. Paraoxonase-1 (PON-1) activity as a diagnostic and prognostic marker in horses and foals.....	40
Materials and Methods.....	40
Results.....	42
Discussion.....	47
III. Validation of a western blot technique for assaying protein carbonylation in canine serum.....	50
Materials and Methods.....	50
Results.....	52
Discussion.....	55
IV. Diagnostic and prognostic role of protein carbonyls (PCOs) in dogs with sepsis or polytrauma in comparison with paraoxonase-1 (PON-1), C-reactive protein (CRP).....	58
Materials and Methods.....	58
Results.....	60
Discussion.....	62
CONCLUSIONS.....	64
Perspectives.....	67
SCIENTIFIC ACTIVITIES.....	68
REFERENCES.....	72

Abstract

Nowadays in veterinary medicine the discrimination between an inflammatory status and a systemic inflammatory response syndrome (SIRS) sometimes related to an infectious disease and with a poorer prognosis, could be difficult, both for the clinicians and the clinical pathologists. An early diagnosis would permit to avoid unnecessary use of antibiotics (mainly to contain the problem of antibiotic resistance) and to establish an appropriate monitoring plan.

The aim of the two first described works was to validate in horses the Paraoxonase-1 (PON-1), a negative acute phase protein already used in dogs; 120 healthy horses of different sex, age and breed were enrolled; then, this protein was evaluated as a possible diagnostic and prognostic markers of SIRS in this species; PON-1 did not seem to be useful to this aim. The prognostic value was not evaluable because of the small amount of survivors in the SIRS group that did not permit a serial evaluation of PON-1 values.

The second part of the thesis is about the Protein Carbonyls (PCOs), that are already used as sepsis markers in humans; a Western Blotting method was initially validated to detect PCOs in canine serum from healthy patients; then, a spectrophotometric method, that could be cheaper and faster than the first one, was employed. With this method, serum from healthy dogs and from dogs with septic or non-septic inflammation was used to measure PCOs. Results between groups were compared to evaluate if PCOs could be considered as possible diagnostic markers of sepsis in association with PON-1 and C-reactive protein (CRP). This marker seemed to be useful to distinguish dogs with sepsis from dogs with sterile inflammation or healthy, but not to give prognostic information.

An increase in the amount of the enrolled dogs would consent to enforce the hypothesis that PCOs could become a reliable support to diagnose sepsis.

Abstract (italian version)

Ad oggi in medicina veterinaria risulta difficile discriminare, sia dal punto di vista clinico che laboratoristico, uno stato infiammatorio da una forma di più grave infiammazione sistemica talvolta correlata ad infezione e con una prognosi peggiore. Una diagnosi precoce potrebbe permettere di evitare l'uso indiscriminato di antibiotici, sempre più diffuso e responsabile dell'annoso problema dell'antibiotico-resistenza, nonché di impostare un corretto protocollo di monitoraggio del paziente.

Lo scopo dei primi due lavori in seguito descritti è stato quello di validare il metodo per la misurazione della Paraoxonase-1 (PON-1), proteina di fase acuta negativa già utilizzata ad esempio in medicina canina, nel cavallo, includendo 120 soggetti sani di vario sesso, età e razza; in seguito, tale proteina è stata valutata come possibile marker diagnostico di sepsi e prognostico in tale specie, senza tuttavia risultare discriminante fra cavalli con e senza SIRS. Il ridotto numero di campioni di pazienti con SIRS sopravvissuti non ha permesso di valutarne il significato prognostico con delle misurazioni seriali.

La seconda parte della tesi è invece incentrata sulla valutazione delle Proteine Carbonilate (PCOs), già utilizzate come marker di sepsi nell'uomo, nel cane; è stato inizialmente validato un metodo Western Blotting per la loro misurazione nel siero di cani sani ed in seguito è stato utilizzato un metodo spettrofotometrico, che risulta più economico e rapido; le PCO misurate in cani sani e in cani con infiammazione settica e non settica sono state quindi valutate come possibile fattore discriminante la presenza di sepsi in associazione a PON-1 e Proteina C-Reattiva (CRP). Tale marker si è dimostrato utile per distinguere i cani con sepsi da quelli sani o con infiammazione non settica ma non sembra poter fornire informazioni utili ai fini prognostici.

Ulteriori studi su un maggior numero di pazienti potranno essere utili per confermare tali risultati.

List of Publications

The works presented in this thesis are based on the following publications:

- I. Ruggerone B, Bonelli F, Nocera I, Paltrinieri S, Giordano A, Sgorbini M. **Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses.** *Veterinary Clinical Pathology* 2018 Mar;47(1):69-77. doi: 10.1111/vcp.12562
- II. Ruggerone B, Paltrinieri S, Giordano A, Scacco L, Pratelli P, Sgorbini M. **Paraoxonase-1 (PON-1) activity as a diagnostic and prognostic marker in horses and foals.** *Under review (Journal of Veterinary Internal Medicine).*
- III. Ruggerone B, Colombo G, Paltrinieri S. **Utility of a western blot technique for the detection of protein carbonyls in canine serum.** *Under review (Journal of Veterinary Science).*
- IV. Ruggerone B, Troia R, Murgia E, Giunti M, Dondi F, Paltrinieri S. **Protein Carbonylation (PCOs) evaluation in dogs with polytrauma or sepsis in association with Paraoxonase-1 (PON-1), C-Reactive Protein (CRP) and outcome.** *To be submitted.*

The contributions of Beatrice Ruggerone to the listed papers are the following:

- I. Took major part in the laboratory work (sample registration, PON-1 measurement), interpreted the results together with supervisor and co-authors, had an important part in writing the paper.
- II. Took major part in the laboratory work (sample registration, PON-1 measurement), interpreted the results together with supervisor and co-authors, had an important part in writing the paper.
- III. Took major part both in the collection of samples (patients clinical assessment, blood collection) and a minor part in the laboratory work; interpreted the results together with supervisor and co-authors, had an important part in writing the paper.
- IV. Took major part in the laboratory work (sample registration, PCO, CRP and PON-1 measurement), collected the samples of healthy dogs, interpreted the results together with supervisor and co-authors, had an important part in writing the paper.

SCIENTIFIC BACKGROUND

Inflammation

The term “inflammation” comes from Latin and literally means “burning”; it is a non-specific response of vascularized tissues to potentially harmful agents with a defensive purpose (Bochsler and Slauson, 2002).

It consists in a sequela of events that occur regardless of the inciting stimulus, developing from vascularized living tissue. Its aim is to dilute, isolate and eliminate the cause of injury.

Without inflammation, animals would not survive their daily interaction with environmental microbes, foreign material and trauma. However, in some instances, excessive and/or prolonged inflammation could be detrimental and even more harmful than the inciting stimulus (Rock et al., 2010).

Inflammation is characterized by a sequela of events, mostly associated with the activation of phagocytes, that generates reactive oxygen species (ROS) and other oxidants (Van Berlo et al., 2010). This induces an oxidative stress, i.e. an imbalance between oxidants and antioxidant defences, due on one hand to the continuous production of oxidative compounds and, on the other hand, on the consumption of antioxidants, whose serum concentration decreases (Sies and Cadenas, 1985).

ROS can induce reversible or irreversible chemical changes (oxidation, nitrosylation and nitrosation) in proteins, lipids and DNA, resulting in diminished biochemical functions (Valko et al., 2007) and in damaging to cells and organs.

The type of pathway induced depends on the nature of the inflammatory trigger. Bacterial pathogens are detected by receptors of the innate immune system, such as Toll-like receptors (TLRs), which are expressed on tissue-resident macrophage and induce the production of inflammatory cytokines (IL-1, IL-6, TNF), chemokines (CXCL8, CCL2) and prostaglandins. These mediators act on target tissues such as local blood vessels by inducing vasodilation and extravasation of neutrophils that destroy pathogens. Moreover, inflammatory cytokines have systemic effects such as the induction to the production of acute phase proteins (e.g. C-reactive protein, coagulation factors, pro-inflammatory prostaglandins) by the liver and the central nervous system.

Viral infections induce the production of type-I interferons (IFN- α , IFN- β) by activating the cytotoxic lymphocytes; parasites lead to the production of histamine, IL-4, IL-5 and IL-13 by mast cells and basophils (Medzhitov, 2010).

The acute phase response

The acute phase response is a medical expression used to indicate the pathophysiological and behavioural changes that may result from a significant round of either acute or chronic inflammation (Bochsler and Slauson, 2002). Although inflammation is mostly localized at the site of interaction between the pro-inflammatory agent and inflammatory cells, it can lead to systemic consequences: behavioural changes, fever, loss of muscle mass or/and adipose tissue, plasma proteins and leukogram alterations, anaemia and endocrine response.

It may occur as:

- Behavioural changes: malaise, anorexia and increased sleep. Some of these symptoms may be of survival value. For example, malaise and somnolence would decrease physical activity and therefore decrease the energy needs, allowing substrates for proteins synthesis used for inflammatory response. Localized sensation of pain in the site of injury promote immobility of the affected part of the body. Sensory nerve fibres are activated by cytokines or by prostaglandin E2 through a specific glycine receptor subtype (GlyR alpha-3). Moreover, tissue swelling secondary to accumulation of exudate or oedema fluid stretches mechanoreceptors leading to the sensation of pain (Ackerman, 2017).
- Fever: IL-1, IL-6 and TNF are often produced during acute inflammatory response. Without crossing blood-brain barrier, these cytokines, along with bacterial lipopolysaccharides (LPS), stimulate the endothelial cells of the central nervous system to produce prostaglandins, such as PGE2, which change the hypothalamic set-point of temperature control. Therefore, thermogenesis increases and thermodispersion decreases, resulting in a risen body temperature. Fever may have value in terms of enhancing immunity against pathogens (Conti et al., 2004).
- Loss of muscle mass and/or adipose tissue: because lipolysis can provide energy substrates, muscle is catabolized and provides amino acids needed for synthesis of proteins during a negative nitrogen balance phase such as anorexia (Dickerson, 2016).

Acute phase proteins (APPs)

Acute phase proteins are plasma proteins synthesized mostly in the liver, whose concentration increases (positive APPs) or decreases (negative APPs) by 25% or more during inflammation. APPs can be also classified as major or minor depending on the magnitude or frequency of increase or decrease.

They are valuable in clinical practice for two reasons: first, they are sometimes more specific than other markers (neutrophilia or pyrexia) to detect inflammation; second, they allow monitoring an inflammatory process either for therapeutic management or establish a prognosis.

The synthesis of APPs is promoted by several cytokines, especially IL-6, through up and down-regulation of transcription (Gabay and Kushner, 1999).

Positive APPs

These proteins may inhibit or modulate the inflammatory response; their production increases within hours and may persist as long as inflammation is present, depending on their half-lives. The most common positive APPs in veterinary medicine are:

- Haptoglobin (Hp): Hp concentration can increase enough to increase the concentration of total proteins, along with other proteins involved in the delayed-response to inflammation. The serum concentration of Hp increases 1-3 weeks after the onset of inflammation as well as the concentration of immunoglobulins (mostly IgG) and complement fractions (C3) (Eckersall and Bell, 2010).
- Fibrinogen: plasma protein produced by hepatocytes; when enzymatic processes in plasma convert prothrombin to thrombin, thrombin then promotes the conversion of soluble fibrinogen to insoluble fibrin. Interactions of fibrin, platelets and endothelial cells help prevent blood loss from blood vessels. It is expected that fibrinogen increases during inflammatory states but, when inflammation is concurrent with coagulation and fibrinolysis, increased fibrinogen consumption may mask increased fibrinogen production and vice-versa (Davalos and Akassoglu, 2012)
- C-reactive protein (CRP) is a positive APP produced by the hepatocytes in response to cytokines such as TNF and IL-1 β . The main functions of CRP are thought to be the promotion of complement binding to facilitate phagocytosis of bacteria, the induction of cytokine release from monocytes, the inhibition of chemotaxis, and the modulation of neutrophil function. CRP

has a short half-life in the dog (Conner et al., 1988) and serum CRP concentrations are not affected by glucocorticoid administration (Martinez-Subiela et al., 2004) and nonsteroidal anti-inflammatory drugs (NSAIDs) (Borer et al., 2003). The serum concentration of CRP rises both in septic and non-septic patients with systemic inflammatory response syndrome (SIRS): tissue destruction or inflammatory stimulation (Eckersall and Conner, 1988) secondary to infectious (Eckersall and Bell, 2010), neoplastic (Tecles et al., 2005), immune mediated (Eckersall and Bell, 2010) and other conditions (Conner et al., 1988). Moreover, CRP concentrations do not have a circadian rhythm in dogs and are not affected by sex, age, or repeated venous blood sampling (Gommerren et al., 2018). Some studies suggested that its level increases according to the severity of inflammation but these levels have not been shown to differ between survivors and non-survivors (Meisner et al., 2006). Taking all of these facts into consideration, CRP could serve as a useful clinical marker for the presence and resolution of systemic inflammation in dogs (Burton et al., 1994; Otabe et al., 2000; Hayashi et al., 2001). CRP may be used to evaluate the severity of ongoing inflammation and to monitor disease progression and the response to treatment. These characteristics have been confirmed in people (Ehl et al., 1997; Jaswal et al., 2003) and in dogs with pancreatitis, ehrlichiosis, leishmaniasis, and steroid-responsive meningitis-arteritis (Gommerren et al., 2018).

- Serum Amyloid A (SAA): it promotes recruitment of inflammatory cells to inflammatory sites; it is induced by cytokines with the plasma concentration increasing up to a 1000-fold. SAA is mostly associated with HDL and can even become the major apolipoprotein of this particle (Myung-Hee et al., 2012). In horses, it has been shown to have clinical utility for diagnosing the presence of inflammation, assessing response to therapy, determining prognosis (Jacobsen et al., 2005; Jacobsen et al., 2007; Belgrave et al., 2013).

Negative APPs

This group includes, among others, albumin, transferrin, Retinol Binding Protein (RBP) and Paraoxonase-1 (that will be described in the next paragraph):

- Albumin: decreased albumin concentration allows the liver to dispose of amino-acids to produce APPs; hypoalbuminemia becomes evident after several days of inflammation and, when

inflammation is the only cause of the hypoalbuminaemic state, hypoalbuminemia is expected to be mild (Stockham and Scott, 2008).

- Transferrin: it is produced by hepatocytes and is the major transport protein for iron; during inflammation it decreases by action of inflammatory mediators such as IL-1; as consequence, the total iron binding capacity (TIBC), which is a measure of plasma capacity to carry iron and depends mainly on serum concentration of transferrin, decreases during inflammation. Its plasma half-life is not established in domestic species; however, decreased as a major of transferrin concentration) may not be seen until inflammation has persisted for at least a week (Ceron et al., 2005).
- RBP: Serum retinol binding protein transports ingested retinol from the intestine to the liver and other tissues. However, during microbial infection—when retinol transport is particularly important—the amount of this protein dramatically decreases; as such it is unclear how retinol is transported when the body is under attack from pathogens (Derebe et al., 2014).

SEPSIS and Systemic Inflammatory Response Syndrome (SIRS)

The Systemic Inflammatory Response Syndrome (SIRS) refers to clinical manifestations of a complex physiologic response to a non-specific insult of either infectious or non-infectious disease (Bone et al., 1992).

In case of SIRS caused by an infection, the term sepsis is indicated (Singer et al., 2016); in dogs and cats, Gram negative bacterial infections are the most common cause of sepsis, with *E. coli* being the most common isolate (Dow et al., 1989; Walket et al., 2000). However, any organism (e.g. fungus, parasite, virus, protozoan) could result in sepsis. Although SIRS is commonly associated with infection, other non-infectious diseases are known to cause systemic inflammation (e.g. pancreatitis, heat stroke, trauma, burns, major surgery) and an early identification of the cause can change the therapeutic approach and the prognosis definition (Sweeney and Wong, 2016).

The manifestations of sepsis vary based on both pathogen (type, load, virulence, site of inoculation) and host factors (genetic factors, anatomic regions of infection and comorbid diseases); sepsis is not only the induction of inflammation, but rather the induction of an imbalance in the immune system such that physiologic homeostasis can no longer be maintained (De Clue, 2017).

The hyper-inflammatory response and the production of cytokine storm during sepsis have been blamed for the morbidity and the mortality associated to this process. In human medicine, a large amount of money has been spent trying to identify treatments to control the hyper-inflammatory response caused by sepsis (Bosman and Ward, 2013).

Whereas in human medicine several markers of SIRS are well known and used (e.g. procalcitonin, protein carbonyls), in veterinary medicine, diagnostic and prognostic aspects still need to be studied; heart rate, respiratory rate, body temperature and white blood cell count are the clinical criteria often used to determine the severity of illness and prognosis in critically ill patients and can be used to obtain the “sepsis score” (Table 1); but these criteria have neither ideal sensitivity nor ideal specificity.

In one study of 500 dogs, mortality was significantly associated with the SIRS score (Okano et al., 2002); however, it is possible that the stress-induced tachypnea and tachycardia could interfere with the score.

SIRS CRITERIA	DOG	HORSES
Body temperature	>39,2° C or <37,2° C	>38,5° C or <37° C
Heart rate (bpm)	>140	> 52
Respiratory rate (apm)	>40	>20
White blood cell count	>19.500/μL or <5.000/μL or >5% bands	>12.500/μL or <5.000/μL

Table 1. SIRS criteria in dogs (Hackett, 2002) and horses (Roy et al., 2017).

Roy et al. (2017) evaluated the prognostic value of the SIRS score in 479 horses during emergency admissions. For a higher score (3 and 4 out of 4), an increased odd of death was found (OR=8,22). If the blood lactate concentration and the colour of the mucous membranes were added to the score, it was possible to best predict the outcome in the population.

The Acute Patient Physiologic and Laboratory Evaluation (APPLE) has been recently validate to stratify mortality risk in hospitalized dogs, independent of the underlying disease, by illness severity (Hayes et al., 2010). 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 (Figures 1 and 2).

The APPLE fast score seems to be significantly associated with an increased risk of death in dogs with SIRS (Giunti et al., 2015).

Unfortunately, the parameters included in the APPLE score are not always available in clinical practice; lactates and SpO₂ need an arterial blood gas test, not always executable.

The simpler method, based on only 4 parameters easily collected, seems to remain for this reason the most widely used.

During sepsis, biochemical changes cause an imbalance in the redox system leading to formation of an oxidant state, which can intensify SIRS (Andrades et al., 2011). This imbalance favours the oxidant state and is associated with reduced plasma and tissue levels of antioxidants such as glutathione, selenium

and the increase of oxidised lipids (Bosmann and Ward, 2013).

				creatinine (mg/dL) 0-0.62	1 0.63-1.35	8 1.36-2.26	9 >2.26
		9 <5.1		wbc (x10 ⁹ /l) 5.1-8.5	2 8.6-18	3 >18	
6 <2.6	7 2.6-3.0	9 3.1-3.2		albumin (g/dL) 3.3-3.5	2 >3.5		
10 <90	4 90-94	1 95-97		SpO ₂ (%) 98-100			
				total bilirubin (mg/dL) 0-0.23	6 0.24-0.46	4 0.47-0.93	3 >0.93
				mentation score 0	5 1	7 2	8 3
				respiratory rate (bpm) <25	3 25-36	5 37-48	6 49-60
				age (years) 0-2	6 3-5	8 6-8	7 >8
3 2	4 1			fluid score 0			
				lactate (mg/dL) <18.0	2 18.0-71.2	3 71.3-90.1	6 >90.1

Fig. 1 Canine APPLE score: calculated by summing the value in the upper left corner of the appropriate cell for each of the 10 parameters listed, with a maximum potential score of 80. The specificity for a score > 30/80 is 89,4%. From Hayes et al., 2010.

7 <84	8 84-102	9 103-164	10 165-273	glucose (mg/dL) >273			
8 <2.6	7 2.6-3.0	6 3.1-3.2		albumin (g/dL) 3.3-3.5	2 >3.5		
				lactate (mg/dL) <18.0	4 18.0-72.1	8 72.2-90.1	12 >90.1
5 <151	6 151-200	3 201-260		platelet count (x10 ⁹ /L) 261-420,000	1 >420		
				mentation score 0	4 1	6 2	7 3
						14 4	

Fig. 2 Canine APPLE fast score: calculated by summing the value in the upper left corner of the appropriate cell for each of the 5 parameters listed, with a maximum potential score of 50. The specificity of a score >22/50 is 80%. From Hayes et al., 2010.

Thus, it is associated to a complex, variable and prolonged host response in which both pro and anti-inflammatory responses are involved and, based on the type of response, can result in inflammation with tissue damage, immunosuppression and infection, or recovery. As a consequence, it is expected that a hyperinflammatory state develops and that OS is higher than during non-septic inflammation (Bosmann and Ward, 2013).

Sepsis and Oxidation

The assumption on which this thesis is based is that OS is bigger during sepsis than in non-infectious inflammatory state. It is well-known that OS caused by sepsis induces a concomitant imbalance between the production of free radicals and endogenous available anti-oxidants (Alonso de Vega et al., 2002). Results obtained by Muehl et al. (2011) from the comparison of sepsis and burned non-septic patients proved that sepsis is associated with a more pronounced free radical production than SIRS alone: the decreased values of erythrocyte glutathione (GSH) and plasma protein sulphhydryl groups (SH) in septic patients show the depletion of anti-oxidant resources at admission caused by prolonged OS, while acute burn patients gradually developed OS during the study period. Moreover, the early activation of granulocytes in septic patients has been demonstrated (Leemans et al., 2004; Muehl et al., 2011).

During the inflammatory process, OS depends not only on the intensity and length of the response, but also by the efficacy of the inflammatory feedback due to specific cytokines (e.g. interferon- and IL-10), the production of glucocorticoids, and from the expression of defence mechanisms in tissues. Chronic activation of inflammatory cells and the consequent secretion of inflammatory mediators (e.g. the proinflammatory cytokines IL-1, IL-6 and TNF) and ROS are nowadays recognized as pathogenic responsible for different chronic degenerative conditions such as cancer, atherosclerosis and neurodegeneration, and in the overall tissue degeneration associated with aging (Galli et al., 2005).

Several potential biomarkers of OS can be identified at the cellular and molecular level: the activation of immune cells as well as OS-related responses are the consequence of the stimulation of specific signaling pathways such as the protein kinase C (PKC)-dependent signaling, p38-MAPK, and the activation of redox-sensitive transcription factors as AP-1 and NF B (Kyaw et al., 2004; Ceaser et al., 2004). These pathways regulate the expression of different genes that lastly produce important biological responses during the inflammatory process (Boullier et al., 2001). These biological responses can lead to necrosis or apoptosis: for instance, lipid oxidation products trigger specific signals involved in pathways of programmed cell death of inflammatory cells. Lipid peroxidation can induce an imbalance in the cell redox with a decrease in intracellular thiol (-SH) that together with other events

(such as an increase in intracellular calcium) can activate proapoptotic genes (Colussi et al., 2000; Galli et al., 2003).

Some of oxidation-sensitive genes can regulate the expression of several inflammatory or anti-inflammatory cytokines, ROS-generating enzymes, and activated biomolecules, some of which act as paracrine factors influencing inflammatory cell and platelet activation, and vascular cell homeostasis (e.g. nitric oxide, leukotrienes, prostaglandins, and thromboxanes) (Kyaw et al., 2004; Chen et al., 2004). Other genes directly or indirectly activated by inflammatory mediators (mainly cytokines and ROS) are responsible of the enzymatic expression in target tissues, but also induce the production of acute phase. OS is universally recognized as a key event in the overall pathophysiology of aging and a unifying pathogenic mechanism for inflammation-based chronic degenerative diseases (Butterfield et al., 2002; Shishehbor et al., 2004; Gackowski et al., 2004). Unfortunately, one of the main biases in several clinical trials is the complete absence or weakness of biomarkers that would provide a consistent support to the achievement of such therapeutic or overall clinical outcomes.

Early diagnosis: how and why it could make the difference

The management of an animal with systemic and severe clinical signs often represents a challenge in clinical practice: veterinarians should give to the owner reliable information both about the prognosis and a cost estimation. The decision to hospitalize a dog or a cat or to euthanize him is strictly correlated to these aspects.

It is also necessary to remember that not in all these cases an antibiotic therapy is necessary. For example, pancreatitis in dogs is almost always sterile and idiopathic (Hess et al., 1998) and antibiotics could be dangerous by changing the gut microbiota and increasing the problem of the drug resistance in the world.

Therefore, it is clear that the use of reliable diagnostic and prognostic markers of sepsis could make the difference in veterinary medicine, where our knowledge is less wide than in humans. To know if a dog has less chances to survive is necessary both for the veterinarian (who will organize a tight monitoring) and for the owner.

To find reliable markers in veterinary medicine is not easy: first of all, it is imperative to use the best technique for their detection and the validation could be more hardworking than in human medicine. Then, adequate reference intervals and cut offs from a healthy population are necessary. Only at this point, it would be possible to evaluate if this potential marker will be a reliable marker. An amount of sick animals calculated using statistical approach should be collected and a right classification based upon clinical signs and test results is necessary.

The use of some reliable markers of sepsis in human medicine is still controversial in veterinary medicine: procalcitonin, for example, could be indicative for the presence of sepsis according to some studies (Goggs et al., 2018), but the ELISA kit currently available for its measurement presents analytical problems (Floras et al., 2014).

Paraoxonase-1 (PON-1)

PON-1 is a calcium containing enzyme that works as a fast negative APP (Feingold et al., 1998). PON-1 is associated with high-density lipoprotein (HDL) and protects low-density lipoprotein (LDL) and high-density lipoprotein (HDL) from peroxidation (Mackness et al., 2004). Moreover, it has anti-inflammatory properties by reducing the production of pro-inflammatory mediators. During the acute phase response, HDLs lose apolipoprotein A1, esterified cholesterol and many HDL-associated enzymes, including PON-1, which is replaced by serum amyloid A (SAA) and ceruloplasmin (Fig. 3). Therefore, PON-1 serum activity decreases in inflammation.

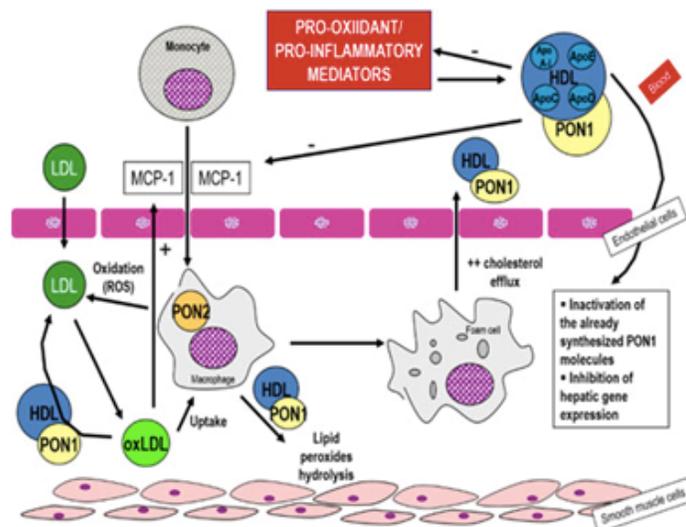


Fig. 3 Schematic representation of the interactions between inflammatory cells, APPs, antioxidant molecules and lipids (reproduced by permission from Dr. G. Rossi, Murdoch University, Perth, Australia).

In people, serum PON-1 activity decreases during oxidative stress (OS) (Novak et al., 2010) and therefore it could be considered a potential biomarker for pathological conditions characterized by OS such as cardiovascular, neurological, liver, renal and orthopaedic disease (Garelnabi and Youlnis, 2015; Milijkovic et al., 2018; Olszewska-Slonina et al., 2018) as well as sepsis and SIRS (Costa and Furlong, 2002; Novak et al., 2010; Li et al., 2013; Bojic et al., 2014; Inal et al., 2015; Marek et al., 2018; Szczeklik et al., 2018).

In the study of Wan Fadzlina et al. (2018), the use of leukocyte counts, procalcitonin, IL-6 and PON were considered in order to understand their prognostic value. A significant difference in PON values between survivors and non survivors has been found.

Its decrease in association to inflammation has been proved also in cattle (Giordano et al., 2013), cat (Tvarijonaviciute et al., 2012a), swine (LaBrecque et al., 2009), horse (Turk et al., 2011; Radakovic et al., 2016) and dog (Tvarijonaciute A et al., 2012; Rossi et al., 2013; Rossi et al., 2014a; Ibbá et al., 2015) but the paraoxon-based method to measure PON-1 activity in serum has been validated only in dogs (Tvarijonaviciute et al., 2012b; Rossi et al., 2013), cattle (Giordano et al., 2013), and in veterinary medicine different methods in absence of a complete preliminary validation study or the establishment of reference intervals (RIs) have been used.

The method validated from Rossi et al. (2013) is precise and accurate, has a variance for intra- and inter-assay repeatability lower than 10%; PON-1 activity decreases only in presence of severe or extreme lipaemia and increases with severe haemolysis. The RI found in healthy dogs ranges from 106,6 to 197,2 U/mL. No significant differences were found between males and females and different age groups.

Moreover, no significant differences were found between dogs with normal CRP and dogs with abnormal CRP or between healthy dogs and dogs with systemic inflammation; this may be due to different kinetics of CRP expression and PON-1 activity changes in serum. In conclusion, this first evaluation in dogs did not confirm the possible utility of PON-1 in discriminating dogs with or without inflammation.

The following study from the same research group (Rossi et al., 2014a) demonstrated that PON-1 was associated to a systemic involvement and significantly decreased only in dogs with severe clinical signs; in dogs with Leishmaniosis, it is expected that the complete normalization of CRP and globulin fractions should not be expected in the first 4-6 weeks post-treatment (Martinez-Subiela et al., 2011; Torres et al., 2011). According to Rossi et al. (2014a), PON-1 normalized earlier than other markers of inflammation, maybe due to its anti-oxidative role (treatments should decrease inflammation and OS in a few days); their conclusion was that PON-1 may work as an early biomarker of responsiveness to treatments and avoid unnecessary prolongation of therapies.

These results confirm that PON-1 is mainly influenced by OS that is more expected during sepsis than during inflammation alone.

Protein Carbonylation

The presence of ROS leads to protein oxidation, and in particular, to the oxidation of protein side chains, with the subsequent generation of protein carbonyl (PCO) groups (aldehydes and ketones) (Dalle-Donne et al., 2003). The amount of PCOs in blood increases under pathological conditions related to oxidative stress. In people, protein carbonylation is the most widely used biomarker for oxidative damage to proteins, since it reflects cellular damage induced by multiple forms of ROS (Dalle-Donne et al., 2005).

As told above, inflammation is characterized by oxidative phenomena and the detection of protein carbonyls (PCO) in biological samples may be used to quantify the level of oxidative stress (OS) associated with inflammation (Colombo et al., 2016). It is expected that, in septic patients, OS is higher than in subjects with inflammation not associated with sepsis (Abu-Zidan et al., 2002) and therefore PCOs may be markers of sepsis. In people, the concentration of plasma protein carbonyl is significantly higher in septic patients compared with controls (Abu-Zidan et al., 2002).

There are many methods used nowadays for the evaluation of the plasmatic concentration of PCOs. Among these, the most employed is based on derivatization of proteins using 2,4-dinitrophenylhydrazine (DNPH) (Levin et al., 1990; Abu-Zidan et al., 2002; Colombo et al., 2016). DNPH reacts with PCO and leads to the formation of 2,4-dinitrophenylhydrazone (DNP), a stable compound that can be detected and quantified through different methods [5]. With the discovery of antibodies able to recognize the DNP adducts, it has been possible to increase the sensitivity of PCO detection. Antibody-based methods have been developed to investigate the concentration of PCO. These methods include dot blot, immunochemistry, ELISA and Western blot (WB) (Buss et al., 1997; Colombo et al., 2012; Wehr and Levine, 2012; Augustyniak et al., 2015).

To the best of our knowledge, in veterinary medicine, few studies have been performed about PCO. For example, Vannucchi et al. (2007) used PCOs along with other markers of oxidation to evaluate the presence and magnitude of oxidative stress associated with pregnancy in healthy dogs. In this study, no significant changes in the concentration of PCOs were found. However, the measurement of PCOs in the cited study was based on an ELISA method not validated in dogs.

Ferreira et al. (2014) evaluated PCOs together with other markers of inflammation in 20 healthy Beagle dogs three hours after the stress due to transport but did not find any significant difference in their concentration before and after the stressful event, differently from Thiobarbituric acid reactive substances (TBARS), Total antioxidant capacity (TAC) and retinol.

Nevertheless, considering the importance of PCOs in human medicine, it would be useful to better assess whether PCO could be a good marker of sepsis also in dogs. As a first step of this approach, however, information on the analytical performances of reliable methods to detect PCOs is needed.

Method validation and Reference Intervals (RIs) establishment

A big part of this project was about the method validation according to already published protocols (Jacobs et al., 1992; Westgard, 2003; Kjølgaard-Hansen and Jensen, 2010; Rossi et al., 2013), both for the PON-1 measurement in equine serum and for PCOs in canine serum.

It is important to underline that if the method validation has not been performed or has not been performed in an adequate manner, the method is not proven to provide reliable data (Bridwell et al., 2010). The method validation involves experimental design to prove that it produces accurate and precise results within the scope of its intended use.

The other preliminary part of the studies was about the RIs establishment for each species. Reference values are used to describe the dispersion of variables in healthy individuals. They are usually reported as population-based RI comprising the 95% of the healthy population (Geffré et al., 2009).

The ASVCP guidelines (Friedrichs et al, 2012) were used to define RIs in these studies. They offer a protocol to determine RIs that meet the minimum requirements for reliability and usefulness. The guidelines focus on health-associated reference values as they relate to quantitative clinical laboratory tests. The aim was to provide RIs that are adequate and useful for clinical interpretation.

AIMS

Aims of the thesis

The aim of this thesis was to evaluate the analytical and biological reliability of innovative markers of SIRS potentially associated with sepsis and characterized by OS. Based on what reported in the paragraphs above, it is expected that OS is less severe in animals with inflammation but without SIRS/sepsis because of a lower level of oxidative substances, compared with animals with inflammation and SIRS/sepsis, that should have a higher level of oxidants (and therefore a lower level of antioxidants). The benefit to identify new reliable markers able to distinguish animals with sterile inflammation from those with sepsis could have a twofold advantage: on one hand, the use of antibiotics could be made only in necessary situations by reducing the widespread of antimicrobials resistance; on the other hand, more early information about the prognosis and the kind of the requested monitoring could be obtained. It is important to remember that antibiotics could change for long periods the gut microbiota and interfere with the immune system of the host. For these reasons, this thesis had the aim to evaluate some markers that, in the future, could become a reliable, cheap and fast support for clinicians.

Moreover, the correlation between these markers and the outcome will be considered in this thesis. Four different studies (studies I and II about PON-1 activity in horses and studies III and IV about PCOs in dogs), each with a well-defined specific aim, were developed and specific aims are reported below.

Specific aims

- I. The first study has been developed with the aim to gain information on analytical performances of a paraoxon-based method to measure PON-1, including the establishment of reference intervals for foals and adult horses. This could be considered as a preliminary step toward investigations about the use of PON-1 in clinical practice as a potential diagnostic and prognostic marker of sepsis.
- II. The sequel of the first study had the aim to compare PON-1 activity in horses classified as septic based on the SIRS score and to investigate the performances of PON-1 activity in terms of sensitivity, specificity and likelihood ratio. Finally, the possible prognostic role of

PON-1 activity was assessed by assessing PON-1 values in sick horses sub-grouped based on the clinical outcome.

- III. Protein Carbonyls (PCOs), already considered markers of severe inflammation in humans, have been considered as possible markers of sepsis in dogs. The aim of the third study was to use a sensitive method such as Western blotting (WB) to detect PCO in canine serum and to investigate, using this method, the actual presence of PCO in canine serum including a preliminary evaluation in sera from dogs with inflammatory diseases.
- IV. The last study had the aim to assess if PCOs in canine serum could be useful to differentiate dogs with sepsis from those with sterile inflammation or from healthy dogs using PON-1 and CRP to assess the presence of oxidative stress and inflammation, respectively; moreover, the possible prognostic role of PCOs by comparing the results obtained at admission with the clinical outcome has been preliminarily investigated.

DESCRIPTION OF STUDIES

I. Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses.

Materials and methods

Case selection

One hundred and twenty horses (40 geldings, 40 stallions, and 40 mares) and 55 foals (27 females, 28 males) were included in this study. The median age in adult horses was 11 years, without significant differences between the age distribution of females, intact males, and geldings. The median age in foals was 47 days, without significant differences in the age distribution between females and males. In addition, horses and foals were grouped by breed (Table 2).

	Foals	Adults	Total
Thoroughbreds		6	6
Trotters	31	46	77
Warmbloods	24	57	81
Draft horses		3	3
Ponies		8	8
Total	55	120	175

Table 2. Classification of the enrolled horses according to the breed.

Horses and foals were classified as clinically healthy on the basis of physical examination, routine biochemistry, and haematology. Horses with particular pathophysiologic conditions (pregnancy, lactation, obesity) were not enrolled. Samples were collected from nonfasted animals sampled during routine health checks. Ten ml were collected into plain tubes without anticoagulant (Venosafe plastic

tubes for serum, Terumo, Europe), followed by centrifugation within 4 h of collection and freezing of serum at 20°C until analysed. Serum PON-1 activity was measured at the Department of Veterinary Medicine, University of Milan. The study was performed within plans of health monitoring and the protocol was approved by the Ethical Committee of the University of Pisa [prot. n. 23506/16]. An owner's written consent to collect samples during health checks and to include the results in this study was also signed.

Measurement of serum PON-1 activity

Serum PON-1 activity was measured spectrophotometrically using an automated analyzer (Cobas Mira; Roche Diagnostic, Basel, Switzerland) and an enzymatic method previously described, which has already been validated in dogs (Rossi et al., 2013) and cattle (Giordano et al., 2013). Six μL of serum were incubated at 37°C with 89 μL of distilled water and 100 μL of reaction buffer. The rate of hydrolysis of paraoxon to p-nitrophenol was measured by monitoring the increase in absorbance at 504nm using a molar extinction coefficient of $18,050 \text{ L mol}^{-1} \text{ cm}^{-1}$. The unit of PON-1 activity expressed as U/mL is defined as 1 nmol of p-nitrophenol formed per minute under assay conditions.

Analytic validation

The intra-assay precision was determined by measuring PON-1 activity in pooled equine sera with low, medium, and high PON-1 activity 20 consecutive times within a single run of each analysis (Westgard, 2003; Kjelgaard-Hansen and Jensen, 2010). The inter-assay variability was assessed on frozen aliquots by analysing the same samples in triplicate on 10 consecutive working days. The mean value, the SD, and the CV ($\text{CV} = \text{SD}/\text{mean} \times 100$) were then calculated. The accuracy was determined using the evaluation of linearity under dilution (LUD) and a spike-recovery test (SRT). The LUD test was performed by measuring PON-1 activity in triplicate on pooled equine serum after serial dilutions with distilled water to obtain samples containing 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, and 0% of serum. The SRT test was performed by adding a pooled equine serum with low PON-1 activity with increasing volumes of a pooled equine serum with high PON-1 activity, followed by the measurement of PON-1 activity in triplicate.

The correlation between the percentage of recovery compared with the expected values of the LUD

and SRT tests were assessed using a least square regression test.

Interference studies

Five serum samples from control horses that appeared non-haemolytic, non-lipaemic, and non-hyperbilirubinemic were pooled in order to obtain a sufficient volume to generate multiple aliquots to be tested with the different interfering substances. The effect of haemolysis, hyperbilirubinemia, and hyperlipaemia was assessed using previously published protocols (Rossi et al., 2013). Specifically, the pooled sample had different concentrations of HGB (Merck KGaA, Darmstadt, Germany), bilirubin (Bil) (Fluka; Sigma-Aldrich, Milan, Italy), and a commercial fat emulsion (Trig) (Lipofundin S 20%; B. Braun Milano SpA, Milan, Italy) added, followed by triplicate measurement of PON-1 activity. Interfering substances were added to each aliquot of the pooled sample in order to obtain final concentrations adequate to simulate the following degrees of haemoglobinemia, lipaemia, and icterus: slight, severe, and extreme.

To further investigate the effect of haemolysis, a haemolysate of equine erythrocytes was also added to the pooled serum (Jacobs et al., 1992). The aim was to assess the influence of intraerythrocytic compounds that could theoretically interfere with PON-1 activity (eg, intraerythrocytic enzymes) or with PON-1 measurements (eg, intraerythrocytic cations that could interfere as cofactors of the in vitro reaction used to measure PON-1 activity). To this aim, equine blood samples collected in EDTA were submitted to the diagnostic laboratory for routine haematology and centrifuged at 2500g for 10 min. The pellet obtained after removal of plasma was then washed twice with phosphate-buffered saline (PBS) to completely remove plasma. After the second wash and a further centrifugation, PBS and buffy coat were removed by aspiration and a hypotonic lysis of RBCs was then performed by adding an equal volume of distilled water to the cell pellet, followed by centrifugation. The final concentration of HGB in the supernatant was verified by a haematology analyser (Sysmex XT-2000iV; Sysmex Corporation, Kobe, Japan). Based on this concentration, the pooled serum was combined with the haemolysate to obtain final HGB concentrations of 10.0, 5.0, 2.5, and 1.0 g/L thus simulating the 1–7% haemolytic rate of the mean RBC mass in a normal equine blood sample.

For all the interferents, the percentage changes of PON-1 activity, compared with the basal sample, with the same volume of distilled water as the volume of interfering solution being added to the serum,

were calculated and plotted vs the concentration of interfering substances to create an interferogram for each substance. Results obtained from the pooled serum with and without the different concentrations of interfering substances were compared using an ANOVA test for repeated measurement (Friedman test). Irrespective of the results of statistical analysis, a 15% variability of mean values were established as an acceptance criterion to assess the clinical utility of the interference study results.

Determination of RIs

The RIs were determined using the Reference Value Advisor macroinstructions (freeware v2.1; <http://www.biostat.envt.fr/spip/spip.php?article63>) for Excel (Microsoft Corp., Redmond, WA, USA), recently validated for use in veterinary laboratories (Geffré et al., 2009). The software performs tests of normality (Anderson–Darling with histograms and Q–Q plots and Box–Cox transformation). Following the Clinical and Laboratory Standards Institute (CLSI) recommendations (2010), the histogram of the RI of PON-1 activity was examined for the initial assessment of distribution and identification of outliers. Dixon’s and Tukey’s tests were used to identify the outliers, with Tukey’s test being more stringent than Dixon’s test. According to the CLSI guidelines (2010), the emphasis was to retain rather than delete outliers. Specifically, near outliers (i.e., values exceeding quartiles I or III minus or plus 1.59 the interquartile range [IQR]) were classified as “suspect” and retained, while far outliers (ie, values exceeding quartiles I or III minus or plus 3.0 9 IQR), if any, were removed (Harris and Boyd, 1990).

The RIs were calculated using the robust method on Box–Cox transformed data, and the 90% CI around the reference limits were determined using a nonparametric bootstrap method.

The possible differences depending on sex, age, and breed were investigated. Specifically, results obtained in foals and adult horses were investigated using a Mann–Whitney U test. The same test was used to investigate the possible differences between male and female foals or in Trotter vs Warmblood horses either in foals or in adults. Results recorded in geldings, mares, and stallions were compared to each other using a Kruskal–Wallis test followed by a Bonferroni post hoc test. The same test was used to compare the results obtained in the different breeds of adult horses. The possible age-related differences were investigated, either in foals or in adult horses, using a regression analysis run in the Reference Value Advisor software cited above.

Results

Analytic validation

Results regarding intra- and inter-assay precision recorded on pooled sera with low, medium, and high PON-1 activity are reported in Table 3. The CVs were < 4% for all the 3 levels of PON-1 in the pooled sera explored in this study.

		High	Medium	Low
Intra-assay	Mean	61.01	34.02	24.04
	SD	0.57	0.55	0.62
	CV	0.93	1.60	2.57
Inter-assay	Mean	62.44	33.71	23.23
	SD	1.64	1.34	0.68
	CV	2.62	3.98	2.91

Table 3. Results of intra- and inter-assay imprecision calculated on pooled sera with high, medium and low paraoxonase-1 activity (U/mL).

Results regarding LUD and SRT are reported in Figure 4. Both tests fitted the linear model ($r^2 = .98$, $P < .001$ for the LUD test; $r^2 = 1.00$, $P < .001$ for the SRT).

Results of the interference studies performed on a pooled serum composed of 5 samples are reported in Figure 5. A significant and progressive decrease of PON-1 activity was found in lipaemic samples: values exceeded the acceptance criterion of $\leq 15\%$ when the concentration of triglycerides was ≥ 5 g/L. Mild bilirubinaemia induced a statistically significant increase of PON-1 activity compared with the baseline value, while the other concentrations did not affect the activity of PON-1. This increase, however, did not exceed the acceptance criterion. Both haemoglobin and haemolysates induced a progressive increase of PON-1 activity that became significant and exceeded the acceptance criteria at values corresponding to very severe haemolysis.

No samples were excluded due to macroscopic abnormalities and therefore all the horses initially selected for inclusion in the study were used to calculate the RIs. Partitioning by age did not reveal significant differences within the group of foals ($P = .180$) or the group of adult horses ($P = .949$) (data not shown).

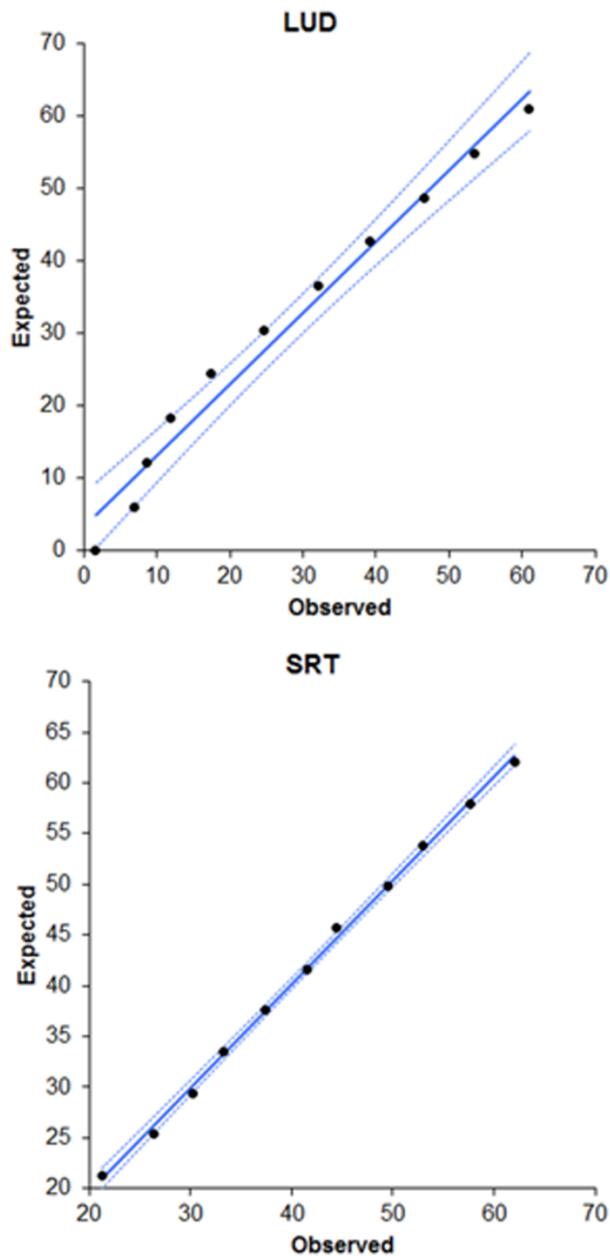


Fig. 4 Linearity under dilution (LUD) of paraoxanase-1 (PON1) activity in a pool of equine sera (60.9 U/mL) progressively diluted (100% to 0%) with distilled water, and Spiking recovery test (SRT) of paraoxanase-1 (PON1) activity in a pool of equine sera with low PON1 activity (21.3 U/mL) spiked with increasing amounts of a pool of equine sera with high PON1 activity (62.0 U/mL). Each data point indicates the mean of a triplicate measurement. The solid line indicates the linear correlation between expected and observed values, dotted lines indicate the 95% Confidence Interval (CI).

Results recorded in male foals were not statistically significantly different ($P = .963$, Figure 6) from those recorded in female foals. The Harris and Boyd test suggests that RIs specific for male and female foals

do not need to be calculated. Conversely, significant sex-related differences ($P = .010$) were found in adults: specifically, results recorded in mares were statistically significantly higher than those recorded in stallions and in geldings (Figure 6). Although the RIs for females, intact males, and geldings overlapped, the Harris and Boyd tests indicates that a sex-specific RI should be used for females, while a common RI could be used for geldings and intact males.

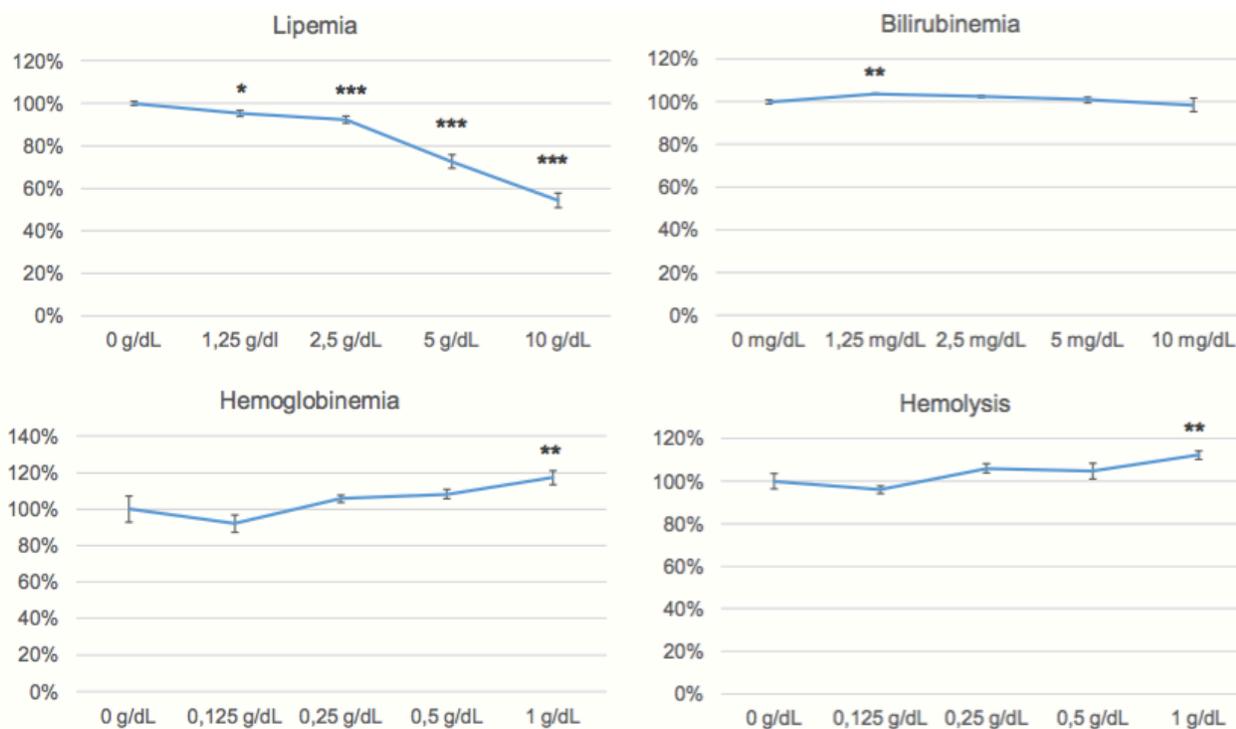


Fig. 5 Effects of increasing concentrations of interfering substances on PON-1 activity determined on pooled equine sera (PON-1 activity of pooled serum: 59,9 U/mL).

Reference intervals

Details about all the RIs recorded in this study are summarized in Table 4, and the data distribution is reported in Figure 7, according to the current guidelines for establishment of RIs (Friedrichs et al., 2012).

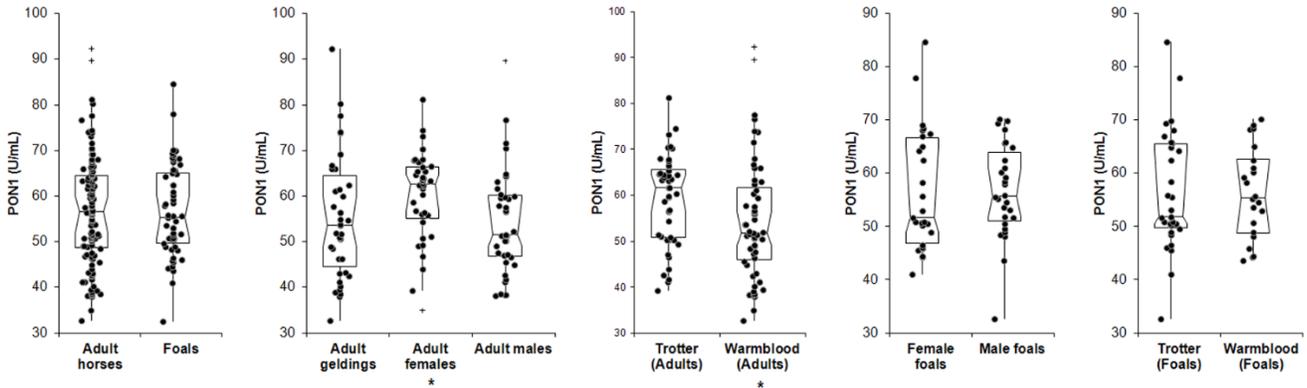


Fig. 6 Comparison of results obtained in adult horses vs foals, stallions vs mares vs geldings, Trotters vs Warmblood adult horses, male vs female foals, and Trotter vs Warmblood foals. The boxes indicate the I–III interquartile range (IQR), the horizontal line indicates the median values, whiskers extend to further observation within quartile I minus $1.5 \times$ IQR or to further observation within quartile III plus $1.5 \times$ IQR. '+' indicates near outliers (i.e., values exceeding quartiles I or III minus or plus $1.5 \times$ IQR). The grey shaded area indicates the RI calculated for the whole population of horses. The asterisk indicates a significant difference (mares vs stallions and geldings; adult Trotters vs Warmbloods).

The RI recorded in the whole combined population of horses was 38.1–80.8 U/mL. The RIs recorded in adults and foals were very similar to each other, and the Harris and Boyd test (Harris and Boyd, 1990) did not indicate the need of establishing separate RIs for adults and foals. Moreover, no statistically significant differences were found between foals and adults (Figure 6).

The analysis of data recorded in adult Draft horses (mean \pm SD: 45.5 ± 12.4 ; median: 38.5; I–III IQR: 38.2–56.2), Ponies (59.5 ± 6.0 ; 60.7; 55.6–62.3), adult Thoroughbreds (60.0 ± 13.8 ; 60.1; 46.6–70.1), Trotters (59.3 ± 9.8 ; 61.6; 39.3–65.6), and Warmbloods (54.4 ± 12.9 ; 51.8; 32.7–61.6) did not reveal statistically significant differences ($P = .083$), but when the analysis was restricted to the 2 breeds represented by a sufficient number of animals (Trotter vs Warmblood horses), a statistically significant difference was found (Figure 6). Also, in this case, breed-specific RIs overlapped but the Harris and Boyd test suggested that RIs specific for Trotter or Warmblood horses should be used.

No statistically significant differences were found between PON-1 activities recorded in Trotter foals compared with Warmblood foals (Figure 7). Also in this case the RIs recorded in Trotter foals and Warmblood foals overlapped. The Harris and Boyd test suggests that RIs specific for Trotter or Warmblood foals are not necessary.

	N	Mean	SD	Median	Min	Max	Outliers	RI	CI Lower limit	CI Upper limit	Distr	Analysis type
Whole population	175	56.6	11.2	56.0	32.6	92.3	2 s	38.1-80.8	32.6-38.6	74.5-92.3	G	NP
Total adult horses	120	56.6	11.7	56.6	32.7	92.3	2 s	38.0-81.2	32.7-38.5	74.5-92.3	G	NP
Stallions	40	54.3	11.7	51.5	38.2	89.6	1 s	38.4-87.7	37.6-40.2	78.9-100.0	G	RT
Mares	40	60.3	9.6	62.7	34.9	81.2	1 s	37.3-77.5	29.7-45.4	74.7-80.4	NG	RT
Geldings	40	55.3	13.3	53.7	32.7	92.3	0	33.2-87.1	30.5-36.7	78.6-95.9	G	RT
Adult Trotters	46	59.3	9.8	61.6	39.3	81.2	0	37.6-78.4	32.5-43.7	75.3-82.0	NG	RT
Adult Warmbloods	57	54.4	12.9	51.8	32.7	92.3	2 s	25.8-78.9	21.0-31.2	72.3-85.1	NG	RT
Foals	55	56.5	10.0	55.2	32.6	84.7	0	38.2-79.9	35.0-41.2	73.7-84.2	G	NP
Male foals	28	56.7	8.8	55.8	32.6	70.2	0	35.7-73.2	29.5-42.3	68.5-76.7	G	RT
Female foals	27	56.6	11.3	51.1	41.0	84.7	0	39.4-92.7	37.8-42.3	75.4-111.5	NG	RT
Trotter foals	31	56.5	11.2	51.9	32.6	84.7	0	35.4-84.5	31.7-40.1	72.5-92.7	NG	RT
Warmblood foals	22	56.4	8.6	55.5	43.7	70.2	0	39.5-76.3	36.6-44.2	70.5-81.5	G	RT

Table 4. RIs established in this study for the whole population of adult horses, for foals or for the subcategories of adult horses. All the values are expressed in U/ml. N= number of horses; SD= standard deviation; CI= confidence interval; Distr= distribution; G= gaussian; NG = non Gaussian; NP= non parametric; s = suspected outlier; RT = Robust Box-cox transformed.

Discussion

Several studies of PON-1 activity in other species have been reported (Turk et al., 2004; Bionaz et al., 2007; Tvarijonavičiute et al., 2012a; Giordano et al., 2013; Rossi et al., 2013; Rossi et al., 2014b; Escribano et al., 2015; Ibbá et al., 2015). A single study in horses is available but no information on the analytic performances of the method employed were provided (Turk et al., 2011). Before investigating the differences between clinically healthy and sick horses and foals a validation study was required to determine the analytic performance of the paraoxon-based method for measurement, that in other species, was preferred over other substrates because they are cheap, rapid to perform, very precise, and accurate (Rossi et al., 2013).

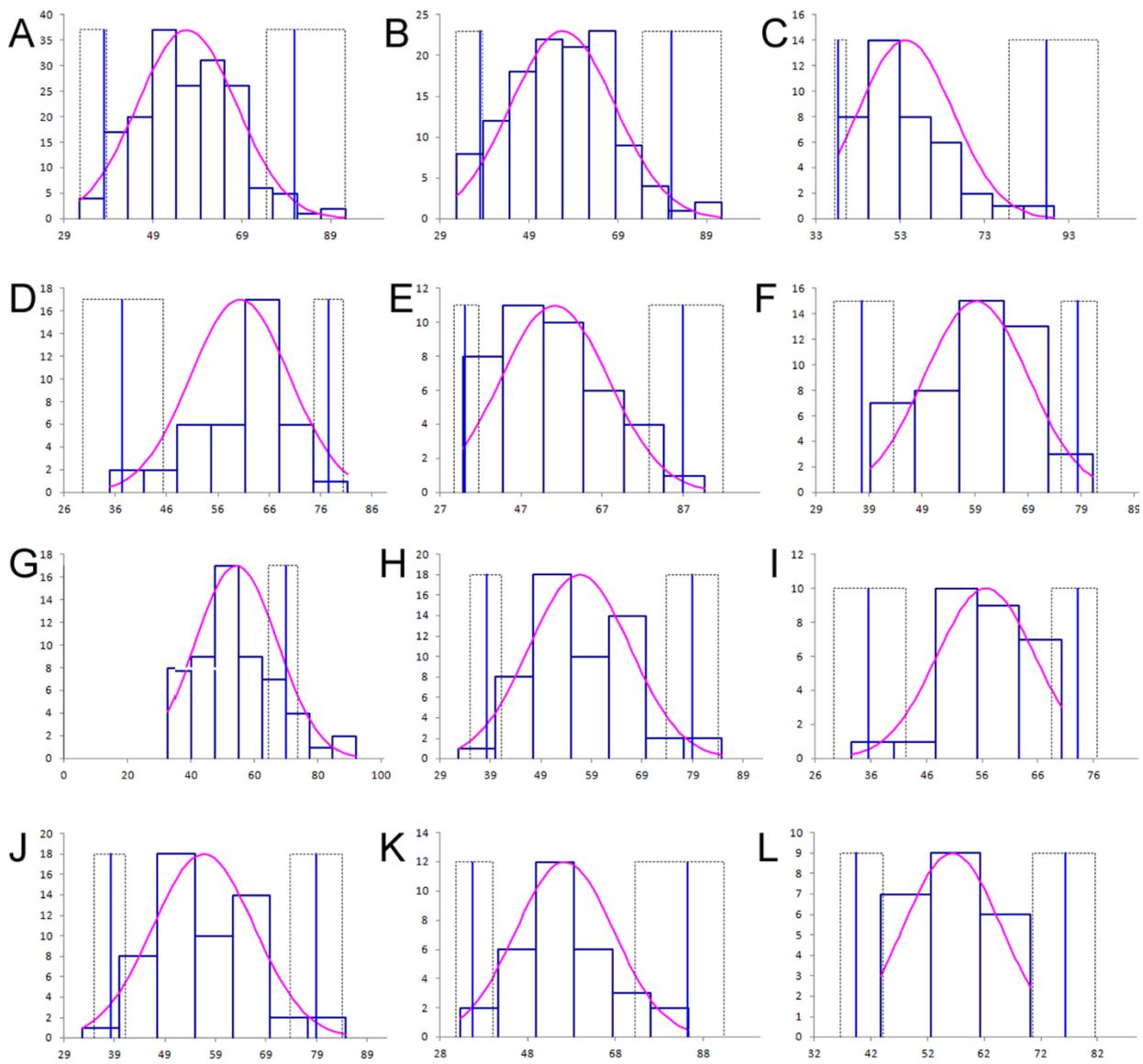


Fig. 7 Distribution of PON-1 values recorded in adult horses, foals and in the various categories of adult horses. The bars indicate the relative frequency of each unit of PON-1 activity. The pink line summarizes the fitted distribution. The vertical light blue lines indicate the upper and lower limit of the reference interval whereas the dashed lines indicate the 90% confidence intervals of each limit. A= whole population (adults and foals); B= adult horses; C= stallions; D= mares; E= geldings; F= Trotter (adults); G= Warmblood (adults); H= foals; I= male foals; J= female foals; K= Trotter (foals); L= Warmblood (foals).

With this study, we demonstrated that this method for the measurement of PON-1 is precise and accurate also on equine serum. Specifically, both intra-assay and inter-assay imprecision were < 4%,

which is considered acceptable for most biochemical analytes (Ricòs et al, 1999) and is similar or, in regards to inter-assay imprecision, lower than that recorded in dogs (Rossi et al., 2013). Currently, a gold standard method for the evaluation of PON-1 does not exist and therefore, accuracy was indirectly estimated through the evaluation of LUD and a SRT which fits the linear model confirming the excellent accuracy of the paraoxon-based method in horses.

Our study demonstrated that the interference of bilirubin is minimal and not clinically relevant. This finding is consistent with previous observations in dogs (Rossi et al., 2013), and considering that hyperbilirubinemia is often seen in sick horses, this finding is very valuable (Engelking, 1993; Barton, 2004). Also the variations in PON-1 activity associated with lipaemia, hemoglobinemia, or haemolysis are similar to those previously recorded in dogs (Rossi et al., 2013); therefore, it is not advised to analyse PON-1 activity in samples with slight to very severe lipaemia or with very severe haemolysis, as a significant bias can occur in PON-1 activity (decrease or increase, respectively) in such samples.

Moreover, we documented that in healthy horses PON-1 activity measured by the paraoxon-based method is lower than that of dogs, cats, and cattle (Tvrijonaviciute et al., 2012a; Giordano et al., 2013; Rossi et al., 2013). It could be hypothesized that different species have a different hepatic or lipid metabolism that influence PON-1 activities, or that every species has its own particular isoforms that reach different serum concentrations. In people, the polymorphism of selected regions of the PON-1 genes may influence the types of enzymatic activity (eg, esterase and/or lactonase) thus explaining the wide individual variability in term of capability to interact with different substrates in vitro (Van Himbergen et al., 2006).

No statistically significant differences were observed between values recorded in adult horses and foals, as reported in calves where PON-1 activity was not significantly different from that of adults (Giordano et al., 2013). In other species, very low PON-1 activity was recorded only in newborns, with significant increases from day 3 to 21 after birth (Giordano et al., 2013), possibly due to the immaturity of the liver, to differences in lipid metabolism, and to a high susceptibility of newborns to OS (Inanami et al., 1999). In our study, foals between 19 and 90 days of age were included. Hence, it would be advisable to assess PON-1 activity in foals younger than 19 days. This information would be particularly interesting when investigating the diagnostic value of PON-1 activity as a biomarker in neonatal septicaemia, typically

occurring a few days after birth (Carter and Martens, 1986; Paradis, 1994).

Partitioning by age did not show any significant association between PON-1 activity in adult horses or foals. This conclusion is similar to that in studies of people, where no significant age-related differences were found (Mueller et al., 1983).

The detection of higher PON-1 activity in mares contrasts with findings in dogs (Rossi et al., 2013) but is consistent with that reported in mice (Ali et al., 2003) and people, where the difference was statistically significant in some studies (Wehner et al., 1987) but not in others (Mueller et al., 1983), which may also be associated with a genetic predisposition (Krisch et al., 1968; Playfer et al., 1976; Carro-Ciampi et al., 1981, Eckerson et al., 1983; Mueller et al., 1983; Kinnunen et al., 2005).

Differences that we found by sex, however, were present only in adult horses and not in foals, maybe because foals were below the age of sexual maturity. This is consistent with previous studies in mice that showed sex-associated differences were testes- but not plasma testosterone-dependent, and that ovariectomy had no effect on PON-1 mRNA expression (Playfer et al., 1976). However, further studies are needed to fully assess the effect of sex in foals, as the number of cases per group was < 40 individuals, the minimum database recommended by the ASVCP guidelines (Friedrichs et al, 2012).

After grouping the horses based on the breed, we found a statistically significant difference only between the Trotters and Warmbloods, with higher PON-1 activity in the Trotter compared with Warmblood horses. This difference, however, was recorded in adult horses but not in foals, although the lack of differences in foals may be due to the low number of observations (Friedrichs et al, 2012). However, the higher values recorded in adult Trotters may also be related to differences in training and exercise intensity that may induce oxidative phenomena (Kinnunen et al., 2005). No statistically significant differences in PON-1 activity were found when other breeds were also included in the statistical comparison. The absence of statistically significant differences for other breeds could be related to the small number of horses in these groups that may have induced a type II statistical artefact (Armitage et al., 1971). Further studies with a higher number of horses in each of these breeds could be useful to identify possible differences associated with variations in exercise and workloads.

Despite the statistically significant differences associated with sex or breed, the RIs recorded in the

different groups of animals overlapped with each other. Nevertheless, according to the Harris and Boyd test, specific RIs in mares, and Trotter and Warmblood horses do not need to be established. However, these latter aspects need to be verified through additional studies on larger study groups as analysis of the reference ranges and of data distribution reveals that the RI generated in the whole population is narrower and has a higher upper reference limit compared with the RIs of stallions, geldings, or Warmbloods. This is likely to be due to the low number of animals per group after partitioning by sex or use. Independent of the need to establish sex- or breed-associated RIs, it is worth noting that the lower reference limit of the intervals was similar for all the categories of partitioning examined in this study. Considering that in many species, sick animals with OS associated with inflammation have very low PON-1 activities compared with the lower reference limit (Giordano et al., 2013; Ibba et al., 2015), it is thus advisable to use a single RI for stallions and geldings or for foals and adults.

One limitation of this study was the absence of other inflammatory markers to better focus on the inflammatory status of horses. For example, Serum Amyloid A concentrations have been shown as useful for screening and preliminary diagnosis in several internal disorders, both in adult horses (Nunokawa et al., 1993; Hulten et al., 1999; Petersen et al., 2004; Copas et al., 2013) and in foals (Hulten et al., 2002; Giguère et al., 2016). Future studies should consider this marker together with PON-1 activity measurement to evaluate its possible role as diagnostic and prognostic marker.

Moreover, to confirm the role of PON-1 as an anti-oxidative marker, the evaluation of oxidative parameters (e.g. reactive oxygen metabolites - d-ROMs) should be considered. D-ROMs are nowadays considered a 'gold standard' for measuring total systemic oxidative status (Celi et al., 2010) and an indirect method to evaluate the free radicals in the serum.

II. Paraoxonase-1 (PON-1) activity as a diagnostic and prognostic marker in horses and foals.

Materials and methods

Samples and study design

This study was done on 171 blood samples from 40 adult horses (24 mares, 11 geldings and 5 stallions) and 7 foals (6 males, 1 female). In order to perform a reliable comparison with reference intervals (RIs) generated in our previous study (Ruggerone et al., 2018), only foals aged 20-100 days of life were included in this study.

Horses and foals were classified as sick when clinical examination and ancillary tests (routine biochemistry and haematology, blood culture, radiographs, ultrasound examination, cytological and bacteriological evaluation of synovial fluid or bronchoalveolar lavage fluid) were consistent with the presence of inflammation, potentially associated with bacterial infections (pneumonia, arthritis, pyometra, omphalitis and pericarditis) or not (neoplasia or colic).

For every sick animal, the “SIRS score” was calculated (Roy et al., 2017) based on the presence of two or more of the following changes: leukopenia or leucocytosis (RI: $5.4-14.3 \times 10^3/\mu\text{L}$), left shift (>10% band neutrophils), hyperthermia or hypothermia (RIs 37.2-38.3°C), tachycardia (RIs: 28-44 bpm), tachypnea (RIs: 8-15 bpm) (Byars and Gonda, 2015; Sharkey and Overmann, 2014).

Sick animals were referred to two different hospitals providing secondary health care; horses and foals already treated with anti-inflammatory or antibiotic drugs were not included in this study. After the first sampling, all the subjects received appropriate treatments according to the diagnosis and the clinical presentation.

Blood samples were drawn within 1h after the admission at the hospital, and additional samples were then collected at 24h intervals until discharge or death or for a maximum of 96h.

Blood samples were collected for the evaluation of CBC and PON-1 activity from the jugular vein using

a sterile syringe and 16G needle. Each blood sample was divided in two aliquots: a 1 mL aliquot was collected in a potassium ethylene diamine tetraacetic (K₂EDTA) test tube and analysed by a cell counter (ProCyte Dx™, IDEXX, USA) within 5 minutes after the collection. A second 2.5 mL aliquot was collected in plain tubes and immediately centrifuged at 2100 relative centrifugal force (RCF) for 10 minutes within 4 hours of collection. The harvested serum was placed in sterile tubes, frozen at -20°C to be transported in cold chain to the Department of Veterinary Medicine of our University, where serum PON-1 activity was measured in a single batch as described below.

The study was performed within plans of health monitoring and during the hospitalization of sick animals: an owner's written consent has also been signed. The protocol was approved by the Ethical Committee of the University of Pisa [prot. n. 23506/16].

Measurement of serum PON-1 activity

The method is the same used for the previous study and described at page in the materials and methods section of study I.

Statistical analysis

Statistical analysis was performed in an Excel spreadsheet using a specific software (Analyse-it, Analyse-it Software Ltd, Leeds, UK).

Results regarding PON-1 activity at the first visit (before treatment) collected from sick horses with a SIRS score equal to or higher than 2, consistent with a systemic response to inflammation (Roy et al., 2017) were compared with those of sick horses with a SIRS score lower than the threshold above, using the Mann-Whitney *U* test. The same test was used to compare the results obtained at first visit from animals that died with those that responded to treatment, including both SIRS and non-SIRS horses. A Fisher exact test was applied to verify the association between categorized PON-1 activity values and SIRS scores. Statistical differences were set for $p < 0.05$.

In order to assess the diagnostic performances of PON-1 activity in detecting horses with SIRS, the number of samples from sick animals with a normal or high SIRS score having, during the first visit, PON-1 activity values within or below the RIs already established (Ruggerone et al., 2018) was counted. Data were then classified as follows:

- True positive results (TP): Animals with PON-1 activity lower than the RIs and with a SIRS score ≥ 2 ;
- False positive results (FP): Animals with PON-1 activity lower than the RIs and with a SIRS score < 2 ;
- True negative results (TN): Animals with PON-1 activity within the RIs and with a SIRS score < 2 ;
- False negative results (FN): Animals with PON-1 activity within the RIs and with a SIRS score ≥ 2 .

The same classification was used to assess the diagnostic performances of PON-1 activity in detecting animals with a negative prognosis; horses that died despite treatments were considered as 'positive' whereas those who survived as 'negative'.

In both cases, TP, TN, FP and FN results were used to calculate sensitivity, specificity and likelihood ratios using standard formulae (Gardner and Greiner, 2006; Jensen and Kjelgaard-Hansen, 2010).

Results from sequential samplings collected after treatments were not statistically compared to each other due to the low number of samples with a comparable follow up. Hence, the analysis of results collected during the follow up was limited to a visual observation of the trend recorded in animals that survived or died despite treatments.

Results

Caseload

The 47 sick animals were affected by one or more of the following: colic (n=15), pneumonia (n=9), wounds (n=5), arthritis, neoplasia, weight loss, neurological signs, fracture, polyarthritis (n=2 each), pleuropneumonia, pyometra, enteritis, sinusitis, evisceration, paraphimosis, omphalitis, oesophageal

obstruction, inguinal hernia, pericarditis and caesarean section (n=1 each).

A SIRS score ≥ 2 was found in 19/40 adult horses and in all the foals (7/7). In horses, the highest score (4/4) was associated with pneumonia (3 cases), while the score was < 2 in the majority of cases of colic and neoplasia. The foals with the highest score (4/4) had pneumonia, polyarthritis and omphalitis.

Nine/40 horses and 1/7 foal died despite treatments or were euthanatized (1/40) due to the worsening of clinical conditions and not for economic reasons. Dead adults were affected by colic (n=3), pneumonia (n=3), inguinal hernia (n=1), neurological signs (n=1) and pleuropneumonia (n=1), whereas the foal had polyarthritis and omphalitis. Four horses died after the first sampling, 1 after the second, 4 after the discharge. The foal died 8 days after the first sampling.

Comparison of results recorded before treatment

At admission, in all the foals (7/7), PON-1 activity values were within the reference limits (lower limit of the RI: 38.2 U/mL). Conversely, in 7/40 adult horses PON-1 activity values were below the lower limit of the RI of adult horses (38.0 U/mL). All of them belonged to the SIRS group. However, when results recorded in males, females or geldings were compared with the lower limit of the specific RI (Males: 38.4 U/mL; Female: 37.3 U/mL; Geldings: 33.2 U/mL), only 6 horses (5 females and 1 gelding) had low PON-1 values.

Considering the whole caseload (e.g. horses and foals merged in a single group), no significant differences were found in PON-1 levels between animals with low or high SIRS score or between animals that survived or died (Figure 8). Contingency analysis showed a statistical association ($P=0.012$) between categorized PON-1 values (within vs below the reference interval) and SIRS scores (higher or lower than 2).

Similarly, no differences were found if the analysis above were restricted to the groups of adult horses, regardless of the gender. Such a comparison was impossible for foals, because all of them had a positive SIRS score and only one patient died during the follow up, the same applies to male adult horses, because only two animals had a positive SIRS score and none died during the follow up, and to geldings,

because only two animals had a positive SIRS score and other two died during the follow up.

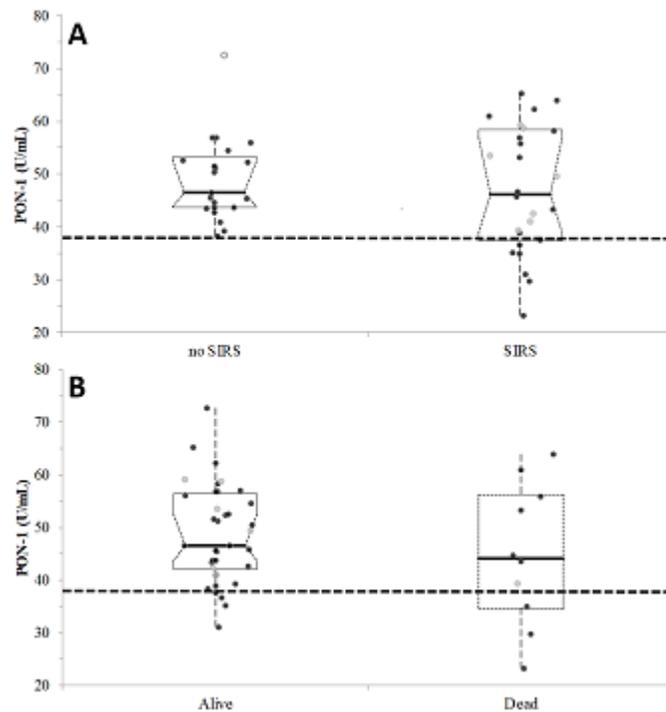


Fig. 8 A) Distribution of results of adult horses (black dots) and foals (grey dots) according to the SIRS Score. B) Distribution of results of adult horses (black dots) and foals (grey dots) according to the outcome. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicate the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR. The white circles indicate the outliers. The dotted line indicates the lower limit of the RI reported in healthy horses.

As noted in Fig. 8A, horses with PON-1 activity lower than the RI were found only in the SIRS group, that, however, included also several horses with normal PON-1 activity. Conversely, horses with either low or normal PON-1 activity were found in both groups formed according to the outcome (Fig. 8B). More specifically, the number of FP, FN, TP, TN recorded in each group, and specificity, sensitivity or positive likelihood ratio calculated are reported in table 5.

As shown in the table, a low PON-1 activity has an absolute specificity for the diagnosis of SIRS (no FP are found, independently on the group and on the corresponding cut-off of each group of gender or age). Conversely, PON-1 activity has a very low sensitivity, since the number of FN (normal PON-1

activity in horses with SIRS) is high, especially in foals and in male adults.

		TP	FN	FP	TN	TOT	Sens (%)	Spec (%)	LR+
SIRS	Foals and adults	7	19	0	21	47	26.9	100.0	n.c.
	Foals	0	7	0	0	7	0.0	n.c.	n.c.
	Adults	7	12	0	21	40	36.8	100.0	n.c.
	Adult males	0	2	0	3	5	0.0	100.0	n.c.
	Adult females	5	10	0	9	24	33.3	100.0	n.c.
	Adult geldings	1	1	0	9	11	50.0	100.0	n.c.
Outcome	Foals and adults	3	7	4	33	47	30.0	89.2	2.78
	Foals	0	1	0	6	7	0.0	100.0	n.c.
	Adults	3	6	4	27	40	33.3	87.1	2.58
	Adult males	0	0	0	5	5	n.c.	100.0	n.c.
	Adult females	3	3	2	16	24	50.0	88.9	4.50
	Adult geldings	0	2	1	8	11	0.0	88.9	0.00

Table 5. Sensitivity (Sens), Specificity (Spec) and positive Likelihood Ratio (LR+) in the examined subpopulation according to the SIRS score and the outcome. TP = true positive, FN = false negative, FP = false positive and TN = true negative, TOT= total number.

The trend reported above occurs also when animals are grouped based on the outcome. In this case, however, the specificity, despite high, is not absolute, and false positive results may occur. However, when PON-1 activity is low, the likelihood to have a poor prognosis is 2.58 to 4.50 times higher than the likelihood to have a response to treatments.

Results recorded during the follow up

Results recorded during the follow up of horses that died despite treatments are summarized in Fig. 9A: four of these horses (4/10) at admission had PON-1 activity values below the lower reference limits, but they died after the first sampling. The remaining 6/10 horses had normal PON-1 activity at admission: one died within the first 24 hours and 5 were repeatedly sampled during the follow up. Two of them had transient decreases of PON-1 activity below the lower limit of the RI, followed by a restoration to normal values before death. None of the dead horses had persistent decreases of PON-1 activity over time. Conversely, also 9 of the 106 samples collected during the follow up from horses that had normal PON-1 activity values and survived the hospitalization period were transiently lower than the lower reference limit (data not shown).

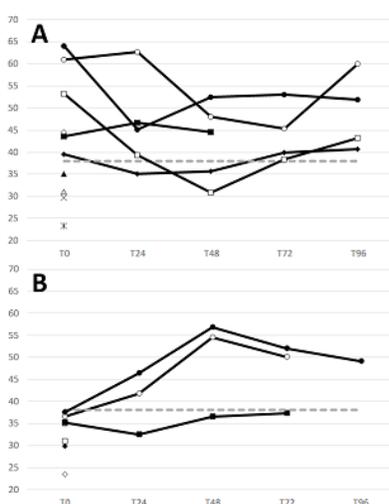


Fig. 9 A) Trend of PON-1 values in adult horses and foals that died despite treatments. The grey dotted line indicates the lower limit of the RIs of PON-1 already reported in healthy horses. B) Trend of PON-1 values in foals or horses that had low PON-1 values at admission, independently on the outcome. The grey dotted line indicates the lower limit of the RIs of PON-1 already reported in healthy horses.

In order to understand whether fluctuations over time of PON-1 activity may be associated or not with a positive follow up, we compared the behaviour of PON-1 activity of all the horses (dead or alive) with low values at admission (Fig. 9B). However, such a comparison was hampered by the fact that the only 4 horses that died early during the follow up had only a single sample. The other three survivors had an increase of PON-1 activity that, however, was not sufficient to restore a normal PON-1 in one of them.

Discussion

In several species, it has already been demonstrated that the antioxidant enzyme paraoxonase-1 (PON-1) decreases in association with oxidative stress that characterizes sepsis (Novak et al., 2010; Turk et al., 2011; Tvarijonaviciute et al., 2012c; Rossi et al., 2014a; Ibba et al., 2015; Radakovic et al., 2016). This study was designed to assess whether PON-1 may work as a diagnostic or prognostic marker in horses. To this aim, a paraoxon-based method recently validated in horses (Ruggerone et al., 2018) has been used.

However, our results support only partially the possible role of PON-1 activity as a diagnostic marker in horses. In order to improve the rigorousness of the study, we compared the results of adults and foals with the age-specific RIs determined in the previous study, although the difference between the lower reference limits of the two age groups (0.2 U/mL) was minimal and probably lower than the analytical precision of the method. However, at the first visit PON-1 activity in sick horses or foals was mostly within the age-specific RIs, except for a few cases. All the horses with low PON-1 activity values at admission had a SIRS score consistent with the presence of systemic inflammation. However, several other horses with a high SIRS score had normal PON-1 activity. Additionally, the group of horses with a poor prognosis included either horses with low PON-1 activity or horses with normal PON-1 activity.

Hence, PON-1 activity may not be a good marker of SIRS in horses, differently to what reported in other species (Giordano et al., 2013; Rossi et al., 2014b). This may suggest that, compared to other species, horses have a different PON-1 metabolism, or a less intense oxidative stress during inflammation. In fact, differently from other inflammatory markers, PON-1 activity decreases only in the presence of oxidative stress (Feingold et al., 1998; Novak et al., 2010). Future studies based also on the measurement of other markers of oxidation (e.g. thiobarbituric acid substances, reactive oxygen species or others) would allow to clarify this aspect. The hypothesis that not all the inflammatory conditions are associated with oxidative stress is supported by results of previous studies in dogs, that found a decreased PON-1 activity only in some, but not all, dogs with increases of other acute phase proteins (Rossi et al., 2013; Rossi et al., 2014b). Furthermore, either horses with localized inflammation (e.g. septic arthritis) or horses with systemic diseases (e.g. pneumonia), which probably have a different magnitude of oxidation, were included in the current study and this may have contributed to the

variability of results among the different horses. Additionally, as in any field study, it was not possible to standardize the time elapsed between the onset of inflammation and the first sampling and therefore the magnitude of inflammation may be different in horses examined just after the onset of clinical signs or a few hours later. Finally, a possible explanation for the lack of differences between results of horses with low and high SIRS score may depend on a poor accuracy of the SIRS score in differentiating horses with sepsis. Differently from the sepsis score used in people (Novak et al., 2010), the SIRS score employs clinical and laboratory parameters that may be associated with sepsis but also with other pathophysiological conditions; it is therefore possible that foals with high SIRS score actually do not have a systemic spread of the septic process and vice versa. Moreover, the reference limits for the parameters included in the SIRS score may vary with the different studies, in accordance with the guidelines for determining the reference intervals (Jacobsen and Andersen, 2007), which recommend adapting the reference intervals to each specific conditions of methods, instruments, sampled population and environment. For example, Lambert et al. (2016) employs wider reference intervals for heart and respiratory rates and narrower intervals for leukocyte counts and the application of these different intervals may slightly modify the composition of groups with and without SIRS. Here again, future studies based also on other markers of systemic inflammation such as serum amyloid A, that in horses works as a major acute phase protein (Perkins et al., 2015), would be useful to more accurately classify horses or foals with SIRS.

In other species, decreased PON-1 activity is a negative prognostic marker (Giordano et al., 2013; Rossi et al, 2014b; Sharkey and Overmann, 2014). This seems not to be true in horses, since the specificity of these changes is not absolute, possibly due to the same hypotheses reported above. Also the comparison of results with horses with positive or negative outcome, however, may have been biased by the low number of observations, on one hand, and by the wide distribution of results in the group of dead horses, on the other. It is then possible that, by increasing the number of horses with a negative prognosis, the difference between groups will become significant.

Independently on the mechanisms responsible for the lack of decreases of PON-1 activity, the fact that all the horses with low PON-1 activity had a high SIRS score may have some practical utility. In practice, when PON-1 activity at admission is lower than the RI, SIRS is present, as demonstrated also by the Fisher test. In these cases, the likelihood to not survive is 2.58 to 4.50 times higher than the likelihood

to respond to treatments, supporting the hypothesis that PON-1 activity may provide useful information in clinical practice. Conversely, a normal PON-1 activity value does not exclude the presence of SIRS or a negative prognosis during the follow up. Therefore, it would be advisable to measure PON-1 activity at admission and to pay particular attention in the management of horses with low PON-1 activity.

The low sensitivity of PON-1 activity, due to the high number of false negative results especially in foals, may depend also on the immaturity of the immune system in foals younger than 1 year (Perkins et al., 2015).

Additionally, this study failed to provide information on the possible utility of sequential measurements of PON-1 activity after the administration of treatments to achieve prognostic information. The number of horses that died was too low to draw any conclusion, as well as the number of horses that had low PON-1 activity at admission but survived. Some of the horses with normal PON-1 activity values at admission that died during the follow up had transient decreases of PON-1 activity below the lower limit of the RI, but this happened also in some of the survivors. In other species, a rapid increase of PON-1 activity was recorded in animals that had abnormal values at admission and responded to treatments (Rossi et al., 2013). Therefore, it would be advisable in the future to increase the caseload, especially regarding the number of dead horses with repeated samplings.

Finally, although in a previous study (Ruggerone et al., 2018) it was found that the RI is different in horses of different breed or use, in the current study we did not have the opportunity to apply breed related RIs because the number of horses with low PON-1 activity per breed was too low to allow a reliable statistical comparison. Therefore, it may be advisable to investigate, by analysing a larger caseload, whether the diagnostic performance may increase by comparing the results of sick horses also with the breed-specific RIs.

Another limitation of this study is that its rationale assumes an anti-inflammatory and anti-oxidative role of PON-1 activity in horse but no other reliable inflammatory markers (e.g. SAA) have been evaluated to better focus on the inflammatory status of each horse. Finally, other statistical approaches required to assess the prognostic performances (e.g. Cox proportional hazard analysis, Kaplan-Meier survival curves, logistic regression models, odd ratios) would have been considered only with a larger caseload.

III. Utility of a western blot technique for the detection of protein carbonyls in canine serum.

Materials and methods

Samples and study design

The first part of the study was focused on the establishment of the procedure. To this aim, 400 µg of serum from one healthy dog were oxidized “in vitro” with 10% (v/v) cigarette smoke extract (CSE) for 1h at room temperature. Even if CSE doesn't seem the most obvious source of oxidation in dogs, it is a proven and reliable method to oxidized serum (Colombo et al., 2016). The CSE-treated serum was separated using Micro Bio-Spin P-6 Gel Columns (Biorad) to eliminate residual CSE, and control and CSE-treated samples were subjected to WB analysis as described below.

Moreover, to assess the repeatability of the method, 7 naïve (i.e. non CSE-treated) serum samples randomly selected from dogs (two healthy dogs as control, three dogs with pyometra, one dog with pyelonephritis and one with systemic parasitic disorder) were analyzed in 5 consecutive working days using the WB method described below. The coefficient of variation (CV) was then established, based on the mean value and the standard deviation of the 5 working sessions using the formula: $CV = SD/Mean \times 100$.

For the third and retrospective part of this study, four serum samples from healthy dogs and fifteen serum samples collected at the Veterinary teaching hospital of the University of Milan from 14 privately owned dogs with diseases potentially associated with inflammation, possibly with a septic base (pyelonephritis, pyometra, pneumonia, discospondilitis, parvovirus and systemic parasitic disease), and stored at the Department of Veterinary Medicine, were analyzed.

Dogs were considered healthy or sick on the basis of physical examination, routine biochemistry and hematology, ultrasound or radiographic examination when necessary. Whole blood was collected from the jugular or the cephalic vein, placed in tubes without anti-coagulant and centrifuged within 2 h of collection to obtain serum that has been frozen at -20 °C until analyzed; EDTA blood was evaluated within 2 h of collection.

Samples were collected during routine veterinary visits for health monitoring and an owner's written consent that allows the use of residual amount of samples for research purposes has been signed for every dog. Therefore, based on the regulation of the Ethical Committee of our Institution (Decision n° 2/2016) a formal approval of the Ethical Committee is not needed.

Western blot analysis

The procedure was described by Levine et al. [5]. Two-hundreds μL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) in HCl 2N, also known as Brady's reagent, were added to 1 ml of 1 mg/ml of serum protein solution. A similar amount of carbonylated human serum albumin (HSA) (0.22 nmol PCO/mg protein) was used as standard during validation assays. DNPH molecule reacts with carbonyl groups leading to the formation of the stable 2,4-dinitrophenylhydrazone (DNP). A blank sample with 200 μL of 2N HCl (without DNPH) with 1 ml of protein sample was also prepared.

Each tube was incubated in the dark at room temperature for one hour and briefly vortexed every 15 min during the incubation.

Tubes were added with 1.2 mL of 20% trichloroacetic acid (TCA), placed on ice for 15 min, and centrifuged at 10.000 x g for 10 min at 4 °C in a micro centrifuge.

The supernatant was then removed and the pellet washed with 1 mL of (1:1) ethanol/ethyl acetate mixture and vortexed in order to remove any free DNPH. These three latter passages were repeated until supernatants was completely transparent.

After the final wash, the protein pellets were resuspended in 500 μL of Laemmli Sample Buffer and incubated at 90 °C for 5-10 min. After cooling at room temperature, samples were centrifuged 5 min at 10.000 x g and supernatants were transferred to a new tube.

Protein samples labelled with 2,4-dinitrophenylhydrazine (3 μg) were run on SDS-PAGE on Tris-HCl 10% resolving gels and electroblotted onto an Immobilon P polyvinylidene difluoride (PVDF, Sigma-Aldrich) membrane and stored at -20 °C for later use. A two-steps immunodetection protocol employing primary 1:40.000 anti-DNP antibodies (rabbit IgG fraction (cod. A6430) from Molecular Probes (Eugene) and 1:80.000 horseradish peroxidase (HRP)-conjugated secondary antibodies [goat anti-rabbit IgG, (cod. G21234) from Molecular Probes] was performed as described in Colombo et al., 2015. The signal was developed with Enhanced Chemiluminescence (ECL) using ChemiDoc Touch Imaging System (Bio-

Rad Laboratories) and PVDF membranes were stained with Amido Black. Chemiluminescent PCO levels and Amido Black images were densitometrically quantified using a specific software (Image Lab Software, Bio-Rad). The ratio between the carbonyl signal intensity and the protein signal intensity defined the relative protein carbonyl content (Colombo et al., 2016). Results are expressed as arbitrary units (A.U.) considering the mean value of controls as reference.

Clinical chemistry

To better focus on the inflammatory and oxidative status of the dogs included in this study, we evaluated the serum concentration of C reactive protein (CRP), the major acute phase protein of dogs (Ceron et al., 2005) and the serum activity of the antioxidant enzyme PON-1, whose metabolism is strictly associated with that of CRP (Ibba et al., 2015). These biochemical analyses were performed using an automated chemistry analyzer (Cobas Mira, Roche Diagnostics) as previously described (Rossi et al., 2013; Rossi et al., 2014a).

Statistical analysis

Results of PCO, CRP and PON-1 were correlated to each other using a spearman correlation test run in an excel-based specific software (Analyse-it v 4.90, Analyse-it software Ltd).

Results

Detection of PCO in canine serum

Western blot showed an evident band of apparent molecular weight of 69 kDa, consistent with carbonylated dog serum albumin (Fig. 10). The intensity of the band was weak in naïve serum but strong in the serum oxidized with CSE and even stronger when the percentage of oxidized serum increases from 5% to 10% (these percentages were obtained by diluting CSE).

In serum oxidized with CSE, the concentration of PCO was 4.10 A.U.. This value was then used as a positive control in the further analysis of results obtained in healthy and sick dogs.

The coefficient of variation of the 5 sequential reading varied from 24% to 36% (median CV = 30%).

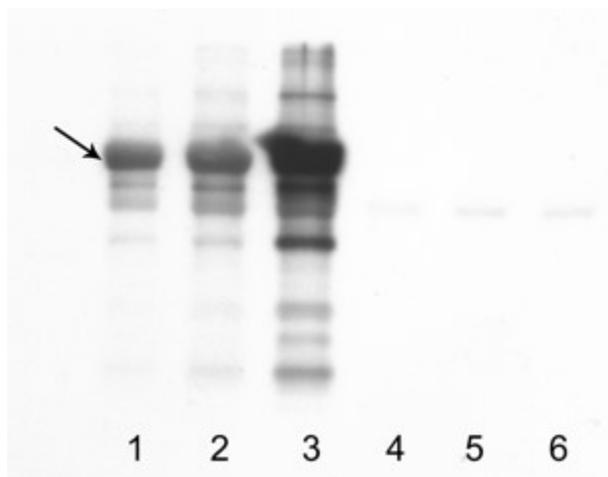


Fig. 10 Results obtained with WB on control samples and samples oxidized with CSE. A band of apparent MW of 69 kDa (black arrow), consistent with carbonylated dog serum albumin, is overt. In lanes 1-3, 10 ug of sample added with DNPH were loaded (1: control, 2: solution with oxidized serum at 5%, 3: solution with oxidized serum at 10%); in lanes 4-6, same quantity and dilution of sera but without DNPH.

Results recorded in healthy and sick dogs

The WB obtained using all the serum samples included in this study is reported in Figure 11. Results obtained after densitometric analysis of positive signals are reported in Table 6.



Fig. 11 Results obtained with WB on the samples collected from healthy dogs (lanes 1-4) and from the clinically sick dogs listed in table 6 (the number of each dog is reported under each lane). The control sample (serum oxidized with CSE) has been loaded on lane 1.

Dog n°	DIAGNOSIS	PCO (A.U.)	CRP n.v. < 10,4 (mg/L)	PON n.v. > 116 (U/mL)
1	Healthy	1.17	Nd ^a	Nd
2	Healthy	0.96	Nd	Nd
3	Healthy	0.82	Nd	Nd
4	Healthy	1.04	Nd	Nd
5	Pyelonephritis	2.23	162.0	106.6
6	Pyelonephritis	1.63	315.1	113.4
7	Pyelonephritis	1.32	194.9	101.5
8	Pyelonephritis	2.16	39.6	113.5
9	Pyelonephritis	2.92	5.6	125.3
10	Angiostrongylus	0.98	100.0	147.7
11	Pyometra	2.66	195.4	114.9
12	Pyometra	0.94	78.0	110
13	Pyometra	0.71	10.7	112.7
14	Pyometra	2.24	1186.3	103.5
15	Pneumonia	0.98	395.0	160.8
16	Pneumonia	2.14	173.3	119.6
17	Parvovirus	33.56	39.3	43.1
18	Parvovirus	1.48	182.9	119.6
19	Discospondilitis	49.65	96.3	75.3

Table 6. Values of PON-1, CRP and PCO in the examined samples. Number 17 and 18 are associated to the same dog before and after the therapy. n.v. = normal value; ^a = Not determined

All the sick dogs had clinical signs consistent with inflammation and all but one (dog number 9) had CRP values that supports the hypothesis of systemic inflammation. With rare exceptions (dogs number 9, 10, 15) PON-1 values were low-normal or lower than the lower limit of the reference interval established in a previous study (Rossi et al, 2013). In 2 cases (dogs number 17 and 19) PON-1 activity was largely lower than the lower limit of the reference interval.

The concentration of PCOs was low and comparable with that of healthy dogs in 2 out of 3 dogs with normal PON-1 activity and increased in one of these dog, that had both CRP and PON-1 activity within the reference interval. Moreover, PCOs were moderately increased, with values consistent with those recorded in the positive control (canine serum oxidized with CSE) in the large majority of dogs with low-

normal PON-1 activity, and severely increased in the two dogs with very low PON-1 activity. However, in sick dogs, PCOs and PON-1 were not correlated to each other ($P = 0.091$; $r = -0.356$). Conversely, the increase of CRP was not apparently associated with changes in PCOs: the CRP value of the two dogs with very high PCOs was only moderately increased, and, on the contrary, PCOs values in dogs with high or very high CRP concentration were low or moderately increased. Also in this case no correlations were found between the two analytes ($P = 0.737$; $r = -0.095$).

Discussion

The discovery of reliable markers of sepsis has a twofold-benefit: an early antibiotic administration is determinant for the survival of septic patients (Dellinger et al., 2013) and, on the other hand, the overuse of broad-spectrum antibiotics in patients with a false-positive sepsis diagnosis can generate drug-resistant pathogens.

Since a clear marker of sepsis is lacking in veterinary medicine, we designed this study to assess whether PCOs may be used to support the suspicion of an oxidative pathogenesis in dogs with inflammation. The first step of this study was to assess whether antibody-based techniques, that have been shown to be able to react with DNP adducts in other species (Colombo et al., 2016) may work in dogs. To this aim, we used a western blot technique already used in other species (Dalle-Donne et al., 2003). With this method, we confirmed the presence of PCOs in oxidized canine serum and we showed that the positive signal increases with the percentage of CSE, suggesting a good linearity of the method. However, the repeatability of the method is not excellent, since the CV is higher than those recommended for most of the analytes currently employed in veterinary medicine (Ricos et al., 1999). Anyway, the magnitude of imprecision could affect the interpretation of PCO results in the pathologic population. The lack of a complete analytical validation cannot allow to correctly interpret the results from pathological samples.

The application of this method to canine sera collected from dogs with inflammation confirmed by increased CRP demonstrated that increases of PCO may occur independently on the magnitude of inflammation. Conversely, increases of PCOs seems to be inversely associated with decreases of PON-

1 activity. The lack of a statistical correlation may depend on the low number of observations, on the possible presence of oxidations not associated with inflammation (as suggested by one single dog with normal CRP and increased PCOs), on the wide individual variability of the response of both the analytes and possibly also by imprecision and inaccuracy of the method. Despite the lack of statistical significance, results of PCOs and PON-1 seem to be inversely proportional to each other in most dogs: increases of PCOs were moderate and slightly lower than values obtained after experimental oxidation of sera with CSE, in dogs with low-normal PON-1 activity and severe in dogs with very low PON-1 activity. In turn, changes in PON-1 activity were not correlated in magnitude or frequency with changes of CRP but this confirms what already demonstrated in other studies (Rossi et al., 2013) and likely depends on the different magnitude of oxidative phenomena associated with inflammation in the different septic patients. In other words, severe decreases of PON-1 activity may be found only in those dogs with sepsis on which oxidative stress is particularly severe. From this standpoint, the similar behavior of PCOs and PON-1 suggests that both the markers identified oxidations only in some of the dogs with systemic inflammation included in this study.

This was a preliminary study focused on the analytical validation of the western blot approach, on which dogs with inflammatory conditions were included only to draw provisional information on the possible practical application of this technique. Therefore, the caseload was composed by a low number of dogs, very heterogeneous in terms of type, etiology, and magnitude of inflammation, on which the diagnosis of sepsis was presumed on the basis of the final diagnosis and/or on the presence of changes in inflammatory markers of inflammation such as CRP and/or PON-1. Despite this limitation, the results are very encouraging and support the hypothesis that increases of PCOs may be found in dogs with sepsis. Based on these results, it would be advisable to perform larger case-control studies, with a better clinical evaluation of sepsis. In veterinary medicine, the presence of sepsis could be defined through clinical or laboratory data that concur to achieve the sepsis score (e.g. heart and respiratory rate, temperature and WBC/ μ L) (Hackett, 2002). Due to the retrospective nature of the current study, it was not possible to collect this information but, in future studies on the clinical utility of PCOs, it would be necessary to better standardize patient's enrolment through the Sepsis score. Moreover, it would be interesting to perform longitudinal studies based on sequential samplings during the follow up, in order to investigate the possible prognostic role of PCOs based on the outcome of the disease.

Another potential limitation of this study is that Western blotting, although still considered the gold standard in many laboratory settings, is time consuming and operator-dependent. This may be a limitation for a large scale application of this method to support a clinical diagnosis of sepsis associated with oxidative stress. However, since the current study clearly demonstrated the possibility to detect PCOs using labelled anti-DNP adducts antibodies, other antibody-based methods that may allow to process larger batches of samples such as ELISA kits or automated immunoturbidimetric systems, would improve the practical applicability of PCO measurement in routine practice.

IV. Diagnostic and prognostic role of protein carbonyls (PCOs) in dogs with sepsis or polytrauma in comparison with paraoxonase-1 (PON-1), C-reactive protein (CRP)

Materials and methods

Caseload

This retrospective study was done on 41 serum samples collected from privately owned dogs (19 males, 2 castrated males, 15 females and 5 neutered females) that underwent clinical examination at the University of Bologna and Milan and stored at the same Institutions; the median age was 48 months (age range: 1 month - 15 years). Fourteen dogs were mongrels, whereas some breeds were more represented: German shepherd (n=5), Golden retrievers (n=4), Pugs (n=2), English bulldogs (n=2), and some others were represented by only one dog (American Staffordshire, Maltese, Doberman Pinscher, Maremmano shepherd, Italian Bloodhound, Leonberger, Miniature Poodle, Yorkshire terrier, Bernese Mountain dog, Jack Russell terrier, English setter, Dachshund, Shetland sheepdog and Airedale terrier). The mean weight was 20,9 Kg (min. 4 kg – max. 44 Kg). Dogs were divided into three groups: 15 of them (7 males, 1 castrated male, 5 females, 2 neutered females; median age 39 months, age range: 6 months – 9 years) were considered healthy on the basis of the absence of abnormalities at physical examination, history and blood test results; 14 dogs (4 males, 7 females, 3 neutered females; median age 36 months; age range: 1 month – 15 years) were considered septic as a result of clinical examination based on the presence of symptoms such as depression, fever or hypothermia, tachycardia, tachypnea and of appropriate tests on the basis of the suspected diagnosis (e.g. cytology consistent with presence of intracellular bacteria, ultrasound examination, radiography, positive blood culture); the remnant 12 dogs (8 males, 1 castrated male, 3 females; median age 48 months; age range: 6 months – 15 years) were polytraumatized secondary to vehicular trauma (n = 10) or falls (n = 2). All these subjects received appropriate treatments according to the diagnosis. For each sick patients, clinical data and outcome were recorded; blood was collected from the cephalic or the jugular vein before starting treatments

and at first presentation for each dog. Blood was then put in plain tubes, centrifuged within 1 hour and partially used for biochemical tests aimed to achieve a diagnosis and to assess the clinical status of the patient. The remnant part of serum was frozen at -20°C and transported in cold chain until the moment of the analysis at the Department of Veterinary Medicine at the University of Milan.

For this research, an informed consent was signed by the owners and an Ethical approval was obtained (DL 26/2014). For the healthy dogs, the study was performed in plans of patients monitoring and didn't request an Ethical Committee approval (Deliberation number 2/2016, www.unimi.it/ateneo/20138.htm).

Clinical chemistry

The serum concentration of CRP and PON-1 were measured in 36 dogs and in all the enrolled dogs, respectively, using an automated chemistry analyser as previously described (Rossi et al., 2013; Rossi et al., 2014a). Serum PON-1 activity was measured spectrophotometrically using the enzymatic method proposed by Feingold et al (1998).

CRP was measured in serum using the automated analyser BT3500 with Biotechnica instruments reagents (Biotechnica Instruments SPA, Roma, Italy).

A protein carbonyl ELISA Kit (Enzo Life Sciences, 3V Chimica, Roma, Italy) was used to measure PCOs in this study. The sample derivatization technique with DNP, already described by Colombo et al. (2016), was used. PCO was measured in all the enrolled dogs.

Statistical analysis

Statistical analysis was performed in an Excel spreadsheet using a specific software (Analyse-it, Analyse-it Software Ltd, Leeds, UK). Non parametric tests were used without assessing the normality of data distribution as recommended for comparisons of small datasets.

Results regarding PCO, PON-1 and CRP were compared in each group of dogs using the Mann-Withney

U test. The same test was used to compare the results obtained at first visit from animals that died with those that survived. Statistical differences were set for $p < 0.05$.

Results

Results of PCO, CRP and PON1

No significant differences were found regarding the age ($P=0.949$), the body weight ($P=0.828$), the proportion of male or female dogs ($P=0.346$) or the proportion of dogs of different size ($P=0.691$) among the three groups.

A significant difference among groups was found for the serum concentration of PCOs and CRP as well as for PON1 activity ($P < 0.001$ for all the analytes, Figure 12).

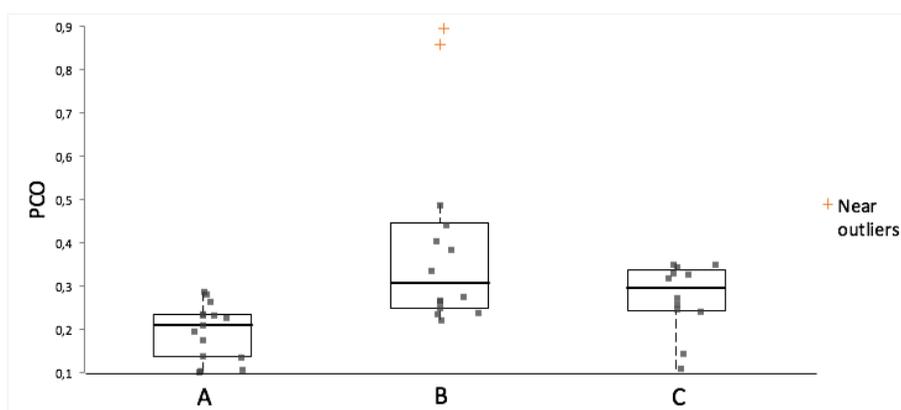


Fig. 12 PCO values (nmol/mg) in the three groups of dogs (A = healthy dogs; B = dogs with sepsis; C = dogs with polytrauma). The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR.

In particular, despite similar median values, the concentration of PCOs was significantly higher in dogs with sepsis (0.40 ± 0.22 , median: 0.31; min-max: 0.22-0.89) than in dogs with trauma (0.27 ± 0.08 ; 0.30; 0.11-0.35; $P=0.005$) and in clinically healthy dogs (0.20 ± 0.06 ; 0.21; 0.10-0.29; $P < 0.001$) but no significant differences between dogs with trauma or clinically healthy dogs ($P=0.237$) (Fig. 13).

Also the serum concentration of CRP was significantly higher in dogs with sepsis (26.00 ± 14.16 ; 20.60;

13.50-60.70) than in dogs with trauma (5.00 ± 7.18 ; 1.46; 0.00-18.60; $P < 0.001$) and in clinically healthy dogs (1.75 ± 1.61 ; 1.54; 0.00-5.22; $P < 0.001$) but no significant differences between dogs with trauma or clinically healthy dogs ($P = 0.740$). In dogs with polytrauma, CRP seems to not be increased, maybe for a short distance from the trauma or because trauma is not necessarily associated with a systemic inflammation detectable by CRP (Fig. 13).

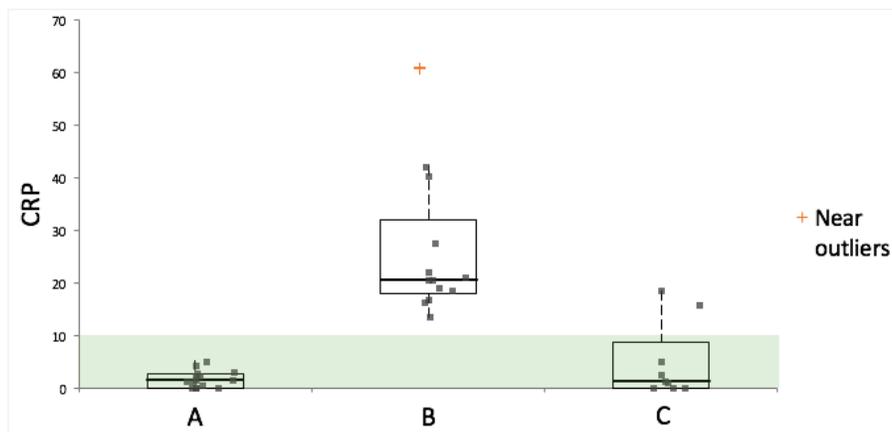


Fig. 13 CRP values (mg/L) in the three groups of dogs (A = healthy dogs; B = dogs with sepsis; C = dogs with polytrauma). The green area indicates the normal value of CRP (< 10 mg/L); the boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR.

Similarly, PON-1 activity was significantly lower in dogs with sepsis (74.6 ± 32.8 ; 71.3; 28.9-152.6) than in dogs with trauma (117.9 ± 23.1 ; 115.3; 89.6-174.0; $P < 0.001$) and in clinically healthy dogs (181.3 ± 22.2 ; 181.7; 140.6-218.0; $P < 0.001$) and a significant difference ($P = 0.001$) was found also between dogs with trauma and clinically healthy dogs (Fig. 14).

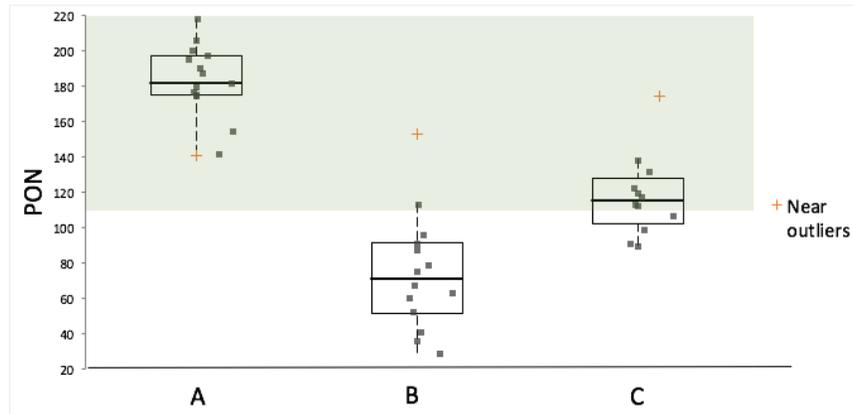


Fig. 14 PON values (U/mL) in the three groups of dogs (A = healthy dogs; B = dogs with sepsis; C = dogs with polytrauma). The green area indicates normal values of PON-1 (>116 U/mL); the boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR.

Moreover, PON1 activity was significantly lower ($P=0.002$) in dogs that died (76.4 ± 34.1 ; 67.6; 36.2-131.5; $n=9$) compared with dogs that survived (140.3 ± 40.3 ; 141.0; 28.9-218.0; $n=32$). Conversely, the concentrations of PCO and CRP were not significantly different in dogs that survived (0.26 ± 0.14 ; 0.25; 0.10-0.86; $n=32$ for PCO; 10.29 ± 15.27 ; 2.74; 0.00-60.70; $n=28$ for CRP) compared with dogs that died (0.37 ± 0.22 ; 0.33; 0.15-0.89; $n=9$ for PCO; 11.84 ± 9.66 ; 16.60; 0.00-+21.10; $n=8$ for CRP). However, the P value was very close to the level of statistical significance for PCO ($P=0.078$) but not for CRP ($P=0.674$).

Discussion

In several species, it has already been proved that the antioxidant enzyme paraoxonase-1 decreases in association with oxidative stress that characterizes sepsis (Novak et al., 2010; Turk et al., 2011; Tvarijonaviciute et al., 2012c; Rossi et al., 2014a; Ibba et al., 2015; Radakovic et al., 2016) and different studies are available about the utility of CRP to distinguish dogs with sepsis from dogs with sterile inflammation (Jitpean et al., 2014; Viitanen et al., 2014). No information is available about the utility of PCO in dogs as diagnostic or prognostic marker, differently from human medicine.

The aim of this study was to evaluate if PCO could be useful to confirm the presence of sepsis in dogs by considering both dogs with severe sterile inflammation and dogs with confirmed sepsis and to assess if its determination could be useful to predict the outcome in canine population. The evaluation of CRP and PON-1 could be useful to better focus on the severity of the inflammatory status in the enrolled patients.

PCOs could be considered, according to our results, a reliable marker to distinguish dogs with sepsis from dogs with sterile inflammation; as reported in the introduction, to establish if a patient needs antibiotics and has a poorer prognosis could be very difficult in veterinary medicine. Even if this is only a preliminary study, these results could be useful to develop new projects about the use of PCO as diagnostic marker of severe inflammation.

However, the best marker to distinguish the three conditions analysed in this study seemed to be the PON-1, because it is lower in dogs with sepsis but differs also between dogs with polytrauma and clinically healthy dogs, even if most dogs with polytrauma had normal values. This result confirmed the association between the presence of OS and the decrease of PON-1. Moreover, a lower value of PON-1 seemed to be predictive of a poorer outcome, differently from PCOs and CRP. These results confirm those of the study of Meisner et al. (2006) who demonstrated that CRP levels did not differ between survivors and non-survivors but differ from the study of Kjelgaard-Hansen et al (2003), in which CRP was considered a potentially useful clinical marker for the presence and resolution of systemic inflammation in dogs.

Unfortunately, the small amount of samples was a limitation of the study: specific statistical tests to assess prognostic markers performances (Cox proportional hazard analysis, Kaplan-Meier survival curves, logistic regression models, odd ratios) were not run for this reason. Future studies should include a bigger amount of samples to better evaluate these possible markers as reliable and useful.

CONCLUSIONS

This thesis aimed to evaluate innovative markers of inflammation in veterinary medicine; this could be useful to distinguish non-infectious inflammation from SIRS and sepsis, that needed a more accurate therapeutic approach and could have a poorer prognosis.

Moreover, to exclude the presence of an infectious process is necessary to avoid the indiscriminate use of antibiotics and to reduce the severe problem of the antibiotics-resistance.

Unfortunately, our studies about PON-1 in horses did not allow to confirm that this protein could be a reliable diagnostic or prognostic marker of sepsis, even if a low PON-1 value should be considered a possible red flag in sick horses and suggest further evaluations. On the other hand, PON-1 seemed to be more reliable to this aim in dogs, as already reported; we identified the presence of carbonylated proteins (PCOs) in serum of dogs under oxidative stress and put a basis for future studies. Specific conclusions for each study are detailed below.

Regarding the first possible marker evaluated in this project (PON-1 activity in equine species, since this analyte has never been validated in horses), it is possible to conclude that the paraoxon-based method for measurement of PON-1 activity is precise and accurate in horses as in other species, and PON-1 activity seems to be lower in horses than in many other domestic species. Despite the presence of some statistically significant differences associated with sex (with higher values in mares) or breed (with higher values in Trotters), the lower reference limits for age-, breed-, or sex-associated RIs are similar to each other. Therefore, in routine practice, it would be advisable to use a single RI for young foals and adult horses, independent of their sex and use.

The second study about PON-1 had the aim to evaluate if the OS cause a significant decrease of PON-1 activity, by comparing PON-1 values in healthy and sick horses. Moreover, by evaluating sequential samples collected after treatment, a possible association of PON-1 with the outcome was considered. Our conclusions were that PON-1 activity have a limited relevance to differentiate horses with and without SIRS or to predict the outcome of the disease. In practice, only horses with low PON-1 values may have SIRS or a negative prognosis, while normal PON-1 values does not exclude these two conditions. This may be due to a different metabolism of paraoxonase in horses, to a less intense magnitude of oxidation associated with sepsis in this species or to the inaccuracy of the SIRS score in identifying horses with SIRS.

The two other studies were about Carbonylated Proteins (PCOs), an already used marker of sepsis and inflammation in humans never validated or tested in dogs.

With the first study, we demonstrated that antibody-based methods to detect DNP adducts associated with protein oxidation may identify the presence of carbonylated proteins in canine serum. Moreover, through a preliminary analysis of samples from dogs with inflammation potentially associated with oxidative stress, we provided evidence that increases of PCOs are evident in dogs in which the activity of the antioxidant enzyme PON-1 is decreased; however, the lack of repeatability and of information about accuracy in absence of a reliable method validation require further studies.

The last study has been developed on a larger caseload of dogs divided into three groups (healthy vs polytrauma vs septic), by using other inflammatory markers (e.g. CRP and PON-1) and a spectrophotometric method for measuring PCOs in canine serum; the aim was to confirm the possible diagnostic or prognostic role of PCOs as a marker of sepsis in dogs. Even if PCO seemed to not correlate with the outcome, our results indicate that it can distinguish dogs with sterile inflammation from those with sepsis. PON-1, differently from our results in horses, seems to have both a prognostic and a diagnostic value and should be preferred as a marker of OS associated with sepsis since it may also provide information about the possible outcome. A limitation of the study was that our assumption was to considered polytrauma dogs an ideal model for sterile inflammation; nevertheless, CRP was not increased in these patients maybe for the absence of systemic inflammation.

Perspectives

The exact mechanism responsible for the different behaviour of PON-1 activity in horses compared with other species deserves to be investigated in future studies based on a larger caseload and on a higher number of horses sampled during the follow up; with a wider panel of tests, that should include other markers of oxidation or of systemic inflammation and/or horses with experimentally-induced SIRS, results could be useful to understand the possible utility of PON-1 activity in clinical practice.

New studies with a larger cohort of dogs and a complete validation study will also be necessary to better focus on the utility of PCOs in this species; our results could be the starting point to develop the knowledge about this possible diagnostic marker. After that, the use of PCOs in clinical practice could become possible in veterinary medicine.

Scientific activities (including studies not focused on the aims of this thesis)

International peer reviewed papers:

- Ruggerone B, Scarpa P, Marelli SP, Polli M. **Genetic evaluation of English bulldogs with cystine uroliths.** *Veterinary Records* 2016 Jul 7. pii: vetrec-2015-103615. doi: [10.1136/vr.103615](https://doi.org/10.1136/vr.103615).
- Ruggerone B, Bonelli F, Nocera I, Paltrinieri S, Giordano A, Sgorbini M. **Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses.** *Veterinary Clinical Pathology* 2018 Mar;47(1):69-77. doi: 10.1111/vcp.12562
- Ruggerone B, Giraldi, M., Paltrinieri, S., Scarpa, P. **Hematological and biochemical reference intervals in Shetland Sheepdogs.** *Veterinary Clinical Pathology* 2018;1-8. doi:10.1111/vcp.12665
- Ruggerone B, Paltrinieri S, Giordano A, Scacco L, Pratelli P, Sgorbini M. **Paraoxonase-1 (PON-1) activity as a diagnostic and prognostic marker in horses and foals.** *Under review (Journal of Veterinary internal Medicine).*
- Ruggerone B, Colombo G, Paltrinieri S. **Utility of a western blot technique for the detection of protein carbonyls in canine serum.** *Under review (Journal of Veterinary Science).*
- Scarpa P, Ruggerone B, Gironi S, Vitiello T, Paltrinieri S. **Hematologic and biochemical RIs in Italian Greyhound.** *Under review (Veterinary Clinical Pathology).*
- Ruggerone B, Troia R, Murgia E, Giunti M, Dondi F, Paltrinieri S. **Protein Carbonylation (PCOs) evaluation in dogs with polytrauma or sepsis in association with Paraoxonase-1 (PON-1), C-Reactive Protein (CRP) and outcome.** *To be submitted.*

Non-peer reviewed papers:

- Ruggerone B, Colombo G, Paltrinieri S. **Analytical aspects of protein carbonyl group in oxidized canine serum.** Abstract on International Journal of Health, Animal Science and Food Safety, 2016 Vol.3 n°1s
- Ruggerone B, Bonelli F, Giordano A, Nocera I, Paltrinieri S, Sgorbini M. **Validation of a Paraoxon-based method for measurement of Paraoxonase (PON-1) activity and establishment of RI in horses.** Abstract on International Journal of Health, Animal Science and Food Safety, 2017 Vol. 4 n°1s.

- Ruggerone B, Troia R, Murgia E, Giunti M, Dondi F, Paltrinieri S. **Diagnosis of sepsis in dogs by measuring carbonylated proteins (PCOs) and paraoxonase (PON-1)**. Abstract on International Journal of Health, Animal Science and Food Safety, 2018 Vol. 5 n° 1
- Ruggerone B, Scarpa P. **Treatment of Chronic Kidney Disease in dogs and cats**. *In press, Veterinaria*

Abstracts:

- Ruggerone B, Scarpa P, Giraldi M, Paltrinieri S. **References intervals in Shetland Sheepdogs: is primary hyperlipidemia a real feature in this breed?** ECVIM-CA Congress 2016, Goetheborg, Sweden.
- Ruggerone B, Colombo, G., Paltrinieri, S. **Preliminary evaluation of a protein carbonyl group in canine serum using a Western Blotting technique**. ECVIM-CA Congress 2017, St. Julian's, Malta.
- Zambarberi J, Giraldi M, Ruggerone B, Faverzani S, Scarpa P. **Symmetric dimethylarginine (SDMA) and nephropathy in dog: diagnostic utility in clinical practice**. ECVIM-CA Congress 2017, St. Julian's, Malta.
- Ruggerone B, Sgorbini M, Giordano A, Bonelli F, Marmorini P, Paltrinieri S. **Serum paraoxonase-1 activity in newborn foals: Age related variations**. ECEIM Congress 2017, Budapest, Hungary.
- Marazzi R, Ruggerone B, Paltrinieri S. **Preliminary investigation on hematological and biochemical parameters as prognostic markers in dogs with severe inflammation**. ECVIM-CA Congress 2018, Rotterdam, The Netherlands.
- Scarpa P, Piazza C, Ruggerone B, Paltrinieri S, Giraldi M. **Big endothelin-1 in cats with CKD: preliminary evaluation**. ECVIM-CA Congress 2018, Rotterdam, The Netherlands.
- Zambarbieri J, Ruggerone B, Martino PA, Scarpa P. **UTI complicate nel cane: prevalenza degli uropatogeni e antibioticoresistenza nel Nord Italia**. Congresso SCIVAC 2018, Arezzo, Italy.

Oral presentations:

- Case report, SIMIV congress, Cremona, 2016
- Innovative markers of inflammation in domestic animals. Winter presentation, University of Milan, 2016.

- Analytical aspects of protein carbonyl group in oxidized canine serum. Vas Days, University of Milan, 2016.
- Case report, SIMIV congress, Cremona, 2017
- Innovative markers of inflammation in domestic animals. Winter presentation, University of Milan, 2017.
- Validation of a paraoxon-based method for measurement of paraoxonase-1 (PON-1) activity and establishment of RI in horses. Vas Days, University of Milan, 2017.
- Paraoxonase-1 (PON-1) activity evaluation as a diagnostic and prognostic marker in horses and foals. Winter presentation, University of Milan, 2018.

Lecturer:

- Participation as speaker in 3 webinars for *Farmina pet food*[®] lecturing about diagnosis and treatment of canine and feline chronic kidney diseases

Co-supervisor of Degree Thesis:

- Selena Luperto: **Quadro infiammatorio e ossidativo in cani esposti ad infezione da Babesia spp.** Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Beatrice Ruggerone. February, 2016.
- Roberto Marazzi: **Preliminary investigation on hematological and biochemical parameters as prognostic markers in dogs with severe inflammation.** Thesis supervisor: Saverio Paltrinieri; Thesis co-supervisor: Beatrice Ruggerone. February, 2018.

Other activities:

During the three years, I had the opportunity to perform and interpret 584 haematological samples, 402 biochemical panels and 76 urine samples of feline and canine patients presented for clinical consultations at the Department of Veterinary Medicine, under the supervision of Prof. Saverio Paltrinieri. I also spent time in daily clinical practice at the Department of Veterinary Medicine under the supervision of Prof. Paola Scarpa, performing both clinical consultations and laboratory works. In this period, I managed 154 primary clinical cases (dogs and cats). I also followed veterinary students

which attended small animal internal medicine clinic and clinical pathology laboratory during my PhD period, tutoring them in their clinical and laboratory training.

Moreover, I organized a weekly Journal Club at the University of Milan about veterinary internal medicine and clinical pathology from May 2017 to May 2018.

Externship periods:

- September 2017: Teaching Veterinary Hospital of the University of Bologna, Ozzano dell'Emilia (BO), tutors Prof. M. Giunti and F. Fracassi
- June 2018: Ospedale Veterinario I Portoni Rossi, Zola Predosa (BO), tutor Dr. F. Procoli
- July 2018: Faculty of Veterinary Medicine, Utrecht University (The Netherlands), tutor Dr. S. Galac.

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