UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI MEDICINA VETERINARIA E SCIENZE ANIMALI

PHD COURSE IN VETERINARY AND ANIMAL SCIENCE
(XXXIV CYCLE)

Clinical applications of Paraoxonase-1 in domestic animals: beyond conventional inflammatory markers

DONATELLA SCAVONE
R12375

Tutor: prof. SAVERIO PALTRINIERI
Coordinator: prof. FABRIZIO CECILIANI

Academic year 2020-2021

SUMMARY

SCIENTIFIC BACKGROUND

Inflammation and oxidative stress

Infectious agents, mechanical trauma, heat or cold, chemicals, toxins, radiation, or other cancerous cells can cause cell injury or death, leading to a complex set of interactions known as acute inflammation (Rock et al., 2010; Ackermann, 2017). Acute inflammation develops within minutes or hours, typically lasts for several hours to a few days and is sustained mainly by the innate immunity (Kumar et al., 2014). The whole process involves vascularized living tissue and is mediated by cellular and extracellular fluidic changes, that eventually result in the accumulation of fluid, electrolytes, plasma proteins and leukocytes in extravascular tissues, clinically reflecting in redness, heat, swelling, pain and loss of function of the concerned tissue. Inflammation is intended to be a protective mechanism, the aim of which is to dilute, isolate and remove the cause of injury and to repair damaged tissues (Ackermann, 2017). This process is tightly regulated by the balance between pro-inflammatory and anti-inflammatory mediators, and it is supposed to be restricted to the site of injury. Loss of the local control or excessively activated responses lead to an exaggerated systemic response, clinically known as systemic inflammatory response syndrome (SIRS) (Davies and Hagen, 1997; Brady and Otto, 2001). The imbalance between pro-inflammatory and anti-inflammatory pathways exacerbates those physiologic mechanisms intended to be protective, making them harmful instead. In this context, uncontrolled systemic vasodilation and increased systemic vascular permeability lead to hypotension and extravascular third spacing, preventing normal homeostatic response required to preserve oxygen delivery. The ultimate result is end-organ hypoperfusion, with anaerobic metabolism and end-organ dysfunction (Davies and Hagen, 1997; Balk, 2014).

According to definitions established by the consensus conference of the Society of Critical Care Medicine and the American College of Chest Physicians, SIRS is a systemic inflammatory process characterized by at least two of the following clinical manifestations: increased or decreased body temperature; increased heart rate; increased respiratory rate or hyperventilation; increased or decreased white blood cell count or again the presence of immature (band) neutrophils (Bone et al., 1992). According to this definition, any insult leading to a generalized activation of the inflammatory reaction in organs remote from the initial insult and inducing the described clinical condition, is a cause of SIRS, be it infectious or not.

In the whole of systemic changes occurring during the acute phase response, changes in the concentration of plasma proteins are included. Pro-inflammatory mediators and cytokines such as TNF, IL-1 and IL-6 are released into blood circulation and act as endocrine mediators on different tissues, inducing or inhibiting the synthesis of the so-called acute phase proteins (APP), in variable ways among different species and depending on the type of stimulus (Ceciliani et al., 2002; Cray et al., 2009). Plasma proteins that increase in concentration are defined as positive APPs, and include: C-reactive protein (CRP), serum amyloid A (SAA), haptoglobin, alpha-1-acid glycoprotein (AGP), ceruloplasmin and fibrinogen; while plasma proteins that decrease in concentration are called negative APPs, such as: albumin, transferrin. The ones having that increase more frequently in a given species are defined as major APPs and are thus the inflammatory markers of choice (Paltrinieri, 2007).

Infection is one of the major triggers of inflammation (Rock et al., 2010). Sepsis was defined as the systemic inflammatory response to the presence of infection, the latter intended as a microbial phenomenon characterized by an inflammatory response to the presence or the invasion of normally sterile host tissue by microorganisms. Thus, the term sepsis represents the subset of SIRS resulting from a confirmed infectious process (Bone et al., 1992). To differentiate sepsis from uncomplicated infection and emphasize the non-appropriate magnitude and detrimental effect of the inflammatory response, a new definition was given, as life-threatening organ dysfunction caused by a dysregulated host response to infection (Singer et al., 2016). However, criteria for diagnosis of sepsis proposed by Bone et al. (1992) are still used in veterinary medicine (Troia et al., 2018). The best described causes of sepsis in the veterinary clinical literature are gram-negative (in particular, enteric) bacteria, but also gram-positive bacteria, as well as fungal, viral and protozoal micro-organisms can trigger SIRS and sepsis mechanisms (Brady and Otto, 2001).

During sepsis, the origin of infection can be difficult to identify in some cases. However, collection of culture specimens is not always simple, especially in patients with severe lung impairment or coagulopathies (Brady and Otto, 2001). Early diagnosis of sepsis and prompt recognition of disease severity are crucial to the rapid administration of antimicrobials (Troia et al., 2018). Early intervention aims to stop the progression of sepsis to severe sepsis, septic shock and multiple organ disfunction. Furthermore, the use of antibiotics in SIRS is controversial, since SIRS can occur without a microbial cause (Brady and Otto, 2001).

Distinction between sepsis and SIRS would be very useful in administration of targeted antimicrobial therapy only when necessary, since antimicrobial resistance is nowadays a widespread and alarmingly growing concern of global public health (WHO, 2014). To this aim in human medicine useful biomarkers of sepsis such as procalcitonin have been developed to diagnose, monitor and predict prognosis in sepsis (Hochreiter et al., 2009; Reinhart and Meisner, 2011; Georgopoulou et al., 2011; Jekarl et al., 2013; Bouadma et al., 2013).

Much of our knowledge about SIRS and sepsis is deduced from human literature and, even if the majority of available information is valuable and applicable, controlled veterinary studies are required to deepen and clarify these aspects in companion animals (Brady and Otto, 2001). In light of these premises, it would be important to detect new biomarkers useful in identifying SIRS and sepsis, in addition to others already investigated. One more reason to look for a good prognostic biomarker would be that euthanasia is never an easy choice, either for the owner or for the vet, who must justify and explain to the owner the reasons leading to such an alternative. A biomarker able to predict mortality would provide scientific evidence supportive in making this choice.

During inflammation activated polymorphonuclear leukocytes seem to be a source of reactive oxygen intermediates (Alonso de Vega et al., 2002). Following activation, neutrophils start the respiratory burst and produce reactive oxygen species (ROS), with different stability, reactivity and permeability to membranes, but all able to modify and damage other molecules (Amulic et al., 2012). Molecular oxygen is reduced to superoxide, which reacts with other molecules creating compounds useful for signalling and antimicrobial activity, but also potentially harmful to the host tissues (Smith, 1994). When production of reactive species exceeds organism's ability to compensate for production and damage, oxidative stress occurs (Galley, 2011).

In human medicine, patients with SIRS were found to have increased plasma levels of lipid peroxidation products and decreased plasma total antioxidant capacity and reduced sulfhydryl groups. These are indexes of plasma redox status, and thus stand for a more severe oxidative stress in patients with SIRS compared to patients without SIRS (Alonso de Vega et al., 2002). Furthermore, the degree of oxidative stress appears to be related to the severity of illness (Alonso de Vega et al., 2000; Alonso de Vega et al., 2002). Oxidative stress is also thought to be related to mortality of patients in human intensive care unit (ICU), independently of the presence of SIRS (Alonso de Vega et al., 2002).

Septic event induces, as well, upregulation of oxidative metabolism in neutrophils with high reactive oxygen and nitrogen species generation (Trautinger et al., 1991; Martins et al., 2003). In particular, sepsis is reported to be associated with higher and earlier oxidative stress compared to SIRS alone (Muhl et al., 2011). Assuming that oxidative stress may be bigger in sepsis than in non-infectious inflammatory disease even in dogs, markers of oxidation could represent viable candidates for distinction between sepsis and SIRS, as well as for predicting mortality, severity stratification of disease and monitoring progress and recovery.

Paraoxonase-1

The paraoxonases are a family of proteins present in many organisms, from invertebrates to mammals (Furlong, 2008). They are probably ancestral enzymes (Furlong, 2008) and descend from a common precursor, with PON-2 being the oldest member, followed by PON-3 and PON-1 (Draganov and La Du, 2004). In humans, paraoxonases are encoded by genes located adjacent to each other on chromosome 7, sharing about 70% homology at the nucleotide level and 65% at the amino acid level (Primo-Parmo et al., 1996).

Paraoxonase-1 (PON-1) was the first discovered and the most studied. It was identified for its ability to hydrolyze paraoxon (diethyl p-nitrophenyl phosphate), the toxic metabolite of parathion, an organophosphate insecticide, and was named after this feature (Draganov et al., 2005).

Aldridge (Aldridge, 1953a; Aldridge, 1953b) in 1953, firstly reported a high rate of paraoxon hydrolysis in rabbit serum and identified two types of enzymes: A-esterases, able to hydrolyze organophosphates and arylesters, and B-esterases, which were on the contrary inhibited by interaction with these substrates. PON-1 was therefore classified as an A-esterase, but it is now recognized that is a lipolactonase with several other enzymatic activities, such as paraoxonase and arylesterase (Mackness and Sozmen, 2021). Accordingly, PON-1 has different substrates, such as organophosphorus triester pesticides and nerve gases, arylesters, lactones, thiolactones, estrogen esters, cyclic carbamates and glucuronide drugs (Mackness and Sozmen, 2021), so that it has been defined as a promiscuous enzyme (Mackness and Mackness, 2015). However, after structure-activity studies, lactonase activity had been considered the native activity of PON-1 and lactones its natural substrates (Khersonsky and Tawfik, 2005).

Initially, the relationship between arylesterase and paraoxonase activity was not completely understood. These activities were thought to be catalyzed by different serum proteins, because in the same individuals, arylesterase showed a unimodal distribution pattern, while paraoxonase did not. Later, when polymorphisms of PON-1 gene were discovered and investigated, it became clear that arylesterase and paraoxonase activity were catalyzed by the same enzyme (Gan et al., 1991), of which different phenotypes with different magnitude of paraoxonase activity existed (Eckerson et al., 1983).

Adkins et al. (1993) firstly defined the genetic bases of polymorphisms, identifying two polymorphic sites in the coding region: Leucine/Methionine in position 55 (polymorphism PON-1 L55M) and Glutamine/Arginine in position 192 (polymorphism PON-1 Q192R). The polymorphic site at position 192 was recognized to largely affect the catalytic efficiency of the enzyme as regards paraoxonase activity. In particular, three phenotypes of paraoxonase activity were attributed to this polymorphism: individuals homozygous for a "low" activity allele (QQ) which were arbitrarily called A-type; individuals homozygous for a "high" activity allele (RR), which were arbitrarily called B-type; and heterozygous AB-type individuals with intermediate activity (Davies et al., 1996). The esterase activity instead appeared to be scarcely affected by this polymorphism (Eckerson et al., 1983). On the contrary, the polymorphic site at position 55 was shown to have little influence on the catalytic properties, but to affect PON-1 expression levels (Garin et al. 1997; Leviev and James, 2000). Other polymorphic sites in the promoter region were subsequently described (James et al., 2000; Brophy et al., 2001a), among which the T108C appeared to have the highest influence on PON-1 expression. The CC genotype was associated with the highest PON-1 levels, the TT genotype with the lowest and heterozygous CT type with intermediate levels. Actually, the effect of PON-1 polymorphism at position 55 appears to be related to the one at 108 position, given that alleles of these polymorphic sites are in strong linkage disequilibrium (in other words, segregate together) (Leviev and James 2000; Suehiro et al., 2000; Brophy et al. 2002). In people, more than 400 single-nucleotide polymorphisms have been identified, even if the effects have not been understood for all of them (Mackness and Sozmen, 2021). However, it is known that genetic variability can produce differences in PON-1 activity of up to 40 times and differences in PON-1 concentration of up to 15 times (Mackness and Sozmen, 2021). Furthermore, the frequency of low activity allele is variable among populations of different ethnic and geographical distribution (La Du, 1988).

It is most important to consider all these aspects when investigating correlation between PON-1 and a certain disease or clinical condition, and it is extremely advisable to evaluate both PON-1 levels and activity, in order to determine the so called PON-1 'status' (Li et al., 1993; Costa et al., 2005a). The sole determination of polymorphisms through genetic analyses does not accurately assess or predict PON-1 status (Kim et al., 2013), and the lack of genetic matching in many studies represents a huge limitation, which often lead to misconceptions in the literature (Camps et al., 2009).

Evaluation of PON-1 activity through just one substrate, like happened in the first studies using only phenyl acetate or paraoxon, does not allow to establish if results are due to coincidental differences in allelic frequencies (Camps et al., 2009). In human medicine, a high-throughput enzyme assay involving two substrates measuring paraoxonase and arylesterase, such as paraoxon and diazoxon, is recommended to this approach and provides an accurate assessment of PON-1 levels and functional genomics (Richter and Furlong, 1999; Richter et al., 2004). Rates of hydrolysis of one substrate are plotted against the other's, obtaining the separation of individuals in the three phenotypes related to the 192 polymorphism. Accordingly, PON-1 activities within a genotype provide information about the PON-1 levels (Costa et al., 2005a) (Figure 1).

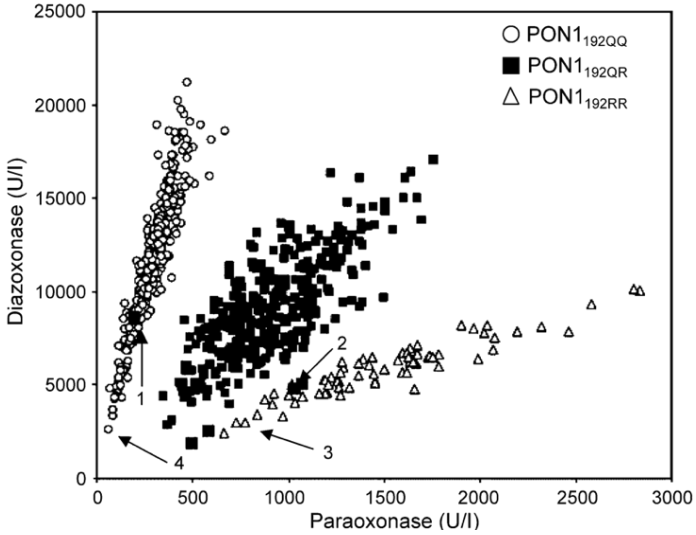


Figure 1. Determination of PON-1 status by plotting of diazoxonase vs. paraoxonase activities in plasma of carotid artery disease cases and controls. The two-substrate assay provides an accurate prediction of PON-1 Q192 genotype (QQ, QR or RR) confirmed by PCR and PON-1 activities. Individuals indicated by arrows showed discordant genotype/phenotype (genotyped as QR heterozygotes but classified as homozygotes by enzyme analyses) because of mutations in one allele resulting in the expression of the other active allele phenotype (Jarvik et al., 2003).

However, in veterinary species the study of polymorphisms is not so accomplished (Ceron et al., 2014) and its importance maybe not so understood. In most of the species of veterinary interest it is not known if the presence of polymorphisms may affect PON-1 activity and its evaluation in specific clinical settings. For example, in bovine PON-1 the use of a two substrate assays showed constant paraoxonase/arylesterase ratio in different breeds, suggesting the lack of phenotypes as described in people (Miyamoto et al. 2005; Kulka et al. 2014, Kulka et al. 2016).

PON-1 is mainly produced by the liver, the organ showing the highest PON-1 gene expression (Pellin et al., 1990), and secreted into blood circulation tightly bound to high-density lipoprotein (HDL) particles (Deakin et al., 2002).

Human PON-1 shows a 354 amino acids structure with a molecular mass of approximately 45 kDa (Furlong et al., 1989; Hassett et al., 1991). PON-1 is a six-blade beta-propeller with hydrophobic leader sequence and an amphipathic helix H2 which binds to HDL (Harel et al., 2004) and in particular to Apolipoprotein A-I, which stabilizes and selectively stimulates lactonase activity (Sorenson et al., 1999; James and Deakin, 2004; Gaidukov and Tawfik, 2005) (Figure 2).

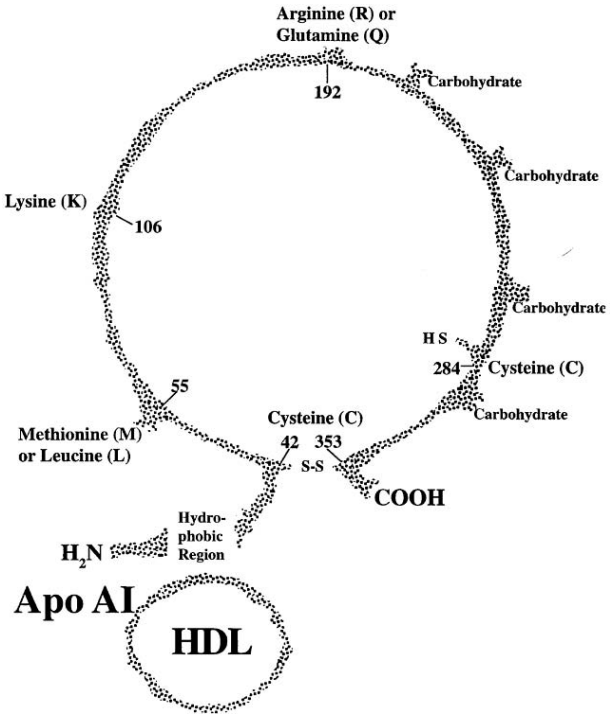


Figure 2. Human PON-1 structure (Furlong, 2008).

PON-1 requires calcium for catalytical activity and stability, therefore has two binding sites relatively committed to these functions (Kuo and La Du, 1998; La Du, 2002; Harel et al., 2004) and the degree of calcium affinity determines PON-1 stability. As a consequence, EDTA impairs calcium-dependent activities, as well as measurement of activity and concentration of PON-1 (Erdös and Boggs, 1961; Mackness and Sozmen, 2021).

In people, PON-1 levels show a high variability among individuals, whereas activity for a given individual is relatively stable over time (Zech and Zurcher, 1974; Costa et al., 2005b),

In young individuals of many species, PON-1 activity is known to be lower than adults. In people, PON-1 levels are very low in newborns and increase until reaching mature levels between 6 months to 2 years of age (Augustinsson and Barr, 1963; Ecobichon and Stephens, 1973; Mueller et al., 1983; Cole et al., 2003; Chen et al., 2003). However, in people a large variability appears to exist among newborn individuals, some of which showed higher levels than some mothers (Furlong et al., 2006). On the contrary, in elderly people a decrease in PON-1 concentration and activity is reported with aging (Milochevitch and Khalil, 2001; Seres et al., 2004; Marchegiani et al., 2006), possibly related to concurrent increased oxidative stress (Harman, 1992; Lu et al., 1999). As regards veterinary species, aging-related changes had not been investigated, but low PON-1 activity in young subjects had been reported in many species. For example, calves up to 120 days of age have significantly lower PON-1 activity compared with adults (Giordano et al., 2013). Mice are reported to reach mature levels between 20 and 25 days after birth (Li et al., 1997). In dogs, young age is likely influencing PON-1 activity, since reference interval calculated for dogs aged 4-6 years was higher than for dogs <3 years (Rossi et al., 2013). On the contrary, in horses, significant difference in PON-1 activity were not detected between adults and foals, at least after 19 days of age (Ruggerone et al., 2018). In cats as well, age-related differences were not detected in a population of cats including cats less than 1 year of age (Rossi et al., 2020). However, even if age-related reference intervals are not available or age-related changes have not been reported, age should always be taken into account as a possible source of error in interpreting low values.

PON-1 can be indirectly measured through assessment of activity, or directly quantified with immunological methods (Ceron et al., 2014). Measurement of PON-1 activity (intended as ability to hydrolyze substrates) through spectrophotometrical method, is more common, probably because of low cost and availability (Ceron et al., 2014). The spectrophotometric assay method based on the rate of p-nitrophenol production measured at 405 nm implemented by Krisch (1968) represented the basis for most of the subsequent methods. The most frequently used substrates are paraoxon (to measure paraoxonase activity), phenyl acetate, 4(p)-nitrophenyl acetate (to measure arylesterase activity), 5-thiobutil butyrolactone or dihydrocoumarin (to measure lactonase activity). The use of different substrates matches the evaluation of different activities.

However, the use of these and many other substrates, as well as the lack of standardized methods to measure PON-1 activity in the years, caused difficulties in comparing results obtained through different assays by different studies and in some cases impaired their interpretation (Camps et al., 2009; Ceron et al., 2014). Paraoxon is unstable and, like other organophosphorus compounds, highly toxic, while phenyl acetate is less toxic and 4-nitrophenyl acetate and 5-thiobutil butyrolactone are not (Camps et al., 2009; Ceron et al., 2014). On the other hand, paraoxon is very useful in the evaluation of the Q192R polymorphism, because as already explained above, it is hydrolyzed at higher rates by individuals with RR phenotype. Accordingly, the other substrates, whose hydrolysis rate is not influenced by Q192R polymorphism, such as phenyl acetate and 4-nitrophenyl acetate can be used to measure arylesterase activity and indirectly estimate PON-1 concentration (Eckerson et al., 1983).

PON-1 was initially studied with respect to the toxicological field (and in particular, organophosphate toxicity). Its levels combined with activity and catalytic efficiency, are a determinant of sensitivity to specific organophosphorus compounds toxicity (Costa et al., 2005a). Later, growing evidence of its relevant role in lipid metabolism shifted the interest on its antioxidant activity and modulation of oxidative stress. Changes in PON-1 activity and concentration had been reported in a wide variety of diseases both inflammatory and non-inflammatory characterized by oxidative stress.

The most studied field in human medicine, especially in early times, had been that of cardiovascular disease. HDL and PON-1 in fact appear to have a central role in atherosclerosis development in people and a negative correlation exist between HDL blood concentration and PON-1 activity and risk of cardiovascular disease (Getz and Reardon, 2004; Mackness et al., 2004b). HDL have many anti-atherosclerotic functions (Gordon et al., 1989; Rubins et al., 1999, Barter and Rye, 2001), such as removal and transport of cholesterol from peripheral tissues to the liver (reverse-cholesterol transport), anti-inflammatory and anti-oxidative action, and regulation of normal endothelial function (Kontush and Chapman, 2006). However, PON-1 activity was demonstrated to predict adverse coronary events independently of HDL levels (Navab et al. 1997; Bhattacharyya et al., 2008). Actually, PON-1 appears to be largely responsible for the antioxidant power of HDL (Mackness et al., 1993; Ahmed et al., 2001; Aviram et al., 1998a; Watson et al., 1995) and among HDL-associated proteins with antioxidant properties, PON-1

appears to be the predominant antioxidant enzyme (Mackness and Mac). HDL isolated from mice deficient of the PON-1 gene or from avian species, which naturally lack PON-1, did not protect LDL from oxidation (Mackness et al., 1996; Laplaud et al., 1998; Shih et al., 1996) or instead enhanced LDL oxidation (Shih et al., 1998; Mackness et al., 1998), whereas HDL isolated from mice overexpressing PON-1 completely suppressed LDL oxidation (Tward et al., 2002). PON-1 metabolizes oxidized phospholipids and lipid hydroperoxides probably through lipo-lactonase activity (Rosenblat et al., 2006). This protects not only LDL, but also HDL and macrophages from oxidative stress (Aviram et al., 1998b; Mackness et al., 1993; Rozenberg et al., 2003; Aviram and Rosenblat, 2004; Rozenberg et al., 2005). Oxidized LDL induce the production of monocyte-chemotatic protein 1, which enhances monocyte/endothelial cell interactions, an initiating step to the inflammatory process that promote atherosclerosis (Klimov et al., 2001; Ferretti et al., 2004). PON-1 inhibits these interactions (Mackness et al., 2004a), reduces oxidized LDL uptake and cholesterol biosynthesis by macrophages (Fuhrman et al., 2002; Rozenberg et al., 2003), promotes HDL-mediated cholesterol efflux from macrophages, an essential process to the attenuation of atherosclerosis development (Von Eckardstein et al., 2001; Rosenblat et al., 2005). PON-1 is in turn inactivated by oxidized lipids (Aviram et al., 1999; Karabina et al., 2005). As a result of these mechanisms, low PON-1 activity or concentration are associated with increased risk or incidence of atherosclerosis and coronary heart disease (McElveen et al., 1986; Mackness et al., 2000; Jarvik et al., 2000; Mackness et al., 2001; Ferrè et al., 2002b). In a prospective study PON-1 activity was found to be 30% lower in men affected by coronary events compared with controls (Mackness et al. (2003).

Clinical applications of PON-1 in veterinary medicine

As regards human medicine, a large literature is available about PON-1 and its role and application in many different clinical contexts, while interest about PON-1 in veterinary species has grown only in the latest years. A brief review of most relevant published works will be described below. As for human medicine, also among studies performed on veterinary species there is quite heterogeneity in assays adopted for PON-1 activity evaluation, which are usually transposed or adapted from human publications, often without a validation phase.

Compared with other veterinary species, in dogs a much larger literature has been produced about PON-1. Different methods using different substrates were validated for dogs (Tvarijonaviciute et al., 2012a). The paraoxon-based method validated with a reference interval of 106.6-197.2 U/mL will be adopted in this thesis (Rossi et al., 2013).

Leishmaniosis, which is reportedly characterized by oxidative stress (Almeida et al., 2013), has by far been the most studied disease. PON-1 activity was reported to decrease in dogs either naturally or experimentally infected with Leishmania spp. compared with healthy controls (Martinez-Subiela et al., 2014; Ibba et al., 2015), being significantly correlated with disease severity (Pardo-Marin et al., 2020). Furthermore, lower PON-1 activity was detected in proteinuric compared with non-proteinuric dogs (Martinez-Subiela et al., 2014). In Leishmania spp. seropositive dogs, dogs with highest antibody titers had significantly lower PON-1 activity, as well as higher positive APP concentration, compared with dogs with low antibody titers (Cantos-Barreda et al., 2018). However, changes in PON-1 activity are not always consistent and a high individual variability, probably related to the magnitude of oxidative phenomena, appeared to exist (Rossi et al., 2014a; Ibba et al., 2015). In leishmaniotic dogs, PON-1 activity increased up to normal values after treatment (Martinez-Subiela et al., 2014; Rubio et al., 2016) earlier than other inflammatory markers like CRP (Rossi et al 2014). Monitoring of its activity was indeed used to compare the efficacy of different treatment protocols (Gonzalez et al., 2019). PON-1 activity was also evaluated in a miscellaneous of infectious diseases characterized by oxidation and inflammation. Dogs with babesiosis has significantly lower PON-1 activity compared with healthy controls (Rossi et al., 2014b; Kules et al., 2016), which significantly increased 6 days after treatment (Rossi et al., 2014b). In dogs experimentally infected with Ehrlichia canis, PON-1 activity significantly decreased after 14 days and increased after 42 days regardless of treatment with rifampicin (Karnezi et al., 2016). In dogs with Dirofilaria immitis, PON-1 activity decreased compared with healthy dogs (Mendez et al., 2014; Carreton et al., 2017) and improvement after treatment, although not always significant, was observed (Mendez et al., 2015; Carreton et al., 2017). In dogs with parvoviral enteritis PON-1 activity significantly decreased compared with healthy dogs (Kocaturk et al., 2015). Studies were performed also in dogs with both experimentally induced and naturally occurring sepsis. A significant decrease in PON-1 activity was reported 48 hours following LPS injection (Tvarijonaviciute et al 2012b) and dogs with naturally occurring sepsis showed significantly lower PON-1 activity compared with healthy controls and with dogs with low-grade systemic inflammation (Torrente et al., 2019).

PON-1 activity was also reported to be significantly lower in dogs with pancreatitis (Tvarijonaviciute et al 2015a), inflammatory bowel disease (Rubio et al., 2017) and atopic dermatitis (Almela et al., 2018) compared with healthy controls. In cardiopathic patients PON-1 activity was reported to progressively decrease with severity of heart failure, either due to chronic mitral valve disease or dilated cardiomyopathy (Prasad et al., 2014; Kulka etal 2017; Rubio et al., 2020). Furthermore, significantly increased PON-1 activity was reported in dogs with hyperadrenocorticism compared with healthy controls, possibly due to a direct influence of glucocorticoids on PON-1 gene expression (Tvarijonaviciute et al 2015b).
A significant negative correlation was found between PON-1 and CRP in many (Martinez-Subiela et al., 2014; Tvarijonaviciute et al 2015a) but not all studies (Torrente et al., 2019). Indeed, PON-1 activity appears to decrease only, or at least particularly, when severe inflammation is present (Rossi et al., 2013) and was in many diseases negatively correlated with clinical severity (Tvarijonaviciute et al 2015a; Torrente et al., 2019; Pardo-Marin et al., 2020). However, even in dogs the role of PON-1 as a negative APP is widely recognized. Usefulness of PON-1 measurement at admission in predicting the outcome of different diseases was evaluated in some studies, but was not confirmed (Rossi et al., 2014a; Torrente et al., 2019).

In cats, few studies are available and mainly deal with metabolic conditions and some infectious diseases. PON-1 activity was evaluated in obese cats, with no differences before and after weight loss but lower arylesterase activity in cats that failed to lose weight (Tvarijonaviciute et al., 2012c). In cats naturally infected with *Hepatozoon felis and Babesia vogeli*, PON-1 activity was significantly lower than in healthy non-infected cats (Vilhena et al., 2017). Cats with feline infectious peritonitis (FIP) showed significantly lower PON-1 lactonase and arylesterase activity compared with healthy cats, but not significantly different compared with cats with other inflammatory diseases. Furthermore, cats with effusive forms of FIP showed significantly lower values compared with non-effusive forms (Tecles et al., 2015). On the contrary, another study evaluating PON-1 activity found significantly lower values in cats with FIP compared not only with healthy controls, but also with cats affected by other diseases (Meazzi et al., 2021). This study used a paraoxon-based method validated in cats with a reference interval of 58-154 U/mL that will be adopted in this thesis. A negative correlation was found between PON-1 activity and alpha-1-acid glycoprotein (AGP) (Rossi et al., 2020), while there is controversy about correlation with SAA. Tecles et al., 2015 found a weak but significant correlation between SAA concentration

and PON-1 activity, while other studies did not find this correlation (Rossi et al., 2020). However, changes in PON-1 activity in the investigated infectious diseases are likely due to inflammation and/or oxidative stress, and PON-1 was recognized as a negative acute phase protein also in this species (Tecles et al., 2015; Vilhena et al., 2017; Rossi et al., 2020).

In horses, there are very few publications regarding PON-1. PON-1 activity was evaluated in horses routinely screened for leptospirosis with different levels of antibody titer against *Leptospira spp.* No significant differences were found among groups with high and low antibody titers, however none of the horses involved showed clinical signs of leptospirosis (Turk et al., 2011). Another study reported significantly lower PON-1 activity in healthy horses infected with *Theileria equi* compared with non-infected controls, along with changes in other markers of oxidative stress (Radakovic et al., 2016). In this case, measurement of PON-1 activity was performed using plasma collected in lithium heparin, which is reported to mildly inhibit PON-1 activity or, however, to provide lower values compared with serum (Ferré et al., 2005). Anyway, the comparison should be considered reliable, because a possible mild underestimation of activity would have equally affected all sample. While both these studies used paraoxon-based methods not validated in the equine species, a precise and accurate paraoxon-based method was later validated by Ruggerone et al. (2018) and a reference interval of 38.1-80.8 U/mL was established for this species, which will be adopted within this thesis.

In cattle, research interest was mainly focused on metabolic changes related to pregnancy and lactation. In dairy cows, PON-1 activity was significantly lower in late pregnancy and early post-partum period compared with the second trimester of pregnancy and the late non-pregnant lactation, potentially due to oxidative stress consequent to negative energy balance, and/or post-partum calcium deficiency, overall reduction of hepatic protein synthesis, especially apolipoprotein A1 (Turk et al., 2004; Turk et al., 2008), or also inflammatory conditions occurring during peripartum (Bionaz et al., 2007). Significantly lower activity was found also in non-pregnant cows with hepatomegaly due to lipomobilization syndrome compared with healthy subjects in the same reproduction period (Turk et al., 2005). Furthermore, PON-1 activity was significantly lower in cows with both clinical and subclinical mastitis compared with controls, suggesting its possible usefulness in diagnosing subclinical disease (Kovacic et al., 2019). A negative correlation was found between PON-1 activity and haptoglobin concentration (Bionaz

et al., 2007) and PON-1 activity was significantly lower in sick calves with diarrhea and respiratory disease compared with healthy age-matched controls, supporting the role of PON-1 as a negative acute phase protein also in this species (Giordano et al., 2013).

In pigs, PON-1 genome was characterized and discovered to be highly expressed in kidney, followed by liver, lung and small intestine, and at very low level in heart, spleen, lymph, muscle, cerebrum, fat, cerebellum or hypothalamus (Xie et al., 2010). Even PON-3 was characterized and found to be ubiquitously expressed in tissues (Labreque et al., 2009). PON-1 activity was evaluated in pigs subjected to total splenectomy and spleen autotransplantation, observing a decrease in activity over following 40 days compared with sham-operated ones, probably due to post-surgical inflammation (Turk et al., 2009). In another study, a three substrates method was validated for pigs and PON-1 activity was measures after experimentally induced inflammation through subcutaneous injection of turpentine oil, finding a decrease after 72 hours post injection, suggesting that PON-1 acts as a negative APP also in this species (Escribano et al., 2015).

AIMS

Inflammation and oxidative stress occur frequently in domestic animals. The diagnosis and monitoring of inflammatory and oxidative conditions are based on robust markers that are so far considered accurate (changes in leukograms and electrophoretic profiles, concentration of some APPs). Unfortunately, most of these markers may lack specificity in some clinical contexts and may not be useful for the early diagnosis of inflammation and/or for the achievement of prognostic information about the clinical outcome. The development of novel biomarkers may provide complementary information to conventional markers, leading to a more comprehensive overview of the pathophysiology of clinical forms, helping in identifying subclinical conditions either *in vivo* or *post mortem*, and improving the management of sick patients.

In the last years' research, interest has grown about the role of PON-1 in the pathogenesis and diagnosis of many diseases characterized by inflammation and/or oxidative stress, both in human and veterinary medicine.

In veterinary species, PON-1 measurement is to date primarily restricted to the research field and has not been yet undertaken in the routine clinical field. However, recent research has highlighted promising utility in many clinical contexts. Since accurate and precise spectrophotometrical assays validated in many species are now available and proved to be cheap, rapid and automated (Rossi et al., 2013), its measurement could easily be included in routine chemistry panel in the near future.

Therefore, the aim of my research was focused on the evaluation of the usefulness of PON-1 as a diagnostic and prognostic marker in different conditions characterized by inflammation and/or oxidative stress in different veterinary species.

More specifically, the following species and scenarios were explored:

- Paraoxonase activity in horses: utility of PON-1 as a marker of inflammation and sepsis;
- Paraoxonase activity in dogs: utility of PON-1 in selected clinical scenarios;
- Paraoxonase activity in cats: PON-1 activity in cats infected with Leishmania spp.;
- Paraoxonase activity in pigs: role of PON-1 as a predictor of lesions before slaughtering;
- Paraoxonase activity in cattle: role of PON-1 as a predictor of clinical or ultrasonographic abnormalities in calves with pneumonia.

METHODS

All the studies included in this thesis were based on the same method of PON-1 measurement described below. However, the selection of cases and the study design differed among the studies, and the details of each experimental design will be described separately for each study.

Method of PON-1 measurement and preparation of control materials

PON-1 activity was measured using a paraoxon-based method already validated in cattle, dogs, horses and cats (Giordano et al., 2013; Rossi et al., 2013; Ruggerone et al., 2018; Rossi et al., 2020), which measures spectrophotometrically PON-1 activity through the enzymatic method proposed by Feingold et al. (1998). Briefly, 6 µL of samples are incubated at 37°C with 89 µL of distilled water and 100 µL of reaction buffer (glycine buffer 0.05 mM, pH 10.5 containing 1 mM of paraoxon-ethyl, purity >90%, and 1 mM of CaCl2). The rate of hydrolysis of paraoxon to p-nitrophenol is measured by monitoring the increase in absorbance at 405 nm using a molar extinction coefficient of 18.050 L·mol-1·cm-1. The unit of PON-1 activity expressed as U/mL is defined as 1 nmol of p-nitrophenol formed per minute under the assay conditions. For the first studies (**studies I, II, III, IV, VI, VII** and **VIII**) we used an automated chemistry analyzer dedicated to research purposes on which the method had been already validated in previous studies (Cobas Mira, Roche Diagnostics, Basel, Switzerland). Later, the method was set and adjusted on a different automated chemistry analyzer (BT 3500, Biotecnica Instruments SPA, Rome, Italy) used for the hospital diagnostic routine. Thus, the measurement of PON-1 activity was included in the most frequently required biochemistry panels in dogs admitted to the hospital, giving access to a wide range of data regarding routinely examined animals which were used for **study V**.

Since quality control samples for PON-1 measurements are not commercially available, frozen aliquots of horse serum to be used as control were created (Ceron et al., 2014). Horses reportedly show PON-1 values lower than dogs or cats and higher than livestock (Rossi et al., 2013; Giordano et al., 2013; Ruggerone et al., 2018; Rossi et al., 2020). For this reason, equine serum with intermediate values was preferred to other species as quality control material. Leftovers of horse serum samples received at the university teaching hospital laboratory for routine analyses were collected. On each sample PON-1 activity was measured in triplicate before and after 1:2 dilution, with an automated analyzer (Cobas Mira, Roche diagnostic, Basel, Switzerland) using the enzymatic method already explained and validated in equine medicine (Ruggerone et al., 2018).

Results and volume of each sample were recorded, and samples were stored within the day of collection at -20°C. Samples collection lasted until we reached a calculated volume of about 30 mL for each of two pools: a high-level pool (calculated average PON-1 value = 55.24 U/mL) and a-low level pool (calculated average PON-1 value = 33.99 U/mL). Pools were created merging samples and, on each of the two, 20 measurements of PON-1 activity were performed, in order to calculate average (59.3 U/mL for high-level pool and 37.7 U/mL for low-level pool) and standard deviation (1.2 and 1.5 respectively). Then, 150 µL aliquots were created and stored at -20°C, to be used as control samples for future PON-1 measurements on horse serum.

Statistical analysis

In all studies, statistical analysis was performed using an Excel spreadsheet and a specific software (Analyse-it, Analyse-it Software Ltd, Leeds, UK) using statistical tests considered more appropriate to each study design, as detailed below. For all statistical tests, significance was set for $P<0.05$.

DESCRIPTION OF THE STUDIES

PARAOXONASE ACTIVITY IN HORSES

Rationale and aims

The main aim of studies on equine PON-1 was to assess the potential role of this molecule as a diagnostic or prognostic biomarker in spontaneous or experimentally induced systemic inflammatory reaction syndrome (SIRS) and sepsis (**studies I** and **II**, respectively). These studies represented the continuation and conclusion of a project already started at our Institution in collaboration with other veterinary Institutions (University of Pisa and School of Veterinary Medicine and Animal Science of the São Paulo State University), where samples were collected and (**study II**) the experimental protocol was designed and run. Both studies were completed by measuring PON-1 activity on collected samples and analyzing results, and were subsequently published in the Journal of Veterinary Medicine [doi:10.1111/jvim.15722] and in BMC Veterinary Research [doi:10.1186/s12917-020-02629-4], respectively, and will be now described separately.

I. Paraoxonase-1 activity evaluation as a diagnostic and prognostic marker in horses

Study design

The study was performed on 172 blood samples from 58 horses (36 mares, 17 geldings, and 5 stallions) referred to 3 different hospitals providing secondary health care. Horses were classified as sick on the basis of clinical examination and ancillary tests (routine serum biochemistry and hematology, radiographs, ultrasound examination, and cytological and bacteriological evaluation of synovial fluid or bronchoalveolar lavage fluid). The following data were recorded in order to classify and divide the sick horses in SIRS-positive or SIRS-negative groups (Roy et al., 2017): presence of abnormal leukocyte count as leukopenia or leukocytosis (reference interval [RI], 5.0-12.5 × 103/μL), left shift (RI, >10% band neutrophils), hyperthermia or hypothermia (RI, 37.0°C-38.5°C), tachycardia (RI, >52 beats per minute [bpm]), and tachypnea (RI, >20 breaths per minute). Horses with 0 or 1 abnormal criterion were included in the SIRS-negative group, whereas horses with ≥2 abnormal criteria were included in the SIRS-positive group (Roy et al., 2017). Retrospectively, sick horses also were divided into survivors and non-survivors. Animals were considered survivors if they were discharged from the hospital, whereas they were considered non-survivors if they died or were humanely euthanized because of severe medical prognosis rather than for economic reasons.

Although all the sick horses included in the study were admitted to the hospitals within 30 hours after the onset of the disease, it was not possible to standardize the time elapsed from the onset of clinical signs and the first sampling or to collect a complete history regarding the pre-admission period. After the first sampling (T0), all of the horses received appropriate treatment based on the actual diagnosis and clinical presentation.

Blood samples were collected by the investigators within 1 hour after admission at the hospital, and additional samples then were collected at 24-hour intervals until discharge or death or for a maximum of 96 hours. Lipemic samples were excluded from the study. Blood samples were collected from the jugular vein for the determination of complete blood count and PON-1 activity using a sterile syringe and 16G needle. Each blood sample was divided into 2 aliquots: a 1-mL aliquot was collected in potassium ethylene diamine tetra-acetic (K2EDTA) and analyzed using a cell counter (ProCyte Dx, IDEXX, Westbrook, Maine) within 5 minutes after collection. A second 2.5 mL aliquot was collected in plain tubes and centrifuged at 2100 relative centrifugal force for 10 minutes within 4 hours of collection. The harvested serum was placed in sterile tubes, frozen at -20°C to be transported to the laboratory, where serum PON-1 activity was measured in a single batch as described above. The study was performed during the regular course of hospitalization of the animals and with the owner's written consent. Therefore, a formal authorization by the Institutional ethical committee was not needed (decision 2/2016, Ethical committee of the University of Milan).

Results for PON-1 activity upon admission to the hospital from SIRS-positive horses were compared with results from the SIRS-negative horses, using the Mann-Withney U test. The same test was used to compare the results obtained upon admission between survivors and non-survivors. A Fisher exact test was used to verify the association between categorized PON-1 results (within vs. below the RI) and SIRS-positive or negative classification or outcome (survivors vs. non-survivors). To assess diagnostic performance of PON-1 activity in detecting SIRS-positive horses, the numbers of samples from SIRS-positive and SIRS-negative subjects that upon admission had PON-1 activity within or below the RI established in the previous study by Ruggerone et al. (2018) were counted. Data then were classified as follows:

- True-positive (TP): Sick horses with PON-1 activity lower than the RI and SIRS-positive;
- False-positive (FP): Sick horses with PON-1 activity lower than the RI and SIRS-negative;
- True-negative (TN): Sick horses with PON-1 activity within the RI and SIRS-negative; and
- False-negative (FN): Sick horses with PON-1 activity within the RI and SIRS-positive.

The same classification scheme was used to assess the diagnostic performance of PON-1 activity in detecting animals with a poor prognosis, considering as "positive" the non-survivors and as "negative" the survivors. In both instances, TP, TN, FP, and FN results were used to calculate sensitivity, specificity and likelihood ratios using standard formulas (Gardner et al., 2006; Jensen and Kjelgaard-Hansen, 2010). Results from sequential samplings collected after treatment were not statistically compared to each other because of the low number of samples that had 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 treatment.

Results

The 58 sick horses were grouped as follows based on the final diagnosis: obstructive or strangulated colic (n=34), other gastrointestinal diseases (n=4:3/4, enteritis; 1/4, esophageal obstruction), bacterial pneumonia or pleuropneumonia (n=4), trauma (musculoskeletal or skin or both; n=8), neoplasia (n=2), and paraphimosis, neurological signs, pyometra, sinusitis, pericarditis and Cesarean section (n=1 each).

The SIRS-positive horses consisted of 35/58, whereas 23/58 were classified as SIRS-negative. The SIRS-negative horses had obstructive (7/23) or strangulated (4/23) colic, trauma (4/23 horses: 1/4, mild fracture; 3/4, wound), 2/23 neoplasia and granulomatous enteritis, respectively, and 1/23 paraphimosis, pyometra, sinusitis, and choke, respectively. The SIRS-positive horses had obstructive (4/35) and strangulated (19/35) colic, 4/35 pneumonia or pleuropneumonia (3/4, pneumonia; 1/4, pleuropneumonia), 3/35 severe trauma, 1/35 pericarditis, Cesarean section, and granulomatous enteritis associated with dermatitis, respectively. The SIRS score was 4 in 3/35 horses, all affected by pneumonia or pleuropneumonia, 3 in 15/35 animals (1/15 obstructive and 10/15 strangulated colic; other gastrointestinal disease, 1/15; trauma, 2/15; pneumonia, 1/15), and 2 in 17/35 (3/17 obstructive and 9/17 strangulated colic; trauma, 3/17; 1/17, pericarditis and Cesarean section, respectively).

Eighteen of 58 horses died despite treatment. Nine of these 18 horses were euthanized because of worsening clinical condition and not for economic reasons and 9/18 died spontaneously. Non-survivor horses were affected by obstructive (n=3) or strangulated colic (n=9), pneumonia or pleuropneumonia (n=4), trauma and other gastrointestinal diseases (n=1 each). Thirteen horses died or were euthanized after the first sampling, 1 after the third sample, and 4 after the fifth and last sample.

At admission, 11/58 horses had PON-1 activity lower than the lower limit of the RI of adult horses (38.0 U/mL). However, when results recorded in males, females or geldings were compared with the lower limit of the specific RI (males, 38.4 U/mL; females, 37.3 U/mL; geldings, 33.2 U/mL), only 10 horses (9 females and 1 gelding) had low PON-1 activity.

No differences were found in PON-1 activity between SIRS-negative (mean ± SD, 49.1 ± 8.2 U/mL; median, 46.5 U/mL; I-III interquartile range, 43.6-54.3 U/mL) versus SIRS-positive horses (45.3 ± 11.9 U/mL; 45.8 U/mL; 35.4-54.6 U/mL; P=0.21) and between survivors (47.9 ± 9.1 U/mL; 46.5 U/mL; 39.9-54.8 U/mL) versus non-survivors (44.4 ± 13.5 U/mL; 44.1 U/mL; 33.3-53.4 U/mL; P=0.27) (Figure 3).

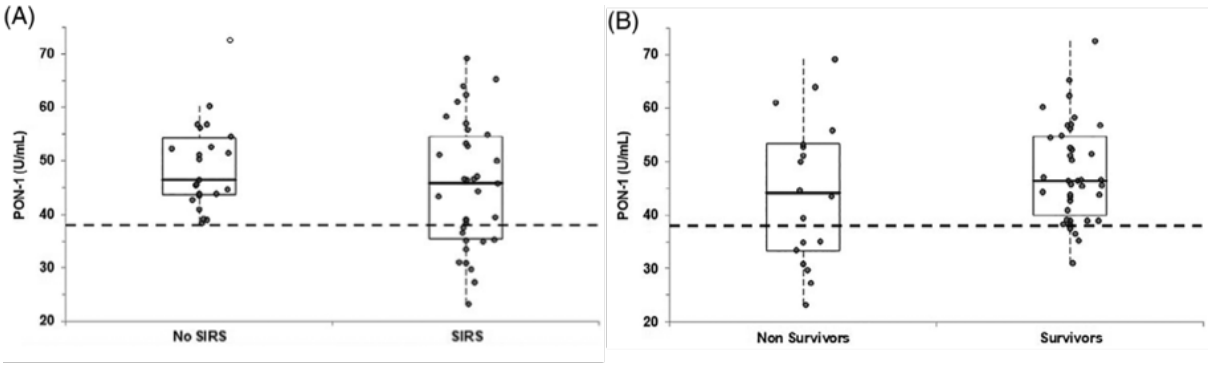


Figure 3. A, Distribution of results according to the systemic inflammatory response syndrome (SIRS) status. B, Distribution of results according to the outcome. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicate the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or to further observation within quartile III plus 1.5 × IQR. The white circles indicate the outliers. The dotted line indicates the lower limit of the RI reported in healthy horses.

Contingency analysis showed a significant association between categorized PON-1 activity and SIRS classification (positive versus negative; P=0.002) and between categorized PON-1 activity and outcome (P=0.02). No significant differences were found when the analysis was restricted to geldings (SIRS-negative: 46.2 ± 6.4 U/mL; 44.6 U/mL; 42.9-50.4 U/mL; n=11; versus SIRS-positive: 44.9 ± 15.7 U/mL; 44.4 U/mL; 30.7-54.4 U/mL; n=6; P=0.81; survivors: 44.5 ± 7.7 U/ mL; 43.8 U/mL; 38.9-49.2 U/mL; n=12; versus non-survivors: 48.9 ± 15.1 U/mL; 50.0 U/mL; 38.8-58.5 U/mL; n=5; P=0.38). Conversely, in mares, PON-1 activity was significantly lower (P=0.05) in SIRS-positive horses (44.8 ± 11.2 U/mL; 45.8 U/mL; 35.4-54.5 U/mL; n=27) compared with SIRS-negative horses (53.9 ± 9.1 U/mL; 54.6 U/mL; 48.9-56.9 U/mL; n=9).

As noted in Figure 3, horses with PON-1 activity lower than the RI were found only in the SIRS-positive group, that, however, also included several horses with normal PON-1 activity.

Conversely, either horses with low PON-1 activity or horses with normal PON-1 activity were found in both groups on the basis of outcome (Figure 3).

The number of FP, FN, TP, and TN recorded in each group, and specificity, sensitivity or positive likelihood ratio calculated are reported in Table 1.

Table 1. Sensitivity (Sens), specificity (Spec), and positive likelihood ratio (LR+) in the examined subpopulation according to the SIRS status and the outcome.

		TP	**FN**	**FP**	**TN**	**TOT**	**Sens (%)**	**Spec (%)**	**LR+**
SIRS	Whole caseload	10	25	0	23	58	28.6 (16.3-45.1)	100.0 (85.7-100.0)	n.c.
	Stallions	0	2	0	3	5	0.0 (0.0-65.8)	100.0 (43.9-100.0)	n.c.
	Mares	8	19	0	9	36	29.6 (19.5-48.5)	100.0 (70.1-100.0)	n.c.
	Geldings	2	4	0	11	17	33.3 (9.7-70.0)	100.0 (74.1-100.0)	n.c.
Outcome	Whole caseload	7	11	4	36	58	38.9 (20.3-63.4)	90.0 (76.9-98.0)	3.89
	Stallions	0	0	0	5	5	n.c.	100.0 (56.6-100.0)	n.c.
	Mares	6	7	3	20	36	46.2 (23.2-70.9)	87.0 (67.9-95.5)	3.54
	Geldings	1	4	1	11	17	20.0 (3.6-62.4)	91.7 (64.6-98.5)	2.40

FN, false negative; FP, false positive; n.c., not computable; SIRS, systemic inflammatory response syndrome; TN, true negative; TOT, total number; TP, true positive. The 95% confidence intervals of Sens, Spec, is reported in brackets.

As shown in Table 1, low PON-1 activity has an absolute specificity for the diagnosis of SIRS (no FP were found, independent of group and corresponding cutoff). Conversely, PON-1 activity has a variably low sensitivity, because the number of FN (normal PON-1 activity in SIRS-positive horses) was high, especially in male adults.

When animals are grouped based on outcome, specificity, although high, is not absolute, and FP results may occur. However, when PON-1 activity is low, the likelihood to have a poor prognosis is 2.40 to 3.89 times higher than the likelihood to have a positive response to treatment.

Results recorded in the 36 horses repeatedly sampled during follow-up are reported in Table 2.

Table 2. Results of PON-1 activity (U/mL) recorded in sequential samplings of the 36 horses that were survivors (S) or non-survivors (NS) at the end of the study period and that were sampled during the follow up. Results below the lower reference limit are reported in bold.

N	*	T0	T24	T48	T72	T96
9	S	**35.2**	**32.5**	**36.5**	**37.3**	
10	S	**36.6**	41.7	54.5	50.1	
11	S	**37.5**	46.4	56.9	51.9	49.1
12	S	38.3	44.2	40.2	42.8	
15	S	38.9	**30.5**	39.5	37.7	43.6
17	S	39.2	49.5			
20	S	42.7	45.9	46.1		
21	S	43.3	**34.0**	43.1	42.5	42.9
22	NS	43.6	46.7	44.5		
23	S	43.8	43.8	44.5		
24	S	43.8	40.6			
25	S	44.4	**37.4**	**34.0**	**37.0**	38.4
27	S	45.4	**37.6**	**35.5**	40.6	39.2
28	S	45.6	49.4	52.3	55.3	
29	S	45.8	51.5			
31	S	46.5	46.9	49.1	47.4	53.4
32	S	46.6	**37.0**	**33.9**	38.4	50.9
34	S	47.1	39.4	44.6	42.4	38.3

N	*	T0	T24	T48	T72	T96
36	S	50.4	**35.3**	46.1		
38	S	51.2	46.3	53.4	52.4	
39	S	51.5	47.6			
40	S	52.3	64.4	60.6	61.5	62.1
41	S	52.6	45.8	58.7	40.5	42.8
43	NS	53.2	39.4	**30.8**	38.3	43.1
44	S	54.6	63.8	60.2	56.9	
45	S	54.9	52.4	54.7	58.3	
46	NS	55.9	44.9	43.6	**37.5**	**36.7**
48	S	56.9	45.9	47.3	55.3	
50	S	57.0	57.0	60.4		
51	S	58.3	50.9	63.4	58.3	58.3
52	S	60.3	43.1	49.6	49.0	45.5
53	NS	61.0	62.6	48.1	45.4	59.9
54	S	62.3	42.7	33.6	38.4	45.0
55	NS	64.0	45.1	52.5	53.1	51.9
56	S	65.3	59.2	59.7	48.8	63.0
58	S	72.6	57.2	68.3	65.7	54.5

Sampling was repeated in 5 horses that had normal PON-1 activity upon admission but died despite treatment (non-survivors). Analysis of sequential samplings indicated that none of these horses had persistent decreases of PON-1 activity over time: only 1 of these horses had low PON-1 activity before death, whereas 1 had a transient decrease in PON-1 activity 24 or 48 hours after treatment, but PON-1 activity returned to within normal limits at the last 2 samplings. However, a transient decrease followed by rapid normalization also was observed in 6 of the 28 survivors, which had normal values upon admission and were sampled at least for 48 hours. Only 3 survivors that had low PON-1 activity upon admission were repeatedly sampled during follow-up. In all 3 horses, activity increased over time and in 2 of them PON-1 activity rapidly returned to within normal limits.

Discussion

In several species, the activity of PON-1 decreases in association with oxidative stress that characterizes sepsis (Bionaz et al., 2007; Novak et al., 2010; Tvarijonaviciute et al., 2012; Rossi et al., 2013; Rossi et al., 2014a; Escribano et al 2015). Our study was designed to assess whether the activity of the antioxidant enzyme PON-1 may serve as a diagnostic or prognostic marker in horses. To this aim, a paraoxon-based method recently validated in horses was used. However, our results only partly support a possible role for PON-1 as a diagnostic marker in horses, because low PON-1 activity may be associated with positive SIRS status, whereas normal PON-1 activity does not rule out negative SIRS status.

All of the horses with low PON-1 activity upon admission were also SIRS-positive, whereas several SIRS-positive horses had normal PON-1 activity. Moreover, the horses with poor prognosis included either horses with low PON-1 activity or horses with normal PON-1 activity. Hence, PON-1 activity may be a good marker of SIRS in horses only when results recorded upon admission are low, as opposed to what has been reported in other species (Rossi et al., 2014a; Giordano et al., 2013). This difference may suggest that, compared with other species, horses have different PON-1 metabolism, or less severe oxidative stress during inflammation. Different from other inflammatory markers, PON-1 activity decreases only if oxidative stress is present (Feingold et al., 1998; Novak et al., 2010). Future studies including measurement of other markers of oxidation (eg, thiobarbituric acid substances, reactive oxygen species) may allow clarification. The hypothesis that not all inflammatory conditions are associated with oxidative stress is supported by results of previous studies in dogs that found decreased PON-1 activity only in some, but not all, dogs with increases in other APP (Rossi et al., 2013; Rossi et al., 2014b). Moreover, the horses included in our study were affected by different diseases which likely have different severity of oxidation, which could have contributed to the variability of the results. Although most horses were affected by colic, the study population was heterogeneous in terms of type of disease, the pathogenesis of which may or may not have included oxidative phenomena. Unfortunately, the number of cases in the different disease categories was not homogeneous and a reliable statistical comparison of results obtained in the different groups was not possible. Additionally, 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. In people, sepsis is the condition mostly associated with oxidation (Mackness et al., 2004a).

The lack of differences of PON-1 activity between SIRS-negative and SIRS-positive horses may be related to the fact that SIRS may or may not be associated with sepsis. Thus, it might be possible that SIRS-positive horses actually do not have systemic spread of the septic process and vice versa. Future studies based on other markers of systemic inflammation such as serum amyloid A, that in horses acts as a major APP (Jacobsen et al., 2007), or based on reliable tests to definitely classify the SIRS-positive horses as septic or non-septic would be useful to more accurately classify horses or foals with SIRS. In other species, decreased PON-1 activity is a negative prognostic marker and may predict outcome (Rossi et al., 2014b; Giordano et al., 2013; Atay et al., 2014). This does not seem to be true in horses, because the specificity of decreased PON-1 was not absolute, possibly because of the same factors described above. However, comparison of results from horses with positive or negative outcome also may have been biased by the low number of observations, on one hand, and by the wide distribution of results in the non-survivors on the other hand. It is possible that, with increasing numbers of horses with a negative prognosis, these differences would become significant. Independent of the mechanisms responsible for the lack of decreases in PON-1 activity, the fact that all of the horses with low PON-1 activity also were positive for SIRS may have some practical utility. In routine practice, when PON-1 activity upon admission is lower than the RI, according to our data, SIRS is always present, as demonstrated also by the Fisher test, and the likelihood for the horse to not survive is 2.40 to 3.89 times higher than the likelihood to respond to treatment, supporting the hypothesis that PON-1 activity may provide useful information in clinical practice. Conversely, normal PON-1 activity does not exclude the presence of SIRS or a possible negative prognosis despite treatment during follow-up. Therefore, it would be advisable to measure PON-1 activity upon admission and pay particular attention to the management of horses with low PON-1 activity.

Additionally, our study failed to provide information on the possible utility of sequential measurement of PON-1 activity after administration of treatment to achieve prognostic information. The number of horses that died and were repeatedly sampled was too low to draw any conclusions, as was the number of horses that had low PON-1 activity upon admission and survived. Some of the horses with normal PON-1 activity at admission that died during follow-up had transient decreases of PON-1 activity below the lower limit of the RI, but this also happened in some of the survivors. In other species, a rapid increase of PON-1 activity was recorded in animals that had abnormal activity at admission and responded to treatment (Rossi et al., 2013).

Therefore, it would be advisable in the future to increase the number of animals, and especially the number of non-survivors with repeated samplings.

Finally, although a previous study (Ruggerone et al., 2018) demonstrated that the RI varies among horses of different breeds or use, we did not have the opportunity to apply breed-related reference intervals because the number of horses with low PON-1 activity per breed was too low to allow a reliable statistical comparison. Therefore, it also may be advisable to investigate, by analyzing a larger caseload, whether diagnostic performance could improve by comparing the results of sick horses with breed-specific reference intervals.

II. Measurement of Paraoxonase-1 activity in horses with experimentally induced endotoxemia

Study design

The study was performed on 66 plasma samples from six non-pregnant 6-9 years old clinically healthy Quarter Horse mares, with mean body weight 425 (± 20) kg, belonging to the São Paulo State University (Unesp), School of Veterinary Medicine and Animal Science, herd and previously sampled during another study (Oliveira-Filho et al., 2012). The study was submitted and approved by the São Paulo State University, Institutional Animal Care and Use Committee Institutional Animal Care and Use Committee (108-A/2007). All experiments on animals were carried at the São Paulo State University (Unesp). In these animals, endotoxemia was induced by intravenous infusion of 30 ng/kg of LPS (E. coli O55:B5, Sigma-Aldrich, St. Louis, MO, USA) in 300 ml of 0.9% sterile NaCl over 30 min. Blood samples were collected through a 14G catheter aseptically inserted in the right jugular vein at time 0 h (immediately before LPS infusion) and at several time intervals from 2 to 240 h after infusion. Heparinized samples were used to perform a cell blood count (only at some time point) and then centrifuged. Aliquots of plasma were used to measure total protein, serum iron and fibrinogen, and then stored at -80°C until the analysis. Part of the aliquots were transferred on dry ice to the University of Milan for the measurement of PON-1. Details of the hematological and biochemical parameters that were investigated in the previous study (Oliveira-Filho et al., 2012), as well as the clinical score and laboratory results recorded at the different time points selected for the current study are reported in Table 3.

Table 3. Mean ± standard deviation regarding the clinical score and the main clinico-pathological changes recorded in the previous study (Oliveira-Filho et al., 2012) in the time points selected for inclusion in this study.

Time	WBC x10^3/µL	PMN x10^3/µL	PCV (%)	Hb (g/dL)	Iron (µg/dL)	Fibrinogen (mg/dL)	Protein (g/dL)	CLINICAL SCORE
0	8.3 ± 1.2	4.4 ± 0.8	36.5 ± 2.7	10.8 ± 2.1	105.2 ± 15.0	300.0 ± 63.2	7.2 ± 0.4	0.00 ± 0.00
2	nd	nd	36.3 ± 2.9	nd	103.4 ± 17.8	250.0 ± 54.8	7.1 ± 0.4	1.67 ± 0.26
6	9.8 ± 3.0	8.0 ± 0.3	37.2 ± 3.0	12.4 ± 1.3	70.5 ± 10.4	300.0 ± 89.4	7.4 ± 0.5	0.67 ± 0.27
12	nd	nd	36.8 ± 3.3	nd	36.4 ± 4.9	283.3 ± 160.2	7.4 ± 0.3	0.00 ± 0.00
24	13.3 ± 2.7	9.1 ± 2.5	35.8 ± 4.1	11.5 ± 1.5	66.7 ± 31.7	350.0 ± 54.8	7.2 ± 0.4	0.00 ± 0.00
36	nd	nd	35.3 ± 3.7	nd	142.6 ± 37.8	383.3 ± 147.2	7.2 ± 0.5	0.00 ± 0.00
48	11.9 ± 1.4	7.4 ± 1.5	35.3 ± 2.3	11.5 ± 1.2	146.5 ± 22.8	350.0 ± 104.9	7.1 ± 0.4	0.00 ± 0.00
72	11.2 ± 2.1	6.6 ± 1.2	35.3 ± 1.8	11.3 ± 1.0	115.9 ± 30.5	383.3 ± 132.9	7.2 ± 0.6	0.00 ± 0.00
168	9.5 ± 1.1	5.4 ± 0.9	35.7 ± 2.9	11.6 ± 1.0	113.1 ± 28.5	366.7 ± 103.3	7.5 ± 0.7	0.00 ± 0.00
240	9.2 ± 1.1	4.7 ± 1.1	35.5 ± 2.1	11.6 ± 1.2	102.6 ± 20.5	316.7 ± 98.3	7.7 ± 0.5	0.00 ± 0.00

WBC, white blood cells; PMN, polymorphonuclear leukocytes; PCV, packed cell volume; Hb, hemoglobin; nd, not determined

To summarize, all horses subjected to LPS infusion showed clinical signs related to endotoxemia, such as depression, muscle fasciculations, intestinal hypomotility and mild-to-moderate abdominal pain and they had a significant increase in the clinical score proposed by Moore et al. (2007) based on heart and respiratory rate and of body temperature from 1 to 6 h ($P<0.01$). Starting from 12 h and up to 240 h post-infusion, clinical score was reported as normal. Conversely, neutrophilic leukocytosis, lymphopenia and decreased plasma iron were detectable between 6 and 24 h, then gradually normalized over time, with the return to normal levels at 72 h (Oliveira-Filho et al., 2012). All animals showed no sequelae at the end of the study and returned to the activities they performed before the study. On the basis of clinical score and laboratory data, the current study was done on samples collected at time 0, 2, 6, 12, 24, 36, 48, 72, 168, and 240 h after infusion, to concentrate the analysis in samples that had the most prominent clinical or laboratory changes and to include two additional samples (168 and 240 h) to assess possible long-term effects on PON-1 activity. Plasma PON-1 activity was measured as described above and data recorded in the different sampling times were compared to each other using a non-parametric ANOVA test for repeated measurements (Friedmann test) followed by the Wilcoxon signed rank test to compare paired results recorded at each sampling time. The

Spearman test was used to investigate the possible correlation between mean PON-1 activity and mean values of the clinical or laboratory changes recorded in the previous study (Oliveira-Filho et al., 2012).

Results

Table 4 and Figure 4 report PON-1 activity measured at scheduled time points. A progressive decrease in PON-1 activity from baseline was observed from 24 h after the end of LPS infusion. The Friedmann test evidenced that results collected at the different sampling times were significantly different to each other (P<0.001). Mean PON-1 values measured from 48 h to 168 h post-infusion were significantly lower than those recorded in the samples collected in the first 24 h and especially from the baseline values. After this transient decrease, at 240 h post-infusion PON-1 activity returned to similar baseline values, significantly higher than lowest ones observed 72- and 168-h post-infusion.

Table 4. Mean, standard deviation, median and range of plasma PON-1 activity for scheduled time points before (T0) and after (T2-T240) LPS administration in healthy horses (n=6).

Sampling time (hours)	**PON-1 (U/mL)**		
	mean ± SD	median	min-max
0	33.7 ± 6.3	35.6	28.8-38.4
2	34.6 ± 8.2	31.5	30.0-42.7
6	33.0 ± 7.9	31.7	27.3-36.5
12	33.5 ± 5.4	33.3	29.1-36.4
24	31.9 ± 8.8	33.1	22.5-39.7
36	27.6 ± 5.8	28,8	23.4-32.6
48	27.2 ± 5.8	27.5	22,2-32,3
72	23.5 ± 6.4	24.6	16.4-29.2
168	24.8 ± 5.3	24,5	20.9-29.7
240	30.6 ± 5.1	32.4	24.5-34.5

Asterisks indicate values significantly different (P<0.05) from reported time points

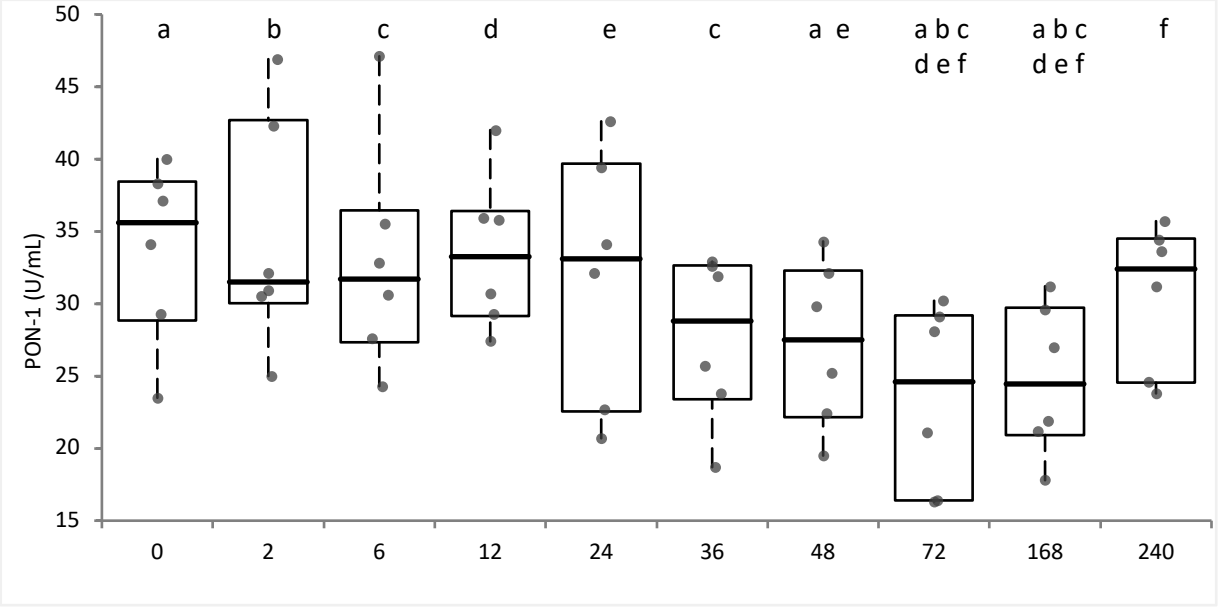


Figure 4. Plasma PON-1 activity (U/mL) measured at scheduled time points before (T0) and after (T2-T240) LPS administration in individual healthy horses (n=6). The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicate the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or to further observation within quartile III plus 1.5 × IQR. Time points that are significantly different to each other are indicated by the same letters on the top of boxplots.

Moreover, PON-1 activity was negatively correlated with fibrinogen ($P<0.001$, $rs=-0.899$) and positively correlated with the PCV ($P=0.008$; $rs=0.779$). No other significant correlations with laboratory parameters were found. Similarly, the clinical score was not correlated with PON-1 activity.

Discussion

PON-1 has an established role in modulating oxidative stress, which is a major promoter and mediator of the systemic inflammatory response occurring in sepsis (Draganov et al., 2010). The activity of PON-1 during septic inflammation has been evaluated in other species (Novak et al., 2010; Tvarijonaviciute et al., 2012; Torrente et al., 2019; Giordano et al., 2013; Iftimie et al., 2019), but not in horses yet. We investigated plasma PON-1 activity following intravenous injection of 30 ng/kg of LPS (E. coli O55:B5) in healthy horses included in a previous study. The authors of the previous study demonstrated significant clinical changes caused by endotoxemia within the first 12h post-infusion, significant changes in WBC counts 6h and marked neutrophilia at 24h after LPS infusion. Then, all these changes normalized in all horses after 48 h.

Plasma iron concentration significantly decreased to lowest values at 12 h post-infusion and raised again starting from 24 h, returning to baseline values at 36 h. As expected, we observed a decrease in PON-1 activity. Differently from clinical signs and changes in WBC and plasma iron reported in the previous study (Oliveira-Filho et al., 2012), PON-1 activity recorded in the present study did not show significant changes in the first 12 h. On the contrary, a significant decrease in PON-1 activity was recorded starting from 48h post-infusion, when all the other clinical and analytical parameters had returned to normal reference intervals. The simultaneous decrease in PON-1 activity and recovery from clinical signs is in contrast with those studies that in people reported PON-1 increase when patients with naturally occurred sepsis clinically improved (Novak et al., 2010; Sans et al., 2012; Bojic et al., 2014). The same studies however (Sans et al., 2012; Bojic et al., 2014), reported a sustained decrease in PON-1 activity for several days, which we also observed. Our study is based on an experimental model of endotoxemia, which does not completely mimic the conditions and pathologic pathways of naturally occurring endotoxemia. In the previous study (Oliveira-Filho et al., 2012), the evaluation of short-term effects of LPS infusion (i.e. 0–6h after LPS administration) was based on clinical changes and not on a complete SIRS score, that also includes the evaluation of WBC count. However, all the horses had at least 2 alterations of clinical parameters consistent with SIRS status evaluation proposed by Roy et al. (2017) and therefore it can be assumed that LPS administration induced a systemic inflammatory reaction. Our results agree with other papers assessing PON-1 activity in experimentally induced endotoxemic animals. In dogs, Tvarijonaviciute et al. (2012) reported normalization of clinical signs in 48h after LPS administration (although clinical signs occurred earlier compared with horses included in our study), and decreased PON-1 activity at 30 h, which progressed and became significant at 48h compared to 24h; a similar trend to what we observed. These authors did not measure PON-1 activity over 48h post LPS administration, so a comparison of long-term normalization of PON-1 activity cannot be made. Feingold et al. (1998) found in Syrian hamsters a progressive decrease in PON-1 activity, already significant 8h after LPS administration, which even in this case persisted at least up to the last sampling at 48h. These and our results seem to state that experimentally induced endotoxemia induces changes in PON-1 activity that does not have a rapid resolution, contrarily to clinical signs, that normalize earlier. This latter discrepancy may explain the lack of significant correlations between PON-1 activity and the clinical score. Our data seem to state that endotoxemia triggers changes in PON-1 activity later and for a longer period, compared with leukocyte count and plasma iron concentration.

Again, this delayed response may explain the lack of correlations between PON-1 activity and many laboratory changes, except for fibrinogen, which as expected for a positive APP, negatively correlated with PON-1 activity, and for the PCV that increased soon after LPS infusion likely due to dehydration and then decreased to normal values, thus paralleling the late decrease of PON-1. This late PON-1 response could be interpreted as a delayed occurrence of inflammatory oxidations and oxidative stress, possibly due to a different PON-1 metabolism during inflammation in horses compared to other species, as suggested by Ruggerone et al. (2020). Otherwise, it may also be possible that anti-inflammatory oxidations occurred in the first 12h post-LPS infusion, determining PON-1 activity fluctuations not wide enough to produce significant changes, possibly because of a less severe oxidative stress in horses compared to other species (Ruggerone et al., 2020). A reduced hepatic synthesis, due to cytokine down regulation or to liver function impairment after LPS administration, could have determined the subsequent noticeable decrease of PON-1 activity from the third day up to the seventh. Feingold et al. (1998) simultaneously evaluated also liver PON-1 mRNA expression and observed an earlier and greater decrease in mRNA level, starting from 4h post-LPS administration and reaching lowest values at 16 h, followed by a progressive increase which did not reach baseline values at 48h. This led to the hypothesis that acute changes in PON-1 production mildly affect serum levels, possibly because of a relatively long half-life of the enzyme (Feingold et al., 1998). Liver expression of PON-1 mRNA was not evaluated in our study, but a similar mechanism would explain the delayed and sustained decrease in PON-1 activity that followed clinical recovery and normalization of other laboratory parameters. Investigation of PON-1 liver expression in the future may clarify the causes of delayed and prolonged decrease of PON-1. Furthermore, PON-1 activity is dependent on the lipid and protein composition of HDL (James and Deakin, 2004), and correlation with lipid profile and other markers of inflammation such as serum amyloid A, may help acquiring knowledge about PON-1 metabolism in this species. Biomarkers of sepsis so far evaluated in horses include interleukin-1β (IL-1β), interleukin-10 (IL-10), interleukin-6 (IL-6), serum amyloid A (SAA), soluble CD14 (sCD14) and procalcitonin (Bonelli et al., 2017). Measurement of sCD14 provided contrasting results: no significant differences in concentration were recorded among time in experimental settings (Bonelli et al., 2017), while sCD14 was higher in spontaneous clinically endotoxemic than in nonendotoxemic horses, showing however no correlation with LPS concentration (Fogle et al., 2017). In many studies involving different types of experimentally induced endotoxemia in horses, cytokine expression was rapidly upregulated.

Following LPS infusion, IL-1β and IL-6 gene expression peaked at 60 and 90 min respectively and decreased to still significantly higher levels than baseline 3 h post infusion (Nieto et al., 2009). In a similar way, serum TNF-α increases within 30min, peaks at 60 min, and decreases 2–3 h from endotoxin administration (Nieto et al., 2009; Poulin et al., 2009). Even if studies about SAA in experimentally induced endotoxemic horses are not available, blood SAA concentration is reported to increase up to 1000 times 6h after inflammatory stimuli and decrease within 12h after stimulus removal (Long et al., 2020; Nunokawa et al., 1993; Jacobsen et al., 2006). It is not known if infection causes an earlier or persistent increase in SAA concentration compared with non-infectious inflammation, but there is evidence that infections are particularly effective in producing strong SAA responses (Taylor, 2015), especially in neonatal sepsis (Witkowska-Piłaszewicz et al., 2019). Since SAA is produced by the liver in response to cytokines stimulation, it is reasonable to think its increase in circulation be delayed. A decrease in PON-1 activity would be expected together with the early increase in SAA levels, since during the acute phase response the latter displaces PON-1 in HDL (Van Lenten et al., 1995) and these two APPs were reported to be inversely correlated (Kotani et al., 2013). However, cytokine-dependent hepatic regulation could have major impact on PON-1 activity rather than displacement by SAA (Han et al., 2006), and this would explain the delayed decrease of PON-1 activity, as reported above. Comparing changes in PON-1 activity detected in this study and changes in other markers investigated in experimentally induced endotoxemic horses, it seems that other markers normalize before PON-1 decreases. In fact, CCL-2 and IL-10 concentrations increased, peaked and decreased within 24h post-infusion. Procalcitonin as well showed a similar trend, but its concentration, even if decreased, was still statistically higher than baseline 24 h post-infusion (Bonelli et al., 2017). However, endotoxin is a single factor implicated in the development of sepsis, and it is well known that differences exist between experimentally induced and clinical endotoxemia and sepsis (Tadros and Frank, 2012). In humans, PON-1 activity and CCL-2 concentration have an inverse correlation during natural infection (Iftimie et al., 2016) and CCL-2 decreases together with the increase of PON-1 and the resolution of naturally occurring septic processes after several days of hospitalization (Sans et al., 2012). Investigation and comparison of changes in these markers during natural infection in horses would clarify their behavior, relationship and usefulness in this species.

This study has some limitations. Firstly, the long storage of samples that may have in part affected the magnitude of PON-1 activity. A previous study demonstrated that 6 months storage at -20°C can artifactually slightly increase PON-1 activity in dogs (Rossi et al., 2013). However, no information on storage stability at -80°C, or storage studies on equine PON-1 are available. However, as a support of the hypothesis of a storage effect, PON-1 activity was lower than the reference intervals (Ruggerone et al., 2018) in most of the horses at T0 despite the lack of any clinical or laboratory abnormality associated with inflammation. Nevertheless, this effect, if any, was present for all the samples included in this study and despite possible storage artifacts, fluctuations of PON-1 activity consistent with endotoxin-induced inflammation were present. A second limitation is the low caseload, coupled with the lack of a control group inoculated with a sham solution: this approach, that however, is in line with other studies based on a complex study design and is supported also by ethical reasons. However, also in this case, such a low but well standardized caseload, on which post-inoculation results were compared with baseline values recorded before LPS administration, allowed to have a better overview of the possible effect of LPS infusion on PON-1 activity compared with in field studies, on which several variables cannot allow to evaluate the direct effect of sepsis on clinical and laboratory parameters. Undoubtedly, following an inflammatory stimulus of septic type, such as the administration of endotoxins, PON-1 activity decreases, as reported for other species. According to our results, plasma iron concentration appears to be an earlier marker of sepsis onset and positive resolution compared to PON-1 activity. However, all horses showed a normalization of PON-1 activity to baseline values 10days after LPS administration. As claimed by Oliveira-Filho et al. (2012), the inflammatory model adopted by the authors is safe and efficient and caused an acute systemic inflammation without prolonged inflammatory effects. As noted above, in naturally occurring sepsis, clinical and pathological conditions are not as well standardized. Therefore, in natural situations, where the extent and severity of inflammation (Draganov et al., 2010) are very variable, PON-1 might prove to be useful as a diagnostic or prognostic marker of sepsis. In this case, measurement of PON-1 activity could be useful where limitations of iron measurement in detecting systemic inflammation exist, for example during corticosteroid administration, iron supplementation, hemolysis and age-related variability (Borges et al., 2007).

Conclusive remarks

In conclusion, our studies describe for the first time PON-1 activity in horses with ongoing endotoxemia caused by experimental LPS administration and evidence how PON-1 results may be handled in horses with spontaneous SIRS potentially induced by sepsis. As expected, LPS infusion induced a progressive transient decrease of PON-1 activity, which became maximum from 3 days to 7 days post-infusion, followed by a normalization to pre-infusion levels the tenth day. The inflammation model employed in our experimental study does not mimic the variety and severity of septic diseases occurring in the clinical practice. Therefore, further studies are warranted to investigate changes in PON-1 activity during natural onset of endotoxemia in horses. However, our data suggest that measurement and monitoring of PON-1 activity might be useful to evaluate progression of the septic process and recovery in endotoxemic horses and possibly to diagnose it. From this perspective, the study on spontaneous SIRS suggests that horses with low PON-1 activity likely have SIRS and may have a negative prognosis, whereas normal PON-1 activity does not allow exclusion of SIRS or negative prognosis. This may be a consequence of different metabolism of PON-1 in horses, lower magnitude of oxidation associated with SIRS in this species or the sensitivity of the assessment of SIRS status in identifying SIRS-positive horses. The exact mechanism responsible for this difference should be investigated in future studies based on a higher number of horses, on a higher number of horses sampled repeatedly during follow-up, and on a wider panel of tests that should include other markers of oxidation or systemic inflammation and horses with experimentally induced SIRS, by which several variables typical of field studies may be standardized.

PARAOXONASE ACTIVITY IN DOGS

Rationale and aims

The role of PON-1 in canine inflammation has been already described in several studies either alone or in comparison with some, but not all the markers that may be routinely used for diagnostic or prognostic purposes in dogs with inflammation. Therefore, within this thesis we preferred to investigate the potential utility of PON-1 in dogs in selected clinical scenarios. More specifically, three separate studies were performed:

- We first compared the diagnostic potential of PON-1 and CRP with that of carbonylated proteins (PCOs), recently proposed as an ancillary marker of inflammation in dogs, by completing a study already started within a previous PhD thesis in collaboration with the Department of Veterinary Medicine of the University of Bologna (**study III**). This study was published in the journal Veterinary Sciences [https://www.mdpi.com/2306-7381/8/6/93].
- We assessed the ability of PON-1 and CRP in detecting oxidative phenomena associated with seropositivity for Borrelia burgdorferi *sensu lato*, within a serosurveillance plan for Borrelia burgdorferi *sensu lato* in dogs living in the area of Valchiavenna (Northern Italy), leaded by the section of parasitology of our Department (**study IV**). The results of this study were presented as a poster at the 74th Congress of Società Italiana delle Scienze Veterinarie (SISVet) in June 2021.
- We evaluated the potential utility of PON-1 as a screening test in the population of dogs admitted to the Veterinary Teaching Hospital of the University of Milan, to assess whether PON-1 values may identify dogs affected by severe diseases, dogs requiring hospitalization to achieve complete recovery, or dogs with a negative outcome (**study V**). This study has been completed in late 2021 and will be soon submitted for publication to a peer reviewed journal.

III. Comparison of protein carbonyl, Paraoxonase-1 and C-reactive protein as diagnostic and prognostic markers of septic inflammation in dogs

Study design

This retrospective study was done on 92 serum samples collected from privately owned dogs (37 males, 10 castrated males, 29 females and 16 neutered females) that underwent clinical examination at the University of Bologna and Milan. Samples were stored at the same Institutions at −80 ◦C for a maximum of 12 months. The median age of the dogs included in the study was 36 months (age range: 1 month–15 years). Thirty-six dogs were mongrels, whereas the other dogs were: German Shepherds (n=8), Golden Retrievers (n=4), Cavalier King Charles Spaniels, Labrador Retrievers, American Staffordshire Terriers, Italian Bloodhounds (n=3 for each breed), Pugs, English Bulldogs, Miniature Poodles, Doberman Pinschers, Maremmano-Abruzzese Sheepdogs, Jack Russell Terriers, Rottweilers (n=2 for each breed), Maltese, Leonberger, Yorkshire Terrier, Bernese Mountain Dog, English Setter, Dachshund, Shetland Sheepdog, Airedale Terrier, Australian Shepherd, Great Dane, Shih Tzu, Cocker Spaniel, Chihuahua, Weimaraner, Italian Pointer, Dogo Argentino, Saluki, Rhodesian Ridgeback, Spanish Greyhound (n=1 for each breed). Dogs were divided into the following three groups:

- Group A, clinically healthy: 35 dogs (12 males, 7 castrated males, 10 females, 6 neutered females; median age 24 months, age range: 6 months–13 years) that were considered healthy on the basis of normal physical examination, history and blood test results;
- Group B, septic: 34 dogs (12 males, 2 castrated males, 12 females, 8 neutered females; median age 60 months; age range: 1 month–15 years) that were considered septic as a result of the presence of symptoms such as abnormal mentation, fever or hypothermia, tachycardia, tachypnoea and of appropriate tests on the basis of the suspected diagnosis (e.g., inflammatory leukogram, cytology consistent with presence of intracellular bacteria, abdomen ultrasound examination, thorax X-rays, positive blood culture) (Hauptmann et al., 1997);
- Group C, non-septic inflammation or trauma: 23 dogs (13 males, 1 castrated male, 7 females, 2 neutered females; median age 24 months; age range: 3 months–15 years) polytraumatized after motor vehicle accidents (n=15) or falls (n=3) or blunt trauma of unknown origin (n=5): in these dogs, sepsis was excluded based on history and collateral tests reported above.

Serum samples collected within two hours since first presentation and before starting any treatment or just after the administration of treatment were included in this study. Then, all the dogs from Groups B and C received appropriate treatments according to the diagnosis. For each sick patient, clinical data and outcome were recorded. All the samples were collected from client-owned dogs for diagnostic purposes or during routine examinations; an informed consent about the use of residual amounts of samples for research purposes was signed by the owners. Therefore, according to the regulations of our Institution, it was not necessary to require a formal authorization to the Institutional animal care committee (Decision of the institutional committee no. 2/16 dated 15/02/16). The serum activity of PON-1 was measured in all the samples included in this study as previously described. The serum concentration of CRP was measured in 87 serum samples (35 from Group A, 32 from Group B and 20 from Group C) using the automated analyzer BT3500 (Biotecnica Instruments SPA, Roma, Italy) using an immunoturbidimetric method provided by the manufacturer of the analyzer. The serum concentration of PCO was measured in 41 samples (15 from Group A, 14 from Group B and 12 from Group C) using a commercially available ELISA kit (Enzo Life science, 3V Chimica, Roma, Italy) following manufacturer's instructions. The plate was read with an automated plate reader (Dasit multiscan, Dasit, Cornaredo, Milan, Italy) using a wavelength of 450 nm. A regression standard curve was then designed by plotting the lot specific nmoL/mg protein carbonyl concentration of the standards, against their absorbances. The concentration of PCO in each sample was then calculated by interpolating the absorbance of each sample with the standard curve. The mortality rates recorded in the two pathologic groups were compared to each other using a Pearson chi-square test. Results regarding PCO, PON-1 and CRP were compared in each group of dogs using the Kruskal-Wallis test, followed by a Mann-Whitney U test to assess the differences between single groups. The Mann-Whitney U test was used also to compare the results obtained at admission from animals that died with those that survived. These comparisons were performed either on the whole caseload or on sick dogs (Groups B and C), in order to draw information more relevant to the clinical application of the test. A Spearman's correlation test was used to correlate the results of PCO, PON-1 and CRP in the 36 samples on which all the analytes were measured.

In order to assess the discriminating power of the three analytes in detecting dogs with sepsis or with a poor outcome and to establish the optimal diagnostic cut-off, for each numerical value recorded in the study (operating point), we classified as true or false positive the dogs with or without sepsis or the dogs that died or not, that had PCO or CRP values higher and PON-1 values

lower than each operating point, and as true or false negative the dogs with or without sepsis or the dogs that died or not that had PCO or CRP values lower and PON-1 values higher than each operating point. Sensitivity and specificity and the positive likelihood ratio were calculated for each analyte and for each operating point, using standard formulae (Gardner and Greiner, 2006; Christenson, 2007), and receiver operating characteristic (ROC) curves were designed by plotting sensitivity versus (1–specificity) (Gardner and Greiner, 2006). The Youden index (i.e., the operating point that maximizes the difference between true positives and false positives) and the operating points characterized by the highest LR+ and by absolute specificity were then calculated (Ruopp et al., 2008). Additionally, the evaluation of sensitivity and specificity and ROC curve analyses were performed first by also including the control group and then excluding the control group.

Results

The mortality rate was significantly lower (P=0.027) in the non-septic group (3/23; 13.0%) than in the septic group (14/34; 41.2%). No significant differences were found regarding the age (P=0.150) or the proportion of male or female dogs (P=0.211) among the three groups.

A significant difference among groups was found for the serum concentrations of PCO and CRP as well as for PON-1 activity (P<0.001 for all the analytes, Table 5, Figure 5).

Table 5. Mean ± SD, median (between parenthesis) and min-max range of PCO, CRP and PON-1 in dogs with sepsis, trauma and clinically healthy dogs.

	PON-1 (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)
SEPSIS	96.4 ± 44.4 (88.7) *** ††† (‡) 14.1-180.9	24.22 ± 12.47 (20.60) *** ††† (‡‡‡) 5.66-60.70	0.40 ± 0.22 (0.31) *** 0.22-0.89
TRAUMA	126.6 ± 25.1 (111.5) *** 89.6-184.4	9.46 ± 9.36 (6.17) * 0.00-30.60	0.27 ± 0.08 (0.30) ** 0.11-0.35
HEALTHY	176.8 ± 24.4 (179.5) 126.0-220.6	2.88 ± 3.76 (1.72) 0.00-20.50	0.20 ± 0.06 (0.21) 0.10-0.29

*** P<0.001 vs Clinically healthy; ** P<0.01 vs Clinically healthy; * P<0.05 vs clinically healthy; ††† P<0.001 vs trauma; ‡‡‡ P<0.001 vs trauma when only dogs with sepsis and trauma were compared to each other; ‡ P<0.05 vs trauma when only dogs with sepsis and trauma were compared to each other

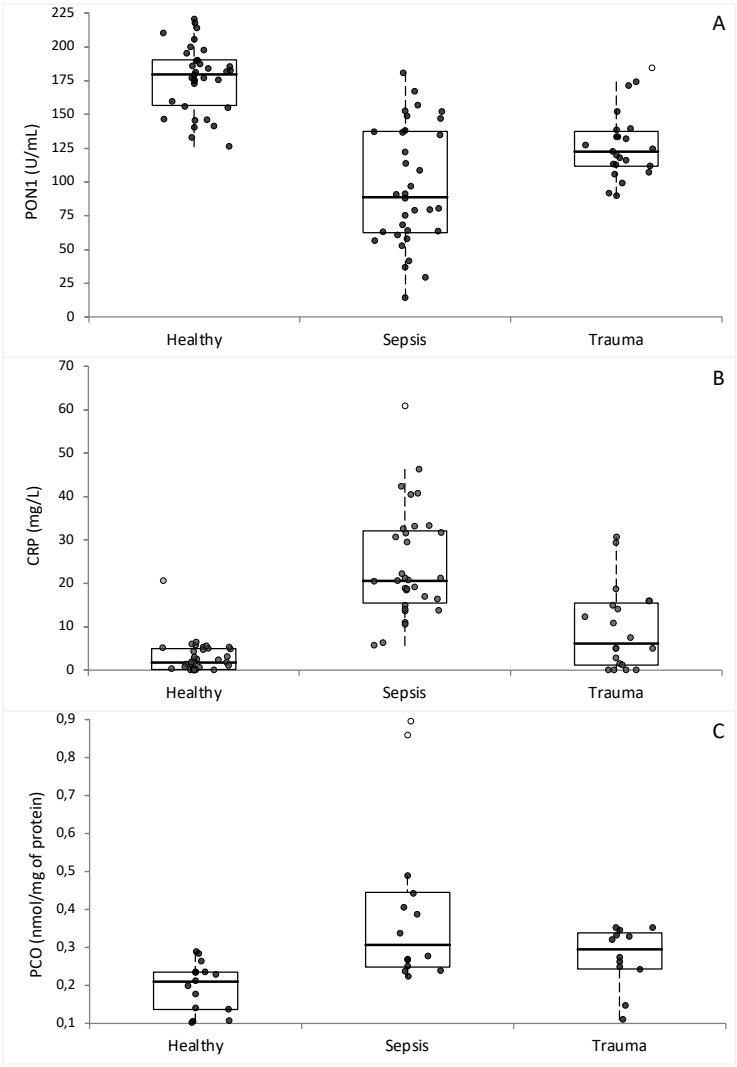


Figure 5. Serum PON-1 activity (A) and concentration of CRP (B) and PCO (C) in clinically healthy dogs, in dogs with sepsis and in those with polytrauma. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or within quartile III plus 1.5 × IQR. Black dots indicate the results that are not classified as outliers. White dots indicate near outliers (values exceeding the third quartile ± (1.5 × IQR)) and grey dots indicate far outliers (values exceeding the third quartile ± (3.0 × IQR)).

In particular, the concentration of PCO was significantly higher in dogs with sepsis and in dogs with trauma than in clinically healthy dogs, but no significant differences were found between dogs with trauma or sepsis. Conversely, the serum concentration of CRP was significantly higher in dogs with sepsis than in dogs with trauma and in clinically healthy dogs; a significant difference was also found between dogs with trauma and clinically healthy dogs. Similarly, PON-1 activity was significantly lower in dogs with sepsis than in dogs with trauma and in clinically healthy dogs, and a significant difference was found also between dogs with trauma and clinically healthy dogs.

The same differences were found when only results from dogs with sepsis or trauma were compared to each other, although the level of significance was lower than in the previous comparison for PON-1 ($P<0.05$). On the whole caseload, PON-1 was negatively correlated either with PCO ($P<0.001$, $r=-0.594$) or with CRP ($P<0.001$, $r=-0.510$) while PCO and CRP were positively correlated to each other ($P=0.007$, $r=0.440$) (Figure 6).

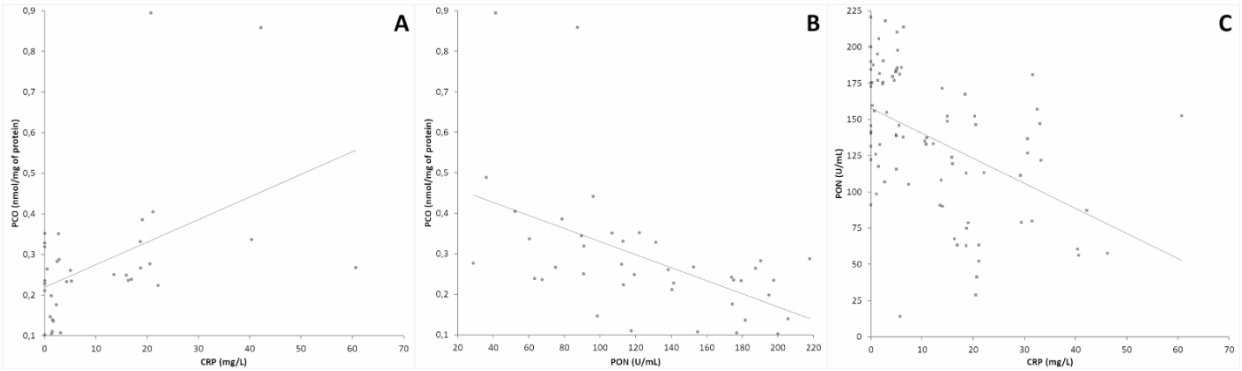


Figure 6. Correlations between CRP and PCO (A), PON-1 and PCO (B), and CRP and PON-1 (C).

The ROC curve analysis (Figure 7) on the whole caseload demonstrated that all the three analytes had a discriminating power for sepsis ($P<0.001$ compared with the line of no discrimination) (Table 6). However, the area under the curve (AUC) of PON-1 and of CRP were significantly higher than the AUC of PCO, but not significantly different to each other ($P=0.990$).

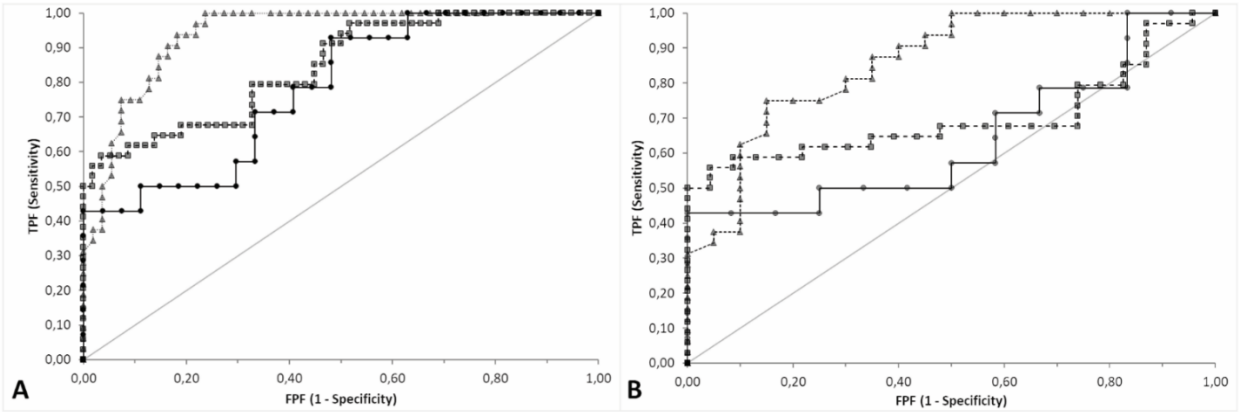


Figure 7. Comparison of ROC curves to support a diagnosis of sepsis designed for PCO (black circles, continuous line), CRP (black triangles, dotted line) and PON-1 (black squares, dashed line). The graph on the left (A) includes the results of the three groups of dogs, the graph on the right (B) includes only the results of dogs with sepsis or trauma. The grey line indicates the line of no discrimination. TPF, true positive fraction; FPF, false positive fraction.

Table 6. Area under the ROC curves (AUCs), Youden index and operating points characterized by the highest LR+ and by absolute specificity for PON-1, CRP and PCO to support a diagnosis of sepsis, calculated either including or excluding clinically healthy dogs from the analysis.

	Whole population			**Only sick dogs**		
	PON-1 (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)	**PON-1** (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)
AUC (%)	95 (87-104) *	95 (91-100) *	78 (63-93)	90 (76-104) *	90 (79-100) *	63 (41-86)
Youden index	98.6 (Y: 0.554)	5.64 (Y: 0.764)	0.23 (Y: 0.447)	91.5 (Y: 0.515)	15.90 (Y: 0.600)	0.35 (Y: 0.429)
Max LR+	90.9 (LR^+ 30.7)	29.20 (LR^+ 20.6)	0.35 (LR^+ 11.6)	91.1 (LR^+ 12.8)	29.20 (LR^+ 7.5)	0.35 (LR^+ 5.1)
100% Sp	89.6	30.60	0.35	89.6	30.30	0.35

* P<0.05 compared with PCO

Results were substantially similar when the comparison above was repeated excluding clinically healthy dogs, despite all the AUCs were slightly lower than those recorded in the previous comparison and only the ROC curve of PON-1 and of CRP, but not that of PCO, had a discriminating power (P=0.001, P<0.001 and P=0.115, respectively). As in the previous comparison, the AUCs of PON-1 and CRP were significantly higher than that of PCO (P=0.034 and P=0.035, respectively), but not significantly different to each other (P=0.928). CRP had the highest Youden index, although the cut-off to discriminate septic vs. non-septic dogs was higher when clinically healthy dogs were excluded from the analysis, as expected. The same occurred for PCO and PON-1, whose optimal cut-offs were, respectively, higher and lower when clinically healthy dogs were excluded from the analysis. Conversely, all the tests had the highest LR+ or absolute specificity at similar cut-offs regardless of the presence or absence of clinically healthy dogs, but the LR+ was obviously lower when clinically healthy dogs were excluded.

Overall, PON-1 activity was significantly lower in dogs that died compared with dogs that survived (Table 7, Figure 8). Similarly, the concentration of CRP was significantly different in dogs that died compared with dogs that survived, although with lower level of significance. Conversely, despite the P value was very close to the level of statistical significance (P=0.078), the concentration of PCO was not significantly different in dogs that survived compared with dogs that died.

Table 7. Mean ± SD, median (between parenthesis) and min-max range of PCO, CRP and PON-1 in dogs that died and dogs that survived, both including and excluding clinically healthy dogs.

	PON-1 (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)
DEAD	79.1 ± 39.0 (67.6) *** ††† 14.1-167.4; n=17	17.36 ± 11.70 (18.50) * 0.00-40.60; n=16	0.37 ± 0.22 (0.33) 0.15-0.89; n=9
ALIVE (ALL GROUPS)	146.6 ± 40.9 (146.7) 28.9-220.6; n=74	11.25 ± 13.24 (5.20) 0.00-60.70; n=70	0.26 ± 0.14 (0.25) 0.10-0.86; n=32
ALIVE (SEPSIS-TRAUMA)	121.1 ± 34.3 (121.7) 14.1-106.4; n=40	19.07 ± 14.22 (15.35) 0.00-60.70; n=36	0.32 ± 0.16 (0.28) 0.11-0.86; n=17

*** P<0.001 vs Alive (all the groups); * P<0.05 vs Alive (all the groups); ††† P<0.001 vs Alive (only dogs with sepsis or trauma)

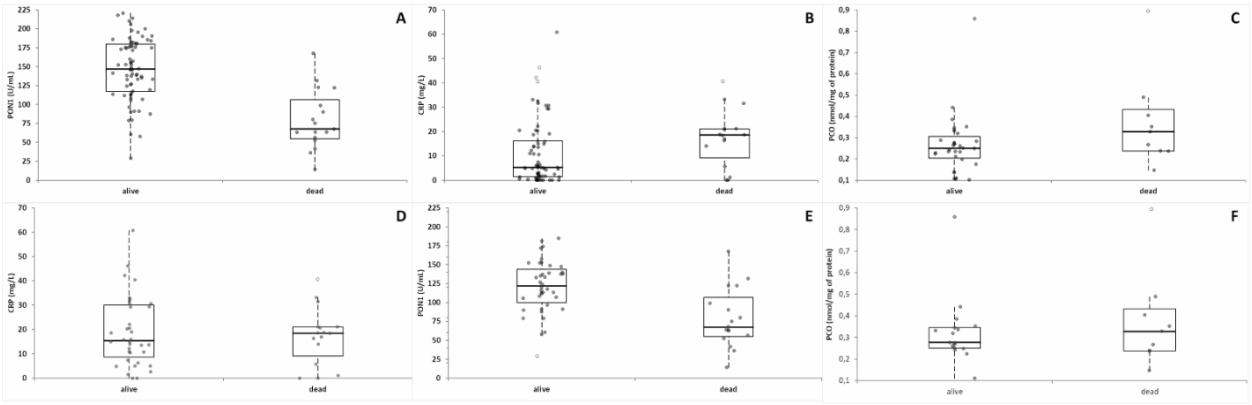


Figure 8. Serum activity of PON-1 (A, E) and concentration of CRP (B, D) *and* PCO (C, F) in dogs that survived or died during the follow up. The analysis was performed either on the whole caseload (upper graphs) or after exclusion of clinically healthy dogs (lower graphs). The boxes indicate the I-III 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. Black dots indicate the results that are not classified as outliers. White dots indicate near outliers (values exceeding the third quartile ± [1.5 x IQR]), and grey dots indicate far outliers (values exceeding the third quartile ± [3.0 x IQR]).

Excluding healthy controls from the analysis, PON-1 activity was still significantly lower in dogs that died compared with dogs that survived, and the concentration of PCO was still not significantly different (P=0.726) in dogs that survived compared with dogs that died. On the contrary, the concentration of CRP showed no significant differences (P=0.897) in dogs that died compared with dogs that survived.

Based on the ROC curve analysis on the whole caseload (Table 8, Figure 9) only the AUC of PON-1 had a discriminating power (P<0.001 compared with the line of no discrimination) and was significantly higher than the AUC of CRP (P<0.001).

Table 8. AUCs, Youden index and operating points characterized by the highest LR+ and by absolute specificity for PON-1, CRP and PCO to support a poor outcome, calculated either including or excluding clinically healthy dogs from the analysis.

	Whole population			**Only sick dogs**		
	PON-1 (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)	**PON-1** (U/mL)	**CRP** (mg/L)	**PCO** (nmol/mg of protein)
AUC (%)	90 *** (82-99)	54 (38-70)	70 (48.90)	82 *** (68-96)	39 (22-57)	54 (27-82)
Youden index	132.7 (Y: 0.635)	13.90 (Y: 0.454)	0.35 (Y: 0.351)	90.9 (Y: 0.531)	15.90 (Y: 0.243)	0.35 (Y: 0.268)
Max LR+	57.7 (LR^+ 22.1)	15.90 (LR^+ 2.9)	0.44 (LR^+ 7.1)	57.7 (LR^+ 11.8)	15.90 (LR^+ 1.5)	0.44 (LR^+ 3.8)
100% Sp	28.9	60.70	0.86	28.9	60.70	0.86

*** vs CRP

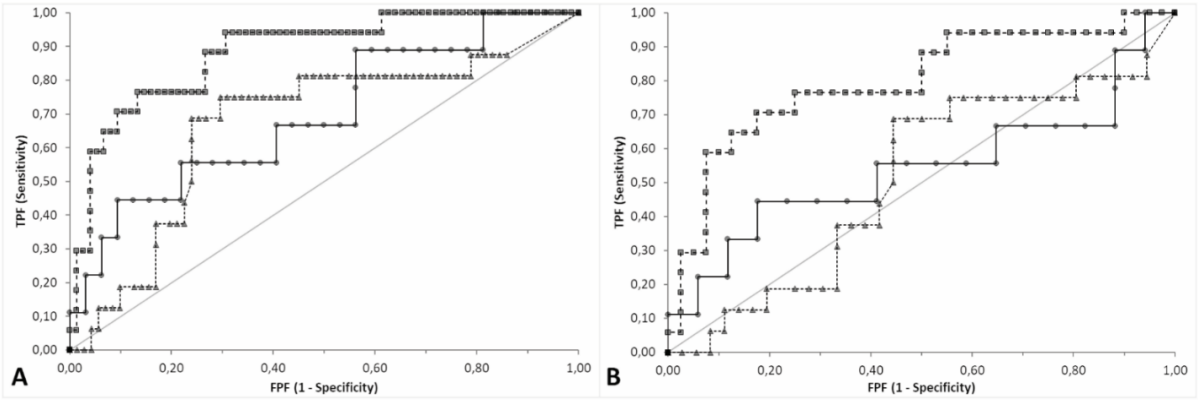


Figure 9. Comparison of ROC curves to support a poor outcome designed for PCO (black circles, continuous line), CRP (black triangles, dotted line) and PON-1 (black squares, dashed line). The graph on the left (A) includes the results of the three groups of dogs, the graph on the right (B) includes only the results of dogs with sepsis or trauma. The grey line indicates the line of no discrimination. TPF, true positive fraction; FPF, false positive fraction.

The AUC of PCO and CRP did not significantly differ from the line of no discrimination although the P value of PCO was close to the level of significance (P=0.068). No significant differences were found between the AUCs of PCO and CRP (P=0.218) or of PCO and PON-1 (P=0.062). The exclusion of clinically healthy dogs provided the same results: although the AUCs were lower for all the analytes, only the AUC of PON-1 was significantly different from the line of no discrimination (P<0.001) and significantly higher than the AUC of CRP (P<0.001).

The AUC of PCO and CRP did not significantly differ from the line of no discrimination and were not significantly different to each other (P=0.339) as well as no significant differences were found between the AUCs of PCO and PON-1 (P=0.069). The highest Youden index was found for PON-1, although the cut-off to discriminate dead vs alive dogs was lower when clinically healthy dogs were excluded from the analysis, as expected. The same occurred for PCO and CRP whose optimal cut-offs were higher when clinically healthy dogs are excluded than when clinically healthy dogs are included in the analysis. All the tests had the highest LR+ or absolute specificity at similar cut-offs, independent of the presence or absence of clinically healthy dogs. However, the LR+ were obviously lower when clinically healthy dogs were excluded, and the maximum specificity was detected only at very high values of CRP or PCO or at very low values of PON-1.

Discussion

In several species, it has already been proved that sepsis induces an acute phase reaction, that in turn is characterized by an increase of acute phase proteins such as CRP (Ceron et al., 2005; Cray et al., 2009; Tothova et al., 2016), and by oxidative phenomena. The latter may induce protein oxidation, leading to the increase of the serum concentration of PCO (Abu-Zidan et al., 2002; Dalle Donne et al., 2005) and to the decrease of antioxidant compounds, including the enzyme paraoxonase-1 (Feingold et al., 1998; Cabana et al., 2003; Novak et al., 2010). Different studies are available about the utility of CRP to distinguish dogs with sepsis from dogs with sterile inflammation (Yamamoto et al., 1994; Martinez-Subiela et al., 2002; Fransson et al., 2004; Jitpean et al., 2014; Viitanen et al., 2014) but only a recent study demonstrated that PON-1 may be useful to discriminate dogs with sepsis from dogs with non-septic inflammation, although ultimately, CRP and albumin may better predict the outcome (Torrente et al., 2019) and no information is available about the utility of PCO in dogs as diagnostic or prognostic marker, differently from human medicine (Takeda et al., 1984; Goode et al., 1995; Amorim de Oliveira et al., 2017; Novak et al., 2010; Bavunoglu et al., 2016; Camps et al., 2017; Bednarz-Misa et al., 2019).

Therefore, the aim of this study was to evaluate which analyte (CRP, PCO or PON-1) better differentiates dogs with sepsis from those with polytrauma or from healthy dogs and predicts the outcome in dogs in septic conditions. In the case a possible diagnostic or prognostic role could be demonstrated, the evaluation of CRP, PCO and/or PON-1 could be recommended as a tool to better focus on the severity of the inflammatory status in the enrolled patients.

To this aim, two different approaches have been followed. First, the comparison of results, the evaluation of sensitivity and specificity and the ROC curves analysis was performed by including the whole caseload, composed by both sick and clinically healthy dogs, in order to simulate the use of the markers for screening purposes independently of a pre-test probability of disease. Then, the comparison was performed excluding the control group, to simulate the use of the markers for diagnostic purposes in animals on which a diagnosis of a condition potentially associated with sepsis has already been formulated.

This study confirmed the possible role of CRP in supporting a clinical diagnosis of sepsis, as already evidenced by previous studies (Ceron et al., 2005; Fransson et al., 2007; Gebhardt et al., 2009), since, regardless of the inclusion or the exclusion of clinically healthy dogs, the highest concentration of CRP was found in dogs with sepsis and the ROC curve analysis demonstrated that CRP had the highest AUC. However, CRP concentration was increased also in dogs with trauma, that likely had an hyperacute, although non-infectious, inflammation. This confirmed that increases of CRP may occur in different inflammatory conditions and are thus not specific for sepsis (Ceron et al., 2005; Jergens et al., 2003; Tecles et al., 2005; Raila et al., 2011; Polizopoulou et al., 2015; Fazio et al., 2015; Hindenberg et al., 2020) and slightly decreased the diagnostic performance of CRP when the test was applied only on sick dogs. A similar trend was observed for PON-1, that was significantly lower in septic dogs than in the other two groups and decreased also in dogs with polytrauma compared with controls. The AUC of the ROC curve referred to PON-1, in agreement with what reported by Torrente et al. (2019), was slightly lower but not significantly different from that of CRP, although, also in this case, the diagnostic performance of the test decreased, but remained acceptable, when only sick dogs were considered. From this perspective and compared with PCO, CRP and PON-1 seem to be better markers to differentiate the three conditions considered in this study. This hypothesis is confirmed both by the group comparisons and by the analysis of the ROC curves. Additionally, the LR+ of CRP and, to a lesser extent, of PON-1 were notably higher than that of PCO either on the whole caseload or when the tests were applied to discriminate groups of sick dogs. However, the concentration of CRP characterized by absolute specificity was relatively high, and the PON-1 activity characterized by absolute specificity was relatively low, compared with the upper and lower limits of the reference interval, respectively. Hence, moderate increases of CRP or moderate decreases of PON-1 may not differentiate dogs with sepsis from dogs with non-septic conditions.

Moreover, the comparison of results from dogs that died during the follow up with survivors suggests that the decrease of PON-1 and, to a lesser extent, the increase of CRP may predict a poorer outcome. More specifically, the analyte that better differentiated dogs with a poor outcome in clinical settings on which the likelihood of death is high, was found to be PON-1 activity. In fact, the concentration of CRP did not show significant differences between survivors and non-survivors when healthy controls, which are obviously not supposed to die, were excluded from the analysis. Ultimately, when data were examined on the whole caseload, results were in contrast with those of Meisner et al. (2006), who demonstrated that CRP levels did not differ between survivors and non-survivors but were in agreement with 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 induced by infectious agents in dogs. Our results also agree with those of Torrente et al. (2019), although these authors reported increased CRP as a better predictor of negative outcome than decreased PON-1. These discrepancies may be explained by the different composition of the caseload, as demonstrated by the subsequent analysis after exclusion of clinically healthy dogs, that highlighted a superior role of PON-1 compared with CRP in predicting the outcome. This clinical scenario, however, is much more adherent to what may happen in practice, when prognostic information is needed in dogs with a high pre-test probability of disease. In any case, based on the analysis of LR+ and cut-off values characterized by maximum specificity, it should be stressed that either PON-1 or CRP may be excellent indicators of a poor prognosis only if recorded values are respectively extremely low or high compared with the refence intervals. Independently of all the comments above or of the inclusion/exclusion of clinically healthy dogs, PCO seems not to predict the outcome in dogs.

A limitation of this study is that the presence of oxidation was not confirmed by the measurement of oxidants such as reactive oxygen species, or other indirect reliable markers of oxidation, such as thiobarbituric acid reactive substances or malondialdehyde (Da Silva et al., 2016; Bottariet al., 2015). However, the study was focused on markers that can be easily used in routine practice and that are known to be associated with oxidation. This association is further confirmed by the negative correlation found, as expected, between PCO and PON-1. Ultimately, the comparison of results of CRP, PCO and PON-1 confirm the hypothesis that in dogs with sepsis oxidative phenomena are stronger than in dogs with non-septic inflammation, as already demonstrated for people (Povoa, 2008; Lisboa et al., 2008; Cals et al., 2009; Povoa et al., 2011; Teggert et al., 2020).

This hypothesis is supported also by the positive correlation between CRP and PCO and by the negative correlation between PCO and the other two markers. However, the magnitude of oxidation seems to affect the kinetic of the two analytes in different ways. More specifically, oxidation of proteins, which is responsible for the increased concentration of PCO, seems to occur in any inflammatory condition able to determine an increase of CRP, including those not associated with infectious agents. On the contrary, the decrease of PON-1 activity, which depends on a more complex mechanism involving decreased hepatic production and displacement of circulating PON-1 from oxidized lipoproteins (Rossi et al., 2013), seems to occur only when inflammation is more severe, as it usually happens in sepsis. Independent of the possible mechanisms by which oxidation differently affects the two compounds, based on our results, PCO cannot be considered reliable markers to distinguish dogs with sepsis from dogs with non-septic inflammation or predict the outcome, while PON-1 and CRP may be considered adequate markers to support a clinical diagnosis of sepsis and provide prognostic information on the possible outcome. A second limitation of the study was the lack of clinical information about severity of disease and organ dysfunction for many dogs. These aspects would have been remarkable to evaluate, but unfortunately, as a retrospective study, the retrieval of such information was frequently troublesome. Finally, another limitation was the lack of repeated sampling during the follow up. This would be particularly important to assess a possible prognostic role of PCO not detected by a single measurement at admission. Fluctuations over time of PCO concentration may have provided prognostic information, as demonstrated for other markers with a limited prognostic role at admission, such as calprotectin, procalcitonin, CRP or PON-1 (Troia et al., 2018; Ibba et al., 2015; Nielsen et al., 2007; Alexandrakis et al., 2017; Kanno et al., 2019; Thames et al., 2019).

IV. C-reactive protein, Paraoxonase-1 activity and serum protein electrophoresis in dogs seropositive for Borrelia burgdorferi *sensu lato*

Study design

The collection of samples was performed in collaboration with a private veterinary facility located in Chiavenna. Inclusion criteria for dogs were age older than 8 months and having spent at least one summer in the area of Valchiavenna (Northern Italy). Overall, 151 dogs living or frequently attending the area and admitted to the facility throughout the period from April to October 2019 were included in a serosurveillance plan for Borrelia burgdorferi *sensu lato*. Ethical approval was granted by decision n°2/2016 of the Ethical committee of the University of Milan and written informed consent of the owner to use samples was obtained. Dogs underwent physical examination and different diagnostic procedures based on the clinical condition and the reason for the visit. Therefore, the caseload included both sick dogs, affected by a wide range of diseases, and healthy dogs undergoing health checks and/or vaccination. Signalment and origin of the dogs were recorded.

Fasting blood samples were taken from the cephalic or jugular vein and collected into plain tubes (PROMED®, FL Medical S.r.l) and tubes were centrifuged within 15 minutes. Serum was collected in 1.5 ml Eppendorf tubes, transported on ice to the Veterinary Teaching Hospital of Lodi and stored at -20°C until analyses were performed. Samples were processed at the end of the period of collection in a single batch to reduce inter-assay variability. Serum level of anti-Borrelia IgM and IgG class antibodies was determined using an indirect immunofluorescence assay (MegaFLUO® BORRELIA canis, MEGACOR Diagnostik GmbH, Hörbranz, Austria) according to manufacturer's instructions. Briefly, 20 µL of serum samples were diluted 1:64 with phosphate-buffered saline (PBS) and incubated in duplicate with pre-prepared slides for 30 minutes at 37°C. After washing with PBS, fluorescein-labelled anti-dog IgG and IgM conjugates were added to wells and incubated again for 30 minutes at 37°C. Slides were washed with PBS, mounted with anti-fluorescence medium and observed using a fluorescence microscope at 400x magnification. Sera showing a fluorescent signal at a titer of 1:64 were considered positive and serially diluted on a two-fold basis until negative results were obtained, to determine the antibody titer. Negative and positive control samples were included on each slide.

18/151 (11.9%) dogs, 11 of which clinically healthy, tested seropositive for anti-Borrelia IgM and/or IgG (threshold antibody titer 1:64). In particular, 9.3% (14/151) tested positive for IgM, 6.6% (10/151) tested positive for IgG and 4% (6/151) tested positive for both. None of seropositive dogs was vaccinated for B burgorferi.

To evaluate inflammatory and oxidative status, based on immunofluorescence results and clinical data, 56 samples were selected in order to create four groups:

- Healthy seronegative (n=22): seronegative dogs without clinical or clinico-pathological abnormalities;
- Sick seronegative (n=17): seronegative dogs with clinical or clinico-pathological abnormalities consistent with inflammatory conditions of different etiology and severity;
- Healthy seropositive (n=11): seropositive dogs without clinical or clinico-pathological abnormalities;
- Sick seropositive (n=6): seropositive dogs with clinical or clinico-pathological abnormalities consistent with inflammatory conditions of different etiology and severity.

Median age of dogs was 5 years (age range: 8 months-16 years), 22 were males, 8 castrated males, 15 females and 11 neutered females. Eighteen dogs were mongrels, while the other dogs were: Golden Retriever (n=7), Border Collie (n=6), German Shepherd (n=3), Bergamasco Shepherd, Cocker Spaniel, English Setter, Labrador Retriever, Siberian Husky (n=2 for each breed), Alaskan malamute, Australian Kelpie, Australian Shepherd, Belgian Shepherd, Boxer, Dogo Argentino, English Bulldog, French Bulldog, Maltese Dog, Pit Bull, Shih Tzu, Welsh Terrier (n=1 for each breed). Serum C-reactive protein (CRP) concentration was measured with an automated chemistry analyzer (BT3500, Biotecnica Instruments SPA, Rome, Italy) with an immunoturbidimetric assay and manufacturer's reagents. Serum protein concentration was measured using the same analyzer with the biuret colorimetric method and manufacturer's reagents. Serum PON-1 activity was measured as described above. Serum protein electrophoresis was performed on agarose gel using an automated analyzer (Hydrasys, Sebia Italia S.r.l., Bagno a Ripoli, Florence, Italy) and specific manufacturer's reagents (Hydragel 15 β1-β2). The Mann-Whitney U tests was used to compare the results recorded in seropositive vs. seronegative dogs or in symptomatic vs. non-symptomatic dogs. The Kruskal-Wallis test was used to compare the results of the four groups of dogs cited above.

Results

Overall, sick dogs had significantly higher CRP levels (mean ± SD, 7.42 ± 7.70 mg/L; median, 4.60 mg/L; I-III interquartile range, 0.86-11.57 mg/L; n=23) (P=0.004) compared with healthy dogs (2.18 ± 2.66 mg/L; 1.17 mg/L; 0.03-3.39 mg/L). Similarly, significantly lower PON-1 activity was observed in sick dogs (158.43 ± 30.53 U/mL; 162.90 U/mL; 139.05- 183.73 U/mL) compared with healthy dogs (197.85 ± 31.99 U/mL; 198.80 U/mL; 176.40-222.53 U/mL) (P<0.001).

Comparison of CRP concentration and PON-1 activity between groups of seropositive and seronegative dogs are shown in Table 9 and Figure 10. The concentration of CRP was significantly higher only in seronegative sick compared with seronegative healthy dogs (P=0.006). PON-1 activity was significantly lower in seronegative sick (P<0.001) and seropositive sick (P=0.006) compared with seronegative healthy dogs. Furthermore, PON-1 activity was significantly lower in seropositive healthy compared with seronegative healthy dogs (P=0.029).

Table 9. Mean ± SD, median (between parenthesis), I-III interquartile range and min-max range (between parenthesis) of serum CRP concentration and PON-1 activity in B. burgdorferi seronegative and seropositive sick and healthy dogs.

	CRP (mg/L)	**PON-1** (U/mL)
NEGATIVE SICK	7.95 ± 8.63 (4.06) * 0.41-13.17 (0.00-26.30); n=17	57.42 ± 34.8 (154.50) ** 133.80-185.90 (94.90-215.50); n=17
NEGATIVE HEALTHY	1.83 ± 2.47 (0.75) 0.00-3.25 (0.00-7.96); n=21	208.33 ± 32.64 (217.20) 197.19-229.55 (122.80-265.40); n=22
POSITIVE SICK	5.92 ± 4.35 (5.16) 2.57-8.84 (0.95-13.1); n=6	161.32 ± 14.57 (163.55) * 150.90-181.40 (138.80-181.40); n=6
POSITIVE HEALTHY	2.85 ± 2.98 (1.74) 0.33-6.1 (0.00-7.70); n=11	176.87 ± 17.69 (180.00) *** 163.60-190.98 (149.10-200.30); n=11

* P<0.01 vs seronegative healthy; ** P<0.001 vs seronegative healthy; *** P<0.05 vs seronegative healthy

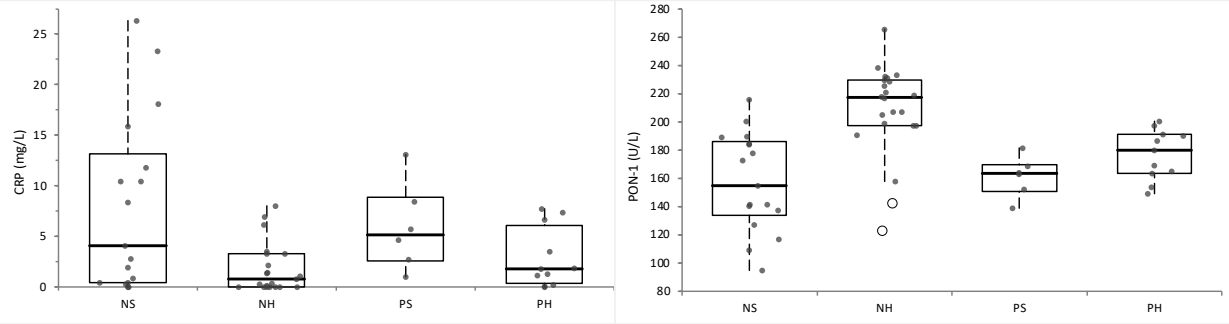


Figure 10. Serum CRP concentration (left) and PON-1 activity (right) in B. burgdorferi seronegative and seropositive sick and healthy dogs. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or within quartile III plus 1.5 × IQR. Grey dots indicate the results that are not classified as outliers. White dots indicate near outliers (values exceeding the third quartile ± (1.5 × IQR)). NS, negative sick; NH, negative healthy; PS, positive sick; PH, positive healthy.

Overall, albumin concentration (4.23 ± 1.05 g/L; 3.87 g/L; 3.54-4.73 g/L; P=0.038) and albumin/globulin ratio (1.54 ± 0.60; 1.58; 0.97-1.97; P=0.008) were significantly lower in sick dogs compared with healthy dogs (albumin: 4.75 ± 0.99 g/L; 4.62 g/L; 4.23-5.05 g/L; A/G ratio: 1.96 ± 0.41; 1.98; 1.71-2.21) and β1-globulin and β2-globulin concentrations were significantly higher in sick dogs (β1-globulin: 0.38 ± 0.122 g/L; 0.37 g/L; 0.29- 0.46 g/L; P=0.024; β2-globulin 0.87 ± 0.34 g/L; 0.82 g/L; 0362-1.06 g/L; P=0.011) compared with healthy dogs (β1-globulins: 0.32 ± 0.09 g/L; 0.29 g/L; 0.26-0.35 g/L; β2-globulins: 0.68 ± 0.18 g/L; 0.63 g/L; 059-0.71 g/L). On the contrary, α1-, α2 and γ-globulins did not show significant differences between sick and healthy dogs.

Among seronegative and seropositive groups, significant differences in total protein, albumin and α1-globulins concentration were not detected. Sick dogs showed higher α2-globulins compared with healthy dogs, but significant differences were detected only between seropositive sick and seropositive healthy dogs (P=0.016). Both β1- and β2-globulins were significantly higher in seropositive sick dogs compared with seropositive (P=0.001; P<0.001) and seronegative (P=0.003; P<0.001) healthy dogs, and also in seropositive sick compared with seronegative sick dogs (P=0.003; P=0.002). On the contrary, γ-globulins were significantly higher in seropositive sick compared with seronegative healthy dogs (P=0.021). Albumin/globulin ratio was significantly lower in seropositive sick compared with seropositive dogs (P=0.022) and seronegative healthy dogs (P=0.006).

Discussion

Overall and independent of seropositivity, sick dogs had significantly higher CRP levels and significantly lower albumin concentration, albumin/globulin ratio and PON-1 activity compared with healthy dogs. These results support the presence of inflammatory and/or oxidative processes in dogs that were classified as sick on a clinical or clinico-pathological basis. However, PON-1 activity, CRP values and globulin fractions recorded in sick and healthy dogs showed partial overlapping, likely due to the heterogeneity of clinical conditions affecting sick dogs. However, it is not possible to definitely exclude that clinically healthy dogs were affected by ongoing subclinical disease. Similarly, as expected, among seronegative dogs, sick subjects had significantly higher CRP levels and significantly lower PON-1 activity than seronegative healthy dogs. On the contrary, electrophoresis didn't show significant differences between seronegative healthy and sick dogs for any of the protein fraction or albumin/globulin ratio.

Among seropositive dogs, more than half (65%, 11/17) did not show clinical signs and were sampled as part of health checks and/or vaccinations. Symptoms of Lime disease are reportedly present only in 5-10% of infected dogs and are mainly represented by lethargy, anorexia, fever, depression, lymphadenopathy, lameness and joint swelling (Parry, 2016). However, seropositive clinically sick dogs included in our study did not show signs of arthritis and were affected by clinical conditions apparently not linked to B. burgdorferi infection, such as dermatitis (n=4), keratoconjunctivitis (n=1), keratoconjunctivitis and mitral valve disease (n=1).

Unexpectedly, significant differences in CRP concentration were not observed between seropositive sick and healthy dogs, even if values tended to be higher in seropositive sick dogs. This overlap is likely due to low CRP values detected in sick dogs (rather than to the detection of high values in healthy dogs), which were affected by low grade inflammatory localized diseases reported above. The concentrations of α2-, β1- and β2-globulins were significantly higher in seropositive sick compared with healthy dogs. This finding was expected, because acute phase protein, complement and immunoglobulins migrate in these fractions (Tappin et al., 2011). Furthermore, β1- and β2-globulins were significantly higher in seropositive sick than seronegative sick dogs, as to suggest that inflammatory phenomena in seropositive dogs could be more prominent than in seronegative dogs. Albumin/globulin ratio was predictably lower in seropositive sick compared with both seropositive and seronegative healthy. This finding is more likely due to an increase in globulin fractions rather than to a decrease in albumin in sick dogs, given that significant differences were detected for globulin fractions and not for albumin.

Among healthy dogs, mean and median values of CRP were higher in seropositive compared with seronegative dogs but differences were not significant. Different from CRP, PON-1 activity was significantly lower in seropositive than in seronegative healthy dogs. Even if the low numerosity of seropositive healthy dogs has to be considered, the graphical distribution of samples is noticeably tight (Figure 10). This is an interesting finding, which leads to the hypothesis that clinically occult inflammatory and/or oxidative phenomena, possibly due to *B. burgdorferi* infection, may occur in clinically healthy seropositive subjects. Obviously, it must be considered that indirect tests, such as serology, demonstrate a previous contact with the pathogen but does not necessarily imply the presence of active infection (Little et la., 2010). Similarly, it is not possible to exclude that healthy dogs were affected by clinically latent inflammatory processes due to other unknown causes. Direct tests, such as bacterial culture, cytology and PCR, were not performed in our study due to difficulties in collecting bioptic samples and limited diagnostic performance of direct tests themselves. In persistently infected animals, spirochetes are predominantly present in tissues (Krupka and Straubinger, 2010). Culture of tissue samples such as skin or synovial fluids, requires long time for bacterial growth and most importantly can have low sensitivity because of low bacterial load in tissues, as well as polymerase chain reaction (PCR) and cytology (Littman, 2020). Performing skin biopsy increases chances of spirochetes detection if a punch sample is collected near the site of tick bite (Krupka and Straubinger, 2010). This was not feasible in dogs included, and PCR was not performed on blood samples because Borrelia is very rarely found in blood (Chang et al, 1996). Detection by direct methods is reportedly difficult and of little practical relevance (Pantchev et al., 2015) and serology is the only recommended modality to evaluate exposure to B. burgdorferi (Littman et al., 2018). However, to better interpret these results, it would have been advisable to confirm the presence of *B. burgdorferi* infection and exclude other possible causes of oxidation in seropositive dogs, especially coinfections such as *Anaplasma spp*. (Greig and Armstrong, 2006), *Ehrlichia spp*. (Little et al., 2010), *Babesia spp*., and possibly *Bartonella spp*., *Hepatozoon canis*, *Dirofilaria spp*. and *L. infantum* (Otranto et al., 2009).

In our study, the simultaneous decrease of PON-1 activity and lack of increase in CRP values could be explained by the presence of oxidative responses developed by the host towards Borrelia (Showman et al., 2016), which contribute to keep infection latent, as demonstrated for other pathogens causing persistent infection, like Leishmania spp. (Almeida et al., 2013).

It has to be noted however, that despite significantly lower compared with other groups, PON-1 values were within the reference interval established by Rossi et al. (2013).

The numerosity of seropositive samples represents a limitation of this study. However, considering the prevalence of Lyme borreliosis in our country and the number of dogs included in the study, the number of dogs that tested seropositive could not have been much higher. Further studies including a wider caseload of dogs might increase the number of seropositive subjects and possibly confirm our preliminary findings.

V. Measurement of Paraoxonase-1 activity in hospitalized dogs

Study design

The study was performed on 554 serum samples sent to the Laboratory of Clinical Pathology of the University Veterinary Teaching Hospital throughout the period from April to September 2021. Samples were collected as part of routine diagnostic procedures from 401 privately owned dogs admitted to the different units of the hospital: Cardiology (n=50), Hospitalization (n=83), Internal Medicine (n=75), Oncology (n=71), Neurology (n=17), Radiology (n=19), Reproduction (n=25), Surgery (n=42), Ultrasonography (n=19). Overall, median age of dogs included in the study was 100 months (age range: 1 month-17 years and 10 months), with no meaningful differences of distribution among different hospital units. For 7 dogs, sex was not reported, among the others 160 were males, 42 castrated males, 102 females and 90 neutered females. For 10 dogs breed was unknown, 109 dogs were mongrels, whereas the other dogs were: Labrador Retriever (n=20), French Bulldog (n=18), Poodle (n=16), Golden Retriever (n=15), Jack Russell Terrier, German Shepherd (n=13 for each breed), Bernese Mountain Dog (n=11), Boxer (n=10), Dachshund, Cocker Spaniel (n=9 for each breed), Rottweiler (n=8), Dobermann, Maltese, Weimaraner (n=7 for each breed), Cavalier King Charles Spaniel, Chihuahua (n=6 for each breed), American Staffordshire Terrier, (n=5), Border collie, German Pointer, Dogo Argentino, Fox Terrier, Pitbull, Shih Tzu, Brittany, West Highland White Terrier (n=4 for each breed), Akita Inu, Beagle, Bracco Italiano, English Setter, Yorkshire Terrier, Maremmano-Abruzzese Sheepdog, Pug, Siberian Husky (n=3 for each breed), Miniature Poodle, Bolognese, Boston Terrier, Bull Terrier, English Bulldog, Dalmatian, Greyhound, Australian Shepherd, Belgian Shepherd, Rhodesian Ridgeback, Whippet (n=2 for each breed), American Bulldog, Bobtail, Entlebucher Mountain Dog, Cane Corso, Drahtaar, Dogue de Bordeaux, Flat coated retriever, Foxhound, Leonberger, Saluki, Czechoslovakian Wolfdog, Caucasian Shepherd Dog, Italian Greyhound, Doberman Pinscher, Pointer, Pomeranian, Samoiedo, Schnauzer, Segugio Italiano, Shar Pei, Shetland Sheepdog, Springer Spaniel, Newfoundland (n=1 for each breed).

Dogs underwent different diagnostic procedures and pharmacological/surgical treatment including hospitalization, based on clinical status and diagnosed disease. When it was necessary or required by the veterinary, sampling was repeated after admission during the follow up, to monitor clinical condition until discharge or death/euthanasia. In the latter case, when allowed by the owner, necropsy was performed to improve diagnosis. An informed consent about the

use of residual amounts of samples for research purposes was signed by the owners. Therefore, according to the regulations of our Institution, it was not necessary to require a formal authorization to the Institutional animal care committee (Decision of the institutional committee no. 2/16 dated 15/02/16). Fasting blood samples were collected from the jugular, cephalic or saphenous vein in potassium ethylene diamine tetra-acetic (K2EDTA) tubes for complete blood count using an automated analyzer (Sysmex XN-V, Sysmex Corporation, Kobe, Japan), and in plain tubes for biochemical analyses. Serum was separated within 15 minutes from collection and the parameters requested by referral veterinarians were measured within the day using an automated chemistry analyzer (BT 3500, Biotecnica Instruments SPA, Rome, Italy) and reagents provided by the manufacturer. Serum PON-1 activity was measured on residual volume of samples using the same instrument and the adapted method described above. For each patient, data regarding signalment, history, clinical examination and referral hospital unit were recorded. Further samples were collected from the same dogs when required by different clinical conditions for diagnostic purposes, to perform further laboratory analyses such as coagulation tests, parasitology tests, urinalysis, body fluid analysis, molecular tests, and cytological or histological examination of any lesion. The frequency of PON-1 values lower than reference interval in samples coming from different hospital units was firstly evaluated regardless of diagnosis/availability of clinical information and hospitalization of patients. Second, PON-1 values at admission were evaluated based on symptoms severity (acute or severe vs. chronic or mild), need for hospitalization (hospitalization or discharge) and clinical outcome (discharge with good prognosis, death, or euthanasia). Finally, PON-1 values recorded at first sampling and subsequently during follow up were evaluated among hospitalized dogs.

Statistical analysis was performed in an Excel spreadsheet using a specific software (Analyse-it v. 5.66, Analyse-it Software Ltd., Leeds, UK). Statistical differences were set for $P<0.05$.

The Mann-Whitney U test was used to compare the results obtained at admission from dogs that died with dogs that survived. Comparison between more than two groups (e.g. comparison between hospital units) was performed using the Kruskal-Wallis test, followed by the Wilcoxon signed rank test to compare paired groups. A Pearson chi-square test was used to compare the frequency rates of PON-1 values within or lower than reference interval recorded in two or more groups. To assess the discriminating power of PON-1 in detecting dogs requiring hospitalization, a receiver operating characteristic curve (ROC) was designed, classifying for each numerical value recorded in the study (operating point), as true or false positive dogs requiring or not

hospitalization respectively, that had PON-1 values lower than reference interval, and as true or false negative the dogs requiring or not hospitalization that had normal PON-1 values. Sensitivity, specificity and positive likelihood ratio were calculated for each operating point, using standard formulae (Gardner and Greiner, 2006; Christenson, 2007), and receiver operating characteristic (ROC) curves were designed by plotting sensitivity versus (1–specificity) (Gardner and Greiner, 2006). The Youden index (i.e., the operating point that maximizes the difference between true positives and false positives) and the operating points characterized by the highest LR+ and by absolute specificity were then calculated (Ruopp et al., 2008).

Results

Results of PON-1 activity in samples sent from the different hospital units are reported in Table 10 and Figure 11. The Hospitalization unit showed significantly lower PON-1 values compared with all other units, except for Ultrasonography, and, overall, the highest number and frequency of values lower than reference interval (27/52, 51.9% of total low PON-1 values) (27/133, 20.3% of samples from Hospitalization unit). The same statistical analysis was repeated excluding follow up samples collected from the same dogs and considering for each dog only results of the first sample submitted at the laboratory (Table 11 and Figure 12).

Table 10. Mean ± SD, median (between parenthesis), I-III interquartile range and min-max range (between parenthesis) of serum PON-1 activity in samples sent from the different hospital units.

	PON-1 (U/mL)		**PON-1** (U/mL)
Cardiology	195.91 ± 57.47 (192.50) ** 159.33-238.08 (63.34-330.00); n=66	Neurology	210.20 ± 52.41 (194.50) * 171.00-246.25 (134.40-351.00); n=18
Surgery	205.32 ± 55.24 (205.00) ** 173.00-240.00 (80.73-346.00); n=46	Oncology	184.91 ± 45.24 (184.00) * *** 155.37-216.25 (61.70-314.00); n=126
Hospitalization	162.86 ± 57.95 (163.00) 122.67-204.33 (18.50-309.00); n=133	Radiology	196.01 ± 42.39 (191.00) * 162.80-228.20 (130.40-292.00); n=21
Ultrasonography	185.34 ± 43.09 (180.90) 158.93-223.47 (58.30-259.00); n=21	Reproduction	202.34 ± 37.84 (198.20) ** 179.30-225.67 (127.80-281.00); n=25
Internal medicine	185.08 ± 56.32 (188.00) * 152.45-223.33 (23.80-320.00); n=98		

* P<0.01 vs Hospitalization; ** P<0.001 vs Hospitalization; *** P<0.05 vs Surgery

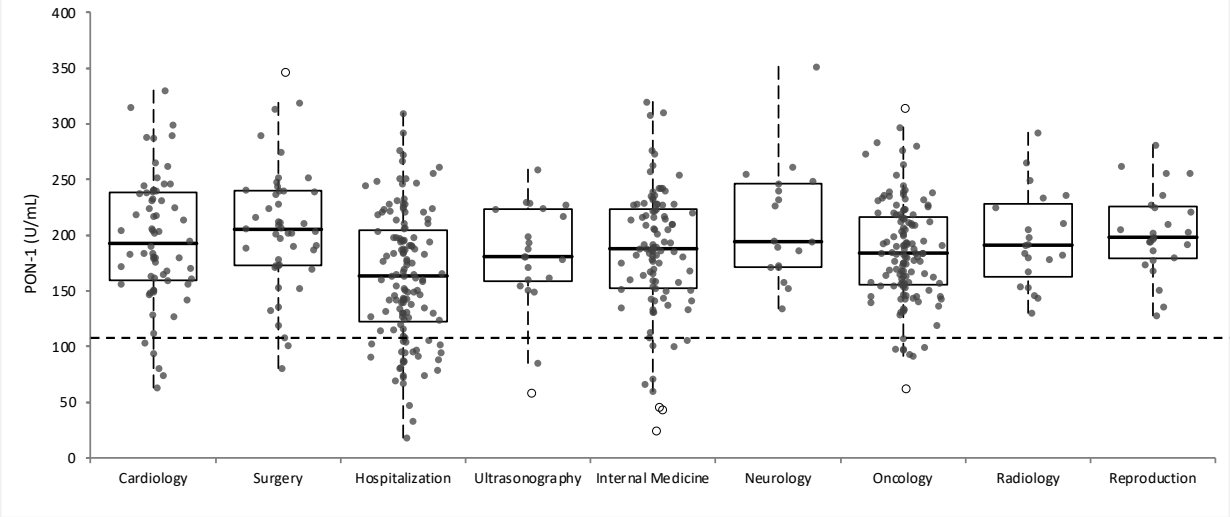


Figure 11. Serum PON-1 activity in samples sent from the different hospital units. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR. Black dots indicate the results that are not classified as outliers. White dots indicate near outliers (values exceeding the third quartile ± [1.5 x IQR]). The dotted line indicates the lower limit of the RI reported in healthy dogs.

Table 11. Mean ± SD, median (between parenthesis), I-III interquartile range and min-max range (between parenthesis) of serum PON-1 activity in samples sent from the different hospital units excluding follow up samples.

	PON-1 (U/mL)		**PON-1** (U/mL)
Cardiology	200.62 ± 58.35 (204.00) * 165.55-238.78 (63.34-330.00); n=55	Neurology	213.62 ± 51.90 (195.00) * 172.33-247.00 (134.40-351.00); n=17
Surgery	205.45 ± 53.28 (206.50) * 173.00-240.00 (80.73-346.00); n=42	Oncology	190.82 ± 47.21 (189.60) * 157.36-224.75 (91.20-314.00); n=84
Hospitalization	167.88 ± 62.20 (165.00) 130.00-221.00 (18.50-309.00); n=93	Radiology	196.71 ± 43.36 (191.20) ** 159.50-230.38 (130.40-292.00); n=20
Ultrasonography	185.14 ± 49.96 (188.00) 160.68-224.17 (58.30-259.00); n=19	Reproduction	202.34 ± 37.84 (198.20) * 179.30-225.67 (127.80-281.00); n=25
Internal medicine	184.14 ± 56.08 (184.90) *** 151.03-222.58 (42.80-320.00); n=80		

* P<0.01 vs Hospitalization; ** P<0.05 vs Hospitalization; *** P<0.05 vs Surgery

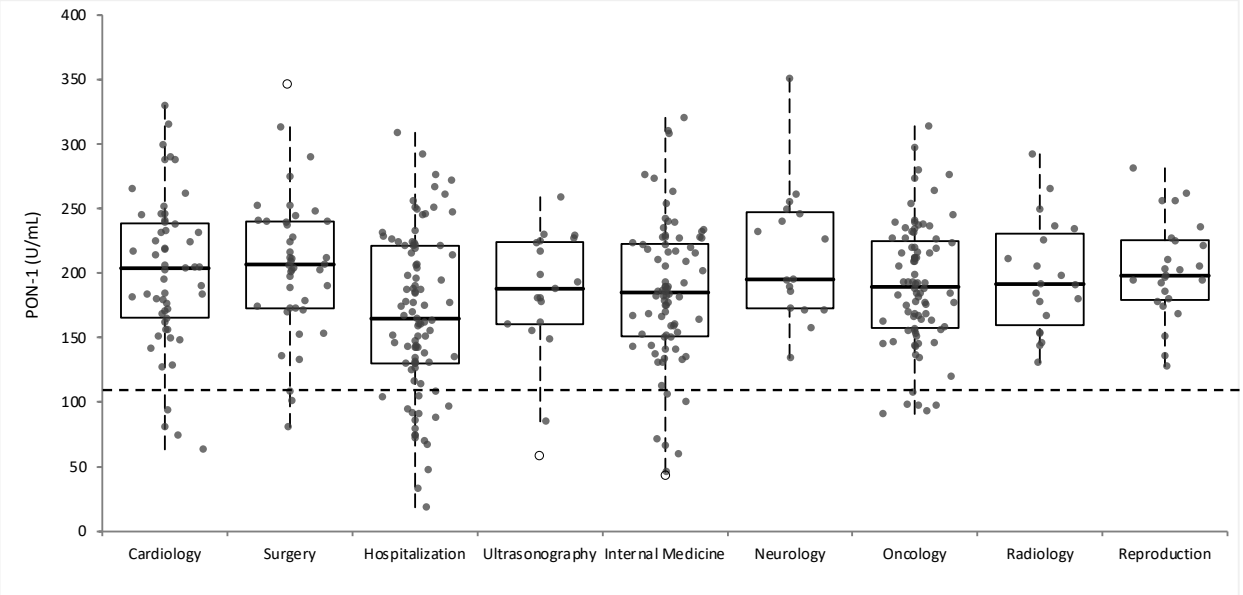


Figure 12. Serum PON-1 activity in samples sent from the different hospital units excluding follow up samples.

The Hospitalization unit still showed significantly lower PON-1 values compared with all other units, except for Ultrasonography and Internal Medicine, and overall still had the highest number and frequency of values lower than reference interval (17/37, 45.9% of total low PON-1 values) (17/93, 18.3% of samples from Hospitalization unit).

For 409/435 samples collected at admission, it was possible to distinguish, based on symptoms severity, two groups of dogs: dogs affected by acute or severe disease (e.g. life-threatening or septic conditions, acute infectious diseases, pulmonary edema, neoplastic disease with systemic involvement or at end stage) and dogs affected by chronic or mild disease (e.g. health checks, non-traumatic orthopedic disease, neoplastic disease without complications or systemic involvement). PON-1 activity was significantly lower in dogs with acute or severe disease (mean ± SD, 173.86 ± 59.57 U/mL; median, 177.00 U/mL; I-III interquartile range, 136.67-221.33 U/mL; n=113) compared with dogs with chronic or mild disease (193.87 ± 53.06 U/mL; 192.95 U/mL; 162.59-228.00 U/mL; n=296) ($P<0.05$) (Figure 13).

In 378 cases information about hospitalization or discharge of dogs was available. PON-1 activity was significantly lower in hospitalized dogs (152.26 ± 64.13 U/mL; 150.00 U/mL; 97.83-192.63 U/mL; n=65) compared with non-hospitalized dogs (195.52 ± 51.40 U/mL; 193.00 U/mL; 163.67-228.33 U/mL; n=313) ($P<0.001$) (Figure 13).

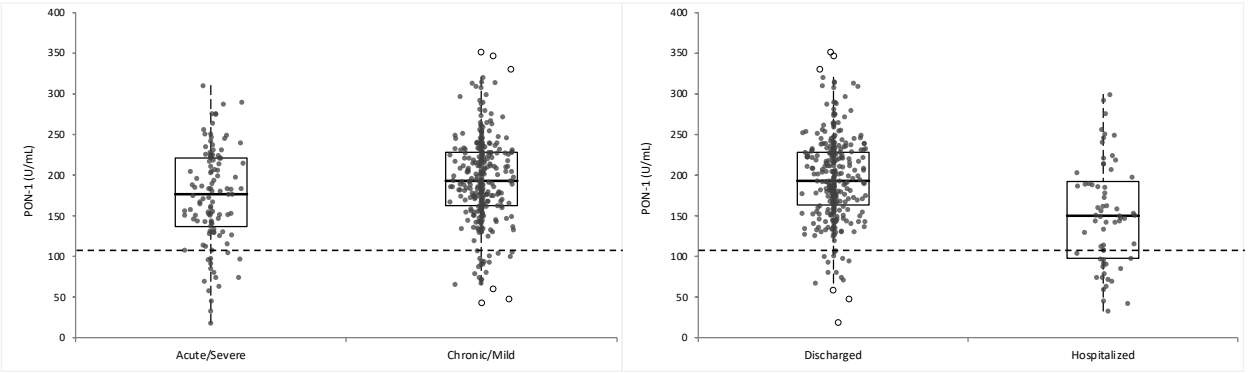


Figure 13. Serum PON-1 activity in dogs with acute/severe and chronic/mild clinical conditions (left) and in hospitalized and non-hospitalized dogs (right).

The ROC curve analysis designed to assess the discriminating power of PON-1 in detecting dogs requiring hospitalization, showed an AUC (0.72; 95%CI=0.62-0.78) significantly different from the line of no discrimination ($P<0.001$) (Figure 14). The optimal cut-off for clinical decision was 119.50 U/mL, with 95.2% specificity, 35.4% and 7.38 LR+. The highest LR+ (14.4) and absolute specificity were reached at cut-offs of 45.7 U/mL and 32.8 U/mL respectively.

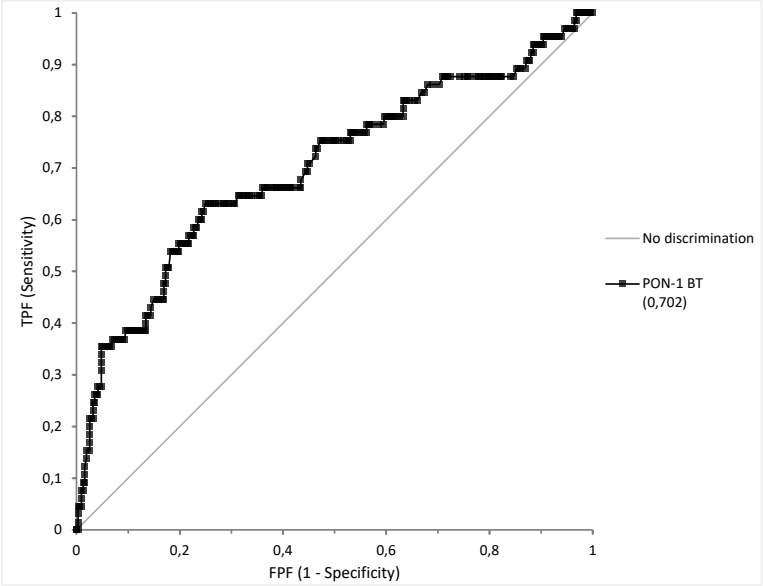


Figure 14. ROC curve designed to assess the discriminating power of PON-1 in detecting dogs requiring hospitalization. The grey line indicates the line of no discrimination. TPF, true positive fraction; FPF, false positive fraction.

For 103 cases information regarding outcome was available and PON-1 activity was compared between dogs with a positive (improvement of clinical condition and or discharge) or a negative (death or euthanasia) outcome, regardless of hospitalization. No significant differences were observed between survivors (162.97 ± 56.78 U/mL; 162.00 U/mL; 130.00-206.42 U/mL; n=55) and non-survivors (163.23 ± 66.84 U/mL; 161.35 U/mL; 109.58-206.47 U/mL; n=48), even considering as separated groups dogs that spontaneously died (158.38 ± 84.59 U/mL; 156.00 U/mL; 86.65 ± 213.63 U/mL; n=19) and dogs subjected to euthanasia (166.40 ± 53.59 U/mL; 167.20 U/mL; 136.83-196.33 U/mL; n=29) (Figure 15).

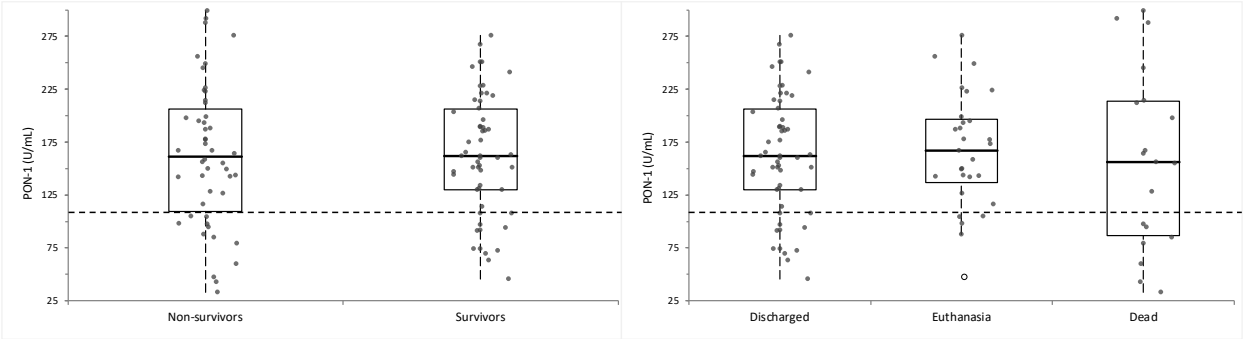


Figure 15. Serum PON-1 activity in dogs with positive (improvement of clinical condition and or discharge) or negative (death or euthanasia) outcome (left), considering as separated groups dogs that died and dogs subjected to euthanasia (right).

The same trend was observed restricting the analysis to 47 samples collected at admission from 40 hospitalized dogs (Table 12, 13 and 14). Dogs that were hospitalized more than once, for a different clinical condition or after a significant period of time were considered as distinct events.

Table 12. Signalment, clinical data and PON-1 activity of dogs that were discharged following hospitalization.

N	**Breed**	**Sex**	**Age** (months)	**Diagnosis**	**PON-1** (U/mL)
1	Siberian Husky	M	67	Intervertebral disc disease	210
2	WHWT	FN	168	Diabetes	311
3	Mongrel	FN	141	Chronic kidney disease	167
4	Dobermann	M	22	Patent ductus arteriosus	323
5	English setter	MC	126	Tracheal collapse and laringeal paralysis	273
5*	English setter	MC	129	Lung carcinoma	293
6	Mongrel	F	138	Otitis	209
7	Labrador retriever	FN	-	Phlegmon	117
8	Shih tzu	M	135	Politrauma	187
8*	Shih tzu	M	137	Exudative dermatitis	125
9	Mongrel	F	78	Polyneuropathy	269
10	Labrador retriever	M	100	Politrauma	108
11	Drahtaar	M	95	Urolithiasis	160
12	Mongrel	MC	-	Politrauma	134
13	Fox terrier	FN	132	Lung carcinoma	292
14	Mongrel	FN	24	Hypoadrenocorticism	45.7
15	Golden retriever	F	2	Bilateral ectopic ureter	96.7
16	French bulldog	MC	127	Intracranic neoplasia	224
17	Mongrel	M	125	Multiple mast cell tumor	153
18	Maltese	M	121	IMHA	95.7
18*	Maltese	M	122	IMHA	177
19	WHWT	M	8	Portosystemic shunt	67.27
20	Mongrel	M	119	Rodenticide poisoning	115.9
21	Labrador retriever	F	134	Heart failure	93.8
22	Labrador retriever	FN	150	Linfoma	213
23	Mongrel	M	3	Portosystemic shunt	173
24	Setter irlandese	M	95	Politrauma	181
25	Mongrel	-	132	Splenic angiosarcoma, heart failure	260
26	Mongrel	FN	192	Hypothyroidism and heart failure	246
27	Amstaff	F	9	Pyometra	139

F, female; FN, neutered female; M, male; MC, castrated male; IMHA, immune-mediated hemolytic anemia; WHWT, West Highland White Terrier; '*' indicates second hospitalization of the same dog

Table 13. Signalment, clinical data and PON-1 activity of dogs that were subjected to euthanasia following hospitalization.

N	**Breed**	**Sex**	**Age** (months)	**Diagnosis**	**PON-1** (U/mL)
28	German Shepherd	M	117	Cauda equina syndrome	173
29	Pinscher	M	147	Squamous cell carcinoma	138
30	Leonberger	M	66	Histiocytic neoplasia	98.1
31	Fox terrier	MC	180	Polyostotic lesions	142.5
32	Poodle	MC	108	Transitional cell carcinoma	218
33	Fox terrier	FN	132	Lung carcinoma	283
2*	WHWT	FN	168	Diabetes	219
4*	Dobermann	M	26	Patent ductus arteriosus	249
17*	Mongrel	M	128	Multiple mast cell tumor	116
25*	Mongrel	-	132	Splenic angiosarcoma, heart failure	209

FN, neutered female; M, male; MC, castrated male; WHWT, West Highland White Terrier; '*' indicates second hospitalization of the same dog

Table 14. Signalment, clinical data and PON-1 activity of dogs that spontaneously died following hospitalization.

N	**Breed**	**Sex**	**Age** (months)	**Diagnosis**	**PON-1** (U/mL)
34	Cavalier King	FN	129	Chronic kidney disease, pancreatitis	258
35	Mongrel	M	142	Splenic angiosarcoma, heart failure	184
36	Epagneul breton	M	85	Acute renal failure	155
37	Cocker spaniel	M	157	Enterectomy for jejunal neoplasia	85.1
38	Jack Russel	M	57	Mitral and tricuspid dysplasia	156.4
39	Jack Russel	M	147	Pulmonary edema	156
40	-	M	72	Gastric dilatation and volvulus	193.6

FN, neutered female; M, male

Significant differences were not observed between survivors (178.45 ± 56.01 U/mL; 173.00 U/mL; 141.00-218.33 U/mL; n=30) and non-survivors (181.97 ± 76.25 U/mL; 175.00 U/mL; 116.91-247.17 U/mL; n=17), even considering as separated groups dogs that died (169.73 ± 52.14 U/mL; 156.40 U/mL; 155.17-192.00 U/mL; n=7) and dogs subjected to euthanasia (184.56 ± 60.54 U/mL; 191.00 U/mL; 136.17-221.50 U/mL; n=10) (Figure 16).

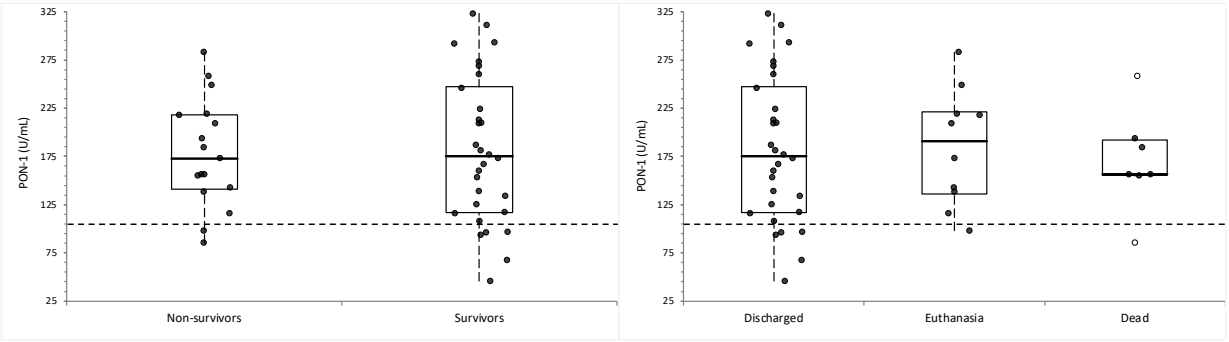


Figure 16. Serum PON-1 activity in hospitalized dogs with positive or negative outcome (left), considering as separated groups dogs that spontaneously died and dogs subjected to euthanasia (right).

In 21 cases, PON-1 measurement was repeated at different time points after admission and during hospitalization follow up until discharge or death/euthanasia (Table 15, Figure 17).

Table 15. PON-1 activity measured at multiple time points during hospitalization.

N	Outcome	T0	T1	T2	T3	T4	T5	T6	T7	T8	T9
2	Discharged	311	219								
4	Discharged	323	249								
5	Discharged	273	293								
7	Discharged	117	124	180	199	186					
8	Discharged	187	125								
10	Discharged	108	184	120	198						
12	Discharged	134	95.3	81.6	88.6	103	101	166	124	197	196
12*	Discharged	91.8	94.9	106	110	183	228	211			
14	Discharged	45.7	23.8								
15	Discharged	96.7	87.2	80.7							
17	Discharged	153	116								
18*	Discharged	95.7	177								
20	Discharged	115.9	163.1	162							
21	Discharged	93.76	103.5								
25	Discharged	260	209								
28	Euthanasia	173	119	149	127						
29	Euthanasia	138	118.1								
33	Euthanasia	283	198	220							
34	Dead	258	181	225	237	149.2					
35	Dead	184	107								
36	Dead	155	142								

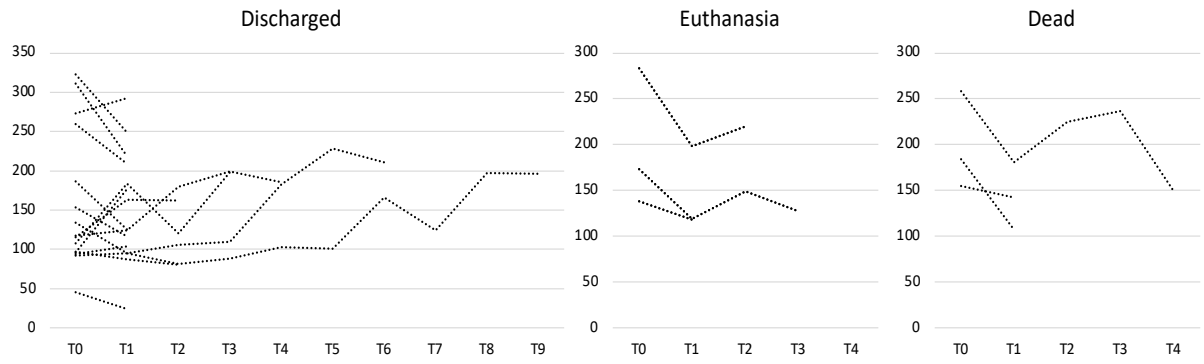


Figure 17. Graphical representation of PON-1 activity measured at multiple time points during hospitalization in dogs with a positive or a negative outcome.

A univocal trend was not observed among dogs with different outcome, and given the impossibility to analyze paired data for all cases at each time point, results of PON-1 activity at admission were compared with results at last sampling in dogs with a positive or a negative outcome (Table 16, Figure 18).

Table 16. PON-1 activity at admission and at last sampling in dogs with a positive (improvement of clinical condition and or discharge) or a negative (death or euthanasia) outcome.

	Survivors (n=15)		**Non-survivors** (n=6)	
	T0	**T-last**	**T0**	**T-last**
mean ± SD	160.37 ± 88.77	169.93 ± 69.44	198.50 ± 58.47	140.22 ± 32.22
median	117.00	186.00	178.50	134.50
I-III IQR	95.87-247.83	117.50-210.67	153.58-260.08	117.18-153.27
min-max	45.70-323.00	23.80-293.00	138.00-283.00	107.00-198.00

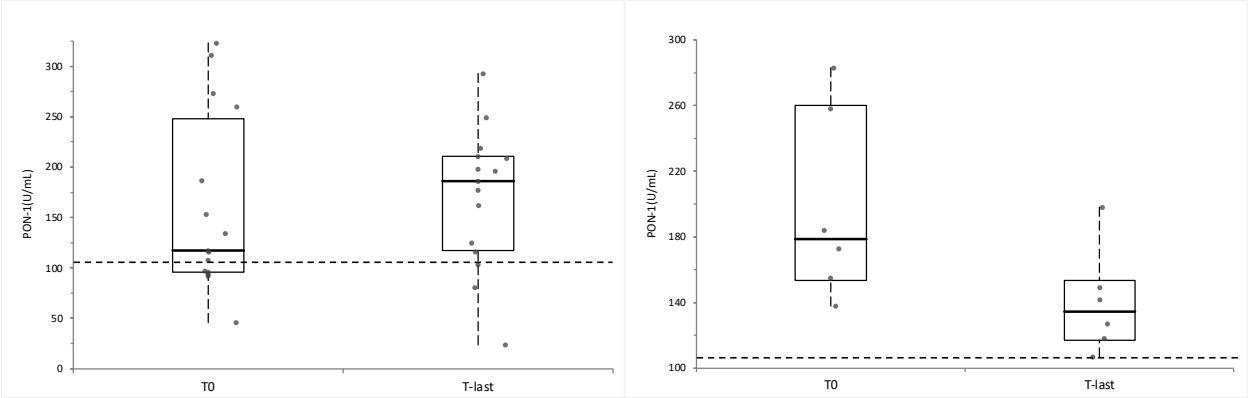


Figure 18. PON-1 activity at admission and at last sampling in dogs with a positive (improvement of clinical condition and or discharge) (left) or a negative (death or euthanasia) (right) outcome.

Significant differences between admission and last sampling were not observed among survivors, whereas in dogs with a negative outcome PON-1 values measured at last sampling were significantly lower than at admission (P=0.031).

The same trend was observed comparing values recorded at admission and in second samplings performed within 15 days (mean 3.8 ± 4.1 days, median 2 days) (Table 17, Figure 19). PON-1 values measured in second samplings was significantly lower than at admission (P=0.031).

Table 17. PON-1 activity at admission and at second samplings performed within 15 days in dogs with a positive (improvement of clinical condition and or discharge) or a negative (death or euthanasia) outcome.

	Survivors (n=10)		**Non-survivors** (n=6)	
	T0	**T-1**	**T0**	**T-1**
mean ± SD	120.96 ± 70.69	127.20 ± 58.07	198.50 ± 58.47	144.20 ± 37.30
median	102.35	113.75	178.50	130.50
I-III IQR	93.60-118.42	94.26-177.58	153.58-260.08	117.18-182.42
min-max	45.70-311.00	23.80-219.00	138.00-283.00	107.00-198.00

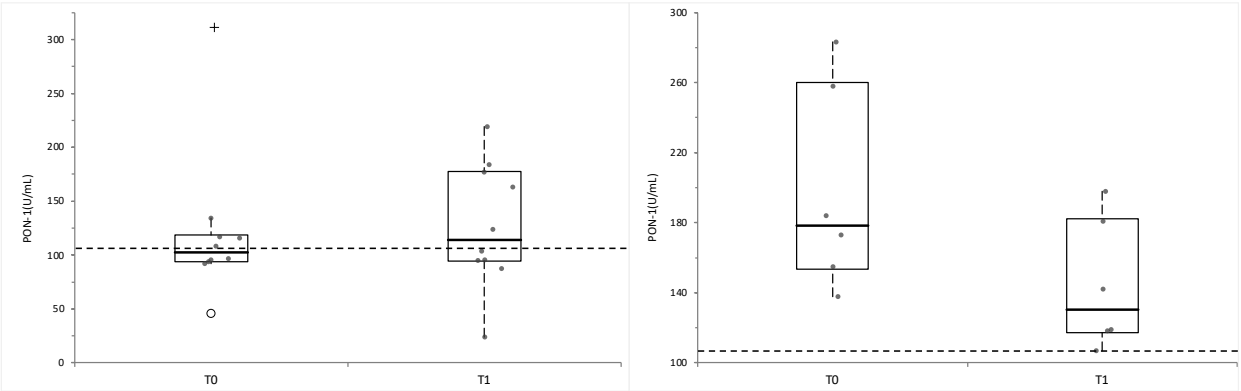


Figure 19. PON-1 activity at admission and at second samplings performed within 15 days in dogs with a positive (improvement of clinical condition and or discharge) (left) or a negative (death or euthanasia) (right) outcome.

Discussion

Among hospital units, the Hospitalization unit showed significantly lower PON-1 values. This finding is not surprising, since this unit usually manages dogs in emergency first visit or dogs requiring hospitalization and monitoring referred by other units. Clinical conditions leading to hospitalization are usually severe and often characterized by oxidative and/or inflammatory phenomena, which are expected to cause a decrease in PON-1 activity (Torrente et al., 2018; Ruggerone et al., 2021; Ruggerone et al., 2020). Likewise, this group showed the highest frequency of values lower than reference interval. It must be noted that this unit included the highest number of samples and, most importantly, samples subsequently collected from the same dogs during follow up. Hospitalized dogs are usually subjected to repeated sampling over monitoring period. As a result, it is more likely that dogs with low PON-1 activity at admission, had low values at subsequent samplings until clinical normalization and discharge, or death. On the contrary, other units usually manage scheduled visits and/or second opinion visits, and do not usually manage severe or life-threatening conditions and do not require strict monitoring, at least in the near future. An exception to this assumption is represented by chronic conditions, such as metabolic or oncologic diseases, which require periodic clinical checks. However, to overcome these possible sources of bias, statistical analysis was repeated excluding follow up samples and considering for each dog only samples collected at admission and results were very similar. Overall, our results confirmed that the evaluation of PON-1 activity is primarily useful for clinical conditions related to emergency and/or hospitalization.

Therefore, to investigate the usefulness of PON-1 as marker of disease severity and progression, values measured at admission were evaluated based on clinical severity of disease, hospitalization and outcome. PON-1 activity was significantly lower in dogs with acute or severe disease (classified based either on the type of disease – ie. diseases that are known to induce more severe clinical conditions – or based on the clinical records of each single dog that allowed to detect severe clinical presentations also in some case of diseases usually associated with chronic course) compared with dogs with chronic or mild disease, and in hospitalized compared with non-hospitalized dogs, regardless of hospital units. These results further confirm that PON-1 could be a useful marker of disease severity and that hospitalization might be required when admission values are particularly low. In particular, the test had the highest LR+ and absolute specificity at cut-offs of 45.7 U/mL and 32.8 U/mL respectively.

Contrarily to what reported in other studies and other species (Bojic et al., 2014; Ruggerone et al., 2021; Ruggerone et al., 2020; Li et al., 2013; Draganov et al., 2010), no significant differences of PON-1 activity at admission were observed between dogs with a positive or a negative outcome, neither considering dogs that died and dogs subjected to euthanasia as one or different groups. The lack of differences is likely due to the dispersion among groups of PON-1 values, which mostly fell within reference interval regardless of the outcome. Accordingly, severe clinical conditions characterized by low values can improve if adequately treated, as well as more chronic conditions characterized by poor outcome can show normal values.

Among hospitalized dogs with positive outcome, five dogs showed low PON-1 activity at admission. One dog (n°19) was brought to visit because of neurologic signs and was diagnosed with portosystemic shunt. In this case, low PON-1 values could be due to decreased PON-1 synthesis related to insufficient hepatic mass. In human patients with chronic liver disease, PON-1 activity is correlated with the degree of liver damage and is lower in patients with cirrhosis than in those with hepatitis (Ferré et al., 2002a). This correlation is supposed to be a consequence of hepatic dysfunction or abnormal HDL synthesis and/or secretion (Marsillach et al., 2010; Ferré et al., 2002a). Dog n°15 was a two month old pup diagnosed with bilateral ectopic ureter. Even if a correlation with the clinical condition could not be excluded, low PON-1 activity could be imputable to the young age. Several studies in people showed low serum PON-1 activity and low liver genic expression at birth, which progressively increase reaching a plateau between 6 to 15 months of age (Ecobichon e Stephens, 1973; Mueller et al., 1983; Cole et al., 2003). In other species as well, PON-1 activity is reportedly lower in young individuals compared with adults (Giordano et al., 2013; Ruggerone et al., 2018). Dog n°21 was affected by severe right dilated cardiomyopathy with tricuspid insufficiency and was hospitalized because of ventricular tachycardia. Despite most studies focus on left-sided heart disease, a negative correlation between PON-1 activity and severity of heart failure is known to exist in dogs (Mahadesh et al., 2014; Kulka et al., 2017; Rubio et al., 2020). Following discharge, a third control sampling was performed after two months of treatment and PON-1 activity was within reference interval, according with clinical improvement. Dog n°18 was affected by primary immune-mediated hemolytic anemia, a hyperacute and severe clinical condition which was promptly treated with immunosuppressive therapy with rapid clinical improvement and normalization of PON-1 values two weeks after hospitalization. Finally, dog n°14 was hospitalized because of severe weakness associated to hypotension and bradycardia consequent to hypoadrenocorticism.

In this case, which showed the lowest values of our caseload, decrease of PON-1 activity could possibly be due to decreased hepatic synthesis secondary to hepatic hypoperfusion and hypoxia (Peterson et al., 1996; Van Lanen and Sande, 2014). Interestingly, significantly increased PON-1 activity was reported in dogs with hyperadrenocorticism compared with healthy controls (Tvarijonaviciute et al 2015b), possibly due to a direct influence of glucocorticoids on PON-1 gene expression (Bin Ali et al., 2003; Tvarijonaviciute et al 2015b). Unfortunately, except for a second sampling two days after hospitalization and one day after corticosteroid treatment, still showing low values, a long time follow up to verify the possible normalization of PON-1 activity was not available for this patient.

In 21 cases of hospitalization, PON-1 measurement was repeated at multiple time points after admission. A univocal visual trend in time was not observed among hospitalized dogs with different outcome, even if dogs that died or underwent euthanasia appeared to show a decrease of PON-1 activity in time (Figure 17). However, none of them reached values below reference interval, and decrease of PON-1 activity in time was observed also in dogs with a positive outcome, possibly representing physiological fluctuations of enzyme activity in time. Notably, an increase in PON-1 activity in time was observed only in dogs with a positive outcome and not in those with a negative outcome. To statistically verify the trend among repeated sampling, admission values were compared with those recorded at second and last sampling. Contrarily to hospitalized dogs with a positive outcome, in dogs with a negative outcome PON-1 values measured at second and last sampling were significantly lower than admission levels. These findings may be relevant for clinicians monitoring hospitalized patients, suggesting that increase of PON-1 activity may parallel or possibly predict the improvement of clinical conditions, as reported for dogs with leishmaniosis (Rossi et al., 2014a). However, effective evaluation of PON-1 activity trend in time is impaired by the lack of standardization in number and time of multiple sampling among patients. In many cases included in our study, repeated samplings were limited due to financial restraints of the owners, or not performed because not required for monitoring purpose, for example in case of clinical improvement and discharge. To evaluate PON-1 trend and correlation with clinical symptoms in hospitalized dog in a more accurate way, it would be advisable to perform repeated samplings at standardized multiple time points, possibly including also post-discharge follow up, in a wider caseload.

Conclusive remarks

Regardless of disease involved, PON-1 activity can decrease in particularly severe clinical conditions, and hospitalization may be recommended when low values are detected at admission. However, our results suggest that a single measurement of PON-1 activity is not predictive of the outcome, while monitoring of PON-1 activity following hospitalization could be useful in predicting outcome and clinical improvement. As regards sepsis in particular, our results confirm that this clinical condition is characterized by severe oxidation and support the role of CRP and PON-1 in confirming a clinical diagnosis of sepsis. Moreover, the measurement of PON-1 and CRP at admission, but not of PCO, may predict the possible outcome of dogs with sepsis. Future studies on a larger caseload or on serial measurements of these markers over time may provide additional information about their prognostic role in septic dogs.

Finally, the presence of oxidative phenomena potentially due to Borrelia infection seemed to emerge in seropositive healthy dogs. However, this finding must be carefully considered, given the low number of samples and considering the fact that seropositivity does not reflect an undergoing infection, but only an immune response to a previous contact with the pathogen. Therefore, studies including a wider caseload are warranted and the concurrent presence of Borrelia infection in seropositive dogs should be confirmed while other possible causes of oxidation should be excluded.

PARAOXONASE ACTIVITY IN CATS

Rationale and aims

Contrarily to dogs, PON-1 activity has been measured only in few studies related to feline health and disease. Studies based on the paraoxon-based method employed in this thesis are limited to the analytical validation of the method and on the possible role of PON-1 in supporting a clinical diagnosis of feline infectious peritonitis. Feline leishmaniosis is an emerging disease, increasingly diagnosed and reported in the last decades in endemic areas and sporadically also in non-endemic areas in rehomed cats (Pennisi et al., 2015). PON-1 activity has never been evaluated in cats with Leishmania infantum infection, whereas many studies in dogs have detected huge changes related to this disease, which is reportedly characterized by oxidative stress (Panaro et al., 1998; Sisto et al., 2001; Almeida et al., 2013). Therefore, we investigated PON-1 activity in cats screened for L. infantum positivity or seropositivity by the Department of Veterinary Medicine of the University of Bari (Study VII). As shown below, results of this study are still preliminary, and the caseload will be furtherly increased in order to prepare a scientific report to be submitted to peer-reviewed journals.

VI. Assessment of Paraoxonase-1 activity in cats infected with Leishmania spp.

Study design

The study was performed on 64 serum samples previously collected during another study performed at the University of Bari in the time frame from June 2017 to August 2018 (Iatta et al., 2019). Six veterinary laboratories distributed throughout Italy received blood and serum samples collected from clinical practitioners for health check analyses. Animal data (i.e. age, sex, breed, clinical signs) and results of laboratory analyses (i.e. hematology and biochemistry, including measurement of serum amyloid A, as a major feline acute phase reactant) were recorded. Samples were subsequently sent on dry ice to the Parasitology Unit of the Department of Veterinary Medicine, University of Bari (Italy) for serological and molecular testing. The protocol of this study was therefore approved by the ethical committee of the Department of Veterinary Medicine of the University of Bari, Italy (Prot. Uniba 7/17). A slightly modified IFAT protocol previously described by Otranto et al. (2009) was used to detect anti-Leishmania infantum antibodies in serum samples.

In particular, after the incubation of serum samples and fluorescein-labelled rabbit anti-cat immunoglobulin G (IgG) the slides were washed by immersion in phosphate-buffered saline three times for 10 min each by shaking. In addition, the conjugated anti-cat IgG was diluted 1:50 (Sigma-Aldrich, Germany). A serum sample from a cat positive for L. infantum by cytological and molecular analyses was used as positive control, and serum samples from 10 healthy cats living in a non-endemic area (Westbrook, Maine, USA), were used as negative controls. Samples were classified as positive when producing a clear cytoplasmic and membrane fluorescence of promastigotes from a cut-off dilution of 1:80, as recommended by LeishVet guidelines (Pennisi et al., 2015). Positive sera were titrated by serial dilutions until negative results were obtained. Genomic DNA was extracted from blood using the GenUP DNA Kit (Biotechrabbit, Germany), following manufacturer's recommendations. The detection of a fragment (120 bp) of L. infantum kDNA minicircle was achieved by qPCR, using primers, probes and protocol described in another study (Francino et al., 2006). For all qPCR tests, DNA extracted from blood samples of a cat positive to L. infantum by cytological examination was used as positive control and DNA extracted from blood samples of 10 healthy cats living in a non-endemic area (i.e., Westbrook, ME 04092 USA) was used as negative control. Samples were scored as positive when a threshold cycle less than 37 was recorded. FeLV and FIV proviral DNAs were tested using primers and protocol previously described (Endo et al., 1997; Stiles et al., 1999). Afterwards, aliquots of samples were transferred on dry ice to the University of Milan, where measurement of serum PON-1 activity was performed as described above. Based on results of serological and molecular testing, cats were classified into four groups:

- negative healthy: cats that tested negative to both IFAT and PCR and did not show clinical signs of disease or abnormalities at clinical examination,
- negative sick: cats that tested negative to both IFAT and PCR and showed history with symptoms of disease or abnormalities at clinical examination,
- positive healthy: cats that tested positive to IFAT and/or PCR and did not show clinical signs of disease or abnormalities at clinical examination,
- positive sick: cats that tested positive to IFAT and/or PCR and showed history with symptoms of disease or abnormalities at clinical examination.

In order to investigate if a correlation may exist between L. infantum positivity and PON-1 activity, results of PON-1 activity in positive and negative cats were compared using the Mann-Whitney U test. The same test was used to compare results from sick and healthy cats. Comparison of results between more than two groups was performed using the Kruskal-Wallis test followed by Bonferroni post-hoc test.

Results and discussion

Overall, among 64 cats, 12 (18.8%) tested positive to IFAT and two (3.1%) to PCR, while 50 cats tested negative to both methods. As regards the presence of clinical abnormalities, 10 cats were classified as healthy because of the absence of clinical signs (Table 18), while 53 showed symptoms (Tables 19 and 20). For one cat (cat n°64), which by the way tested positive at PCR, data regarding clinical status were not available. This cat was therefore excluded from the statistical analyses regarding groups based on clinical status.

Table 18. Signalment, clinical data and results of serological and molecular testing for Leishmania infantum, Feline Immunodeficiency Virus and Feline Leukemia Virus of clinically healthy cats.

N	**BREED**	**SEX**	**AGE** (mth)	**IFAT** (titer)	**PCR**	**FIV**	**FeLV**	**SYMPTOMS / DIAGNOSIS**
1	DSH	M	36	neg	neg	neg	neg	no symptoms
2	DSH	M	24	neg	neg	neg	**pos**	no symptoms
3	DSH	F	13	neg	neg	neg	neg	no symptoms
4	DSH	M	13	neg	neg	neg	neg	no symptoms
5	DSH	M	13	neg	neg	neg	neg	no symptoms
6	DSH	F	28	neg	neg	neg	neg	no symptoms
7	DSH	F	28	neg	neg	neg	neg	no symptoms
8	DSH	F	28	neg	neg	neg	neg	no symptoms
9	DSH	F	24	**160**	neg	neg	neg	no symptoms
10	DSH	F	24	**80**	neg	neg	neg	pregnant

DSH, domestic shorthair; mth, months; M, male, F, female; neg, negative; pos, positive

Table 19. Signalment, clinical data and results of serological and molecular testing for Leishmania infantum, Feline Immunodeficiency Virus and Feline Leukemia Virus of cats with localized pathologic conditions.

N	**BREED**	**SEX**	**AGE** (mth)	**IFAT** (titer)	**PCR**	**FIV**	**FeLV**	**SYMPTOMS / DIAGNOSIS**
11	DSH	M	168	neg	neg	neg	neg	chronic rhinitis
12	DSH	M	108	neg	neg	neg	neg	head and neck pruritus, iatrogenic diabetes
13	DSH	F	60	neg	neg	neg	neg	pruritus, parasitic infection
14	DSH	M	40	neg	neg	neg	neg	asthma
15	M Coon	F	8	neg	neg	neg	neg	nasal polyp, gingivostomatitis
16	M Coon	F	8	neg	neg	neg	neg	nasal polyp
17	M Coon	F	20	neg	neg	neg	neg	nasal polyp
18	DSH	F	12	neg	neg	neg	neg	keratitis, conjunctivitis
19	DSH	F	96	neg	neg	neg	neg	right eye conjunctivitis, right nasal discharge
20	Siberian	M	48	neg	neg	neg	neg	urinary tract infection
21	DSH	F	60	neg	neg	neg	neg	gingivostomatitis
22	DSH	M	36	neg	neg	neg	neg	bladder atony, urinary tract infection
23	British	M	84	neg	neg	neg	neg	cough, nasal discharge
24	DSH	F	132	neg	neg	neg	neg	lymphoplasmacytic enteritis
25	DSH	F	24	neg	neg	neg	neg	stomatitis
26	DSH	M	8	neg	neg	neg	neg	stomatitis
27	DSH	M	42	**160**	neg	neg	neg	gingivostomatitis

DSH, domestic shorthair; mth, months; M Coon, Maine Coon; M, male, F, female; neg, negative; pos, positive

Table 20. Signalment, clinical data, results of serology and molecular tests for L. infantum, FIV and FELV of cats with systemic diseases.

N	**BREED**	**SEX**	**AGE** (mth)	**IFAT** (titer)	**PCR**	**FIV**	**FeLV**	**SYMPTOMS / DIAGNOSIS**
28	DSH	F	159	neg	neg	neg	neg	anorexia, icterus, proteinuria
29	DSH	F	192	neg	neg	neg	neg	weight loss , alopecia, gingivostomatitis, diabetes
30	DSH	F	144	neg	neg	neg	neg	weight loss, anorexia
31	DSH	M	72	neg	neg	**pos**	**pos**	fever, icterus, dysorexia, gingivostomatitis
32	DSH	M	163	neg	neg	neg	neg	weight loss, dysorexia
33	DSH	F	162	neg	neg	neg	neg	gingivostomatitis, chronic vomiting, CKD
34	DSH	M	192	neg	neg	**pos**	neg	weight loss, dysorexia, CKD
35	Ragdoll	M	72	neg	neg	neg	neg	vomiting, abdominal lymphadenomegaly
36	Persian	F	76	neg	neg	neg	neg	weight loss, enteritis
37	DSH	F	132	neg	neg	neg	neg	fever, pneumonia
38	DSH	M	168	neg	neg	neg	neg	weight loss, alopecia, chronic vomiting, CKD
39	DSH	M	72	neg	neg	neg	neg	weight loss, weakness, anorexia, icterus
40	DSH	M	60	neg	neg	neg	neg	weakness, anorexia, vomiting
41	DSH	F	18	neg	neg	neg	neg	fever, stomatitis
42	DSH	M	204	neg	neg	neg	neg	gingivostomatitis, splenomegaly, hepatic fibrosis
43	DSH	M	168	neg	neg	neg	neg	weight loss, gingivostomatitis
44	DSH	M	36	neg	neg	neg	neg	weight loss, weakness, dysorexia, urinary infection
45	DSH	F	26	neg	neg	neg	neg	leukopenia
46	DSH	M	36	neg	neg	neg	neg	anemia
47	DSH	F	73	neg	neg	neg	**pos**	weight loss, weakness, anorexia, icterus
48	DSH	F	48	neg	neg	neg	neg	bronchopneumonia
49	DSH	F	28	neg	neg	neg	neg	vomiting, diarrhea, anorexia
50	DSH	F	48	neg	neg	neg	neg	weakness, fever, neurological signs
51	DSH	M	132	neg	neg	neg	neg	CKD
52	DSH	M	24	neg	neg	neg	neg	weakness, dysorexia
53	DSH	F	11	neg	neg	neg	neg	weakness, dysorexia
54	DSH	F	241	**80**	neg	neg	neg	chronic vomiting
55	Persian	F	108	neg	**pos**	neg	neg	gingivostomatitis, chronic vomiting, CKD
56	DSH	F	24	**80**	neg	neg	neg	anorexia, gingivostomatitis (ulcers and nodules)
57	DSH	F	9	**640**	neg	neg	neg	lymphadenomegaly, alopecia, oral ulcers
58	DSH	M	186	**80**	neg	neg	neg	weakness, weight loss, anorexia, uveitis
59	DSH	M	84	**320**	neg	neg	neg	weakness, uveitis
60	DSH	F	24	**80**	neg	neg	neg	weight loss, dysorexia, gingivostomatitis
61	DSH	M	74	**160**	neg	**pos**	neg	fever, lymphadenomegaly, weight loss, weakness, dysorexia, ulcerative skin lesions, gingivostomatitis
62	DSH	M	48	**80**	neg	neg	neg	subcutaneous abscess, neurological symptoms
63	DSH	F	11	**160**	neg	neg	neg	ocular discharge, keratitis, ulcerative skin lesions
64	DSH	F	180	neg	**pos**	neg	neg	NA

CKD, chronic kidney disease; DSH, domestic shorthair; F, female; M, male; NA, not available

Among healthy cats (n=10), two tested positive to IFAT (cats n°9 and n°10). This finding is not surprising, given that in cats infection is frequently subclinical and clinical disease is rarer than in dogs (Pennisi et al., 2015). It was not possible to establish if these cats were just exposed or subclinically infected, because infection was not confirmed by direct tests. It would have been interesting to test again these subjects with serology after a few months to verify clinical status and possible increase or decrease of antibody titer. Furthermore, one of them was reported as pregnant (n°10), and to test kittens to assess vertical transmission would have been of great interest. Unfortunately, follow-up of these cats was not available. Seventeen cats were affected by pathologic conditions that were classified as localized and most frequently involved the upper airways, the oral cavity, or the lower urinary tract (Table 19). Among these cats only one subject (cat n°27), which was affected by gingivostomatitis, showed a positive IFAT. On the contrary, 36 cats showed clinical signs of different kinds of systemic disease (Table 20). Of these, 9 tested positive to IFAT and one to PCR.

As expected, the most frequently reported symptoms in positive cats were gingivostomatitis, ulcerative lesions of skin and oral cavity, lymphadenomegaly, anorexia, weight loss, chronic vomiting, alopecia, ocular lesions (e.g. uveitis, corneal lesions). Such clinical signs are the same commonly reported in literature in cats with leishmaniosis (Pennisi et al., 2015). As discussed above, clinical disease is relatively rare in cats and is often associated with immunosuppressive conditions and comorbidities such as malignant neoplasia or diabetes mellitus (Pennisi, 2015). One of the 14 positive cats (cat n°61) was coinfected with FIV and showed overt clinical disease. The remaining cats tested negative for retroviral infection but based on available data, it is not possible to exclude that other immunosuppressive factors such as drugs or comorbidities could be present.

As regards biochemistry abnormalities in seropositive or PCR positive cats (Table 21), results were not available for three cats.

Table 21. Main abnormalities of complete blood count, biochemistry and acute phase proteins in Leishmania positive cats.

N	**WBC** x10³/µL 5.5-12	**Hgb** g/dL 9.5-15	**PLT** x10³/µL 130-400	**ALP** U/L <62	**ALT** U/L <70	**Urea** mg/dL 29-60	**Crea** mg/dL <1.70	**Prot** g/dL 5.8-7.7	**Alb** g/dL 2.8-3.7	**A/G** <0.6	**SAA** mg/L >0.5	**PON** U/mL >58
9	**15.3**	12.1	539	57	49	32	1.04	4.6	3.2	0.70	0.2	**56.8**
10	**26.5**	13	526	28	35	53	1.1	**8.8**	**2.7**	**0.44**	**1.8**	65
27	**15.3**	12.9	259	29	**87**	54	1.53	**5.4**	3.2	**0.59**	0.1	91.2
54	11.1	**7.6**	148	52	**84**	**176**	**3.13**	7.2	**2.6**	0.78	0.1	**11.7**
55	**12.6**	10.4	310	22	**133**	33	1.12	7.3	2.9	0.74	**79.4**	88.5
56	**26.9**	**6.9**	491	11	22	54	1.19	**8.7**	**1.7**	**0.24**	**129.1**	**43.6**
57	**23.8**	**9.4**	332	30	**89**	57	1.17	**9.2**	**2.2**	**0.34**	**88.1**	99.2
58	12.1	9.8	139	10	41	54	1.7	**9**	4.2	0.72	**165.1**	**47.9**
59	5.1	11.6	280	17	34	**68**	**2.35**	6.5	2.9	0.81	0.1	72
60	**15.1**	**6.7**	542	-	-	-	-	-	-	-	-	**33.6**
61	**18.8**	**4.7**	**127**	-	-	-	-	-	-	-	-	**24.3**
62	-	-	-	-	-	-	-	-	-	-	-	68.1
63	**19.9**	11.5	179	40	66	36	0.87	**10.2**	**2.3**	**0.29**	**34.8**	76
64	**3.3**	10.5	196	11	34	**74**	**2.11**	**9.7**	2.9	**0.43**	-	**31.5**

Alb, albumin; A/G, albumin/globulin ratio; ALP, alkaline phosphatase; ALT, alanine aminotransferase; Crea, creatinine; Hgb, hemoglobin; PLT, platelets; Prot, total protein; WBC, white blood cells. Values exceeding reference intervals are highlighted in bold

Mild to moderate leukocytosis was often present, including in cats that were classified as healthy based on clinical signs. On the contrary, leukopenia was present only in one case. Mild to moderate anemia was observed in about a third of cases and thrombocytopenia, which by the way was very mild and not reliable without blood smear evaluation, only in one case. Moderate azotemia was present in three cases and mild to moderate increase in ALT activity was observed in four cases. Hyperproteinemia was observed in about half of the cats, often associated with decreased albumin concentration and albumin/globulin ratio. As reported above, all these abnormalities are consistent with Leishmania infection (Pereira and Maya, 2021).

Results of serum SAA concentration and PON-1 activity from positive and negative cats were compared regardless of clinical information (Table 22 and Figure 20).

Table 22. Results of serum SAA concentration and PON-1 activity from Leishmania positive and negative cats.

		SAA (mg/L) (Neg=36; Pos=10)	**PON-1** (U/mL) (Neg=50; Pos=14)
NEGATIVE	mean ± SD (median) min-max (I-III quartile)	16.0 ± 46.9 (0.1) 0.1-222.1 (0.1-1.1)	67.2 ± 24.3 (59.9) 27.8-134.6 (52.2-78.1)
POSITIVE	mean ± SD (median) min-max (I-III quartile)	49.9 ± 61.8 (18.3) 0.1-165.1 (0.1-91.5)	57.8 ± 26.7 (60.9) 11.7-99.2 (33.4-77.0)
P		0.043	0.350

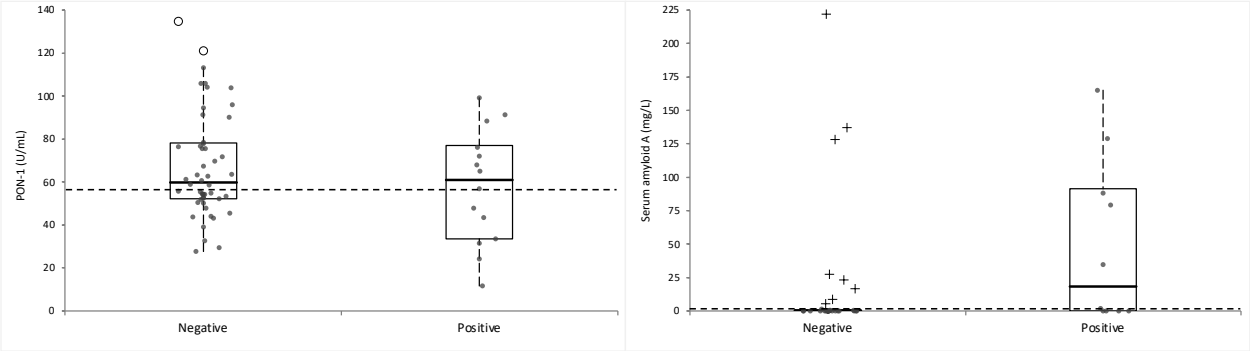


Figure 20. Serum PON-1 activity (left) and SAA concentration (right) in Leishmania negative and positive cats. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 x IQR or to further observation within quartile III plus 1.5 x IQR. Grey dots indicate results that are not classified as outliers, white dots indicate near outliers (values exceeding the third quartile + (1.5 x IQR)), '+' indicates far outliers (values exceeding the third quartile + (3 x IQR)). The dotted line represents the lower reference limit of PON-1 activity and SAA concentration in healthy cats respectively.

As regards PON-1 activity, statistically significant differences between positive and negative cats were not detected. On the contrary, SAA concentration was significantly higher in positive cats than in negative cats, even if P value was close to the cut off for statistical difference. Considering the distribution of PON-1 activity values reported in the figure, we can observe that among negative cats many samples (23/50) showed values lower than the reference interval set for this species in a previous study (Rossi et al., 2020) and this causes the lack of significant differences between groups. Accordingly, many of the negative samples (10/36) showed values of SAA higher than reference interval, even if most of these values are considered outliers compared with the more compact distribution of positive values.

Overall, these results suggest that the distribution of values of PON-1 activity and SAA concentration among positive and negative cats, is likely more dependent on the presence of sick cats in both groups than on the positivity to Leishmania.

The hypothesis explained above, is partly supported by analyzing data from sick and healthy cats regardless of the positivity (Table 23 and Figure 21).

Table 23. Results of serum SAA concentration and PON-1 activity from sick and healthy cats.

		SAA (mg/L) (sick=38; healthy=8)	**PON-1** (U/mL) (sick=53; healthy=10)
SICK	mean ± SD (median) min-max (I-III quartile)	27.8 ± 56.0 (0.1) 0.1-222.1 (0.1-23.7)	66.8 ± 26.6 (62.8) 11.7-134.6 (49.4-89.0)
HEALTHY	mean ± SD (median) min-max (I-III quartile)	2.4 ± 5.8 (0.1) 0.1-16.7 (0.1-1.1)	59.6 ± 10.0 (57.7) 43.8-75.6 (54.0-65.9)
P		0.356	0.591

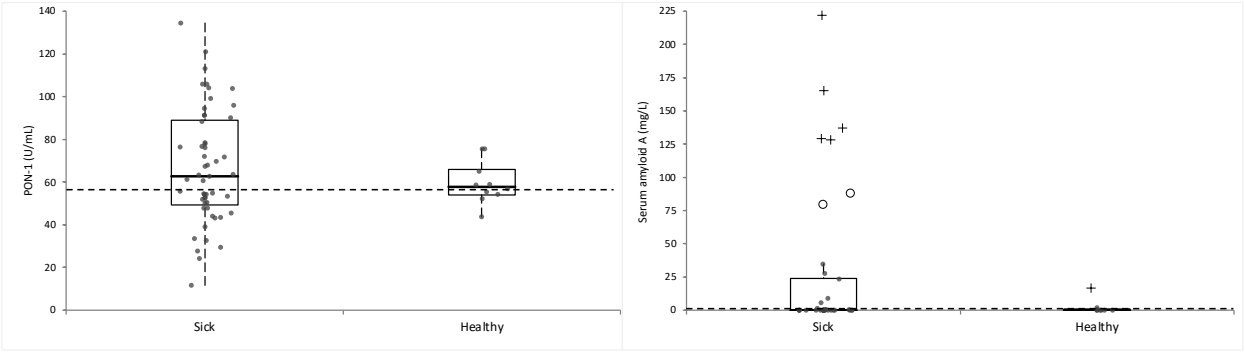


Figure 21. Serum PON-1 activity (left) and SAA concentration (right) in sick and healthy cats.

Despite the absence of significant differences, it is possible to observe that except for rare exceptions, values of PON-1 activity and/or SAA concentration respectively lower and higher than reference intervals were prevalently observed in the group of sick cats. The absence of statistically significant differences is due to the wide distribution of values in the group of sick cats. In particular, among healthy cats, all values were centered close to the median, while an evident dispersion of values was observed among sick cats, which in many cases showed values within or close to reference intervals. This dispersion of values likely derives from the heterogeneous composition of the group of sick cats, which included subjects with more or less severe systemic or localized disease.

This hypothesis was confirmed by analyzing the same data with different grouping. Sick cats were furtherly divided into two groups based on the local or systemic involvement of disease and compared with healthy cats (Table 24 and Figure 22).

Table 24. Results of serum SAA concentration and PON-1 activity from cats grouped based on the local or systemic involvement of disease.

		SAA (mg/L) (L=15; S=23; H=8)	**PON-1 (U/mL)** (L=17; S=36; H=10)
LOCAL (L)	mean ± SD (median) min-max (I-III quartile)	2.6 ± 7.3 (0.1) 0.1-27.5 (0.1-0.2)	73.3 ± 22.6 (67.5) 45.4-120.9 (54.0-91.2)
SYSTEMIC (S)	mean ± SD (median) min-max (I-III quartile)	44.2 ± 67.3 (0.5) 0.1-222.1 (0.1-86.6)	63.7 ± 28.0 (57.7) 11.7-134.6 (43.8-83.6)
HEALTHY (H)	mean ± SD (median) min-max (I-III quartile)	2.4 ± 5.8 (0.1) 0.1-16.7 (0.1-1.1)	59.6 ± 10.0 (57.7) 43.8-75.6 (54.0-65.9)
P		0.055	0.265

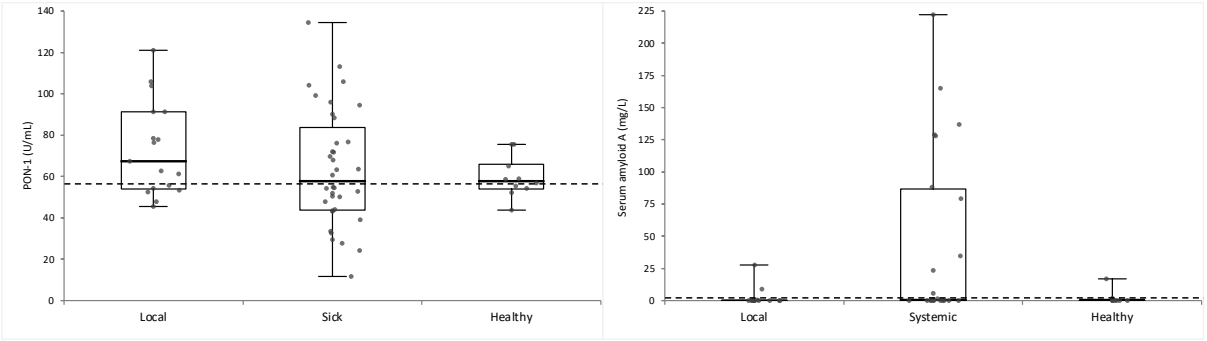


Figure 22: Serum PON-1 activity (left) and SAA concentration (right) in cats grouped based on the local or systemic involvement of disease.

Significant differences were still not observed, even if for SAA concentration the P value was close to the cut off for statistical difference. However, the distribution of data confirmed that cats with localized disease had PON-1 and SAA values most similar to that of healthy cats. Furthermore, PON-1 and SAA values respectively lower and higher than reference interval were frequently detected in cats with systemic disease and only occasionally detected in the group of cats with localized disease as well as in healthy cats. Actually, post-hoc tests showed a significant difference of SAA concentration in cats with localized and systemic disease (P=0.029).

The group of sick cats included many subjects with normal PON-1 activity and SAA concentration. This is not unexpected, since group formation was based on the presence of symptoms related to systemic diseases, that were not necessarily characterized by inflammation or oxidation. Clinical signs like weakness, anorexia, weight loss, vomiting and many others can be due to non-inflammatory systemic disease, such as neoplasia or chronic kidney disease. However, very low and high PON-1 activity and SAA concentration respectively, were observed only in cats with systemic disease, thus confirming what reported in literature about the specificity of both markers for systemic illness characterized by an inflammatory or oxidative etiopathogenesis (Rossi et al., 2013; Rossi et al., 2014a; Meazzi et al., 2021).

Results reported above seem to suggest that abnormalities of SAA and PON-1 could be related to the presence of inflammatory or oxidative systemic illness. To verify if the same trend was present in the groups of positive and negative cats, results from positive and negative sick and healthy cats were compared (Table 25 and Figure 23).

Table 25. Results of serum SAA concentration and PON-1 activity from cats grouped based on clinical status and positivity to Leishmania.

		SAA (mg/L) (NS=30; NH=6; PS=8; PH=2)	**PON-1** (U/mL) (NS=42; NH=8; PS=11; PH=2)
NEGATIVE SICK (NS)	mean ± SD (median) min-max (I-III quartile)	18.6 ± 51.1 (0.1) 0.1-222.1 (0.1-1.7)	68.7 ± 25.9 (62.0) 27.8-134.6 (51.7-90.1)
NEGATIVE HEALTHY (NH)	mean ± SD (median) min-max (I-III quartile)	2.9 ± 6.8 (0.1) 0.1-16.7 (0.1-1.5)	59.3 ± 11.1 (56.9) 43.8-75.6 (53.0-68.6)
POSITIVE SICK (PS)	mean ± SD (median) min-max (I-III quartile)	62.1 ± 63.6 (57.1) 0.1-165.1 (0.1-112.0)	59.6 ± 29.2 (68.1) 11.7-99.2 (35.3-86.4)
POSITIVE HEALTHY (PH)	mean ± SD (median) min-max (I-III quartile)	1.0 ± 1.1 (1.0) 0.2-1.8 (0.2-1.8)	60.9 ± 5.8 (60.9) 56.8-65.0 (56.8-65.0)
P		0.149	0.798

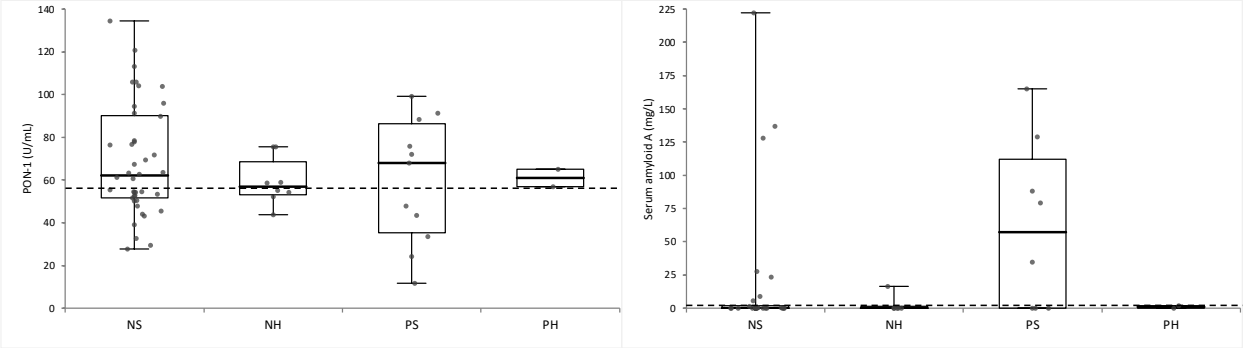


Figure 23. Serum PON-1 activity (left) and SAA concentration (right) in cats grouped based on clinical status and positivity to Leishmania. NS, negative sick; NH, negative healthy; PS, positive sick; PH, positive healthy.

This comparison is strongly affected by the low number of healthy cats in both negative and positive groups, but particularly in the latter which included only two subjects. Anyway, significant differences among groups were not detected and post-hoc test confirmed that differences were not present neither between healthy and sick within positive and negative cats nor between negative and positive within healthy and sick. The same trend discussed above was confirmed for both SAA and PON-1, especially considering the distribution of individual results. In other words, cats with SAA and PON-1 values respectively higher and lower than reference intervals were prevalently detected among sick cats and more rarely among healthy cats, which by the way represented a low number as mentioned above. As regards SAA, the dispersion of values among sick cats appears to be more uniform in positive ones, while the majority of negative cats showed normal or normal to high values, except for some outliers. This finding seems to suggest that negative cats are more frequently affected by non-inflammatory diseases, while positive cats more frequently show inflammatory abnormalities possibly associated to Leishmania infection as discussed previously. On the contrary, the distribution of values of PON-1 activity was more variable, and normal and low PON-1 values were equally observed both in negative and positive healthy cats. This is in line with what reported in dogs. In fact, only dogs with severe clinical forms of leishmaniosis showed decreased PON-1 activity, along with increase of other acute phase proteins such as C reactive protein (CRP). On the contrary, dogs with mild disease showed PON-1 activity within reference intervals, even if increase in CRP concentration was increased (Rossi et al., 2014a). This is likely due to the already mentioned different kinetic of PON-1, whose activity decreases only when inflammation is associated with oxidative phenomena, and other acute phase proteins, whose concentration increases regardless of the

presence of oxidation and severity of inflammation (Kotani et al., 2013). This finding seems to suggest that the evaluation of SAA concentration and PON-1 activity may be a useful tool for clinical scoring and monitoring of clinical disease due to Leishmania in cats, as happens in dogs. In practice, the finding of low PON-1 activity in association with seropositivity to L. infantum should lead to investigate the presence of infection by direct tests.

Considering results from positive healthy cats, it is worth to make another consideration. In dogs, it was observed that positive non-symptomatic subjects showed an activation of oxidative stress (Panaro et al., 2008; Paltrinieri et al., 2010), which was interpreted as a protective response due to infected macrophages activation. Infected macrophages, by producing oxidizing radicals, manage to control intracellular parasites and prevent the dissemination of infection and emergence of symptoms. If clinical disease develops, a greater activation of the oxidative phenomena occurs in association with the appearance of inflammatory lesions, in which the production of ROS by phagocytes is no longer aimed to contain the infection but is a consequence of the uncontrolled activation of inflammatory cells within the lesions. From this point of view, data regarding symptomatic subjects (with the aforementioned limitations), seem to support this latter phenomenon. However, the detection of normal PON-1 activity in the only two clinically healthy subjects seems to suggest that the activation of infected phagocytes for protective purposes does not occur in cats. However, this interpretation is affected by the low number of positive clinically healthy cats, which, despite not showing clinical signs, showed hematochemical signs of inflammation (leukocytosis in both cats and increase in SAA concentration and total protein and decrease in A/G ratio in cat n°10). Furthermore, the lack of conclusive information on the real presence of the parasite in seropositive subjects also does not allow to make further considerations.

Conclusive remarks

Overall, our results seem to suggest that SAA concentration and PON-1 activity could be more affected by the presence of clinical disease than by positivity to Leishmania, even if among positive cats oxidative and inflammatory phenomena appeared variably present.

This study however has some limitations. First of all, the low number of positive cats and in particular of positive healthy subjects. Furthermore, in most cases (12/14), cats tested positive at serology, sometimes with low antibody titers, but the presence of Leishmania was not confirmed by direct tests such as PCR or cytology. In other words, the majority of subjects

included in this study had been definitely exposed to L. infantum, but were not necessarily infected. This consideration may be not so relevant, since a recent study reported that either seropositive cats in which infection was not demonstrated or seropositive cats in which infection was demonstrated by PCR, showed normal SAA concentration despite increased alpha 2 and gamma globulins (Savioli et al., 2021). However, it could be relevant to interpret our results of PON-1 activity, that in other species was hypothesized to vary dependent on exposition, infection, clinical symptoms and protective induced oxidative phenomena (Rossi et al., 2014a, Ibba et al., 2015), with the latter variably activated in infected non-symptomatic compared with symptomatic patients (Panaro et al., 2008; Paltrinieri et al., 2010). Finally, our classification into healthy and sick cats could have affected the distribution of results in the different groups. In fact, groups including sick cats (both negative and positive) were extremely heterogenous in terms of type and severity of disease and clinical signs. Furthermore, given the wide spectrum of possible clinical signs of feline leishmaniosis (Pennisi et al., 2015), it was not possible to relate clinical signs of sick cats with Leishmania infection for certain, only based on seropositivity. In other words, besides non-specific symptoms such as weakness, fever, anorexia or dysorexia and weight loss could possibly be related to Leishmania infection, it was not possible to exclude that cats had only been exposed to Leishmania and that symptoms were due to diseases other than leishmaniosis with systemic involvement. This, by the way, would also explain the overlap of results recorded in sick cats of positive and negative groups.

Further studies with a higher and more standardized caseload are surely warranted. Anyway, what emerged from this study is that lowest PON-1 values were detected in sick positive cats and therefore it can be hypothesized that in cats with signs of systemic disease and low PON-1 activity and/or high SAA concentration, it is worth to consider leishmaniosis among differential diagnoses. On the contrary, for cats in which leishmaniosis is diagnosed, measurement of PON-1 activity and SAA concentration could be considered a useful tool to evaluate possible systemic spread of infection and monitoring disease.

PARAOXONASE ACTIVITY IN PIGS

Rationale and aims

In livestock, clinical information is often incomplete and the extensive use of hematological or biochemical testing is limited due to cost-benefit issues. Therefore, acute phase proteins as surrogate markers of the health status can be very helpful. One of the strategies proposed in the management of the health status of livestock is the measurement of acute phase reactant in random blood samples collected at slaughter, in order to identify animals or groups of animals with subclinical condition that require a more extensive and accurate meat inspection to identify potential pathogens. Therefore, we designed a study to assess whether the measurement of PON-1 in blood collected at slaughter may predict the presence and severity of lesions detected during meat inspection (study VII). The results of this study will be presented as a poster at the 75th Congress of Società Italiana delle Scienze Veterinarie (SISVet) in June 2022.

VII. Paraoxonase-1 activity in slaughtering pigs

Study design

Samples were collected in three different abattoirs in batches of 20 samples per slaughtering session, to have sufficient time to complete the inspection of the carcass and internal organs and move to the laboratory to process samples within the same day. At slaughter, blood samples were collected during jugulation by percolation of whole blood in tubes without anticoagulant. For each sampled animal, data regarding the age group (piglets vs adults) and any information about external lesions or lesions detectable during inspection of the carcass and internal organs (with particular attention to pulmonary and hepatic lesions) were recorded. In a few cases, tissue slices were collected in 10% isosmotic buffered formalin and stored to be submitted for routine histology, if needed. Based on the macroscopical appearance of internal or external organs, the presence of lesions was recorded, and lesions were in turn classified as inflammatory or non-inflammatory. After each sampling session, tubes were transported in cold chain at the laboratory and centrifuged within three hours. Serum was then harvested using a disposable plastic pipette and transferred to an Eppendorf tube to be stored at -20°C until analysis. On each sample, PON-1 was measured using the method described above, and results were used to run the following statistical analysis: a non-parametric t-test for independent data (Mann-Whitney

U test) was used to compare results of adults and piglets, of animals with or without lesions or with <2 or >2 pulmonary lesions. A non-parametric ANOVA test for independent samples (Kruskal-Wallis test) followed by a Mann-Whitney U test to compare each single groups was used for comparisons that involved more than 2 groups (i.e. comparison of results from animals without lesions, with inflammatory lesions or with non-inflammatory lesions, or comparison of results of animals with different numbers of pulmonary lesions, as specified in the results section).

Results and discussion

Overall, 56 animals were included in the study (39 sows, 9 piglets, 8 fattening hogs). Of these, 23 animals did not have external or internal lesions. PON-1 values recorded in these animals were 12.3 ± 2.2 U/mL (median 12.3 U/mL, min-max 8.2-15.9 U/mL). Using the Robust method after Box Cox transformation, that is the statistical approach recommended by the ASVCP guidelines for small datasets (Friedrichs et al., 2012), the reference interval of these animals was 7.2-16.7 U/mL.

The group of animals without lesions did not include piglets. Results recorded in sows (n=20) and fattening hogs (n=3) were not statistically different to each other (P=0.144), but a trend towards lower values in sows, likely not significant due to the low number of fattening hogs, was visually evident from the graph (Figure 24).

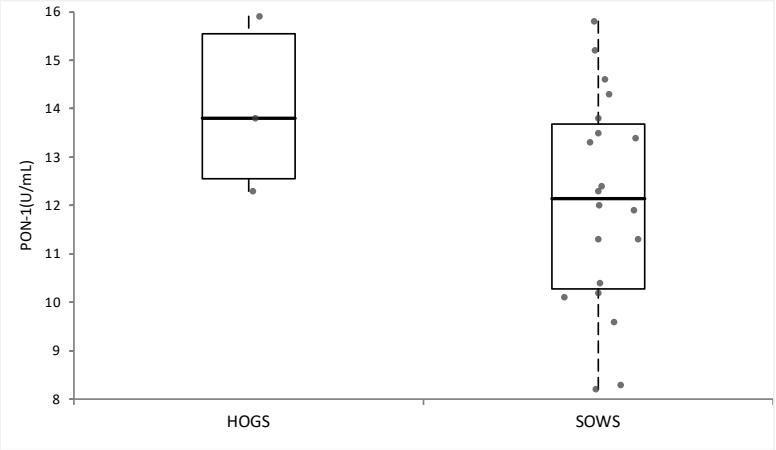


Figure 24. Serum PON-1 activity in fattening pigs and sows included in the study. The boxes indicate the I-III interquartile range (IQR), the horizontal black line indicates the median values, whiskers extend to further observation within quartile I minus 1.5 × IQR or within quartile III plus 1.5 × IQR. Black dots indicate the results that are not classified as outliers.

Results of animals without lesions confirmed that PON-1 activity is lower in swine than in other species, in which values are usually higher than 50-100 U/mL, as reported above. This may suggest that swine PON-1 has a low paraoxonase activity. This is the first study based on paraoxonase activity while other studies investigated lactonase and arylesterase activity (Escribano et al., 2015). It is then possible than in this species the paraoxonase activity is lower than arylesterase activity, as happens in humans (Mackness et al., 1991). Alternatively, it may be supposed that the low PON-1 activity in swine depends on the peculiar lipid metabolism of this species. This hypothesis may be supported by the close interaction between PON-1 and molecules involved in lipid metabolism like HDL (Deakin et al., 2002). On the other hand, the design of this study, based on the collection of blood samples at slaughtering and on the macroscopic inspection of animals and organs, does not allow to exclude that animals without lesions were actually affected by metabolic diseases, or that histological lesions associated with early phases of inflammatory response could be present and not detected, with subsequent activation of oxidative phenomena potentially responsible of decreased PON-1 activity (Pallares et al., 2008). Moreover, the lack of piglets without lesions precluded to assess whether PON-1 activity could be lower in young animals, as already reported in other species (Li et al., 1997; Cole et al., 2003; Giordano et al., 2013). This information would have been important, since the inclusion of piglets in the group of animals without lesions would have likely further decreased the lower limit of the reference interval. In this case, age-specific reference intervals should be created, in order to avoid that low values due to physiological factors like age, may be erroneously interpreted as associated with inflammation. Ultimately, the detection of low values also in adults limited the possibility to diagnose inflammation. Indeed, when the lower limit is low, it is difficult to accurately detect further decreases caused by disease. Another aspect that remains to be elucidated, is the trend to lower values in sows compared with fattening hogs. In other species low PON-1 values have been reported in young animals, as stated above, and in male (Ruggerone et al., 2018), both factors inducing decreased values in fattening hogs compared with sows and not vice versa. As stated above, all these findings should be verified on a larger study population, with equal distribution of male and female subjects. However, if fattening hogs had physiologically higher PON-1 activity than sows, the most likely explanation could be again the influence of a different lipid metabolism in this species (Ferretti e Bacchetti, 2012).

PON-1 activity recorded in the whole group of animals with lesions was 11.9 ± 2.8 U/mL (median 11.6 U/mL, min-max 5.2-17.8 U/mL). This result was not significantly different (P=0.489) from the one recorded in animals without lesions reported above, and only two animals with lesions had lower values than lower limit of the reference interval (Figure 25).

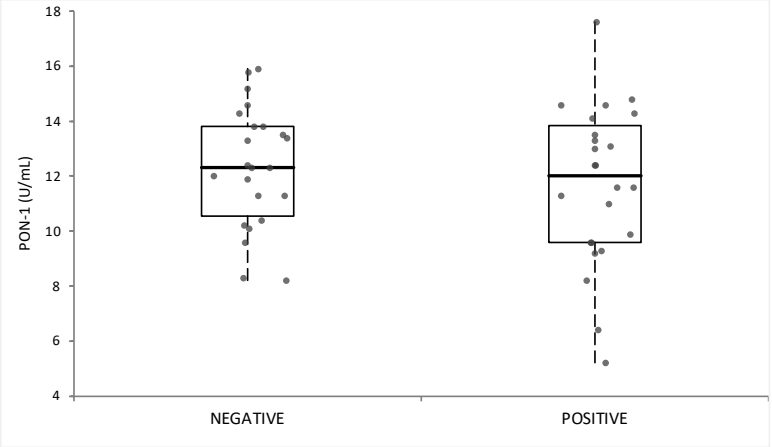


Figure 25. Serum PON-1 activity in pigs with (positive) or without (negative) macroscopically evident lesions at slaughter.

When results from the 9 piglets with lesions (that cannot be compared with results of healthy animals potentially characterized by physiologically low values) are excluded from the whole group, PON-1 activity (11.7 ± 2.9 U/mL; 12.0 U/mL; 5.2-17.6 U/mL) was not significantly different (P=0.443) from that of animals with lesions. In both cases above, the lack of significance may depend on the heterogeneity of the lesions detected at slaughter, either in terms of pathophysiology (presence/absence of inflammation, acute or chronic, local or systemic disease), or in terms of severity. This heterogeneity may account for a different PON-1 response, since in other species it has been demonstrated that not all inflammatory conditions influence serum PON-1 activity (Rossi et al., 2013). From this perspective, the following lesions were reported in our caseload (Table 26).

Table 26. Macroscopical lesions recorded at slaughter.

Type of lesion	**N. of animals**
Lung nodule of possible purulent or parasitic origin	15*
Chronic pleuritis	5
Subacute or chronic pericarditis	4
Milk spot	2
Subacute or chronic catarrhal bronchopneumonia	2
Umbilical hernia	2
Pelvic hematoma	1
Hepatic necrosis	1
Podal lesions	1
TOTAL	33

* In 2 cases associated with milk spot, in 2 cases with umbilical hernia, in 2 cases with podal lesions

As shown in Table 26, lesions were mostly located in the thoracic cavity, where nodular lesions (abscesses or nodules of possible chronic inflammatory or parasitic origin, sometimes associated with extrapulmonary lesions), pleuritis, pericarditis or bronchopneumonia (of different severity or chronicity) were detected. Extra-thoracic lesions were less frequently detected. The heterogeneity of lesions supports the hypothesis that PON-1 activity may be less affected in the presence of mild lesions with moderate or slight associated inflammation. In other species, in fact, only severe lesions or lesions with a more prominent oxidative pathogenesis may influence PON-1 activity (Rossi et al., 2014a).

In order to assess whether PON-1 activity may help to identify animals with specific types of lesions, the following groups of animals based on type of lesions were created and compared: type of inflammation, severity of the lesions and localization of lesions.

Based on the nature of lesions, 8 animals were classified as affected by lesions possibly associated with acute inflammation. This group included animals with evident purulent exudate in pulmonary nodular lesions (n=3), podal lesions (alone or associated with lung lesions) (n=2), pelvic hematoma (n=1), chronic pleuritis associated with enlarged mediastinal lymph nodes (n=1), hepatic necrosis (n=1). The remaining 25 animals were classified as affected by chronic or slight inflammation.

The comparison of these 2 groups and animals without lesions showed slight statistical significance (P=0.047), with lower values in animals with acute inflammation (mean ± SD: 10.18 ± 2.67; median: 9.60; interquartile range: 9.27-11.70) compared with animals with chronic inflammation (12.52 ± 2.64; 12.40, 10.94-14.48; P=0.019) and with animals without lesions (12.34 ± 2.20; 12.30; 10.55-13.80; P=0.027) (Figure 26).

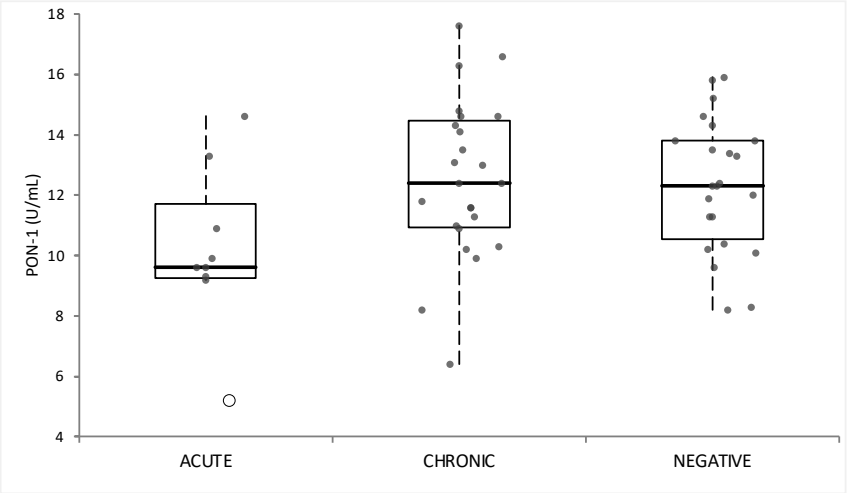


Figure 26. Serum PON-1 activity in pigs with lesion classified as acute or chronic and without lesions at slaughter. White dots indicate near outliers (values exceeding the third quartile ± (1.5 × IQR)).

However, even some animals with slight or chronic inflammation showed very low PON-1 activity (and one piglet with umbilical hernia had values lower than reference interval). Conversely, most animals with acute inflammation showed values comparable with those of the other group, except for one fattening hog with bronchopneumonia, that had PON-1 values lower than reference interval. From this perspective, despite significant difference, the practical utility of PON-1 in identifying animals with acute inflammation at slaughter may be limited. To verify this hypothesis, the ability of PON-1 to discriminate animals with acute inflammation from others was assessed using a Receiver Operating Characteristic (ROC) curve, that was significantly different from the line of no discrimination (P=0.008) but with a moderate area under the curve (76.1%, 95% confidence interval: 56.6%-95.7%) (Figure 27).

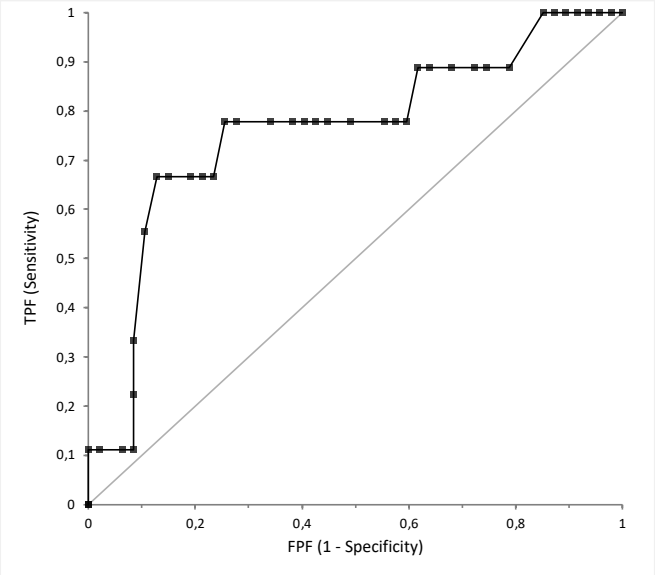


Figure 27. ROC curve designed to assess the discriminating power of PON-1 in detecting pigs with acute inflammation. The grey line indicates the line of no discrimination. TPF, true positive fraction; FPF, false positive fraction.

Moreover, the ROC curve showed that the specificity of PON-1 in identifying animals with acute inflammation was absolute only at values lower than reference intervals (<6.4 U/mL), although values close to the lower limit of the reference interval increased the diagnostic probability (e.g. at PON-1 <9.9 U/mL the specificity is 89.4%, the positive predictive value exceeds 90%, and the positive likelihood ratio is 5.22, indicating that the probability that an animals with PON-1 <9.9 U/mL at slaughter had lesions associated with acute inflammation was 5.22 times higher than the probability that these lesions are not present). However, even at low PON-1 values, the negative predictivity was still low (less than 50%) and the negative likelihood ratio remained close to 1.00.

These results suggest that low PON-1 values before slaughtering may allow to suspect the presence of lesions associated with acute inflammation, but values within the reference interval do not allow to exclude a possible acute pathogenesis. As stated above, this is not surprising since only in some cases of acute inflammation (i.e. only in those associated with severe oxidation) PON-1 activity decreases in serum (Rossi et al., 2014a). From a practical standpoint, however, the low negative predictivity limits the use of this test as a screening test to address the procedure of meat inspection at slaughtering.

As regards the severity of lesions, in 12 cases, lesions were considered as more severe and possibly associated with systemic changes compared with the other 21 cases. The group of "severe" lesions included the 8 cases with suspected acute pathogenesis, 2 cases of bronchopneumonia and 2 cases of pleuritis that, although classified as chronic, were considered particularly severe at *post mortem* examination. Despite the local extension of the lesion, the persistence of inflammatory stimuli may sustain the so-called acute phase reaction, which induces, among other effects, a modulation of hepatic protein synthesis potentially responsible of decreased gene expression of negative acute phase proteins (Ehlting et al., 2021). In other species, the decreased synthesis of PON-1 during prolonged inflammation, along with an increased peripheral consumption due to oxidative phenomena, is considered the main cause of decreased PON-1 activity (Feingold et al., 1998).

However, this comparison did not reveal statistical differences among groups (P=0.578). Despite a visible trend to decreased PON-1 activity in animals with severe inflammation and possible systemic involvement, the values recorded in this group (11.41 ± 3.23; 10.40; 9.43-14.39) were not significantly different from those in which a systemic involvement was less likely (12.15 ± 2.58; 11.80; 10.70-13.77) or from those without lesions (12.34 ± 2.20; 12.30; 10.55-13.80) (Figure 28).

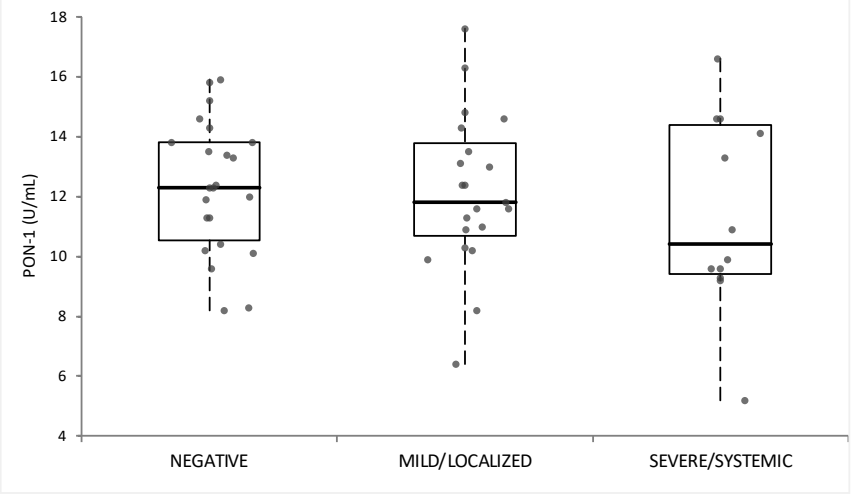


Figure 28. Serum PON-1 activity in pigs without lesions at slaughter and in pigs with lesion classified as causing systemic impact and not.

Since intrathoracic lesions were more frequent, we investigated the possible association between pulmonary lesions and PON-1 activity or between the number of pulmonary lesions and PON-1 activity. These comparisons, however, did not reveal significant differences among groups, neither in terms of localization of lesions (P=0.503) nor in terms of number (P=0.254). More specifically, PON-1 values recorded in 26 animals with intrathoracic lesions (12.10 ± 2.73; 12.10; 10.18-14.33) were similar in magnitude to those recorded in animals without lesions (12.34 ± 2.20; 12.30; 10.55-13.80), while the 7 animals with extra-thoracic lesions had lower values (11.09 ± 3.18; 10.90; 9.35-13.18) but without statistical significance (Figure 29).

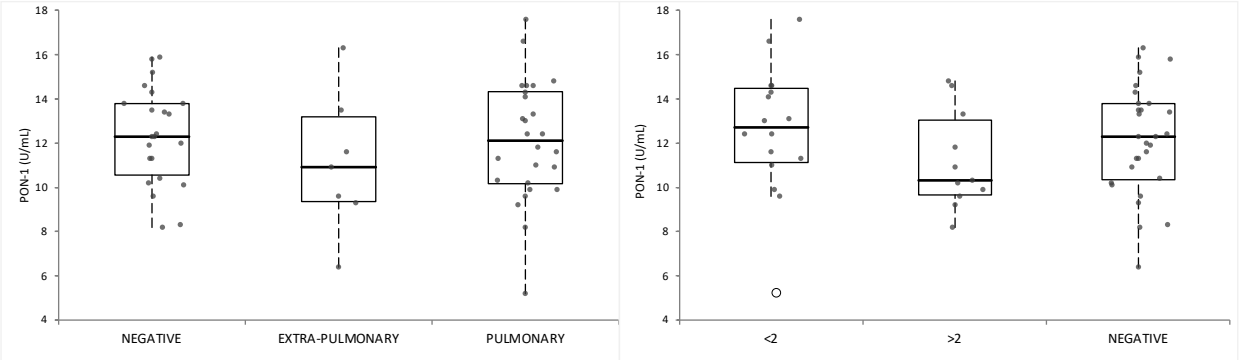


Figure 29. Serum PON-1 activity in pigs without lesions and in pigs with lesions grouped based on the localization (pulmonary or extra-pulmonary) (left) and based on the number of lesions (right). White dots indicate near outliers (values exceeding the third quartile ± (1.5 × IQR)).

Similarly, although 11 animals with more than 2 pulmonary nodules had visually lower values (11.16 ± 2.20; 10.30; 9.65-13.05), compared with the 16 animals with less than 2 lesions (12.58 ± 2.95; 12.70; 11.13-14.48) and with the 29 animals without lesions or with extrapulmonary lesions (12.13 ± 2.46; 12.30; 10.33-13.80), significant differences were not observed (Figure 29). It should be noted, however, that the group of animals with low number of pulmonary lesions also included animals with diffuse pleuritis or bronchopneumonia, that, as noted above, were sometimes very severe. Therefore, the occasional detection of low PON-1 activity in this group likely masked a possible statistical significance.

Conclusive remarks

The analysis of PON-1 activity did not provide encouraging results about the possibility to use this analyte as a surrogate marker of lesions at slaughter to optimize *post mortem* examination. However, some aspects recorded in this study may stimulate future studies.

In particular, our results highlighted some limitations of PON-1 measurement in pigs. Paraoxonase activity is particularly low in this species, and this may hamper the possibility to detect further decreases induced by pathologic processes. From this perspective, it may be important in the future to investigate the need of age-specific reference intervals for young animals, through the analysis of serum from piglets not affected by lesions (lacking in the current caseload). Moreover, it would be interesting to investigate the mechanism responsible for the low paraoxonase activity in pigs, through the analysis of other methods than the paraoxon-based one used in this study. Inclusion of other substrates may reveal whether PON-1 activity in pigs is really low, possibly due to the peculiar fat and lipid metabolism of this species, or depends on the prevalence of lactonase or arylesterase activity over the paraoxonase activity investigated in this study. Regardless of these considerations, low values recorded in all the animals included in this study may have contributed to the lack of significant differences in most of the comparisons performed, suggesting that measuring PON-1 at slaughter may be not relevant in practice. Limitations of this study also included the heterogeneity of lesions included in the caseload, the lack of clinical or productive information useful to definitely classify animals as sick or healthy, and the lack of information about histological findings in the slaughtered pigs. However, the lack of histological data should not be considered a real limitation, since it actually allows to simulate what happens in field, where the identification and the classification of lesions is mostly based on their macroscopic appearance. Anyway, it would be interesting in the future to investigate the ability of serum markers to predict also histological changes. Despite all these limitations, the detection of low PON-1 values in pigs with severe lesions (possibly associated with a systemic response) or with acute inflammation may suggest a possible practical utility of PON-1 measurement at slaughter. However, ROC curve analysis evidenced that although very low PON-1 values may predict the presence of acute lesions, low or normal PON-1 values do not allow to exclude that acute lesions are present. Our results, however, encourage future studies based on a larger caseload and on a more standardized selection of cases.

PARAOXONASE ACTIVITY IN CATTLE

Rationale and aims

The diagnosis and classification of respiratory diseases in cattle is often challenging. Several scoring systems based on clinical signs have been proposed to stage the disease and to achieve prognostic information but none of these systems is considered sufficiently accurate. Additional information may be acquired by ancillary techniques, that are often invasive (e.g. bronchoalveolar lavages or transtracheal washes). Recently ultrasound-based evaluation of pulmonary lesions has shown to have a high accuracy in identifying sick animals and in staging the severity of the lesions, but the application of this technique in routine clinical examination in field may be time consuming, may require expensive instruments and skilled and experienced operators. Therefore, a surrogate marker able to identify sick regardless of clinical score, and to provide preliminary information about the possible presence of lesions, to be further investigated ultrasonographically, would be very useful. Based on its pathophysiology, PON-1 may be a good non-invasive, cheap and rapid diagnostic tool to screen the herd and identify animals that need a more accurate investigation.

VIII. Paraoxonase-1 activity in calves with bovine respiratory disease (BRD)

Study design

The study was performed on 199 blood samples collected from calves living in 9 herds located in the area of Lodi (Lombardy region, Northern Italy). The criteria to include animals in the study were: calves of Italian Friesian breed from farms with recent problems of BRD, of both sexes, aged between 30 and 90 days in the pre-weaning period reared in multiple pens. Blood samples were collected from the jugular vein under informed consent of the farmer within a screening plan for the presence of respiratory disease or, in clinically sick animals, as a part of the diagnostic protocol. Therefore, according to the decision 2/2016 of the Ethical committee of the University of Milan, residual aliquots of the serum used in the diagnostic approach may be used for research purposes without an additional step of approval from the Ethical Committee. Before each sampling, animals were visited and received a complete thoracic ultrasound. TUS Systematic thoracic ultrasound (TUS) is now considered the gold standard for the diagnosis of BRD in calves. Ultrasound examination was performed according to the procedure described by Ollivett and

Buczinski (2016), using a portable ultrasound machine with a linear probe (MyLab Five, Esaotem SpA, Genova, Italy) and vegetable oil was used to remove air from the animal's coat.

The type of lesions observed and their location were noted, and each pathological appearance was assigned a score using the scoring system shown in Table 27 and Table 28 (Ollivett and Buczinski, 2016).

Table 27. Criteria for scoring ultrasound findings and ultrasound appearance of lung lesions.

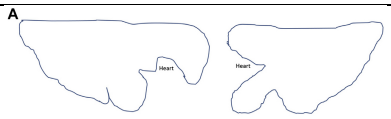
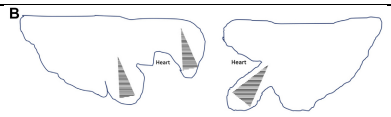
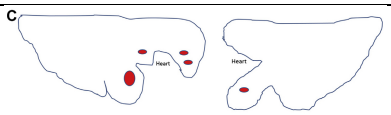
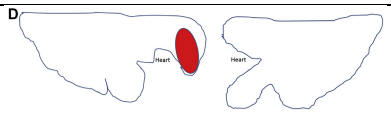
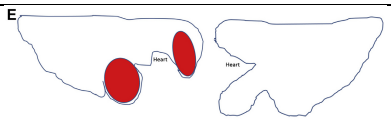
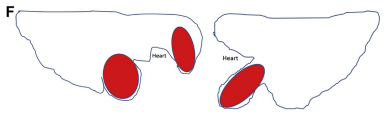
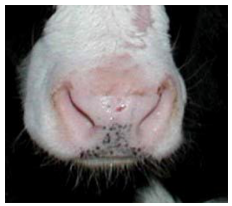
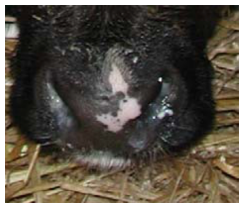
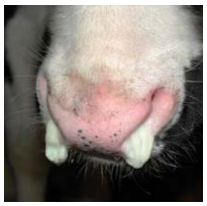
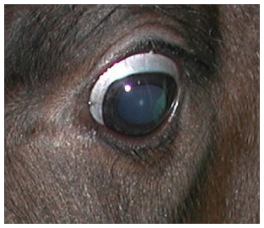
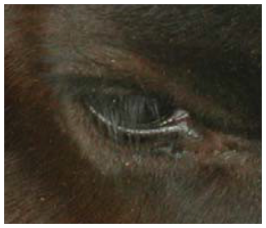
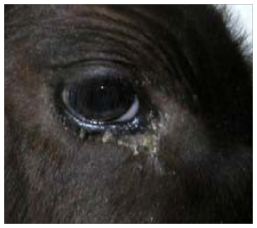
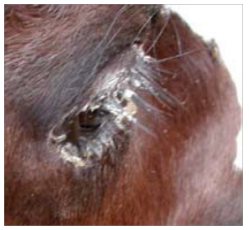
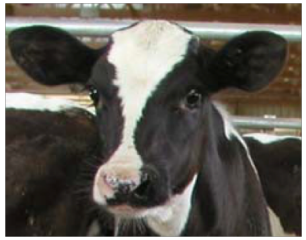
Score	Ultrasound finding	Description	Aspect	Diagnosis
0	Reverberation artefacts	Echogenic pleural line with parallel reverberation artefacts moving away from the probe		Healthy, well-ventilated lung
1	Comet tail artefacts	Hyperechogenic artefact originating in the pleurae and moving vertically away from the probe		Focal pleural roughening or thickening
2	Lobular consolidation	Hyperechoic subpleural lesion at least 1 cm in diameter within properly ventilated lung tissue		Lobular pneumonia
3	Lung consolidation	Consolidated, hepatized and non-aerated portion of lung parenchyma in one lung lobe		Lobar pneumonia affecting one lung lobe
4	Lung consolidation	Consolidated, hepatized and non-aerated portion of lung parenchyma at the level of two lung lobes		Lobar pneumonia affecting two lung lobes
5	Lung consolidation	Consolidated, hepatized and non-aerated portion of lung parenchyma at the level of three lung lobes		Lobar pneumonia affecting three or more lung lobes

Table 28. Calf Respiratory Scoring Criteria (Wisconsin University of veterinary medicine, 2012, Madison).

Calf Health Scoring Criteria				
	0	**1**	**2**	**3**
Rectal temperature	37.8 - 38.3 °C	38.4 - 38.8 °C	38.9 - 39.4 °C	≥ 39.5 °C
Cough	None	Single induced cough	Repeated induced coughs or occasional spontaneous	Repeated spontaneous coughing
Nasal discharge	Normal serous secretion 	Small amount of one-sided cloudy discharge 	Bilateral turbid or mucous discharge 	Abundant bilateral mucopurulent discharge 
Eyes	Normal 	Small amount of eye secretion 	Moderate eye discharge 	Severe eye discharge 
Head and ears	Normal 	Shaking of the ears or head 	Slight drooping of one ear 	Rotation of head or lowered ears 

The following clinical scores were recorded.

Clinical score according to Calf Respiratory Scoring Criteria (CRSC) was determined as follows: each animal received a complete clinical examination, recording following clinical findings: rectal temperature, ear bearing, presence and type of ocular and nasal discharges, presence of cough. Each of the above parameters was assigned a score from 0 to 3 based on the severity of the clinical signs observed as reported in the CRSC table (Table 28) (Ollivett and Buczinski, 2016). Nasal discharge, eye discharge and ear score were assigned without going into the calf pen. Occasional or repeated spontaneous coughing can also be noted from outside the pen, giving the calf respectively 2 or 3 points in that category. Cough induction was performed by tracheal compression and 1 or 2 points were assigned in this category for single or repeated induced cough respectively. Subsequently, rectal temperature was recorded. The value 0 is indicative of a normal condition, while a score of 3 is attributed to a very altered clinical state. A total score of less than 4 is indicative of a healthy calf, and which therefore would not need antibiotic therapy. A value of 4 identifies an animal potentially at risk of developing respiratory disease and which must therefore be monitored. Instead, values > 4 characterize an animal affected by BRD and therefore could require antibiotic treatment. The sum of scores gives a value between 0 and 12 and the values for eye discharge and ear position are taken together. The accuracy of this clinical scoring system was recently assessed using a Bayesian latent class framework (Buczinski et al., 2015). The score sensitivity for detection of active BRD was 62.4% and specificity was 74.1% (Buczinski et al., 2018).

TUS score was determined as follows: animals with no lesions or with comet-tail artifacts were given a score of 0 and 1 respectively and were considered healthy. Animals considered sick were given a score from 2 to 5, corresponding to lobular lesions or lobular lesions of increasing severity with involvement of one, two, three or more than three lung lobes (Ollivett and Buczinski, 2016). Blood was immediately transferred to the laboratory after each session of sampling (approximately 20 calves were sampled in each session) and serum was obtained by centrifugation within three hours. Serum samples were then used to measure PON-1 activity using the method described above. Results obtained in the different classes of the WRSC were compared to each other using a non-parametric ANOVA for unpaired samples (Kruskal-Wallis test), followed by a Mann-Whitney U test to compare the results of paired classes.

The same tests were used to compare the results obtained in the different classes of the TUS scoring system. Conversely, results from animals classified as healthy or sick based on the TUS score were compared to each other using a non-parametric t-test for unpaired samples (Mann-Whitney U test). In order to assess the discriminating power of PON-1 activity to identify sick animals (i.e. animals with a TUS score ≥2) and to establish the optimal diagnostic cut-off, for each numerical value recorded in the study (operating point), we classified as true or false positive the calves that had or not a TUS score ≥2, that had PON-1 values lower than each operating point, and as true or false negative the calves that had or not a TUS score ≥2, that had PON-1 values higher than each operating point. Sensitivity and specificity and the positive likelihood ratio were calculated for each operating point, using standard formulae (Gardner and Greiner, 2006; Christenson, 2007), and Receiver Operating Characteristic (ROC) curves were designed by plotting sensitivity versus (1–specificity) (Gardner and Greiner, 2006). The Youden index (i.e., the operating point that maximizes the difference between true positives and false positives) and the operating points characterized by the highest LR+ and by absolute specificity were then calculated (Ruopp et al., 2008).

Results:

The results of different scoring system and PON-1 activity in healthy animals are summarized in table 29 and 30.

Table 29. Results of clinical examination (RCS), thoracic ultrasound (TUS) and PON-1 activity of healthy animals.

N	RSC	TUS	PON-1 (U/mL)	N	RSC	TUS	PON-1 (U/mL)
1	0	1	98.6	34	3	1	73.5
2	2	1	131.2	35	3	1	93.2
3	6	1	70	36	3	1	63.2
4	2	1	64.5	37	3	1	93.6
5	4	1	42	38	1	1	66.1
6	3	1	87.6	39	2	1	79.1
7	6	1	49.8	40	1	1	28
8	6	1	92.6	41	1	1	54.1
9	2	1	105.3	42	4	1	62.8
10	2	1	38.1	43	3	1	52.1
11	3	1	33.6	44	2	1	63.7
12	3	1	118.1	45	2	1	49.9
13	2	1	30.9	46	1	1	71.6
14	3	1	48.8	47	4	1	77.1
15	2	1	48.2	48	3	1	85.9
16	1	1	31,1	49	4	1	72.3
17	2	1	62,1	50	5	1	69.7
18	2	1	67.3	51	5	1	111.4
19	5	1	87	52	4	1	77.3
20	4	1	109.7	53	2	1	79.4
21	3	1	115.2	54	2	1	106.7
22	3	1	94.4	55	2	1	61.9
23	5	1	81.4	56	3	1	69.6
24	2	0	121.6	57	4	0	96.5
25	4	1	108.3	58	3	1	76
26	2	1	79.3	59	2	1	119.8
27	1	1	77.8	60	3	1	88.7
28	1	1	75.9	61	2	1	119.2
29	4	1	93.8	62	3	1	92.7
30	4	1	62.7	63	0	1	135.3
31	4	1	90.7	64	4	1	126.4
32	6	1	58.8	65	3	1	106.3
33	5	1	98.9	66	1	1	104.2

Table 30. Results of clinical examination (RCS), thoracic ultrasound (TUS) and PON-1 activity of sick animals.

N	RSC	TUS	PON-1 (U/mL)
67	5	3	73.3
68	8	5	95.9
69	3	3	35.7
70	4	5	47.5
71	6	5	70.2
72	2	5	42
73	6	2	112.2
74	6	3	107.5
75	2	2	108.8
76	8	5	77.2
77	4	4	69.7
78	5	5	92.8
79	3	3	106.6
80	3	4	78
81	5	2	69.6
82	5	3	82.2
83	6	5	97.8
84	5	2	67.4
85	2	3	67.5
86	3	3	28.1
87	3	4	47.7
88	5	5	43.5
89	5	2	53.2
90	6	2	70.6
91	4	5	39.6
92	2	5	66.8
93	4	3	51
94	4	2	12.1
95	5	5	29.3
96	5	2	52.8
97	6	4	23.9
98	2	3	44.9
99	2	5	64.5
100	1	5	38.1
101	4	4	78.9
102	1	4	39.5
103	1	5	3.1
104	4	4	15.6
105	1	5	76.2
106	4	3	69.5
107	2	5	42.1
108	4	5	36.8
109	1	3	44.5
110	0	2	14.3
111	0	4	27.4
112	4	3	73.3
113	1	3	36.2
114	3	2	23.2
115	4	2	40.4
116	2	4	26
117	1	2	25
118	0	2	33.8
119	2	2	47.8
120	4	5	53
121	4	3	29.2
122	3	2	46.3
123	3	5	35.8
124	5	2	49
125	4	2	78
126	5	4	69
127	4	5	28.2
128	1	2	71.8
129	5	4	59.4
130	6	5	37.3
131	3	5	59.5
132	6	2	35.3
133	5	3	29
134	2	3	32.7
135	9	3	109.8
136	7	3	65.1
137	6	2	116.4
138	8	2	98.9
139	4	2	78.5
140	2	2	59.5
141	4	5	20.5
142	5	3	67.4
143	5	3	82.5
144	1	5	50.3
145	4	5	68.3
146	3	3	84.8
147	1	2	84.2
148	7	5	74.2
149	1	5	99.3
150	5	5	64
151	3	3	18.2
152	1	4	88.7
153	4	3	100.4
154	0	4	70.3
155	3	5	64.2
156	3	3	65.3
157	1	3	95.4
158	3	3	66.8
159	6	3	47.3
160	4	5	65.9
161	5	2	46.7
162	2	3	68
163	1	3	117.8
164	3	4	57.3
165	2	3	64.4
166	3	3	79.1
167	1	2	47.8
168	1	2	66.6
169	3	5	27.3
170	5	5	86.6
171	5	2	113.1
172	5	2	90.2
173	3	2	79.3
174	6	3	69
175	4	4	68.2
176	3	4	71.6
177	8	4	59.1
178	6	4	27.4
179	3	2	119.6
180	2	2	92.8
181	4	3	82.3
182	5	3	90.4
183	8	5	59.2
184	6	5	64.6
185	5	3	54.8
186	4	2	86.4
187	3	3	56.8
188	1	2	49.1
189	3	2	68.3
190	3	5	75.2
191	3	3	57.9
192	2	4	26.4
193	2	5	92.1
194	1	2	55.8
195	1	5	29.4
196	2	3	36.7
197	4	4	36.9
198	6	5	61
199	3	3	24.9

Results regarding PON-1 activity in calves with different WRSC are reported in Figure 30 and summarized in Table 31. Figure 31 summarizes the correlation between the WRSC and PON-1 activity.

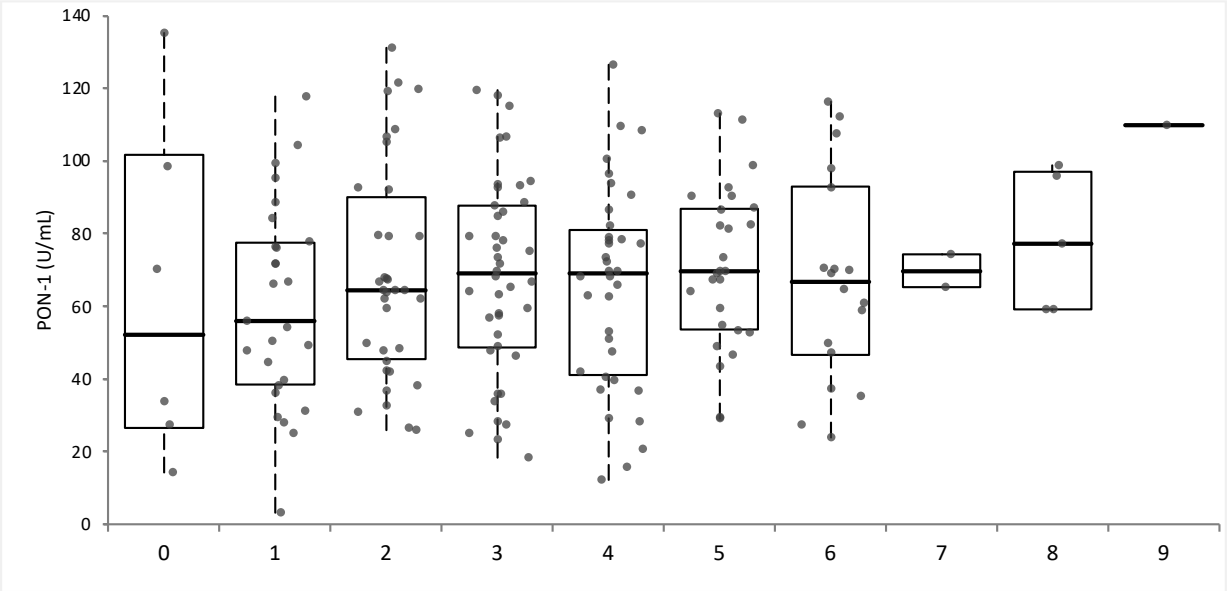


Figure 30. Serum PON-1 activity in calves grouped according to Calf Respiratory Scoring Criteria by Wisconsin University.

Table 31. Serum PON-1 activity in calves grouped according to Calf Respiratory Scoring Criteria by Wisconsin University (WCRSC).

WCRSC	**PON-1** (U/mL)				
	mean ± SD	median	I-III IQR	min-max	n
0	63.28 ± 46.99	52.05	26.31-101.66	14.3-135.3	6
1	60.28 ± 27.78	55.80	38.33-77.53	3.1-117.8	27
2	68.89 ± 29.44	64.50	45.38-89.98	26.0-131.2	35
3	68.33 ± 26.91	68.95	48.71-87.69	18.2-119.6	42
4	63.27 ± 27.79	68.90	41.07-80.88	12.1-126.4	36
5	70.91 ± 22.13	69.60	53.47-86.93	29.0-113.1	27
6	67.32 ± 28.56	66.80	46.47-93.03	23.9-116.4	18
7	69.65 ± 6.43	69.65	65.10-74.20	65.1-74.2	2
8	78.06 ± 19.16	77.20	59-17-96.90	59.1-98.9	5
9	109.80	109.80	109.80-109.80	109.80-109.80	1

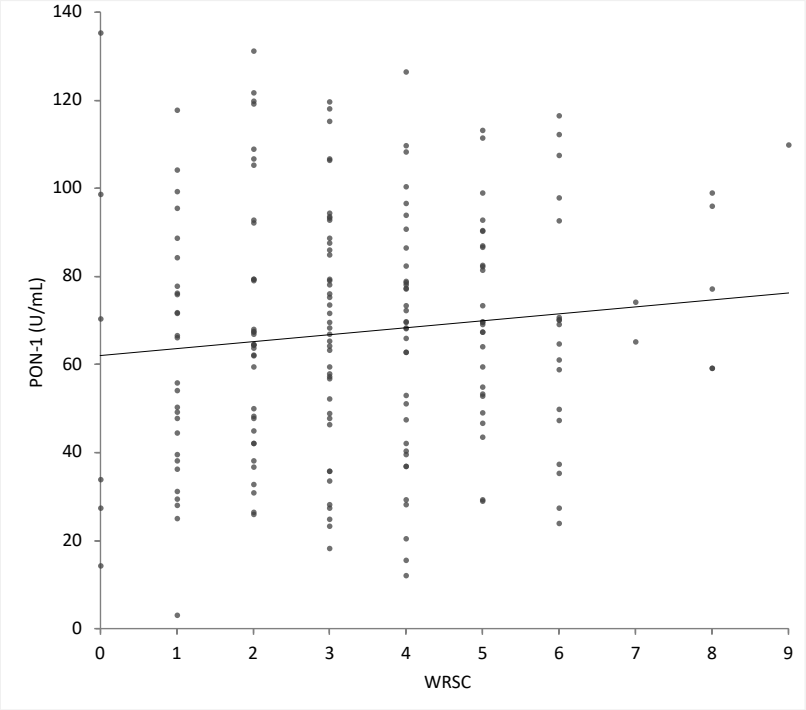


Figure 31. Correlation between WRSC and PON-1 activity.

As shown in Table 31, no significant differences were found in PON-1 activity among different WRSC classes and PON-1 activity seemed to increase with the WRSC score. This hypothesis was confirmed by the trend to a positive correlation between PON-1 activity and the WRSC score, which however, was not statistically significant (P= 0.152; r= 0.102). This finding is somehow unexpected, since, as already described, PON-1 activity is reportedly negatively correlated with disease clinical severity in many diseases of other species (Tvarijonaviciute et al 2015a; Torrente et al., 2019; Pardo-Marin et al., 2020; Rubio et al., 2020). However, it must be considered that the WRCS score mostly refers to symptoms related to the upper airways, and upper way respiratory forms are usually less severe than those affecting lower airways. Indeed, the WRSC score does not have high sensitivity and specificity in detecting BRD (Buczinski et al., 2018). It might be hypothesized that calves given high WRSC scores were not necessarily affected by severe pulmonary lesions leading to oxidative stress and PON-1 activity decrease, and vice versa. This is supported by results regarding PON-1 activity in the different classes of TUS and correlation between TUS and PON-1, which are summarized in Table 32 and Figures 32 and 33.

Table 32. Serum PON-1 activity in calves grouped according to systematic thoracic ultrasound (TUS) score.

TUS	**PON-1** (U/mL)				
	mean ± SD	median	I-III IQR	min-max	n
0	109.05 ± 17.75	109.05	96.50-121.60	96.50-121.60	2
1	79.45 ± 25.98	77.55	62.74-96.85	28.00-135.30	64
2	65.69 ± 29.03 # *	67.00	47.16-85.48	12.10-119.60	36
3	64.52 ± 25.99 # **	66.80	44.57-82.28	18.20-117.80	39
4	52.05 ± 22.39 # ***	58.20	27.40-70.05	15.60-88.70	20
5	57.35 ± 23.62 # ***	60.25	38.03-74.28	3.10-99.30	38

# P<0.05 compared with TUS 0; * P<0.05 compared vs TUS 1; ** P<0.01 compared vs TUS 1; *** P<0.001 compared vs TUS 1

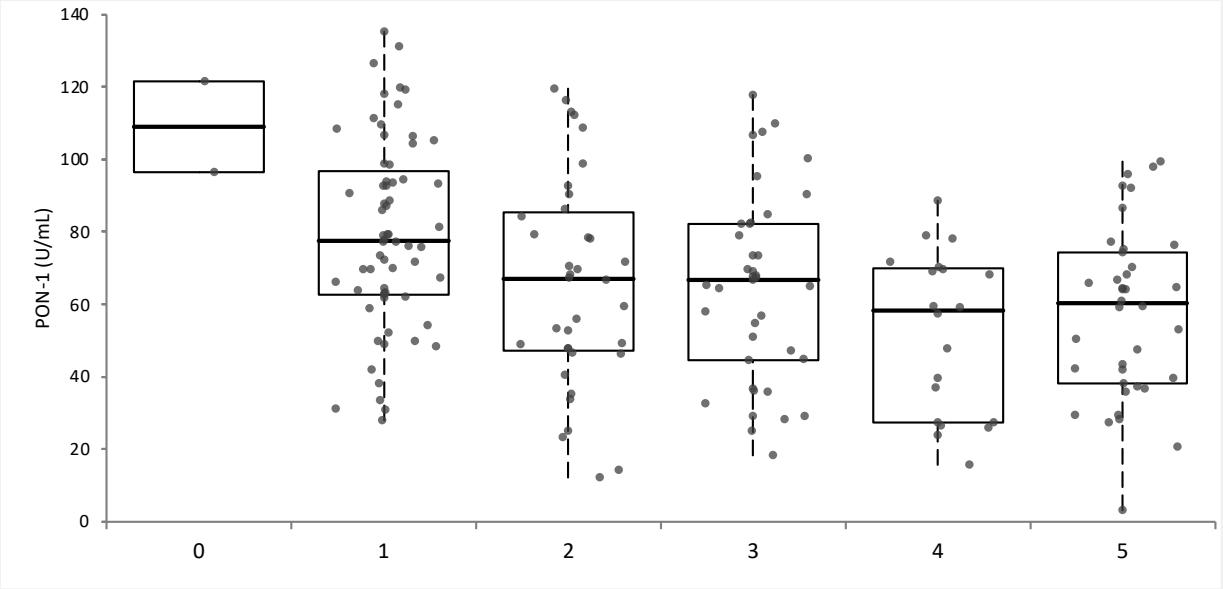


Figure 32. Serum PON-1 activity in calves grouped according to systematic thoracic ultrasound score.

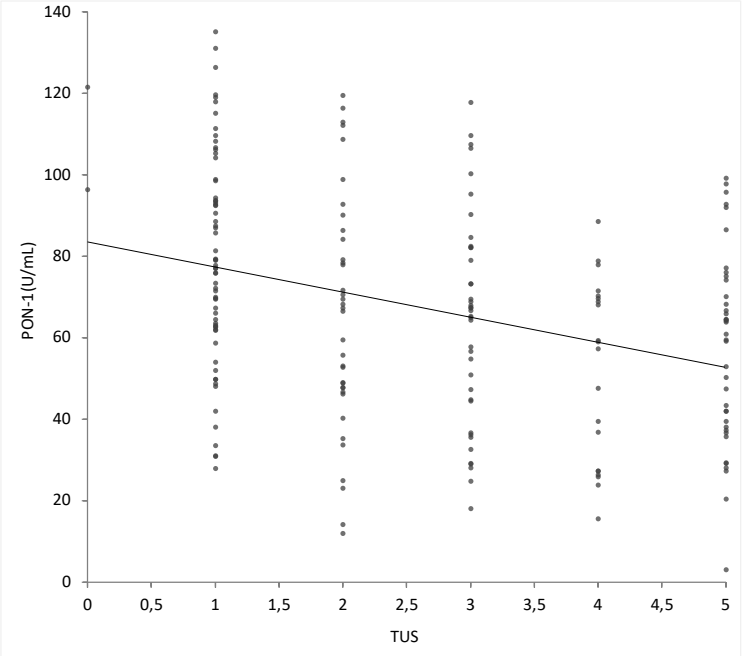


Figure 33. Correlation between TUS and PON-1 activity.

Statistical analysis revealed significant differences between PON-1 activity recorded in the different TUS classes, with values significantly lower in the highest classes compared with the lowest ones. Similarly, a significant negative correlation was found between PON-1 activity and TUS ($P<0.001$; $r=-0.338$). This suggests that, as expected, inflammation and the subsequent oxidative reactions are progressively higher as soon as the TUS score increases. Based on the current knowledge about PON-1 pathophysiology, in these conditions, PON-1 activity might decrease either because of decreased hepatic synthesis (Feingold et al., 1998) or because of displacement from HDL and increased peripheral consumption (Van Lenten et al., 1995).

Results regarding PON-1 activity were analyzed with respect to the presence or absence of disease based on the TUS score and are summarized in Figure 34. Calves with TUS score equal to or higher than 2 were considered diseased.

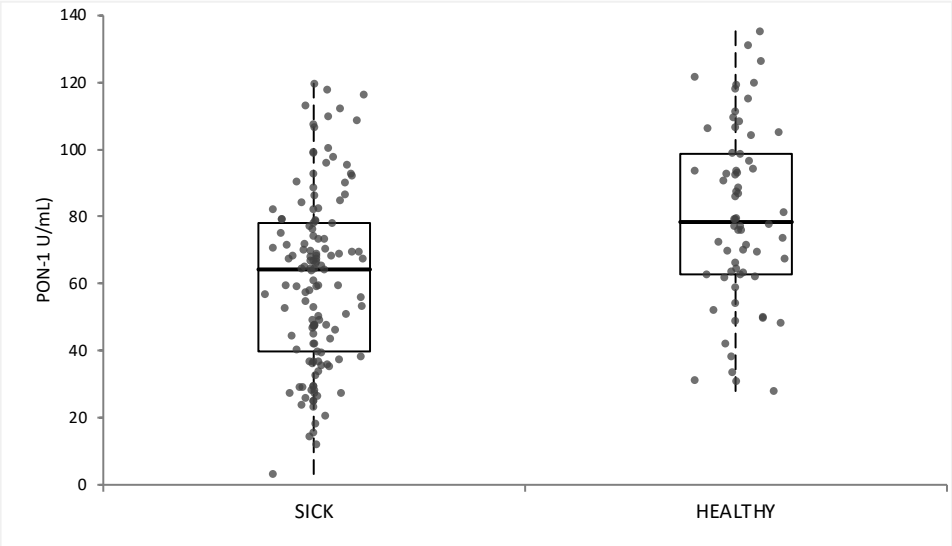


Figure 34. Serum PON-1 activity in calves classified as sick or healthy based on systematic thoracic ultrasound score (TUS).

Briefly, PON-1 activity was significantly lower in clinically sick calves (mean ± SD: 60.91 ± 25.92; median: 64.20; interquartile range: 39.57-78.00; n=133) than in clinically healthy calves (80.34 ± 26.18; 78.45; 28.00-98.63; n=66) (P<0.001). This result is not surprising, based on our current knowledge on the pathophysiology of PON-1 also in this species (Bionaz et al., 2007; Giordano et al., 2013) and is in line with previous preliminary reports about changes in PON-1 activity recorded in sick cows or calves of different age (Giordano et al., 2013).

As an attempt to assess whether the magnitude of changes of PON-1 activity may predict the presence of pulmonary lesions and therefore classify as sick animals with potential respiratory disease, we designed a ROC curve. The curve was significantly different from the line of no discrimination (P<0.001). However, the area under the curve (AUC) was not particularly high (69.8%; 95% CI: 62.1-77.4) (Figure 35). The analysis of the diagnostic parameters designed by the ROC curve evidenced that only at very low PON-1 values (in our caseload 30.9 U/mL), the probability that animals have a respiratory diseases characterized by pathological values of TUS is notably higher than the probability to not have pathological changes (at the threshold above the LR+ was 10.42). Furthermore, the absolute specificity that allows to classify an animal as affected by pulmonary lesions without false positive results was even lower (<28.0 U/mL). Conversely, based on the youden index, PON-1 analysis may maximize its performances at PON-1 values of 69.6 U/mL, when sensitivity and specificity are 64.7% and 66.7% respectively.

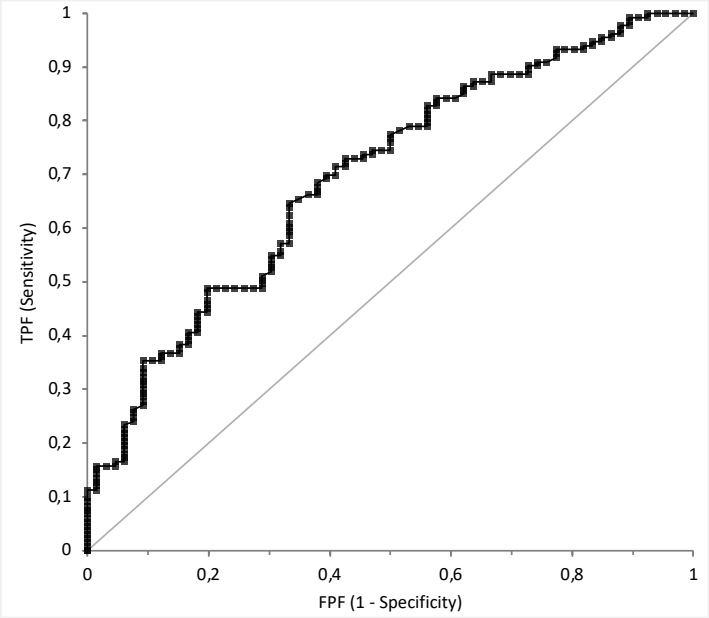


Figure 35. ROC curve designed to assess the discriminating power of PON-1 in detecting sick calves. The grey line indicates the line of no discrimination. TPF, true positive fraction; FPF, false positive fraction.

Conclusive remarks

In conclusion, these results confirm that the measurement of PON-1 may be a rapid tool to identify animals that will likely have a high TUS score. Conversely, this marker does not seem to well correlate with the clinical score, likely because the latter is poorly sensitive or specific. The comparison of results of the WRSC with those of PON-1 suggests that in some cases, animals with severe clinical signs are actually not affected by inflammatory processes so severe to induce oxidation and, vice versa, that oxidative phenomena may be already present despite the absence of severe clinical signs. This is not surprising, since also in other species it has been demonstrated that the severity of the clinical condition may not depend only on the severity and magnitude of the local inflammatory reaction. The design of this study did not allow to fully exploit this hypothesis. However, further studies based on the comparison of results of PON-1 with those of other markers of inflammation (e.g. major bovine acute phase proteins such as haptoglobin or SAA) should be designed to clarify this aspect. Therefore, PON-1 could be used when animals show clinical signs regardless of their severity, or as screening test in herds characterized by high prevalence of respiratory disorders, in order to select those animals that require additional invasive approaches or an ultrasound investigation. The results of this study open new insights about the possible role of PON-1 in assessing health or disease in bovine species.

In particular, it would important, through future studies, to investigate the mechanisms that account for the discrepancy between the clinical status and PON-1 levels, to assess the correlation between PON-1 and other markers of inflammation, and to investigate whether the presence/absence or the magnitude of decrease of PON-1 activity may be associated with some specific type of pulmonary lesions.

CONCLUSIONS

The results collected in the different studies described in this thesis provided further insights about the possible utility of PON-1 as a biomarker in veterinary clinical pathology. More specifically, this thesis confirmed the role of PON-1 as a marker of SIRS, possibly associated with sepsis in several species. This was particularly evident in horses, in which it was demonstrated an inverse relationship between PON-1 activity and clinical or laboratory signs suggestive of SIRS in animals with experimentally induced endotoxemia and with spontaneous diseases. We demonstrated that low PON-1 activity is potentially associated with SIRS (although normal levels of do not allow to exclude SIRS) and potentially indicative of a poor prognosis. In dogs as well, this marker had better diagnostic performance compared with other markers conventionally associated with severe inflammation, like C-reactive protein, or innovative markers of oxidation like protein carbonyls. In addition, PON-1 proved to be useful in identifying subclinical changes potentially consistent with an activation of the innate response, like in dogs with borreliosis or in cats with leishmaniasis. In these conditions, decreases of PON-1 may be observed also in seropositive animals not showing clinical signs, suggesting that host-pathogen interactions could be occurring in the absence of overt disease. We also highlighted the possibility to include PON-1 analysis in laboratory profiles as an ancillary test to support clinical decisions or address further testing. In dogs, PON-1 activity was particularly decreased in subjects admitted to the hospital wards where acute or severe diseases are more likely (e.g. first aid/primary care service, oncology) and in subjects requiring hospitalization in the ICU. A practical recommendation could be to measure PON-1 activity at admission and to hospitalize dogs when values are very low, with following periodical assessment of PON-1 activity during the follow up. Indeed, the lack of increase in PON-1 activity in the first days after hospitalization may be associated with a negative outcome. In calves with respiratory disease, PON-1 values better correlated with ultrasonographic scores consistent with lung inflammation compared with clinical scores. Therefore, measurement of PON-1 activity may be recommended as a screening test, to select animals requiring more thorough clinical examination or time-consuming and laborious approaches like diagnostic imaging. The use of PON-1 as a screening test was also evaluated in swine at slaughter, assessing the ability to predict presence and severity of lesions, but differently from other species, the performances of this analyte as an ancillary test to address further *post mortem* analyses were not satisfactory. However, the multispecies approach followed in this thesis showed that the potential utility of PON-1 varies among species, likely due to species-specific metabolic peculiarities and/or different levels of expression of PON-1 genes.

Similar to what is recommended in people, further studies using substrates other than paraoxon, as well as genetic investigations on PON-1 polymorphisms, should be encouraged in the future in veterinary species, to provide a more extensive overview of the practical utility of PON-1.

In conclusion, the information produced by the different studies allowed to satisfy the general aims of the thesis and may serve as basis for future studies about the additional research questions arisen within this thesis. Overall, this thesis demonstrated that PON-1 may be considered a useful, cheap and rapid diagnostic marker to provide basic information when SIRS or sepsis are clinically suspected. Moreover, it may be advisable to introduce measurement of PON-1 in routine testing in small and large animal practice, either for diagnostic or prognostic purposes or to provide ancillary information that may address clinical decisions.

REFERENCES

Abu-Zidan FM, Plank LD, Windsor JA. (2002). Proteolysis in severe sepsis is related to oxidation of plasma protein. *Eur J Surg*. 168(2):119-123. doi:10.1080/11024150252884359

Ackermann MR. (2017). Inflammation and Healing. In: Zachary JF Ed. Pathologic basis of veterinary disease. Elsevier Saunders Edn. 6th Ed. St. Louis, Missouri, USA 73-131

Adkins S, Gan KN, Mody M, La Du BN. (1993). Molecular basis for the polymorphic forms of human serum paraoxonase/arylesterase: glutamine or arginine at position 191, for the respective A or B allozymes. *Am J Hum Genet*. 52(3):598-608. PMID:7916578

Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. (2001). Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor. *J Biol Chem*. 276(27):24473-24481. doi:10.1074/jbc.M010459200

Aldridge WN. (1953a). Serum esterases. I. Two types of esterase (A and B) hydrolysing p-nitrophenyl acetate, propionate and butyrate, and a method for their determination. *Biochem J*. 53(1):110-117. doi:10.1042/bj0530110

Aldridge WN. (1953b). Serum esterases. II. An enzyme hydrolysing diethyl p-nitrophenyl phosphate (E600) and its identity with the A-esterase of mammalian sera. *Biochem J*. 1953;53(1):117-124. doi:10.1042/bj0530117

Almeida BF, Narciso LG, Melo LM, Preve PP, Bosco AM, Lima VM, Ciarlini PC. (2013). Leishmaniasis causes oxidative stress and alteration of oxidative metabolism and viability of neutrophils in dogs. *Vet J*. 198(3):599-605. doi:10.1016/j.tvjl.2013.08.024

Almela RM, Rubio CP, Cerón JJ, Ansón A, Tichy A, Mayer U. (2018). Selected serum oxidative stress biomarkers in dogs with non-food-induced and food-induced atopic dermatitis. *Vet Dermatol*. 29(3):229-e82. doi:10.1111/vde.12525

Alonso de Vega JM, Díaz J, Serrano E, Carbonell LF. (2000). Plasma redox status relates to severity in critically ill patients. *Crit Care Med*. 28(6):1812-1814. doi:10.1097/00003246-200006000-00021

Alonso de Vega JM, Díaz J, Serrano E, Carbonell LF. (2002). Oxidative stress in critically ill patients with systemic inflammatory response syndrome. *Crit Care Med*. 30(8):1782-1786. doi:10.1097/00003246-200208000-00018

Amorim de Oliveira YP, Pontes-de-Carvalho LC, Couto RD, Noronha-Dutra AA. (2017). Oxidative stress in sepsis. Possible production of free radicals through an erythrocyte-mediated positive feedback mechanism. *Braz J Infect Dis*. 21(1):19-26. doi:10.1016/j.bjid.2016.11.004

Amulic B, Cazalet C, Hayes GL, Metzler KD, Zychlinsky A. (2012). Neutrophil function: from mechanisms to disease. *Annu Rev Immunol*. 30:459-489. doi:10.1146/annurev-immunol-020711-074942

Atay AE, Kaplan MA, Evliyaoglu O, Ekin N, Isıkdogan A. (2014). The predictive role of Paraoxonase 1 (PON1) activity on survival in patients with metastatic and nonmetastatic gastric cancer. *Clin Ter*. 165(1):e1-e5. doi:10.7471/CT.2014.1663

Augustinsson KB, Barr M. (1963). Age variation in plasma arylesterase activity in children. *Clin Chim Acta*. 1963;8:568-573. doi:10.1016/0009-8981(63)90106-2

Aviram M, Billecke S, Sorenson R, Bisgaier C, Newton R, Rosenblat M, Erogul J, Hsu C, Dunlop C, La Du B. (1998a). Paraoxonase active site required for protection against LDL oxidation involves its free sulfhydryl group and is different from that required for its arylesterase/paraoxonase activities: selective action of human paraoxonase allozymes Q and R. *Arterioscler Thromb Vasc Biol*. 18(10):1617-1624. doi:10.1161/01.atv.18.10.1617

Aviram M, Rosenblat M, Billecke S, Erogul J, Sorenson R, Bisgaier CL, Newton RS, La Du B. (1999). Human serum paraoxonase (PON 1) is inactivated by oxidized low density lipoprotein and preserved by antioxidants. *Free Radic Biol Med*. 26(7-8):892-904. doi:10.1016/s0891-5849(98)00272-x

Aviram M, Rosenblat M, Bisgaier CL, Newton RS, Primo-Parmo SL, La Du BN. (1998b). Paraoxonase inhibits high-density lipoprotein oxidation and preserves its functions. A possible peroxidative role for paraoxonase. *J Clin Invest*. 101(8):1581-1590. doi:10.1172/JCI1649

Aviram M, Rosenblat M. (2004). Paraoxonases 1, 2, and 3, oxidative stress, and macrophage foam cell formation during atherosclerosis development. *Free Radic Biol Med*. 37(9):1304-1316. doi:10.1016/j.freeradbiomed.2004.06.030

Balk RA. Systemic inflammatory response syndrome (SIRS): where did it come from and is it still relevant today? (2014). *Virulence*. 5(1):20-26. doi:10.4161/viru.27135

Barter PJ, Rye KA. (2001). Cholesteryl ester transfer protein, high density lipoprotein and arterial disease. *Curr Opin Lipidol*. 12(4):377-382. doi:10.1097/00041433-200108000-00002

Bavunoglu I, Genc H, Konukoglu D, Cicekci H, Sozer V, Gelisgen R, Uzun H. (2016). Oxidative stress parameters and inflammatory and immune mediators as markers of the severity of sepsis. *J Infect Dev Ctries*. 10(10):1045-1052. doi:10.3855/jidc.7585

Bednarz-Misa I, Mierzchala-Pasierb M, Lesnik P, Placzkowska S, Kedzior K, Gamian A, Krzystek-Korpacka M. (2019). Cardiovascular Insufficiency, Abdominal Sepsis, and Patients' Age Are Associated with Decreased Paraoxonase-1 (PON1) Activity in Critically Ill Patients with Multiple Organ Dysfunction Syndrome (MODS). *Dis Markers*. 2019:1314623. doi:10.1155/2019/1314623

Bhattacharyya T, Nicholls SJ, Topol EJ, Zhang R, Yang X, Schmitt D, Fu X, Shao M, Brennan DM, Ellis SG, Brennan ML, Allayee H, Lusis AJ, Hazen SL. (2008). Relationship of paraoxonase 1 (PON1) gene polymorphisms and functional activity with systemic oxidative stress and cardiovascular risk. *JAMA*. 299(11):1265-1276. doi:10.1001/jama.299.11.1265

Bin Ali A, Zhang Q, Lim YK, Fang D, Retnam L, Lim SK. (2003). Expression of major HDL-associated antioxidant PON-1 is gender dependent and regulated during inflammation. *Free Radic Biol Med*. 34(7):824-829. doi:10.1016/s0891-5849(02)01436-3

Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A, Bertoni G. (2007) Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. *J Dairy Sci*. 90(4):1740-1750. doi:10.3168/jds.2006-445

Bojic S, Kotur-Stevuljevic J, Kalezic N, Jelic-Ivanovic Z, Stefanovic A, Palibrk I, Memon L, Kalaba Z, Stojanovic M, Simic-Ogrizovic S. (2014). Low paraoxonase 1 activity predicts mortality in surgical patients with sepsis. *Dis Markers*. 2014:427378. doi:10.1155/2014/427378

Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. (1992). *Chest*. 101(6):1644-1655. doi:10.1378/chest.101.6.1644

Bonelli F, Meucci V, Divers TJ, Wagner B, Intorre L, Sgorbini M (2017). Kinetics of plasma procalcitonin, soluble CD14, CCL2 and IL-10 after a sublethal infusion of lipopolysaccharide in horses. *Vet Immunol Immunopathol*. 184:29-35. doi:10.1016/j.vetimm.2016.12.010

Borges AS, Divers TJ, Stokol T, Mohammed OH. (2007). Serum iron and plasma fibrinogen concentrations as indicators of systemic inflammatory diseases in horses. *J Vet Intern Med*. 21(3):489-494. doi:10.1892/0891-6640(2007)21[489:siapfc]2.0.co;2

Bottari NB, Munhoz TD, Torbitz VD, Tonin AA, Anai LA, Semolin LM, Jark PC, Bollick YS, Moresco RN, França RT, Lopes ST, Stefani LM, Tinucci-Costa M, Da Silva AS. (2015). Oxidative stress in dogs with multicentric lymphoma: Effect of chemotherapy on oxidative and antioxidant biomarkers. *Redox Rep*. 20(6):267-274. doi:10.1179/1351000215Y.0000000037

Bouadma L, Luyt CE, Tubach F, Cracco C, Alvarez A, Schwebel C, Schortgen F, Lasocki S, Veber B, Dehoux M, Bernard M, Pasquet B, Régnier B, Brun-Buisson C, Chastre J, Wolff M. (2010). Use of procalcitonin to reduce patients' exposure to antibiotics in intensive care units (PRORATA trial): a multicentre randomised controlled trial. *Lancet*. 375:463-74. doi:10.1016/S0140-6736(09)61879-1

Brady CA, Otto CM. (2001). Systemic inflammatory response syndrome, sepsis, and multiple organ dysfunction. *Vet Clin North Am Small Anim Pract*. 31(6):1147-vi. doi:10.1016/s0195-5616(01)50097-2

Brophy VH, Hastings MD, Clendenning JB, Richter RJ, Jarvik GP, Furlong CE. (2001a). Polymorphisms in the human paraoxonase (PON1) promoter. Pharmacogenetics. 11(1):77-84. doi:10.1097/00008571-200102000-00009

Brophy VH, Jarvik GP, Furlong CE. (2002). PON1 polimorphisms. In: Costa LG, Furlong CE Eds. Paraoxonase (PON1) in Health and Disease: Basic and Clinical Aspects. Springer. 1st Ed. Boston, MA 53-78

Buczinski S, Fecteau G, Dubuc J, Francoz D. (2018). Validation of a clinical scoring system for bovine respiratory disease complex diagnosis in preweaned dairy calves using a Bayesian framework. *Prev Vet Med*. 156:102-112. doi:10.1016/j.prevetmed.2018.05.004

Buczinski S, L Ollivett T, Dendukuri N. (2015). Bayesian estimation of the accuracy of the calf respiratory scoring chart and ultrasonography for the diagnosis of bovine respiratory disease in pre-weaned dairy calves. *Prev Vet Med*. 119(3-4):227-231. doi:10.1016/j.prevetmed.2015.02.018

Cabana VG, Reardon CA, Feng N, Neath S, Lukens J, Getz GS. (2003). Serum paraoxonase: effect of the apolipoprotein composition of HDL and the acute phase response. *J Lipid Res*. 44(4):780-792. doi:10.1194/jlr.M200432-JLR200

Cals JW, Butler CC, Hopstaken RM, Hood K, Dinant GJ. (2009). Effect of point of care testing for C reactive protein and training in communication skills on antibiotic use in lower respiratory tract infections: cluster randomised trial. *BMJ*. 338:b1374. doi:10.1136/bmj.b1374

Camps J, Iftimie S, García-Heredia A, Castro A, Joven J. (2017). Paraoxonases and infectious diseases. *Clin Biochem*. 50(13-14):804-811. doi:10.1016/j.clinbiochem.2017.04.016

Camps J, Marsillach J, Joven J. (2009). The paraoxonases: role in human diseases and methodological difficulties in measurement. *Crit Rev Clin Lab Sci*. 46(2):83-106. doi:10.1080/10408360802610878

Cantos-Barreda A, Escribano D, Cerón JJ, Bernal LJ, Furlanello T, Tecles F, Pardo-Marín L, Martínez-Subiela S. (2018). Relationship between serum anti-Leishmania antibody levels and acute phase proteins in dogs with canine leishmaniosis. *Vet Parasitol*. 260:63-68. doi:10.1016/j.vetpar.2018.08.010

Carretón E, Cerón JJ, Martínez-Subiela S, Tvarijonaviciute A, Caro-Vadillo A, Montoya-Alonso JA. (2017). Acute phase proteins and markers of oxidative stress to assess the severity of the pulmonary hypertension in heartworm-infected dogs. *Parasit Vectors*. 10(Suppl 2):477. Published 2017 Nov 9. doi:10.1186/s13071-017-2426-8

Ceciliani F, Giordano A, Spagnolo V. (2002). The systemic reaction during inflammation: the acute-phase proteins. *Protein Pept Lett*. 9(3):211-223. doi:10.2174/0929866023408779

Ceron JJ, Eckersall PD, Martýnez-Subiela S. (2005). Acute phase proteins in dogs and cats: current knowledge and future perspectives. *Vet Clin Pathol*. 34(2):85-99. doi:10.1111/j.1939-165x.2005.tb00019.x

Ceron JJ, Tecles F, Tvarijonaviciute A. (2014). Serum paraoxonase 1 (PON1) measurement: an update. *BMC Vet Res*. 10:74. doi:10.1186/1746-6148-10-74

Chang YF, Straubinger RK, Jacobson RH, et al. (1996). Dissemination of Borrelia burgdorferi after experimental infection in dogs. *J Spirochetal Tick-Borne Dis*. 3:80–86.

Chen J, Kumar M, Chan W, Berkowitz G, Wetmur JG. (2003). Increased influence of genetic variation on PON1 activity in neonates. *Environ Health Perspect*. 111(11):1403-1409. doi:10.1289/ehp.6105

Christenson RH; Committee on Evidence Based Laboratory Medicine of the International Federation for Clinical Chemistry Laboratory Medicine. (2007). Evidence-based laboratory medicine - a guide for critical evaluation of in vitro laboratory testing. *Ann Clin Biochem*. 44(Pt 2):111-130. doi:10.1258/000456307780118127

Cole TB, Jampsa RL, Walter BJ, Arndt TL, Richter RJ, Shih DM, Tward A, Lusis AJ, Jack RM, Costa LG, Furlong CE. (2003). Expression of human paraoxonase (PON1) during development. Pharmacogenetics. 13(6):357-364. doi:10.1097/00008571-200306000-00007

Costa LG, Cole TB, Vitalone A, Furlong CE. (2005a). Measurement of paraoxonase (PON1) status as a potential biomarker of susceptibility to organophosphate toxicity. *Clin Chim Acta*. 352(1-2):37-47. doi:10.1016/j.cccn.2004.09.019

Costa LG, Vitalone A, Cole TB, Furlong CE. (2005b). Modulation of paraoxonase (PON1) activity. *Biochem Pharmacol*. 69(4):541-550. doi:10.1016/j.bcp.2004.08.027

Cray C, Zaias J, Altman NH. (2009). Acute phase response in animals: a review. *Comp Med*. 59(6):517-526.

Da Silva AS, Munhoz TD, Faria JL, Vargas-Hérnandez G, Machado RZ, Almeida TC, Moresco RN, Stefani LM, Tinucci-Costa M. (2013). Increase nitric oxide and oxidative stress in dogs

experimentally infected by Ehrlichia canis: effect on the pathogenesis of the disease. *Vet Microbiol*. 164(3-4):366-369. doi:10.1016/j.vetmic.2013.03.003

Dalle-Donne I, Scaloni A, Giustarini D, Cavarra E, Tell G, Lungarella G, Colombo R, Rossi R, Milzani A. (2005). Proteins as biomarkers of oxidative/nitrosative stress in diseases: the contribution of redox proteomics. *Mass Spectrom Rev*. 24(1):55-99. doi:10.1002/mas.20006

Davies HG, Richter RJ, Keifer M, Broomfield CA, Sowalla J, Furlong CE. (1996). The effect of the human serum paraoxonase polymorphism is reversed with diazoxon, soman and sarin. *Nat Genet*. 14(3):334-336. doi:10.1038/ng1196-334

Davies MG, Hagen PO. (1997). Systemic inflammatory response syndrome. *Br J Surg*. 84(7):920-935. doi:10.1002/bjs.1800840707

Deakin S, Leviev I, Gomaraschi M, Calabresi L, Franceschini G, James RW. (2002). Enzymatically active paraoxonase-1 is located at the external membrane of producing cells and released by a high affinity, saturable, desorption mechanism. *J Biol Chem*. 277(6):4301-4308. doi:10.1074/jbc.M107440200

Draganov D, Teiber J, Watson C, Bisgaier C, Nemzek J, Remick D, Standiford T, La Du B. (2010). PON1 and oxidative stress in human sepsis and an animal model of sepsis. *Adv Exp Med Biol*. 2010;660:89-97. doi:10.1007/978-1-60761-350-3_9

Draganov DI, La Du BN. (2004). Pharmacogenetics of paraoxonases: a brief review. Naunyn Schmiedebergs Arch Pharmacol. 369(1):78-88. doi:10.1007/s00210-003-0833-1

Draganov DI, Teiber JF, Speelman A, Osawa Y, Sunahara R, La Du BN. (2005). Human paraoxonases (PON1, PON2, and PON3) are lactonases with overlapping and distinct substrate specificities. *J Lipid Res*. 46(6):1239-1247. doi:10.1194/jlr.M400511-JLR200

Eckerson HW, Wyte CM, La Du BN. (1983). The human serum paraoxonase/arylesterase polymorphism. *Am J Hum Genet*. 1983;35(6):1126-1138. PMID:6316781

Eckerson HW, Wyte CM, La Du BN. (1983). The human serum paraoxonase/arylesterase polymorphism. *Am J Hum Genet*. 35(6):1126-1138.

ecles F, Caldín M, Tvarijonaviciute A, Escribano D, Martínez-Subiela S, Cerón JJ. (2015). Serum biomarkers of oxidative stress in cats with feline infectious peritonitis. *Res Vet Sci*. 100:12-17. doi:10.1016/j.rvsc.2015.02.007

ecles F, Spiranelli E, Bonfanti U, Cerón JJ, Paltrinieri S. (2005). Preliminary studies of serum acute-phase protein concentrations in hematologic and neoplastic diseases of the dog. *J Vet Intern Med*. 19(6):865-870. doi:10.1892/0891-6640(2005)19[865:psosap]2.0.co;2

Ecobichon DJ, Stephens DS. (1973). Perinatal development of human blood esterases. *Clin Pharmacol Ther*. 14(1):41-47. doi:10.1002/cpt197314141

Ehlting C, Wolf SD, Bode JG. (2021). Acute-phase protein synthesis: a key feature of innate immune functions of the liver. *Biol Chem*. 402(9):1129-1145. doi:10.1515/hsz-2021-0209

Endo Y, Cho KW, Nishigaki K, Momoi Y, Nishimura Y, Mizuno T, Goto Y, Watari T, Tsujimoto H, Hasegawa A. (1997). Molecular characteristics of malignant lymphomas in cats naturally infected with feline immunodeficiency virus. *Vet Immunol Immunopathol*. 57(3-4):153-167. doi:10.1016/s0165-2427(97)00004-4

Erdös EG, Boggs LE. (1961). Hydrolysis of paraoxon in mammalian blood. *Nature* 190:716-717. https://doi.org/10.1038/190716a0

Escribano D, Tvarijonaviciute A, Tecles F, Cerón JJ. (2015). Serum paraoxonase type-1 activity in pigs: assay validation and evolution after an induced experimental inflammation. *Vet Immunol Immunopathol*. 163(3-4):210-215. doi:10.1016/j.vetimm.2014.12.002

Fazio F, Casella S, Giannetto C, Giudice E, Piccione G (2015). Characterization of acute phase proteins and oxidative stress response to road transportation in the dog. *Exp Anim*. 64(1):19-24. doi:10.1538/expanim.14-0032

Feingold KR, Memon RA, Moser AH, Grunfeld C. (1998). Paraoxonase activity in the serum and hepatic mRNA levels decrease during the acute phase response. *Atherosclerosis*. 139(2):307-315. doi:10.1016/s0021-9150(98)00084-7

Ferré N, Camps J, Marsillach J, Mackness B, Mackness M, Coll B, Tous M, Joven J. (2005). Comparison of paraoxonase 1 measurements in serum and in lithium-heparin-anticoagulated plasma samples. *Clin Chem*. 51(5):922-923. doi:10.1373/clinchem.2005.048231

Ferré N, Camps J, Prats E, Vilella E, Paul A, Figuera L, Joven J. (2002a). Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. Clin Chem. 48(2):261-8. PMID:11805006

Ferré N, Tous M, Paul A, Zamora A, Vendrell JJ, Bardají A, Camps J, Richart C, Joven J. (2002b). Paraoxonase Gln-Arg(192) and Leu-Met(55) gene polymorphisms and enzyme activity in a population with a low rate of coronary heart disease. *Clin Biochem*. 35(3):197-203. doi:10.1016/s0009-9120(02)00295-3

Ferretti G, Bacchetti T, Busni D, Rabini RA, Curatola G. (2004). Protective effect of paraoxonase activity in high-density lipoproteins against erythrocyte membranes peroxidation: a comparison between healthy subjects and type 1 diabetic patients. *J Clin Endocrinol Metab*. 89(6):2957-2962. doi:10.1210/jc.2003-031897

Ferretti G, Bacchetti T. (2012). Effect of dietary lipids on paraoxonase-1 activity and gene expression. *Nutr Metab Cardiovasc Dis*. 22(2):88-94. doi:10.1016/j.numecd.2011.08.011

Fogle J, Jacob M, Blikslager A, Edwards A, Wagner B, Dean K, Fogle C. (2017). Comparison of lipopolysaccharides and soluble CD14 measurement between clinically endotoxaemic and nonendotoxaemic horses. *Equine Vet J*. 49(2):155-159. doi:10.1111/evj.12582

Francino O, Altet L, Sánchez-Robert E, Rodriguez A, Solano-Gallego L, Alberola J, Ferrer L, Sánchez A, Roura X. (2006). Advantages of real-time PCR assay for diagnosis and monitoring of canine leishmaniosis. *Vet Parasitol*. 137(3-4):214-221. doi:10.1016/j.vetpar.2006.01.011

Fransson BA, Karlstam E, Bergstrom A, Lagerstedt AS, Park JS, Evans MA, Ragle CA. (2004). C-reactive protein in the differentiation of pyometra from cystic endometrial hyperplasia/mucometra in dogs. *J Am Anim Hosp Assoc*. 40(5):391-399. doi:10.5326/0400391

Fransson BA, Lagerstedt AS, Bergsttrom A, Hagman R, Park JS, Chew BP, Evans MA, Ragle CA. (2007). C-Reactive protein, tumor necrosis factor a, and interleukin-6 in dogs with pyometra and SIRS. J. Vet. Emerg. *Crit. Care*. 17,373-381. doi:10.1111/j.1476-4431.2006.00203.x

Friedrichs KR, Harr KE, Freeman KP, Szladovits B, Walton RM, Barnhart KF, Blanco-Chavez J; American Society for Veterinary Clinical Pathology. (2012). ASVCP reference interval guidelines: determination of de novo reference intervals in veterinary species and other related topics. *Vet Clin Pathol*. 41(4):441-453. doi:10.1111/vcp.12006

Fuhrman B, Volkova N, Aviram M. (2002). Oxidative stress increases the expression of the CD36 scavenger receptor and the cellular uptake of oxidized low-density lipoprotein in macrophages from atherosclerotic mice: protective role of antioxidants and of paraoxonase. *Atherosclerosis*. 161(2):307-316. doi:10.1016/s0021-9150(01)00646-3

Furlong CE, Holland N, Richter RJ, Bradman A, Ho A, Eskenazi B. (2006). PON1 status of farmworker mothers and children as a predictor of organophosphate sensitivity. *Pharmacogenet Genomics*. 16(3):183-190. doi:10.1097/01.fpc.0000189796.21770.d3

Furlong CE, Richter RJ, Seidel SL, Costa LG, Motulsky AG. (1989). Spectrophotometric assays for the enzymatic hydrolysis of the active metabolites of chlorpyrifos and parathion by plasma paraoxonase/arylesterase. *Anal Biochem*. 180(2):242-247. doi:10.1016/0003-2697(89)90424-7

Furlong CE. (2008). Paraoxonases: an historical perspective. In Mackness B, Mackness M, Aviram M, Paragh G, Eds. The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism. Springer Eds. Dordrecht. 3-31

Gaidukov L, Tawfik DS. (2005). High affinity, stability, and lactonase activity of serum paraoxonase PON1 anchored on HDL with ApoA-I. *Biochemistry*. 44(35):11843-11854. doi:10.1021/bi050862i

Galley HF. (2011). Oxidative stress and mitochondrial dysfunction in sepsis. *Br J Anaesth*. 107(1):57-64. doi:10.1093/bja/aer093

Gan KN, Smolen A, Eckerson HW, La Du BN. (1991). Purification of human serum paraoxonase/arylesterase. Evidence for one esterase catalyzing both activities. *Drug Metab Dispos*. 19(1):100-106. PMID:1673382

Gardner IA, Greiner M. (2006). Receiver-operating characteristic curves and likelihood ratios: improvements over traditional methods for the evaluation and application of veterinary clinical pathology tests. *Vet Clin Pathol*. 35(1):8-17. doi:10.1111/j.1939-165x.2006.tb00082.x

Garin MC, James RW, Dussoix P, Blanché H, Passa P, Froguel P, Ruiz J. (1997). Paraoxonase polymorphism Met-Leu54 is associated with modified serum concentrations of the enzyme. A possible link between the paraoxonase gene and increased risk of cardiovascular disease in diabetes. *J Clin Invest*. 99(1):62-66. doi:10.1172/JCI119134

Gebhardt C, Hirschberger J, Rau S, Arndt G, Krainer K, Schweigert FJ, Brunnberg L, Kaspers B, Kohn B. (2009). Use of C-reactive protein to predict outcome in dogs with systemic inflammatory response syndrome or sepsis. *J Vet Emerg Crit Care (San Antonio)*. 19(5):450-458. doi:10.1111/j.1476-4431.2009.00462.x

Georgopoulou AP, Savva A, Giamarellos-Bourboulis EJ, Georgitsi M, Raftogiannis M, Antonakos N, Apostolidou E, Carrer DP, Dimopoulos G, Economou A, Efthymiou G, Galanakis N, Galani L, Gargalianos P, Karaiskos I, Katsenos C, Kavatha D, Koratzanis E, Labropoulos P, Lada M, Nakos G, Paggalou E, Panoutsopoulos G, Paraschos M, Pavleas I, Pontikis K,

Poulakou G, Prekates A, Sybardi S, Theodorakopoulou M, Trakatelli C, Tsiaoussis P, Gogos C, Giamarellou H, Armaganidis A, Meisner M; Hellenic Sepsis Study Group. (2011). Early changes of procalcitonin may advise about prognosis and appropriateness of antimicrobial therapy in sepsis. *J Crit Care*. 26(3):331.e1-331.e3317. doi:10.1016/j.jcrc.2010.07.012

Getz GS, Reardon CA. (2004). Paraoxonase, a cardioprotective enzyme: continuing issues. *Curr Opin Lipidol*. 15(3):261-267. doi:10.1097/00041433-200406000-00005

Giordano A, Veronesi MC, Rossi G, Pezzia F, Probo M, Giori L, Paltrinieri S. (2013). Serum paraoxonase-1 activity in neonatal calves: age related variations and comparison between healthy and sick animals. *Vet J*. 197(2):499-501. doi:10.1016/j.tvjl.2013.01.034

González MA, Fragío Arnold C, Fermín Rodríguez M, Checa R, Montoya A, Portero Fuentes M, Rupérez Noguer C, Martínez Subiela S, Cerón JJ, Miró G. (2019). Effect of two treatments on changes in serum acute phase protein concentrations in dogs with clinical leishmaniosis. *Vet J*. 2019;245:22-28. doi:10.1016/j.tvjl.2018.12.020

Goode HF, Cowley HC, Walker BE, Howdle PD, Webster NR. (1995). Decreased antioxidant status and increased lipid peroxidation in patients with septic shock and secondary organ dysfunction. *Crit Care Med*. 23(4):646-651. doi:10.1097/00003246-199504000-00011

Gordon DJ, Probstfield JL, Garrison RJ, Neaton JD, Castelli WP, Knoke JD, Jacobs DR Jr, Bangdiwala S, Tyroler HA. (1989). High-density lipoprotein cholesterol and cardiovascular disease. Four prospective American studies. *Circulation*. 79(1):8-15. doi:10.1161/01.cir.79.1.8

Greig B, Armstrong PJ. (2006). Canine granulocytotropic anaplasmosis (A phagocyto-philum). In: Greene CE, Ed. Infectious diseases of the dog and cat. 3rd edition. Philadelphia: Saunders Elsevier. 219-224

Han CY, Chiba T, Campbell JS, Fausto N, Chaisson M, Orasanu G, Plutzky J, Chait A. (2006). Reciprocal and coordinate regulation of serum amyloid A versus apolipoprotein A-I and paraoxonase-1 by inflammation in murine hepatocytes. *Arterioscler Thromb Vasc Biol*. 26(8):1806-1813. doi:10.1161/01.ATV.0000227472.70734.ad

Harel M, Aharoni A, Gaidukov L, Brumshtein B, Khersonsky O, Meged R, Dvir H, Ravelli RB, McCarthy A, Toker L, Silman I, Sussman JL, Tawfik DS. (2004). Structure and evolution of the serum paraoxonase family of detoxifying and anti-atherosclerotic enzymes. Nat Struct Mol Biol. 11(5):412-9. doi: 10.1038/nsmb767. Epub 2004 Apr 18. Erratum in: Nat Struct Mol Biol. 2004 Dec;11(12):1253. PMID: 15098021.

Harman D. (1992). Role of free radicals in aging and disease. *Ann N Y Acad Sci*. 673:126-141. doi:10.1111/j.1749-6632.1992.tb27444.x

Hassett C, Richter RJ, Humbert R, Chapline C, Crabb JW, Omiecinski CJ, Furlong CE. (1991). Characterization of cDNA clones encoding rabbit and human serum paraoxonase: the mature protein retains its signal sequence. *Biochemistry*. 30(42):10141-10149. doi:10.1021/bi00106a010

Hauptman JG, Walshaw R, Olivier NB. (1997). Evaluation of the sensitivity and specificity of diagnostic criteria for sepsis in dogs. *Vet Surg*. 26(5):393-397. doi:10.1111/j.1532-950x.1997.tb01699.x

Hindenberg S, Bauer N, Moritz A. (2020). Extremely high canine C-reactive protein concentrations > 100 mg/l - prevalence, etiology and prognostic significance. *BMC Vet Res*. 16(1):147. doi:10.1186/s12917-020-02367-7

Hochreiter M, Köhler T, Schweiger AM, Keck FS, Bein B, von Spiegel T, Schroeder S. (2009). Procalcitonin to guide duration of antibiotic therapy in intensive care patients: a randomized prospective controlled trial. *Crit Care*. 13(3):R83. doi:10.1186/cc7903

Hultén C, Sandgren B, Skiöldebrand E, Klingeborn B, Marhaug G, Forsberg M. (1999). The acute phase protein serum amyloid A (SAA) as an inflammatory marker in equine influenza virus infection. *Acta Vet Scand*. 40(4):323-333. doi:10.1186/BF03547012

Iatta R, Furlanello T, Colella V, Tarallo VD, Latrofa MS, Brianti E, Trerotoli P, Decaro N, Lorusso E, Schunack B, Mirò G, Dantas-Torres F, Otranto D. (2019). A nationwide survey of Leishmania infantum infection in cats and associated risk factors in Italy. *PLoS Negl Trop Dis*. 13(7):e0007594. doi:10.1371/journal.pntd.0007594

Ibba F, Rossi G, Meazzi S, Giordano A, Paltrinieri S. (2015). Serum concentration of high density lipoproteins (HDLs) in leishmaniotic dogs. *Res Vet Sci*. 98:89-91. doi:10.1016/j.rvsc.2014.11.011

Iftimie S, Escribano A, Díez-Sans A, Albiciuc I, Hernández-Aguilera A, Fort-Gallifa I, López-Azcona AF, Camps J, Joven J, Castro A. (2019). Influence of Surgical Procedures on Serum Paraoxonase-1-Related Variables and Markers of Inflammation in Hospitalized Patients. *J Invest Surg*. 34(2):216-224. doi:10.1080/08941939.2019.1597223

Iftimie S, García-Heredia A, Pujol I, Ballester F, Fort-Gallifa I, Simó JM, Joven J, Castro A, Camps J. (2016). A preliminary study of paraoxonase-1 in infected patients with an indwelling central venous catheter. *Clin Biochem*. 49(6):449-457. doi:10.1016/j.clinbiochem.2015.11.006

Jacobsen S, Thomsen MH, Nanni S (2006). Concentrations of serum amyloid A in serum and synovial fluid from healthy horses and horses with joint disease. *Am J Vet Res*. 67(10):1738-1742. doi:10.2460/ajvr.67.10.1738

James RW, Deakin SP. (2004). The importance of high-density lipoproteins for paraoxonase-1 secretion, stability, and activity. *Free Radic Biol Med*. 37(12):1986-1994. doi:10.1016/j.freeradbiomed.2004.08.012

James RW, Leviev I, Ruiz J, Passa P, Froguel P, Garin MC. (2000). Promoter polymorphism T(-107)C of the paraoxonase PON1 gene is a risk factor for coronary heart disease in type 2 diabetic patients. *Diabetes*. 49(8):1390-1393. doi:10.2337/diabetes.49.8.1390

Jarvik GP, Jampsa R, Richter RJ, Carlson CS, Rieder MJ, Nickerson DA, Furlong CE. (2003). Novel paraoxonase (PON1) nonsense and missense mutations predicted by functional genomic assay of PON1 status. *Pharmacogenetics*. 13(5):291-295. doi:10.1097/00008571-200305000-00009

Jarvik GP, Rozek LS, Brophy VH, Hatsukami TS, Richter RJ, Schellenberg GD, Furlong CE. (2000). Paraoxonase (PON1) phenotype is a better predictor of vascular disease than is PON1(192) or PON1(55) genotype. *Arterioscler Thromb Vasc Biol*. 20(11):2441-2447. doi:10.1161/01.atv.20.11.2441

Jekarl DW, Lee SY, Lee J, Park YJ, Kim Y, Park JH, Wee JH, Choi SP. (2013). Procalcitonin as a diagnostic marker and IL-6 as a prognostic marker for sepsis. *Diagn Microbiol Infect Dis*. 2013;75(4):342-347. doi:10.1016/j.diagmicrobio.2012.12.011

Jensen AL, Kjelgaard-Hansen M. (2010). Diagnostic test validation. In: Weiss DJ, Wardrop KJ, eds. Schalm's Veterinary Hematology. 6th ed. Ames, IA: Wiley-Blackwell; 1031-1033

Jergens AE, Schreiner CA, Frank DE, Niyo Y, Ahrens FE, Eckersall PD, Benson TJ, Evans R. (2003). A scoring index for disease activity in canine inflammatory bowel disease. *J Vet Intern Med*. 17(3):291-297. doi:10.1111/j.1939-1676.2003.tb02450.x

Jitpean S, Holst BS, Höglund OV, Pettersson A, Olsson U, Strage E, Södersten F, Hagman R. (2014). Serum insulin-like growth factor-I, iron, C-reactive protein, and serum amyloid A for prediction of outcome in dogs with pyometra. *Theriogenology*. 82(1):43-48. doi:10.1016/j.theriogenology.2014.02.014

Kanno N, Hayakawa N, Suzuki S, Harada Y, Yogo T, Hara Y. (2019). Changes in canine C-reactive protein levels following orthopaedic surgery: a prospective study. *Acta Vet Scand*. 61(1):33. doi:10.1186/s13028-019-0468-y

Karabina SA, Lehner AN, Frank E, Parthasarathy S, Santanam N. (2005). Oxidative inactivation of paraoxonase--implications in diabetes mellitus and atherosclerosis. *Biochim Biophys Acta*. 1725(2):213-221. doi:10.1016/j.bbagen.2005.07.005

Karnezi D, Ceron JJ, Theodorou K, Leontides L, Siarkou VI, Martinez S, Tvarijonaviciute A, Harrus S, Koutinas CK, Pardali D, Mylonakis ME. (2016). Acute phase protein and antioxidant responses in dogs with experimental acute monocytic ehrlichiosis treated with rifampicin. *Vet Microbiol*. 2016;184:59-63. doi:10.1016/j.vetmic.2016.01.007

Khersonsky O, Tawfik DS. (2005). Structure-reactivity studies of serum paraoxonase PON1 suggest that its native activity is lactonase. *Biochemistry*. 44(16):6371-6382. doi:10.1021/bi047440d

Kim DS, Marsillach J, Furlong CE, Jarvik GP. (2013). Pharmacogenetics of paraoxonase activity: elucidating the role of high-density lipoprotein in disease. *Pharmacogenomics*. 14(12):1495-1515. doi:10.2217/pgs.13.147

Kjelgaard-Hansen M, Kristensen AT, Jensen AL. (2003). Evaluation of a commercially available enzyme-linked immunosorbent assay (ELISA) for the determination of C-reactive protein in canine serum. *J Vet Med A Physiol Pathol Clin Med*. 50(3):164-168. doi:10.1046/j.1439-0442.2003.00509.x

Klimov AN, Kozhevnikova KA, Kuzmin AA, Kuznetsov AS, Belova EV. (2001). On the ability of high density lipoproteins to remove phospholipid peroxidation products from erythrocyte membranes. *Biochemistry (Mosc)*. 66(3):300-304. doi:10.1023/a:1010203930470

Kocaturk M, Tvarijonaviciute A, Martinez-Subiela S, Tecles F, Eralp O, Yilmaz Z, Ceron JJ. (2015). Inflammatory and oxidative biomarkers of disease severity in dogs with parvoviral enteritis. *J Small Anim Pract*. 56(2):119-124. doi:10.1111/jsap.12250

Kontush A, Chapman MJ. (2006). Functionally defective high-density lipoprotein: a new therapeutic target at the crossroads of dyslipidemia, inflammation, and atherosclerosis. *Pharmacol Rev*. 58(3):342-374. doi:10.1124/pr.58.3.1

Kotani K, Yamada T, Gugliucci A. (2013). Paired measurements of paraoxonase 1 and serum amyloid A as useful disease markers. *Biomed Res Int*. 2013:481437. doi:10.1155/2013/481437

Kotani K, Yamada T, Gugliucci A. (2013). Paired measurements of paraoxonase 1 and serum amyloid A as useful disease markers. *Biomed Res Int*. 2013:481437. doi:10.1155/2013/481437

Kovačić M, Samardžija M, Đuričić D, Vince S, Flegar-Meštrić Z, Perkov S, Gračner D, Turk R. (2019). Paraoxonase-1 activity and lipid profile in dairy cows with subclinical and clinical mastitis. *Journal of Applied Animal Research*. 1;47(1):1-4. doi:10.1080/09712119.2018.1555090

Krisch K. (1968). Enzymatische Hydrolyse von Diäthyl-p-nitrophenylphosphat (E 600) durch menschlichen Serum [Enzymatic hydrolysis of diethyl-p-nitrophenylphosphate (E 600) by human serum]. *Z Klin Chem Klin Biochem*. 6(1):41-45.

Krupka I, Straubinger RK. (2010). Lyme borreliosis in dogs and cats: background, diagnosis, treatment and prevention of infections with Borrelia burgdorferi sensu stricto. *Vet Clin North Am Small Anim Pract*. 40(6):1103-1119. doi:10.1016/j.cvsm.2010.07.011

Kuleš J, de Torre-Minguela C, Barić Rafaj R, Gotić J, Nižić P, Ceron JJ, Mrljak V. (2016). Kuleš J, de Torre-Minguela C, Barić Rafaj R, et al. Plasma biomarkers of SIRS and MODS associated with canine babesiosis. *Res Vet Sci*. 2016;105:222-228. doi:10.1016/j.rvsc.2016.02.011

Kulka M, Bełtowski J, Kluciński W, Orłowska M, Kołodziejska J, Kleczkowski M. (2014). Serum paraoxonase-1 activity of dairy Holstein-Fresian cows in different lactation stages--preliminary study. *Pol J Vet Sci*. 17(1):143-147. doi:10.2478/pjvs-2014-0019

Kulka M, Garncarz M, Parzeniecka-Jaworska M, Kluciński W. (2017). Serum paraoxonase 1 activity and lipid metabolism parameter changes in Dachshunds with chronic mitral valve disease. Assessment of its diagnostic usefulness*. Pol J Vet Sci*. 20(4):723-9. https://doi.org/10.1515/pjvs-2017-0090

Kulka M, Kołodziejska-Lesisz J, Kluciński W. (2016) Serum paraoxonase 1 (PON1) activity and lipid metabolism parameters changes in different production cycle periods of Holstein-Friesian, Polish Red and Norwegian breeds. *Pol J Vet Sci*. 19(1):165-173. doi:10.1515/pjvs-2016-0021

Kumar V, Abbas AK, Aster JC (2014) Acute and chronic inflammation. In: Robbins and Cotran Pathologic Basis of Disease. Elsevier Saunders Edn. 9th Ed. Philadelphia, USA

Kuo CL, La Du BN. (1998). Calcium binding by human and rabbit serum paraoxonases. Structural stability and enzymatic activity. *Drug Metab Dispos*. 26(7):653-660.

La Du BN. (1988). The human serum paraoxonase/arylesterase polymorphism. *Am J Hum Genet*. 43(3):227-229. PMID:2843045

La Du. (2002) Historical considerations. In: Costa LG, Furlong CE Eds. Paraoxonase (PON1) in Health and Disease: Basic and Clinical Aspects. Springer. 1st Ed. Boston, MA 1-25

Labrecque B, Beaudry D, Mayhue M, Hallé C, Bordignon V, Murphy BD, Palin MF. (2009). Molecular characterization and expression analysis of the porcine paraoxonase 3 (PON3) gene. Gene. 443(1-2):110-120. doi:10.1016/j.gene.2009.04.026

Laplaud PM, Dantoine T, Chapman MJ. (1998). Paraoxonase as a risk marker for cardiovascular disease: facts and hypotheses. *Clin Chem Lab Med*. 36(7):431-441. doi:10.1515/CCLM.1998.073

Leviev I, James RW. (2000). Promoter polymorphisms of human paraoxonase PON1 gene and serum paraoxonase activities and concentrations. Arterioscler Thromb Vasc Biol. Feb;20(2):516-21. doi:10.1161/01.atv.20.2.516

lexandrakis I, Tuli R, Ractliffe SC, Tappin SW, Foale RD, Roos A, Slater KJ. (2017). Utility of a multiple serum biomarker test to monitor remission status and relapse in dogs with lymphoma undergoing treatment with chemotherapy. *Vet Comp Oncol*. 15(1):6-17. doi:10.1111/vco.12123

Li WF, Costa LG, Furlong CE. (1993). Serum paraoxonase status: a major factor in determining resistance to organophosphates. *J Toxicol Environ Health*. 40(2-3):337-346. doi:10.1080/15287399309531798

Li WF, Matthews C, Disteche CM, Costa LG, Furlong CE. (1997). Paraoxonase (PON1) gene in mice: sequencing, chromosomal localization and developmental expression. *Pharmacogenetics*. 7(2):137-144. doi:10.1097/00008571-199704000-00007

Li Y, Zhai R, Li H, Mei X, Qiu G. (2013). Prognostic value of serum paraoxonase and arylesterase activity in patients with sepsis. *J Int Med Res*. Jun;41(3):681-7. doi:10.1177/0300060513483412

Lisboa T, Seligman R, Diaz E, Rodriguez A, Teixeira PJ, Rello J. (2008). C-reactive protein correlates with bacterial load and appropriate antibiotic therapy in suspected ventilator-associated pneumonia. *Crit Care Med*. 36(1):166-171. doi:10.1097/01.CCM.0000297886.32564.CF

Little SE, Heise SR, Blagburn BL, Callister SM, Mead PS. (2010). Lyme borreliosis in dogs and humans in the USA. *Trends Parasitol*. 26(4):213-218. doi:10.1016/j.pt.2010.01.006

Littman MP, Gerber B, Goldstein RE, Labato MA, Lappin MR, Moore GE. (2018). ACVIM consensus update on Lyme borreliosis in dogs and cats. *J Vet Intern Med*. 32(3):887-903. doi:10.1111/jvim.15085

Littman MP. (2020) Lyme Borreliosis. In: Bruyette SD Ed. Clinical Small Animal Internal Medicine. John Wiley & Sons, Inc. 1st Ed. Volume II, 941-944

Long A, Nolen-Walston R. (2020). Equine Inflammatory Markers in the Twenty-First Century: A Focus on Serum Amyloid A. *Vet Clin North Am Equine Pract*. 36(1):147-160. doi:10.1016/j.cveq.2019.12.005

Lu CY, Lee HC, Fahn HJ, Wei YH. (1999). Oxidative damage elicited by imbalance of free radical scavenging enzymes is associated with large-scale mtDNA deletions in aging human skin. *Mutat Res*. 423(1-2):11-21. doi:10.1016/s0027-5107(98)00220-6

Mackness B, Davies GK, Turkie W, Lee E, Roberts DH, Hill E, Roberts C, Durrington PN, Mackness MI. (2001). Paraoxonase status in coronary heart disease: are activity and concentration more important than genotype?. *Arterioscler Thromb Vasc Biol*. 21(9):1451-1457. doi:10.1161/hq0901.094247

Mackness B, Durrington P, McElduff P, Yarnell J, Azam N, Watt M, Mackness M. (2003). Low paraoxonase activity predicts coronary events in the Caerphilly Prospective Study. *Circulation*. 107(22):2775-2779. doi:10.1161/01.CIR.0000070954.00271.13

Mackness B, Durrington PN, Mackness MI. (1998). Lack of protection against oxidative modification of LDL by avian HDL. *Biochem Biophys Res Commun*. 247(2):443-446. doi:10.1006/bbrc.1998.8803

Mackness B, Hine D, Liu Y, Mastorikou M, Mackness M. (2004a). Paraoxonase-1 inhibits oxidised LDL-induced MCP-1 production by endothelial cells. *Biochem Biophys Res Commun*. 318(3):680-683. doi:10.1016/j.bbrc.2004.04.056

MacKness B, Mackness MI, Durrington PN, Arrol S, Evans AE, McMaster D, Ferrières J, Ruidavets JB, Williams NR, Howard AN. (2000). Paraoxonase activity in two healthy populations with differing rates of coronary heart disease. *Eur J Clin Invest*. 30(1):4-10. doi:10.1046/j.1365-2362.2000.00580.x

Mackness B, Mackness MI. (2008). Paraoxonase-1 and cardiovascular disease. In: Mackness B, Mackness M, Aviram M, Paragh G, Eds. The Paraoxonases: Their Role in Disease Development and Xenobiotic Metabolism. Springer Eds. Dordrecht. 51-60

Mackness M, Durrington P, Mackness B. (2004b). Paraoxonase 1 activity, concentration and genotype in cardiovascular disease. *Curr Opin Lipidol*. 15(4):399-404. doi:10.1097/01.mol.0000137227.54278.29

Mackness M, Mackness B. (2015). Human paraoxonase-1 (PON1): Gene structure and expression, promiscuous activities and multiple physiological roles. *Gene*. 567(1):12-21. doi:10.1016/j.gene.2015.04.088

Mackness M, Sozmen E. (2021). A critical review on human serum Paraoxonase-1 in the literature: truths and misconceptions. *Turkish Journal of Biochemistry*. 46(1):3-8. https://doi.org/10.1515/tjb-2020-0186

Mackness MI, Arrol S, Abbott C, Durrington PN. (1993). Protection of low-density lipoprotein against oxidative modification by high-density lipoprotein associated paraoxonase. *Atherosclerosis*. 104(1-2):129-135. doi:10.1016/0021-9150(93)90183-u

Mackness MI, Arrol S, Durrington PN. (1991). Substrate specificity of human serum paraoxonase. *Biochem Soc Trans*. 19(3):304S. doi:10.1042/bst019304s

Mackness MI, Mackness B, Durrington PN, Connelly PW, Hegele RA. (1996). Paraoxonase: biochemistry, genetics and relationship to plasma lipoproteins. *Curr Opin Lipidol*. 7(2):69-76. doi:10.1097/00041433-199604000-00004

Marchegiani F, Marra M, Spazzafumo L, James RW, Boemi M, Olivieri F, Cardelli M, Cavallone L, Bonfigli AR, Franceschi C. (2006). Paraoxonase activity and genotype predispose to successful aging. *J Gerontol A Biol Sci Med Sci*. 61(6):541-546. doi:10.1093/gerona/61.6.541

Marsillach J, Aragonès G, Mackness B, Mackness M, Rull A, Beltrán-Debón R, Pedro-Botet J, Alonso-Villaverde C, Joven J, Camps J. (2010). Decreased paraoxonase-1 activity is associated with alterations of high-density lipoprotein particles in chronic liver impairment. Lipids Health Dis. 9:46. doi:10.1186/1476-511X-9-46

Martinez-Subiela S, Cerón JJ, Strauss-Ayali D, Garcia-Martinez JD, Tecles F, Tvarijonaviciute A, Caldin M, Baneth G. (2014). Serum ferritin and paraoxonase-1 in canine leishmaniosis. *Comp Immunol Microbiol Infect Dis*. 37(1):23-29. doi:10.1016/j.cimid.2013.10.004

Martins PS, Kallas EG, Neto MC, Dalboni MA, Blecher S, Salomão R. (2003). Upregulation of reactive oxygen species generation and phagocytosis, and increased apoptosis in human neutrophils during severe sepsis and septic shock. *Shock*. 20(3):208-212. doi:10.1097/01.shk.0000079425.52617.db

McElveen J, Mackness MI, Colley CM, Peard T, Warner S, Walker CH. (1986). Distribution of paraoxon hydrolytic activity in the serum of patients after myocardial infarction. *Clin Chem*. 32(4):671-673.

Meazzi S, Paltrinieri S, Lauzi S, Stranieri A, Brentali I, Ferriani R, Rossi G, Giordano A. (2021). Role of paraoxonase-1 as a diagnostic marker for feline infectious peritonitis. *Vet J*. 272:105661. doi:10.1016/j.tvjl.2021.105661

Meisner M, Adina H, Schmidt J. (2006). Correlation of procalcitonin and C-reactive protein to inflammation, complications, and outcome during the intensive care unit course of multiple-trauma patients. *Crit Care*. 10(1):R1. doi:10.1186/cc3910

Méndez JC, Carretón E, Martínez S, Tvarijonaviciute A, Cerón JJ, Montoya-Alonso JA. (2014). Acute phase response in dogs with Dirofilaria immitis. *Vet Parasitol*. 204(3-4):420-425. doi:10.1016/j.vetpar.2014.05.016

Méndez JC, Carretón E, Martínez-Subiela S, Tvarijonaviciute A, Cerón JJ, Montoya-Alonso JA. (2015). Acute phase protein response in heartworm-infected dogs after adulticide treatment. *Vet Parasitol*. 209(3-4):197-201. doi:10.1016/j.vetpar.2015.02.036

Milochevitch C, Khalil A. (2001). Study of the paraoxonase and platelet-activating factor acetylhydrolase activities with aging. *Prostaglandins Leukot Essent Fatty Acids*. 65(5-6):241-246. doi:10.1054/plef.2001.0320

Miyamoto T, Takahashi Y, Oohashi T, Sato K, Oikawa S. (2005). Bovine paraoxonase 1 activities in serum and distribution in lipoproteins. *J Vet Med Sci*. 67(3):243-248. doi:10.1292/jvms.67.243

Moore JN, Norton N, Barton MH, Hurley DJ, Reber AJ, Donovan DC, Vandenplas ML, Parker TS, Levine DM. (2007). Rapid infusion of a phospholipid emulsion attenuates the effects of endotoxaemia in horses. *Equine Vet J*. 39(3):243-248. doi:10.2746/042516407x173343

Mueller RF, Hornung S, Furlong CE, Anderson J, Giblett ER, Motulsky AG. (1983). Plasma paraoxonase polymorphism: a new enzyme assay, population, family, biochemical, and linkage studies. *Am J Hum Genet*. 35(3):393-408. PMID:6305189

Mühl D, Woth G, Drenkovics L, Varga A, Ghosh S, Csontos C, Bogár L, Wéber G, Lantos J. (2011). Comparison of oxidative stress & leukocyte activation in patients with severe sepsis & burn injury. Indian J Med Res. 134(1):69-78. PMID:21808137

Navab M, Hama-Levy S, Van Lenten BJ, Fonarow GC, Cardinez CJ, Castellani LW, Brennan ML, Lusis AJ, Fogelman AM, La Du BN. (1997). Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio [published correction appears in J Clin Invest 1997 Jun 15;99(12):3043]. *J Clin Invest*. 99(8):2005-2019. doi:10.1172/JCI119369

Nielsen L, Toft N, Eckersall PD, Mellor DJ, Morris JS. (2007). Serum C-reactive protein concentration as an indicator of remission status in dogs with multicentric lymphoma. *J Vet Intern Med*. 21(6):1231-1236. doi:10.1892/07-058.1

Nieto JE, MacDonald MH, Braim AE, Aleman M. (2009). Effect of lipopolysaccharide infusion on gene expression of inflammatory cytokines in normal horses in vivo. *Equine Vet J*. 41(7):717-719. doi:10.2746/042516409x464780

Novak F, Vavrova L, Kodydkova J, Novak F Sr, Hynkova M, Zak A, Novakova O. (2010). Decreased paraoxonase activity in critically ill patients with sepsis. *Clin Exp Med*. 10(1):21-25. doi:10.1007/s10238-009-0059-8

Nunokawa Y, Fujinaga T, Taira T, Okumura M, Yamashita K, Tsunoda N, Hagio M. (1993). Evaluation of serum amyloid A protein as an acute-phase reactive protein in horses. *J Vet Med Sci*. 55(6):1011-1016. doi:10.1292/jvms.55.1011

Oliveira-Filho JP, Badial PR, Cunha PH, et al. Lipopolysaccharide infusion up-regulates hepcidin mRNA expression in equine liver. *Innate Immun*. 2012;18(3):438-446. doi:10.1177/1753425911420181

Ollivett TL, Buczinski S. (2016). On-Farm Use of Ultrasonography for Bovine Respiratory Disease. *Vet Clin North Am Food Anim Pract*. 2016;32(1):19-35. doi:10.1016/j.cvfa.2015.09.001

Otranto D, Paradies P, de Caprariis D, Stanneck D, Testini G, Grimm F, Deplazes P, Capelli G. (2009). Toward diagnosing Leishmania infantum infection in asymptomatic dogs in an area where leishmaniasis is endemic. *Clin Vaccine Immunol*. 2009;16(3):337-343. doi:10.1128/CVI.00268-08

Pallarés FJ, Martínez-Subiela S, Seva J, Ramis G, Fuentes P, Bernabé A, Muñoz A, Cerón JJ. (2008). Relationship between serum acute phase protein concentrations and lesions in finishing pigs. *Vet J*. 177(3):369-373. doi:10.1016/j.tvjl.2007.04.019

Paltrinieri S, Ravicini S, Rossi G, Roura X. (2010). Serum concentrations of the derivatives of reactive oxygen metabolites (d-ROMs) in dogs with leishmaniosis. *Vet J*. 186(3):393-395. doi:10.1016/j.tvjl.2009.08.019

Paltrinieri S. (2007). Early biomarkers of inflammation in dogs and cats: the acute phase proteins. *Vet Res Commun*. 31 Suppl 1(Suppl 1):125-129. doi:10.1007/s11259-007-0107-3

Panaro MA, Brandonisio O, de Caprariis D, Cavallo P, Cianciulli A, Mitolo V, Otranto D. (2008). Canine leishmaniasis in Southern Italy: a role for nitric oxide released from activated macrophages in asymptomatic infection?. *Parasit Vectors*. 1(1):10. doi:10.1186/1756-3305-1-10

Panaro MA, Lisi S, Mitolo V, Acquafredda A, Fasanella A, Carelli M G, and Brandonisio O. (1998). Evaluation of Killing, Superoxide Anion and Nitric Oxide Production by Leishmania Infantum-Infected Dog Monocytes. *Cytobios.* 95(380):151-60. PMID:10093201

Pantchev N, Pluta S, Huisinga E, Nather S, Scheufelen M, Vrhovec MG, Schweinitz A, Hampel H, Straubinger RK. (2015). Tick-borne Diseases (Borreliosis, Anaplasmosis, Babesiosis) in German and Austrian Dogs: Status quo and Review of Distribution, Transmission, Clinical Findings, Diagnostics and Prophylaxis. *Parasitol Res*. 114 Suppl 1:S19-S54. doi:10.1007/s00436-015-4513-0

Pardo-Marin L, Ceron JJ, Tecles F, Baneth G, Martínez-Subiela S. (2020). Comparison of acute phase proteins in different clinical classification systems for canine leishmaniosis. *Vet Immunol Immunopathol*. 219:109958. doi:10.1016/j.vetimm.2019.109958

Parry N. (2016). Canine borreliosis: epidemiology, pathogenesis, clinical signs, and diagnostics. *Companion Animal*. 21(6):323-31. doi:10.12968/coan.2016.21.6.323

Pellin MC, Moretto A, Lotti M, Vilanova E. Distribution and some biochemical properties of rat paraoxonase activity. *Neurotoxicol Teratol*. 1990;12(6):611-614. doi:10.1016/0892-0362(90)90071-j

Pennisi MG, Cardoso L, Baneth G, Bourdeau P, Koutinas A, Miró G, Oliva G, Solano-Gallego L. (2015). LeishVet Update and Recommendations on Feline Leishmaniosis. *Parasit Vectors.* 8(1):302. doi:10.1186/s13071-015-0909-z

Pereira A, Maia C. (2021). *Leishmania* infection in cats and feline leishmaniosis: An updated review with a proposal of a diagnosis algorithm and prevention guidelines. *Curr Res Parasitol Vector Borne Dis*. 1:100035. doi:10.1016/j.crpvbd.2021.100035

Peterson ME, Kintzer PP, Kass PH. (1996). Pretreatment clinical and laboratory findings in dogs with hypoadrenocorticism: 225 cases (1979-1993). *J Am Vet Med Assoc*. 208(1):85-91. PMID:8682712

Polizopoulou ZS, Koutinas CK, Cerón JJ, Tvarijonaviciute A, Martínez-Subiela S, Dasopoulou A, York MJ, Roman IF, Gandhi M, Patel S, O'Brien PJ. (2015). Correlation of serum cardiac troponin I and acute phase protein concentrations with clinical staging in dogs with degenerative mitral valve disease [published correction appears in Vet Clin Pathol. 2016 Dec;45(4):732]. *Vet Clin Pathol*. 44(3):397-404. doi:10.1111/vcp.12278

Poulin Braim AE, MacDonald MH, Bruss ML, Grattendick KJ, Giri SN, Margolin SB. (2009). Effects of intravenous administration of pirfenidone on horses with experimentally induced endotoxemia. *Am J Vet Res*. 70(8):1031-1042. doi:10.2460/ajvr.70.8.1031

Póvoa P, Teixeira-Pinto AM, Carneiro AH; Portuguese Community-Acquired Sepsis Study Group SACiUCI. (2011). C-reactive protein, an early marker of community-acquired sepsis resolution: a multi-center prospective observational study. *Crit Care*. 15(4):R169. doi:10.1186/cc10313

Póvoa P. (2008). Serum markers in community-acquired pneumonia and ventilator-associated pneumonia. *Curr Opin Infect Dis*. 21(2):157-162. doi:10.1097/QCO.0b013e3282f47c32

Prasad AJ, Krueger M, Krueger M. (2014). Decreased level of serum paraoxonase (PON) activity in dogs with dilated cardiomyopathy (DCM). *J Vet Med Anim Health*. 6(9):245-50. doi:10.5897/JVMAH2011.028

Primo-Parmo SL, Sorenson RC, Teiber J, La Du BN. (1996). The human serum paraoxonase/arylesterase gene (PON1) is one member of a multigene family. *Genomics*. 33(3):498-507. doi:10.1006/geno.1996.0225

Radakovic M, Davitkov D, Borozan S, Stojanovic S, Stevanovic J, Krstic V, Stanimirovic Z. (2016). Oxidative stress and DNA damage in horses naturally infected with Theileria equi. *Vet J*. 217:112-118. doi:10.1016/j.tvjl.2016.10.003

Raila J, Schweigert FJ, Kohn B. (2011). C-reactive protein concentrations in serum of dogs with naturally occurring renal disease. *J Vet Diagn Invest*. 23(4):710-715. doi:10.1177/1040638711407896

Reinhart K, Meisner M. (2011). Biomarkers in the critically ill patient: procalcitonin. *Crit Care Clin*. 27(2):253-263. doi:10.1016/j.ccc.2011.01.002

Richter RJ, Furlong CE. Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics*. 1999;9(6):745-753.

Richter RJ, Jampsa RL, Jarvik GP, Costa LG, Furlong CE. (2004). Determination of paraoxonase 1 status and genotypes at specific polymorphic sites. *Curr Protoc Toxicol*. Chapter 4:Unit4.12. doi:10.1002/0471140856.tx0412s19

Rock KL, Latz E, Ontiveros F, Kono H. (2010). The sterile inflammatory response. *Annu Rev Immunol*. 28:321-342. doi:10.1146/annurev-immunol-030409-101311

Rosenblat M, Gaidukov L, Khersonsky O, Vaya J, Oren R, Tawfik DS, Aviram M. (2006). The catalytic histidine dyad of high density lipoprotein-associated serum paraoxonase-1 (PON1) is essential for PON1-mediated inhibition of low density lipoprotein oxidation and stimulation of macrophage cholesterol efflux. *J Biol Chem*. 281(11):7657-65. doi: 10.1074/jbc.M512595200

Rosenblat M, Vaya J, Shih D, Aviram M. (2005). Paraoxonase 1 (PON1) enhances HDL-mediated macrophage cholesterol efflux via the ABCA1 transporter in association with increased HDL binding to the cells: a possible role for lysophosphatidylcholine. *Atherosclerosis*. 179(1):69-77. doi:10.1016/j.atherosclerosis.2004.10.028

Rossi G, Giordano A, Pezzia F, Kjelgaard-Hansen M, Paltrinieri S. (2013) Serum paraoxonase 1 activity in dogs: preanalytical and analytical factors and correlation with C-reactive protein and alpha-2-globulin. *Vet Clin Pathol*. 42(3):329-341. doi:10.1111/vcp.12073

Rossi G, Ibba F, Meazzi S, Giordano A, Paltrinieri S. (2014). Paraoxonase activity as a tool for clinical monitoring of dogs treated for canine leishmaniasis. *Vet J*. 2014;199(1):143-149. doi:10.1016/j.tvjl.2013.10.007

Rossi G, Kuleš J, Rafaj RB, Mrljak V, Lauzi S, Giordano A, Paltrinieri S. (2014b). Relationship between paraoxonase 1 activity and high density lipoprotein concentration during naturally occurring babesiosis in dogs. *Res Vet Sci*. 97(2):318-324. doi:10.1016/j.rvsc.2014.07.010

Rossi G, Meazzi S, Giordano A, Paltrinieri S. (2020). Serum paraoxonase 1 activity in cats: analytical validation, reference intervals, and correlation with serum amyloid A and alpha-1-acid glycoprotein. *J Vet Diagn Invest*. 32(6):844-855. doi:10.1177/1040638720949638

Roy MF, Kwong GPS, Lambert J, Massie S, Lockhart S. (2017). Prognostic value and development of a scoring system in horses with systemic inflammatory response syndrome. *J Vet Intern Med*. 31: 582-592 doi:10.1111/jvim.14670

Rozenberg O, Rosenblat M, Coleman R, Shih DM, Aviram M. (2003). Paraoxonase (PON1) deficiency is associated with increased macrophage oxidative stress: studies in PON1-knockout mice. *Free Radic Biol Med*. 34(6):774-784. doi:10.1016/s0891-5849(02)01429-6

Rozenberg O, Shih DM, Aviram M. (2003). Human serum paraoxonase 1 decreases macrophage cholesterol biosynthesis: possible role for its phospholipase-A2-like activity and

lysophosphatidylcholine formation. *Arterioscler Thromb Vasc Biol*. 23(3):461-467. doi:10.1161/01.ATV.0000060462.35946.B3

Rozenberg O, Shih DM, Aviram M. (2005). Paraoxonase 1 (PON1) attenuates macrophage oxidative status: studies in PON1 transfected cells and in PON1 transgenic mice. *Atherosclerosis*. 181(1):9-18. doi:10.1016/j.atherosclerosis.2004.12.030

Rubins HB, Robins SJ, Collins D, Fye CL, Anderson JW, Elam MB, Faas FH, Linares E, Schaefer EJ, Schectman G, Wilt TJ, Wittes J. (1999). Gemfibrozil for the secondary prevention of coronary heart disease in men with low levels of high-density lipoprotein cholesterol. Veterans Affairs High-Density Lipoprotein Cholesterol Intervention Trial Study Group. *N Engl J Med*. 341(6):410-418. doi:10.1056/NEJM199908053410604

Rubio CP, Martinez-Subiela S, Tvarijonaviciute A, Hernández-Ruiz J, Pardo-Marin L, Segarra S, Ceron JJ. (2016). Changes in serum biomarkers of oxidative stress after treatment for canine leishmaniosis in sick dogs. *Comp Immunol Microbiol Infect Dis*. 2016;49:51-57. doi:10.1016/j.cimid.2016.09.003

Rubio CP, Saril A, Kocaturk M, Tanaka R, Koch J, Ceron JJ, Yilmaz Z. (2020). Changes of inflammatory and oxidative stress biomarkers in dogs with different stages of heart failure. *BMC Vet Res*. 16(1):433. doi:10.1186/s12917-020-02650-7

Rubio CP, Yilmaz Z, Martínez-Subiela S, Kocaturk M, Hernández-Ruiz J, Yalcin E, Tvarijonaviciute A, Escribano D, Ceron JJ. (2017). Serum antioxidant capacity and oxidative damage in clinical and subclinical canine ehrlichiosis. *Res Vet Sci*. 2017;115:301-306. doi:10.1016/j.rvsc.2017.06.004

Ruggerone B, Bonelli F, Nocera I, Paltrinieri S, Giordano A, Sgorbini M. (2018) Validation of a paraoxon-based method for measurement of paraoxonase (PON-1) activity and establishment of RIs in horses. *Vet Clin Pathol*. 47(1):69-77. doi:10.1111/vcp.12562

Ruggerone B, Paltrinieri S, Giordano A, Scavone D, Nocera I, Rinnovati R, Spadari A, Scacco L, Pratelli P, Sgorbini M. (2020). Paraoxonase-1 activity evaluation as a diagnostic and prognostic marker in horses and foals. *J Vet Intern Med*. 2020;34(2):949-954. doi:10.1111/jvim.15722

Ruopp MD, Perkins NJ, Whitcomb BW, Schisterman EF. (2008). Youden Index and optimal cut-point estimated from observations affected by a lower limit of detection. *Biom J*. 50(3):419-430. doi:10.1002/bimj.200710415

Sans T, Rull A, Luna J, Mackness B, Mackness M, Joven J, Ibañez M, Pariente R, Rodriguez M, Ortin X, Masdeu G, Camps J. (2012). Monocyte chemoattractant protein-1 and paraoxonase-1 and 3 levels in patients with sepsis treated in an intensive care unit: a preliminary report. *Clin Chem Lab Med*. 50(8):1409-1415. doi:10.1515/cclm-2011-0896

Savioli G, Archer J, Brianti E, Benelli G, Schnyder M, Iatta R, Otranto D, Cantacessi C. (2021). Serum amyloid A levels and alpha 2 and gamma globulins on serum protein electrophoresis in cats exposed to and infected with Leishmania infantum. *Parasit Vectors*. 14(1):217. doi:10.1186/s13071-021-04710-9

Seres I, Paragh G, Deschene E, Fulop T Jr, Khalil A. (2004). Study of factors influencing the decreased HDL associated PON1 activity with aging. *Exp Gerontol*. 39(1):59-66. doi:10.1016/j.exger.2003.08.001

Shih DM, Gu L, Hama S, Xia YR, Navab M, Fogelman AM, Lusis AJ. (1996). Genetic-dietary regulation of serum paraoxonase expression and its role in atherogenesis in a mouse model. *J Clin Invest*. 97(7):1630-1639. doi:10.1172/JCI118589

Shih DM, Gu L, Xia YR, Navab M, Li WF, Hama S, Castellani LW, Furlong CE, Costa LG, Fogelman AM, Lusis AJ. (1998). Mice lacking serum paraoxonase are susceptible to organophosphate toxicity and atherosclerosis. *Nature*. 394(6690):284-287. doi:10.1038/28406

Showman AC, Aranjuez G, Adams PP, Jewett MW. (2016). Gene bb0318 Is Critical for the Oxidative Stress Response and Infectivity of Borrelia burgdorferi. *Infect Immun*. 84(11):3141-3151. Published 2016 Oct 17. doi:10.1128/IAI.00430-16

Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, Bellomo R, Bernard GR, Chiche JD, Coopersmith CM, Hotchkiss RS, Levy MM, Mashall JC, Martin GS, Opal SM, Rubenfeld GD, Van der Poll T, Vincent JL, Angus DC. (2016). The third international consensus definitions for Sepsis and septic shock (Sepsis-3). *JAMA* 315:801-10. doi:10.1001/jama.2016.0287

Sisto M, Brandonisio O, Panaro M A, Acquafredda A, Leogrande D, Fasanella A, Trotta T, Fumarola L, Mitolo V. (2001). Inducible Nitric Oxide Synthase Expression in Leishmania-Infected Dog Macrophages. *Comp Immunol Microbiol Infect Dis*. 24(4):247-254. doi:10.1016/s0147-9571(01)00013-3

Smith JA. (1994). Neutrophils, host defense, and inflammation: a double-edged sword. *J Leukoc Biol*. 56(6):672-686. doi:10.1002/jlb.56.6.672

Sorenson RC, Bisgaier CL, Aviram M, Hsu C, Billecke S, La Du BN. (1999). Human serum Paraoxonase/Arylesterase's retained hydrophobic N-terminal leader sequence associates with HDLs by binding phospholipids : apolipoprotein A-I stabilizes activity. *Arterioscler Thromb Vasc Biol*. 19(9):2214-2225. doi:10.1161/01.atv.19.9.2214

Stiles J, Bienzle D, Render JA, Buyukmihci NC, Johnson EC. (1999). Use of nested polymerase chain reaction (PCR) for detection of retroviruses from formalin-fixed, paraffin-embedded uveal melanomas in cats. *Vet Ophthalmol*. 2(2):113-116. doi:10.1046/j.1463-5224.1999.00066.x

Suehiro T, Nakamura T, Inoue M, Shiinoki T, Ikeda Y, Kumon Y, Shindo M, Tanaka H, Hashimoto K. (2000). A polymorphism upstream from the human paraoxonase (PON1) gene and its association with PON1 expression. *Atherosclerosis*. 150(2):295-298. doi:10.1016/s0021-9150(99)00379-2

Tadros EM, Frank N. (2012). Effects of continuous or intermittent lipopolysaccharide administration for 48 hours on the systemic inflammatory response in horses. *Am J Vet Res*. 73(9):1394-1402. doi:10.2460/ajvr.73.9.1394

Takeda K, Shimada Y, Amano M, Sakai T, Okada T, Yoshiya I. (1984). Plasma lipid peroxides and alpha-tocopherol in critically ill patients. *Crit Care Med*. 12(11):957-959. doi:10.1097/00003246-198411000-00007

Tappin SW, Taylor SS, Tasker S, Dodkin SJ, Papasouliotis K, Murphy KF. (2011). Serum protein electrophoresis in 147 dogs. *Vet Rec*. 168(17):456. doi:10.1136/vr.d88

Taylor S. (2015). A review of equine sepsis. *Equine Vet Educ*. 27(2):99-109. doi:10.1111/eve.12290

Teggert A, Datta H, Ali Z. (2020). Biomarkers for Point-of-Care Diagnosis of Sepsis. *Micromachines (Basel)*. 11(3):286. doi:10.3390/mi11030286

Thames BE, Barr JW, Suchodolski JS, Steiner JM, Heilmann RM. (2019). Prospective evaluation of S100A12 and S100A8/A9 (calprotectin) in dogs with sepsis or the systemic inflammatory response syndrome. *J Vet Diagn Invest*. 31(4):645-651. doi:10.1177/1040638719856655

Torrente C, Manzanilla EG, Bosch L, Villaverde C, Pastor J, de Gopegui RR, Tvarijonaviciute A. (2019). The diagnostic and prognostic value of paraoxonase-1 and butyrylcholinesterase activities compared with acute-phase proteins in septic dogs and stratified by the acute patient physiologic and laboratory evaluation score. *Vet Clin Pathol*. 2019;48(4):740-747. doi:10.1111/vcp.12807

Tothova C. Nagy O. Kovac G (2016). Serum proteins and their diagnostic utility in veterinary medicine: A review. *Vet Med*. (9): 475-496 doi: 10.17221/19/2016-VETMED

Trautinger F, Hammerle AF, Pöschl G, Micksche M. (1991). Respiratory burst capability of polymorphonuclear neutrophils and TNF-alpha serum levels in relationship to the development of septic syndrome in critically ill patients. *J Leukoc Biol*. 49(5):449-454. doi:10.1002/jlb.49.5.449

Troia R, Giunti M, Goggs R. (2018). Plasma procalcitonin concentrations predict organ dysfunction and outcome in dogs with sepsis. *BMC Vet Res*. 14(1):111. Published 2018 Mar 27. doi:10.1186/s12917-018-1427-y

Turk R, Habuš J, Flegar-Meštrić Z, Svetina A, Mojčec V, Perkov S, Belić M, Starešina V, Turk N. (2011). Serum platelet-activating factor acetylhydrolase and paraoxonase-1 activity in horses infected with Leptospira spp. *Acta Trop*. 118(2):97-100. doi:10.1016/j.actatropica.2011.03.002

Turk R, Juretić D, Geres D, Svetina A, Turk N, Flegar-Mestrić Z. (2008). Influence of oxidative stress and metabolic adaptation on PON1 activity and MDA level in transition dairy cows. *Anim Reprod Sci*. 108(1-2):98-106. doi:10.1016/j.anireprosci.2007.07.012

Turk R, Juretic D, Geres D, Turk N, Rekic B, Simeon-Rudolf V, Svetina A. (2004). Serum paraoxonase activity and lipid parameters in the early postpartum period of dairy cows. *Res Vet Sci*. 76(1):57-61. doi:10.1016/j.rvsc.2003.08.001

Turk R, Juretić D, Gereš D, Turk N, Simeon-Rudolf V, Rekić B, Svetina A. (2005) . Oxidative stress in dairy cows - serum paraoxonase activity related to hepatomegaly. *Croatica chemica acta*. 15;78(3):375-8.

Turk R, Vnuk D, Svetina A, Flegar-Meštrić Z, Bottegaro NB, Juretić D. (2009). Anti-oxidative/anti-inflammatory paraoxonase activity and lipid alterations after total splenectomy and autologous spleen transplantation in pigs. *Veterinarski Arhiv.* 79:1-10.

Tvarijonaviciute A, Caldin M, Martinez-Subiela S, Tecles F, Pastor J, Ceron JJ. (2015b). Serum paraoxonase 1 and butyrylcholinesterase in dogs with hyperadrenocorticism. *Vet J*. 203(2):262-263. doi:10.1016/j.tvjl.2014.12.002

Tvarijonaviciute A, Ceron JJ, Holden SL, Morris PJ, Biourge V, German AJ. (2012). Effects of weight loss in obese cats on biochemical analytes related to inflammation and glucose homeostasis. *Domest Anim Endocrinol*. 42(3):129-141. doi:10.1016/j.domaniend.2011.10.003

Tvarijonaviciute A, García-Martínez JD, Caldin M, Martínez-Subiela S, Tecles F, Pastor J, Ceron JJ. (2015a). Serum paraoxonase 1 (PON1) activity in acute pancreatitis of dogs. *J Small Anim Pract*. 56(1):67-71. doi:10.1111/jsap.12297

Tvarijonaviciute A, Kocaturk M, Cansev M, Tecles F, Ceron JJ, Yilmaz Z. (2012). Serum butyrylcholinesterase and paraoxonase 1 in a canine model of endotoxemia: effects of choline administration. *Res Vet Sci*. 93(2):668-674. doi:10.1016/j.rvsc.2011.09.010

Tvarijonaviciute A, Tecles F, Caldin M, Tasca S, Cerón J. (2012a). Validation of spectrophotometric assays for serum paraoxonase type-1 measurement in dogs. *Am J Vet Res*. 73(1):34-41. doi:10.2460/ajvr.73.1.34

Tward A, Xia YR, Wang XP, Shi YS, Park C, Castellani LW, Lusis AJ, Shih DM. (2002). Decreased atherosclerotic lesion formation in human serum paraoxonase transgenic mice. *Circulation*. 106(4):484-90. doi: 10.1161/01.cir.0000023623.87083.4f

Van Lanen K. Sande A. (2014). Canine hypoadrenocorticism: Pathogenesis, Diagnosis and Treatment. *Topics in Companion Animal Medicine*. 29,88-95. doi:10.1053/j.tcam.2014.10.001

Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, La Du BN, Fogelman AM, Navab M. (1995). Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J Clin Invest*. 96(6):2758-2767. doi:10.1172/JCI118345

Viitanen SJ, Laurila HP, Lilja-Maula LI, Melamies MA, Rantala M, Rajamäki MM. (2014). Serum C-reactive protein as a diagnostic biomarker in dogs with bacterial respiratory diseases. *J Vet Intern Med*. 28(1):84-91. doi:10.1111/jvim.12262

Vilhena H, Tvarijonaviciute A, Cerón JJ, Vieira L, Pastor J, Silvestre-Ferreira AC. (2017). Acute phase proteins response in cats naturally infected with Hepatozoon felis and Babesia vogeli. *Vet Clin Pathol*. 46(1):72-76. doi:10.1111/vcp.12451

von Eckardstein A, Nofer JR, Assmann G. (2001). High density lipoproteins and arteriosclerosis. Role of cholesterol efflux and reverse cholesterol transport. *Arterioscler Thromb Vasc Biol*. 21(1):13-27. doi:10.1161/01.atv.21.1.13

Watson AD, Berliner JA, Hama SY, La Du BN, Faull KF, Fogelman AM, Navab M. (1995). Protective effect of high density lipoprotein associated paraoxonase. Inhibition of the biological activity of minimally oxidized low density lipoprotein. *J Clin Invest*. 96(6):2882-2891. doi:10.1172/JCI118359

Witkowska-Piłaszewicz OD, Żmigrodzka M, Winnicka A, Miśkiewicz A, Strzelec K, Cywińska A. (2019). Serum amyloid A in equine health and disease. *Equine Vet J*. 51(3):293-298. doi:10.1111/evj.13062

World Health Organization. (2014). Antimicrobial resistance: global report on surveillance. World Health Organization

Xie S, Li J, Chen Y, Wang C, Zhang H, Mo D. (2010). Sequence identification, chromosomal mapping and tissue specific expression of the porcine paraoxonase 1 (PON1) gene. *Mol biol rep*. 37(3):1347-53. doi:10.1007/s11033-009-9514-6

Yamamoto S, Shida T, Okimura T, Otabe K, Honda M, Ashida Y, Furukawa E, Sarikaputi M, Naiki M. (1994). Determination of C-reactive protein in serum and plasma from healthy dogs and dogs with pneumonia by ELISA and slide reversed passive latex agglutination test. *Vet Q*. 16(2):74-77. doi:10.1080/01652176.1994.9694422

Zech R, Zürcher K. (1974). Organophosphate splitting serum enzymes in different mammals. *Comp Biochem Physiol B*. 48(3):427-433. doi:10.1016/0305-0491(74)90277-6

SCIENTIFIC ACTIVITIES

SCIENTIFIC PRODUCTION CONCERNING PHD PROJECT

Author or Co-author of peer reviewed papers:

- Ruggerone B, Paltrinieri S, Giordano A, Scavone D, Nocera I, Rinnovati R, Spadari A, Scacco L, Pratelli P, Sgorbini M. (2020). Paraoxonase-1 activity evaluation as a diagnostic and prognostic marker in horses and foals. Journal of veterinary internal medicine, 34(2), 949–954. https://doi.org/10.1111/jvim.15722
- Scavone D, Sgorbini M, Borges AS, Oliveira-Filho JP, Vitale V, Paltrinieri S. (2020). Serial measurements of Paraoxonase-1 (PON-1) activity in horses with experimentally induced endotoxemia. *BMC veterinary research*, 16(1), 422. https://doi.org/10.1186/s12917-020-02629-4
- Ruggerone B, Scavone D, Troìa R, Giunti M, Dondi F, Paltrinieri S. (2021). Comparison of Protein Carbonyl (PCO), Paraoxonase-1 (PON1) and C-Reactive Protein (CRP) as Diagnostic and Prognostic Markers of Septic Inflammation in Dogs. *Veterinary sciences*, 8(6), 93. https://doi.org/10.3390/vetsci8060093

Author or Co-author of manuscript in preparation:

- Scavone D, Bettoni V, Stefanello D, Scarpa P, Paltrinieri S. (2022). Measurement of Paraoxonase-1 activity in hospitalized dogs*. To be submitted*
- Paltrinieri S, Scavone D, Elitzur R, Iatta R, Otranto D. (2022). Assessment of Paraoxonase-1 activity in cats infected with Leishmania spp*. To be submitted*
- Scavone D, Nesossi A, Scanziani E, Stella S, Paltrinieri S. (2022). Paraoxonase-1 activity in slaughtering pigs*. To be submitted*
- Ferrulli V, Scavone D, Pravettoni D, Paltrinieri S. (2022). Paraoxonase-1 activity in calves with bovine respiratory disease (BRD)*. To be submitted*

Author or Co-author of congress abstracts:

- Scavone D, Zanzani S, Raffa C, Paltrinieri S (2021). C-reactive protein, Paraoxonase-1 activity and serum protein electrophoresis in dogs seropositive for Borrelia burgdorferi sensu lato. *74° Convegno Sisvet*, 23-26th June 2021, online
- Scavone D, Nesossi A, Scanziani E, Stella S, Paltrinieri S (2022). Paraoxonase-1 (PON-1) activity as a screening test in pigs at slaughter. *75° Convegno Sisvet*, 15-18th June 2022, Lodi, Italy

OTHER SCIENTIFIC PRODUCTION NON-CONCERNING PHD PROJECT

Author or Co-author of peer reviewed papers:

- Stranieri A, Scavone D, Paltrinieri S, Giordano A, Bonsembiante F, Ferro S, Gelain ME, Meazzi S, Lauzi S. (2020). Concordance between Histology, Immunohistochemistry, and RT-PCR in the Diagnosis of Feline Infectious Peritonitis. *Pathogens (Basel, Switzerland)*, 9(10), 852. https://doi.org/10.3390/pathogens9100852
- Scavone D, Lauzi S, Stranieri A, Tramontano G, Ratti G, Paltrinieri S (2022). Evaluating the presence of domestic cat hepadnavirus viraemia in cats with biochemical alterations suggestive of liver disease. *The Veterinary record, e1626*. Advance online publication. https://doi.org/10.1002/vetr.1626

Author or Co-author of congress abstracts:

- Sala G, Ratti G, Ferrulli V, Scavone D, Stranieri A, Giordano A, Boccardo A, Pravettoni D, Lauzi S (2022). First detection of "Candidatus Mycoplasma haemolamae" in alpaca (Vicugna pacos) in Italy. *31st World Buiatric Congress*, 4-8th September 2022, Madrid, Spain
- Cafiso A, Villa L, Bazzocchi C, Gazzonis AL, Scavone D, Raffa C, Lauzi S, Manfredi MT, Zanzani A (2022). Risk of Lyme borreliosis and Ixodes ricinus bite in dogs: a serological survey in high-risk cluster for resident human population in Lombardy region. *75° Convegno Sisvet*, 15-18th June 2022, Lodi, Italy
- Ratti G, Stranieri A, Scavone D, Meazzi S, Giordano A, Paltrinieri S, Lauzi S (2022). Detection of Domestic cat Hepadnavirus in cats with cavitary effusions. *75° Convegno Sisvet*, 15-18th June 2022, Lodi, Italy

Co-supervisor of Degree Thesis:

- Colli A, Misurazione della 8-idrossi-2'-deossiguanosina (8-OHdG): validazione analitica e valutazione del potenziale ruolo come biomarker di sepsi nel cane (2019 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)
- Raffa C, Prevalenza della sieropositività per Borreliosi in Valchiaveanna e indagine sullo stato infiammatorio nei cani positivi (2019 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)
- Tramontano G, Ricerca di Hepadnavirus nel siero di gatti con epatopatia (2020 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)

- Elitzur R, Assessment of Paraoxonase-1 activity in cats infected with Leishmania spp. (2021 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)
- Bettoni V, Utilità prognostica della misurazione di Paraoxonasi-1 (PON-1) in cani ospedalizzati (2021 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)
- Nesossi A, Relazione tra attività di Paraoxonasi-1 e lesioni rilevate al macello nel suino (2022 - Degree Thesis in Veterinary Medicine, Supervisor Prof. Saverio Paltrinieri)

ACKNOWLEDGEMENTS

Thanks to the ones that I love and that make me feel happy and lucky every day.

The first acknowledgement goes to my parents, as usual my certainties, the most important people of my life. No need to write, I owe everything to you.
Thanks to my brothers Andrea, for always being ready to help me, especially in my incompetencies, and little Pino, for the laughs, the sensitivity and his pure heart.
Thanks to my grandparents, for always reminding me what it means to be a good person, I miss you every day. Thanks to Roberto, for always being with me, besides the distance.

Thanks to Saverio, an outstanding and out of the ordinary person, in everything. Thank you for being brilliant and enterprising, for always caring of everything, for making the hospital real, for being a remarkable teacher and equally an honest and humble man, and for bringing wisdom and stupidity together every time. Thanks for your trust, your time and for always being there.

Thanks to Alessia, for your wisdom and practical sense, your strength and comprehension, for taking care of the lab, for teaching me and most of all for always caring about the human side of people.

Thanks to Piero, for helping and teaching me and always being kind, cheerful and generous.
Thanks to Valeria, Sara, Stefano, Tiziana and Sonia for the help and the time spent together.
Thanks to the students who attended the lab, for the gratifications and for your willingness to learn. Thanks to Virginia, my light in the dark.
Thanks from the heart to all of you, for your time and for making the lab a happy and special place.

Thanks to all the other colleagues I had the opportunity to know and work with in these years. In particular, thanks to the pathology section, to Mario for teaching me almost all I know about cytology and always being available and supportive, and to the PhD students Andrea, Francesco, Luca for the remarkable skills, and most importantly for your kindness and friendship. Thanks as well to dr. Galicci, Anna, Gabriele and Filippo for being skilled, professional and always warm and cheerful at the same time.

It was a pleasure to work and spend time outside of work with you all.

Thanks to Beatrice, for helping and inspiring me, for always being available, supportive and truthful. We have been missing you every day.

Thanks to Alice, Marta, Valeria and Francesca, for being like sisters to me and for being the ones I can always trust and rely on.

Thanks to Gianmarco, someone that turned out to be the opposite of what I believed. Thank you for the time spent together, for always being spontaneous, for your passion and dedication, for your patience, your willingness to build and most of all for changing my mind.

Finally,
Thanks to Angelica, my mentor, my friend, my (cursing) guardian angel. You pushed me and guided me through this journey, from degree thesis until today. Thank you for spending your time on me, even if you think it was not enough, you taught me more than anyone else. Thank you for always being curious and proactive, there's no way to make you understand how talented you are. Thank you for all that you shared with me in these years, from the worst to the most beautiful days.