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CLINICO-PATHOLOGICAL INVESTIGATION OF SERUM PROTEINS IN ODONTOCETES

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Summary

ABSTRACT

An initial assessment in stranded marine mammals, including evaluation of clinico-pathological variables, is a preliminary and critical step to define treatment and assessing the suitability of the animals for rehabilitation. Serum protein electrophoresis (SPE) is the most reliable method to determine the distribution of serum protein fractions and it is considered an essential step to evaluate the health status of animals, providing clinical useful information. The measurement of APPs in association to serum proteins' fractions can supplement and extend the baseline information obtained from the complete blood cell count, fibrinogen, and standard serum chemistry panel.

The thesis is divided in two main chapters, the first one focused on the serum protein electrophoresis (SPE) and the second one on the acute phase proteins evaluation

In the first chapter, 38 under human care bottlenose dolphin serum samples were screened with agarose gel electrophoresis (AGE) to determine the reference intervals (RIs) of the serum proteins. Four main protein fractions were evident in all the animals tested: albumin, α-globulins, β-globulins, and γ-globulins. The RIs for the serum protein fractions were: albumin 45.0 ± 4.0 g/L, α-globulins 8.0 ± 1.0 g/L, β-globulins 5.0 ± 2.0 g/L, and γ-globulins 7.0 ± 2.0 g/L. Compared to previously published data in free ranging bottlenose dolphins, in our samples the concentration of total protein, α-globulins, and γ-globulins were slightly lower, while the concentration of albumin and the albumin/globulins ratio were slightly higher. The lower concentration of ''inflammatory'' proteins associated to a higher concentration of albumin and the consequent higher albumin/globulins ratio reported in our study could reflect a lower antigenic stimuli in the animals housed in aquaria compared to the free-ranging populations. Moreover, in 8 electropherograms, we noticed that the base of the albumin peak was wider compared to the electropherograms of the other animals. For this reason, the same serum samples used for AGE were evaluated also with capillary zone electrophoresis (CZE), a more sensitive electrophoretic method. With CZE 9 out of 38 samples showed a double albumin peak. However, all these samples except 2 had an albumin peak wider than that observed with AGE in dolphins classified as non bisalbuminemic by CZE; furthermore a wider albumin peak was also noted with AGE in one sample with normal CZE profile. We report for the first time the presence of hereditary bisalbuminemia in two groups of related bottlenose dolphins identified by means of CZE and we confirm that AGE could fail in the identification of this alteration.

To understand the genetic basis of bisalbuminemia, the albumin gene of 15 bottlenose dolphins belonging to two distinct families were reconstructed by direct comparison of its full length cDNAs with the provisional sequence of bottlenose dolphin albumin gene. Eighteen albumin gene variations were identified in the bottlenose dolphins studied (15 non-synonimous and 3 synonymous). In order to identify the non-synonimous variations able to cause bisalbuminemia, the genotype-phenotype correlations within the two families were studied. Two heterozygous non-synonymous variations that co-segregate with the ''bisalbuminemia'' phenotype detected by SPE were identified: c.483C>G p.Phe146Leu in exon 4 and c.487T>C p.Tyr163His in exon 5. The genetic analysis of bottlenose dolphins' albumin gene showed a significant polymorphism and two mutations associated with bisalbuminemia. Moreover, we were able to identify the autosomal codominant trait of this condition in dolphins, a similar pattern of inheritance to that in humans. The in silico analysis and the comparison between dolphin and human variations support the hypothesis that the variation p.Tyr163His could be more likely responsible for bisalbuminemia.

In the second chapter double radial immunodiffusion (DRI), western blot (WB) analysis, and spectrophotometric measurement using immunologic or enzymatic assays were employed on serum samples of bottlenose dolphins and striped dolphins to validate, establish RIs, and evaluate the diagnostic accuracy of two positive acute phase proteins (C-reactive protein, CRP and serum amyloid-A, SAA) and one negative APP (serum paraoxonase-1, PON-1). With DRI none of the antibodies (Abs) against CRP and SAA cross-reacted with the serum samples of bottlenose dolphins and striped dolphins. Both the anti-SAA Abs tested were latex-conjugated, because produced for automatic immunoturbidimetric assays. The presence of latex associated to the Abs may have interfered to the migration of the Abs across the agarose gel.

WB analysis for anti-CRP antibodies showed a weak positivity for striped dolphins and a pattern of positivity in the serum samples of bottlenose dolphins similar to those observed in dog, with multiple bands. However, we are not able to exclude the possibility that this pattern may represent an unspecific signal. The discouraging result obtained with the automated measurement of dolphins CRP (0.00 mg/L) seemed to confirm the hypothesis that the anti-human CRP Ab used does not recognize the cetaceans' CRP, based also on the low homology of the amino acid sequence. On contrary, the SAA is highly conserved between different species. The automated measurement of SAA provided results with good precision; the SAA concentration in the whole set of bottlenose dolphins samples was 8.7 ± 11.8 mg/L. In addition, for the SAA concentration no differences were noted between different storage time, between the sex of the animals, and between pregnant and non-pregnant animals. The lack of differences in SAA concentration between males and females,

and pregnant and non-pregnant animals allowed us to establish the SAA RIs using samples from the whole population instead of establish partitioned RIs .Moreover, a stability in SAA concentration in serum samples with long storage time was demonstrated.

PON-1 activity was determined using 4 different substrates using enzymatic assays. The PON-1 activity using paraoxon as substrate provided results with good. The PON-1 activity in the whole set of bottlenose dolphins samples was 6.7 ± 4.6 U/L. As for the concentration of SAA, no differences in PON-1 activity were noted, based on sex, and between pregnant and non-pregnant animals. On contrary, the PON-1 activity for the long storage samples was significantly lower compared to the short storage samples. To evaluate the genetic influence of the single nucleotide polymorphisms (SNPs) in the PON-1 activity, we sequenced the two most studied SNPs of human PON-1 gene, the Q192R and the L55M. Based on the sequence analysis, all the dolphins were homozygous for methionine in L55M SNPs and for arginine in Q192R SNPs. Despite all the animals are homozygous for the phenotype associated to a higher paraoxonase activity in humans, the bottlenose dolphins' PON-1 activity is low and it seems not useful to discriminate between healthy and diseased animals. The PON-1 activity using 4-nitrophenyl acetate (4-nPA) as substrate was higher compare to those obtained using paroxon, providing results with good precision and accuracy but no significant difference were noted between healthy dolphins and diseased dolphins. However, our results are based on a limited number of animals so we cannot exclude that, including a higher number of animals with different diseases, a more drastic change in PON activity will be evident.

1. INTRODUCTION

1.1 Acute phase response

The first reaction of the organisms to different pathological conditions is an innate, aspecific response that precede specific immune response (Gruys et al., 2005). The term 'acute phase response' (APR) is referred to a nonspecific and complex reaction of an organism that occurs shortly after any tissue damage (Cerón et al., 2005). Conversely from the specificity of cellular and humoral immunity, the changes induced by APR are non-specific and occur in response to several conditions (Jaye and Waites, 1997) such as infection, trauma, neoplasia, inflammation, and stress (Cray, 2012). The systemic reaction of APR includes neurological, endocrine, and metabolic perturbations. The consequence of these perturbations are fever, leucocytosis, increased release of several hormones, activation of the clotting, complement and kinin-forming pathways, and drastic rearrangement of plasma protein synthesis (Koj, 1996). Macrophages, blood monocytes, and dendritic cells localized in the site of tissue damage are the cells primarily involved in the starting of APR (Cassatella, 1995). These cells are activated when particular chemical structures in damaged tissue or infectious agents are presented for detection (Cassatella, 1995). At least 15 different cytokines, secreted by activated leukocytes and other cells, are involved in APR (Gruys et al., 2005). These cytokines are classified in three groups, based on the effect pathways: (1) cytokines that primarily act as positive or negative growth factor for a variety of cells (IL-2, IL-3, IL-4, IL-7, IL-10, IL-11, IL-12, and granulocyte-macrophage stimulating factor); (2) cytokines with pro-inflammatory properties (TNF-α, TNF-β, IL-1 α, IL-β, IL-6, IFN α, IFN-γ, IL-8, and macrophage inhibitory protein-1); and (3) factors with anti-inflammatory activity (IL-1 receptor antagonist, soluble IL-1 receptors, TNF-α binding protein, and IL-1 binding protein) (Gruys et al., 2005).

The APR is associated to some changes in the organism that can be measured with some laboratory tests: (1) a decrease of blood plasma low and high density lipoproteins-bound cholesterol and leukocyte numbers; (2) an increased values of ACTH and glucocorticoids; (3) an activation of the complement and blood coagulation system; (4) a decreased serum levels of calcium, zinc, iron, vitamin A, and α-tocopherol; and (5) a change in concentration of several plasma proteins, the acute phase proteins (APPs) (Dinarello, 1989, 1983; Gruys et al., 1994). Few hours after a pathogen stimulus, in the liver, a change in the protein synthesis' pattern happens such as the increased synthesis of some proteins (positive APPs). Hepatic mRNA up-regulation for positive APPs is

associated with a decreased synthesis of normal blood proteins such as transthyretin, retinol binding protein, cortisol binding globulin, transferrin, and albumin, which represent the negative APPs (Blackburn, 1994; Dinarello, 1989, 1983; Gruys et al., 1994; Ingenbleek and Young, 1994; Kushner et al., 1981). The changes in blood plasma composition induced by APR are beneficial to the organism because they prevent microbial growth and they help the restoring of homeostasis (Gruys et al., 2006). Some APPs opsonize micro-organisms and activate complement, others scavenge cellular remnants and free radicals, or neutralize proteolytic enzymes (Gruys et al., 2006).

1.2 Electrophoresis

Serum protein electrophoresis (SPE) is the most reliable method to determine the distribution of serum protein fractions and it is considered, together with a basic hematological and biochemical profile, an essential step to evaluate the health status of animals, providing clinically useful information. The interpretation of kinetics of total protein and albumin and globulin fractions is receiving increased attention in domestic and wild animals in which a typical pathologic pattern could be identified in several diseases, such as inflammatory diseases. With SPE, it is possible to separate the serum proteins into 4-6 categories based on the protein's feature to migrate through cellulose acetate or agarose gel in an electrical field. With SPE, the degree of migration toward the anode is based on the electrical charge of the proteins and their mass and shape. In the serum of domestic mammals, albumin it is a small and very anionic protein, thus for these reasons, it has the faster migration. Globulins are bigger than albumin, but their negative charges caused an anodal migration. Moreover, some immunoglobulins are large and positive charge, so they migrate toward the cathode or do not migrate. The pH of the electrophoresis medium affects the charge and consequently the migration of proteins (Stockham and Scott, 2008b). Electrophoresis using cellulose acetate allows recognizing 5-9 protein bands, whereas using agarose allows recognizing 10-15 protein bands. A protein band may represent one protein or several proteins with the same migration pattern. With these techniques, it is possible to identify the album and five globulins regions: α_1, α_2, β_1, β_2, γ in most of the domestic mammals (Stockham and Scott, 2008b). Capillary zone electrophoresis (CZE) is an attractive alternative to the gel-based methods' electrophoresis (Bossuyt, 2006) because CZE allows a better peak resolution compared to AGE and eliminates some of its artefacts (Wijnen and van Dieijen-Visser, 1996). CZE combines the separation principles of conventional electrophoresis with the advanced instrumental design of high-performance liquid or gas chromatography and capillary technology (Wijnen and van Dieijen-Visser, 1996). With CZE, the separation of the protein fractions occurs in free solution in a narrow-bore fused silica capillary that is exposed to a high voltage. For this technique, an alkaline borate-

based buffer (pH 10) is used because at alkaline pH, serum proteins are negatively charged because the net charge of the proteins depends on the isoelectric point and by the pH of the buffer (Bossuyt, 2003). When voltage is applied, two forces act on the proteins. First, the proteins, that are negatively charged, are attracted toward the anode. Second, an electro-osmotic force is involved. The internal surface of the fused-silica capillaries is negatively charged when the electrolyte solution inside is at pH 10. For this reason, the cations of the solution near the capillary wall migrate toward the cathode, pulling electrolyte solution with them: this is the electro-osmotic flow (Bossuyt, 2006). These two forces act in opposite directions and in CZE, the force of the electro-osmotic flow surpasses the force of the electrical field so all proteins are carried toward the cathode (Bossuyt, 2006). Prealbumin and albumin are more resistant to the electro-osmotic flow than the γ-globulins because they are more acidic. Therefore, prealbumin and albumin are the last to reach the cathode. The proteins are detected and quantified at the cathodal end of the capillary by measurement of the absorbance at 200 to 214 nm (Bossuyt, 2006). The absorbance at 200-214 nm is directly proportional to the number of peptide bonds and the borate buffer does not interfere with the UV detection (Bossuyt, 2006). With CZE it is possible to identify the albumin and 4 fractions of globulins referred to as α_1, α_2, β, γ globulins (Bossuyt, 2003). With serum protein electrophoresis, it is not possible to identify the amount of a single protein but rather the amount of a group of proteins with the same electrophoretic mobility (Cray et al., 2009). The APPs are contained in the globulins fractions: α globulins include α_1-antitrypsin and α_1–acid glycoprotein (AGP), and haptoglobin (Hp); β-globulins include transferrin, serum amyloid-A (SAA), and γ-globulins are composed primary by IgG, even if also C-reactive protein (CRP) is present (Johnson et al., 1999; Stockham and Scott, 2008b).

1.3 Acute phase proteins

Recently, in the small animal medicine, studies on the role of APPs as markers of infectious, inflammatory and neoplastic diseases have proliferated (Eckersall and Schmidt, 2014) and at least 40 different plasma proteins have been identified as APPs (Schrödl et al., 2016). Not only the 'classical APPs' increase during APR, but also other non-protein molecules such as total serum sialic acid (Gopaul and Crook, 2006) or other proteins such as the APR-inducing cytokines or the hormones ghrelin, leptin, and gonadotropins (Maruna et al., 2005; Owen-Ashley et al., 2006). For this reason, in 2008, it has been proposed to replace the term 'acute phase proteins' with the term 'acute phase reactant' (Paltrinieri, 2008). In the present thesis, our efforts were focused in the validation of the classical APPs, for this reason we used the term 'acute phase proteins' instead of 'acute phase reactants'.

APPs are a group of blood proteins that change in concentration in animals subjected to external or internal challenges, such as infection, inflammation, surgical trauma or stress (Murata et al., 2004). APPs are synthesized primarily by hepatocytes (Cray et al., 2009) stimulated by cytokines (Petersen et al., 2004) or endogenous glucocorticoids (Paltrinieri, 2008). The APPs are components of the non-specific innate immune response, and their concentration in the blood are proportional to the severity of the disorder and/or the extent of tissue damage (Murata et al., 2004). APPs are classified based on the direction of changes in positive APPs, when their synthesis is upregulated and the concentration in blood increase, or in negative APPs when their synthesis is downregulated and the concentration in blood decrease, after a pathogen stimulus (Schrödl et al., 2016). Positive APPs are further classified as major, moderate or minor based on the magnitude of increase during the APR: major APPs showed an increase of 10- to 100-fold, moderated APPs showed an increase of 2- to 10-fold, and minor APPs have only a slight increase (Cerón et al., 2005). Moreover also the velocity of the concentration's increase vary between major, moderate, and minor positive APPs: major APPs usually have a marked increase within 48 hours after a pathogen stimulus and often have a rapid decline after the cease of the pathogen stimulus due to their short half-life (Johnson et al., 1999). Moderate and minor APPs usually increase more slowly but their increased concentration is more prolonged in duration. For this reason, moderate and minor APPs are usually detected increased during chronic inflammatory process (Cerón et al., 2005). The major, moderate, and minor APPs are different between species (Kent, 1992). For example, the major APP in ruminants is Hp (Conner et al., 1989); the major APP in dogs and men is CRP (Bürger et al., 1992; Eckersall et al., 1991; Pepys and Baltz, 1983); the major APP in horse is SAA (Pepys et al., 1989); the major APP in cats is AGP (Paltrinieri, 2008); and the major APPs in pigs are Hp, SAA, and major acute phase protein (MAP) (Petersen et al. 2004).

The CRP is named for its binding capacity to pneumococcal somatic C-polysaccharide. CRP is synthetized by the hepatocytes, it has a molecular size of approximately 115 kDa and consists of five non covalently associated polypeptides subunits (Pepys and Baltz, 1983). It is a member of the highly conserved pentraxin protein family and contributes to innate immunity against infection and to handling of autologous ligands, probably helping to prevent development of autoimmunity. The autologous ligands of CRP include phospholipids and ribonucleoproteins from necrotic and apoptotic cells (Thompson et al. 1999). The human SAA is has a molecular size of approximately 180 kDa (Pepys and Baltz, 1983) since SAA is normally found in a complex with lipoproteins (Petersen et al., 2004). The molecular size of SAA decrease after denaturation, and the molecular size of the denaturated SAA is between 9 and 14 kDA in different species (Hultén et al., 1997; Nunokawa et al., 1993; Pepys and Baltz, 1983). Serum paraoxonase-1 (PON-1) is synthetized

primarily by the hepatocytes and a portion is secreted into the plasma, where it is associated with high density lipoprotein (HDL) (Costa et al., 2005). The name PON-1 is given by one of the most substrate used for the evaluation: paraoxon, the toxic metabolite of the insecticide parathion. However, PON-1 hydrolyzes the active metabolites of several other organophosphorus insecticides, as well as nerve agents such as sarin, soman and VX (Draganov et al., 2004). Based on the several functions of PON-1, several substrates can be used to evaluate the different activities of this enzyme.

The use of APPs as marker of homeostasis' perturbation provides some advantages compared with the leukocyte count. The diagnostic sensitivity of APPs is higher (Kjelgaard-Hansen et al., 2003); the stability of APPs in serum/plasma samples is higher than the cellular components of blood, so it is possible to measure APPs in frozen serum/plasma samples (Solter et al., 1991); the change in APPs concentration is faster than changes in WBC counts (Kjelgaard-Hansen et al., 2003). One limitation of the APPs is the poor diagnostic specificity, for this reason they cannot be used as primary diagnostic test for a particular disease (Cerón et al., 2005). The APPs were successfully used to detect subclinical diseases and to monitor clinical evolution and to assess response to treatment (Cerón et al., 2005). In dogs an increase of APPs was demonstrated in cases of infectious diseases (Yamamoto et al., 1994), surgery (Christensen et al., 2015; Yamamoto et al., 1993), gastrointestinal disease (Otabe et al., 2000), autoimmune diseases (Caspi et al., 1987), endocrine diseases (McGrotty et al., 2003; Sasaki et al., 2003), and neoplasia (Caspi et al., 1987; Yuki et al., 2011). In addition, a correlation has been described between APPs levels and the severity of disease (Matijatko et al., 2007; Rikihisa et al., 1994; Yamamoto et al., 1994; Yule et al., 1997). Moreover, the measurement of APPs is proposed also as a test for liver function because, in end-stage liver, there is a decrease in the production of APPs (Kent, 1992). The combined measurement of several APPs provides more information than the evaluation of a single protein. In 2006 Gruys and colleagues proposed the 'APP value index' in which a major and moderate positive APPs are combined to a major and moderate negative APPs (Gruys et al., 2006). This index was used as prognostic factor in human patients with multiple myeloma (Dupire et al., 2012) and as acute phase index in pigs (Toussaint et al., 1995).

1.4 Infectious diseases of marine mammals

The cetaceans are primarily or secondary affected by several diseases.

Different cetacean's species are susceptible to distemper-like diseases due to cetacean morbillivirus (CeMV). CeMV consists of three strains: the porpoise morbillivirus (PMV), the dolphin

morbillivirus (DMV), and the pilot whale morbillivirus (PWMV) (Van Bressem et al., 2014). PMV and DMV are closely related each other, and are more closely related to the ruminant morbilliviruses and measles virus than to the distemper virus (Osterhaus et al., 1995). Most of the morbilliviruses are lymphotropic and epitheliotropic (Osterhaus et al., 1995). The first site of replication is the lymphoid tissues, after the infected lymphocytes carries the virus through the lymphatic system to the epithelial cells, which are infected (Delpeut et al., 2014; Ludlow et al., 2015). The lesions in the acute fatal infection are multifocal to diffuse interstitial broncho-pneumonia, necrosis of type 1 pneumocytes and bronchiolar epithelial cells, interstitial oedema, hyperplasia of type 2 pneumocytes, and formation of large syncytia in the alveolar and bronchiolar lumina. Intracytoplasmatic and intranuclear inclusion bodies can be present in the affected cells. The lymphoid tissues are characterized by lymphoid depletion and necrosis of the germinal centres, with the presence of syncytial cells (Van Bressem et al., 2014). If the animals survive the acute infection, they may succumb to opportunistic infections such as *Toxoplasma gondii*, herpesviruses, bacteria, and fungi due to immunosuppression. The typical lesions of the acute infection can be absent or obscured by the inflammation secondary to the opportunistic pathogens. The lesion indicative of sub-acute infection is a non-suppurative demyelinating meningoencephalitis, often focally distributed (Van Bressem et al., 2014). The animals that survive to the acute and sub-acute infection can succumb later because of the secondary infections, or from complications of CNS infection. The cause of the death is usually multifactorial (Van Bressem et al., 2014). Lesions and virus in the brain can be detected in the animals that survived the DMV systemic infection (Domingo et al., 1995; Ludlow et al., 2015; Soto et al., 2011). The lesions of the brain form are localized mostly in the cerebral cortex, the subcortical white matter, and the thalamus (Domingo et al., 1995), where it is possible to identify morbilliviral genome and/or antigens (Di Guardo and Mazzariol, 2016). The histological lesions noticed are similar to the lesions described in the subacute sclerosing panencephalitis (Garg, 2008) and in the old dog encephalitis (Headley et al., 2009). The DMV infection is the only variant that is associated to the 'brain only form' (Van Bressem et al., 2014).

Papillomaviruses are epitheliotropic viruses. The infection induces the proliferation of the epithelium of skin and mucosae causing warts, papillomas, and condylomas (Howley and Lowry, 2001). Three type of papillomaviruses, named type 1, type 2, and type 3, are capable to induce genital papillomatosis in dolphins from Europe and Atlantic USA (Rector et al., 2008). It seems that the development of the lesions in affected animals is associated to immunosuppressive conditions (Antonsson and Hansson, 2002).

Cetacean poxviruses are the etiologic agents responsible of tattoo skin disease. This pathology is characterized by the presence of typical, irregular, grey, black or yellowish, stippled cutaneous lesions (Van Bressem et al., 2003). As for papillomavirus's lesions, also the development of tattoo skin disease is associated to immunosuppressive status: juvenile animals or odontocetes in poor health conditions are more susceptible (Van Bressem et al., 2009). In healthy animals, the tattoo skin disease usually doesn't have high mortality rate (Van Bressem et al., 2003), however it can kill neonates and calves without protective immunity (Van Bressem et al., 1999), and also the co-infection with fungi or bacteria increases the mortality (Smith et al., 1983; Van Bressem et al., 2003).

Brucellosis is a zoonotic disease that affects reticuloendothelial, reproductive, musculoskeletal, and cutaneous system. In humans, it is reported that can cause generalized infection and septicaemia (Corbel, 1997). Three species of Brucella have marine mammals as preferred host: *Brucella delphini* for dolphins, *Brucella phocoenae* for porpoises, and *Brucella pinnipedialis* for seals (Foster et al., 2007; Groussaud et al., 2007). The transmission can be both horizontal and vertical (Van Bressem et al., 2009). The inflammatory lesions are localized in placenta with consequent aborts, testes, mammary gland, lung, sub-cutis, joints, liver, spleen, lymph nodes, and central nervous system (Corbel, 1997; Dagleish et al., 2008; Davison et al., 2009; Foster et al., 2007, 2002; González et al., 2002; Groussaud et al., 2007). The inflammation in central nervous system and the consequent damages may induce stranding of animals (Davison et al., 2009; Hernández-Mora et al., 2008).

Toxoplasma gondii is an obligate intracellular protozoan parasite that can infest marine mammals as intermediate hosts (Dubey et al., 2003; Miller et al., 2008), even if only cats and wild felids are the definitive hosts. Animals, usually immunosuppressed due to morbillivirus infection or other immunosuppressive diseases (Di Guardo et al., 1995), can be infected by ingestion of contaminated food/water or transplacentally (Van Bressem et al., 2009). The inflammatory lesions are usually located in lymph nodes, adrenal glands, myocardium, lungs, central nervous system (Van Bressem et al., 2009).

1.5 Evaluation of cellular and humoral immune responses in marine mammals

Increasing knowledge in cellular and humoral immune response is a fundamental step in understanding the relationship between infectious pathologies and the immune system in marine

mammals. From 1978, different assays were proposed to evaluate the cellular and humoral immune response.

The lymphocyte transformation assay allows the qualitative and quantitative evaluation of non-specific and antigen-specific cellular immune response. Several protocols have been proposed in the past years. In 1978 Colgrove demonstrated *in vitro* that concavalin A (Con A) induced the most intense lymphocyte proliferation compared to phytohemagglutinin (PHA) and pokeweed mitogen (PWM) (Colgrove, 1978). However inter-individual differences of white blood cells and the inhibitory effect of elevated blood cortisol levels during transportation or handling of animals hindered the standardization of lymphocyte transformation assay (De Guise et al., 1996; Noda et al., 2006). For these reasons, it was proposed to isolate the leukocytes, with different techniques such as density gradient centrifugation. The isolated leukocytes are successfully stimulated by Con A, PHA, and PWM, while the B cell mitogen lipopolysaccharide fails to induce detectable proliferation (Lahvis et al., 1993).

Natural killer (NK) cells plays an important role in innate immunity against viral infections. To evaluate the role of these cells, chromium release assay and detection of necrotic cells by flow cytometry have been validated (Beineke et al., 2010). The phagocytosis and generation of reactive oxygen species represent other defence mechanisms of innate immunity. The kinetics of these leukocyte functions, particularly *in vitro* ingestion of latex beads and hydrogen peroxide production have been evaluated in beluga whales and in bottlenose dolphins using flow cytometry (De Guise et al., 1996; Noda et al., 2003).

The superoxide generation of neutrophils of bottlenose dolphins is upregulated by phorbolmyristicacetate *in vitro* and the incubation with superoxide dismutase causes a dismutation of oxygen radicals in cultured dolphin leukocytes (Noda et al., 2003; Shiraishi et al., 2002). Immunoglobulin (Ig)A, IgG, and IgM have been purified or identified in the plasma of dolphins and whales by cross-reacting markers (Andrésdóttir et al., 1987; Cavagnolo, 1979; Nash and Mach, 1971; Travis and Sanders, 1972). Moreover cDNA of the heavy chains of IgA, IgG and IgM coding genes of the bottlenose dolphin have been cloned and sequenced (Lundqvist et al., 2002; Mancia et al., 2007, 2006). To investigate serum Ig levels by radial immunodiffusion assay and by ELISA, species-specific antibodies against cetacean IgG have been produced (Nollens et al., 2007; Ruiz et al., 2009; Taylor et al., 2002).

1.6 Evaluation of cytokines in marine mammals

In addition, the investigation of cytokine expression and acute phase responses give important information about the functionality of lymphoid cells in marine mammals. Also the use of recombinant cytokines enables the production of specie-specific antibodies for developing immunological assays and to study the possible therapeutic application in whales and dolphins (Denis and Archambault, 2001; Manire and Rhinehart, 2000; St-Laurent and Archambault, 2000).

Quantification of cytokine expression enables the investigation of leukocyte functionality and characterization of inflammatory response (Beineke et al., 2010). IL-1α, IL-1β, and IL-1 receptor antagonist (ra) cDNA have been cloned and sequenced from mitogen-stimulated leukocytes of bottlenose dolphins (Beineke et al., 2010). The receptor binding of IL-1 is highly conserved, while the remaining structure have a minor degree of homology (Beineke et al., 2010). To assess the cellular immune response in bottlenose dolphins an IL-2 receptor expression assay was developed (Erickson et al., 1995) and the mRNA of the IL-2 precursor molecule of the beluga whales was cloned and sequenced (St-Laurent et al., 1999). In bottlenose dolphins it was demonstrated also the existence and the function of the pro-inflammatory cytokines tumour necrosis factor (TNF)-α (St-Laurent et al., 1999). In 1996 King et colleagues demonstrated a conserved biological activity of the cytokine in different terrestrial and aquatic mammal species (King et al., 1996). Moreover the coding regions of the IL-1β, IL-6, and TNF-α gene of the beluga whale have been sequenced, and the cytokine-specific rabbit antisera have been produced (Denis and Archambault, 2001; St-Laurent and Archambault, 2000). In harbour porpoise the detection and quantification of mRNA of IL-1β, IL-2, IL-4, IL-6, IL-10, and TNF-α have been performed by PCR (Beineke et al., 2004), and the increase of IL-10 was seen in harbour porpoises suffering from long lasting infectious (Beineke et al., 2007). The identification of IL-2, IL-4, IL-10, IL-12, IL-13, IL-18, TNF-α, TGF-β , and interferon (IFN)-γ was performed using RT-PCR in bottlenose dolphins, pacific white-sided dolphins, and beluga whales (Sitt et al., 2008).

1.7 Acute phase reaction in marine mammals

The evaluation of health status of marine mammals is difficult because the classical clinical signs of illness used for human and domestic animals are difficult to recognize or of difficult interpretation. For this reason, in the past years, several efforts were done to identify laboratory markers of disease in these animals. First markers tested were white blood cell counts, erythrocyte sedimentation rate, and body temperature (Bossart et al., 2001). However, a change in these parameters are not seen in

early stage of diseases. For this reason, efforts are direct at identifying inflammation at earlier stage (King et al., 2001). The APPs have demonstrated their role as early markers of inflammation in both human and veterinary medicine, so based on the results obtained in humans and companion animals, several positive APPs were tested in marine mammals while no information are available for negative APPs. One limitation in the application of APPs in marine mammals is the lack of specie-specific Abs, moreover the knowledge about APP in one species cannot be readily extrapolated to another species, in which healthy levels, response to inflammation or infection, and diagnostic and prognostic potential may be different (Kjelgaard-Hansen et al., 2011). In bottlenose dolphins (*Tursiops truncatus*) three APPs (CRP, SAA, and Hp) were tested, even if not always complete validation study was performed (Cray et al., 2013; Segawa et al., 2013); in florida manatees (*Trichechus manatus latirostris*) five different APPs were tested: AGP, CRP, Hp, fibrinogen, and SAA (Harr et al., 2006); in harbor seals (*Phoca vitulina*) Hp and CRP were tested (Funke et al., 1997; Kakuschke et al., 2010; Rosenfeld et al., 2009). The aims of these studies were to evaluate the feasibility of the assays to measure the APPs and to determine the RIs. In bottlenose dolphins, the authors established the RIs for the three APPs (Cray et al., 2013) and demonstrated that Hp levels in the serum increase under inflammatory conditions (Segawa et al., 2013). In the florida manatee, SAA was the APP with the highest diagnostic sensitivity and specificity (90% for both sensitivity and specificity) in the detection of inflammatory diseases, the diagnostic specificity of Hp and fibrinogen were 93% and 95% respectively while the diagnostic sensitivity for these two APPs were 60% and 40% respectively. The Abs used for the determination of the concentration of AGP and CRP did not cross-react in this specie (Harr et al., 2006). In the harbor seals, an Ab to identify the CRP was produced (Funke et al., 1997) and the Hp was demonstrated to be a sensitive marker of the health/disease status (Kakuschke et al., 2010).

1.8 Validation process

The major steps in the establishment of reference intervals are

1. Select criteria for reference individuals
2. Establish a reference sample group
3. Collect and process the samples
4. Determine the reference values
5. Determine the reference distribution
6. Determine the reference limits and reference intervals (Stockham and Scott, 2008a).

The analytical properties of one assay are five: analytical precision, analytical accuracy, analytical specificity, detection limit, and analytical sensitivity.

- ANALYTICAL PRECISION is the ability of an assay to get the same result if a sample is analyzed several times and it is frequently expressed in clinical laboratories by the coefficient of variation (CV) of the method. The CV is expressed as a percentage and it is calculated by this formula: (standard deviation/mean)*100. The CV is determined from replicate analysis within an assay run (intra-assay CV) and between different runs of the same assay (inter-assay CV). The intra-assay CV is the expected error (Stockham and Scott, 2008a).

- ANALYTICAL ACCURACY represents the closeness of the agreement between the measured value of an analyte and its 'true value'. To determine the analytical accuracy it is possible to compare the values obtained by the 'new' assay to the values obtained by the reference method or it is possible to use standard solutions (Stockham and Scott, 2008a).

- ANALYTICAL SPECIFICITY is the ability of an assay to detect only the substance of interest (Stockham and Scott, 2008a).

- DETECTION LIMIT represent the smallest concentration or quantity of an analyte that can be detected with reasonable certainty. It represents the ability of one assay to discriminate the 'background noise' from a true change because of the presence of an analyte (Stockham and Scott, 2008a).

- ANALYTICAL SENSITIVITY is how much change of the analyte is needed for the assay to detect the change (Stockham and Scott, 2008a).

After the analytical validation of a new assay, it is important to determine how likely if a laboratory test result is outside the RIs, the animal has a certain disorder and if a laboratory test resultis within the RIs, the animal does not have a certain disease or pathologic state. This evaluation is called 'diagnostic validation'. The test results can be classified as true positive, true negative, false positive, and false negative. After the test results are classified as true positive, true negative, false positive, or false negative, it is possible to determine the diagnostic sensitivity, the diagnostic specificity, the diagnostic accuracy, the predictive value of positive test, and the predictive value of negative test (Stockham and Scott, 2008a).

- DIAGNOSTIC SENSITIVITY is the probability that a test results positive in diseased animals. The diagnostic sensitivity must be high in the screening test. To calculate the diagnostic sensitivity the formula is (true positive/(true positive + false negative))*100 (Stockham and Scott, 2008a).

- DIAGNOSTIC SPECIFICITY is the probability that a test results negative in non-diseased animals. A test with high diagnostic specificity is used to confirm a positive result obtained with a screening test (test with high diagnostic sensitivity). To calculate the diagnostic specificity the formula is (true negative/(true negative + false positive))*100 (Stockham and Scott, 2008a).

- DIAGNOSTIC ACCURACY is the frequency with which a test correctly classifies an animal as having or not having the disease. To calculate the diagnostic accuracy the formula is (true positive + true negative/(true positive + true negative + false positive + false negative))*100 (Stockham and Scott, 2008a).

- PREDICITVE VALUE OF A POSITIVE RESULT is the probability that a positive test result indicates that the animal has the disease. To calculate the predictive value of a positive result the formula is (true positive/(true positive + false positive))*100 (Stockham and Scott, 2008a).

- PREDICITVE VALUE OF A NEGATIVE RESULT is the probability that a negative test result indicates that the animal does not have the disease. To calculate the predictive value of a positive result the formula is (true negative/(true negative + false negative))*100 (Stockham and Scott, 2008a).

RECEIVER OPERATING CHARACTERISTIC (ROC) CURVES display the relationship between a true positive rate and a false positive rate. ROC curves can be used to compare the diagnostic accuracy of assays and to establish a decision threshold to rule in or rule out a diagnosis. To compare the diagnostic accuracy of different assays it is possible to visually analyse the ROC curves or to calculate the area under the ROC curves of the different assays (Stockham and Scott, 2008a).

Results of laboratory tests on patient samples would be very difficult to interpret without reference intervals (RIs). RIs are an integral component of laboratory diagnostic testing and clinical decision-making and represent the estimated distribution of reference values from healthy populations of comparable individuals (Friedrichs et al., 2012). RIs usually comprised the 95% of a healthy reference population, and the criteria used to establish health (selection or inclusion criteria) should be established (Friedrichs et al., 2012). The confidence intervals (CI) is an estimation of the uncertainty of the limits and is generally narrower for large sample sizes (Friedrichs et al., 2012). To determine RIs using non parametric methods with 95% CI, a minimum of 120 individuals is recommended. In this case the 2.5th fractile serves as lower reference limit and the 97.5th fractile as upper reference limit (Horn and Pesce, 1983). If fewer than 120 samples are available, alternative

methods are required to determine 95% CI. For example, robust methods are recommended when the reference samples are ≥ 40 and ≤ 120 (Horn et al., 1998), but also parametric methods can be used when the reference samples are ≥ 40 and ≤ 120 if the data have or can be transformed to Gaussian distribution (Friedrichs et al., 2012). When the reference samples are ≥ 20 and ≤ 40, RI should be calculated by methods that are robust or parametric, but to highlight the incertainty, 90% CI should be calculated (Friedrichs et al., 2012). If the reference samples are ≤ 20 RI should not be determined and it is better to report a table of ascending values along with histogram and mean or median values (Friedrichs et al., 2012).

2. AIMS

In order to increase the knowledge on inflammatory markers and to provide more sensitive tools to evaluate health status in odontocetes, we focused our work on two main topics: serum protein electrophoresis (SPE) and acute phase proteins (APPs). Thus, the present thesis is dived in two chapters: the first chapter is focused on the generation of RIs for SPE in under human care bottlenose dolphins, on the identification of bisalbuminemia and on the evaluation of the genetic bases of this disorder; while the second chapter is focused on the evaluation of APPs in dolphins.

More in detail, in order to increase the knowledge on SPE, the aims of the first chapter were:

- To establish the RIs for serum protein fractions evaluated with agarose gel electrophoresis (AGE) in bottlenose dolphins kept under human care.

Since AGE electropherogram of some dolphins showed a wider base of the albumin peak, all the animals were investigated also with CZE, in order

- To report the presence of bisalbuminemia and to evaluate the sensitivity and specificity of AGE in the diagnosis of this disorder compared to CZE.
- To investigate the polymorphisms in the albumin gene in two families of bottlenose dolphins in which bisalbuminemia was found.
- To identify the mutations responsible for bisalbuminemia and to identify the inheritance pattern of this condition in two families of bottlenose dolphins.

In the present work, two positive APPs: CRP and SAA, and one negative APP, the PON-1 were studied. The aims of the second chapter were:

- To assess the cross-reactivity of commercially available anti-human and anti-canine antibodies (Abs) used in immunological automated assays for SAA and CRP in striped dolphins (*Stenella ceoruleoalba*) and bottlenose dolphins (*Tursiops truncatus*).
- To validate automated assays for the measurement of SAA and CRP in bottlenose dolphins.
- To validate methods to measure PON-1 with different substrates, identify the one(s) more suitable to use in bottlenose dolphins, and to define the RIs. This part of the project was partially carried out in Murdoch University, Perth, Western Australia.
- To identify the presence of single nucleotide polymorphisms (SNPs) in the PON-1 gene, and to evaluate if the SNPs can influence the PON-1 activity.

3. MATERIALS AND METHODS

Since the present study has two different chapters, named 'serum protein electrophoresis, and 'acute phase proteins', this section has been divided in two main paragraphs to give better details on the methodology used.

3.1 Serum Protein Electrophoresis

3.1.1 Establishment of reference intervals for serum protein fraction in bottlenose dolphins

Animals and samples

Serum samples from bottlenose dolphins with normal clinical history, physical examination and complete haematological and biochemical exams were used. The exclusion criteria were: diseased animals, clinically healthy animals with abnormality in the haematological and biochemical exams and animals that are receiving medical treatment. A total of 38 dolphins were enrolled: 20 dolphins were males and 18 females. The median age was 18 years, minimum 1 year, maximus 51 years. They were housed in Acquario di Genova (14 animals), Oltremare in Riccione (11 animals), Mediterraneo Park in Malta (8 animals), and Zoomarine Italy (7 animals). Peripheral blood samples were obtained from individual animal during the veterinary procedures to evaluate health status of the animals. The animals were housed and handled in agreement with the Italian and Maltese Zoo directive law (DL 73/2005 and S.L.439.08 respectively) and all the samples were obtained according to D.M. 469/2001, which establishes the management objectives and prescriptions to maintain the species *Tursiops truncatus* under human care. Blood was collected in plain tubes; serum was obtained by centrifugation of blood samples at 1500g for 10 minutes. All serum samples were visually inspected and were not grossly hemolyzed or lipemic; thus, all samples were stored at -20°C until analysis.

Agarose gel electrophoresis

AGE was performed as already described (Giordano and Paltrinieri, 2010) using an automated system and kits provided by the manufacturer of the instrument (Sebia Italia Srl, Bagno a Ripoli, Firenze, Italy). Briefly, a 0.8% agarose gel was run in Trisbarbital buffer at pH 8.5, with migration time of 7 minutes at 800 V. Gels were stained with amido Schwarz, destained, and dried for scanning by the appropriate gel scanner. Data where then transferred to the software program and

visually inspected to correct the possible errors in fractions separation generated by the automated software (Phoresis, Sebia Italia Srl, Bagno a Ripoli, Firenze, Italy).

Total protein

Total protein (TP) concentration was determined by the biured method on an authomated spectrophotometer (Cobas Mira, Roche Diagnostics, Basel, Switzerland), and absolute values for each electrophoretic fraction were calculated based on TP and percentage of the fraction.

Data analysis

The RIs were obtained using an Excel spreadsheet with the Reference Value Advisor (v.2.1) set of macroinstructions as reported by Geffré and colleagues (Geffré et al., 2011). The software performs the computations recommended by the International Federation of Clinical Chemistry-Clinical and Laboratory Standards Institute (2008) such as the descriptive statistics (mean, median, standard deviation (SD), minimum and maximum values), the tests of normality (Anderson–Darling with histograms and Q–Q plots and Box–Cox transformation) and the outlier analysis. Both Dixon–Reed and Tukey tests were used, and outliers classified as 'suspected' were retained, as recommended by the ASVCP guidelines (Friedrichs et al., 2012). RIs were calculated using standard and robust methods on both non-transformed and transformed data. The software indicates the best method to define the RIs based on data distribution. A non-parametric bootstrap method was used to calculate the 90% confidence interval.

For the intra-assay and inter-assay repeatability of AGE, a pooled dolphin's serum sample was used.

3.1.2 Identification of bisalbuminemia

Animals and samples

For the identification of bisalbuminemia and to evaluate the diagnostic sensitivity and specificity of the AGE in comparison to CZE in the diagnosis of this disorder, the same serum samples used in the 'Establishment of reference intervals for serum protein fraction in bottlenose dolphins' chapter were used.

Agarose gel electrophoresis

The AGE was performed as described in the 'Establishment of reference intervals for serum protein fraction in bottlenose dolphins' chapter.

Capillary Zone Electrophoresis

CZE was performed with the MINICAP system 6 kit by SEBIA (Sebia Italia Srl, Bagno a Ripoli, Firenze, Italy), designed for the separation of serum protein in alkaline buffer (pH 9.9) into four major fractions. The MINICAP performed all analysis automatically to obtain a protein profile for qualitative and quantitative analysis. A sample diluted with buffer was prepared and injected by aspiration at the anodic end of the capillary. A high voltage protein separation was then performed and direct detection of the proteins was made at 200 nm at the cathode end of the capillary. The instrument recorded the absorbance corresponding to each electrophoretic fraction and sent the data to the software (Phoresis, Sebia Italia Srl, Bagno a Ripoli, Firenze, Italy), which converted the absorbance in peaks. The capillaries were immediately washed with a wash solution and prepared for the next analysis with buffer. The electrophoretograms were interpreted visually to screen for any pattern abnormality.

Electrophoretic profiles obtained with CZE and AGE were firstly visually analyzed to identify the presence of a double peak in the albumin region. Since only an apparently wider albumin peaks were noted in some AGE electrophoretic profiles, the ratio between base and height (b/h) of the albumin peak was calculated and each point-value recorded in the whole data set was used to calculate the ROC curve and to define the 'ideal' cut-off value able to identify this abnormality in AGE electrophoresis. For this analysis, the true positive samples were considered those with a double peak in CZE.

The difference in TP, albumin percentage and absolute value between bisalbuminemic and normal animals was evaluated by Mann-Whitney test while the differences between the albumin percentage obtained by CZE and AGE were analyzed with a Wilcoxon signed-rank test for paired data. All statistical analysis was performed using standard statistical software (IBM SPSS Statistics 22.0).

3.1.3 Genetic bases of bisalbuminemia

Animals and samples

A total of 15 animals from two distinct families (nine from family A and six from family B) were used. Five animals in family A (Tt5, Tt6, Tt7, Tt8, Tt9) and two animals in family B (Tt10, Tt14) were known to be bisalbuminemic based on CZE analysis. Four animals, in both family A (Tt1, Tt2, Tt3, Tt4) and in family B (Tt10, Tt11, Tt12, Tt13) were wild founders, while all the other dolphins (Tt5, Tt6, Tt7, Tt8, Tt9 in family A; Tt14, Tt15 in family B) were born under human care. The animals were permanently housed in aquaria and dolphinaria (Acquario di Genova and Oltremare, Italy, and Mediterraneo Park, Malta) under conditions approved by Italian and Maltese

laws (Italy: Zoo directive DL 73/2005, and DM 469/2001; Malta: Zoo directive 94 S.L.439.08 and the European Association of Aquatic Mammals (EAAM) 2009 ''Standards and Guidelines for the Management of Bottlenose Dolphins (*Tursiops* sp) Under Human Care'').

Peripheral blood samples (10 cm^3 collected into EDTA for haematology) were collected in accordance with relevant guidelines and regulations: 13 animals were sampled from the ventral fluke veins during routine veterinary health examinations without restraint according to DM 469/2001. Once used for the complete blood cell count exam, the remaining blood samples were used for DNA extraction. Two dolphins (Tt10, Tt11) had died prior to commencement of the study, thus archived frozen tissue obtained during the necropsy were used (liver for Tt10 and muscle for Tt11).

DNA extraction

Genomic DNA was extracted using QIAamp DNA Mini Kit (Quiagen, Milan, Italy) following the manufacturer's instruction. Twenty microliters of proteinase K were added to 200 µl of each sample. Then, 200 µl buffer AL was added to the sample, mixed by pulse-vortexing for 15 seconds and incubated at 56°C for 10 minutes. Two hundreds µl of ethanol (96%-100%) were added to the samples and then carefully applied to the QIAamp Mini spin column in a collection tube without wetting the rim. The spin columns were centrifuged at 6000 x g for 1 minute and then placed in a clean collection tube and the tubes containing the filtrate were discharged. Carefully, 500 µl of buffer AW1 were added without wetting the rim and centrifuged at 6000 x g for 1 minute. QIAamp Mini spin columns were then placed in a clean tube, 500 µl of buffer AW2 were added and centrifuged at 20,000 x g for 3 minutes. Finally, the QIAamp Mini spin columns were placed in a clean tubes and 200 µl of distilled water were added. After incubation at room temperature for 1 minute, the tubes were centrifuged at 6000 x g for 1 minute. Amount of DNA extracted from peripheral whole blood ranged from 10.4 ng/µl to 50.4 ng/µl, while DNA extracted from tissues ranged from 119.6 ng/µl (liver) to 362.8 ng/µl (muscle).

Albumin gene mutation and gene reconstruction

The structure of the bottlenose dolphin albumin gene was reconstructed by direct comparison of its full length cDNAs with the provisional sequence of bottlenose dolphin albumin gene (Gene ID: XP_004322082.1). The coding region of the albumin gene was screened for alterations by direct sequencing of all samples. Polymerase chain reaction (PCR) primers flanking each exon of the albumin gene were designed by PRIMER3 (http://www.genome.wi.mit.edu). PCR amplifications were performed in a final volume of 25 µl, containing 50-100 ng of genomic DNA, 5x Colorless

GoTaq® Reaction Buffer (Promega, Fitchburg, WI, USA), 400 nmol/L each primer, 100Mmol/L deoxinucleotide triphosphates (Invitrogen, Waltam, MA, USA), and 0.5 U of GoTaq® DNA Polymerase (Promega). PCR amplification for exon 10 was performed in a final volume of 25 µl, containing 50-100 ng of genomic DNA, 5x AS Reaction Buffer (Fisher Molecular Biology, Rome, Italy), 500 nmol/L $MgCl_2$, 400nmol/L each primer, 100 Mmol/L deoxynucleotide triphosphates (Invitrogen), and 0.5 U of Taq DNA Polymerase (Fisher Molecular Biology). Cycling conditions (denaturations at 95°C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 45 seconds) were repeated for 35 cycles. PCR primers and conditions are reported in table 3.1.3.A. Sequencing of the albumin gene was performed using the automatic sequencer 3130ABI PRISM Genetic Analyzer (Applied Biosystem, Foster City, CA, USA). Chromas 1.5 software (Technelysium, Suth Brisbane, Queensland, Australia) and LASERGENE package computer programs (DNASTAR, Madison, WI, USA) were used to edit, assemble, and translate sequence.

The obtained albumin sequences were compared with reference sequence XP_004322082.1 and analyzed in order to identify non-synonymous and synonymous substitutions (variation in DNA sequences with or without change in amino acid sequences). For non-synonymous substitutions, the correlation between genotype and ''bisalbuminemic'' phenotype was investigated with the pedigree of the two families, in order to identify both the mutation responsible for bisalbuminemia and the pattern of inheritance.

Table 3.1.3.A: Oligonucleotide primers used for PCR amplification and DNA sequencing of the exons of dolphin albumin gene. Abbreviation: TD, touch-down; bp, base pairs.

Exons	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Products size	Annealing temperature
Exon 1	agcgctgatctccctctatt	ctcactattcgtttcacctagga	340 bp	55°C
Exon 2	atcttggagcaaatgaatacct	aaccagtaccattagaaacgtt	225 bp	55°C
Exon 3	tctagcatagcaacctactgt	gctatacccacttttgagcg	344 bp	55°C
Exon 4	ggaaaacaaaagtctgccca	tgtcattagaacagaagcttcaa	383 bp	58°C
Exon 5	tggaggttctggggagaatgt	ggggaaaagacggaggaagg	367 bp	TD 70°C-60°C
Exon 6	ggggtggtctgcgtaaaatt	tgttactgatgctctggttttca	322 bp	60°C
Exon 7	tgccctaagagaacaatttggt	ctcaaccactctcagttgttct	400 bp	55°C
Exon 8	gaggaagcacagcaaagtca	ggcaacatgaagattgtagcaa	372 bp	58°C
Exon 9	ttgggagtgcggagacttag	agcacagagttgaaaccatctt	369 bp	55°C
Exon 10	gaaacacaacctgcatctgg	agacccaaagcagccaaaa	300 bp	TD 70°C-60°C
Exon 11	cagtttcttgcctggctgaa	acacacattagcccaccctt	282 bp	55°C
Exon 12	acatttgttttggtcctttgga	ttctgctggacctagcctaaga	490 bp	55°C
Exon 13	agcatcttaagtagcagcgat	gcaacagcaaaggaaattagaca	298 bp	55°C
Exon 14	agtggtgctaatattttcccc	ctggtcagttgatctttgct	189 bp	58°C

Secondary structure prediction

Three different primary structures of albumin, named wild type Albumin, Phe146Leu Albumin, and Tyr163His Albumin, were characterized in order to identify differences in secondary structures. Each protein sequence was subjected to consensus secondary structure prediction using PSIRED (Jones, 1999).

3.2 Acute phase proteins

3.2.1 Positive APPs

Animals and samples

To investigate the cross reactivity of the antibodies to measure the positive APPs 10 serum samples (4 from bottlenose dolphins and 6 from striped dolphins) were used. Bottlenose dolphins' samples were collected from animals under human care in according to Zoo directive DL 73/2005, and DM 469/2001 and were stored in the Mediterranean Marine Mammals Tissue Bank of the University of

Padua, while striped dolphins' samples were collected from stranded animals during the normal veterinary procedures for the health assessment and were stored in the Department of Comparative Medical Science of the University of Teramo. All the samples were stored at -20°C until analysis.

The automatic measurement of positive APPs was performed on 74 serum samples collected from bottlenose dolphins under human care. In these animals, in addition to the determination of RIs, differences between samples with long (more than 1 year at -80°C) and short (less than 1 year at -80°C) storage, between male and female animals, and between pregnant and non-pregnant animals were evaluated.

Cross-reactivity evaluation

To assess the cross-reactivity, seven antibodies against CRP and 2 antibodies against SAA were tested with double radial immunodiffusion (DRI) and/or western blot (WB) analysis. The different Abs used, the companies, the clones, the target species, and the species in which the Abs were validated are reported in table 3.2.1.A.

Table 3.2.1.A: antibodies used for double radial immunodiffusion (DRI) and western blot analysis (WB).

| Antibody	Produced by	Clone	Produced in	Target specie	Validated in	Methodology tested
CRP	Randox, Crumlin, UK	300626	Goat	Human	Human, dog	DRI, WB
CRP	Biodevice, Santarcangelo di Romagna, Rimini, Italy	CRP005A	Non specified	Non specified	Human	DRI
CRP	Aptec, Sint Niklaas, Belgium	CRP024 / PG4448	Goat	Non specified	Human	DRI
CRP	Real Time, Viterbo, Italy	1172	Non specified	Non specified	Human	DRI
CRP	Tridelta, Maynooth, Ireland	51KT010b	Non specified	Dog	Human, dog	DRI
CRP	Ben S.r.l., Milano, Italy	B1424Y	Goat	Dog	Human	DRI
CRP	Life Diagnostics, West Chester, PA, USA	C-G1513A	Goat	Dog	Dog	DRI, WB
SAA	Eiken Chemical Co., Tokio, Japan	09006	Mouse (monoclonal), Rabbit (polyclonal), Goat	Human	Human, horse, cat	DRI, WB
SAA	Tridelta, Maynooth, Ireland	24CC017	Multispecies	Non specified	Multispecies	DRI

Double radial immunodiffusion

DRI is an immunological technique used in the identification of antibodies or antigens. In our work, 0.1% agarose gel were cut to form a series of wells in the gel (Figure 3.2.1.A). Several different tests were performed, changing the volume and the dilution of the serum samples and of the Abs used. As controls, a healthy human serum sample, serum samples from dogs with high and normal CRP concentration, and serum samples from cats with high and normal SAA concentration were used. In the central well 20 µl of Ab and in the peripheral wells 12 µl of serum were added or, alternatively, 20 µl of serum in the central well and 12 µl of Abs in the peripheral wells. All the different tests were summarized in table 3.2.1.B.

After the Abs and serum samples were putted in the wells, the gels were left for 24 hours in the dark in a moist chamber. During this time, the antigens present in the serum and the antibodies each diffuse out of their respective wells. Where the two diffusion fronts meet, if any of the antibodies recognize any of the antigens, they will bind to the antigens and form an immune complex. The immune complex precipitates in the gel to give a thin white line (precipitation line), which is a visual signature of antigen recognition. We conducted the experiments testing in parallel 6 different serum samples, when the Ab was placed in the central well, or 6 different Abs, when the serum was placed in the central well. When more than one well is used, there are many possible outcomes based on the reactivity of the antigen and Ab selected. The zone of equivalence line may give full identity (when a continuous line is present), partial identity (when a continuous line with a spur at one end), or a non-identity (when the two lines cross completely) (Figure 3.2.1.B).

Table 3.2.1.B: double radial immunodiffusion tests performed. In the central wells, 20 µl of antibody and in the peripheral wells 12 µl of serum were added or, alternatively, 20 µl of serum in the central wells and 12 µl of antibodies in the peripheral wells. In each test, each serum sample/antibody in the central will react with the antibodies/serum samples present in the six peripheral wells.

Test number	Central wells	Peripheral wells
1	CRP Randox undiluted CRP Randox diluted 1:2 SAA Tridelta undiluted SAA Tridelta diluted 1:2 SAA Eiken undiluted SAA Eiken diluted 1:2	Bottlenose dolphin Striped dolphin Human Dog high CRP Dog normal CRP Cat high SAA
2	Striped dolphin Striped dolphin Bottlenose dolphin Human Dog high CRP Dog normal CRP Cat high SAA Cat normal SAA	CRP Randox undiluted CRP Randox diluted 1:2 SAA Eiken undiluted SAA Eiken diluted 1:2 SAA Eiken diluted 1:3 Physiological saline
3	Striped dolphin Striped dolphin Bottlenose dolphin Human Dog high CRP Dog normal CRP Cat high SAA Cat normal SAA	CRP biodevice undiluted CRP Aptec undiluted CRP realtime undiluted CRP Tridelta undiluted CRP Ben undiluted SAA Eiken undiluted
4	CRP biodevice undiluted CRP Aptec undiluted CRP realtime undiluted CRP Tridelta undiluted CRP Ben undiluted SAA Eiken undiluted	Bottlenose dolphin Dog high CRP Striped dolphin Dog high CRP Human Dog high CRP
5	CRP Life Diagnostic diluted 1:100 CRP Life Diagnostic diluted 1:1000	Bottlenose dolphin Dog high CRP Striped dolphin Dog high CRP Human Dog high CRP
6	CRP Life Diagnostic diluted 1:5 CRP Life Diagnostic diluted 1:10	Bottlenose dolphin Dog high CRP Striped dolphin Dog high CRP Human Dog high CRP

Figure 3.2.1.A: 0.1% agarose gel was cut to form 7 wells. In the central wells 20 µl of antibody and in the peripheral wells 12 µl of serum were added or, alternatively, 20 µl of serum in the central wells and 12 µl of antibodies in the peripheral wells. Asterisks: precipitation lines.
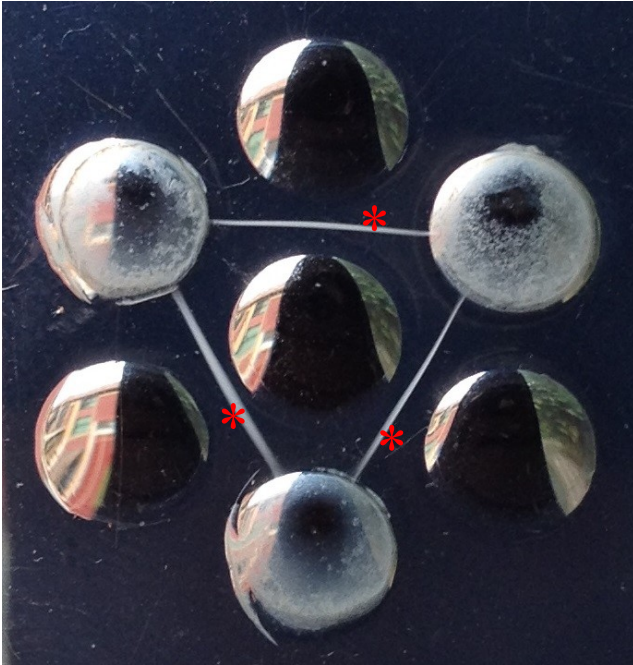
Figure 3.2.1.B: interpretation of the DRI cross-reaction when the antibody is placed in the central well and the serum samples in the peripheral wells.
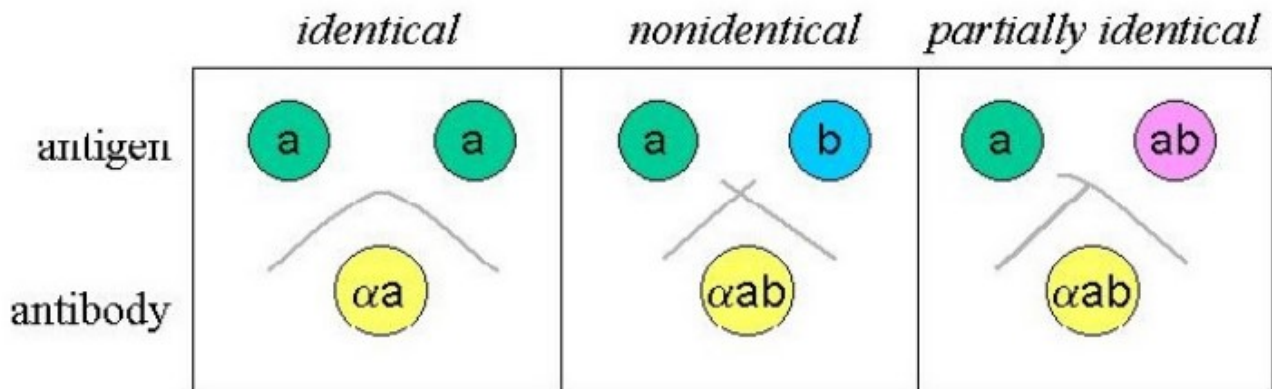
Western Blot analysis

The polyacrylamide gel allows separating proteins with a molecular weight (MW) between 4 kDa and 200 kDa. The optimal concentration of the polyacrylamide in the gel is based on the MW of the proteins of interest. A higher concentration of polyacrylamide is indicated for the low MW proteins; on the contrary, a lower concentration of polyacrylamide is better for the high MW proteins.

With this technique, it is possible to separate the proteins based only on their MW. In our work, the separation of the serum proteins were performed using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli protocol (Laemmli, 1970). 20 µl of working solution (4 µl of serum or Ab plus 11 µl of water plus 5 µl of loading buffer) was loaded in the gel and run in a running buffer (TRIS-base 25mM, glycine 192m, 0.1% SDS; pH 8.3) at 80V for 30 minutes and then at 100V for 2 hours at room temperature.

After molecular weight separation, proteins were transferred to a nitrocellulose membrane using a trans-blot (Trans Blot turbo, BIO-RAD Transfer system, Hercules, CA, USA). The nitrocellulose membrane was then covered with a blocking solution (5% goat milk and 0.05% tween) for 1 hour at room temperature. After this, the membrane was washed to complete the blocking of aspecific binding site.

The nitrocellulose membrane was then incubated with the primary antibody (SAA or CRP) (Table 3.2.1.A) diluted 1:1000 for 1 hour in the dark at room temperature, washed, and then incubated with the secondary antibody for 45 minutes in the dark at room temperature. The dilution was 1:4000 for the secondary antibody against SAA and 1:3000 for the secondary antibody against CRP. The dilution of primary and secondary Abs were performed with 5% goat milk, PBS, and tween. After the incubation with the secondary antibody the nitrocellulose membrane was washed. The washing procedure consisted in three 5 minutes incubations in PBS buffer 0.1% tween. The membrane was then incubated with chemioluminescent AP substrate (Immobilon TM Western, Millipore, Billerica, MA, USA) for 5 minutes in the dark at room temperature. The chemoluminescent reaction was detected using x-ray (CL-X posure TM film, Thermo Fisher Scientific, Waltham, MA, USA). All the different tests were summarized in table 3.2.1.C.

Table 3.2.1.C: Western blot tests performed.

Test number	Lane	Samples	Volume of samples	Volume H_2O	Volume loading buffer
1	1	Marker			
	2	SAA calibrator	4 µl (undiluted)	11 µl	5 µl
	3	Empty			
	4	Cat high SAA	4 µl (undiluted)	11 µl	5 µl
	5	Empty			
	6	Cat normal SAA	4 µl (undiluted)	11 µl	5 µl
	7	Empty			
	8	Bottlenose dolphin-1	4 µl (undiluted)	11 µl	5 µl
	9	Striped dolphin-1	4 µl (undiluted)	11 µl	5 µl
	10	Marker			
2	1	Marker			
	2	Standard SAA	4 µl (undiluted)	11 µl	5 µl
	3	Marker			

	4	Standard SAA	4 µl (diluted 1:2)	11 µl	5 µl
	5	Marker			
	6	Standard SAA	4 µl (diluted 1:4)	11 µl	5 µl
	7	Marker			
	8	Standard SAA	4 µl (diluted 1:8)	11 µl	5 µl
	9	Standard SAA	4 µl (diluted 1:16)	11 µl	5 µl
	10	Marker			
3	1	Marker			
	2	Cat high SAA	4 µl (undiluted)	11 µl	5 µl
	3	Cat normal SAA	4 µl (undiluted)	11 µl	5 µl
	4	Bottlenose dolphin-1	4 µl (undiluted)	11 µl	5 µl
	5	Striped dolphin-1	4 µl (undiluted)	11 µl	5 µl
	6	Marker			
	7	Standard SAA	4 µl (undiluted)	11 µl	5 µl
	8	Standard SAA	4 µl (diluted 1:2)	11 µl	5 µl
	9	Standard SAA	4 µl (diluted 1:4)	11 µl	5 µl
	10	Standard SAA	4 µl (diluted 1:8)	11 µl	5 µl
4	1	Marker			
	2	Dog high CRP	5 µl (undiluted)	10 µl	5 µl
	3	Dog high CRP	5 µl (diluted 1:2)	10 µl	5 µl
	4	Dog normal CRP	5 µl (undiluted)	10 µl	5 µl
	5	Bottlenose dolphin-1	5 µl (undiluted)	10 µl	5 µl
	6	Marker			
	7	Striped dolphin-1	5 µl (undiluted)	10 µl	5 µl
	8	Standard CRP	10 µ (undiluted)l	5 µl	5 µl
	9	Standard CRP	5 µl (undiluted)	10 µl	5 µl
	10	Standard CRP	5 µl (undiluted)	10 µl	5 µl
5	1	Marker			
	2	Dog high CRP	4 µl (diluted 1:20)	11 µl	5 µl
	3	Dog normal CRP	4 µl (diluted 1:20)	11 µl	5 µl
	4	Bottlenose dolphin-1	4 µl (diluted 1:20)	11 µl	5 µl
	5	Striped dolphin-1	4 µl (diluted 1:20)	11 µl	5 µl
	6	Marker			
	7	Dog high CRP	4 µl (diluted 1:40)	11 µl	5 µl
	8	Dog normal CRP	4 µl (diluted 1:40)	11 µl	5 µl
	9	Bottlenose dolphin-1	4 µl (diluted 1:40)	11 µl	5 µl
	10	Striped dolphin-1	4 µl (diluted 1:40)	11 µl	5 µl
6	1	Marker			
	2	CRP calibrator n°2	4 µl (diluted 1:10)	11 µl	5 µl
	3	Dog high CRP	4 µl (diluted 1:20)	11 µl	5 µl
	4	Striped dolphin-1	4 µl (diluted 1:20)	11 µl	5 µl
	5	Bottlenose dolphin-1	4 µl (diluted 1:20)	11 µl	5 µl
	6	Bottlenose dolphin-2	4 µl (diluted 1:20)	11 µl	5 µl
	7	Bottlenose dolphin-3	4 µl (diluted 1:20)	11 µl	5 µl
	8	Striped dolphin-2	4 µl (diluted 1:20)	11 µl	5 µl
	9	Striped dolphin-3	4 µl (diluted 1:20)	11 µl	5 µl
	10	Striped dolphin-4	4 µl (diluted 1:20)	11 µl	5 µl
7	1	Marker			
	2	CRP calibrator n°2	4 µl (diluted 1:20)	11 µl	5 µl
	3	CRP calibrator n°2	4 µl (diluted 1:50)	11 µl	5 µl
	4	Empty			
	5	Empty			
	6	Striped dolphin-5	4 µl (diluted 1:100)	11 µl	5 µl
	7	Empty			
	8	Bottlenose dolphin-4	4 µl (diluted 1:20)	11 µl	5 µl
	9	Striped dolphin-6	4 µl (diluted 1:20)	11 µl	5 µl
	10	Empty			

Automated measurement of CRP

The CRP concentration was measured according to the protocol of Klenner and colleagues (Klenner et al., 2010) validated in dogs. We used an assay from Randox Laboratories (High Linearity CRP, Randox Laboratories Ltd., Crumlin, UK) that consisted of a high-linearity test using an anti-human CRP antibody. The assay was run on an automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) according to the manufacturers' recommendations and was calibrated with test specific calibration material. Briefly, 12 µl of serum were diluted in 150 µl of buffer composed by polyethylene glycol, TRIS-HCl, and NaCl. The anti-CRP latex conjugated antibody was added to the solution. The antigen-antibody complex precipitates and the concentration of CRP was proportional to the increase in absorbance read after 160 seconds at 340nm of wavelength.

Automated measurement of SAA

The SAA concentration was measured according to Christensen and colleagues' assay, validated in dogs, cats, and horses (Christensen et al., 2012). The assay is based on latex-conjugated monoclonal anti-human SAA antibody (Eiken Chemical Co., Tokio, Japan) and was measured using the automated spectrophotometer ILab300 plus (Instrumentation laboratory, Rome, Italy). Briefly, 3 µl of serum were diluted with 3 µl of water and added to the 50 mmol/litre Good's buffer solution, the SAA latex conjugated antibody was added to the solution. The antigen-antibody complex precipitates and the concentration of SAA is proportional to the increase in absorbance read after 296 seconds at 620nm of wavelength. Mann-Whitney test was used to asses if there was differences between storage time, sex, and pregnant and non-pregnant animals.

3.2.2 Negative APP

PON-1 can be measured based on its activity by spectrophotometric assays. Usually, in veterinary medicine, PON-1's activity is measured using a paraxon-based enzymatic method. However PON-1 is considered having the ability to hydrolyze many other substrates such as other organophosphorous compounds, non phosphorous arylesters (arylesterase activity) and also lactones (lactonase activity). For these reasons, PON-1 activity can be measured by different substrates such as paroxon, phenyl acetate, 4-nitrophenyl acetate, or dihydrocumarin.

Animals and samples

For the measurement performed in University of Milan, Italy, 62 serum samples collected from under human care bottlenose dolphins were used. In these animals, in addition to the determination

of RIs, differences between samples with long (more than 1 year at -80°C) and short (less than 1 year at -80°C) storage, between male and female animals, and between pregnant and non-pregnant animals were evaluated.

For the tests performed in Murdoch University, Perth, Western Australia, serum samples from 3 healthy bottlenose dolphins, 3 diseased bottlenose dolphins, 2 healthy indo-pacific bottlenose dolphins *(Tursiops aduncus)*, and 4 diseased indo-pacific bottlenose dolphins were used. These serum samples were kindly provided by Dr. David Blyde, and were collected from animals kept under human care or hospitalized at Sea World-Gold Coast, Queensland, Australia and stored at –20°C until analysis were performed. As controls, serum samples from healthy dogs were used.

Paraoxonase activity

The paraoxonase activity was evaluated using paraoxon (Sigma-Aldrich, St. Luis, MO, USA) as substrate using automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) according to the protocol of Feingold and colleagues (Feingold et al., 1998) modified by Rossi and colleagues (Rossi et al., 2013). Briefly, 6 µl of serum plus 89 µl of water were added to 100 µl of reaction buffer. The reaction buffer is composed by glycine buffer 0.05 mmol, pH 10.5 containing 1mmol paraoxon-ethyl and 1 mmol $CaCl_2$. The rate of hydrolysis of paraoxon to *p*-nitrophenol was measured by monitoring the increasing in absorbance at 504 nm wavelength using a molar extinction coefficient of 18,050 L/mol/cm^{-1}. PON-1 activity expressed as U/mL was defined as 1 nmol of *p*-nitrophenol formed per minute under the assay condition. Mann-Whitney test was used to asses if there were differences between storage time, sex, and pregnant and non-pregnant animals. Since PON-1 is an enzyme associated with high density lipoproteins (HDL), we decided to evaluate the concentration of HDL. The HDL concentration was measured following the protocol of Rossi and colleagues (Rossi et al., 2014), using an automated spectrophotometer (ILab300, Instrumentation Laboratory, Bedford, MA, USA).

Arylesterase activity

The evaluation of arylesterase activity was performed at Murdoch University, Perth, Western Australia. To evaluate the arylesterase activity two different substrates were used: (1) 4-nitrophenyl acetate (4-nPA) (Sigma-Aldrich, St. Luis, MO, USA) using and automated spectrophotometer (Cobas Mira, Roche Diagnostic, Basel, Switzerland) and a manual one (UV-1800 Shimadzu Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) and (2) phenyl acetate (PA) (Sigma-Aldrich, St. Luis, MO, USA) using the manual spectrophotometer, according to the protocol proposed by Tvarijonaviciute and colleagues (Tvarijonaviciute et al., 2012).

Because 4-nPA is insoluble in water, 63 mg of this compound was dissolved in 10 mL of methanol and stored at 2° to 8°C. This stock solution can be kept for approximately 1 week. Afterward, 1 mL of this solution was slowly added to 20 mL of distilled water with strong agitation to prevent precipitation. The aqueous solution was freshly prepared each day.

Twenty µL of serum samples were mixed with 288 µL of buffer containing 50 mmol Tris-HCl and 1mmol $CaCl_2$ (pH 8.0) and then 72 µL of freshly made substrate containing 2.5mmol 4-nPA in distilled water was added. After 100 seconds, the reaction was monitored at 405 nm at 37°C for 210 seconds in an automated spectrophotometer. Molar extinction coefficient used to calculate the rate of hydrolysis was 14,000 mol^{-1} cm^{-1}. Because of the lack of a criterion-referenced standard or commercially available certified reference material for PON-1 activity, the assay precision was evaluated by 10 consecutive repeat measurement of PON-1 activity in 3 serum samples with low, medium, and high activity and the assay accuracy was evaluated indirectly through the assessment of linearity under dilution (LUD) using a bottlenose serum sample that was serially diluted with the working solution without the substrate.

In the manual spectrophotometer 52.6 µL of serum samples were mixed with 758 µL of buffer containing 50 mmol Tris-HCl and 1mmol CaCl2 (pH 8.0) and then 189.5 µL of freshly made substrate containing 2.5 mmol 4-nPA in distilled water was added. After 100 seconds, the reaction was monitored at 405 nm at 25°C for 225 seconds with the manual spectrophotometer. As a blank, the buffer without serum and substrate was used.

For the evaluation of arylesterase activity using PA, only a manual spectrophotometer with UV laser for the detection of the reaction was used. Serum samples were diluted with the buffer consisting of 20mmol Tris-HCl and 1mmol $CaCl_2$ (pH 8.0). In a quartz cuvette, a variable amount of serum was added to 800 µL of freshly made substrate buffer containing Tris-HCl 20mmol, 1mmol $CaCl_2$, and 1mmol PA (pH 8.0). The reaction was monitored for 60 or 320 seconds, at 260 nm at 25°C with a manual spectrophotometer; molar extinction coefficient used to calculate the rate of hydrolysis was 1,310 $mol^{-1}cm^{-1}$. As a blank, the buffer without serum was used. All the test conditions were summarized in table 4.2.2.D.

Lactonase activity

The evaluation of lactonase activity was performed at Murdoch University, Perth, Western Australia, using dihydrocoumarin (DC) (Sigma-Aldrich, St. Luis, MO, USA) as substrate. A manual spectrophotometer (UV-1800 Shimadzu Spectrophotometer, Shimadzu Corporation, Kyoto, Japan) with UV laser for the detection of the reaction was used. We followed the test conditions

reported by Draganov and colleagues (Draganov et al., 2000): in a quartz cuvette containing 800 µL of 1 mmol DC in 50 mmol Tris-HCl (pH 8.0) 1 mmol CaCl$_2$, 10 to 40 µL of serum were added. The hydrolysis of DC was monitored for 60 or 180 seconds by the increase in UV absorbance at 270 nm at 25°C. The molar extinction coefficient used to calculate the activity of PON 1 was 1295 mol^{-1} cm^{-1}. As a blank, the buffer without serum was used. All the test conditions were summarized in table 4.2.2.E.

Genetic polymorphisms of PON-1 gene

For this study, the same DNA samples previously extracted for the determination of the mutations responsible of bisalbuminemia were used.

The structure of the two most studied single nucleotide polimorphisms (SNPs) in the PON-1 gene, the Leucine/Methionine in position 55 (L55M) and Glycine/Arginine in position 192 (Q192R) (Mackness and Mackness, 2015), was reconstructed by direct comparison of its full length cDNAs with the sequence of *Tursiops truncatus* gene (Gene ID: XM_019927639.1). The coding region of the PON-1 gene was screened for alterations by direct sequencing of all samples. The nucleotides' sequence of the PCR primers was taken from literature. They were previously used to detect the L55M PON-1 SNP ((Khoshi et al., 2009) and the Q192R PON-1 SNP (Browne et al., 2007) in human. The primers were tested in DNA extracted from human and dolphins to evaluate if the molecular weight and the nucleotide sequence of the PCR amplificate were the same in all the species. PCR amplification for L55M SNP was performed in a final volume of 25 µl, containing 50-100 ng of genomic DNA, 5x AS Reaction Buffer (Fisher Molecular Biology), 500 nmol/L MgCl$_2$, 400nmol/L each primer, 100 Mmol/L deoxynucleotide triphosphates (Invitrogen), and 0.5 U of Taq DNA Polymerase (Fisher Molecular Biology). PCR amplifications for Q192R SNP were performed in a final volume of 25 µl, containing 50-100 ng of genomic DNA, 5x Colorless GoTaq® Reaction Buffer (Promega), 400 nmol/L each primer, 100Mmol/L deoxinucleotide triphosphates (Invitrogen), and 0.5 U of GoTaq® DNA Polymerase (Promega). Cycling conditions (denaturation at 95°C for 30 seconds, annealing for 30 seconds, and extension at 72 °C for 45 seconds) were repeated for 40 cycles for both the SNPs. PCR primers and conditions are reported in table 3.2.2.A. Sequencing of the PON-1 SNPs was performed using the automatic sequencer 3130ABI PRISM Genetic Analyzer (Applied Biosystem). Chromas 1.5 software (Technelysium) and LASERGENE package computer programs (DNASTAR) were used to edit, assemble, and translate sequence.

Table 3.2.2.A: Oligonucleotide primers used for PCR amplification and DNA sequencing of the dolphin's PON-1 gene. Abbreviation: bp, base pairs.

SNPs	Forward Primer (5'- 3')	Reverse Primer (5'- 3')	Products size	Annealing temperature
L55M	cctgcaataatatgaaacaacctg	tgaaagacttaaactgccagtc	171 bp	50°C
Q192R	gggacctgagcacttttatggc	catcgggtgaaatgttgattcc	176 bp	50°C

4. RESULTS

4.1 Serum Protein Electrophoresis

4.1.1 Establishment of reference intervals for serum protein fraction in bottlenose dolphins

Reference intervals for serum protein fractions based on AGE are presented in table 4.1.1.A. Intra-assay and inter-assay repeatability of AGE were reported in table 4.1.1.B. Four main protein fractions were evident in all the animals tested: albumin, α-globulins, β-globulins, and γ-globulins (Figure 4.1.2.A-a). Moreover, in 8 electropherograms, at a visual inspection, we noticed that the base of the albumin peak was wider compared to the electropherograms of the other animals.

Table 4.1.1.A: reference intervals for total protein and protein fractions (concentration and percentage) in bottlenose dolphins. Abbreviations. TP: total protein; SD: standard deviation; LRL: lower reference limit; URL: upper reference limit; CI: confidence interval; S: suspected; R: removed.

	Mean	Median	SD	Min	Max	LRL 90% CI	URL 90% CI	Outliers	Statistical Method
TP (g/L)	65.5	66.7	5.4	50.8	74.0	54.4 (36.1-56.5)	73.5 (72.1-74.8)	2S	BOX-COX transformed
Albumin (g/L)	45.0	45.0	4.0	36.9	54.1	37.0 (35.0-39.0)	53.0 (52.0-55.0)	0	Untransformed
α-globulins (g/L)	8.0	8.0	1.0	5.3	11.4	5.0 (5.0-6.0)	11.0 (10.0-12.0)	1S	BOX-COX transformed
β-globulins (g/L)	5.0	5.0	2.0	2.6	9.3	2.0 (1.0-3.0)	8.0 (7.0-9.0)	0	Untransformed
γ-globulins (g/L)	7.0	7.0	2.0	0.0	11.3	2.0 (1.0-4.0)	11.0 (10.0-12.0)	1S	BOX-COX transformed

Albumin (%)	69.5	69.6	3.4	61	77.3	62.3 (60.8-63.7)	76.4 (74.5-78.2)	1S	Untransformed robust method
α-globulins (%)	12.0	12.1	2.1	8.3	16.8	7.6 (6.6-8.5)	16.4 (15.3-17.4)	0	Untransformed
β-globulins (%)	7.6	6.9	2.1	3.9	13.5	4.2 (3.7-4.7)	12.6 (11.3-14.4)	1S	BOX-COX transformed
γ-globulins (%)	11.2	11.3	2.6	4.2	15.9	6.1 (4.9-7.5)	16.8 (15.4-18)	1R, 1S	Untransformed robust method
Albumin/ globulins ratio	2.3	2.3	0.4	1.6	3.4	1.7 (1.6-1.8)	3.3 (3.0-3.7)	1S	BOX-COX transformed

Table 4.1.1.B: intra-assay (8 runs) and inter-assay (8 runs) imprecision recorded for AGE of bottlenose dolphin pooled serum samples reported as coefficient of variations (mean values ± SD) for each fraction.

	Intra-assay	Inter-assay
Albumin	1.2 % (60.9 ± 0.73 g/L)	2.5 % (64.13 ± 1.6 g/L)
α-globulins	2.9 % (14.1 ±0.4 g/L)	5.7 % (12.0 ± 0.7 g/L)
β-globulins	3.8 % (9.1 ± 0.4 g/L)	4.0 % (8.6 ± 0.3 g/L)
γ-globulins	3.4 % (15.9 ± 0.5 g/L)	4.8 % (15.4 ± 0.7 g/L)

Figure 4.1.1.A: Graphic representation of the data distribution for total protein, albumin/globulins ratio, albumin (g/L and %), α-globulins (g/L and %), β-globulins (g/L and %), and γ-globulins (g/L and %). The box represents the interquartile range (IQR) defined by 25th (Q1) and 75th (Q3) percentiles with the vertical line representing the median. The horizontal lines are the limits of outlies distribution according to Tukey rule. Suspect outliers are indicated in orange and outliers in red.
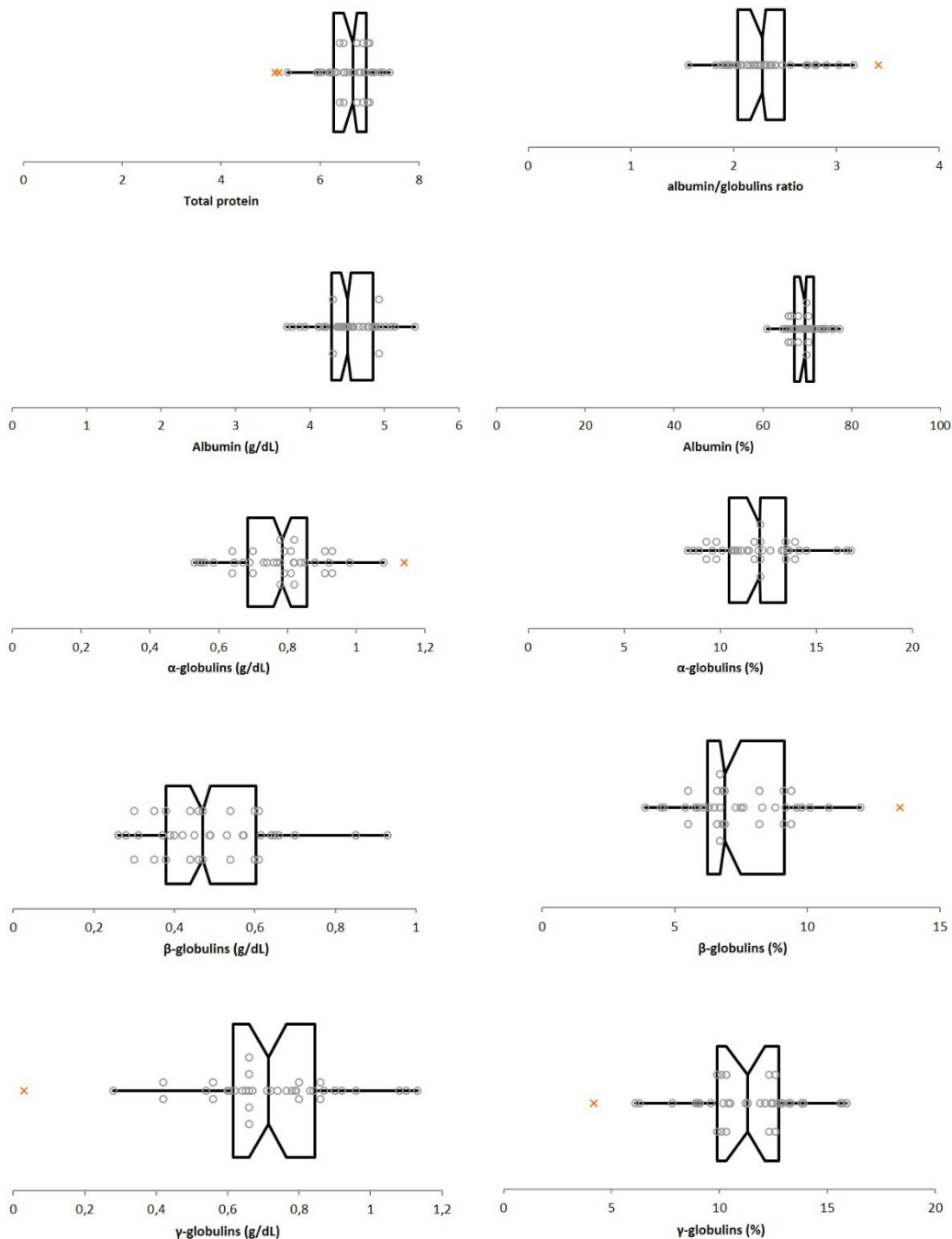
Figure 4.1.1.B: Histograms of the data distribution for total protein (g/L), albumin/globulins ratio, albumin (%) α-globulins (%), β-globulins (%), and γ-globulins (%).
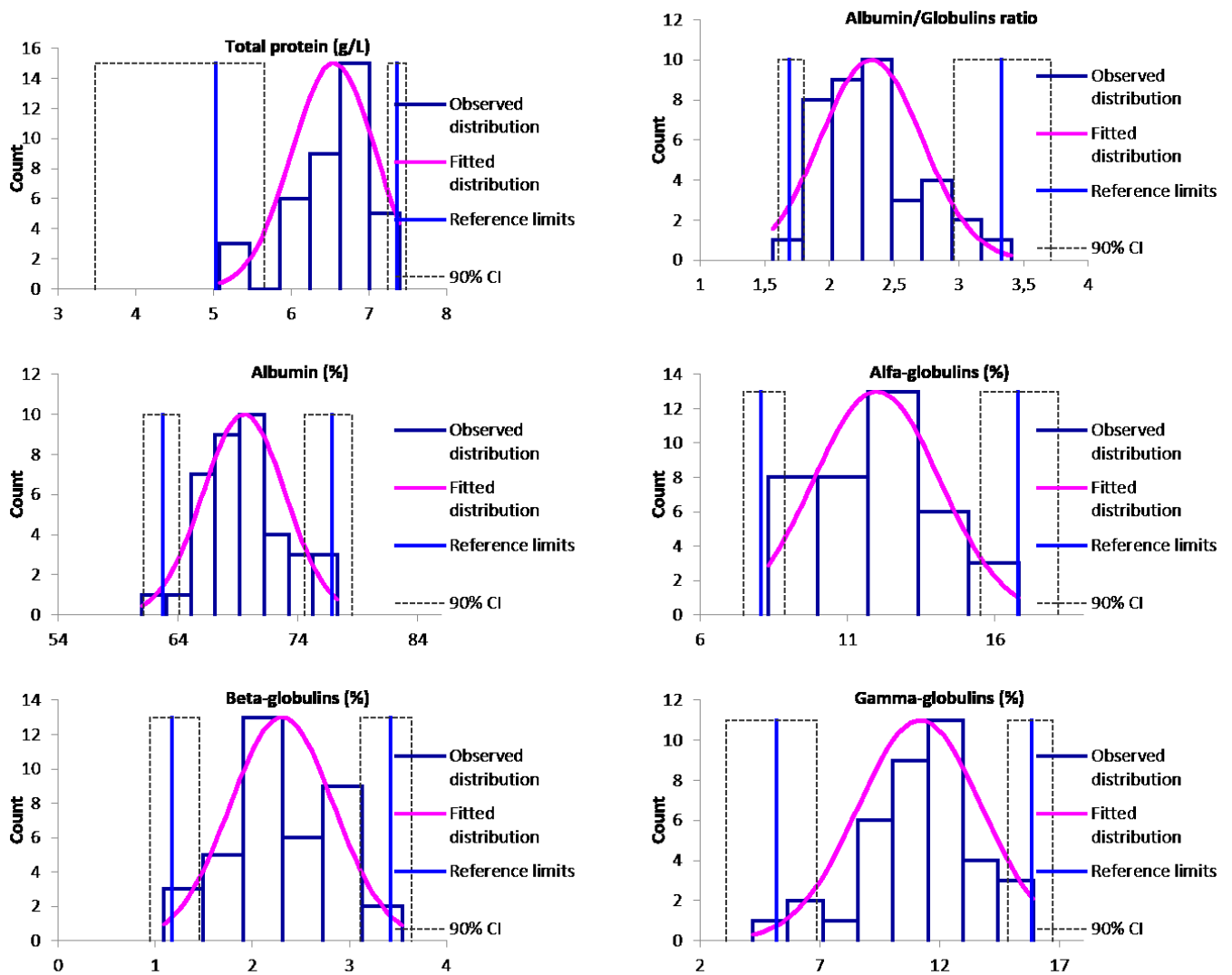
4.1.2 Identification of bisalbuminemia

At the visual examination, no double peaks were noted in AGE profiles while 9 out of 40 samples (22%) showed a double albumin peak in electrophoretic profile obtained with CZE. However, all these samples except 2 had an albumin peak wider than that observed with AGE in dolphins classified as non bisalbuminemic by CZE (Figure 4.1.2.A); furthermore a wider albumin peak was also noted with AGE in one sample with normal CZE profile.

The ROC curve analysis showed that when the base/height (b/h) ratio of albumin peak was equal or higher than 0.25, the sensitivity and specificity of AGE to detect bisalbuminemic samples were 87% and 63% respectively with an area under the curve (AUC) of 0.773.

TP levels, albumin percentage and absolute values were not significantly different between normal and bisalbuminemic dolphins, but the albumin percentages obtained with CZE were significantly lower compared to AGE, either considering the whole data set (P<0.001) and only bisalbuminemic samples (P=0.012) (Table 4.1.2.A).

The bisalbuminemic dolphins belong to two distinct families (Figure 4.1.2.B), they were both male and female animals, and this phenotype was evident both in parents and in offspring. In the first family, all the siblings derived from the same normal sire were bisalbuminemic, whereas in the second family bisalbuminemia was present in a sire and in two out of three siblings.

Table 4.1.2.A: Values of total protein and albumin in bisalbuminemic and normal bottlenose dolphins. Values showed represent mean, standard deviation (median and min-max values) of total protein (TP) and albumin (percentage and absolute values) obtained using CZE and AGE. *P<0.05 vs albumin percentage and absolute value measured with AGE. **P<0.001 vs albumin percentage and absolute value measured with AGE.

	CZE tot N=38			AGE tot N=38	
	TP (g/L)	Albumin (%)	Albumin (g/L)	Albumin (%)	Albumin (g/L)
Bisalbuminemic (N=9)	66.5±7.1 (68.3; 50.8-74.0)	64.1±3.4* (64.1; 58.6-68.1)	42.4±3.70* (43.2; 33.7-46.1)	70.1±3.6 (69.9; 64.6-76.0)	45.5±3.6 (43.2; 38.6-49.4)
Normal	64.7±5.8 (64.9; 48.0-72.7)	63.3±3.3** (62.9; 57.7-72.0)	40.7±3.4** (41.3; 30.9-45.9)	69.3±3.4 (69.5; 61.0-77.3)	45.2±3.9 (45.0; 36.9-54.1)
Total	65.0±6.0 (66.5; 48.0-74.0)	63.4±3.3** (63.5; 57.7-72.0)	41.0±3.5** (41.7; 30.0-45.9)	69.5±3.4 (69.5; 61.0-77.3)	45.2±3.9 (45.0; 36.9-54.1)

Figure 4.1.2.A: Electropherograms of serum samples of unaffected and bisalbuminemic bottlenose dolphins. **a:** Agarose gel electropherogram of serum sample of unaffected bottlenose dolphins, showing no abnormality, and **b:** serum sample from bisalbuminemic bottlenose dolphins, with a wider albumin band compared to normal one. **c:** capillary zone electropherogram of serum sample of unaffected bottlenose dolphins and **d:** from bisalbuminemic bottlenose dolphins, with a double albumin peak.
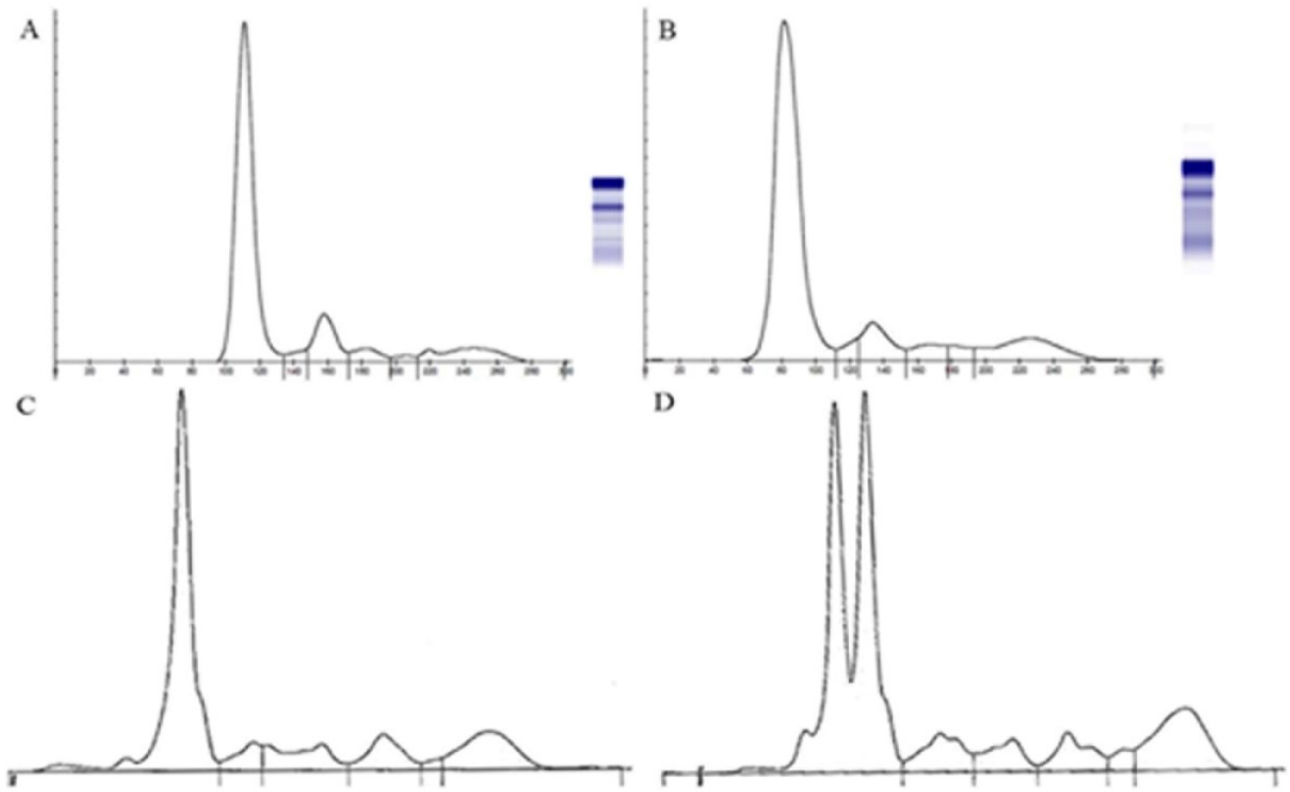
Figure 4.1.2.B: Pedigree of two groups of related bottlenose dolphins (family A on the top, family B on the bottom). Female are indicated by circles, males by squares. A black circle indicates affected dolphins, a white symbol illustrates unaffected dolphins. Red symbols indicate wild founders.
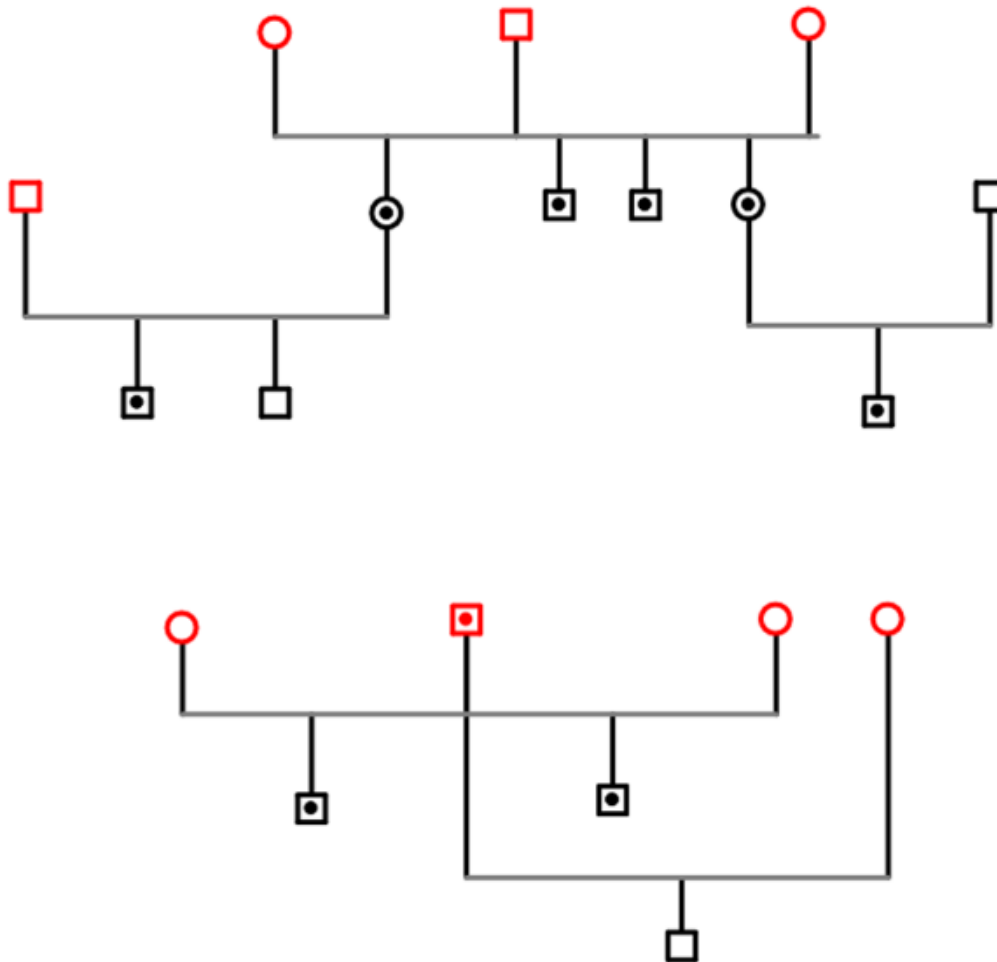
## 4.1.3 Genetic bases of bisalbuminemia

Mutations in the albumin gene

Eighteen albumin gene variations were identified in bottlenose dolphins studied; three variations were synonymous and 15 were non-synonimous (Table 4.1.3.A). The synonymous variation were identified in exons 3, 8, and 9. The non-synonymous variations were identified in exons 4, 5, 6, 7, 8, 9, 10, 12, and 13. Exons 1, 2, 11, and 14 did not show any variation (Figure 4.1.3.A). In order to identify non-synonimous variations able to cause bisalbuminemia, the genotype-phenotype correlations within the two families were studied. Two heterozygous non-synonymous variations that co-segregate with the ''bisalbuminemia'' phenotype detected by SPE were identified:

c.483C>G p.Phe146Leu in exon 4 and c.487T>C p.Tyr163His in exon 5. All bisalbuminemic dolphins were heterozygote for both variations and all non-bisalbuminemic dolphins were homozygotes (Figure 4.1.3.B).

Table 4.1.3.A: Synonymous and non-synonymous substitutions identified in the 15 related bottlenose dolphins and number of heterozygous dolphins for each substitution in the two families. In bold the substitutions that co-segregate with phenotype ''bisalbuminemia''.

	Exons	Protein change	Family A	Family B
Synonymous substitution	Exon 3	p.Ile49Ile	5	3
	Exon 8	p.Phe332Phe	3	0
	Exon 9	p.Ala359Ala	3	2
Non-synonymous substitution	Exon 4	p.Asp119Glu	5	2
	Exon 4	**p.Phe146Leu**	5	2
	Exon 5	**p.Tyr163His**	5	2
	Exon 5	p.Gly189Ala	6	3
	Exon 6	p.Thr207Ala	3	2
	Exon 6	p.Leu208Val	3	2
	Exon 7	p.His270Tyr	3	2
	Exon 8	p.His303Asp	4	2
	Exon 8	p.Leu327Pro	4	2
	Exon 8	p.Leu328Ile	4	2
	Exon 9	p.Asp356Glu	4	1
	Exon 10	p.Arg401Gln	4	1
	Exon 10	p.Glu419Asp	3	0
	Exon 12	p.Arg482Gln	5	1
	Exon 13	p.Ala575Thr	3	0

Figure 4.1.3.A: distribution of the mutations identified in albumin gene in 15 bottlenose dolphins. The map of the gene were derived from Minchetti and colleagues (Minghetti et al., 1986), modified in Minchiotti and colleagues (Minchiotti et al., 2008) and it is adapted for bottlenose dolphins. Boxes and lines represent exons and introns, respectively. The open box indicates the completely untraslated exon 15. In bold, mutations that co-segregate with phenotype ''bisalbuminemia'', in red the synonymous substitutions.
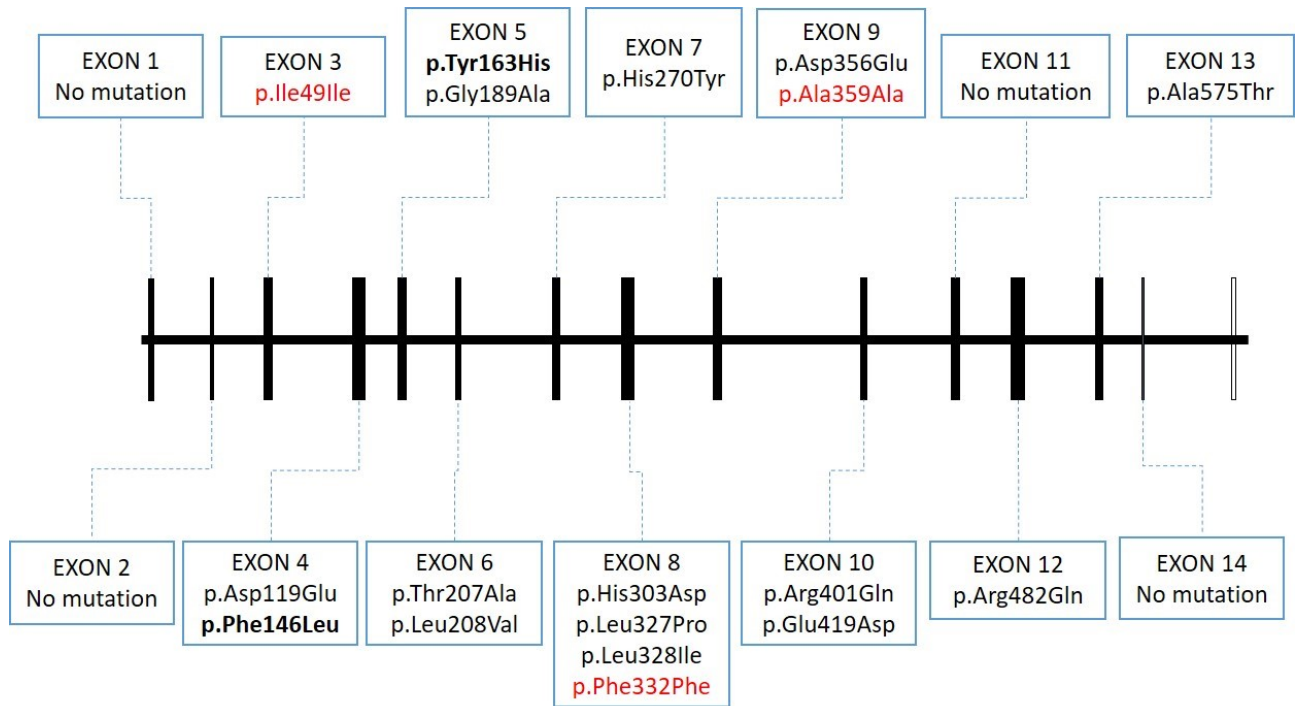
Figure 4.1.3.B: Electropherograms showing the mutations identified in exon 4 and exon 5. Mutation sites were shown with an asterisk. Panel A: sequences of homozygous allele C in exon 4. Panel B: sequence of heterozygous (C/G) alleles in exon 4. Panel C: sequences of homozygous allele G in exon 4. Panel D: sequences of homozygous allele T in exon 5. Panel E: sequences of heterozygous (T/C) alleles in exon 5. Panel F: sequences of homozygous allele C in exon 5.
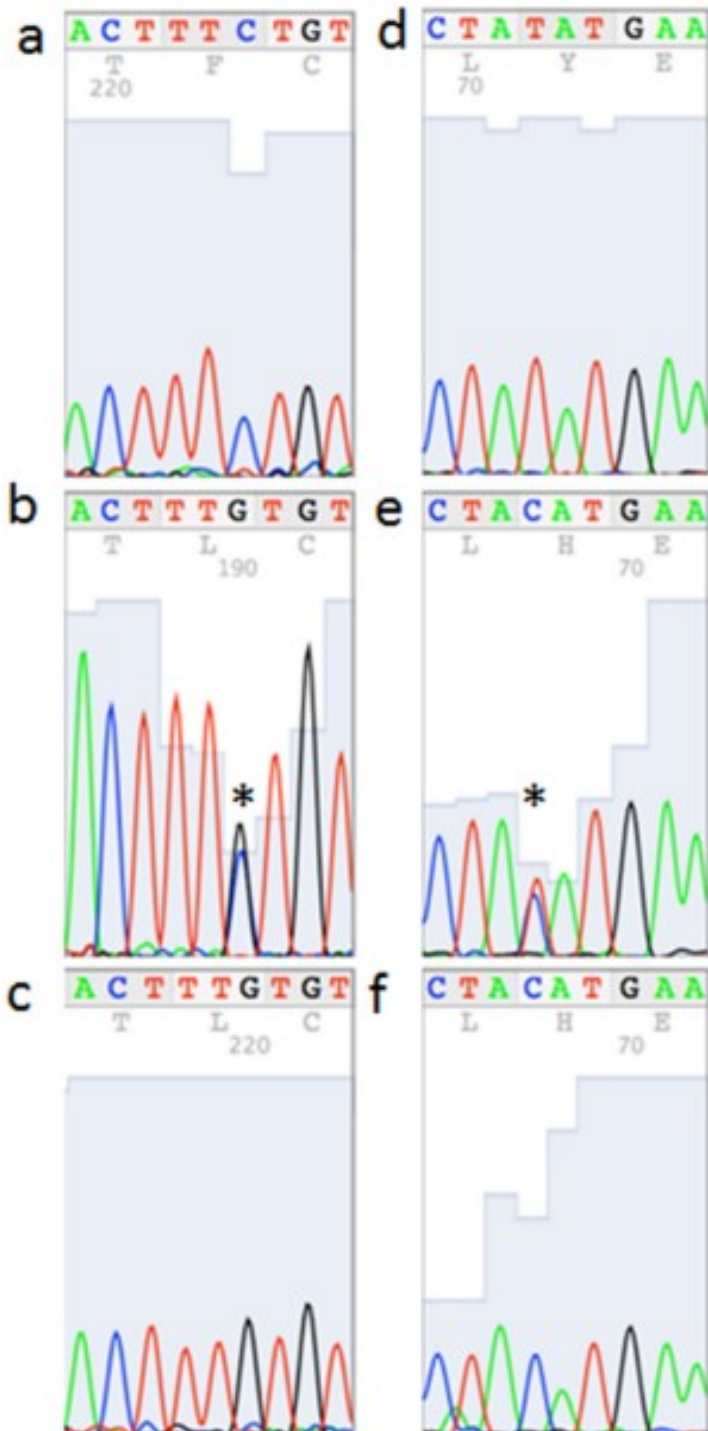
Secondary structure prediction

The amino acid change p.Phe146Leu identified in exon 4 is a missense variation that changes a nonpolar amino acid, phenylalanine, into another nonpolar amino acid, leucine. The variation p.Tyr163His is a missense variation that changes tyrosine, an amino acid with a polar side group, into histidine, a positively charged, aromatic amino acid. This amino acid change can cause a variation in the secondary and/or tertiary structure of the albumin protein. The in silico analysis of these two mutations identified mutation p.tyr163His as the more likely cause of bisalbuminemia in these dolphin families. PSIRED was used to predict the secondary structure of wild-type albumin and of the two albumin isoforms with the p.Phe146Leu and p.Tyr163His amino acid variations. The observed secondary structure of the three proteins show a high degree of similarity in terms of helices and β-sheets (Figure 4.1.3.C). The p.Phe146Leu albumin protein showed differences from the wild-type albumin in positions 290 to 304, especially in protein binding (Figure 4.1.3.C).

Figure 4.1.3.C: PSIPRED prediction of secondary structure maps of dolphin wild-type albumin (Panel A) in comparison with dolphin albumin with the variation p.Phe146Leu (Panel B) and p.Tyr163His (Panel C). Feature predictions are colour coded into the sequence according to the sequence feature key shown below.
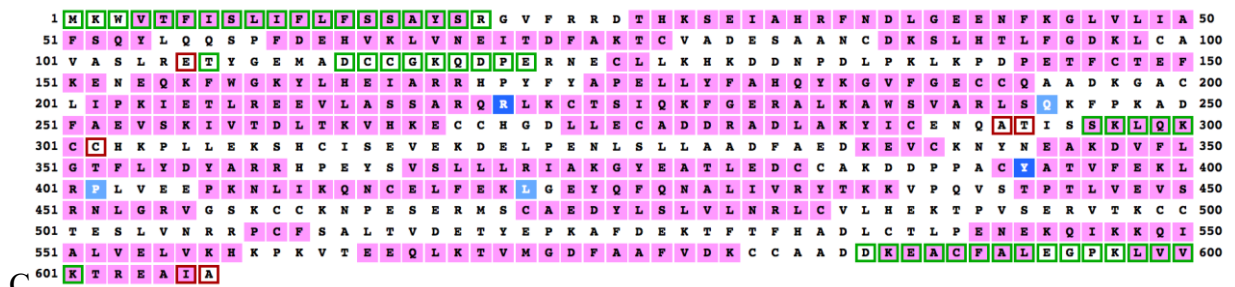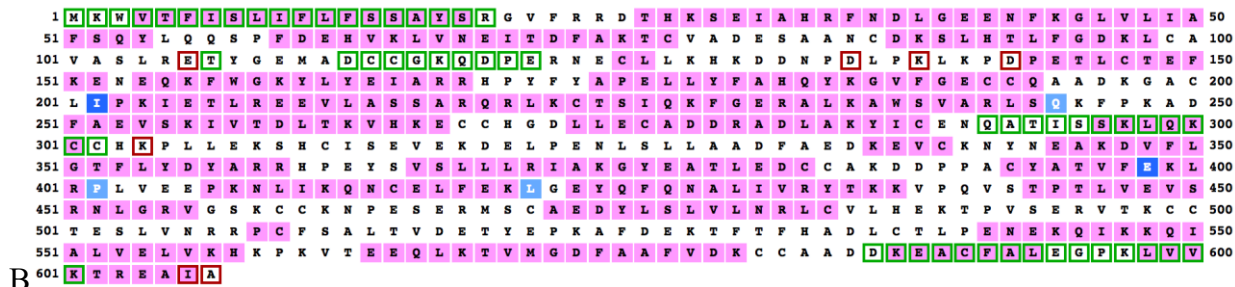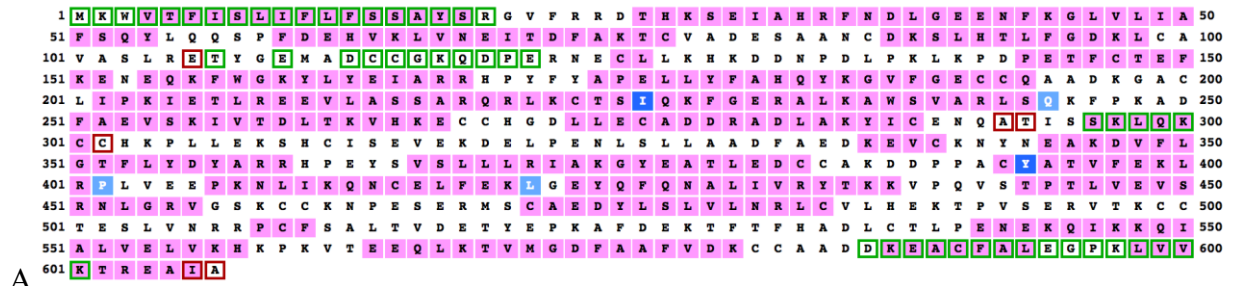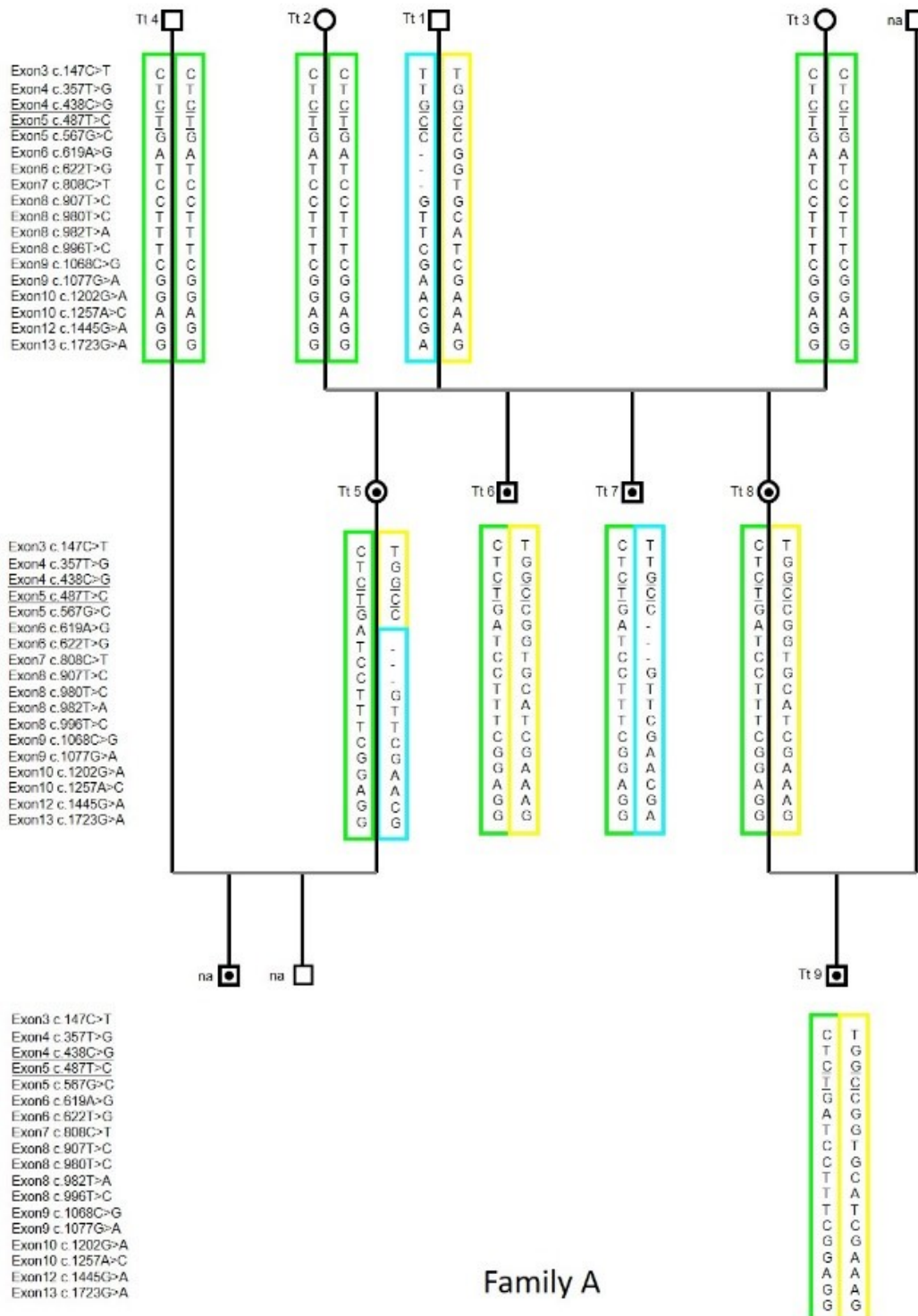
Pattern of inheritance and haplotype analysis

Nine bottlenose dolphins that were analyzed belonged to family A: the founder group was composed of three males (Tt1, Tt4, and 1 non examined animal) and two females (Tt2 and Tt3), none of which were bisalbuminemic; the first generation was composed of two males (Tt6 and Tt7) and two females (Tt5 and Tt8), all of which were bisalbuminemic; and one second generation was composed by 1 male (Tt9) which was bisalbuminemic. Six bottlenose dolphins belonged to family B: the founder group that was composed of one bisalbuminemic male (Tt10) and three non bisalbuminemic females (Tt11, Tt12, and Tt13); and the first generation composed of three males, two bisalbuminemic (Tt14 and 1 non examined animal) and one non-bisalbuminemic (Tt15). Based on pedigree analysis, and autosomal codominant inheritance pattern was identified (Figure 4.1.2.B). The reconstruction of haplotype block for the dolphin albumin chromosomal locus showed three different haplotype blocks for this region, named haplotype 1, 2, and 3. All the wild founders, except for Tt1 and Tt10, were homozygous for haplotype 1 and they were all caught in the surrounding waters of Gulf of Mexico from 1986 to 2003. The wild founder Tt10, caught in the Gulf of Mexico in 2003, and dolphins Tt6, Tt8, Tt9, And Tt14, were heterozygous for haplotype 1 and 2. This indicates the two mutations identified in this study as the responsible for bisalbuminemia. In contrast, dolphin Tt1, caught in 1974 in the Adriatic Sea, was heterozygous for haplotype 2 and 3. In particular, haplotype 3 is very distinctive because it carries a deletion in exons 6 and 7 of the albumin gene. Finally, in dolphins Tt5 and Tt7 a crossing over between haplotype 2 and 3 was evident (Figure 4.1.3.D).
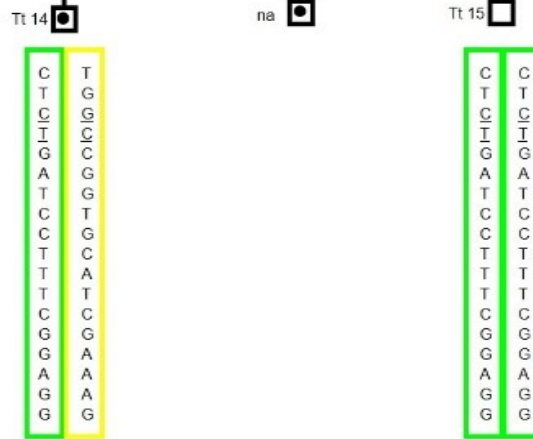
Figure 4.1.3.D: pedigree of the two families of Bottlenose dolphins with the observed phenotype. Family A and family B. Females are indicated by circles, males by squares. A black symbol indicates a bisalbuminemic dolphin, a white symbol illustrates an unaffected dolphin. The reconstruction of haplotypes showed presence of three different haplotypes: haplotype 1 is indicated by green rectangle; haplotype 2 is indicated by yellow rectangle, and haplotype 3 is indicated by blue rectangle. Among dolphins with a homozygous haplotype, 6 are wild founders (Tt2, Tt3, Tt4, Tt11, Tt12, and Tt13). In tt5 crossing-over between haplotype 2 and 3 is observed.
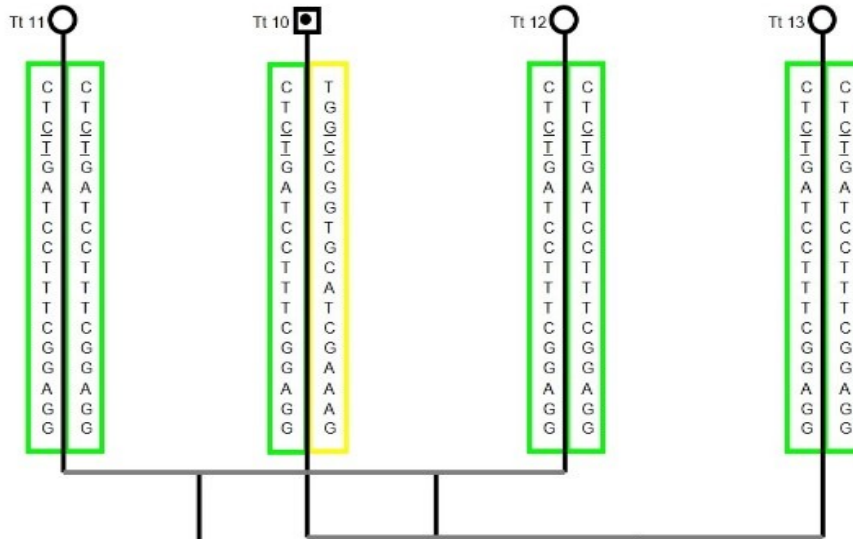
Family A

Family B

4.2 Acute phase proteins

4.2.1 Positive APPs

Cross reactivity evaluation

Double radial immunodiffusion

To evaluate the cross-reactivity of the Abs for the determination of the concentration of CRP and SAA using DRI technique, 6 different tests were performed.

In test number 1, the Ab against CRP produced by Randox undiluted and the Ab against CRP produced by Randox diluted 1:2 cross-reacted with the serum of dog with high CRP concentration. No other precipitation lines were evident.

In test number 2, the Ab against CRP produced by Randox undiluted and the Ab against CRP produced by Randox diluted 1:2 cross-reacted with the serums of dog with high CRP concentration, of cat with high SAA concentration, and of cat with normal SAA concentration. No other precipitation lines were evident.

In test number 3, the Ab against CRP produced by Realtime undiluted cross-reacted with the serums of healthy human, dog with high CRP concentration, dog with normal CRP concentration, cat with high SAA concentration, and cat with normal SAA concentration. The Abs against CRP produced by Biodevice and produced by Aptec cross-reacted with serums of dog with high CRP concentration, cat with high SAA concentration, and cat with normal SAA concentration. No other precipitation lines were evident.

In test number 4, the Abs against CRP produced by Biodevice, by Aptec, by Tridelta, and by Randox cross-reacted with the serum of dog with high CRP concentration. No other precipitation lines were evident.

In test number 5, there were not precipitation lines using the Ab against CRP Life Diagnostics diluted 1:100 and diluted 1:1000 and all the serum samples tested.

In test number 6, the Ab against CRP Life Diagnostics diluted 1:5 and 1:10 cross-reacted with the serum of dog with high CRP concentration. No other precipitation lines were evident.

Western Blot analysis

To evaluate the cross-reactivity of the Abs for the determination of the concentration of CRP and SAA using WB technique, 7 different tests were performed using 2 Abs against CRP and 1 Ab against SAA.

Using two Abs against CRP tested (Randox and Life Diagnostics), the multimeric forms of CRP were evident in dog: a pentamer (~150kDa), tetramer (~ 100kDa), trimer (~ 75 kDa,), dimer (~ 45 kDa). In bottlenose dolphins, a pattern of positivity similar to those observed in dog is present. In striped dolphins, a different pattern is evident with only trimer and dimer forms show a positive reaction to the Ab. The Ab against SAA tested (Eiken Ltd.) did not show any cross-reaction with the serum samples of bottlenose dolphins, but a weak positivity in serum from striped dolphins was visible. However, for all the Abs used, unspecific signals were evident.

Automated measurement of CRP

The automated measurement of CRP provided discouraging results, indeed in all the animals tested, the concentration of CRP was 0.00 mg/L.

Automated measurement of SAA

In the entire population of bottlenose dolphins, the RIs for the SAA were (mean ± SD) 8.7 ± 11.8 mg/L (median: 0.8 mg/L; min-max: 0.0-39 mg/L) (figure 4.2.1.A). No differences were noted between different storage time (long storage: mean ± SD 15.7 ± 26.9 mg/L, median 6.8 mg/L, min-max 0.0-128.7 mg/L; short storage: mean ± SD 13.1±22.3 mg/L, median 0.9 mg/L, min-max 0.0-87.9 mg/L); between the sex of the animals (female: mean ± SD 14.9 ± 27.3 mg/L, median 1.3 mg/L, min-max 0.0-128.7 mg/L; male: mean ± SD 13.9±20.9 mg/L, median 5.4 mg/L, min-max 0.0-84.7 mg/L); and between pregnant and non-pregnant animals (pregnant: mean ± SD 7.2 ± 10.9 mg/L, median 0.0 mg/L, min-max 0.0-30.0 mg/L; non-pregnant: mean ± SD 20.0 ± 36.1 mg/L, median 7.6 mg/L, min-max 0.0-128.7 mg/L) (figure 4.2.1.B). No statistical differences were present between the SAA concentration in samples with long storage time and short storage time (P=0.57), between the SAA concentration of male and female animals (P=0.76) and between the SAA concentration of pregnant and non-pregnant animals (P=0.51).

Figure 4.2.1.A: Graphic representation of the data distribution for SAA concentration. The box represents the interquartile range (IQR) defined by 25th (Q1) and 75th (Q3) percentiles with the vertical line representing the median. The horizontal lines are the limits of outliers distribution according to Tukey rule. Suspect outliers are indicated in orange and outliers in red.
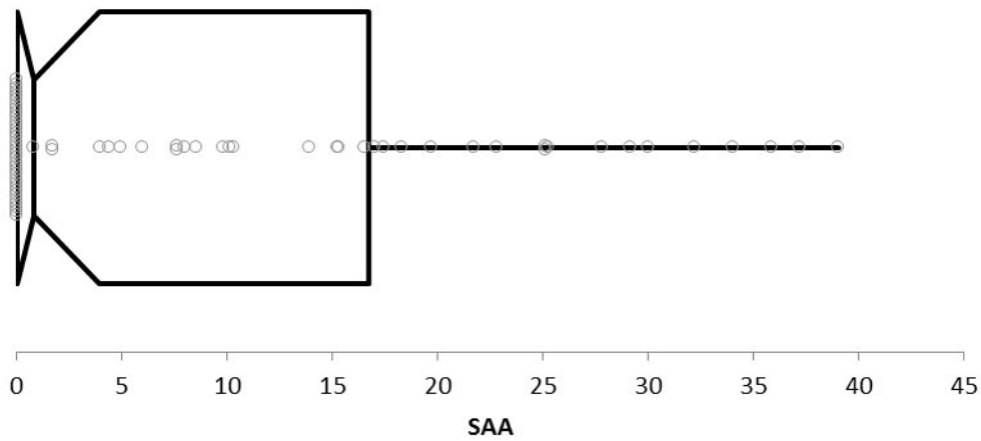
Figure 4.2.1.B: SAA concentration in serum samples with long and short storage time, in serum samples from male and female, and in serum samples from pregnant and non-pregnant animals.
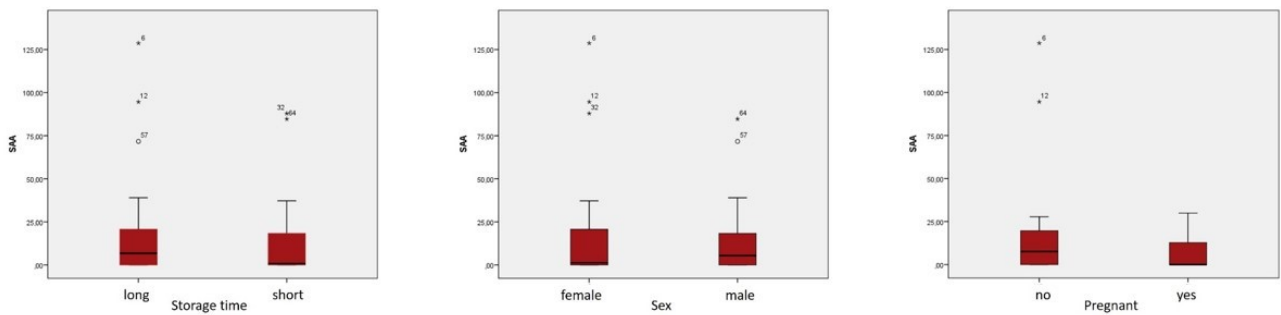
4.2.2 Negative APP

Automated measurement of PON-1

In the entire population of bottlenose dolphins the RIs for the PON-1 activity using paraoxon ad substrates were (mean ± SD) 6.7 ± 4.6 U/L (median: 7.3 U/L; min-max: 0.0-14.6 U/L) (figure 4.2.2.A). As for the concentration of SAA, no statistical differences were present between the PON-1 activity of female and male animals (female: mean ± SD 6.2 ± 4.5 U/L, median 6.3 U/L, min-max 0.0-14.6 U/L; male: mean ± SD 7.4 ± 4.6 U/L, median 8.3 U/L, min-max 0.0-13.9 U/L) (P=0.21) and between the PON-1 activity of pregnant and non-pregnant animals (pregnant: mean ± SD 4.2 ± 3.9 U/L, median 5.2 U/L, min-max 0.0-10.4 U/L; non pregnant: mean ± SD 5.5 ± 5.3 U/L, median 5.3 U/L, min-max 0.0-14.6 U/L) (P=0.76). On contrary the PON-1 activity for the long storage samples was significantly lower compared to the short storage samples PON-1 activity (long storage: mean ± SD 4.6 ± 4.5 U/L, median 3.8 U/L, min-max 0.0-14.6 U/L; short storage: mean ± SD 8.4 ± 3.9 U/L, median 8.9 U/L, min-max 0.0-13.9 U/L) (P=0.001) (figure 4.2.2.B). The mean ± SD HDL concentration was 90.4 ± 53.6 mg/dL (median 63.5 mg/dL; min-max 7.3-206.5 mg/dL)

Table 4.2.2.A: reference intervals for SAA concentration and paraoxonase PON-1 activity in bottlenose dolphins. Abbreviations. SD: standard deviation; LRL: lower reference limit; URL: upper reference limit; CI: confidence interval; Out: outliers; S: suspected; R: removed.

	Mean	Median	SD	Min	Max	LRL 90% CI	URL 90% CI	Outliners	Statisitcal Methods
SAA (mg/L)	8.7	0.8	11.8	0	39	0.0 (0.0-0.0)	37.7 (33.1-39.0)	4R, 1S	Non parametric
PON-1 (U/L)	6.7	7.3	4.6	0	14.6	0.0 (0.0-0.0)	14.5 (13.5-14.6)	0	Non parametric

Figure 4.2.2.A: Graphic representation of the data distribution for PON-1 activity using paraoxon as substrate. The box represents the interquartile range (IQR) defined by 25th (Q1) and 75th (Q3) percentiles with the vertical line representing the median. The horizontal lines are the limits of outliers distribution according to Tukey rule. Suspect outliers are indicated in orange and outliers in red.
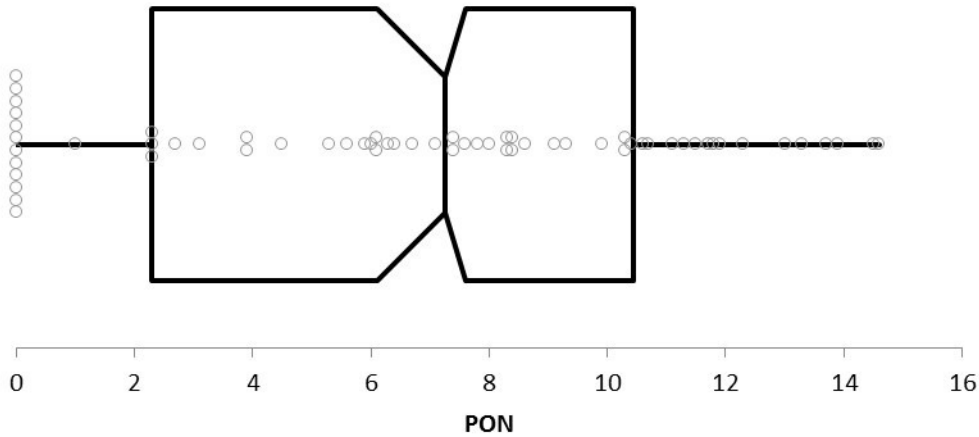
Figure 4.2.2.B: PON 1 activity using paroxone as substrate in serum samples with long and short storage time, in serum samples from male and female, and in serum samples from pregnant and non-pregnant animals.
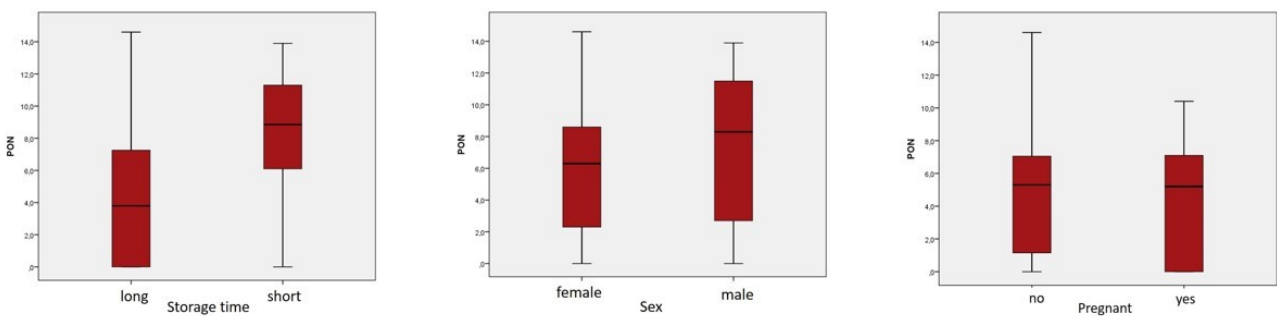
The automatic measurement of PON-1 activity using 4-nPA as substrate provides results with good precision but low accuracy. The results for intra-assay precision was reported in table 4.2.2.B. The accuracy of the assay was determined by LUD. The least square linear regression on mean values of LUD demonstrated a good correlation with $r^2 = 0.87$, however the correlation was affected by three serum samples with different dilution that provide quite the same results. (Graphic 4.2.2.A). Based on Mann-Whitney test, no significant difference were noted between healthy dolphins (mean ± SD: 186.2 ± 20.4 U/L, median: 187.2 U/L; min-max: 154.9-209.6 U/L) and diseased dolphins (mean ± SD: 177.5 ± 14.3 U/L, median: 177.5 U/L; min-max: 156.2-194.2 U/L) (P=0.57).

Table 4.2.2.B: intra-assay precision of the paraoxonase-1 (PON-1) enzymatic activity using 4-nPA as substrate determined with 10 consecutive repeat measurements on bottlenose dolphin's serum samples with low, medium, and high PON-1 activity.

	Low	Medium	High
Min-Max (U/L)	130.6-135.0	134.4-137.2	152.3-155.1
Mean value (U/L)	132.4	135.9	154.1
SD (U/L)	1.3	0.8	0.8
CV (%)	1.0	0.6	0.5

Graphic 4.2.2.A: Linearity under dilution (LUD) of PON-1 activity using 4-nPA as substrate in serially diluted serum sample with basal PON-1 activity of 193.4 U/L. Each data point indicates the mean of a triplicate measurement. The line indicates the linear correlation between expected and observed values.
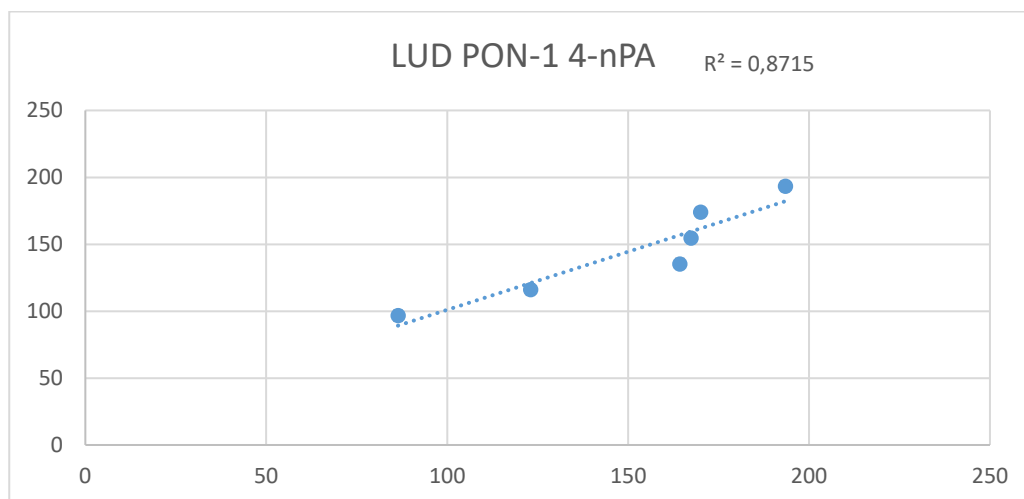
Manual measurement of PON-1

The arylesterase activity using 4-nPA as substrate was also determined with a manual spectrophotometer. The test conditions and the PON-1 activity obtained were reported in table 4.2.2.C.

Moreover, the arylesterase activity of PON-1 using PA as substrate and the lactonase activity of PON-1 using DC as substrate were evaluated with the manual spectrophotometer, since the automated spectrophotometer was not equipped with the UV light, and the correct wavelength to evaluate the hydrolysis of PA is 260 nm and the hydrolysis of DC is 270 nm. The PON-1 activity measured using PA provides results with high variability, in 6 tests no reactions were evident, and 4 tests provided negative activity while using DC no reactions were evident. The test conditions and the PON-1 activity were reported in table 4.2.2.D (PA) and 4.2.2.E (DC).

Table 4.2.2.C: test conditions and results of the evaluation of the PON-1 arylesterase activity using 4-nitrophenyl acetate as substrate. Offshore means animals that live in deep water, far from the coastline.

Animal	Serum's dilution	Serum amount (µl)	Reading time (sec)	Activity (U/L)
Offshore healthy	1:5	52.6	225	87.44
Offshore healthy	1:10	52.6	225	64.35
Offshore healthy	1:20	52.6	225	47.79
Offshore healthy	1:5	52.6	225	37.62
Offshore healthy	1:10	52.6	225	18.81
Offshore healthy	1:20	52.6	225	9.41
Offshore healthy	1:10	52.6	225	23.81
Offshore healthy	1:12	52.6	225	21.02
Offshore healthy	1:14	52.6	225	21.22
Offshore healthy	1:16	52.6	225	16.45

Offshore healthy	1:18	52.6	225	13.76
Offshore healthy	1:20	52.6	225	14.44
Offshore healthy	undiluted	52.6	225	184.65
Offshore healthy	1:5	52.6	225	87.18
Offshore healthy	1:10	52.6	225	64.09
Offshore healthy	1:20	52.6	225	47.52

Table 4.2.2.D: test conditions and results of the evaluation of the PON-1 arylesterase activity using phenyl acetate as substrate. Offshore means animals that live in deep water, far from the coastline.

Animal	Serum's dilution	Serum amount (µl)	Reading time (sec)	Activity (U/L)
Offshore healthy	1:3	20	60	No reaction
Offshore healthy	1:3	40	60	No reaction
Offshore healthy	1:3	60	60	2442.14
Offshore healthy	1:3	80	60	No reaction
Offshore healthy	Undiluted	10	60	No reaction
Offshore healthy	Undiluted	20	60	No reaction
Offshore healthy	Undiluted	30	60	No reaction
Offshore healthy	1:1.5	60	60	1659.27
Offshore healthy	1:1.5	60	60	3888.05
Offshore healthy	1:1.5	60	60	288.85
Offshore healthy	1:3	60	320	1165.27
Offshore healthy	1:3	60	320	1240.76
Offshore healthy	1:3	60	320	774.66
Offshore healthy	1:3	60	320	623.66

Animal	Dilution	Serum amount (µl)	Reading time (sec)	Activity (U/L)
Offshore healthy	1:1.5	60	320	53.26
Offshore healthy	1:40	20	60	-7870.23
Offshore healthy	1:40	40	60	-641.22
Offshore healthy	1:40	40	60	-1410.69
Offshore healthy	1:40	40	60	-1282.44

Table 4.2.2.E: test conditions and results of the evaluation of PON-1 lactonase activity using dihydrocoumarin as substrate. Offshore means animals that live in deep water, far from the coastline.

Animal	Dilution	Serum amount (µl)	Reading time (sec)	Activity (U/L)
Offshore healthy	Undiluted	10	60	No reaction
Offshore healthy	Undiluted	20	60	No reaction
Offshore healthy	Undiluted	40	60	No reaction
Offshore healthy	Undiluted	20	180	No reaction

Genetic polymorphisms of PON-1 gene

Since the primers used to detect SNPs L55M and Q192R were designed to amplify human DNA, we tested the primers on human and dolphin DNA to verify the cross-reactivity of the primers. The figure 4.2.2.C showed the obtained results that indicated the length of the amplicon is the same for dolphins and human. In order to verify also that the DNA sequence is correct we sequenced the obtained PCR products of humans and dolphins confirming that the PCR specifically amplified respectively humans and dolphin PON-1 gene.

Based on the sequence analysis, all the dolphins were homozygous for Metionin in L55M SNPs and for Arginin in Q192R SNPs.

Figure 4.2.2.C: PCR products of SNPs L55M (A) and Q192R (B). The bands of the human's L55M and Q192R had the same length of the dolphin's L55M and Q192R. DNA Marker-100bp (DNA Ladder, Invitrogen) Abbreviation. H: human; D: dolphin; -: negative control.
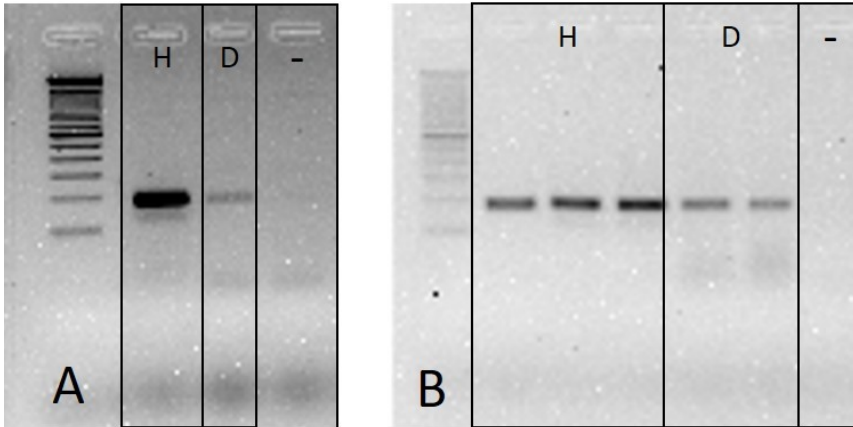
5. DISCUSSION

5.1 Serum Protein Electrophoresis

5.1.1 Establishment of reference intervals for serum protein fraction in bottlenose dolphins

Serum protein electrophoresis is the most reliable method to determine the distribution of serum protein fractions and it is considered, together with a basic haematological and biochemical profile, an essential step to evaluate the health status of animals, providing clinical useful information. The interpretation of kinetics of total proteins and albumin and globulin fractions is receiving increased attention also in marine mammals in which, as in terrestrial mammals, a typical pathologic pattern could be identified in several diseases, such as inflammatory diseases (King et al., 2001). The measurement of serum protein's fractions can be performed with different techniques, such as cellulose acetate electrophoresis (CAE), AGE, and CZE. Nowadays, in human medicine, CZE has replaced classical AGE, due to its higher resolution. However, the higher cost of CZE, compared to the most traditional techniques, hampered its wide diffusion also in veterinary laboratories. So, we decided to determine the RIs of serum protein's fractions in bottlenose dolphins kept under human care using AGE because it is the more common methods to measure the serum proteins' fraction in companion animals (Giordano and Paltrinieri, 2010) and also in dolphins (Schwacke et al., 2009).

Compared to previously published data in free ranging bottlenose dolphins (Schwacke et al., 2009), in our samples the concentration of TP, α-globulins, and γ-globulins were slightly lower; while the concentration of albumin and the albumin/globulins ratio were slightly higher. Haptoglobin and the anti-inflammatory proteins α1-antitrypsin, α1-antichymotripsin, and α2-macroglobulin migrate in the α-globulins fraction, while the IgG and CRP migrate in the γ-globulins fraction(Stockham and Scott, 2008b). Albumin acts as a negative acute phase protein since the synthesis of this protein is decreased during an inflammation(Stockham and Scott, 2008b). The lower concentration of ''inflammatory'' proteins associated to a higher concentration of albumin and the consequent higher albumin/globulins ratio reported in our study could reflect a lower antigenic stimuli in the animals housed in aquaria compared to the free-ranging populations. Moreover, in the paper of Schwacke and colleagues (Schwacke et al., 2009) the possible non-healthy individuals were not excluded from the studied population, so it is possible that the RIs proposed did not represent a strictly disease-free population. On contrary, the serum samples used in our work were collected from animals with normal clinical history and physical examination

The RIs for serum proteins' fraction reported in the present study extend the toolbox of diagnostic and monitoring tools available for dolphins. The current study is limited by the relatively small sample size. In the future, when larger sample sets will be available, the development of RIs for under human care bottlenose dolphins associated with age and sex may be possible. The intra-assay imprecision for AGE of bottlenose dolphins serum samples was between 1.2% and 3.8% and inter-assay imprecision was between 2.5% and 5.7% for the different protein fractions (table 4.1.1.B). This data are similar to the intra- and inter-assay imprecision recorded for AGE of canine and feline serum samples (Giordano and Paltrinieri, 2010). It is possible that the variability recorded in this study had influenced the RIs of the poorly represented serum protein fractions, but this happens also for the other species, for which SPE is widely used.

Moreover, at a visual examination of the AGE electropherograms, in 7 animals wider albumin peaks were evident. For this reason, we decided to investigate all the serum samples with CZE because it allows a better visualization of the albumin fraction. However, we were not able to perform the inter-assay and intra-assay repeatability and coefficient of variation on CZE, so despite the presence of data regarding the absolute values and the percentage of the different serum protein fractions, we cannot determine the RIs of bottlenose dolphins' serum proteins obtained with this technique.

5.1.2 Identification of bisalbuminemia

The analysis of CZE profile revealed the presence of a double albumin peak in 9 bottlenose dolphins from two distinct families, thus we report for the first time the presence of hereditary bisalbuminemia in two groups of related bottlenose dolphins identified by means of CZE and we confirm that AGE could fail in the identification of this alteration, as already reported in human medicine (Jaeggi-Groisman et al., 2000, Kalambokis et al., 2002). In human medicine, CZE has replaced classical AGE, due to its higher resolution. The difference in resolution is mainly due to the different analytic method: while in AGE, proteins migrate toward anode in a solid phase and in an alkaline buffer with low voltage, in CZE proteins rapidly move in a liquid phase toward the cathode thanks to the high voltage applied. This allow a better separation of proteins with similar physiochemical characteristics, thus generating multiple sub-peaks or narrower peaks (Bossuyt et al., 2003). When CZE was introduced routinely in human medicine laboratories, an increased number of bisalbuminemia cases was detected (Jaeggi-Grosiman et al., 2000), based on the improved separation of the albumin, α_1-globulin, and α_2-globulin fractions. In our work, this technique clearly identified a double albumin peak at the visual analysis of the electrophoretic profile in 9 samples whereas with AGE only 8 profiles revealed a wider peak compared to the

normal one, but never an albumin double peak was detected. Furthermore, with AGE, an albumin peak apparently wider than normal was noted also in one sample with normal CZE profile, demonstrating as the visual interpretation of AGE profiles could lead to both false negative and false positive detection of bisalbuminemia. However, the visual identification of a ''wider'' peak could be considered a subjective method. Thus, we calculate the ratio between the length of the base and the height of the albumin peak with the aims to define a cut-off and to establish a more accurate and objective method to identify bisalbuminemia in AGE electrophoretic profile. However, also with this approach the diagnostic accuracy was fair with a low specificity.

As expected, no significant differences in TP concentration, albumin percentage and absolute values between affected and normal dolphins was noted, but CZE albumin were significantly lower compared to AGE. In literature, an opposite situation is reported with higher albumin values obtained with CZE in dogs and cats (Giordano and Paltrinieri, 2010). Nevertheless, it is possible that these data, despite the significant differences, are not clinically relevant. In literature, RIs for TP and serum protein fractions are available for free ranging bottlenose dolphins (Goldstein et al., 2006; Schwacke et al., 2009b): compared to our results obtained with both methods, in free-ranging dolphins TP seemed higher and albumin absolute values lower, suggesting a higher concentration of globulins in these animals, thus a tendency to an inflammatory status, as already suggested (King et al., 2001). All these data highlight the need to define appropriate RIs for different electrophoretic methods for bottlenose dolphins under human care.

The application of higher resolution techniques, such as CZE, can result in an increased number of ''abnormal'' profiles and thus a deeper knowledge of the clinical importance of these new profiles is required. In previous works in CZE validation in companion animals, an unusual albumin peak was observed in sera from clinically healthy cats never been reported previously, likely because of the low resolution of traditional electrophoretic techniques (Giordano and Paltrinieri, 2010). Thus, the correct interpretation of these kinds of data is essential to differentiate normal to pathological conditions. In human medicine, the diagnostic implications of the presence of bisalbuminemia in clinical entities are uncertain: it could be a sign of acquired disorders and it is correlated with several pathological conditions, like pancreatic and hepatic diseases (pancreatitis, pancreatic pseudocyst, hepatic cirrhosis) or lymphoproliferative diseases (monoclonal gammopathy, multiple myeloma) (Ejaz et al., 2004). In our case, all the sampled dolphins were clinically healthy, without any other alteration in haematological or biochemical parameters and bisalbuminemia was detected accidentally, during the routine evaluation of the health status. Furthermore, no drugs were administered to the affected animals, except for an integration of folic acid in two of them, thus we

could exclude the possibility that the abnormal electrophoretic pattern identified is due to a pathological condition or drugs administration.

The inherited form of human bisalbuminemia is usually discovered by chance and apparently does not seem associated with pathological conditions. Genetically, bisalbuminemia is due to a mutation in the albumin gene transmitted as an autosomal codominant trait and it has been reported in various human populations around the world, with significant differences in frequency in terms of race and location, with a higher incidence in small, isolated population groups (Minchiotti et al., 2008). All the dolphins sampled in our study were living under human care and, from a reproductive point of view, they include wild founders and their progeny of first and second generation maintained in four groups separated in different facilities; for this reason the high incidence of this inherited disorder is somehow concentrated and not surprising.

Since the protein synthesis is governed by a single copy gene codominantly expressed, heterozygous subjects carrying point mutations usually show the presence of the normal and the variant proteins. Based on the pedigree of affected dolphins, we could only suppose the same inheritance pattern in bottlenose dolphins. Over the last three decades, more than 60 different albumin variants have been characterized in people, being the vast majority reflecting single-base changes in the structural gene mainly with mutations in hypermutable CpG dinucleotides (Kragh-Hansen et al., 2013). Rarely, the presence of bisalbuminemia may have clinical impact due to the effect of mutation on ligand-binding: three mutations (p.Leu90Pro, p.Arg242His, and p.arg242Pro) form strong bindings sites for triiodothyronine (T3) or thyroxine (T4), causing the familiar dysalbuminemic hyperthyroxinemia syndrome (Galliano et al., 1990; Petersen et al., 1994). Other mutations seem to increase the binding capacity of long-chain fatty acids, but without clinical consequences (Nielsen et al., 1997). However, in sampled dolphins, apparently, no signs of altered hormone or lipid binding capacity were evident based on the absence of laboratory abnormalities. To investigate the possible mutations responsible of bisalbuminemia, the inheritance pattern of this disorder and the possible alteration in the albumin functions, the genetic analysis of the albumin gene of 15 dolphins belonging to 2 families were carried out.

5.1.3 Genetic bases of bisalbuminemia

The genetic analysis of bottlenose dolphins' albumin gene showed a significant polymorphism and two mutations associated with bisalbuminemia. Moreover, we were able to identify the autosomal codominant trait of this condition in dolphins, a similar pattern of inheritance to that in humans. By analysing samples from two dolphin families in which a recurrence of bisalbuminemia had been detected by serum protein electrophoresis, 18 variations in the coding sequence, three of which are

synonymous and 15 are non-synonimous, were identified. Non-synonimous variations are nucleotide changes that alter the amino acid sequence of a protein, thus, these substitutions could be the cause of bisalbuminemia in dolphins as in humans (Kragh-Hansen et al., 2013). The mutation p.Phe146Leu in exon 4 changes phenylalanine, a neutral, nonpolar, amino acid, into leucine, another nonpolar amino acid containing an α-amino group, an α-carboxylic acid group, and an isobutyl side chain. Based on the secondary structure study, this mutation could induce a disordered protein binding. Usually, bisalbuminemia is detected with standard electrophoretic methods under non-denaturing conditions when the mutation induces a change in charge placed on the surface of the protein, while mutations affecting binding properties are less likely causes of bisalbuminemia (Kragh-Hansen et al., 2013). Therefore, we could presume that this mutation is less likely to be the cause of bisalbuminemia, even if we cannot exclude the possible effect on the binding capacity of this albumin variant. In contrast, the variation p.Tyr163His in exon 5 is a missense variation that changes tyrosine, an amino acid with a polar side group classifying it as hydrophobic, into histidine, which contains an α-amino, a carboxylic acid group, and a side chain imidazole, classifying it as a positively charged, aromatic amino acid. The in silico analysis and the comparison between dolphin and human variations showed that this variation has a homolog variation in human albumin, p.Tyr164Cys, verified to be able to cause bisalbuminemia and named ''Asola'' by Minchiotti and co-authors (Minchiotti et al., 1995). These data support the hypothesis that the variation p.Tyr163His could be more likely responsible for bisalbuminemia.

The identification of the same mutation in humans and dolphins could be due to recurring bisalbuminemias being linked to hypermutable CpG regions present in both species (Kragh-Hansen et al., 2013). This hypothesis is supported by the detection of the same mutation in bisalbuminemic dolphins from two unrelated families. In humans, the identification of the same albumin variant in a distant ethnic group has also been reported (Galliano et al., 1999) and, as it is unlikely that an evolutionary advantage derived from the mutation, our findings confirmed the presence of conserved hypermutable sites in albumin genes.

This evaluation of the causative defect of bisalbuminemia is of interest because, in some circumstances, the mutations could affect the ligand-binding activity. Usually this effect is small, but some human variants increase the albumin binding capacity for triiodothyronine and thyroxine, leading to a clinically relevant euthyroid hyperthyroxinemia (Petersen et al., 1994). Other mutations affect the albumin affinity for endogenous and exogenous ligands such as long-chain fatty acid, copper, nickel, and some drugs (e.g. diazepam, warfarin), but no clinical consequences have been reported (Minchiotti et al., 2008). In humans, the ''Asola'' albumin, homologous to the variant

reported here, is not reported to cause these types of alterations, and the dolphins enrolled in our study were clinically healthy. Thus, we can suppose that the mutation p.Tyr163His recognized in this work does not modify albumin function.

The haplotype analysis revealed that in the two families there are three different haplotypes. The majority of wild founders had the same homozygous haplotype (haplotype 1) and they were all caught from 1986 to 2003 in the surrounding waters of Gulf of Mexico. The only wild founder caught in the Gulf of Mexico that showed a heterozygous haplotype was Tt10, a subject with a very heterogeneous composition of alleles both homozygosity and heterozygosity with a differential pattern: he was heterozygous for two different haplotype, each of which carries the two mutations identified in this work. This is consistent with the fact that Tt10 was the progenitor carrying the causative mutation of bisalbuminemia in family B. In contrast, Tt1 had a heterogeneous haplotype consistent with the fact that it was caught in 1974 in the Adriatic Sea, a different area than where the other wild founder dolphins analyzed in this study were from. This dolphin had two different haplotypes, each of which carries the two mutations identified in this work: it was homozygous for both mutations, not bisalbuminemia, but all his siblings were bisalbuminemic. These data suggest that dolphins Tt1 and Tt10 were the ancestors who introduced the mutation responsible for bisalbuminemia into family A and family B, respectively. Dolphin Tt10 was bisalbuminemic, and was heterozygous at the two mutations: 66% of his descendants were bisalbuminemic. Dolphin Tt1 was not bisalbuminemic, because it was homozygous for the two mutations and all his denscendants are bisalbuminemic. It remains unclear if haplotype 2, which carries the mutation responsible for bisalbuminemia in both families analyzed in this paper, identified in the dolphin Tt1 and Tt10, was inherited from a common ancestor or is the consequence of different mutation, and the resulting same haplotype is the effect of natural selection. The fact that dolphins Tt1 and Tt10 were wild founders caught in distant areas, the Adriatic Sea and the Gulf of Mexico respectively, suggested that they did not have a common ancestor. We could therefore speculate that mutations leading to bisalbuminemia in these two dolphins are located in hypervariable regions of the genome of the dolphins, as also happens in humans.

5.2 Acute Phase Proteins

The measurement of APPs in association to serum proteins' fractions can supplement and extend the baseline informations obtained from the complete blood cell count, fibrinogen, and standard serum chemistry panel (Isaza et al., 2014). In our study, two positive APPs, CRP and SAA, and one

negative APP, PON-1, were investigated. The concentration of the positive APPs were determined by immunoturbidimetric methods, while the activity of the negative APP was based on enzymatic activity.

5.2.1 Positive APPs

Cross-reactivity evaluation

Double radial immunodiffusion

To identify at least one Ab to measure the concentration of CRP in the serum samples of bottlenose dolphins and striped dolphins, several Abs were tested. None of the Abs tested cross-reacted with the serum samples of bottlenose dolphins or striped dolphins. Moreover, when the Abs were placed in the central wells and the serum samples in the peripheral wells, the precipitation lines were evident only between the Abs and the serum of dog with high concentration of CRP, except for the test number 5, in which the Ab were diluted 1:100 and 1:1000, and no precipitation lines were evident. Conversely, when the serum samples were placed in the central wells and the Abs in the peripheral wells, more serum samples cross-reacted with the Abs. For example in test number 1 and 2 the same serum samples and the same Abs were used (table 3.2.1.B). In the test number 1, the Abs were placed in the central wells and the serum samples in the peripheral wells, in the test number 2 the serum samples were placed in the central wells and Abs in the peripheral wells. In test number 1, the precipitation lines were evident between the Abs produced by Randox and the serum samples of dog with high concentration of CRP. In test number 2, the precipitation lines were evident between the Abs produced by Randox and the serum samples of dog with high concentration of CRP, cat with normal concentration of SAA, and cat with high concentration of SAA. The capacity of the central wells was 20 µl, while the capacity of the peripheral wells was 12 µl. The higher number of serum samples that cross-reacted with the Abs when the serum samples were placed in the central wells could indicate a low analytic sensitivity of the DRI, which needs a high amount of antigens to provide significant precipitation lines. The lack of precipitation lines when the Ab against CRP produced by Randox was highly diluted (1:100 and 1:1000) confirms the low analytic sensitivity.

No precipitation lines were evident when the Abs against SAA were tested, whether the Abs or the serum samples were placed in the central well. Both the Abs tested were latex-conjugated, because produced for automatic immunoturbidimetric assays. The presence of latex associated to the Abs may have interfered to the migration of the Abs across the agarose gel.

Western blot analysis

The WB analysis is a high specific technique that allows separating the proteins based only by their size thanks to the treatment with SDS that linearize and make negative the proteins (Sanders et al., 2016). Once separated, the protein bands are transferred to a porous membrane by electroblotting. The porous membrane enables access to antibodies to the protein bands for immunodetection (Burnette, 1981). Thus, this test is considered one of the most reliable for the evaluation of antibodies cross-reactivity. In our work, we confirmed the cross-reaction of the Abs against CRP tested with the serum samples of human, dogs and cats, while the pattern of positivity in the serum samples of bottlenose dolphins and striped dolphins was difficult to interpret: in bottlenose dolphins the pattern was similar to those observed in dog, with different multimeric CRP forms and in striped dolphins only dimer and trimer forms were positive. However, we are not able to exclude the possibility that the multiple bands are not only related to different CRP forms, but they may represent an unspecific signal. The anti-CRP Abs used for both DRI and WB were specific for human CRP or canine CRP and no Abs against dolphins' CRP are commercially available. It is possible that the anti-CRP Abs do not recognize the cetaceans CRP, based also on the low homology of the amino acid sequence between the bottlenose dolphins CRP and the human and canine CRP (64%). The discouraging results obtained with the automated measurement of dolphins CRP seemed to confirm this conclusion.

The anti-human Ab against SAA tested cross-reacted with the serum samples of striped dolphins but not with the serum samples of bottlenose dolphins. The SAA is highly conserved between different species (Uhlar and Whitehead, 1999) and the homology of the amino acid sequence between the bottlenose dolphins' SAA and the human's SAA is 75.4%, no data regarding the homology of the amino acid sequence between the striped dolphins' SAA and the human's SAA is present. However, a previous work (Segawa et al., 2013) reported as, in bottlenose dolphins only one circulating highly alkaline SAA isoform is present (SAA-3), unlike other terrestrial mammal, except for pig, and they supposed that SAA in dolphins may have different functions compared to other mammals based on their different evolutionary processes. Thus, we cannot exclude that our different WB result in bottlenose and striped dolphins could be related to some differences in isoform distribution between these two species.

To further analyse the possible cross-reaction of commercial anti-CRP and anti-SAA Abs, we decided to measure the concentration of CRP and SAA using an immunoturbidimetric assay. We used 74 serum samples of healthy bottlenose dolphins to obtain the RIs, to assess if the storage time

affects the concentration of these positive APPs and to evaluate if exists differences between male and female and between pregnant and non-pregnant animals.

Automated measurement of CRP

The concentration of CRP in bottlenose dolphins determined by the automated spectrophotometer Cobas Mira was 0.00 mg/dL. The result it is not surprising and confirm the lack of cross-reaction proved with DRI and WB. In a previous work (Cray et al., 2013), the authors provided the RIs of CRP for bottlenose dolphins, using a different antibody which was also tested in our work, but only in DRI and WB. It is possible that the two Abs are directed against different epitopes of CRP with different level of homology and conservation between species. This data underlines the importance of the complete validation process when immunological assay were validated in new species.

Based on the results of the cross-reactivity evaluation and of the automated measurement, we can conclude that the CRP cannot be used as health marker in bottlenose dolphin and striped dolphin.

Automated measurement of SAA

The RIs for SAA obtained in our work are quite lower compared to the previously published RIs for bottlenose dolphins (Cray et al., 2013), manatee (*Trichecus manatus*) (Harr et al., 2006), and terrestrial mammals (Christensen et al., 2012). These data reflect the importance that each laboratory create its own specific RIs.

No differences were evident between long or short storage time, males or females, and pregnant animals or non-pregnant animals. Partitioning into subclasses should be based on physiologic differences that are expected to results in important clinical differences in RI. Partitioning favours homogenous subpopulations, decreasing variability between individuals and narrowing the RIs. However, partitioning should be considered only if there are at least 40 individuals within each subclass (Friedrichs et al., 2012). The management of under human care dolphins is regulated by strictly laws, moreover few dolphinaria meet the condition to house dolphins in the Mediterranean Sea. For these reasons, it is difficult to obtain a large number of bottlenose dolphins serum samples. The lack of differences in SAA concentration between males and females, and between pregnant and non-pregnant animals allows establishing the SAA RIs using a larger number of samples instead of establish the SAA RIs partitioned for sex or pregnant status, decreasing the number of samples in each subgroups. Further studies will be focused to investigate if the age of animals could affect the SAA concentration or not, as it happens in human, where no age-related changes were noted (Hijmans and Sipe, 1979). The stability of SAA over time was previously evaluate in human

(Rothkrantz-Kos et al., 2003), horses (Hillström et al., 2010; Pepys et al., 1989) and cows (Tóthová et al., 2012), with different results. In humans and horses no differences over time were evident for frozen samples or samples stored at +4°C and at room temperature, on contrary in cows a decrease in SAA concentration was evident from storage-day 2 in frozen samples. In our work no difference in SAA concentration in long storage time samples compared to the short storage time samples was evident, this allows to put together samples collected in different time, to increase the cohort of samples used to generate the RIs.

Unfortunately, the tested animals were all healthy, so we could not evaluate the change in concentration in diseased animals. The only test in a non-healthy animal was performed in a striped dolphins stranded because of a wound in the caudal peduncle. For this animal 3 serum samples collected in 3 consecutive days were available. Interestingly the concentration of SAA increased from the first to the last sample and the increase was proportionally to the deterioration of the clinical condition of the animal, reaching the maximum concentration of 128.0 mg/dL the day of the death (Bonsembiante et al., 2017). The maximum concentration reached in the diseased animal was lower compared to the SAA concentration in diseased manatees (Harr et al., 2006). Thus, we could speculate that the dolphins have lower concentration of SAA than manatees, and that also the magnitude of concentration's increase in diseased animals is lower.

5.2.2 Negative APP

The activity on PON-1 has never been evaluated in marine mammals before, so our results were compared to the RIs of dogs (Rossi et al., 2013) and pigs (Escribano et al., 2015). We used two different substrates (paraoxon and 4-nPA) in an automated spectrophotometer and three substrates (4-nPA, PA, and DC) in a manual spectrophotometer.

Using paraoxon as substrate, the RIs of dolphins (6.7 ± 4.6 U/L) were lower compared to the RIs of dogs (Rossi et al., 2013). Since the PON-1 is a negative APP, the low activity measured in our work hampered the possibility to appreciate an additional decrease in diseased animals. One possible explanation for the low PON-1 activity using paraoxon as substrate in bottlenose dolphins is the lack of the paraoxonase activity of this enzyme and consequently a possible non-specific reaction to the substrates. Moreover, PON-1 is an enzyme associated with high density lipoproteins (HDL) that protects low density lipoproteins (LDL) and HDL from peroxidation (Aviram et al., 1998; Mackness et al., 2004). No data are present regarding the lipid metabolism in marine mammals, so we measured the HDL in the serum of the bottlenose dolphins to evaluate if the low activity of PON-1 could be explained by a low HDL concentration. The mean concentration in HDL was comparable to the concentration of bovine (Crociati et al., 2017), and the evaluation of the PON-1

activity in these animals had been performed successfully (Kulka et al., 2014). The different evolution of these aquatic mammals and their peculiar environment could lead to a different lipid metabolism in dolphins compared to terrestrial mammals, so the enzymes associated to the HDL are instable and the measurement can be a challenge.

In human medicine two SNPs are known to be able to influence the PON-1 activity: when leucine is changed with methionine in position 55 (L55M) and/or when glycine is changed with arginine in position 192 (Q192R) in the PON-1 gene, the paraoxonase activity of PON-1 is increased (Mackness and Mackness, 2015). The coding region of the paraoxon gene was screened for alterations by direct sequencing of all samples and all the animals were homozygous for methionine in position 55 and for arginine in position 192. Despite all the animals are homozygous for the phenotype that in human is associated to a higher paraoxonase activity, the PON-1 bottlenose dolphins activity is low. Further study will be focused on the promoter region of the PON-1 gene, because in the bovine seven SNPs were identified and five of them are associated with variability in serum PON-1 activity (Silveira et al., 2015). Moreover, Mahrooz and colleagues demonstrated that, in human with the QR or RR phenotypes in position 192, when the concentration of NaCl in the Tris/HCl were increased from 1 mmol to 1 mol, also the PON-1 paraoxonase activity increase (Mahrooz et al., 2014). Thus, the salt-stimulated PON-1 paraoxonase activity was evaluated also in dolphins, but no differences were evident compared to the normal (with 1mmol NaCl) paraoxonase activity (data not shown), despite all the animals were homozygous RR in position 192. This data further suggested that the Q192R SNP does not influence the paraoxonase PON-1 activity in dophins.

Since the PON-1 activity using paraoxon as substrate in dolphins is low, other substrates were tested, to evaluate if the PON-1 in dolphins could have higher arylesterase or lactonase activity rather than paraoxonase activity. The PON-1 activity using 4-nPA as substrate were higher compared to those obtained using paroxon, thus more suitable to use as a negative APP. Arylesterase activity in bottlenose dolphins appeared to be higher also compared to other animals, such as dogs (Tvarijonaviciute et al., 2012) and pigs (Escribano et al., 2015) (186.2 ± 20.4 U/L vs. 5.32 ± 0.7 U/L and 51.0 ± 16.0 U/L, respectively). Unfortunately, no differences were noted between healthy and diseased animals and these data obviously hampered the use of 4-nPA as substrate to evaluate the activity of PON-1 as a negative APP to evaluate the health status in bottlenose dolphins, differently from what happens in pigs (Escribano et al., 2015). However, our results are based on a limited number of animals with toxoplasmosis or that were found stranded without a specific disease. We cannot exclude that, including a higher number of animals with

different disease (e.g infectious diseases that marked affected immune system in dolphins such as morbillivirus), a more drastic change in PON-1 activity will be evident.

The arylesterase activity using PA and the lactonase activity using DC as substrates could not be performed in the automated spectrophotometer due to the lack of instrument with UV laser. The PON-1 activity measured using PA provides results with high variability, in 6 tests no reaction were evident, and 4 tests provided negative activity. This high variability could be related to the non-automated procedures for the measurement, which leads to pipetting errors and non-standardized time of reading, or to a low stability of the reagents. Therefore, based on these technical limitations, it is difficult to properly evaluate if PA could be considered a useful substrate for PON activity. Moreover, the possibility to adapt this assay to automated analyser is mandatory for higher specimen throughout without any pipetting errors. As regards the lactonase activity using DC, no reactions were evident, despite the changing in serum amount and reading time. Consequently, we can hypothesize that the dolphin's PON-1 does not have a lactonase activity.

6. CONCLUSIONS

In the present thesis, new data regarding the serum proteins of odondocetes were provided.

Firstly, the RIs of the SPE using AGE for under human care dolphins were established. Moreover, in 8 samples a wider albumin peak was evident. To investigate this abnormality, CZE were performed in all the samples, and in nine of them a double albumin peak was noticed. The visual appearance of CZE profiles was different compared to AGE in our samples, as previously reported for dogs and cats (Giordano and Paltrinieri, 2010). The differences between CZE and AGE profiles likely resulted from the different analytical principle of the two methods and from the higher resolution of CZE (Giordano and Paltrinieri, 2010). The application of higher resolution techniques, such as CZE, can result in an increased number of ''abnormal'' profiles, and thus the knowledge that the CZE profiles are different from the AGE profiles is important, and training in interpretation of CZE data and profiles and more confident interpretations in clinical practice are necessary. Since bisalbuminemia was confirmed by CZE analysis, the next step of the study was focused on the identification of the mutations responsible and the inheritance pattern of this disorder. We found that the albumin gene possessed a significant degree of polymorphisms and we identified two mutations potentially responsible of inherited bisalbuminemia, one of this homologs to a mutation already reported in human medicine and named Albumin Asola (Minchiotti et al., 1995). Moreover, we found that bisalbuminemia was inherited in autosomal codominant manner.

The second part of the thesis was focused on the study of APPs. Our RIs for the positive APPs were different compared to the previously published RIs for bottlenose dolphins (Cray et al., 2013). The difference in the two RIs underlines the importance that each laboratory establishes its own RIs. Moreover, based on the lack of cross-reactivity demonstrated for the anti-CRP Abs, it is important that all the assays developed for new species undergo to a complete validation process, especially when immunological assay has to be validated. We determined the RIs for SAA; but it was not possible to evaluate this assay in diseased animals, so we could not determine the diagnostic accuracy of this assay. Further studies will be focused on the evaluation of the diagnostic performance of SAA including diseased animals to determine if the concentration of SAA increases in case of infectious pathologies.

In addition, we evaluated the activity of the negative APP PON-1 in both healthy and diseased animals. The tests with paraoxon and 4-nPA as substrates provided results that have good precision. The paraoxonase assay was tested only in healthy animals, but due to the low activity demonstrated, it seems that this assay could not be used to discriminate between healthy and diseased animals.

The arylesterase activity of PON-1 was evaluated in both healthy and diseased animals. Unfortunately, no differences were noted between healthy and diseased animals and these data obviously hampered the use of 4-nPA as substrate to evaluate the activity of PON-1 as a negative APP, differently from what happens in pigs (Escribano et al., 2015). However, our results are based on a limited number of animals that were found stranded without a specific disease. We cannot exclude that, including a higher number of animals with diseases which markedly affected immune system a more drastic change in PON activity will be evident.

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List of original publications regarding the Ph.D project

1. Gili C., **Bonsembiante F.**, Bonanni R., Giordano A., Ledda S., Beffagna G., Paltrinieri S., Sommer M., Gelain M.E. Detection of hereditary bisalbuminemia in bottlenose dolphins (*Tursiops truncatus*, Montagu 1821): comparison between capillary zone and agarose gel electrophoresis. BMC Vet Res. 2016; 12:172.

Abstract. *Hereditary bisalbuminemia is a relatively rare anomaly characterized by the occurrence of two albumin fractions on serum protein separation by electrophoresis. In human medicine, it is usually revealed by chance, is not been clearly associated with a specific disease and the causative genetic alteration is a point mutation of human serum albumin gene inherited in an autosomal codominant pattern. This type of alteration is well recognizable by capillary zone electrophoresis (CZE), whilst agarose gel electrophoresis (AGE) not always produces a clear separation of albumin fractions. The aims of this study is to report the presence of this abnormality in two separate groups of related bottlenose dolphins and to compare the results obtained with capillary zone and agarose gel electrophoresis. Serum samples from 40 bottlenose dolphins kept under human care were analyzed. In 9 samples a double albumin peak was evident in CZE electrophoresis while no double peak was noted in AGE profile. Since only an apparently wider albumin peaks were noted in some AGE electrophoretic profiles, the ratio between base and height (b/h) of the albumin peak was calculated and each point-value recorded in the whole set of data was used to calculate a receiver operating characteristic curve: when the b/h ratio of albumin peak was equal or higher than 0.25, the sensitivity and specificity of AGE to detect bisalbuminemic samples were 87 and 63 %, respectively. The bisalbuminemic dolphins belong to two distinct families: in the first family, all the siblings derived from the same normal sire were bisalbuminemic, whereas in the second family bisalbuminemia was present in a sire and in two out of three siblings. We report for the first time the presence of hereditary bisalbuminemia in two groups of related bottlenose dolphins identified by means of CZE and we confirm that AGE could fail in the identification of this alteration.*

2. Gili C.*, **Bonsembiante F***, Beffagna G, Mazzariol S, Gelain M.E.. Mutations and polymorphism in bottlenose dolphin (Tursiops truncatus, Montagu 1821) albumin gene: First identification of mutations responsible for inherited bisalbuminemia. Res Vet Sci. 2017; 114:12-17. (*Gili C. and Bonsembiante F. equally contributed to this article).

Abstract. *Hereditary bisalbuminemia is an asymptomatic and heterozygous condition in a range of species characterized by the presence of two serum albumin fractions with different*

electrophoretic mobility resulting in a bicuspid pattern on serum electrophoresis. Bisalbuminemia has been diagnosed by electrophoresis in two bottlenose dolphin (Tursiops truncatus) families, but causative mutations and the inheritance pattern have not been identified. The aims of this work are: to investigate polymorphisms of the bottlenose dolphin albumin gene and to identify mutations causative of bisalbuminemia; to identify the inheritance pattern in two bottlenose dolphin families. Coding regions of the albumin gene were screened for mutations in 15 bottlenose dolphins kept under human care from two distinct families. Eighteen albumin mutations (three synonymous and 15 non-synonymous) were identified. Two non-synonymous variations co-segregated with bisalbuminemic phenotype: p.Phe146Leu in exon 4 and p.Tyr163His in exon 5. The amino acid change in exon 5 was associatedwith the secondary and/ or tertiary structure variation of the protein and has been reported as causative of bisalbuminemia in humans. Pedigree analysis of the dolphin families showed an autosomal codominant inheritance pattern. In this work, the mutations potentially responsible for bisalbuminemia were identified and confirmed the autosomal codominant trait in bottlenose dolphins.

3. **Bonsembiante F**.*, Centelleghe C.*, Rossi G., Giglio S, Madeo E., Gelain M.E., Mazzariol S. Clinico-pathological findings in a striped dolphin (Stenella coeruleoalba) affected by rhabdomyolysis and myoglobinuric nephrosis. J Vet Med Sci. 2017. (*Bonsembiante F. and Centelleche C. equally contributed to this article),

Abstract. A striped dolphin (Stenella coeruleoalba) calf stranded alive because of a Salter-Harris fracture type 1 of a caudal vertebra and remained in a provisional rehabilitation facility for 3 days where the fracture stabilization was attempted, but he died the day after bandaging. Serum and urine samples were collected during hospitalization (days 1, 2 and 3 serum and day 2 urine). Serum analysis 29 showed increased urea, alanine transaminase, aspartate transaminase, and serum amyloid A values, while creatinine was below the lower limit. Urine analysis showed urinary protein-to-creatinine ratio of 5.3 with glomerular proteinuria. Postmortem analyses demonstrated a severe rhabdomyolysis and myoglobinuric nephrosis, suggestive of capture myopathy syndrome. We report, for the first time, the clinico-pathological changes during this condition in a striped dolphin.

List of original publications regarding other research projects

4. Benali S.L., Lees G.E., Nabity M.B:, Mantovani R., **Bonsembiante F.**, Aresu L. De novo expression of human leukocyte antigen-DR (HLA-DR) and loss of beta-catenin expression in tubular epithelial cells: a possible event in epithelial-mesenchymal transition in canine renal diseases. Vet J. 2013; 198:229-234.

Abstract. Tubulointerstitial fibrosis (TIF) plays a central role in the progression to end-stage renal disease. Tubular epithelial cells (TECs) undergo epithelial-mesenchymal transition (EMT) and may contribute to the progression of TIF. Using immunohistochemistry, the primary aim of this study was to assess the expression of β-catenin, human leukocyte antigen-DR (HLA-DR) and vimentin in renal biopsies from dogs with spontaneous kidney diseases of varying severities. Morphological diagnosis, severity of inflammation, TIF, HLA-DR expression and clinico-pathological variables were compared in dogs with renal injury to identify any potential relationship between the different factors; β-catenin down-regulation was used as a marker of EMT. Fibrosis, HLA-DR expression, serum creatinine concentration (SCr), and urine protein-to creatinine ratio (UPC) were all increased and β-catenin expression decreased in dogs with primary glomerular disease compared with dogs with acute tubular necrosis. HLA-DR expression by TECs was positively correlated to fibrosis, inflammation, UPC, and SCr. β-catenin expression was negatively correlated to fibrosis, inflammation, and HLA-DR expression. The progression of renal failure correlated closely with tubulointerstitial damage. De novo HLA-DR expression associated with β-catenin down-regulation by TECs may represent a possible step in the progression of TIF and EMT.

5. **Bonsembiante F.**, Benali S.L., Trez D., Aresu L., Gelain M.E. Histological and immunohistochemical characterization of feline renal cell carcinoma: a case series. J Vet Med Sci. 2016; 178:1039-1043.

Abstract. Four feline renal cell carcinomas (RCCs) were examined using histopathological ind immunohistochemical procedures. Specimens were classified by predominant histological pattern according to WHO criteria. A panel of antibodies including β-catenin, C-KIT, VEGF, VGF-R2 and double immunostaining for vimentin/cytokeratin and for E-cadherin/CD10 was selected to characterize the tumors. Neoplasms were classified as tubular (3/4) and papillary (1/4). Neoplastic epithelial cells were cytokeratin, vimentin, E-cadherin, VEGF-R2 positive and C-KIT negative; 3 cases were β-catenin positive, whereas only two tumors were CD10 and

VEGF positive. No correlation with histotype was evident. Our results confirm the low frequency of RCCs in cats and suggest a histological pattern similar to canineRCCs. In contrast, a peculiar immunohistochemical profile different from both canine and human RCCs is identified.

6. Zorzan E., Da Ros S., Musetti C., Shahidian L.Z., Coelho N.F., **Bonsembiante F.**, Létard S., Gelain M.E., Palumbo M., Dubreuil P., Giantin M., Sissi C., Dacasto M. Screening of candidate G-quadruplex ligands for the human c-KIT promotorial region and their effects in multiple in-vitro models. Oncotarget. 2016; 7:21658-21675.

Abstract. *Stabilization of G-quadruplex (G4) structures in promoters is a novel promising strategy to regulate gene expression at transcriptional and translational levels. C-KIT proto-oncogene encodes for a tyrosine kinase receptor. It is involved in several physiological processes, but it is also dysregulated in many diseases, including cancer. Two G-rich sequences able to fold into G4, have been identified in C-kit proximal promoter, thus representing suitable targets for anticancer intervention. Herein, we screened an ''in house'' library of compounds for the recognition of these G4 elements and we identified three promising ligands. Their G4-binding properties were analyzed and related to their antiproliferative, transcriptional and post-transcriptional effects in MCF7 and HGC27 cell lines. Besides C-KIT, the transcriptional analysis covered a panel of oncogenes known to possess G4 in their promoters. From these studies, an anthraquinone derivative (AQ1) was found to efficiently downregulate C-KIT mRNA and protein in both cell lines. The targeted activity of AQ1 was confirmed using C-KIT-dependent cell lines that present either C-KIT mutations or promoter engineered (i.e., a155, HCN1.2 and ROSA cells). Present results indicate AQ1 as promising compound for the target therapy of C-KIT-dependent tumors, worth of further and in depth molecular investigations.*

7. Perazzi A., **Bonsembiante F.**, Gelain M.E., Patruno M., Di Iorio F., Migliorati A., Iacopetti I. Cytology of the healthy canine and feline ocular surface: comparison between cytobrush and impression technique. Vet Clin Path. 2017; 46: 164-171.

Abstract. *Impression cytology (IC) is a noninvasive technique in which filters are used to sample superficial layers of ocular epithelium. The aim of this study was to compare cytology specimens obtained by IC and cytobrush from healthy canine and feline eyes. Dogs and cats were prospectively sampled using polytetrafluorethylene filters on the right eye, and cytobrus on the left eye. Wright-Giemsa-stained specimens were evaluated by 2 observers. Cellularity, preservation, and morphology of cells and presence of goblet and inflammatory cells were scored with a 4-grade scale. Inter-observer agreement and effects of topical anesthesia were analyzed. In 20 canine IC samples, 10 showed good cellularity (score 2-3) and 13 good*

preservation. Superficial epithelial cells (SEC) were present in 13/20 of IC, while basal-intermediate cells (BIC) were seen in 14/20. In 6/20 and 7/20, goblet and inflammatory cells were noted, respectively. In 20 cats, 15 of IC showed good cellularity and 14 good preservation, and SEC were present in 16/20 of IC and BIC in 17/20. In 13/20 and 3/20 cats, goblet cells and inflammatory cells were noted, respectively. Canine cytobrush specimens appeared well preserved (9/20) and had good cellularity (8/20). In feline cytobrush specimens good preservation and cellularity were observed in 16/20 and 14/20, respectively. In both species, all cell types were present without a clear separation. There was moderate to fair agreement about cellular morphology in IC between observers. Specimens obtained with and without anesthesia were comparable. Impression cytology allowed collection of samples with maintained cytoarchitecture, while cytoplasmatic and nuclear details were often difficult to evaluate.

8. Banzato T., **Bonsembiante F.**, Aresu L., Zotti A. Relationship of diagnostic accuracy of renal cortical echogenicity with renal histopathology in dogs and cats, a quantitative study. BMC Vet Res. 2017; 13:24.

Abstract. *Renal cortical echogenicity is routinely evaluated during ultrasonographic investigation of the kidneys. Both in dog and cat previous ex-vivo studies have revealed a poor correlation between renal echogenicity and corresponding lesions. The aim of this study was to establish the in-vivo relationship between renal cortical echogenicity and renal histopathology. Thirty-eight dogs and fifteen cats euthanized for critical medical conditions were included in the study. Ultrasonographic images of both kidneys were acquired ante mortem at standardized ultrasonographic settings. The echogenicity was quantified by means of Mean Gray Value (MGV)ofthe renal cortexmeasuredwith ImageJ. A complete histopathological examination of both kidneys was performed. Five kidneys were excluded because histopathology revealed neoplastic lesions. Only samples affected by tubular atrophy showed statistically different values in dog, and histopathology explained 13% of the total variance. MGV was not correlated neither to the degeneration nor to the inflammation scores. However, significant differences were identified between mildly and severely degenerated samples. Overall, the classification efficiency of MGV to detect renal lesions was poor with a sensitivity of 39% and a specificity of 86%. In cats, samples affected by both tubular vacuolar degeneration and interstitial nephritis were statistically different and histopathology explained 44% of the total variance. A linear correlation was evident between degeneration and MGV, whereas no correlation with inflammation was found. Statistically significant differences were evident only between normal and severely degenerated samples with a sensitivity of 54.17% and a specificity of 83.3% and MGV resulted scarce to discriminate renal lesions in this species. Renal cortical echogenicity*

shows low relevance in detecting chronic renal disease in dog whereas it results worth to identify severe renal damage in cat.

9. Schrank M., **Bonsembiante F**., Fiore E., Bellini L., Zamboni C., Zappulli V., Stelletta C., Mollo A. Approccio diagnostico alla mastopatia fibrocistica in una capra: rilievi temrografici, ecografici ed isto-patologici. Large Animal Review. 2017; 23:33-37.

Abstract. The mammary gland diseases are frequent problems in goats and sheep breeding. Mastitis, abscesses due to traumatic events, neoplasia, and cystic lesions are the main problems that could affect udder and nipple. This clinical case is about a pet spayed female goat with an increased volume of the right quarter and nipple (25 cm length, 6 cm width), mammary lymphadenopathy (diameter of 5 cm), no mammary gland secretion, pain during milking procedures, and no opened duct. Inflammatory processes such as mastitis and abscesses were rule out based on the thermographic and ultrasonographic investigations, complete blood cells count, serum biochemistry, and fine needle aspiration of the fluid for bacteriological culture. The histological diagnosis of fibrocystic mastopathy were performed after the mastectomy. In human medicine, these lesions are associated to the breast cystic lesions syndrome, that must be differentiated from a neoplastic lesion. It is reported that in goats and sheep that these lesions have a multifactorial etiology: hormonal imbalance between oestrogens and progesterone, genetic causes, and bacteria. In the present case, no bacteria are present, and the absence of the reproductive apparatus rules out the hormonal imbalance. Further studies are needed to better characterize these lesions frequently reported in goats.

Oral presentations-International congress

1. Benali S.L., Lees G.E., Nabity M.B., **Bonsembiante F.**, Aresu L. De-novo HLA-DR expression in canine renal tubular epithelium: a possible event in epithelial-mesenchymal transition. 31st congress of European Society of Veterinary Pathology. London (UK), 04-07/09/2013.

2. **Bonsembiante F.**, Perazzi A., Gelain M.E., Patruno M., Migliorati A., Iacopetti I. Cytology of the healthy canine and feline ocular surface: comparison between cytobrush and impression technique. 17th congress of European Society of Veterinary Clinical Pathology. Lisbon (PO), 09-12/09/2015.

3. Beffagna G., Sammarco A., **Bonsembiante F.**, Cavicchioli L., Ferro S., Gelain M.E., Zappulli V. The Wnt/beta catenin pathway in canine mammary carcinoma as a potential target of anti-cancer drugs: an in vivo study. 27th congress of European Society of Veterinary Pathology. Bologna (IT), 7-10/09/2016.

4. **Bonsembiante F.**, Martini V., Bonfanti U., Casarin G., Gelain M.E. Comparison between whole slide imaging and optical microscopy in the cytomorphological evaluation of canine lymphoma samples. 18th congress of European Society of Veterinary Clinical Pathology. Nantes (FR), 18-23/10/2016.

5. Gili C., **Bonsembiante F.**, Beffagna G., Mazzariol S., Gelain M.E. Hereditary bisalbuminemia in Bottlenose dolphins (*Tursiops truncatus*, Montagu 1821). 45th annual symposium European Association for Aquatic Mammals. Genova (IT), 8-11/03/2017.

6. **Bonsembiante F.**, Beffagna G., Gili C., Rossi G., Giordano A., Paltrinieri S., Gelain M.E. Paraoxonase-1 (PON1) in Bottlenose dolphins *(Tursiops truncatus)*: serum activity and genetic polymorphisms. 18th International symposium of the World Association of Veterinary Laboratory Diagnosticians. Sorrento (IT), 7-10/06/2017.

Oral presentations-National congress

1. **Bonsembiante F.**, Benali S.L., Trez D., Aresu L., Gelain M.E. Histological and immunohistochemical characterization of feline renal cell carcinoma: a case series. 11th

Congresso Nazionale dell'Associazione Italiana dei Patologi Veterinari (AIPVet), Pisa (IT), 16-18/06/2014.

2. Gili C., **Bonsembiante F.,** Beffagna G., Mazzariol S., Gelain M.E. Mutations and polymorphism in albumin gene of bottlenose dolphin (*Tursiops truncatus*): the first identification of mutations responsible of inherited bisalbuminemia. 13th Congresso Nazionale dell'Associazione Italiana dei Patologi Veterinari (AIPVet), Palermo (IT), 13-16/06/2016.

3. **Bonsembiante F.,** Deganello A., Perazzi A., Gelain M.E., Iacopetti I. Impression cytology of healthy ocular surface in horses: comparison with cytobrush technique. 14th Congresso Nazionale dell'Associazione Italiana dei Patologi Veterinari (AIPVet), Napoli (IT), 28/06-01/07 2017.

Posters

1. **Bonsembiante F.,** Centelleghe C., Mazzariol S., Zanetti R., Gelain M.E. Cross-reactivity evaluation of commercial antibodies against leukocyte sub-populations in striped dolphins (S*tenella coeruleoalba).* 5th convegno Nazionale della Ricerca nei Parchi, Bussolengo (IT) 10-12/10/2014.

2. **Bonsembiante F.,** Centelleghe C., Rossi G., Soloperto S., Giglio S., Madeo E., Di Bello A., Mazzariol S. Capture myopathy after surgery in striped dolphin *(Stenella coeruleoalba)*: a case report. 29th congress of European Cetacean Society (ECS), Malta (M) 22-25/03/2015.

3. Gelain M.E:, **Bonsembiante F.,** Rossi G., Giordano A., Di Guardo G., Paltrinieri S., Mazzariol S. Cross-reactivity evaluation of commercial antibodies against acute phase proteins in striped dolphins *(Stenella coeruleoalba)* and bottlenose dolphins *(Tursiops truncatus).* 29th congress of European Cetacean Society (ECS), Malta (M) 22-25/03/2015.