Universidade de Trás-os-Montes e Alto Douro

Acute Phase Proteins in Feline Medicine

Acute phase proteins response in feline natural infection by hemotropic mycoplasmas (hemoplasmas), in feline pyometra and in feline spontaneous malignant mammary tumors

Tese de Doutoramento em Ciências Veterinárias

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Vila Real, maio de 2020

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Abstract

The acute phase response (APR) is a component of the host innate defense system, characterized by a very sensitive and non-specific systemic reaction of the organism, that develops when animals are exposed to an inflammatory stimulus of different etiology. The APR is a very fast reaction, which develops before stimulation of the specific immune response, and often even before the development of clinical signs, therefore is considered one of the earliest markers of any pathological process or disease. The APR purposes are to prevent further tissue damage, remove the cause of the insult, and to restore the normal function and the homeostasis of the organism. Fever, leukocytosis and changes in concentrations of several serum proteins – designated acute phase proteins (APP) - are considered the principal hallmarks of the APR.

Acute phase proteins are serum proteins which concentrations are altered by more than 25% in relation to their basal level in response to an inflammatory stimulus. During the APR, serum concentration of some proteins will increase - positive APP, and of others will decrease - negative APP. Based on the magnitude and behavior of increase, positive APP are classified as minor, moderate or major responders. Each species has its characteristic positive and negative APP. In the cat, serum amyloid A (SAA) and α1-acid glycoprotein (AGP) are considered major positive APP, haptoglobin (Hp), ceruloplasmin (Cp) and fibrinogen (Fb) are considered moderate APP, and albumin is considered the principal negative APP. Determination of APP profiles that include at least one positive major, one positive moderate and one negative APP are recommended over determination of single APP, in order to obtain a better differentiation between pathological states and to provide more information on the evolution of the disease.

The APP are being increasingly used in human and in veterinary medicine as biomarkers of general health screening, and of diagnosis, prognosis and monitoring of the evolution and response to treatment of different diseases. In addition, recent investigations described new

clinically useful analytes that present an APP behavior, and also have led to the discovery of possible new clinical applications of the APP, namely in treatment of different diseases. Even though the APP are routinely used in clinical practice in human medicine, their clinical application in veterinary medicine, particularly in feline medicine, is still scarce. Therefore, the main aim of this investigation was to contribute to the knowledge of the clinical applications of APP in feline medicine, through the study of the APP response in different feline conditions, including inflammatory, infectious and neoplastic diseases. To the authors' knowledge, these were the first studies to comprehensively evaluate the APP response in cats naturally infected with hemoplasmas, including infections with one agent and co-infections with different species of hemoplasmas, in cats with pyometra and in cats with spontaneous malignant mammary tumors. All these feline conditions induced and APP response.

The APP response in cats naturally infected (single and co-infections) with hemotropic mycoplasmas was evaluated through determination of serum concentrations of SAA, Hp and albumin in 48 cats infected with hemoplasmas, and in 10 healthy control cats. Of the 48 infected animals, 25 cats were infected with *Candidatus Mycoplasma haemominutum*, 12 cats were co-infected with *Candidatus Mycoplasma haemominutum* and *Mycoplasma haemofelis*, 7 cats were co-infected with *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma haematoparvum*-like, and 4 cats were infected with other *Mycoplasma* species. The overall group of infected cats (including symptomatic and asymptomatic animals) had significantly higher Hp and lower albumin than controls, and a tendency for higher concentrations of SAA was also observed in infected cats than in controls. Symptomatic cats had significantly higher SAA and Hp, and lower albumin than asymptomatic animals, and also than controls. Asymptomatic cats had significantly higher Hp than controls. According with these results, feline natural infection with hemoplasmas is associated with development of an APP response, and Hp could be a clinically useful biomarker of the subclinical infections with hemotropic mycoplasmas. The magnitude of the APP response was similar in cats infected with one agent and cats co-infected with different species of hemoplasmas.

The APP response in feline pyometra, at diagnosis and during the post-operative period, was assessed by determination of an APP panel composed by two positive - SAA and Hp, and one negative APP - albumin, in serum samples of 23 queens with pyometra and of 13 healthy control queens submitted to elective ovariohysterectomy. The APP were determined before surgery in all queens included in the study (n = 36), and were also determined in 11 queens of

the pyometra group at days two and 10 after surgery. At diagnosis, queens with pyometra had serum concentrations of SAA and Hp significantly higher, and of albumin significantly lower than controls. Moreover, concentrations of APP were significantly different, with a tendency to return to physiologic levels, at day 10 after surgery than before surgery. These results suggest that APP could be potentially useful biomarkers in diagnosis and assessment of the post-operative period in feline pyometra.

The APP response, their relation with clinical and histological findings, and their prognostic value in feline spontaneous malignant mammary tumors were evaluated through determination of an APP panel, that included two positive - SAA and Hp, and four negative APP - albumin, butyrylcholinesterase (BChE), insulin-like growth factor 1 (IGF1) and paraoxonase 1 (PON1), in serum samples of 50 queens with mammary adenocarcinomas and of 12 healthy control cats. According with our results, feline malignant mammary tumors are associated with an APP response, and different clinical and histological features of mammary tumors influence significantly the inflammatory response, including bigger size, ulceration, lymphovascular neoplastic invasion, metastasis in regional lymph nodes and distant organs, advanced clinical stage of the disease, histological type, higher histological grade, necrosis and higher proliferative activity. Furthermore, some of these analytes proved to have prognostic value. At diagnosis, increases in serum Hp and decreases in albumin may suggest tumor metastization; and increases in SAA during the course of the disease may suggest development of metastasis in distant organs. Decreased serum albumin at diagnosis was associated with a longer survival, and serum BChE < 1.15 μmol/ml.min at diagnosis was associated with a shorter survival time on multivariate analysis.

Key Words: acute phase proteins, acute phase reaction, feline, hemotropic mycoplasmas (hemoplasmas), mammary tumor, pyometra

Resumo

A resposta de fase aguda (RFA) é um componente do sistema imunitário inato do hospedeiro, caracterizada por uma reação sistémica muito sensível e inespecífica do organismo, que se desenvolve quando os animais são expostos a um estímulo inflamatório de diferentes etiologias. A RFA é uma reação muito rápida, que se desenvolve antes da resposta imunitária específica, muitas vezes mesmo antes do desenvolvimento de sinais clínicos, pelo que é considerada um dos primeiros marcadores de qualquer processo ou doença. Os objetivos da RFA são limitar as lesões dos tecidos, eliminar a causa da agressão e restaurar a função normal e a homeostasia do organismo. O desenvolvimento de febre, de leucocitose e de alterações nas concentrações de diversas proteínas séricas - designadas proteínas de fase aguda (PFA) - são considerados os principais biomarcadores da RFA.

As PFA são proteínas séricas cujas concentrações são alteradas em mais de 25% em relação ao seu nível basal em resposta a um estímulo inflamatório. Durante a RFA, a concentração sérica de algumas proteínas aumenta - PFA positivas, e de outras diminui - PFA negativas. De acordo com a magnitude e o comportamento do aumento, as PFA positivas são classificadas como reativos menores, moderados ou maiores. Cada espécie tem as suas PFA positivas e negativas características. No gato, a proteína sérica amilóide A (PSAA) e a glicoproteína α1 ácida (GPA) são consideradas PFA positivas maiores, a haptoglobina (Hp), a ceruloplasmina (Cp) e o fibrinogénio (Fb) são consideradas PFA moderadas, e a albumina é considerada a principal PFA negativa. A determinação de perfis de PFA que incluam pelo menos uma PFA positiva maior, uma PFA positiva moderada e uma PFA negativa é recomendada sobre a determinação de PFA individuais, porque permite uma melhor diferenciação entre estados patológicos e uma melhor avaliação da evolução da doença.

A utilização clínica das PFA tem vindo a aumentar nos últimos anos em medicina humana e, embora em menor extensão, também em medicina veterinária. As PFA são usadas na avaliação do estado geral de saúde, e como biomarcadores de diagnóstico, prognóstico e

monitorização da evolução e da resposta ao tratamento de diferentes doenças. Mais, para além das PFA "clássicas", estudos recentes identificaram novos analitos clinicamente úteis que apresentam um comportamento de PFA, e permitiram também a descoberta de possíveis novas aplicações clínicas das PFA, nomeadamente da sua aplicação / modulação no tratamento de diferentes doenças.

As PFA são usadas rotineiramente na prática clínica em medicina humana, no entanto, a sua aplicação clínica na medicina veterinária, particularmente na medicina felina, ainda é limitada. O principal objetivo desta investigação foi contribuir para o conhecimento da utilização clínica das PFA na medicina felina, através do seu estudo em diferentes processos, nomeadamente doenças inflamatórias, infecciosas e neoplásicas. Que tenhamos conhecimento, estes foram os primeiros estudos a avaliar a resposta das PFA em gatos infectados de forma natural por hemoplasmas (infecções com um agente e co-infecções com diferentes espécies de hemoplasmas), em gatas com piómetra e em gatas com tumores mamários malignos espontâneos. Todos estes processos induziram uma resposta das PFA.

A resposta das PFA em gatos infectados de forma natural por micoplasmas hemotrópicos (infeção com um agente ou co-infecções), foi avaliada através da determinação das concentrações séricas de PSAA, Hp e albumina em 48 gatos infectados com hemoplasmas e em 10 gatos saudáveis. Dos 48 animais infectados, 25 gatos estavam infectados com *Candidatus Mycoplasma haemominutum*, 12 gatos estavam co-infectados com *Candidatus Mycoplasma haemominutum* e *Mycoplasma haemofelis*, 7 gatos estavam co-infectados com *Candidatus Mycoplasma haemominutum* e *Candidatus Mycoplasma haematoparvum*-like e 4 animais estavam infectados com outras espécies de *Mycoplasma*. O grupo de gatos infectados (n = 48, incluindo animais sintomáticos e assintomáticos) apresentou concentrações séricas de Hp e albumina significativamente mais altas do que os controlos; e também uma tendência para apresentar valores séricos de PSAA mais elevados do que animais do grupo controlo. Os gatos sintomáticos apresentaram valores de PSAA e Hp significativamente mais altos, e de albumina mais baixos do que os animais assintomáticos, e também que os controlos. Os gatos assintomáticos apresentaram os valores de Hp significativamente mais elevados do que os controlos. Estes resultados mostram que a infecção natural por hemoplasmas em gatos está associada ao desenvolvimento de uma resposta das PFA, e que a Hp pode ser um biomarcador das infecções subclínicas. A magnitude da resposta das PFA foi semelhante em gatos infectados com um agente e em gatos co-infectados com diferentes espécies de hemoplasmas.

A resposta das PFA em gatas com piómetra, no momento do diagnóstico e no período pós-cirúrgico, foi avaliada através da determinação de um painel de PFA composto por duas PFA positivas - PSAA e Hp, e uma PFA negativa – albumina, no soro de 23 gatas com piómetra e de 13 gatas saudáveis submetidas a ovariohisterectomia eletiva. A resposta das PFA foi determinada antes da cirurgia em todas as gatas incluídas no estudo (n = 36), e também em 11 gatas doentes nos dias dois e 10 após a cirurgia. No momento do diagnóstico, as gatas com piómetra apresentaram concentrações séricas de PSAA e Hp significativamente mais elevadas, e de albumina significativamente menores do que as gatas do grupo controlo. Além disso, nas gatas doentes (n = 11), as concentrações de PFA foram significativamente diferentes, com tendência a retornar aos níveis fisiológicos, no dia 10 após a cirurgia em relação ao momento do diagnóstico. Estes resultados sugerem que as PFA poderão ser biomarcadores potencialmente úteis no diagnóstico e avaliação do período pós-cirúrgico da piómetra felina.

A resposta das PFA, a influência de diferentes características clínicas e histológicas, e o seu valor prognóstico nos tumores mamários malignos espontâneos em gatas foram avaliados através da determinação de um painel de PFA que incluiu duas PFA positivas - PSAA e Hp, e quatro PFA negativas - albumina, butirilcolinesterase (BCE), fator de crescimento semelhante à insulina do tipo 1 (FCSI1) e paraoxonase 1 (PON1), em amostras de soro de 50 gatas com adenocarcinomas mamários e de 12 gatas saudáveis. De acordo com os nossos resultados, os tumores mamários felinos malignos induzem uma resposta das PFA, e diferentes características clínicas e histológicas dos tumores mamários influenciam significativamente a resposta inflamatória, incluindo maior tamanho, ulceração, invasão neoplásica linfovascular, metástases nos linfonodos regionais e órgãos à distância, estadio clínico da doença, tipo histológico, grau histológico, necrose e atividade proliferativa. Além disso, alguns destes analitos provaram ter valor prognóstico. Ao diagnóstico, o aumento da Hp sérica e a diminuição da albumina podem sugerir metastização do tumor; e o aumento da PSAA durante o curso da doença pode sugerir o desenvolvimento de metástases em órgãos à distância. No momento do diagnóstico, a presença de uma diminuição da albumina sérica foi associada a um maior tempo de sobrevida, e uma concentração sérica de BCE < 1,15 μmol / ml.min foi associada a um menor tempo de sobrevida em análise multivariada.

Palavras-chave: felino, micoplasmas hemotrópicos (hemoplasmas), piómetra, proteínas de fase aguda, reação de fase aguda, tumor mamário

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Abbreviations, acronyms and symbols

AA - amyloid A

ACTH - adrenocorticotropic hormone

AGP – alpha 1-acid glycoprotein

AKI - acute kidney injury

Apo - apolipoprotein

APP - acute phase proteins

APR – acute phase response / reaction

AUC – area under the curve

B. vogeli – Babesia vogeli

BChE – butyrylcholinesterase

BW – body weight

CI – confidence interval

CKD - chronic kidney disease

cm - centimeter

CMhm - *Candidatus Mycoplasma haemominutum*

CMhp – *Candidatus Mycoplasma haematoparvum*-like

CMt - *Candidatus Mycoplasma turicensis*

Cp – ceruloplasmin

CRP - C-reactive protein

D. immitis - Dirofilaria immitis

DFI - disease-free interval

dl – deciliter

DL – detection limit

DSH - domestic short-hair

EDTA - ethylenediamine tetra acetic acid

ELISA - enzyme linked immunosorbent assay

Fb – fibrinogen

FCoV - feline coronavirus

FeLV - feline leukemia virus

FIP - feline infectious peritonitis

FIV - feline immunodeficiency virus

fPLI - feline pancreatic lipase immunoreactivity

g – gram

g - relative centrifugal force

H. felis – Hepatozoon felis

Hb – hemoglobin

HIV – human immunodeficiency virus

Hp – haptoglobin

HR – hazard ratio

IBD - inflammatory bowel disease

IFNγ - interferon γ

IGF1 - insulin-like growth factor 1

IL – interleukin

IFNγ - interferon γ

iNOS - nitric oxide synthases

IQR – interquartile range

IRIS – International Renal Interest Society

IU – international units

k – Cohen´s k coefficient

Kg – kilogram

L – liter

MAP – major acute phase protein

max – maximum

mg - miligram

Mhc – *Mycoplasma haemocanis*

Mhf - *Mycoplasma haemofelis*

min – minimum

min – minute

ml – milliliter

mm - milimeter

MST – median survival time

M1 macrophages – type 1 macrophages

M2 macrophages – type 2 macrophages

ng – nanogram

NGAL - neutrophil gelatinase-associated lipocalin

NO - nitric oxide

P – *P*-value or probability value

PCR – polymerase chain reaction

PON1 – paraoxonase 1

RIA - radioimmunoassays

RNA – ribonucleic acid

SAA - serum amyloid A

SAP – serum amyloid P

SCC – squamous cell carcinoma

SD – standard deviation

SIRS - systemic inflammatory response Syndrome

SPE – serum protein electrophoresis

spp. – species

SLE – systemic lupus erythematosus

TSA – total sialic acid

TIBC - total iron binding capacity

TNFα - tumor necrosis factor alpha

TNM – tumor-(lymph)node-metastasis staging system

WBC - white blood cells

WHO - World Health Organization

µg – microgram

µm – micrometer

µmol – micromole

ρ – Spearman correlation coefficient

°C – degree Celsius

Chapter 1

Introduction

Acute Phase Response

The innate immune system represents the first defense line of the organism to an injury, and precedes the specific immune reactions (Rumbo et al., 2004; Martin and Jakob, 2008; Park et al., 2017). The acute phase response or acute phase reaction (APR) is a component of the host innate defense system; it is characterized by a very sensitive and non-specific systemic reaction, triggered by a series of local and systemic events that develop when organisms are exposed to an acute or chronic inflammatory stimulus of different etiology, including infection, stress, trauma, endocrine diseases, metabolic diseases or immunological diseases (Baumann and Gauldie, 1994; Evans et al., 1998; Kajikawa et al., 1999; Sasaki et al., 2003; Petersen et al., 2004; Piñeiro et al., 2007; Korman et al., 2012; Cray, 2012; Seyhan et al., 2014; Schüttler and Neumann, 2015; Bielas et al., 2017). Tumors are also able to originate a similar APR by the host immune system in the absence of an exogenous inflammatory stimulus (Martin et al., 1999; Selting et al., 2000; Itoh et al., 2009).

The APR is a very fast reaction, which develops before the specific immune response, and often even before the development of clinical signs (Martínez-Subiela et al., 2011; Munhoz et al., 2012; Canisso et al., 2014; Brown et al., 2015; Zhang et al., 2015). Therefore, the APR is considered one of the earliest markers of any pathological process or disease. The APR purposes are to prevent further tissue damage, remove the cause of the injury, and to restore the normal function and the homeostasis of the organism (Baumann and Gauldie, 1994; Suffredini et al., 1999; Ceciliani et al., 2002).

The molecular pathogenesis APR is well determined in humans and in laboratory animals, but information on other species, including in felines, is scarce (Pannen and Robotham, 1995; Ramadori and Christ, 1999; Paltrinieri, 2008). The APR begins in the injured tissues with the synthesis and release of pro-inflammatory cytokines from inflammatory cells, mainly macrophages and monocytes, but also fibroblasts, endothelial cells, platelets, keratinocytes, lymphocytes, dendritic cells and adipocytes (Gabay and Kushner, 1999; Bengmark, 2004;

Cray et al., 2009; Cray, 2012). These cytokines are also produced by some tumor cells and by cells present in the tumor microenvironment (Kim et al., 2010; Kulbe et al., 2012) The interleukin (IL)-1, IL-6 and tumor necrosis factor α (TNFα) are the main mediators of the APR (Koj et al., 1988; Yamashita et al., 1994; Lawrence et al., 1995; Murata et al, 2004; Brodzki et al., 2015). These cytokines influence several organs involved in homeostasis, and induce a wide range of neuroendocrine, metabolic, hematopoietic and hepatic alterations in order to originate a rapid and intense state of protective response (Baumann and Gauldie, 1994; Ceciliani et al., 2002; Murata et al., 2004).

The APR is characterized by different clinical hallmarks. Fever, leukocytosis and changes in concentrations of several serum proteins – designated acute phase proteins (APP) - are considered the principal hallmarks (Kushner, 1988; TerWee et al., 1998; Kjelgaard-Hansen and Jacobsen, 2011; Chervier et al., 2012; Kokotovic et al., 2017), however, increased serum concentrations of adrenocorticotropic hormone (ACTH), cortisol, catecholamines, retinol and glutathione, decreased serum thyroxine, iron and zinc concentrations, thrombocytosis, tissue lipolysis, hepatic lipogenesis, gluconeogenesis, muscular and bone protein catabolism, lethargy, depression and hyporexia / anorexia are also clinical and laboratorial features of the APR (Ceciliani et al., 2002; Cerón et al, 2005).

Acute Phase Proteins

Acute phase proteins are serum proteins which concentrations are altered by more than 25% in relation to their basal level in response to an inflammatory stimulus of different etiologies (Gabay and Kushner, 1999; Paltrinieri, 2007). Acute phase proteins are described in all animal species (Cray, 2012). It is estimated that the APR induces changes in more than 200 APP (Cray, 2012). The modulation of the protein concentrations is usually achieved by alterations in their synthesis, mainly in the liver, which is the principal organ responsible for the synthesis of APP (Cerón et al, 2005). However, APP can also be produced in other tissues, including the leucocytes, bone marrow, adipose tissue, lungs, kidneys, intestine, spleen, heart, prostate gland, mammary gland, peritoneum and salivary glands among others (Ramadori et al., 1985; Molmenti et al., 1993; Dobryszycka, 1997; Vreugdenhil et al., 1999; Fournier et al., 2000; Lin et al., 2001; Eckersall et al., 2001; Poland et al., 2002; Jabs et al., 2003; Paltrinieri et al., 2012; Soler et al., 2013). Some tumor cells are also capable of producing APP (Abdullah et al., 2009; Moshkovskii, 2012). Synthesis of APP at the local of the initial lesion in addition to the hepatic production of APP contributes to the control of the tissue damage and to the maintenance of the homeostasis (Cerón et al., 2005).

The alteration in the protein synthesis occurs by two different response types, and involves pro-inflammatory cytokines and endogenous glucocorticoids (Peterson et al, 2004; Paltrinieri, 2008). In the type I response, the cytokines IL-1, IL-6 and TNFα act in a synergistic manner: TNFα increases the available amino acids for the hepatic protein synthesis, through mobilization of peripheral amino acids by stimulation of muscular proteolysis; IL-1 modulates hepatic protein synthesis through a stimulatory activity in conjunction with glucocorticoids on the synthesis of positive APP, and an inhibitory effect on the synthesis of negative APP; and the IL-6 facilitates the release of APP in the circulation (Paltrinieri, 2008). In the type II response, IL-6 and IL-6 type cytokines are the principal inducers of APP synthesis, while IL-1 does not act as an inducer of APP release, and can have an inhibitory action in some type II APP (Petersen et al., 2004).

Besides serum, during the APR the APP can also be exuded or eliminated to other body fluids in which their activity can be also assessed, including cavity effusions, urine, cerebrospinal fluid, saliva, synovial fluid, amniotic fluid, interstitial fluids, fluids from bronchoalveolar lavage, milk, endometrial fluid, meat juice, tears, aqueous and vitreous humor, among others (Hu et al., 2006; Kiropoulos et al., 2007; Pyörälä et al., 2011; Bai et al., 2011; Soler et al., 2013; Koss et al., 2014; Brodzki et al., 2015; Arlas et al., 2015; Bundgaard et al., 2016; Hazuchova et al., 2017).

In response to the APR, serum concentrations of some proteins increase, designated positive APP, while of other proteins decrease, classified as negative APP (Kajikawa et al., 1999; Tecles et al., 2009; Heegaard et al., 2011; Méndez et al., 2014). Some authors propose the replacement of the term APP by the term acute phase reactants, which also include non-protein molecules whose concentrations also change during the APR, and other proteins involved in the APR but traditionally considered separately from APP (Topper and Prasse, 1998; Fulop, 2007; Rossi and Paltrinieri, 2009; Topciu Shufta et al., 2010; Garman et al., 2018).

Positive APP includes a large number of proteins, mostly α- and β-globulins (Cerón et al., 2005; Cray et al., 2009). C-reactive protein (CRP), α1-acid glycoprotein (AGP), serum amyloid A (SAA) and haptoglobin (Hp) are considered the principal positive APP, however, many other proteins were reported as positive APP, including fibrinogen (Fb), ceruloplasmin (Cp), complement fractions C3 and C4, lipopolysaccharide binding protein, α1-antitrypsin, pig major APP, hepcidin and ferritin (Duthie et al., 1997; Paltrinieri, 2007; Tecles et al., 2009; Pomorska-Mól et al., 2013; Bohn, 2015; Javard et al., 2017). Each positive APP has specific functions, and most have more than one function, but in general are involved in regulation of the immune and inflammatory responses, transport of different molecules, protection against infection and other insults, and repair and recovery of damaged tissue (Cerón et al., 2005; Paltrinieri, 2008). By definition, the APR develops only during the first days of the disease process, however, prolonged increases in APP occur in chronic diseases (Rikihisa et al., 1994; Tóthová et al., 2010; Korman et al., 2012). Chronic increases in APP can, in some cases, contribute to underlying tissue damage and further complications, such as amyloid protein deposition in amyloidosis and progression of heart disease (Koenig and Rosenson, 2002; Lu et al., 2014; Sato et al., 2014).

Each species has its characteristic positive and negative APP (Cerón et al., 2005; Paltrinieri, 2008, Eckersall and Bell, 2010) (table 1). Moreover, the magnitude of change is variable depending on the protein and species (Cerón et al., 2005; Paltrinieri 2008). Based on the magnitude and behavior of increase, positive APP are classified as minor, moderate or major responders (Cerón et al., 2005; Paltrinieri 2008). In dogs, differences in the magnitude and in the time course of response of positive major, moderate and minor, and of negative APP are well described (Cerón et al., 2005). A major APP, e.g. CRP, is characterized by a low serum concentration in healthy dogs (< 1 μg/L), that rises dramatically by 10 to 100-fold in response to an inflammatory stimulus, reaches a serum peak in 24-48 hours, and then declines rapidly during the recovery phase due to the short half-life of the protein; moderate APP, e.g. Hp, increase 5 to 10-fold, peak at days 2-3 after the injury, and decrease more slowly than major APP, due to their longer half-lives; minor APP gradually increase by 50-100% of its basal concentration (Cerón et al., 2005; Cerón et al, 2008; Eckersall and Bell, 2010).

In cats, the behavior of positive and negative APP during the APR is similar to dogs, however, the magnitude of increase in serum concentrations of positive APP is less marked (Kajikawa et al., 1999; Sasaki et al., 2003, Tamamoto et al., 2009). In this species, positive major APP, e.g. SAA, typically increases 5 to 20-fold in response to an inflammatory stimulus, while moderate APP, e.g. Hp, increase up to 5-fold in comparison to its basal concentrations (Kajikawa et al., 1999; Sasaki et al., 2003). Interestingly, despite the magnitude of increase (in terms of fold-increase) during the APR being higher in positive major than in positive moderate APP, basal serum concentrations of the latter are usually higher than of positive major APP; moreover, the amount of moderate APP synthesized during the APR is usually higher than of major APP (Kajikawa et al., 1999).

The magnitude of increase and the peak of increase were also reported to vary according with the type and intensity of the stimulus (Kajikawa et al., 1999; Sasaki et al., 2003, Tamamoto et al., 2009). In general, the magnitude of change is correlated with the severity of disease, being more significant in severe cases, and therefore with prognosis (Murata et al., 2004; Cerón et al., 2005; Kann et al., 2012). The magnitude of change might also be greater in symptomatic cases than in asymptomatic processes (Cerón et al., 2005).

Table 1.1. Principal positive major and moderate acute phase proteins in different species

Species	Major APP	Moderate APP
Cat	SAA, AGP	Hp, Cp, Fb
Dog	CRP, SAA	Hp, AGP, Cp, Fb
Horse	SAA	Hp, AGP, CRP, Cp,Fb
Cow	SAA, Hp	AGP, Fb, Cp
Pig	CRP, MAP, SAA	Hp, Fb

(Cerón et al, 2005; Cerón et al, 2008; Eckersall and Bell, 2010; Kjelgaard-Hansen and Jacobsen, 2011)

AGP – α1-acid glycoprotein, APP – acute phase proteins, Cp – ceruloplasmin, CRP – C-reactive protein, Fb – fibrinogen, Hp – haptoglobin, MAP – major acute phase protein, SAA – serum amyloid A

Serum amyloid A was reported to increase earlier than AGP and Hp in healthy cats with induced inflammation and in diseased cats submitted to surgery for urinary diversion (Kajikawa et al., 1999). Serum amyloid A concentrations started to increase approximately 3 to 6 hours after spay surgery in healthy cats (Sasaki et al., 2003), significant increases were detected 8 hours after experimentally induced inflammation (Kajikawa et al., 1999), and peak concentrations were detected 24 to 48 hours after induced inflammation and spay surgery in healthy cats, and after surgery for urinary diversion in diseased animals (Kajikawa et al., 1999; Sasaki et al., 2003). Significant increases in serum concentrations of AGP and Hp were detected at 24 hours, and peak serum concentrations at 48 hours after experimental induced inflammation and surgery for urinary diversion (Kajikawa et al., 1999).

Albumin is the most studied negative APP in most species, including the cat (McMillan, 1989; Ottenjann et al., 2006; Kocaturk et al., 2010; Gerou-Ferriani et al., 2011; Sadek et al., 2017); paraoxonase-1 (PON1) (Tvarijonaviciute et al., 2012a; Tecles et al., 2015), adiponectin (Tvarijonaviciute et al., 2011a), insulin-like growth factor-1 (IGF1) (Tvarijonaviciute et al., 2011a), Butyrylcholinesterase (BChE) (Tvarijonaviciute et al., 2012a) transferrin (Caro et al., 2013; Bohn, 2015; Sampaio et al., 2015; Gest et al., 2015), apolipoprotein-A1 (Escribano et al., 2016), apolipoprotein-H (Sellar et al., 1993), retinol binding protein (Tvarijonaviciute et al., 2012b), transthyretin (Piechotta et al., 2012) and leptin (Don et al., 2001) are other negative APP. Their use in veterinary clinical pathology is not frequent, except for albumin (Eckersall and Bell, 2010).

The information on negative APP in the cat is scarce, even for albumin (Paltrinieri, 2008). Although the decrease of albumin has been proven in different feline inflammatory

conditions, it is not known if this decrease is associated with extravasation of albumin from blood vessels to the inflamed tissues or through intestinal losses, or to a decrease in liver synthesis (Ottenjann et al., 2006; Paltrinieri, 2008; Gerou-Ferriani et al., 2011). Besides albumin, PON1 and BChE were also proved to be negative APP in cats (Da Silva et al., 2010a,b; Costa et al., 2010; Vilhena et al., 2017).

Different factors have been suggested to explain the down-regulation of negative APP, including the increase in amino acids available to the synthesis of positive APP associated with the decrease in synthesis of negative APP; the increase in the biologically active free fraction of vitamins, hormones and oligoelements available for tissues, as most of these molecules are transported by negative APP; and also the decrease in proteins with an inhibitory activity, which is considered a pro-inflammatory mechanism (Ceciliani et al., 2002; Paltrinieri, 2008; Cray et al., 2009). In humans, nutritional factors have also been associated with the decrease in concentrations of negative APP; however, currently it is believed that the APR has a stronger effect in negative APP synthesis than nutrition (Ingenbleek and Carpentier, 1985; Schlossmacher et al., 2002; Fuhrman et al., 2004).

The group of APP, both positive and negative, tends to increase continuously with the inclusion of newly discovered molecules implicated in inflammatory processes (Murata et al, 2004), or other molecules involved in non-inflammatory processes that assume a typical APP behavior like antithrombin III, which may act as an APP in the cat (Paltrinieri, 2008).

Major APP are considered the marker of choice for clinical purposes (Paltrinieri, 2008); nonetheless, determination of APP profiles that include at least one positive major, one positive moderate and one negative APP have been recommended over determination of single APP, to better differentiate between pathological states and to provide more information on the evolution of the disease (Cerón et al., 2005; Cerón et al., 2008).

Protein electrophoresis can provide generic information on presence of inflammatory disorders, since most APP will migrate as α- or β-globulins (Gouffaux et al., 1975; Cerón et al., 2005; Paltrinieri, 2008). However, other proteins also migrate to the α- and β-globulins regions, and consequently the information related with the precise protein responsible for the increase is not provided (Paltrinieri 2008). For this reason, individual APP assays are more sensitive than protein electrophoresis (Gouffaux et al., 1975; Cerón et al., 2005).

Serum amyloid A

Serum amyloid A is an acute phase apolipoprotein of the high density lipoprotein plasmatic fraction (Murata et al, 2004). It is a small protein with a molecular weight of 15 kD (Petersen et al., 2004; Cerón et al., 2005). Serum amyloid A is associated with detoxification of endotoxins, has immunomodulatory activities, presents chemotactic properties for inflammatory cells, downregulates the inflammatory processes and is involved in lipid metabolism and transport (Murata et al., 2004; Cerón et al., 2005; Paltrinieri, 2008). Different isoforms of SAA have been described in humans, laboratory animals, pigs, horses, cattle and dogs (Petersen et al., 2004; Gruys et al., 2005; Kjelgaard-Hansen et al., 2007). In the cat, is considered the fastest responsive APP to inflammatory stimuli (Kajikawa et al., 1999).

Alpha 1 acid glycoprotein

Alpha 1 acid glycoprotein, previously known as orosomucoid, is a highly glycosylated protein that belongs to the lipocalin family, a group of extracellular binding proteins specific for hydrophobic molecules and with immunomodulating properties (Fournier et al., 2000; Murata et al., 2004; Paltrinieri, 2008), performing an important role in determining resistance or susceptibility to infectious diseases (Paltrinieri, 2008). Alpha 1-acid glycoprotein inhibits neutrophil activation, stimulate IL-1 receptor antagonist secretion by macrophages, inhibit platelet aggregation and lymphocyte proliferation, modulate the production of anti-inflammatory cytokines by peripheral blood leucocytes and inhibit complement activity (Murata et al., 2004; Cerón et al., 2005; Paltrinieri, 2008).

Haptoglobin

Haptoglobin is an α-globulin constituent which binds plasmatic free hemoglobin (Hb), which is toxic and pro-inflammatory, reducing the oxidative damage associated with hemolysis (Petersen et al., 2004; Murata et al., 2004; Cerón et al., 2005). Haptoglobin has a variety of immunomodulatory effects, including inhibition of mast cell proliferation, prevention of spontaneous maturation of epidermal Langherhans cells, suppression of T-cell proliferation, inhibition of granulocyte chemotaxis, phagocytosis and bacterial activity, and has a bactericidal effect in infected wounds by binding hemoglobin and consequently limiting the availability of iron for bacterial growth (Murata et al, 2004; Cerón et al, 2005).

C-reactive protein

C-reactive protein was the first APP described in humans infected with *Streptococcus* spp. and *Staphylococcus* spp., and with acute rheumatic fever, and was originally named for its ability to bind the C-polysaccharide of *Streptococcus pneumoniae* (Tillett and Francis, 1930). It plays an important role in innate immunity, induction of cytokines, as a binding receptor of immunoglobulins, inhibition of chemotaxis and modulation of neutrophil function, opsonization of bacteria, fungi and parasites, activation of complement and stimulation of phagocytosis (Cerón et al., 2005; Cray 2012; Moutachakkir et al., 2017). It is considered as one of the most important positive APP in humans and several animal species, including dogs (Sierra et al., 2004; Lelubre et al., 2013; Pomorska-Mól et al., 2013; Christensen et al., 2015; Gommeren et al., 2018). It is not considered a reactant of the acute phase response in cats (Barker and Valli, 1998; Kajikawa et al., 1999). However, significant increases in CRP concentrations were described in cats naturally infected with feline immunodeficiency virus (FIV), feline leukemia virus (FeLV) and co-infected with FIV and FeLV after treatment with sub-cutaneous administration of feline interferon-ω (Leal et al., 2014).

Influence of preanalytic and biological factors in determination of feline APP

Several studies investigated the influence of age and gender in physiologic concentrations of APP in human medicine and in laboratory animals, obtaining contradictory results (Yamamoto et al., 1985; Miller et al., 1999; Yamada et al., 2001; Imhof et al., 2003; Rogowski et al., 2004). In companion animals, the influence of age and gender on concentrations of APP was also investigated by different studies. In dogs, no significant differences in concentrations of APP were reported according to gender or age (Yamamoto et al., 1994; Kuribayashi et al., 2003a; Kuribayashi et al., 2003b), except for lower APP concentrations observed in very young puppies (under 3months of age) (Yuki et al., 2010; Abreu et al., 2018). In cats, contradictory results concerning influence of age and gender in APP concentrations were described. Higher concentrations of SAA, but not of AGP, Hp or albumin, were observed in older animals in the study of Kann et al. (2012). In this study, the increase in SAA activity was attributed to the higher incidence of subclinical diseases in geriatric cats. On the other hand, no relation was detected between SAA or Hp and age in the study of Campbell et al. (2004), nor in Cp activity in healthy cats in the study of Fascetti et al. (2002). Similarly, some authors reported gender-related differences in concentrations of SAA,

AGP, Hp and Cp (Fascetti et al., 2002; Kann et al., 2012), while no significant differences in concentrations of SAA, AGP and Hp were detected in the study of Kajikawa et al. (1999).

To the authors´ knowledge, the information concerning the influence of other preanalytic and biological factors, besides age and gender, in determination of feline APP is lacking. Different studies have shown that other preanalytic factors, including storage, anticoagulants, hemolysis, lipemia and bilirrubinemia; and also biological parameters such as breed, pregnancy, living conditions, environment and different treatments (Tecles et al., 2002; Kuribayashi et al., 2003a,b;Martínez-Subiela et al., 2004; Cerón et al., 2005; Martínez-Subiela and Cerón, 2005) influence concentrations of APP in dogs . The information obtained in those canine studies is usually extrapolated to interpretation of APP results in feline medicine. However, future studies should be performed in feline samples in order to obtain precise information for cats.

Clinical Applications of Acute Phase Proteins in Feline Medicine

Acute phase proteins (APP) are being increasingly used in human and veterinary medicine in general health screening, and in diagnosis, prognosis, monitoring the evolution and response to treatment of different diseases (Cray et al., 2009; Eckersall and Bell, 2010; Kjelgaard-Hansen and Jacobsen, 2011; Ceciliani et al., 2012; Eckersall and Schmidt, 2014; Schrödl et al., 2016).

Several studies in feline medicine evaluated APP in diseased cats, including animals with different diseases, usually comparing diseased animals (considered as a group that included animals with different diseases) with healthy controls (Kajikawa et al., 1999; Sasaki et al., 2003; Hansen et al., 2006; Tamamoto et al., 2008; Kann et al., 2012). Some of these studies also evaluated the changes in concentrations of serum APP in "groups" of diseases, i.e. inflammatory, infectious, endocrine and neoplasia among others; and also in individual cases, i.e. renal failure, pancreatitis, diabetes *mellitus*, hyperthyroidism and FLUTD, among others, but usually including a small number of animals affected by each individual disease (Kajikawa et al., 1999; Sasaki et al., 2003; Hansen et al., 2006; Tamamoto et al., 2008; Kann et al., 2012). In these studies, APP were reported to increase significantly in different feline diseases, including infectious diseases, inflammatory diseases, feline lower urinary tract disease, chronic kidney disease, injury, lymphoma, mesothelioma, hyperthyroidism and diabetes *mellitus* among others (Sasaki et al., 2003; Hansen et al., 2006; Tamamoto et al., 2008). However, no significant increases were detected in cats with liver diseases, enteritis, oral disorders (Sasaki et al., 2003), bronchopneumonia, gastroenteritis, rhinitis (Tamamoto et al., 2008), neoplasia (although only four animals were evaluated, two of which presented adenomas) and endocrinopathies (Hansen et al., 2006). The lack of increase of APP in these diseases was suggested to be associated with development of a focal inflammation rather than a systemic process, and with a decrease in hepatic synthesis of APP in the cases of cats with liver diseases (Sasaki et al., 2003; Tamamoto et al., 2008). These results showed that APP are useful clinical biomarkers in feline medicine, but not in all feline diseases, and highlight the

necessity of evaluation of the APP response in individual diseases to determine the clinical value of individual APP or of APP patterns in diagnosis, monitoring the evolution of disease and response to treatment, and in prognosis of different feline diseases.

Acute phase proteins in feline infectious diseases

The infectious and inflammatory diseases are reported to be the feline diseases in which greater changes in APP were observed, however, similar changes were also reported in individual cases of cats with other diseases, such as disseminated neoplasias (Duthie et al., 1997; Hansen et al., 2006; Tamamoto et al., 2008; Hazuchova et al., 2017).

The diagnosis of FIP is difficult, and the definitive diagnosis is usually established by histopathology of affected tissues, detection of viral antigens by immunochemistry and / or identification of viral RNA by PCR-based methods in histopathology or effusion samples (Pederson, 2014; Tasker, 2018). However, in the clinical practice, when tissue and effusion samples or immunostaining and PCR methods are not available, a high index of suspicion of FIP may be obtained based on the animal´s signalment, clinical history, clinical signs and laboratory tests, including determination of serum and effusion concentrations of APP (Duthie et al., 1997; Bence et al., 2005; Giori et al., 2011; Stranieri et al., 2018).

In fact, the exposure to, or infection with feline coronavirus (FCoV), and the disease feline infectious peritonitis (FIP) are the feline conditions in which the clinical value of APP has been studied more extensively (Gouffaux et al., 1975; Duthie et al., 1997; Hazuchova et al., 2017). Several studies evaluated the APP response, particularly AGP, in asymptomatic cats naturally and experimentally exposed or infected with feline coronavirus (FCoV), and in cats naturally affected or with experimentally induced feline infectious peritonitis (FIP) (Gouffaux et al., 1975; Stoddart et al., 1988; Giordano et al., 2004; Paltrinieri et al., 2007a). Feline APP, particularly AGP, determined in serum, plasma or effusion samples were proved to be clinically useful ancillary tests in diagnosis of FIP (Duthie et al., 1997; Paltrinieri et al., 2007b; Giori et al., 2011; Hazuchova et al., 2017).

Increases in serum AGP, SAA, Hp, Fb and transferrin, and decreases in albumin were observed in cats with natural disease and also in diseased cats with experimental infection (Gouffaux et al., 1975; Stoddart et al., 1988; Duthie et al., 1997; Giordano et al., 2004; Paltrinieri et al., 2007b; Rossi and Paltrinieri, 2009; Giori et al., 2011; Hazuchova et al., 2017). Increases in AGP, SAA and Hp, and decreases in albumin were also detected in effusion samples of cats with natural and experimentally-induced disease (Gouffaux et al., 1975; Duthie et al., 1997; Hazuchova et al., 2017). A tendency for higher serum and peritoneal fluid concentrations of AGP was reported in effusive than in noneffusive FIP by Bence et al., (2005), however, no significant differences in serum AGP, SAA and Hp were detected between cats with the wet and the dry forms of FIP in the study of Giordano et al., (2004). Moreover, serum concentrations of AGP, SAA, Hp and total sialic acid (TSA, considered an acute phase reactant) were significantly higher in FIP diseased cats than in FCoV exposed or infected animals, and no significant differences were observed between FCoV exposed cats and healthy control animals (Giordano et al., 2004; Paltrinieri et al., 2007b; Rossi and Paltrinieri, 2009). Nonetheless, transient increases in serum AGP, SAA and Hp were described in asymptomatic FCoV exposed and infected cats, with greater changes occurring in catteries with high prevalence of FCoV infection than in animals from groups with a low prevalence (Giordano et al., 2004; Paltrinieri et al., 2007a; Paltrinieri et al., 2014). These changes in APP were detected before and after appearance of clinical cases of FIP in the catteries, showing the development of an APR even in FCoV exposed and infected cats that did not develop the disease (Giordano et al., 2004; Paltrinieri et al., 2007a). Moreover, increases in serum concentrations of AGP were detected in individual cats exposed to / or infected with FCoV from catteries with high and very high prevalence for FCoV, in the absence of changes in other inflammatory markers, including albumin, α2 and γ globulins, albumin-to-globulins ratio, IL4, IL12 and IFNγ, showing that AGP increase earlier than other markers of inflammation (Paltrinieri et al., 2014). This inflammatory response in asymptomatic FCoV exposed and infected cats was suspected to be associated with viral spread among the cats of the cattery, and suggested to be a consequence of an increase in the viral load of non-mutated viral strains, or a protective response against the infection with mutated FCoV strains (Giordano et al., 2004; Paltrinieri et al., 2007a). Furthermore, persistent increases in SAA and AGP (but not of Hp) were observed in one FCoV exposed cat that posteriorly developed FIP, showing an APP response before manifestation of clinical signs (Giordano et al., 2004). Also increases in α2 globulins in FCoV exposed cats, and decreases

in cats that developed FIP were described, suggesting that other negative APP or changes in electrophoretic mobility of proteins could be implicated in pathogenesis of FIP (Giordano et al., 2004).

As previously stated, AGP is considered the most clinically useful APP in cats with FIP (Duthie et al., 1997; Paltrinieri et al., 2007b; Giori et al., 2011; Hazuchova et al., 2017). Serum concentrations of AGP were reported to achieve higher concentrations in FIP than in other feline diseases according with some studies, including diseases with similar clinical presentations to FIP, namely conditions that cause effusions; however, similar increases in AGP in serum and effusion samples were also reported in cats with other pathologic conditions, including animals with FIV-associated disease, septic processes and disseminated neoplasias (Duthie et al., 1997; Paltrinieri et al., 2007b; Rossi and Paltrinieri, 2009; Giori et al., 2011; Hazuchova et al., 2017). This overlap in concentrations of AGP, and also of SAA and Hp, between cats with FIP and cats with septic processes and disseminated neoplasias, was lower in effusion samples than in serum samples (Hazuchova et al., 2017). Because of this overlap in concentrations of AGP observed between cats with FIP and cats with other diseases, it cannot be used as a single test for FIP; however, according with different studies, elevated concentrations of AGP in serum, plasma or effusion samples are a reliable aid in the diagnosis of FIP when the clinicopathological findings are suggestive of the disease (Duthie et al., 1997; Paltrinieri et al., 2007b; Giori et al., 2011; Hazuchova et al., 2017). Moreover, serum, plasma or effusion concentrations of AGP > 1.5 g/l showed higher sensitivity, specificity, overall efficacy, and concordance with immunohistochemistry in differentiating FIP from other diseases with similar clinical presentations, namely diseases that cause cavitary effusions, than other parameters, including clinical history, clinical signs, albumin:globulin ratio, serum and effusion concentrations of SAA and Hp, analysis of effusion, serum protein electrophoresis, serology or histopathology (Duthie et al., 1997; Giori et al., 1997; Hazuchova et al., 2017) (table 1.2). Furthermore, AGP (and also SAA and Hp) in effusion showed higher sensitivity and specificity than AGP in serum to differentiate FIP from other diseases with similar clinical signs (Hazuchova et al., 2017) (table 1.2).

Table 1.2. Performance of different diagnostic tests in differentiating feline infectious peritonitis (FIP) from other diseases with similar clinical presentation

Study	Sample	Parameter	*Cut-off*	Sensitivity %	Specificity %	Overall efficacy	AUC	k
Duthie *et al*., 1997[#]	S, P, E	AGP	> 1.5 g/l	85	100	90	-	-
	S, P, E	Hp	> 4.0 g/l	40	86	54	-	-
	S, P, E	A:G ratio	< 0.7	87	85	87	-	-
Giori *et al*., 2011[†]	S	AGP	> 1.5 mg/ml	100	100	-	-	1.0
	-	Hx, Sx	-	62.5	0	-	-	-0.4
	E	An. effusion	-	50	0	-	-	-0.52
	S	SPE	-	37.5	50	-	-	0.25
	-	Serology	-	nd	nd	-	-	-0.08
	-	Histopat.	-	37.5	100	-	-	0.09
Hazuchova *et al*., 2017[‡]	S	AGP	2260 µg/ml	85	90	-	0.899	-
		SAA	97.3 µg/ml	55	87	-	0.800	-
		Hp	2.0 mg/ml	55	82	-	0.777	-
	E	AGP	1550 µg/ml	93	93	-	0.950	-
		SAA	43.6 µg/ml	71	91	-	0.885	-
		Hp	2.1 mg/ml	79	87	-	0.870	-

A – albumin; AGP – α1 acid glycoprotein; An. effusion – analysis of effusion; AUC – area under the curve; E – effusion; G – globulins; Histopat – histopathology; Hp – haptoglobin; Hx – clinical history; k – Cohen´s k coefficient (used to determine the concordance between different parameters and immunohistochemistry); P – plasma, S – serum; SAA – serum amyloid A; SPE – serum protein electrophoresis; Sx – clinical signs

- histopathology used as the definitive diagnostic method, n= 48 cats with FIP, n= 21 cats with other diseases presenting clinical signs consistent with FIP; † - immunohistochemistry used as the definitive diagnostic method, n=12 cats with FIP, n=4 cats with other diseases presenting clinical signs consistent with FIP; ‡ - immunohistochemistry or a sophisticated statistical method using machine learning methodology with concepts from game theory, n= 20 cats with FIP, n=68 cats with other diseases presenting clinical signs consistent with FIP.

Another study evaluated the clinical value of serum concentrations of AGP in the diagnosis of FIP, based in the pre-test probability of FIP determined on history and clinical signs, according with the Bayesian approach (Paltrinieri et al., 2007b). According with the results obtained, when the pre-test probability of FIP is high, moderate increases in serum AGP (1.5 – 2.0 mg/ml) showed as an adequate biomarker to discriminate cats with FIP from cats with other diseases, while higher increases in serum AGP (>3.0 mg/ml) were consistent with FIP in

cats with a low pre-test probability of the disease (Paltrinieri et al., 2007b). Furthermore, the likelihood ratio of detecting a serum concentration of AGP of 3 mg/ml and of 4 mg/ml was approximately 3.5 and 8.0 times higher (respectively) in a cat with FIP than in a cat with other disease (Paltrinieri et al., 2007b). The discriminating power of TSA was also evaluated in cats with FIP (Rossi and Paltrinieri, 2009). Serum TSA showed a moderate discriminating power for FIP (area under the ROC curve of 0.65), and a likelihood ration higher than 3.0 only for high (> 800 mg/l) TSA concentrations (Rossi and Paltrinieri, 2009).

The APR has also been evaluated in other feline infectious diseases. Acute phase proteins were also shown to be clinically useful in cats infected with retrovirus. Increases in serum concentrations of SAA, AGP, CRP and Hp were described in diseased cats with feline immunodeficiency virus (FIV) associated illness (natural and experimental infection) (Duthie et al., 1997; Gil et al., 2014; Taffin et al., 2015). Moreover, the increases observed in AGP and Hp in FIV diseased cats were of similar magnitude to those observed in FIP diseased cats (Duthie et al., 1997). Increases in concentrations of AGP, but not of SAA or CRP, were also described in diseased cats naturally infected with feline leukemia virus (FeLV) (Leal et al., 2014). However, no significant increases in concentrations of SAA, AGP or CRP were observed in symptomatic cats naturally co-infected with FIV and FeLV (Leal et al., 2014).

In FIV diseased cats (natural infection), the plasma viral RNA load was proved to influence the concentrations of SAA, but not of AGP, Hp or albumin; however, no association between concentrations of APP and viral load were detected in FIV infected asymptomatic animals (Kann et al., 2014). The APP were proved to have prognostic value in individuals infected with human immunodeficiency virus (HIV), being associated with HIV RNA load and disease progression (Feldman et al., 2003a,b; Lau et al., 2006; Mehta et al., 2006; Xu et al., 2018). However, in cats, APP, including SAA, AGP, Hp and albumin, showed no prognostic value during the asymptomatic or symptomatic phase of infection with FIV (Duthie et al., 1997; Kann et al., 2014).

Changes in concentrations of APP during treatment of diseased cats naturally infected with FIV and / or FeLV were also reported. While decreases in concentrations of SAA were detected in FIV infected cats after treatment with the antiretroviral compound (R)-9-(2-phosphonylmethoxypropyl)-2,6-diaminopurine (Taffin et al., 2015); increases in concentrations of SAA, AGP and CRP were described in diseased cats naturally infected with

FIV, FeLV and co-infected with FIV and FeLV after treatment with feline interferon-ω subcutaneous licensed protocol (Leal et al., 2014; Gil et al., 2014); and no changes in concentrations of SAA, AGP and CRP were observed in diseased cats naturally infected with FIV after treatment with a protocol of oral administration of feline interferon-ω (Gil et al., 2014).

Alpha-1-acid glycoprotein is a heavily glycosylated protein, with a carbohydrate content of approximately 45% of its molecular weight, which plays an important role in the immunomodulating functions of AGP (Fournier et al., 2000). Different glycoforms of AGP were described in human serum (Van Dick et al., 1995), and the glycan heterogeneity of AGP was proved to change with different physiological and pathological conditions, including different inflammatory conditions in humans, laboratory animals and cats (Venembre et al., 1993; Van Dick et al., 1995; Jørgensen et al., 1998; Kishino et al., 2002; Ceciliani et al., 2004). It was suggested that the biological activity of AGP in pathologic conditions could be exerted by the increase in its serum concentration and also by the modifications in its glycan content; and that the glycosylation patterns of AGP in the different diseases could be clinically useful as specific biomarkers (Ceciliani et al., 2004; Pocacqua et al., 2005). In healthy cats, AGP was proved to lack L-fucose residues on its surface, to present a low carbohydrate branching degree, and to contain an elevated level of sialic acid, particularly linked to galactose in position α(2–6) (Ceciliani et al., 2004; Pacacqua et al., 2005). The glycan branching degree and the fucosylation level did not change in FCoV exposed cats, in FIP diseased animals, in FIV asymptomatic or symptomatic cats, in FeLV asymptomatic cats or in cats with FeLV associated lymphoma (Ceciliani et al., 2004; Pacacqua et al., 2005). Also no significant differences in the degree of sialylation of AGP were detected in FCoV exposed cats, however, the expression of sialic acid, in both α(2–3) and α(2–6) galactose linked positions, decreased significantly in FIP diseased cats (Ceciliani et al., 2004). A simultaneous decrease in fecal shedding of FCoV and an increase in AGP´s α(2–3) linked sialic acid were also reported in FCoV asymptomatic infected cats approximately one month after occurrence of FIP in cats from the same cattery (Paltrinieri et al., 2008). Moreover, AGP was detected by flow cytometry in circulating leucocytes of healthy and diseased cats, and the proportion of animals with positive monocytes and granulocytes was significantly higher in cats with inflammatory conditions than in control animals, but no significant increases were detected in FIP diseased animals or in FCoV seropositive cats (Paltrinieri et al., 2012). Furthermore, the

concentrations of serum TSA were negatively correlated with FCoV antibody titer and with the degree of AGP sialylation (Rossi and Paltrinieri, 2009). These facts suggest that hypersialylation of AGP and increases in TSA concentrations may be involved in host-FCoV interaction, protecting infected cats from development of FIP. On the other hand, no correlations were detected between AGP and TSA serum concentrations in cats with FIP, suggesting that other sialylated proteins besides AGP might be involved (Rossi and Paltrinieri, 2009).

In FeLV infected cats, a decrease in sialic acid was detected in asymptomatic animals, while an increase in both α(2–3) and α(2–6) galactose linked positions was detected in cats with lymphoma (Pocacqua et al., 2005). The increase in the degree of AGP sialylation in cats with lymphoma was however suggested to be potentially associated with the neoplasia rather than the FeLV infection, as described in human patients with cancer, since the over-sialylation renders malignant cells a "self" status, thus acting as a defense mechanism preventing the activation of the immune system (Moule et al., 1987; Pocacqua et al., 2005; Zhang et al., 2017). Changes in sialylation pattern of AGP were also described in FIV infected cats, however the changes varied widely among individuals (Pocacqua et al., 2005). Increases in α(2-3) linked position were detected in FIV asymptomatic cats, while no significant changes in the acid sialic content were detected in cats with advanced disease (Pocacqua et al., 2005).

Increases in serum AGP were described in cats with experimental ocular and intravenous infection with *Chlamydia psitacci* (TerWee et al., 1998). However, the increases in serum AGP were only detected in cats that developed ocular or systemic clinical signs, and none of the infected cats that remained asymptomatic presented increases in serum AGP during the period of the study (41 days after experimental infection) (TerWee et al., 1998). Furthermore, no significant changes in serum concentrations of AGP and SAA were detected in cats with natural *Chlamydiae* subclinical infection (Ström Holst et al., 2011). However, despite median SAA concentrations were in the reference range, in cats with high anti-*Chlamydiae* antibody titers, concentrations of SAA were significantly higher in animals that were actively shedding *Chlamydiae* than in the non-shedder cats (Ström Holst et al., 2011).

A significant increase in concentrations of SAA was also described in cats experimentally infected with *Mycoplasma haemofelis* (Mhf) and in cats experimentally infected with *Candidatus Mycoplasma haemominutum* (CMhm) (Korman et al., 2012). Moreover, infection

with Mhf was associated with significantly higher increases in SAA than infection with CMhm, which was attributed to the higher pathogenecity of Mhf over CMhm (Korman et al., 2012). Significant increases in AGP were also observed in cats infected with Mhf, but not in those infected with CMhm, which was also suggested to be related to the higher pathogenecity of Mhf when compared with CMhm (Korman et al., 2012). Increases in serum Hp were also observed in Mhf and CMhm infected cats, however the changes observed during the study period were not significant (Korman et al., 2012). The APP, particularly SAA, were also shown to be clinically useful in monitoring response to treatment and in differentiation of an acute / clinical from a chronic / subclinical state in feline experimental infection with hemoplasmas (Korman et al., 2012).

Furthermore, the co-infection with hemoplasmas and feline immunodeficiency virus (FIV) was reported to influence the APP response in Mhf and CMhm experimentally-infected cats; Mhf and FIV co-infected cats presented significantly higher concentrations of serum Hp than Mhf infected cats; and cats co-infected with CMhm and FIV presented lower concentrations of AGP than cats infected with CMhm (Korman et al., 2012).

An APP response was also described in cats seropositive to *Dirofilaria immitis* and its endosymbiont bacterium *Wolbachia pipientis*, with significant increases in serum concentrations of SAA, Hp and Cp detected in seropositive cats that presented clinical signs compatible with dirofilariosis (Silvestre-Ferreira et al., 2017). Moreover, significant increases in serum Hp were also detected in asymptomatic seropositive animals (Silvestre-Ferreira et al., 2017). Nonetheless, no significant correlations were detected between serum concentration of APP and *D. immitis* or *Wolbachia* antibody titers (Silvestre-Ferreira et al., 2017).

Increases in α1, α2, β and γ globulins, and decreases in serum albumin and in plasmatic butyrylcholinesterase concentrations were also described in symptomatic cats experimentally infected with *Trypanosoma evansi* (Da Silva et al., 2010a,b; Costa et al., 2010). Decreases in plasmatic butyrylcholinesterase (BChE) and in blood and brain tissue acetylcholinesterase were suggested to be a possible cause for the neurological signs, such as instability and incoordination of the hind limbs, presented by some of the infected animals (Da Silva et al., 2010a).

Significant increases in concentrations of SAA and Hp, and decreases in PON1 were described in *Hepatozoon felis* infected cats with clinical signs compatible with feline hepatozoonosis, and significant increases in serum Hp and decreases in PON1 were also detected in asymptomatic cats infected with *H. felis* (Vilhena et al., 2017). Significant increases in Hp and decreases in PON1 were also reported in symptomatic and asymptomatic cats infected with *Babesia vogeli* (Vilhena et al., 2017). A tendency to increase was also observed in SAA concentrations of symptomatic cats infected with *B. vogeli*, but not in asymptomatic infected animals (Vilhena et al., 2017).

Serum amyloid A was also described as a clinically useful biomarker in diagnosis of the feline systemic inflammatory response syndrome (SIRS) due to sepsis and trauma (Troìa et al., 2017). Cats with sepsis presented concentrations of SAA significantly higher than cats with trauma; however, using a cut-off value > 81 mg/L to diagnose feline sepsis, only a moderate value (AUC = 0.76) was observed (Troìa et al., 2017). Serum concentrations of SAA at admission showed no prognostic value in cats with SIRS; however, the association with other biomarkers, including serum bilirubin and evaluation of neutrophil toxic changes was suggested to improve the potential of SAA in the diagnosis and prognosis of feline sepsis (Troìa et al., 2017).

Acute phase proteins in feline inflammatory and metabolic diseases

As in humans and dogs, an APP response was also reported in cats with chronic kidney disease (CKD) (Sasaki et al., 2003; Carrero and Stenvinkel, 2010; Raila et al., 2011; Tamamoto et al., 2013; Javard et al., 2017). Significant increases in SAA and hepcidin, and significant decreases in total iron binding capacity (TIBC) were detected in cats with CKD, while no significant differences in concentrations of Hp or ferritin were detected between diseased and healthy control cats (Sasaki et al., 2003; Gest et al., 2015; Javard et al., 2017). Moreover, concentrations of SAA and hepcidin showed a significant positive correlation with serum creatinine, while serum TIBC presented a significant negative correlation with creatinine (Javard et al., 2017). These associations suggest that advanced stages of CKD may be associated with more severe inflammation, however, no significant differences in concentrations of SAA, Hp or hepcidin were detected between cats in IRIS CKD stages 2, 3 or 4, and also no significant associations were detected between concentrations of APP at

diagnosis and survival time of cats with CKD (Javard et al., 2017). The group of diseased cats with anemia had significantly higher concentrations of SAA and lower TIBC than the cats without anemia, and concentrations of SAA and hepcidin were significantly associated with lower hematocrit, while TIBC was significantly positively correlated with hematocrit, suggesting that inflammation may contribute to the development of anemia in cats with CKD, probably related with iron sequestration (Javard et al., 2017). In this study, two cats were presented due to episodes of acute decompensation of CKD, and presented among the most elevated concentrations of APP (SAA, Hp and hepcidin) of all cats included in the study, suggesting that APP could also be biomarkers of the feline acute kidney injury (AKI) (Javard et al., 2017).

Neutrophil gelatinase-associated lipocalin (NGAL) is a glycoprotein expressed during inflammatory responses and epithelial damage, including release from the renal tubular cells after an insult (Wasung et al., 2015; Lindberg et al., 2016). Serum and urinary NGAL are biomarkers of early renal damage and progression of CKD in humans and dogs (Hsu et al., 2014; Steinbach et al., 2014; Wasung et al., 2015; Patel et al., 2016). Urinary NGAL and urinary NGAL-to-creatinine ratio were also proved to be clinically useful biomarkers of feline CKD (Wang et al., 2017). Significantly higher concentrations of urinary NGAL and higher urinary NGAL-to-creatinine ratios were detected in cats with CKD IRIS stages 3 and 4 than in cats with CKD IRIS stage 2, and also than controls; while no significant differences in plasma NGAL concentrations were detected between diseased cats and controls, or between diseased cats in different IRIS stages of CKD (Wang et al., 2017). Significant positive correlations were also described between urinary NGAL and urinary NGAL-to-creatinine ratio and serum concentrations of creatinine (Wang et al., 2017). Moreover, higher concentrations of urinary NGAL and higher urinary NGAL-to-creatinine ratios were associated to a shorter survival time in cats with CKD (Wang et al., 2017).

Acute phase proteins were proved to be clinically useful biomarkers of diagnosis and prognosis of human and canine pancreatitis (Rau et al., 2000; Mansfield et al., 2008; Sato et al., 2017). The information concerning the APP response in feline pancreatitis is scarce. Marked increases in concentrations of SAA and increases in AGP were reported in individual cats (Hansen et al., 2006; Tamamoto et al., 2009). Additionally, concentrations of SAA showed to be a useful biomarker in diagnosis and in monitoring the evolution of the disease and response to treatment in one clinical case of a cat with pancreatitis followed during a

period of 831 days after diagnosis (Tamamoto et al., 2009). In the same case report, concentrations of SAA showed a superior clinical value in monitoring the evolution of the disease and response to treatment than the changes in white blood cell (WBC) count and concentrations of feline trypsin-like immunoreactivity (Tamamoto et al., 2009). Discrepancies between SAA concentrations and WBC and band neutrophil counts were also reported in cats with different diseases, suggesting that WBC and neutrophil counts are insufficient for detection of inflammation, and that other markers of inflammation, such as APP, should also be used (Tamamoto et al., 2009). Furthermore, significantly lower concentrations of serum albumin were reported in cats with inflammatory bowel disease (IBD) and increased concentrations of feline pancreatic lipase immunoreactivity (fPLI), suggestive of concomitant pancreatitis, than in cats with IBD and serum fPLI in the reference range (Bailey et al., 2010).

Serum concentrations of Hp were significantly higher in cats affected with plasmacytic gingivostomatitis than in healthy control animals (Polkowska et al., 2018). Moreover, serum concentrations of Hp proved to be a clinically useful parameter to evaluate the response to treatment, and also as a useful prognostic factor for determination of the duration of the appropriate treatment (Polkowska et al., 2018).

Concentrations of acute phase reactants were also assessed in a study that aimed to evaluate changes in haemostatic analytes in cats with naturally occurring liver diseases, including hepatic lipidosis and inflammatory, degenerative and neoplastic diseases (Dircks et al., 2012). Despite the probable consumption due to activation of coagulation and fibrinolysis, significant increases in some analytes were detected in cats with liver diseases, including fibrinogen, α2 plasmin inhibitor and coagulation factor V (Dircks et al., 2012). According with these results, fibrinogen and α2 plasmin inhibitor presented an APP behavior in cats with liver diseases, as described in other conditions in humans, dogs and horses (Schultz and Arnold, 1990; Ueyama et al., 1992; Topper and Prasse, 1998; Mischke, 2005; Caldin et al., 2009; Kum et al., 2013; Dircks et al., 2012). Moreover, coagulation factor V was also suggested to act as an APP in cats since significant increases in coagulation factor V paralleled those of fibrinogen, and were also detected in cats with hepatic lipidosis and inflammatory liver diseases (Dircks et al., 2012). Furthermore, protein C activity was significantly decreased in cats with hepatic lipidosis and inflammatory, neoplastic and degenerative diseases, suggesting that this analyte could be considered a negative APP in cats,

as occurs in humans and dogs (Dircks et al., 2012; Christiaans et al., 2013; Fletcher et al., 2016).

Increases in serum concentrations of AGP (in 14 out of 14 cats tested), Hp (in 13 out of 14 cats) and ferritin (in 12out of 19 cats), and decreases in albumin (in 18 out of 21 cats) and TIBC (in 6 out of 19 cats) were described in cats with abscesses, pyothorax and fat necrosis (Ottenjann et al., 2006). In the same study, no significant correlations were detected between serum APP and hematocrit values, red blood cell counts, hemoglobin concentration or red blood cells indices, with exception of an inverse correlation between concentrations of Hp and the number of aggregated reticulocytes (Ottenjann et al., 2006).

Acute phase proteins in feline obesity

Obesity is a common and increasing disorder in human and in companion animal medicine (Wang and Lim, 2012; Montoya-Alonso et al., 2017; Chandler et al., 2017). Epidemiological studies reported that 11.5% to 39% of cats presented overweight or obesity (Lund et al., 2005; Courcie et al., 2010; Cave et al., 2012; Courcier et al., 2012; Corbee, 2014). Overweight and obesity were associated with development of different diseases in humans and companion animals (Kopelman, 2000; German, 2006; Zoran, 2010; Bischoff et al., 2017; Chandler et al., 2017). In cats, overweight and obesity have been associated with reduced sensitivity to glucose and diabetes *mellitus*, hepatic lipidosis, dermatologic disorders, urolithiasis, arthritis and neoplasia among other diseases (German, 2006; German et al., 2010; Cave et al., 2012; Loftus and Wakshlag, 2014).

In the past, adipose tissue was considered an inert local of lipid accumulation, however, currently, adipose tissue is considered an active endocrine organ with recent studies proving evidence that adipose tissue and other cells in the adipose tissue microenvironment synthesize a series of metabolic active substances, generally designated adipokines, that include inflammatory mediators, cytokines, steroid hormones, growth factors, proteins and regulators of metabolism (Antuna-Puente et al., 2008; Radin et al., 2009; German et al., 2010). Due to the increased synthesis of pro-inflammatory mediators by the adipocytes and by other cells that invade the adipose tissue, including monocytes and macrophages, obesity originates a state of chronic low grade systemic inflammation, which contributes to development of obesity-related diseases (Aguilar-Valles et al., 2015; Bastien et al., 2015; Hamper, 2016).

In humans, different studies described increased activity of inflammatory markers, including IL6, TNFα and different APP in overweighted and obese individuals, and also a trend of these analytes to return to physiologic values after weight loss (Hanusch-Enserer et al., 2003; Manco et al., 2007; Vallianou et al., 2010; Siervo et al., 2012; Sobieska et al., 2013). Similar results were also observed in overweighted and obese dogs (German et al., 2009; Wakshlag et al., 2011; Vitger et al., 2017; Barić Rafaj et al., 2017). However, the APP response was typically mild, and APP concentrations remained in the reference range, as observed in other studies in which no significant increases in APP were detected in overweighted or obese dogs, nor significant changes in APP activity were observed in animals after weight loss or weight gain programs (Tvarijonaviciute et al., 2011b; Tvarijonaviciute et al., 2012b,c,d; Adolphe et al., 2014; Hillström et al., 2015). Information regarding the APP response in overweight and obese cats is scarce. Although feline adipocytes were reported to produce APP *in vitro,* no significant changes in SAA or Hp serum concentrations were observed in obese cats, and no significant changes occurred in SAA, Hp, BChE or PON1 activity after weight loss (Tvarijonaviciute et al.,2012e). Furthermore, although no significant differences in serum IGF1 were detected between obese and lean cats (Tvarijonaviciute et al., 2012f), its concentrations increased significantly in obese cats after weight loss (Tvarijonaviciute et al., 2012f). Moreover, serum AGP was reported to be higher in cats with experimentally induced hyperlipidemia than in healthy controls (Zini et al., 2010). Further studies, evaluating these and other APP, and with use of more sensitive assays for APP determinations are needed to clarify the biological role of overweight and obesity in systemic inflammation of cats.

Acute phase proteins in feline surgery

Significant increases in SAA, AGP, Hp, Cp and Fb were reported in male and female cats submitted to spay surgery and surgery for urinary diversion (Kajikawa et al., 1999; Sasaki et al., 2003; Tamamoto et al., 2008; Moldal et al., 2012; Conceição et al., 2018). These studies evaluated the dynamics of the APP response during the post-operative period, showing that APP could be clinically useful biomarkers to monitor the post-operative period.

In the studies reported by Kajikawa et al. (1999) and Tamamoto et al. (2008), feline SAA showed to be a faster reactant than AGP and Hp. In these studies, concentrations of SAA reached a peak at 21 to 48 hours after surgery, and decrease thereafter, achieving basal concentrations between the days eight to 13 post-surgery; while concentrations of feline AGP and Hp increased later, with significant increases in comparison with basal levels detected at 24 hours after the inflammatory stimulus, peak concentrations at 48 to 72 hours, and the decline in serum concentrations also occurred later than SAA. Significantly higher concentrations of SAA and of Fb were also observed in male cats 24 hours after orquiectomy in comparison with pre-surgery values in the study of Moldal et al. (2012). In the study of Conceição et al. (2018), increases in serum Cp were observed one hour after surgery in queens submitted to ovariectomy, significant increases were observed 24 hours after surgery in comparison with basal values, peak concentrations occurred 48 hours after surgery, and values similar to basal concentrations were observed at day 10 post-surgery; significant increases and peak concentrations of serum Hp were observed at 72 hours post-surgery; while no significant differences in concentrations of transferrin were observed during the post-operative period. Interestingly, and in contrast to previous research, in this study significant increases in comparison with baseline values and peak concentrations in AGP were only observed in the day 10 after surgery.

In cats, as in other species, APP have also been used to compare the inflammatory response between surgical techniques (Grande et al., 2002; Freeman et al., 2010; Martínek et al., 2012; Haraguchi et al., 2017). No significant differences in concentrations of SAA or Fb were detected during the 24 hours after surgery in male cats submitted to orquiectomy under general anesthesia with or without local anesthesia (Moldal et al., 2012). During the post-operative period, serum concentrations of AGP and Hp were significantly higher in queens submitted to ovariectomy through miniceliotomy technique than in queens neutered by laparoscopy, serum transferrin was significantly higher in female cats spayed by miniceliotomy, while no significant differences in Cp activity were observed between the two techniques (Conceição et al., 2018).

Acute phase proteins in feline oncology

An association between chronic inflammation and cancer was proved in several tumors in humans and animals (Hanahan and Weinberg, 2011; Raposo et al., 2015; Carvalho et al., 2016). Therefore, as a clinical hallmark of the systemic inflammatory reaction, APP have been used as biomarkers of different tumors in human and veterinary medicine (Dowling et al., 2012; Chase et al., 2012; Tamamoto et al., 2013). Furthermore, an active role in carcinogenesis has been attributed to APP, including a modulation of the immune response of the organism to the presence of the tumor, with both positive and negative contributions to the host defense system being described (Samak et al., 1982; Stamatiadis et al., 1990; Dempsey and Rudd, 2012; Conrad et al., 2016), and also a promotion of the neoplastic cells invasive capacity and development of metastasis (Tamamoto et al., 2014; Tamamoto et al., 2017).

Acute phase proteins have been widely used in diagnosis, treatment monitoring and prognosis of different tumors in human medicine (Weinstein et al., 1984; Krecicki and Leluk, 1992; Falconer et al., 1995; Kocsis et al., 2011; Ma et al., 2017). Serum APP have also been used as biomarkers of several canine tumors (Tecles et al., 2005; Nielsen et al., 2007; Alexandrakis et al., 2017). The APP have also been evaluated in feline oncology, however the information is scarce.

Serum amyloid A and AGP concentrations were proved to increase in cats with tumors (considering groups of cats with different tumors) (Sasaki et al., 2003; Selting et al., 2000), however, no significant differences in AGP activity were detected between cats with carcinomas, sarcomas or discrete round-cell tumors (Selting et al., 2000). Increased concentrations of SAA at the time of diagnosis was also proved to be a significant negative prognostic factor in cats with tumors (also considering a group of cats with different tumors), with cats with elevated SAA concentrations (> 0.82 mg/l) at diagnosis presenting a significantly shorter median survival time than cats with concentrations of SAA at diagnosis in the reference range (≤ 0.82 mg/l) (Tamamoto et al., 2013). However, the magnitude of increase in SAA concentrations was not significantly correlated with survival time (Tamamoto et al., 2013).

The APP proved to be clinical useful biomarkers of feline lymphoma at diagnosis, in monitoring the response to treatment and in evaluation of a complete remission state. Significant increases in serum AGP, SAA and Hp, and decreases in albumin were detected in cats with lymphoma at diagnosis (considering groups of cats with lymphoma in different anatomical sites, clinical stages and immunophenotypes) (Correa et al., 2001; Gerou-Ferriani et al., 2011; Winkel et al., 2015). Moreover, a gradual decline in SAA and AGP activity was observed during treatment, with diseased cats achieving concentrations of these APP similar to those of healthy cats at 12 weeks of treatment, when all cats were in complete remission of the disease (Winkel et al., 2015). Nonetheless, serum AGP activity of cats with lymphoma did not change significantly during treatment and provided no useful information considering survival time in cats with lymphoma in the study of Correa et al. (2001).

As previously stated, an increase in the degree of sialylation of AGP was described in FeLV positive cats with alimentary lymphoma, with the increase in sialic acid expression of AGP attributed to the neoplasia rather than the FeLV infection, since the change was not observed in Felv positive cats that did not develop lymphoma (Pocacqua et al., 2005). Moreover, changes in glycosylation of AGP were also described in humans with lymphoma (Kremmer at al., 2004) The glycan moiety of AGP was suggested to play an important biological role in the immunomodulatory function of this APP in feline lymphoma (Pocacqua et al., 2005).

In vitro studies proved that feline SAA promotes synthesis of metalloproteinase-9, a matrix degrading protease associated with tumor infiltration and development of metastasis, and stimulates cell infiltration of selected mammary- and lymphoma-derived cell lines (Tamamoto et al., 2014; Tamamoto et al., 2017). These studies suggest that besides being a biomarker of tumors in cats, feline SAA may play a role in mammary and lymphoma tumorigenesis, and probably also in other tumors, by enhancing tumor invasive capacity and consequently promoting metastases development *in vivo*.

Serum amyloid A and feline amyloidosis

Amyloidosis is a generic designation attributed to a group of protein-misfolding disorders that are caused by an extracellular deposition of highly insoluble amyloid fibrils in various tissues of the organism (Knowles et al., 2014; Watanabe et al., 2015). The diseases are caused by conformational changes that occur in proteins that are soluble under physiological conditions,

and that acquire an insoluble β-pleated sheet conformation (Segev et al., 2012). At least 28 different proteins have been shown to be amyloidogenic in humans and animals (Murakami et al., 2014) and are associated with disorders such as Alzheimer disease in humans and cognitive dysfunction in animals, prion diseases, type II diabetes mellitus, familial amyloid polyneuropathy, familial mediterranean fever, familial amyloid cardiomyopathy and reactive amyloid A (AA) amyloidosis, among others (Westemark et al., 2011; Woldemeskal, 2012; Youssef et al., 2016).

Amyloidosis diseases can be localized or systemic. In the localized forms, the amyloid fibrils are deposited in specific organs in which precursor proteins are synthesized, such as the brain (Head et al., 2005; Youssef et al., 2016) and pancreas (Westermark et al., 1987; Jurgens et al., 2011; Zini et al., 2016). In the systemic diseases, such as AA amyloidosis, serum precursor proteins circulate in the blood and polymerize to form insoluble amyloid fibrils that are deposited throughout the organism (Hazenberg et al., 2004; Murakami et al., 2014). In human medicine, systemic amyloidosis are classified in three different types, namely immunoglobulin-associated (primary), reactive (secondary) and senile (heredofamilial) (Segev et al., 2012).

In domestic cats, localized and systemic forms of amyloidosis were described. Localized diseases include deposits of amyloid proteins in pancreas, in brain and in calcifying epithelial odontogenic tumors (amyloid producing odontogenic tumors) (Westermark et al., 1987; Gardner et al., 1994; Head et al., 2005; Zini et al., 2016). Feline systemic amyloidosis, including familial amyloidosis of Abyssinian and Siamese cats, are mostly of the reactive or secondary amyloidosis type, with deposits composed of the protein amyloid A (AA-amyloidosis), an amino-terminal fragment of SAA (DiBartola et al., 1985; DiBartola et al., 1986; Niewold et al., 1999; Woldemeskel, 2012). AA-amyloidosis can be idiopathic, or associated with chronic inflammatory, infectious or neoplastic diseases, in which the persistent increases in concentrations of SAA predispose to partial cleavage of certain isoforms of SAA into fragments that have an increased propensity to form fibrillar aggregates of amyloid, which are deposited systemically, mainly in the kidney, liver, and spleen (DiBartola and Benson, 1989; Asproni et al., 2013). Familial amyloidosis in Abyssinian and Siamese cats are inherited diseases with a high frequency among some pedigree lineages in these feline breeds (DiBartola et al., 1986; van der Linde-Sipman et al., 1997). Different isoforms of amyloid A protein were reported in Abyssinian and in Siamese cats, which were

suggested to be amyloidogenic and associated with the different patterns of amyloid deposition, which occurs mainly in the kidney in Abyssinians and in the liver in Siamese cats; however, other factors than genetic were suggested to be involved in feline familial amyloidosis (Johnson et al., 1989; Niewold et al., 1999; van Rossum et al., 2014; Paltrinieri et al., 2015).

Different studies assessed concentrations of SAA in serum and urine of cats in order to evaluate if changes in these analytes were associated with development of amyloidosis. Significantly higher concentrations of SAA were reported in healthy Abyssinian cats than in healthy cats of other breeds (DiBartola et al., 1989). Significantly higher concentrations of SAA were also reported in Abyssinian cats with amyloidosis than in hospitalized cats with other diseases, and also than in healthy Abyssinian cats, nevertheless, a reliable differentiation of healthy and affected Abyssinian cats based on SAA concentrations could not be obtained due to the wide range of SAA concentrations in these two groups of animals (DiBartola et al., 1989). In contrast, no significant differences in SAA concentrations were detected between healthy Abyssinian cats and Abyssinian cats with familial amyloidosis in the study of Paltrinieri et al. (2015), however, peaks in concentrations of SAA were detected more frequently in cats that developed the disease than in healthy Abyssinian cats, even in the absence of clinical signs, suggesting that Abyssinian cats might have a hypersensitivity to mild inflammatory stimuli, or a delayed clearance of SAA. Moreover, serum amyloid A and urinary protein-to-creatinine ratio showed no clinical value in early identification of Abyssinian cats with familial amyloidosis (Paltrinieri et al., 2015). However, increases in urinary concentration of SAA were associated with development of amyloidosis in Abyssinian cats; thus, urinary SAA-to-creatinine ratio proved to be a clinical useful biomarker for prediction of familial amyloidosis development in Abyssinian cats (Paltrinieri et al., 2015).

Future Perspectives

Laboratory methods for determination of acute phase proteins

Determination of APP is routinely used in human medicine (Hedegaard et al., 2015; Zhang et al., 2019). However, despite the clinical applications in veterinary medicine, their use in the clinical practice, namely in feline medicine, is not consistently implemented. Determination of AGP in FIP is probably the only exception (Giori et al., 2011; Hazuchova et al., 2017).

Different methods have been validated for determination of APP, including colorimetric techniques and several immunological methods such as radioimmunoassays (RIA), enzyme linked immunosorbent assays (ELISA), radial immunodiffusion assays, immunonephelometry assays and immunoturbidimetric assays; however, most of these methods are time-consuming and relatively expensive, and therefore are not routinely used in practice (Cerón et al., 2005; Paltrinieri, 2008). Other methods, such as two-dimensional gel electrophoresis, high performance liquid chromatography, western blotting and lectin staining, that permit detection of qualitative or structural alterations in APP, are only available for research (Paltrinieri, 2008). The lack of standardization of methods used for determination of APP between laboratories also contributes to the limitation of their use in feline practice (Cray et al., 2009). Recently, the validation of immunoturbidimetric tests for measuring feline APP should reduce the cost per test, and consequently promote a wider use of APP measurements (Hansen et al., 2006; Paltrinieri, 2008). Another future challenge for veterinarians would be the development of high throughput techniques, such as protein microarray assays, which would allow simultaneous measurements of thousands of samples per batch, as already validated for other species (Paltrinieri, 2008).

Proteomics

The progresses in molecular biology achieved in the last decades allowed the development of new areas of investigation, including the omics field of research. The term omics includes different areas of investigation, including genomics, transcriptomics, lipidomics, glycomics, proteomics and metabolomics, which have a general aim to perform a collective characterization and quantification of biological molecules in a sample, and to analyze the interactions between these biological molecules (Eckhart et al., 2012; Hamper, 2016). The use of biomarkers profiles, including those obtained from multi-omics studies (including combinations of genes, proteins, metabolites or other molecules) have proved better diagnostic accuracy / increased sensitivity and specificity than use of single biomarkers in diagnosis, prognosis and monitoring evolution and response to treatment of several diseases (Eckhart et al., 2012; Liu et al., 2014; Li et al., 2015; Montague et al., 2015; Ress et al., 2015). However, factors including time, cost and scarcity of commercial availability of methodology limit the application of this approach in clinical practice (Gasser et al., 2011; Liu et al., 2014; Ceciliani et al., 2014).

Proteomics refers to the global analysis of the expression, location, function and interaction of all proteins, i.e. the proteome, that are expressed by an organism in physiological or pathological conditions, and consequently, allows determination of profiles of biomarkers that are expressed in a given condition (Liu et al., 2014; Bujak et al., 2015). Proteomic analysis can be applied in different fluids, including whole blood, serum, plasma, urine, saliva, semen, tears and milk among others, and also in tissue samples (McLean et al., 2007; Bang et al., 2011; Gao et al., 2013; Chiaradia et al., 2013; Vernocchi et al., 2014; Stetson et al., 2015; Meachem et al., 2015; Franco-Martínez et al., 2019; Agrawi et al., 2019). The comparative proteomic analysis of samples from control and diseased animals, allows the detection of differentially expressed proteins and other molecules in health and disease (Tvarijonaviciute et al., 2012b), allowing the detection of individual molecules or profiles of molecules, including APP and other acute phase reactants, which could be used as biomarkers of a given disease.

The application of proteomics has been extensively studied in human medicine, however, its application in veterinary medicine research, namely in feline medicine, has been limited (Matharoo-Ball et al., 2008; Kycko and Reichert, 2014; Li et al., 2017; Geyer et al., 2017;

Ceciliani et al., 2014). Proteomic analyses showed that cats with pancreatic adenocarcinoma had significantly higher serum concentration of pre-apolipoprotein (Apo) A1 than healthy control animals, although no significant differences were detected between cats with pancreatic carcinoma and cats with pancreatitis; and cats with pancreatic disease (pancreatitis and pancreatic carcinoma) showed a tendency to present higher serum AGP and lower serum Apo A1 than healthy cats (Meachem et al., 2015). Cats with degenerative joint disease showed an up-regulation of major serum proteins within the classical complement component of the immune system, such as C1s, C1r and C3, and a decrease in serum clusterin (a regulator of the complement system) than control animals (Gao et al., 2013). Proteomic studies performed in feline urine samples showed that urine fibronectin concentration was higher in cats with idiopathic cystitis than in cats with bacterial urinary tract infection, urolithiasis and healthy cats (Lemberger et al., 2011); identified eight potential biomarkers (including low-molecular weight peptides and proteins) of early renal damage (Jepson et al., 2013); and reported that cats with chronic kidney disease associated with tubular damage presented increased expression of retinol-binding protein, cystatin M and apolipoprotein-H, and decreased expression of uromodulin and cauxin than healthy cats (Ferlizza et al., 2015). Proteomic studies in domestic felines have also been performed in tissues, including visual cortex (Van der Bergh et al., 2003; Van der Bergh et al., 2006), tissues from reproductive organs such as placenta, ovaries, oocytes and spermatozoa (Bang et al., 2011; Bang et al., 2013; Vernocchi et al., 2014; Stetson et al., 2015), and liver samples of cats inoculated with endotoxins (bacteria derived lipopolysaccharide) (Crouser et al., 2006).

Acute phase proteins as therapeutic agents or therapeutic targets of disease

A more recent line of investigation in the field of APP is related with the modulation of its concentration, either locally or systemically, in treatment of different diseases. Different studies, mainly performed in laboratory animal models of human diseases, reported administration of APP as therapeutic agents of different diseases, while others showed that decreasing concentrations or the effects of APP could be beneficial in treatment of other conditions (Pepys et al., 2006; Linke et al., 2017; Piling and Gomer, 2018).

In a mice model of systemic lupus erythematosus (SLE, NZB/NZW mice model), CRP showed to be protective against the disease, with NZB/NZW animals expressing a human CRP transgene (hCRPtg/BW) presenting less proteinuria and longer survival than NZB/NZW mice (Szalai et al., 2003). Moreover, administration of CRP prior to SLE onset delayed manifestation of proteinuria and prolonged survival, and administration of CRP during the acute manifestation of disease significantly decreased proteinuria in both NZB/NZW and MRL/lpr mice models of SLE (Du Clos et al., 1994; Rodriguez et al., 2005; Rodriguez et al., 2006). Administration of CRP, before and after induction of disease in a mice model of nephrotoxic nephritis was also effective in the control of proteinuria and glomerular lesions through an IL-10 dependent anti-inflammatory process (Rodriguez et al., 2005). However, a more recent investigation failed to reproduce the beneficial effects of a single administration of CRP in murine models of SLE and nephrotoxic nephritis (Carlucci et al., 2010).

Administration of human CRP was associated with an increase in cardiac tissue damage in a rat model of acute myocardial infarction, indicating that CRP is a mediator of ischemic myocardial injury (Griselli et al., 1999). The administration of inhibitors of CRP in rats undergoing acute myocardial infarction prevented the increase in infarct size and improved cardiac function (Pepys et al., 2006; Szalai et al., 2014). These facts suggest that therapeutic inhibition of CRP might provide cardioprotection in acute myocardial infarction, and possibly in other clinical conditions in which increased CRP and CRP mediated complement activation might exacerbate tissue injury (Pepys et al., 2006; Szalai et al., 2014).

Serum amyloid P (SAP, also designated pentraxin2) is a member of the pentraxin family of proteins, as CRP and pentraxin3, and is considered a positive APP in mice (Pepys et al., 1979; Hutchinson et al., 2000). Several studies, performed in laboratory animals and also in human patients suggested that SAP deficiency might be involved in pathogenesis of fibrosing diseases, and that SAP might be a therapeutic agent used in these conditions. In a mouse model of bleomycin induced pulmonary fibrosis, SAP knockout mice developed a more persistent inflammatory response and increased pulmonary fibrosis than wildtype animals (Pilling and Gomer, 2014). Furthermore, lower SAP serum concentrations were detected in human patients with pulmonary fibrosis and other fibrosing diseases such as myelofibrosis, scleroderma and mixed connective tissue disease (Pilling et al., 2003; Murray et al., 2011; Verstovsek et al., 2016). In rats, systemic or local administration of SAP reduced the bleomycin-induced pulmonary fibrosis, and in a delayed administration protocol also

promoted an improvement in clinical signs (Pilling et al., 2007; Murray et al., 2010). Beneficial effects of administration of SAP were also observed in clinical trials in humans, with improvements in lung function being observed in patients affected by pulmonary fibrosis, and a reduction in bone marrow fibrosis and improvement in bone marrow function observed in myelofibrosis affected patients (Verstovsek et al., 2015; Raghu et al., 2018).

On the other hand, reducing the concentrations of SAP might be a therapeutic strategy in treatment of other clinical conditions, including wound healing, treatment of amyloidosis and *Mycobacterium* spp. infection. Serum amyloid P inhibits differentiation of monocytes into fibrocytes, which are important in the process of wound cicatrization (Bucala et al., 1994; Mori et al., 2005). Local and systemic injection of SAP was shown to delay wound healing in mice (Naik-Mathuria et al., 2008); SAP removal from the wound using a SAP-binding hydrogel was proved to accelerate the healing process in skin wounds in pigs (Gomer et al., 2009); and in human medicine, burned patients with low serum levels of SAP had shorter complete healing time and higher survival of skin grafts than patients with higher SAP concentrations (Zhang YM et al., 2011). In animal models of amyloidosis, the induction of the disease was retarded in SAP knockout mice (Botto et al., 1997), and treatment with anti-SAP antibodies was able to remove amyloid deposits and was associated with an improvement in organ function in mice and humans (Bodin et al., 2010; Richard et al., 2015; Richards et al., 2018). Type 1 macrophages (M1 macrophages) express nitric oxide synthases (iNOS), produce nitric oxide (NO) and secrete pro-inflammatory cytokines, which are associated with microbicidal capacity against different agents, including *Mycobacterium* spp. (Biswas and Mantovani, 2010; Sica and Mantovani, 2012; Bertolini et al., 2016). Moreover, M1 macrophages are more effective in the induction of the inflammatory response and clearance of infectious agents than type 2 macrophages (M2 macrophages) (Labonte et al., 2014; Bertolini et al., 2016). Serum amyloid P potentiates the differentiation of monocytes into M2 macrophages, and stimulates the conversion of macrophages from a pro-inflammatory to an anti-inflammatory phenotype (Zhang W et al., 2011; Xiang et al., 2017). In an *in vitro* study in human macrophages, SAP was shown to potentiate the proliferation of *Mycobacterium smegmatis* and *Mycobacterium tuberculosis*, and the use of compounds that inhibited the SAP functions was associated with a decrease in proliferation of these agents (Xiang et al., 2017), suggesting that inhibition of SAP might be clinically useful in treatment of *Mycobacterium* infections, and possibly of other infectious agents.

Administration of recombinant human SAA1 in a mice model of sepsis improved reduction of inflammation and prolonged survival in animals with induced SAA1 deficiency (Linke et al., 2017).

Decreases in Cp synthesis have been associated with neurodegenerative diseases in human medicine, such as Parkinson disease and Alzheimer, by increasing iron accumulation and oxidative stress in the substantia nigra (Vassiliev et al., 2005; Ayton et al., 2013; Zhao et al., 2018). Ceruloplasmin knockout mice develop a neurodegenerative disorder similar to Parkinson disease, supporting the role of Cp in etiopathogenesis of Parkinson disease; and infusion of Cp attenuated neurodegeneration and iron accumulation in substantia nigra in a mouse model (1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine mouse model) of Parkinson disease (Ayton et al., 2013). Similarly, restoration of Cp expression in Cp knockout mice through injection of an exogenous expression plasmid into the brain ventricle alleviated neuronal damage in the hippocampus in a mice model of Alzheimer´s disease (Zhao et al., 2018).

To the authors´ knowledge, no studies regarding the use of APP as therapeutic agents or targets have been performed in cats. However, future investigations might find similar clinical applications of APP in feline medicine as those examples described above in laboratory animals and humans.

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Chapter 2

Objectives

General Objectives

The APP are being increasingly used in human and in veterinary medicine as biomarkers of general health screening, and of diagnosis, prognosis and monitoring of the evolution and response to treatment of different diseases. In addition, recent investigations described new clinically useful analytes that present an APP behavior, and also have led to the discovery of possible new clinical applications of the APP, namely in treatment of different diseases. Even though the APP are routinely used in clinical practice in human medicine, their clinical application in veterinary medicine, particularly in feline medicine, is still scarce. Therefore, the main aim of this investigation was to contribute to the knowledge of the clinical applications of APP in feline medicine, through the study of the APP response in different feline conditions including natural infection with hemoplasmas, pyometra and spontaneous malignant mammary tumors, in order to clarify their potential usefulness and promote their routine use in the clinical practice.

Specific Objectives

1. Study acute phase proteins response in cats naturally infected by hemotropic mycoplasmas (hemoplasmas)

1.1. To evaluate the APP response in cats naturally infected with hemotropic mycoplasmas, including infection with one agent of hemoplasmas and co-infections with different species of hemoplasmas, at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin and albumin.

1.2. To investigate the APP response in cats naturally infected with *Candidatus Mycoplasma haemominutum*, at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin and albumin.

1.3. To assess the APP response in cats naturally co-infected with *Candidatus Mycoplasma haemominutum* and *Mycoplasma haemofelis*, at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin and albumin.

1.4. To study the APP response in cats naturally co-infected with *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma haematoparvum*-like, at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin and albumin.

2. Study acute phase proteins response in cats with pyometra

2.1. To investigate the APP response in cats with pyometra, determined at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin and albumin.

2.2. To evaluate the APP response in cats with pyometra, during the post-operative period, through monitoring serum concentrations of serum amyloid A, haptoglobin and albumin.

3. Study acute phase proteins response in cats with spontaneous malignant mammary tumors

3.1. To investigate the APP response in cats with spontaneous malignant mammary tumors, at diagnosis, through determination of serum concentrations of serum amyloid A, haptoglobin, albumin, butyrylcholinesterase, insulin-like growth factor 1 and paraoxonase 1.

3.2. To study the influence of clinical and histological features of mammary carcinomas in the feline inflammatory response.

3.3. To evaluate the prognostic value of serum amyloid A, haptoglobin, albumin, butyrylcholinesterase, insulin-like growth factor 1 and paraoxonase 1 in feline mammary carcinomas.

3.4. To assess the biological role of butyrylcholinesterase, insulin-like growth factor 1 and paraoxonase 1 as APP in cats.

Chapter 3

Serum acute phase proteins response in cats naturally infected by hemotropic mycoplasmas (hemoplasmas)

Vilhena H, Tvarijonaviciute A, Cerón JJ, Pastorinho MR, Martinez-Subiela S, Pastor J, Silvestre-Ferreira AC. Acute phase proteins response in cats naturally infected by hemotropic mycoplasmas. Comp Immunol Microbiol Infect Dis. 2018;56:1-5

Vilhena H, Tvarijonaviciute A, Ceron JJ, Vieira L, Pastorinho R, Pastor J, Silvestre-Ferreira AC. Serum acute phase proteins in *Mycoplasma* spp. infected cats. Poster communication. Proceedings of the 25th European College of Veterinary Internal Medicine – Companion Animals Congress, pp 164, Lisbon, Portugal, 10th to 12th September 2015

Abstract

Background: Acute phase proteins (APP) have proved to be useful biomarkers in several feline infectious diseases, including experimental single infections with *Mycoplasma haemofelis* and *Candidatus Mycoplasma haemominutum*. However, the APP response in cats naturally infected with hemoplasmas and in cats co-infected with different species of hemotropic mycoplasmas has not been investigated.

Objectives: The aim of the present study was to investigate the APP response in cats naturally infected by one or various species of hemotropic mycoplasmas.

Materials and Methods: Serum concentrations of serum amyloid A (SAA), haptoglobin (Hp) and albumin were determined in 48 cats naturally infected with hemoplasmas and in 10 healthy control cats. Of the 48 infected animals, 25 cats were infected with *Candidatus Mycoplasma haemominutum*, 12 cats were co-infected with *Candidatus Mycoplasma haemominutum* and *Mycoplasma haemofelis*, 7 cats were co-infected with *Candidatus Mycoplasma haemominutum* and *Candidatus Mycoplasma haematoparvum*-like, and 4 cats were infected with other *Mycoplasma* species.

Results: The overall group of infected cats (including symptomatic and asymptomatic animals) had significantly higher Hp and lower albumin than controls. A tendency for higher concentrations of SAA in infected cats than in controls was also observed (although the difference was not significant). Symptomatic cats had significantly higher SAA and Hp, and lower albumin than asymptomatic animals, and also than controls. Asymptomatic cats had significantly higher Hp than controls. Concentrations of APP were not significantly different between single infected and co-infected cats.

Conclusions: According with these results, an APP response was detected in cats naturally infected (single and co-infections) by hemotropic mycoplasmas. Hemoplasmosis should be considered when alterations in APP are detected in diseased cats with compatible clinical signs. Furthermore, a subclinical infection should be considered in apparently healthy cats from endemic areas with increased Hp.

Key Words: acute phase proteins, albumin, feline, haptoglobin, hemoplasmas, hemotropic mycoplasmas, serum amyloid A.

Introduction

Hemotropic mycoplasmas (hemoplasmas) are small epierythrocytic bacteria that infect a wide variety of mammalian species, including domestic cats (Sykes, 2010). Formerly considered as *Eperythrozoon* and *Haemobartonella* species, hemoplasmas were reclassified into the genus *Mycoplasma* (Foley and Pedersen, 2001; Neimark et al., 2001). Five hemoplasmas species were reported to infect cats - *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (CMhm), *Candidatus Mycoplasma turicensis* (CMt), *Candidatus Mycoplasma haematoparvum*-like (CMhp) and *Mycoplasma haemocanis* (Mhc) (Foley and Pedersen, 2001; Neimark et al., 2001; Willi et al., 2005; Sykes et al., 2007; Bergmann et al., 2017). These agents have a worldwide distribution; prevalence of infection presents geographical variation, however, CMhm is reported as the most prevalent agent in most studies (Sykes, 2010; Willi et al., 2007; Barker and Tasker, 2013; Walker Vergara et al., 2016). Co-infections with two or more hemoplasmas species are frequently described (Peters et al., 2008; Sykes et al., 2008; Martínez-Díaz et al., 2013; Aquino et al., 2014; Ghazisaeedi et al., 2014).

From the five agents reported to infect cats, Mhf is considered the most pathogenic, causing hemolytic anemia in immunocompetent cats, which can be severe and life-threatening in some cases (Tasker et al., 2009; Baumann et al., 2013; Ghazisaeedi et al., 2014). *Candidatus Mycoplasma haemominutum* and CMt are considered less pathogenic, but can also cause anemia in infected cats, mainly when associated with immunosuppressant conditions (Willi et al., 2006; Reynolds and Lappin, 2007; Weingart et al., 2016). Clinical information on feline infection with Mhc and CMhp is lacking.

Acute phase proteins (APP), which are serum proteins whose concentrations are altered when animals are exposed to an inflammatory stimulus, are being increasingly used in human and veterinary medicine in diagnosis, prognosis, treatment monitoring, and in general health screening (Eckersall and Bell, 2010; Kann et al., 2012; Parrinello et al., 2015). It was suggested that the APP profiles should include at least one positive major, one positive

moderate, and one negative APP in order to better differentiate between pathological states and to provide more information on the evolution of the disease (Cerón et al., 2008). In the cat, serum amyloid A (SAA) is considered a positive major APP, haptoglobin (Hp) a positive moderate APP, and albumin the principal negative APP (Cerón et al., 2005; Eckersall and Bell, 2010).

Serum concentrations of APP have proved to be useful biomarkers in several feline infectious diseases (Duthie et al., 1997; TerWee et al., 1998; Kann et al., 2014; Leal et al., 2014; Hazuchova et al., 2017; Silvestre-Ferreira et al., 2017), including experimental single infections with Mhf and CMhm (Harvey and Gaskin, 1978; Korman et al., 2012). Nonetheless, to the authors´ knowledge, the APP response in cats naturally infected with hemoplasmas or the APP response in cats co-infected with different species of hemotropic mycoplasmas has not been investigated. Thus, the aim of the present study was to investigate the APP response by measuring SAA, Hp and albumin concentrations in cats naturally infected by one or various species of hemotropic mycoplasmas (hemoplasmas).

Materials and Methods

The present study was approved by the Scientific Council of the School of Agricultural and Veterinary Sciences of the University of Trás-os-Montes and Alto Douro, Portugal.

Cats and samples

The present study was performed using serum samples from previous research of prevalence of hemoplasmas and agents of vector-borne diseases in cats from the North and Center regions of Portugal (Martínez-Díaz et al., 2013; Vilhena et al., 2013; Vieira et al., 2014). Briefly, diseased cats that were presented to veterinary medical centers from the North and Center regions of Portugal that required blood analyses as part of their diagnostic plan, and apparently healthy cats that required hematological analysis for elective surgical procedures,

geriatric check-ups or determination of feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) infection status were randomly selected, without inclusion or exclusion criteria, and included in the prevalence studies (Martínez-Díaz et al., 2013; Vilhena et al., 2013; Vieira et al., 2014). Identification and medical data of each cat was collected, including gender, age, breed, living conditions, and clinical data related with owners information, clinical examination and results of complementary diagnostic exams. Blood samples were collected at admission from jugular or saphenous veins, centrifuged (10 min, 2000 x g) and serum was stored at -20°C until analysis. All animals were tested by real-time PCR for Mhf, CMhm, CMt, CMhp (Martínez-Díaz et al., 2013), *Babesia vogeli*, *Babesia canis*, *Hepatozoon felis*, *Hepatozoon canis*, *Leishmania infantum*, *Toxoplasma gondii*, *Anaplasma* spp., *Ehrlichia* spp. and *Rickettsia* spp. (Vilhena et al., 2013), and by ELISA for anti-*Dirofilaria immitis* and anti-*Wolbachia* antibodies (Vieira et al., 2014). Of the samples available from the prevalence studies referred above, samples from cats infected with hemoplasmas (single and co-infections) and without other concomitant diseases, including negative results to all the agents tested; and samples from cats that showed no abnormalities on clinical examination and results of complementary diagnostic exams, including negative results for all agents tested and for FIV and FeLV, were selected for the present research.

The overall population of hemoplasmas infected cats was grouped according with the infectious agent(s) (table 3.1). In addition, the overall population of infected cats, of cats mono-infected with CMhm, of cats co-infected with CMhm and Mhf, and of cats co-infected with CMhm and CMhp, were further subdivided into two subgroups: asymptomatic cats, and cats with clinical signs and clinicopathological abnormalities compatible with hemoplasmosis (table 3.1). These subgroups were made based on owner information, clinical examination and results of complementary diagnostic exams. Clinical signs and clinicopathological abnormalities considered compatible with hemoplasmosis included anemia (considered when the hematocrit < 25%) and its clinical manifestations such as mucosal pallor, weakness, depression, tachypnea and tachycardia; and anorexia, weight loss, dehydration, fever, icterus, splenomegaly, leucocytosis, leucopenia, hyperbilirrubinemia, prerenal azotaemia and elevated serum concentration of alanine aminotransferase (Sykes, 2010; Tasker, 2010; Barker and Tasker, 2013). Due to the low number of animals included in the study, only alterations in APP concentrations are presented for cats infected with CMt, co-infected with CMhm, Mhf and CMhp and co-infected with CMhm, Mhf, CMt and CMhp. Information about presence or absence of clinical signs in these cats is presented in table 3.1.

Table 3.1. Cats infected with hemoplasmas (single and co-infections) included in the study

	Total (n)	**Asymptomatic** (n)	**Symptomatic** (n)
Single infections			
CMhm	25	19	6
CMt	1	1	-
Co-infections			
CMhm + Mhf	12	8	4
CMhm + CMhp	7	-	7
CMhm + Mhf + CMhp	2	1	1
CMhm + Mhf + CMt + CMhp	1	1	-
Overall population	48	30	18

CMhm - *Candidatus Mycoplasma haemominutum*; CMhp - *Candidatus Mycoplasma haematoparvum*-like; CMt - *Candidatus Mycoplasma turicensis*; Mhf - *Mycoplasma haemofelis*

Acute phase proteins assays

Acute phase proteins determinations were performed within two years after collection in all cases, at the Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, University of Murcia, Spain. Serum concentration of SAA, Hp and albumin were determined in all samples.

Serum amyloid A concentrations were determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical Co., Tokyo, Japan) previously validated for use in cats (Hansen et al., 2006). Serum concentrations lower than 5.0 µg/ml were considered normal for cats; limit of detection was set at 0.38 µg/ml (Hansen et al., 2006). Serum Hp concentrations were determined by use of the hemoglobin-binding method with the use of a commercial kit (Tridelta Development Ltd., Kildare, Ireland) previously validated for use in cats (Tvarijonaviciute et al., 2012). Serum concentrations lower than 3.0 g/l were considered normal; limit of detection considered was 0.0088 g/l (Tvarijonaviciute et al., 2012). Serum albumin was performed using a commercially available kit (Albumin OSR 6102; Olympus Life and Material Science Europe GmbH, Irish branch, Ennis, Ireland) following instructions of the manufacturer. Serum albumin concentrations ranging from 2.5 to 3.6 g/dl were considered normal for the species. All analyses were performed on an automated biochemistry analyzer (Olympus AU600, Olympus Diagnostica, GmbH, Freiburg, Germany), and showed an inter- and intra-assay imprecision lower than 15%, and the dilution of the samples resulted in linear regression equations with correlation coefficients close to one.

Statistical analysis

Results are shown as medians and inter quartile range (IQR) unless otherwise stated and were calculated using routine descriptive statistical procedures and software (Graph Pad Prism, version 6, GraphPad Software Inc., California, USA). Concentrations with results lower than the detection limit were set as equal to the detection limit for further statistical analysis. Normality of distribution for each group was assessed by the Shapiro-Wilk test. As data were not normally distributed, the Mann-Whitney U-Test or the Kruskal-Wallis one-way Analysis of Variance on Ranks, followed by Dunn´s multiple comparison test, were used to compare groups of samples. *P* values < 0.05 were considered significant.

Results

Serum samples from a total of 58 cats were included in the present study. Of these, 48 cats were naturally single or co-infected with Mhf, CMhm, CMt and CMhp, and 10 apparently healthy cats were used as controls (table 3.1). The infected population was composed of 37 males and 11 females, mainly domestic short-haired cats (n=41), with ages ranging from one to 15 years of age (mean 3.3 years, standard deviation 3.1 years). Control cats were also mainly males (n=7), all domestic short-haired cats, with ages ranging from one to 10 years old (mean 4.2 years, standard deviation 3.3 years).

Serum concentrations of SAA, Hp and albumin of the overall population of hemoplasmas infected cats, of the different groups and subgroups of hemoplasmas infected animals and of the healthy control cats are presented in table 3.2. Differences in serum concentrations of APP in the overall population of *Mycoplasma* spp. infected, of CMhm infected, of CMhm and Mhf co-infected, of CMhm and CMhp co-infected cats and of healthy control animals are also presented in table 3.2. Significant differences in serum concentrations of APP between infected cats and healthy control animals, and also between infected symptomatic and infected asymptomatic cats were detected in all groups.

Table 3.2. Serum concentrations of serum amyloid A (SAA), haptoglobin (Hp) and albumin in the different groups and subgroups of hemoplasmas infected and of control cats. Significant differences in concentrations of APP between groups and subgroups of infected and control cats are presented in bold

	SAA (μg/ml)			**Hp** (g/l)			**Albumin** (g/dl)		
	Total cats	Symptomatic	Asymptomatic	Total cats	Symptomatic	Asymptomatic	Total cats	Symptomatic	Asymptomatic
***Mycoplasma* species infected cats** (n=48)									
Median	0.38	**6.60***c**	**0.38c**	**4.50*****	**5.61***c**	**3.57**c**	**2.74***	**2.53**a**	**2.84a**
IQR	0.38-2.70	**0.39-57.95**	**0.38-1.00**	**3.04-5.60**	**4.73-5.95**	**2.51-4.58**	**2.34-3.00**	**2.05-2.81**	**2.45-3.22**
CMhm infected cats (n=25)									
Median	0.38	**14.80***b**	**0.38b**	**4.18*****	**5.76***a**	**3.57**a**	**2.74***	**2.05**a**	**2.84a**
IQR	0.38-2.20	**2.20-73.08**	**0.38-1.10**	**2.50-5.74**	**4.41-5.98**	**2.48-5.02**	**2.13-3.00**	**1.82-2.62**	**2.45-3.33**
CMhm and Mhf co-infected cats (n=12)									
Median	0.79	**8.95**a**	**0.38a**	**4.08*****	**5.11*****	**3.41***	2.86	2.72	3.03
IQR	0.38-10.13	**3.45-46.10**	**0.38-1.00**	**3.16-4.60**	**4.31-5.91**	**2.19-4.40**	2.55-3.28	2.55-4.91	2.53-3.28
CMhm and CMhp co-infected cats (n=7)									
Median	0.38	0.38	-	**5.31*****	**5.31*****	-	**2.76***	**2.76***	-
IQR	0.38-67.8	0.38-67.80	-	**4.39-5.95**	**4.39-5.95**	-	**2.50-2.93**	**2.50-2.93**	-
Controls (n=10)									
Median	0.38	-	-	1.39	-	-	3.05	-	-
IQR	0.38-0.39	-	-	1.23-1.91	-	-	2.88-3.33	-	-

CMhm - *Candidatus Mycoplasma haemominutum*; CMhp - *Candidatus Mycoplasma haematoparvum*-like; Mhf - *Mycoplasma haemofelis*; IQR – interquartile range.

* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ when compared with controls; a - $P < 0.05$, b - $P < 0.01$, c - $P < 0.001$ between infected symptomatic and asymptomatic cats of the same group.

Overall population of hemoplasmas infected cats (n=48)

When the overall population of hemoplasmas infected cats was considered, serum concentrations of Hp were significantly higher and of albumin were significantly lower than of controls ($P < 0.001$ and $P < 0.05$, respectively). Serum concentrations of SAA tended to be higher than of controls, however the difference was not significant ($P = 0.059$). The subgroup of symptomatic cats presented significantly higher serum concentrations of SAA and Hp, and significantly lower albumin than the asymptomatic group ($P < 0.001$, $P < 0.001$ and $P < 0.05$, respectively), and also than the control group ($P < 0.001$, $P < 0.001$ and $P < 0.01$, respectively). Moreover, serum concentrations of Hp were significantly higher in infected asymptomatic animals than in controls ($P < 0.01$) (table 3.2).

Candidatus Mycoplasma haemominutum **infected cats (n = 25),** ***Candidatus Mycoplasma haemominutum*** **and** ***Mycoplasma haemofelis*** **co-infected cats (n = 12), and** ***Candidatus Mycoplasma haemominutum*** **and** ***Candidatus Mycoplasma haematoparvum*****-like co-infected cats (n = 7)**

The CMhm infected cats, the CMhm and Mhf co-infected cats and the CMhm and CMhp co-infected animals had significantly higher concentrations of Hp than controls ($P < 0.001$ in all cases). The CMhm infected and the CMhm and CMhp co-infected animals also had significantly lower albumin than controls ($P < 0.05$ in both cases). No significant differences in concentrations of SAA were detected between either group of infected cats and controls ($P = 0.084$, $P = 0.105$ and $P = 0.272$, respectively).

When the symptomatic cats of the different groups were compared to the control group, the CMhm infected and the CMhm and Mhf co-infected cats had concentrations of SAA significantly higher ($P < 0.001$ and $P < 0.01$, respectively). The symptomatic cats of the three groups had significantly higher serum Hp than controls ($P < 0.001$ in all cases); and the symptomatic CMhm infected and the symptomatic CMhm and CMhp co-infected cats had significantly lower albumin than controls ($P < 0.01$ and $P < 0.05$, respectively).

Symptomatic CMhm infected cats presented significantly higher values of SAA and Hp, and lower values of albumin than asymptomatic CMhm infected cats ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). Concentrations of SAA were also significantly higher in the symptomatic CMhm and Mhf co-infected cats than in the asymptomatic cats ($P < 0.05$). Furthermore, the CMhm infected asymptomatic cats and the CMhm and Mhf co-infected asymptomatic animals presented higher Hp serum concentrations than controls ($P < 0.01$ and $P < 0.05$, respectively).

No significant differences in the concentrations of SAA, Hp or albumin were detected between the groups of CMhm infected cats and the groups of co-infected animals (CMhm and Mhf co-infected cats and CMhm and CMhp co-infected cats). Similarly, concentrations of APP were not significantly different between the asymptomatic or between the symptomatic cats of the different groups of infected animals.

Sixteen out of the 48 infected asymptomatic and symptomatic cats (33.3%) had their FIV and FeLV infection status determined. Eight cats were seronegative for both virus, while eight cats were FIV and / or FeLV seropositive. No significant differences in serum concentrations of APP were detected between cats non-infected and infected with FIV and / or FeLV. Serum concentrations of SAA, Hp and albumin (presented as medians with IQR) of the cats non-infected and infected (respectively) with retrovirus were: SAA (6.40 [2.20-20.73] µg/ml, 6.85 [1.00-60.60] µg/ml; $P = 0.620$), Hp (5.94 [4.13-6.00] g/l, 5.06 [4.14-5.58] g/l; $P = 0.128$) and albumin (2.53 [2.26-2.77] g/dl, 2.72 [2.27-3.02] g/dl; $P = 0.101$).

A significant positive weak correlation between age and concentration of Hp was detected in the population of hemoplasmas infected cats ($P = 0.004$, Spearman $\rho = 0.412$) and in the group of CMhm infected animals ($P = 0.025$, Spearman $\rho = 0.306$). No significant associations were detected between age and concentrations of SAA, Hp or albumin in the other groups or subgroups of infected cats. No significant influence of gender was detected in APP concentrations in the different groups or subgroups of hemoplasmas single or co-infected cats.

Considering the groups of cats with other single and co-infections that were represented by a low number of animals (total n = 4; table 3.1), SAA values higher and albumin values lower than the reference range were observed only in the symptomatic CMhm, Mhf, and CMhp co-infected cat. However, Hp concentrations were higher than the reference interval in all the cats (symptomatic and asymptomatic).

Discussion

In the present study, an APP response was evaluated in cats naturally infected with hemotropic mycoplasmas. To the authors' knowledge, this is the first study to comprehensively evaluate the APP response in cats naturally infected with hemotropic mycoplasmas, and in cats with hemoplasmas co-infections.

The results of this study demonstrated that natural single and co-infections with hemoplasmas in cats are associated with increased SAA and Hp concentrations, and decreased albumin values when compared with healthy control animals. Similarly, in the experimental study reported by Korman et al. (2012), Mhf and CMhm single infections were associated with significant increases in concentrations of SAA. However, available data about Hp behavior in hemoplasmas infection in cats are contradictory. While increased serum Hp concentrations were detected in cats experimentally infected with Mhf in the study of Harvey and Gaskin (1978), as occurs in our study, no significant changes in Hp activity were detected in cats experimentally infected with Mhf or CMhm (despite some cats manifested increases in Hp values) in the report of Korman et al. (2012). These variations could be attributed to the influence of other factors besides APR, such as hemolysis that produces a decrease in Hp (Kann et al., 2012; Kuleš et al., 2014).

In the present study, significant increases in SAA concentrations and significant decreases in albumin values in cats infected with hemoplasmas were associated with presence of clinical signs. Serum Hp concentrations were also significantly increased in infected symptomatic cats, but also in infected asymptomatic animals, suggesting that Hp may be useful in detection of subclinical infection with hemoplasmas. Similar SAA and Hp behavior was detected in

Hepatozoon felis and *Babesia vogeli* infected, and in *Dirofilaria immitis* seropositive cats (Silvestre-Ferreira et al., 2017; Vilhena et al., 2017).

Infection with Mhf was also reported to induce a greater SAA response than infection with CMhm, which was attributed to the higher pathogenecity of Mhf when compared with CMhm (Tasker et al., 2009; Korman et al., 2012). However, in our study, concentrations of SAA, Hp and albumin were not significantly different between CMhm infected cats and the groups of co-infected animals.

Feline immunodeficiency virus infection does not affect SAA concentrations following CMhm or Mhf experimental infection, and Hp concentrations following CMhm experimental infection. However, in Mhf experimental infection, FIV-infected cats have a significant greater Hp concentration than non-FIV-infected animals (Korman et al., 2012). In the present study, no significant influence of retrovirus infection (FIV and / or FeLV) was detected in the APP response of hemoplasmas naturally infected cats. Nonetheless, only a small number of cats had their FIV / FeLV infection status determined. Further investigations with a greater number of animals would be required to better evaluate the influence of retrovirus in the APP response of cats infected with hemotropic mycoplasmas in natural conditions.

Age has been associated with significant increases in concentrations of SAA (but not of AGP and Hp, and also not with significant decreases in albumin) in cats, which was suggested to be related with the higher incidence of subclinical diseases in geriatric cats (Kann et al., 2012). Significant positive weak correlations between age and serum concentrations of Hp were detected in the overall population of infected cats and in the group of CMhm infected cats; however, no other significant associations were detected between the concentrations of APP in the other groups or subgroups of cats included in the study. Gender was described to be another factor to influence the feline APP response (Kann et al., 2012). In the present study, no significant differences in concentrations of APP were detected between the males and females infected with hemoplasmas.

The retrospective nature of the present study originated several limitations, including the low number of cats included in some groups and subgroups of infected cats and of controls. In addition, although only cats infected with hemoplasmas and without any other evident diseases were included in this study, the retrovirus infection status was not determined in all the cats. Also in the present study, serial measurements of APP were not determined in the

hemoplasmas infected cats. The APP have proved to be useful biomarkers in monitoring the evolution of the infection and the response to treatment in experimental conditions (Korman et al., 2012). The same is probably valid for feline hemoplasmas infection in natural conditions, but further studies are required to confirm it.

Conclusions

The obtained results indicate that feline natural infection with hemotropic mycoplasmas is associated with development of an APP response. Infected symptomatic cats presented higher serum concentrations of SAA and Hp, and lower albumin values than healthy cats. Infected cats in the subclinical state of infection only showed increases in Hp. No significant differences in APP serum concentrations were detected between cats infected with one agent and cats co-infected with two hemoplasmas species.

Hemoplasmosis should be considered as a possible cause when an APP response is detected in a diseased cat with compatible clinical signs. Moreover, increases in serum Hp concentration in apparently healthy cats living in endemic areas could raise the suspicion of a subclinical infection by hemotropic mycoplasmas.

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Chapter 4

Acute phase proteins response in cats with pyometra

Vilhena H, Figueiredo M, Cerón JJ, Pastor J, Miranda S, Craveiro H, Pires MA, Tecles F, Rubio CP, Dabrowski R, Duarte S, Silvestre-Ferreira AC, Tvarijonaviciute A. Acute phase proteins and antioxidant responses in queens with pyometra. Theriogenology 2018;115:30-37

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Abstract

Background: Acute phase proteins (APP) have proved to be clinically useful biomarkers of pyometra in different species. Despite pyometra is considered one of the most important feline reproductive diseases, information on the APP response in queens with pyometra is lacking.

Objectives: This study aimed to evaluate the APP response at diagnosis and during the post-operative period in feline pyometra.

Materials and Methods: An APP panel including two positive - serum amyloid A (SAA) and haptoglobin (Hp), and one negative APP – albumin, were determined in serum of 23 queens with pyometra at diagnosis, and of 13 healthy control queens submitted to elective ovariohysterectomy. The APP were also evaluated in serum of 11 queens of the pyometra group at days two and 10 after surgery.

Results: At diagnosis, queens with pyometra had serum concentrations of SAA and Hp significantly higher, and of albumin significantly lower than controls. Moreover, concentrations of APP were significantly different, with a tendency to return to physiologic levels, at day 10 after surgery than before surgery.

Conclusions: According to these results, an APP response was detected in queens with pyometra. In addition, APP tended to return to physiologic values after surgery in the queens that recovered from the disease. Therefore, our results suggest that APP could be potentially useful biomarkers in diagnosis and assessment of the post-operative period in feline pyometra.

Key Words: acute phase proteins, acute phase reaction, albumin, feline, haptoglobin, serum amyloid A

Introduction

Pyometra is considered as one of the most important feline reproductive diseases (Scott et al., 2002; Hagman et al., 2014). It is characterized by a suppurative inflammatory process in the endometrium and accumulation of purulent content in the uterine lumen (Misirlioglu et al., 2006; Hollinshead and Krekeler, 2016).

It should be considered an emergency due to the risk of development of severe and life-threatening complications, including septicemia, endotoxemia, azotemia, uterine rupture, peritonitis and shock (Brady et al., 2000; Scott et al., 2002; Demirel and Acar, 2012; Majoy et al., 2013). Ovariohysterectomy is considered the treatment of choice in most cases (Biddle and Macintire, 2000; Hollinshead and Krekeler, 2016). Most queens recover successfully after surgery; however pre-, intra- and post-operative complications may develop (Kenney et al., 1987; Stanley and Pacchiana, 2008), indicating the necessity of new biomarkers for disease evaluation and treatment monitoring.

The acute phase proteins (APP) response is a component of the innate immune reaction of the organism, and is a very fast reaction which develops before stimulation of the specific immune response, and in many cases even before the onset of clinical signs; therefore, it can be considered one of the earliest markers of disease (Cerón et al., 2005).

Acute phase proteins are being increasingly used in both human and veterinary medicine in general health screening, as well as in diagnosis, prognosis and in monitoring progression and response to treatment of several diseases (Dayer et al., 2007; Kjelgaard-Hansen and Jacobsen, 2011). Serum concentrations of APP have proved to be clinically useful biomarkers of pyometra in different species, including bitches, cows and mares (Børresen and Skrede, 1980; Dabrowski et al., 2009; Brodzki et al., 2015; Dabrowski et al., 2015; El-Bahr and El-Deeb, 2016).

To the author`s knowledge, information on the APP response of queens with pyometra is lacking. Thus, the main objective of the present study was to investigate the APP response in feline pyometra, at diagnosis and during the post-operative period, by measuring serum concentrations of serum amyloid A (SAA), haptoglobin (Hp) and albumin.

Materials and Methods

The study was approved by the Scientific Council of Vasco da Gama Universitary School, Coimbra, Portugal.

Animals

A total of 36 female cats that were presented to three veterinary medical centers from Portugal – Baixo Vouga Veterinary Hospital, Universitary Veterinary Hospital of Coimbra and Veterinary Policlinic of Aveiro – between January 2014 and June 2017 were enrolled in the present study. Identification and clinical data, including age, breed, weight, reproductive history, history of contraceptives administration, clinical signs and results of complementary diagnostic exams at the time of diagnosis, and whenever possible during the post-ovariohysterectomy period, were recorded for all cats.

Of these, 13 female cats presented for elective ovariohysterectomy, which were considered healthy based on clinical history, physical examination and results of complementary diagnostic exams, composed the control group. Blood analyses were performed in all cases as part of the pre-anesthetic examination. All queens were of domestic short-haired breed, with ages ranging from six months to eight years old (mean 1.9 years, SD 2.3 years), and body weight (BW) ranging from 2.2 to 4.0 Kg (mean 2.9 Kg, SD 0.5 Kg).

Twenty-three female cats with a presumptive pre-surgical diagnosis of pyometra, established in all cases based on clinical history, results of physical examination and complementary

diagnostic exams, including abdominal ultrasonography (Mindray DC-70 ultrasound system, Mindray Bio-Medical Electronics Co., Schenzhen, China) composed the pyometra group (Hollinshead and Krekeler, 2016). Queens with pyometra and with concomitant uterine conditions (including cystic endometrial hyperplasia, endometritis and endometrial adenocarcinoma) were enrolled in this research, while queens presenting other diseases were excluded. All queens were of domestic short-haired breed, with ages ranging from one to 20 years (mean 6.7 years, SD 4.6 years), and BW ranging from 1.7 to 5.3 Kg (mean 3.5 Kg, SD 0.9 Kg). Most of these queens presented an open-cervix pyometra (n = 21) and history of prolonged progestogens administration (n = 17). All cats showed leukocytosis and/or signs of toxicity in blood smear evaluation, indicating the presence of an active inflammation and possible septicemia.

All the queens of the diseased and of the control groups were submitted to ovariohysterectomy according with standard surgical procedures (Howe, 2006). Routine histopathology of the reproductive organs was performed in all the queens included in the study by a methodology previously described (Saraiva et al., 2015), at the Laboratory of Histology and Anatomical Pathology of the University of Trás-os-Montes and Alto Douro, to confirm pyometra in the diseased cats, and to exclude uterine pathology in the queens of the control group.

Blood samples

Whole blood samples (approximately 2.5 ml) were collected by jugular venous puncture before surgery and in the post-operative period when clinically indicated and at owners consent. One ml of whole blood was transferred into ethylenediamine tetra acetic acid (EDTA) tubes (K3EDTA tubes, Aquisel, Barcelona, Spain) for hematology analysis. The remaining sample was placed into plain tubes (Vacuette Z serum clot activator, Greiner Bio-One International GmbH, Kremsmünster, Austria), left to clot at room temperature for 15 minutes, centrifuged (10 min, 2000 x g), and supernatant used for biochemical analyses. Remaining samples were frozen at -20ºC. Acute phase proteins were determined in the remaining serum samples that were collected for clinical purposes. No blood samples were collected exclusively for this study.

Blood samples were collected in the day of the surgery (T0), before surgery and before administration of any medication in all animals of the diseased and of the control groups included in the study (n = 36). Blood samples were also collected in 11 queens of the pyometra group at days two (T1) and 10 (T2) after ovariohysterectomy. The diseased queens that were monitored in the post-operative period had ages ranging from one to 20 years (mean 6.9 years, SD 5.6 years) and BW ranging from 1.7 to 5.0 Kg (mean 3.5 Kg, SD 0.9 Kg).

Acute phase proteins

Acute phase proteins determinations were performed at the Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, University of Murcia, Spain. Only hemolysis and lipaemia free samples were used for analysis, since significant analytical interference with APP determinations have been reported (Martínez-Subiela and Cerón, 2005; Tecles et al., 2007).

Serum amyloid A concentrations were determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical Co., Tokyo, Japan). Briefly, the SAA proteins present in the serum sample react with the anti-SAA antibodies present in the reagent latex, causing agglutination and changing the turbidity of the sample, proportionally to the concentration of SAA. The method was previously validated for use in cats (Hansen et al., 2006). Serum concentrations lower than 5.0 µg/ml were considered normal for cats; limit of detection was set at 0.38 µg/ml (Hansen et al., 2006). Serum Hp concentrations were determined by use of the hemoglobin-binding method with the use of a commercial kit (Tridelta Development Ltd., Kildare, Ireland). The peroxidase activity of free hemoglobin is inhibited at low pH; however, in this method, the free hemoglobin present in the reagent combines with the Hp molecules of the sample, and the peroxidase activity of the bound hemoglobin is preserved at a low pH, proportionally to the concentration of serum Hp. The method was previously validated for use in cats (Tvarijonaviciute et al., 2012). Serum concentrations lower than 3.0 g/l were considered normal for the species; limit of detection considered was 0.0088 g/l (Tvarijonaviciute et al., 2012). Serum albumin was performed using a commercially available kit (Albumin OSR 6102; Olympus Life and Material Science Europe GmbH, Irish branch, Ennis, Ireland) following instructions of the manufacturer. Serum albumin concentrations ranging from 2.5 to 3.6 g/dl were considered normal for the species. All analyses were performed on an automated biochemistry analyzer (Olympus AU600, Olympus Diagnostica, GmbH, Freiburg, Germany).

Statistical analysis

Results are shown as medians with interquartile range (IQR) unless otherwise stated and were calculated using routine descriptive statistical procedures and software (Graph Pad Prism, version 6, GraphPad Software Inc., California, USA). D'Agostino & Pearson omnibus normality test was used to assess normality. As majority of data were not normally distributed, differences in serum concentrations of APP between the groups of diseased and of control cats before surgery were evaluated using Mann-Whitney test. Friedman test followed by the Dunn's multiple comparison test was used to evaluate differences in analytes between the different sampling time-points in queens with pyometra submitted to ovariohysterectomy. Values of $P < 0.05$ were considered significant.

Results

The queens of the pyometra group (n = 23) were significantly older ($P < 0.001$) and presented significantly higher weight ($P = 0.039$) than the queens of the control group (n = 13).

Serum concentrations of SAA, Hp and albumin of the diseased and of the control queens before ovariohysterectomy (T0) are presented in table 4.1. Diseased queens presented significantly higher concentrations of SAA and Hp, and significantly lower concentrations of albumin than control queens ($P < 0.001$ in all cases). However, six queens of the diseased group presented, at diagnosis, concentrations of SAA within the laboratory's reference range (< 5 µg/ml), despite presenting increased serum Hp (> 3g/l).

Table 4.1. Serum concentrations (presented with medians and IQR) of acute phase proteins of queens with pyometra (n=23) and of control queens (n=13) at diagnosis (T0). Significant differences between groups are presented in bold.

	Pyometra group	**Control group**	***P***
SAA (µg/ml)	63.60 (0.40-92.20)	0.38 (0.38 – 0.38)	< **0.001**
Hp (g/l)	4.64 (4.14 – 4.90)	2.09 (1.60 – 2.76)	< **0.001**
Albumin (g/dl)	2.28 (1.96 – 2.84)	3.12 (2.58 - 3.22)	< **0.001**

Hp – haptoglobin, IQR – interquartile range, SAA – serum amyloid A

Fifteen out of the 23 queens with pyometra (65.2%) also presented an endometrial adenocarcinoma diagnosed at abdominal ultrasound and / or histopathology, including three cats with *in situ* carcinomas and 12 animals with papillary serous adenocarcinomas. Age of queens with pyometra without endometrial adenocarcinoma ranged from one to 11 years (mean 6.1 years, SD 3.9 years), and BW ranged from 1.7 to 5.3 Kg (mean 3.6 Kg, SD 1.2 Kg). Age of the queens with pyometra and with a concomitant endometrial adenocarcinoma ranged from two to 20 years (mean 7.0 years, SD 4.9 years), and BW ranged from 2.5 to 5.0 Kg (mean 3.5 Kg, SD 0.6 Kg). Age and weight were not significantly different between the two groups ($P > 0.05$ in both cases). However, queens with pyometra without endometrial adenocarcinoma and queens with pyometra and endometrial adenocarcinoma were significantly older than controls ($P = 0.023$ and $P = 0.005$, respectively); and weight of cats with pyometra and endometrial adenocarcinoma, but not of cats without adenocarcinoma, was significantly higher than of control queens ($P = 0.028$ and $P = 0.230$, respectively). Serum concentrations of SAA, Hp and albumin of the groups of queens with pyometra with and without concomitant endometrial adenocarcinomas are presented in table 4.2. No significant differences were detected between the two groups in any of the evaluated biomarkers.

Table 4.2. Serum concentrations (presented with medians and IQR) of acute phase proteins of queens with pyometra (n=8) and of queens with pyometra and endometrial adenocarcinoma (n=15) at diagnosis (T0).

	Pyometra group	**Pyometra and endometrial adenocarcinoma group**	***P***
SAA (μg/ml)	32.30 (0.38 – 83.88)	80.80 (5.90 – 96.40)	0.105
Hp (g/l)	4.28 (3.42 – 4.75)	4.63 (4.20 – 4.81)	0.480
Albumin (g/dl)	2.24 (2.02 – 2.75)	2.37 (1.96 – 2.77)	0.457

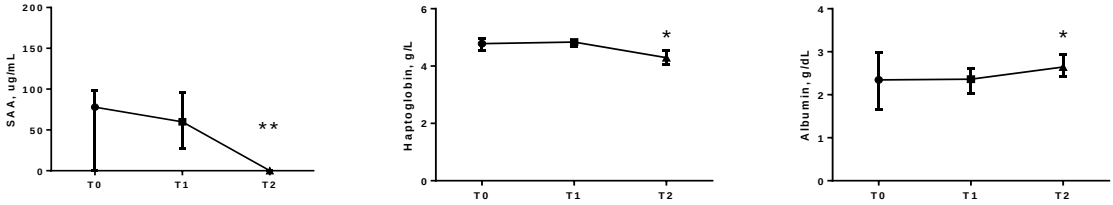
Hp – haptoglobin, IQR – interquartile range, SAA – serum amyloid A

One of the 11 queens of the diseased group which were monitored in the post-operative period developed severe complications (multiple organ failure secondary to septic shock), and died at day 11 post-surgery. At day 10 post-ovariohysterectomy still presented an APP response, characterized by increased SAA (85.9 μg/ml) and Hp (4.5 g/l), and decreased albumin (2.1 g/d). This animal was excluded from the statistical analysis of the evolution of APP in the post-operative period of queens with pyometra.

Serum concentrations of APP of the 10 queens with pyometra that recovered from the disease before surgery (T0), at day two (T1) and at day 10 (T2) after ovariohysterectomy are

presented in figure 4.1. Concentrations of SAA and Hp were significantly lower ($P < 0.01$ and $P < 0.05$, respectively), and of albumin significantly higher ($P < 0.05$) at T2 in comparison with T0. At day 10 post-surgery, these 10 queens presented concentrations of SAA and albumin in the reference range; however, serum Hp, despite being significantly lower than before surgery, was still above the reference range in all queens.

Figure 4.1. Serum concentrations of acute phase proteins before surgery (T0), and at days two (T1) and 10 (T2) after surgery in queens with pyometra (n=10)



* $P < 0.05$ vs. before surgery (T0), ** $P < 0.01$ vs. before surgery (T0), Hp – haptoglobin, SAA – serum amyloid A

Discussion

To the authors` knowledge, this is the first study to comprehensively evaluate the APP response in queens with pyometra.

Acute phase protein responses were already described in mares, cows and bitches with pyometra (Børresen and Skrede, 1980; Dabrowski et al., 2009; Brodzki et al., 2015; Dabrowski et al., 2015; El-Bahr and El-Deeb, 2016). However, to the authors´ knowledge, APP have not been evaluated in queens with pyometra. In the present study, the APP response in feline pyometra was evaluated through an APP profile that included one positive major (SAA), one positive moderate (Hp) and one negative (albumin) APP. Determination of APP profiles, which should include at least one positive major, one positive moderate and one negative APP has been recommended over determination of individual APP, in order to provide a better differentiation between pathological states and more information on the evolution of the disease (Cerón et al., 2008).

The results obtained indicate that an APP response occurs in feline pyometra, since significantly higher serum concentrations of SAA and Hp, and lower albumin were detected in queens with pyometra when compared with healthy control queens. These results are in accordance with previous studies, which described the occurrence of systemic inflammatory response syndrome (SIRS) and increases in inflammatory markers such as 15-keto-(13,14)-dihydro-PGF2α and 6-keto- PGF1α in queens with pyometra (Hagman et al., 2009; Jursza-Piotrowska and Siemieniuch, 2016). In the cat, SAA is considered a positive major APP, characterized by an early and marked increase, and a rapid decline in serum concentrations after an inflammatory stimulus; while Hp is classified as a positive moderate APP, characterized by a moderate and gradual increase and decrease in serum concentrations after an inflammatory stimulus. Albumin is considered the principal negative APP, characterized by a decrease in serum concentrations in inflammation (Eckersall and Bell, 2010; Kann et al., 2012). In this study, magnitude of increase in concentrations of SAA was more pronounced than of Hp. Although caution should be taken if SAA is used as a unique APP, as in this study a lack of increment of this protein was detected in six queens with pyometra. Similarly, SAA concentrations in the reference range were described in some cats with inflammatory diseases (Tamamoto et al., 2008). We consider that this fact could be explained by three possible hypotheses: the method used, which was initially designed for human serum presents a lower sensitivity with feline samples as previously reported (Hansen et al., 2006; Tamamoto et al., 2008), feline SAA possess different isoforms, as previously described in humans and other animal species (Jacobsen et al., 2006; Soler et al., 2011; Li et al., 2012), that could be responsible for the lack of cross-reactivity of antibodies used in the reagents, or the cyclic changes that occur in the uterus during the consecutives estrous cycles and during pregnancy makes this organ less reactive to pathologic stimulus, and consequently a systemic response is only observed in the advanced stages of the disease. Further studies should be undertaken in order to elucidate why SAA is not increased in some cases of inflammatory feline diseases, including pyometra. However, serum Hp was increased in all these six queens at diagnosis.

Furthermore, different studies have described the APP as useful biomarkers in monitoring the post-ovariohysterectomy period in bitches with pyometra (Dabrowski et al., 2007; Dabrowski et al., 2009; Yuki et al., 2010; Dabrowski et al., 2015), similar to what was observed in queens in the present study. In our study, significant decreases in concentrations of a major (SAA) and a moderate APP (Hp), and increases in albumin were detected in the day 10 after

surgery when compared with serum values before surgery, in those cats that presented an adequate response to the treatment. The queen that died of the disease still presented an APP response with elevated SAA and Hp, and decreased albumin concentrations at day 10 post-surgery. This APP dynamic represents a typical APP response, and suggests that APP can be potentially useful biomarkers in monitoring the post-operative period in feline pyometra.

Older age was associated with higher concentrations of SAA (but not of other APP) in cats, which was suggested to be due to the higher incidence of subclinical diseases in geriatric cats (Kann et al., 2012). In the present study, queens of the pyometra group were significantly older than queens of the control group. This difference is related with the fact that feline pyometra is more frequent in middle aged to older queens (Nak et al., 2009; Hagman et al., 2014), while elective ovariohysterectomy is frequently performed at younger ages. However, the reported influence of age (Kann et al., 2012) is not sufficient to explain the magnitude of differences in concentrations of APP between diseased and control queens obtained in this study.

Obesity proved to influence biomarkers of inflammation in cats (Tanner et al., 2007; Tvarijonaviciute et al., 2012; Laflamme, 2012). In the present study, significant differences in body weight were detected between diseased and control cats, which were also mainly related with the difference in the age of the animals studied. However, the magnitude of differences in concentrations of APP reported in feline obesity cases (Tanner et al., 2007; Tvarijonaviciute et al., 2012; Laflamme, 2012) are lower than those observed between diseased and healthy animals included in this study.

The etiology of pyometra is multifactorial (Potter et al., 1991; Miller et al., 2003; Agudelo, 2005; Payan-Carreira et al., 2013; Pires et al., 2016). The repeated uterine exposure to endogenous progesterone and / or exogenous progestogens is considered to be the main factor implicated, however, other factors, including uterine neoplasia have been suggested to be involved in pyometra development in queens (Potter et al., 1991; Miller et al., 2003; Agudelo, 2005; Payan-Carreira et al., 2013; Pires et al., 2016). In the present study, 15 queens of the pyometra group presented a concomitant endometrial adenocarcinoma. Serum amyloid A was reported to be a biomarker of uterine neoplasia in women (Cocco et al., 2009; Cocco et al., 2010). In our study, SAA concentrations were higher in queens with pyometra and a concomitant endometrial adenocarcinoma than in female cats with pyometra without a

concomitant endometrial neoplasia, however, the differences were not significant. This fact could be attributed to the great variation in SAA concentrations in each group. Similarly, no significant differences in serum concentrations of Hp or albumin were detected between the two groups. These data suggest that inflammatory processes occurring in feline pyometra are of similar magnitude regardless of origin, although further long scale studies should be required in order to confirm these observations.

The lack of post-operative samplings in the control group is a limitation of our study, since may hinder a possible additive effect of operative stress on APP. However, as previously stated, APP were determined in the remaining serum samples that were collected for clinical purposes, and no blood samples were collected exclusively for this study. Due to the difficulties in owner compliance for taking blood samples in cats that are recovering correctly from an elective surgical procedure, no post-operative samples were available from queens of the control group.

Conclusions

According with the obtained results, an APP response was detected in queens with pyometra. In addition, APP tended to return to physiologic values after surgery in the queens that recovered from the disease. Therefore, our results suggest that APP could be potentially useful biomarkers in diagnosis and assessment of the post-operative period in feline pyometra.

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Chapter 5

Acute phase proteins response in cats with spontaneous malignant mammary tumors

Vilhena H, Tvarijonaviciute A, Cerón JJ, Figueira AC, Miranda S, Ribeiro A, Canadas A, Dias-Pereira P, Rubio CP, Franco L, Tecles F, Cabeças R, Pastor J, Silvestre-Ferreira AC. Acute phase proteins and biomarkers of oxidative status in feline spontaneous malignant mammary tumors. Vet Comp Oncol 2019;17:394-406

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Vilhena H, Ceron JJ, Figueira AC, Tvarijonaviciute A, Tecles F, Miranda S, Gärtner F, Pastor J, Silvestre-Ferreira AC. Serum acute phase proteins in feline malignant mammary tumors. Oral communication. Research communications of the 24th European College of Veterinary Internal Medicine – Companion Animals Congress Mainz, Germany, 4th to 6th September 2014. J Vet Intern Med 2015;29:423–484

Abstract

Background: Acute phase proteins (APP) change in human breast cancer and in canine mammary tumors, reflecting the associated inflammatory response. However, this information is lacking in feline mammary tumors.

Objectives: The aims were to investigate the APP response in feline spontaneous malignant mammary tumors, to evaluate their relation with clinical and histological findings, and to assess their prognostic value.

Materials and Methods: An APP panel including two positive - serum amyloid A (SAA) and haptoglobin (Hp), and four negative APP - albumin, butyrylcholinesterase (BChE), insulin-like growth factor 1 (IGF1) and paraoxonase 1 (PON1) were determined in serum of 50 queens with mammary carcinomas and of 12 healthy control cats.

Results: At diagnosis, diseased queens presented significantly higher SAA and Hp, and lower albumin and BChE than controls. Different tumor features, including bigger size, ulceration, lymphovascular neoplastic invasion, metastasis in regional lymph nodes and distant organs, advanced clinical stage of the disease, histological type, higher histological grade, necrosis and higher proliferative activity significantly influenced concentrations of APP. Increases in serum Hp and decreases in albumin were significantly associated with presence of neoplastic vascular emboli, metastasis in regional lymph nodes and / or in distant organs; and increases in SAA during the course of the disease were significantly associated with development of distant metastasis. Higher concentrations of albumin and lower serum BChE (< 1.15 μmol/ml.min) at diagnosis were associated with a shorter survival time on multivariate analysis. A tendency for a shorter survival time was also observed in diseased queens with increased concentrations of SAA and decreased serum IGF1 at diagnosis.

Conclusions: According with our results, feline malignant mammary tumors are associated with an APP response. Different clinical and histological features significantly influenced the inflammatory response. Furthermore, some of these analytes proved to have prognostic value. At diagnosis, increases in serum Hp and decreases in albumin may suggest tumor metastization; and increases in SAA during the course of the disease may suggest development of metastasis in distant organs. Decreased serum albumin at diagnosis was associated with a longer survival, and serum BChE < 1.15 μmol/ml.min at diagnosis was associated with a shorter survival time on multivariate analysis.

Key Words: albumin, butyrylcholinesterase, haptoglobin, insulin-like growth factor 1, paraoxonase 1, serum amyloid A.

Introduction

Mammary tumors are among the most frequent neoplasias in female cats (Vascellari et al., 2009; Egenvall et al., 2010). Approximately 80 to 90% are malignant, and most of these present an aggressive behavior and a poor prognosis (Hayes et al., 1981; Ito et al., 1996; Millanta et al., 2002; Figueira et al., 2014). Reported survival times from diagnosis of queens with malignant mammary tumors vary significantly from a few days to several years (Viste et al., 2002; Preziosi et al., 2002; De Campos et al., 2016).

Several studies characterized feline mammary tumors and evaluated related prognostic factors, most of them on clinical, histopathologic and molecular features of these neoplasias (Hayes et al., 1981; Seixas et al., 2011; Hughes and Dobson, 2012; Mills et al., 2015; Zappulli et al., 2015). However, information on clinical value of serum biomarkers related with inflammation in diagnosis, evolution of disease, response to treatment and in prognosis of feline mammary tumors is scarce.

Acute phase proteins (APP) are serum proteins which concentrations are altered in acute or chronic inflammatory conditions of different etiology, including neoplastic diseases (Kann et al., 2012). The APP response is a very fast reaction, which develops before stimulation of the specific immune response, and in many cases even before the onset of clinical signs; consequently, can be considered one of the earliest markers of inflammatory processes or diseases (Petersen et al., 2004; Cerón et al., 2005; Gruys et al., 2005). In the cat, serum amyloid A (SAA) is considered a positive major APP, haptoglobin (Hp) a positive moderate APP, and albumin the main negative APP (Kann et al., 2012; Eckersall and Bell, 2010). Butyrylcholinesterase (BChE) is a serum choline esterase related with inflammation (Da Silva et al., 2010; Zivkovic et al., 2015; do Carmo et al., 2015), and is considered a negative APP in dogs and cats (Da Silva et al., 2010; Tvarijonaviciute et al., 2012a). Insulin-like growth factor 1 (IGF1) is a polypeptide hormone with an important role in the growth and differentiation of different tissues, including the mammary gland (Rinderknecht and Humbel, 1978; Mol et al.,

1996; Ordás et al., 2004), and is also considered a negative APP in dogs (Tvarijonaviciute et al., 2011; Dabrowski et al., 2015). Paraoxonase 1 is an enzyme synthesized by the liver, with antioxidative properties, protecting high- and low-density lipoproteins from peroxidation (Mackness et al., 1991; Aviram et al., 1998), and with anti-inflammatory properties, reducing the production of pro-inflammatory mediators (Watson et al., 1995). It is considered a biomarker of oxidative stress and also a negative APP in dogs and cats (Tvarijonaviciute et al., 2012a; Tecles et al., 2015; Vilhena et al., 2017).

Acute phase proteins have been increasingly studied as biomarkers of neoplastic diseases in human and in veterinary medicine, including in human breast cancer and in canine mammary tumors (Tecles et al., 2009; Planellas et al., 2009; Dowling et al., 2012; Bobin-Dubigeon et al., 2015; Wulaningsih et al., 2015; Machado et al., 2015). However, to the author´s knowledge, there is no information on the APP response in feline mammary tumors.

Thus, the main aim of the present study was to investigate the possible changes in APP in queens with spontaneous malignant mammary tumors. In addition, changes in these analytes related with clinical and histological findings and their prognostic value were evaluated.

Materials and Methods

The present study was approved by the Scientific Council of the School of Agricultural and Veterinary Sciences of the University of Trás-os-Montes and Alto Douro, Vila Real, Portugal.

Animals and samples

Fifty female cats that were presented to three veterinary medical centers from Portugal – Baixo Vouga Veterinary Hospital, University Veterinary Hospital of Coimbra and Veterinary Policlinic of Aveiro – between January 2011 and January 2016 due to the presence of single or multiple masses in the mammary glands, and that were submitted to mastectomy, were included in the study (table 5.1). Queens that presented, at diagnosis, concomitant diseases,

other types of tumors, history of previously diagnosed neoplasia (mammary or other tumors) or with benign mammary lesions were excluded from this research. Data from queens that developed other diseases or other malignant tumors during the follow-up period, and data from queens that were submitted to chemotherapy were only considered for determinations of APP at presentation, but were excluded from the survival analysis.

In all diseased animals included in the study, physical and clinical examination including hematology, serum biochemistry profile, lymph node evaluation, thoracic radiology, abdominal ultrasonography and histopathology of the excised mammary tumors were performed at admission and also in control visits when clinically indicated and at owners consent. Recommended post-surgical control protocol included clinical reevaluations at one and three months after surgery, and then at every three months until death of the animal, or when local disease or distant metastasis were detected. Identification and clinical parameters evaluated included age, breed, weight, neuter status, history of contraceptives administration, tumor size, tumor ulceration, regional lymph node neoplastic invasion, presence of distant metastasis, clinical stage, disease-free interval (DFI; interval of time from surgical treatment to detection of either local tumor recurrence or metastasis in the regional lymph nodes or other organs) and survival time from diagnosis.

Whole blood samples were collected in all the diseased queens included in the study before surgery, and whenever possible and clinically indicated, in subsequent control visits, into EDTA tubes (K3EDTA tubes, Aquisel, Barcelona, Spain) for hematology and into tubes without anticoagulant (Vacuette Z serum clot activator, Greiner Bio-One International GmbH, Kremsmünster, Austria) for serum biochemistry. Within 20 minutes after collection, these were centrifuged (10 min, 2000 x g) and supernatant was used for analyses. Remaining serum samples were stored after use at −80°C. Acute phase proteins were determined in the remaining serum samples that were collected for clinical purposes; no blood samples were collected exclusively for this study. Lymph node evaluation was performed by lymph node palpation and cytology of the enlarged lymph nodes by fine needle aspiration; and thoracic radiology (three projections) and abdominal ultrasonography were performed for clinical staging at diagnosis, and during the follow-up period whenever possible and clinically indicated. Clinical stage was determined according to the modified World Health Organization (WHO) staging criteria (McNeill et al., 2009) (table 5.2).

Serum samples from 12 queens considered clinically healthy based on clinical history, physical examination and results of complementary diagnostic exams, and that required hematological analysis for elective surgical procedures, geriatric check-ups or determination of feline immunodeficiency virus (IV) and feline leukemia virus (FeLV) infection status, were used as controls (table 5.1).

Histopathology

Histopathology analyses were performed at the Laboratory of Veterinary Pathology of the Institute for the Biomedical Sciences Abel Salazar, Porto University (ICBAS-UP), Portugal. After surgery, samples were fixed in 10% buffered formalin, routinely processed and 3µm sections were cut and stained with haematoxylin and eosin. Histological classification of feline mammary tumors was independently performed by two pathologists based on the criteria of the WHO for the histological classification of mammary tumors of domestic animals (Misdorp et al., 1999).

The histological parameters analyzed included tumor type and grade, presence of neoplastic vascular invasion, presence of tumor necrosis and assessment of mitotic counts (determined in 10 high-power fields). Tumor resection margins were assessed in all cases, and classified as tumor infiltrated margins (< 1 mm), narrow tumor free surgical margins (≥ 1 mm to < 3 mm) or as tumor free surgical margins (≥ 3 mm). Queens with tumor narrow free surgical margins or with tumor infiltrated margins were submitted to a second surgery to obtain tumor free wide surgical margins whenever possible and clinically indicated. In queens with multiple tumors, the characteristics of the nodule with larger dimension and with higher histological grade were considered for statistical analysis.

Histological grade of mammary carcinomas was determined in accordance to the Nottingham histological grading system, based on the assessment of three histological features, namely tubule formation, mitotic counts and nuclear pleomorphism (Elston and Ellis, 1991). Mammary carcinomas were classified as grade I (well differentiated), grade II (moderately differentiated) or grade III (poorly differentiated) (Elston and Ellis, 1991).

Table 5.1. Characterization of the female cats with mammary carcinomas (n = 50) and controls (n = 12) included in the study

	Cats with mammary carcinomas	Control cats
Breed		
Domestic short hair	47	11
Persian	3	1
Reproductive status		
intact	25	7
neutered	25	5
Age (years)		
mean (SD)	11.9 (3.2)	7.8 (5.1)
min-max	3-19	1-15
Weight (Kg)		
mean (SD)	3.8 (0.8)	3.7 (0.6)
min-max	2.3-6.3	2.9-5.1
Contraceptives[†]		
Yes	36	1
No	2	8
Tumor size (cm)		
mean (SD)	3.2 (1.8)	-
min-max	0.3-8.0	-
Histopathology		
tubulopapillary	23	-
solid	16	-
cribriform	5	-
mucinous	4	-
SCC	2	-
DFI (months) (n=33) [††]		
median	6.0	-
IQR	4.0-11.0	-
min-max	1.0-28.0	-
MST (months) (n=44) [†††]	11.0	-
IQR	5.0-19.0	-
min-max	0.5-54.0	-

DFI – disease free interval; IQR – interquartile range; max – maximum; MST – median survival time; min – minimum; SCC – squamous cell carcinoma; SD – standard deviation; [†]Data related with history of contraceptives administration was available from 38 queens of the diseased group and from nine queens of the control group. [††]Information concerning the disease free interval was available from 33 diseased queens. [†††]Only queens with tumor related death (n=42) were considered for determination of median survival time.

Table 5.2. Clinical staging of cats with mammary carcinomas according to the modified World Health Organization (WHO) staging criteria (McNeill et al., 2009)

Clinical Stage	T	N	M
I	T_1	N_0	M_0
II	T_2	N_0	M_0
III	$T_{1,2}$	N_1	M_0
	T_3	$N_{0,1}$	M_0
IV	Any T	Any N	M_1

M - distant metastasis; M_0 - no evidence of metastasis; M_1 - evidence of metastasis; N - regional lymph node; N_0 - no histologic/cytologic metastasis; N_1 - histologic/cytologic metastasis; T - primary tumor; T_1 - < 2 cm maximum diameter; T_2 - ≥ 2 to < 3 cm maximum diameter; T_3 - ≥ 3 cm maximum diameter

Acute phase proteins analysis

Acute phase proteins determinations were performed at the Interdisciplinary Laboratory of Clinical Analysis (Interlab-UMU), University of Murcia, Spain. Only serum samples free of hemolysis and lipemia were used for analysis, since significant analytical interference with APP determinations have been reported (Martínez-Subiela and Cerón, 2005; Tecles et al., 2007). Serum concentration of SAA, Hp, albumin, BChE, IGF1 and PON1 were determined in all samples.

Serum amyloid A concentrations were determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical Co., Tokyo, Japan). Briefly, the SAA proteins present in the serum sample react with the anti-SAA antibodies present in the reagent latex, causing agglutination and changing the turbidity of the sample, proportionally to the concentration of SAA. The method was previously validated for use in cats (Hansen et al., 2006). Serum Hp concentrations were determined by use of the hemoglobin-binding method with the use of a commercial kit (Tridelta Development Ltd., Kildare, Ireland). The peroxidase activity of free hemoglobin is inhibited at low pH; however, in this method, the free hemoglobin present in the reagent combines with the Hp molecules of the sample, and the peroxidase activity of the bound hemoglobin is preserved at a low pH, proportionally to the concentration of serum Hp. The method was previously validated for use in cats (Tvarijonaviciute et al., 2012b). Serum albumin was determined using a commercially available kit (Albumin OSR 6102; Olympus Life and Material Science Europe GmbH, Irish branch, Ennis, Ireland) following instructions

of the manufacturer. Serum BChE concentrations were determined by spectrophotometry. Briefly, in this method, the activity of BChE was determined through the assessment of the hydrolysis of the substrate butyrylthiocoline, and using 5,5′-dithiobis-2-nitrobenzoic acid as a chromophore (Tecles et al., 2000). The method was previously validated for use in cats (Tvarijonaviciute et al., 2012b). Serum concentrations of IGF1 were determined using an automated solid-phase, enzyme-labeled immunochemiluminescent assay (Immulite System; Siemens Health Diagnostics, Malvern, USA). Briefly, the assay uses highly-specific antibodies anti-IGF1 present in the reagent to detect serum IGF1, in an acid environment to separate IGF1 from IGF binding proteins. The method was previously validated for use in cats (Tvarijonaviciute et al., 2012b). Serum PON1 concentrations were determined by spectrophotometry. The assay determines PON1 activity by measuring the non-enzymatic hydrolysis of p-nitrophenylacetate (substrate) into p-nitrophenol (Tvarijonaviciute et al., 2012b). The method was previously validated for use in cats (Tvarijonaviciute et al., 2012b). All analyses were performed on an automated biochemistry analyzer (Olympus AU600, Olympus Diagnostica, GmbH, Freiburg, Germany).

Statistical analysis

Results are shown as medians with interquartile range (IQR) unless otherwise stated. Concentrations of APP with results lower than detection limit were set as equal to the detection limit for further statistical analysis. D'Agostino & Pearson omnibus normality test was used to assess normality. As most data were not normally distributed, a Kruskal-Wallis test followed by the Dunn's multiple comparison test were used. Differences in concentrations of the analytes between cats with mammary tumors at presentation and healthy control cats were assessed. In addition, the possible changes in analytes according with clinical and histological features of the neoplasms, namely tumor size, tumor ulceration, regional lymph node neoplastic invasion, presence of distant metastasis, clinical stage, tumor histological type and grade, presence of neoplastic vascular invasion, presence of necrosis and mitotic counts, were evaluated in the population of cats with mammary tumors, and also in comparison with healthy cats. Spearman correlation coefficient was used to determine correlations between concentrations of APP and size of tumor. Furthermore, in the diseased queens, differences between concentrations of APP at presentation and at different time points during the evolution of the disease were also assessed. The Cox proportional hazards model

was used to investigate the prognostic value of serum analytes on overall survival by adjusting for confounding factors. Results of the multivariate analyses are presented as hazard ratios (HR) with 95% confidence intervals (CI). Overall survival was plotted using the Kaplan-Meier method, with statistical analysis by means of the Log rank test. In order to discover the best cut-off point for those variables that were influenced by clinical outcome, different values were assessed, and the value that separated most significantly Kaplan-Meier curves of two resulting groups were selected. Statistical analysis was performed using Graph Pad Prism (version 6, GraphPad Software Inc., California, USA) and SPSS (version 19.0, SPSS, IL, USA). A *P* value < 0.05 was used to determine the level of statistical significance.

Results

Characterization of the diseased (n = 50) and healthy control (n = 12) cats is presented in table 5.1. Most of the animals included in the study were domestic short-hair cats (47 out of the 50 diseased cats, and 11 out of the 12 controls), and approximately half in each group were intact and half were neutered at presentation. Most of the queens of the diseased group had history of contraceptives administration. No statistically significant differences were detected between body weight of the two groups of cats (*P* = 0.886), but control cats were significantly younger than diseased queens (*P* = 0.006). Most of the diseased queens presented tubulopapillary (n = 23) or solid (n = 16) carcinomas. The group of queens with carcinomas presented a median DFI of six months, and a median survival time (MST) of 11 months.

Acute phase proteins at presentation

Data, presented as medians and IQR, of serum concentrations of APP in queens with malignant mammary carcinomas at presentation and controls are presented in table 5.3. Serum concentrations of the positive APP SAA and Hp were significantly higher, and of the negative APP albumin and BChE were significantly lower in cats with malignant mammary tumors than in healthy control cats.

Table 5.3. Serum concentrations (medians and IQR) of acute phase proteins in queens with mammary carcinomas (n = 50) at presentation and in control cats (n = 12). Significant differences are presented in bold

	Mammary carcinoma group	Control group	*P*
SAA (μg/ml)	0.4 (0.38 - 15.2)	0.38 (0.38-0.38)	**0.023**
Hp (g/l)	3.8 (2.7 - 5.5)	3.1 (2.7 - 3.6)	**0.027**
Albumin (g/dl)	2.8 (2.4 - 3.0)	3.1 (2.8 - 3.2)	**0.019**
BChE (μmol/ml·min)	1.5 (1.0 - 1.8)	2.4 (1.6 - 2.6)	**0.002**
IGF1 (ng/ml)	300 (220 – 423)	280 (129 - 427)	0.549
PON1 (IU/ml)	3.5 (2.8 - 4.1)	3.8 (2.8 - 4.8)	0.402

BChE – butyrylcholinesterase, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, PON1 – paraoxonase 1, SAA – serum amyloid A

Acute phase proteins at presentation in accordance to clinical and histopathological features of mammary tumors

Correlations between the size of the tumor and concentrations of APP are presented in table 5.4. Data, presented as medians and IQR, of serum concentrations of APP of cats with malignant mammary carcinomas at presentation in accordance to clinical parameters - tumor size, tumor ulceration, regional lymph node neoplastic invasion, presence of distant metastasis and clinical stage; and histological parameters – tumor type and grade, presence of neoplastic vascular invasion, presence of necrosis and mitotic counts are presented in tables 5.5.1 and 5.5.2.

Serum concentrations of SAA and Hp were significantly positively correlated, and albumin, BChE, IGF1 and PON1 were significantly negatively correlated with tumor dimensions, however, the correlation coefficients were only weak to moderate. Concentrations of Hp were significantly higher, and of BChE and PON1 were significantly lower in queens with tumors ≥ 2 cm than in queens with tumors < 2 cm. Moreover, concentrations of SAA and Hp were significantly higher, and of albumin and BChE were significantly lower in queens with tumors ≥ 2 cm than in control queens.

Queens with ulcerated tumors showed significantly higher serum Hp concentrations than queens with non-ulcerated tumors and than controls; and also significantly lower albumin than controls.

Queens with neoplastic vascular emboli presented significantly lower albumin than queens without evidence of neoplastic vascular invasion. No other significant differences in any of the evaluated analytes were detected between queens with and without neoplastic vascular invasion, metastasis in the regional lymph nodes or metastasis in distant organs. However, compared with healthy controls, diseased queens with neoplastic vascular invasion and cats with metastasis in distant organs presented significantly higher Hp and lower albumin, and queens with metastasis in regional lymph nodes had significantly higher Hp.

No significant differences were detected in concentrations of APP between diseased queens in different clinical stages. However, when compared with controls, stages higher than I showed higher SAA values, stages higher than II showed lower values for albumin and BChE, and stage IV showed higher values for Hp.

Cats with tubulopapillary adenocarcinomas presented significantly lower albumin and BChE than controls, and significant decreases in serum BChE were detected in animals of the group of other carcinomas when compared with controls. Queens with mammary carcinomas with higher histological grade presented significantly higher Hp and lower albumin and BChE than controls.

Queens with tumor necrosis had significantly higher Hp than queens without evidence of necrosis, and also significantly higher Hp and lower albumin than controls.

Concentrations of albumin tended to decrease in queens with tumors with higher mitotic counts, while serum BChE tended to decrease in tumors with lower proliferation activity.

Table 5.4. – Correlations between size of mammary tumor and serum concentrations of acute phase proteins. Significant correlations are presented in bold

	ρ	***P***
SAA (μg/ml)	**0.278**	**0.048**
Hp (g/l)	**0.304**	**0.032**
Albumin (g/dl)	**-0.277**	**0.049**
BChE (μmol/ml·min)	**-0.437**	**0.002**
IGF1 (ng/ml)	**-0.448**	**0.001**
PON1 (IU/ml)	**-0.358**	**0.011**

BChE – butyrylcholinesterase, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, PON1 – paraoxonase 1, SAA – serum amyloid A, ρ - Spearman correlation coefficient

Table 5.5.1. Serum concentrations of acute phase proteins (medians with IQR) in cats with mammary carcinomas according with clinical parameters

		n	**SAA** (μg/ml)	**Hp** (g/l)	**Alb** (g/dl)	**BChE** (μmol/ml·min)	**IGF1** (ng/ml)	**PON1** (IU/ml)
Tumor size	< 2 cm	14	0.38 (0.38-0.60)	**3.27 (2.67-3.50)a**	2.94 (2.66-3.10)	**2.00 (1.60-2.30)a**	387.0 (281.5-467.5)	**4.07 (3.67-4.99)b**
	≥ 2 cm	36	**0.70 (0.38-35.63)*** **	**4.77 (3.03-5.62)*a**	**2.77 (2.25-2.97)***	**1.30 (0.75-1.66)**a**	272.0 (194.0-372.0)	**3.36 (2.72-3.72)b**
Ulceration	positive	19	**0.80 (0.38-29.50)*** **	**5.27 (3.80-5.62)**c**	**2.60 (2.18-2.94)****	**1.30 (1.05-1.65)****	286.0 (211.5-371.5)	3.38 (2.70-3.72)
	negative	31	**0.38 (0.38-3.10)****	**3.28 (2.51-4.81)c**	2.87 (2.69-3.06)	**1.60 (0.95-2.10)***	323.0 (223.0-423.0)	3.61 (2.84-4.61)
Ln. invasion	positive	27	**0.70 (0.38-12.0)*** **	**4.76 (2.92-5.54)***	2.81 (2.28-2.99)	**1.35 (0.93-1.80)***	306.0 (231.5-412.5)	3.41 (2.65-3.88)
	negative	23	**0.40 (0.38-47.5)****	3.44 (2.74-5.47)	2.87 (2.47-3.00)	**1.50 (1.00-2.20)***	279.5 (210.3-423.0)	3.60 (2.92-4.32)
Distant mets.	positive	10	**0.38 (0.38-16.65)****	**4.77 (4.20-5.52)***	**2.84 (2.39-2.91)***	**1.30 (0.60-1.45)****	332.0 (250.3-565.0)	2.81 (2.65-3.52)
	negative	40	**0.40 (0.38-7.90)*** **	3.53 (2.74-5.50)	2.85 (2.40-2.99)	**1.60 (1.03-2.00)***	299.5 (218.5-420.0)	3.60 (2.86-4.46)
Clinical stage	I	7	0.38 (0.38-0.50)	3.04 (2.71-3.40)	3.01 (2.80-3.17)	2.20 (1.53-2.50)	412.0 (307.3-527.3)	3.81 (3.42-5.15)
	II	3	**0.40 (0.38-66.50)***	3.75 (3.00-5.47)	2.92 (2.67-2.99)	1.40 (0.80-2.30)	338.0 (221.3-440.5)	3.54 (2.47-3.99)
	III	30	**0.40 (0.38-15.20)*** **	3.85 (2.66-5.69)	**2.76 (2.26-2.95)***	**1.60 (1.00-1.80)***	279.5 (188.0-363.0)	3.59 (2.87-4.61)
	IV	10	**0.38 (0.38-16.65)****	**4.77 (4.20-5.52)***	**2.84 (2.39-2.91)***	**1.30 (0.60-1.45)****	332.0 (250.3-565.0)	2.81 (2.65-3.52)

Alb – albumin, BChE – butyrylcholinesterase, DFI – disease free interval, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, PON1 – paraoxonase 1, IQR – interquartile range, Ln – lymph node, Mets – metastasis, MST – median survival time, SAA – serum amyloid A.

Significant differences are presented in bold: * $P < 0.05$ with controls, ** $P < 0.01$ with controls, *** $P < 0.001$ with controls, a and b – $P < 0.05$ between tumors < 2 cm and tumors ≥ 2 cm, c – $P < 0.01$ between ulcerated and non-ulcerated tumors.

Table 5.5.2. Serum concentrations of acute phase proteins (medians with IQR) in cats with mammary carcinomas according with histopathological parameters

		n	**SAA** (μg/ml)	**Hp** (g/l)	**Alb** (g/dl)	**BChE** (μmol/ml·min)	**IGF1** (ng/ml)	**PON1** (IU/ml)
Histopathology	tubulopapillary	23	**0.38 (0.38-0.95)****	3.44 (2.66-4.76)	**2.82 (2.56-2.93)***	**1.30 (1.00-1.80)***	258.5 (205.3-396.5)	3.64 (2.71-3.98)
	solid	16	**0.38 (0.38-25.55)****	4.99 (3.14-6.32)	2.86 (2.06-3.09)	1.75 (1.30-2.05)	371.5 (297.5-420.0)	3.39 (2.92-4.09)
	cribriform	5	**0.70 (0.38-122.1)****	3.43 (2.88-5.23)	2.87 (1.98-3.10)	1.80 (1.00-2.75)	375.5 (176.3-542.5)	4.00 (2.78-4.95)
	other tumors	6	**8.80 (0.38-98.40)*****	5.51 (2.77-6.10)	2.95 (2.23-3.01)	**1.40 (0.70-1.60)***	292.5 (229.0-448.0)	3.38 (2.74-4.00)
Grade #	I	4	**0.70 (0.38-1.03)***	3.09 (2.38-6.59)	2.91 (2.79-2.94)	1.00 (0.18-2.13)	219.5 (168.5-313.3)	3.80 (3.07-3.87)
	II	14	**0.40 (0.38-57.0)*****	3.47 (2.79-5.46)	2.74 (2.54-3.02)	1.60 (1.10-2.20)	358.5 (221.5-433.0)	3.89 (3.38-4.77)
	III	26	**0.38 (0.38-3.00)****	**4.64 (2.66-5.43)***	**2.84 (2.30-2.91)***	**1.50 (1.00-1.80)***	303.0 (217.3-391.8)	3.36 (2.70-3.78)
Vasc. Invasion	positive	24	**0.38 (0.38-1.20)****	**4.39 (3.27-5.43)***	**2.78 (2.23-2.87)**a**	**1.30 (1.00-1.63)****	279.5 (217.3-420.0)	3.32 (2.64-3.73)
	negative	26	**0.90 (0.38-86.18)*****	3.48 (2.68-5.60)	**2.92 (2.59-3.06)a**	**1.60 (0.90-2.20)***	318.0 (214.0-423.0)	3.69 (3.11-4.46)
Necrosis	positive	38	**0.38 (0.38-8.80)*****	**4.64 (3.27-5.63)*b**	**2.78 (2.37-2.96)***	**1.40 (1.10-1.80)***	302.5 (212.8-425.5)	3.54 (2.71-4.59)
	negative	12	**0.50 (0.38-62.70)*****	**2.80 (2.03-3.88)b**	2.94 (2.62 -3.06)	**1.50 (0.50-2.20)***	286.0 (232.0-399.0)	3.57 (2.85-3.89)
Mitotic counts	0-9	7	**2.00 (0.38-57.68)****	3.85 (2.10-6.37)	2.76 (2.02-3.00)	**1.25 (0.40-1.53)***	304.0 (265.3-363.0)	3.06 (2.43-3.53)
	10-19	10	**0.70 (0.40-6.88)*****	3.57 (3.27-6.71)	2.93 (2.79-2.98)	**1.35 (0.43-1.93)***	244.0 (200.5-407.0)	3.86 (3.55-4.57)
	≥ 20	33	**0.38 (0.38-2.65)****	4.31 (2.74-5.52)	**2.73 (2.28-2.98)***	1.60 (1.10-1.95)	323.0 (211.8-430.5)	3.59 (2.75-4.33)

Alb – albumin, BChE – butyrylcholinesterase, DFI – disease free interval, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, IQR – interquartile range, MST – median survival time, PON1 – paraoxonase 1, SAA – serum amyloid A, Vasc. – vascular.

- According with the Nottingham grading system,[41] histological grade was not determined for mucinous and squamous cell carcinomas.

Significant differences are presented in bold: * $P < 0.05$ with controls, ** $P < 0.01$ with controls, *** $P < 0.001$ with controls, a – $P < 0.05$ between cats with and without neoplastic vascular invasion, b- $P < 0.05$ between tumors with and without necrosis.

Acute phase proteins at follow-ups

Differences in concentrations of APP (presented as medians and IQR) in queens with mammary carcinomas without distant metastasis at presentation, and concentrations at the time of diagnosis of distant metastasis are presented in table 5.6 (n = 11). Serum concentrations of SAA were significantly higher at time of detection of distant (thoracic or abdominal) metastasis than at diagnosis.

Table 5.6. Serum concentrations (medians and IQR) of acute phase proteins in queens with mammary carcinomas at diagnosis and at time of detection of distant metastasis (n = 11). Significant differences are presented in bold

	Diagnosis[#]	**Distant Metastasis**[##]	***P***
SAA (μg/ml)	**0.74 (0.38-1.50)**	**27.20 (0.66-44.78)**	**0.035**
Hp (g/l)	3.90 (3.31-4.91)	4.55 (3.49-6.62)	0.106
Albumin (g/dl)	2.76 (2.45-3.15)	2.72 (2.57-2.98)	0.898
BChE (μmol/ml·min)	1.35 (0.55-1.98)	1.15 (1.03-1.78)	0.813
IGF1 (ng/ml)	286.0 (203.0-456.0)	217.0 (179.0-383.5)	0.148
PON1 (IU/ml)	3.60 (2.41-4.76)	3.80 (3.06-4.46)	0.769

– Data at time of diagnosis of queens with mammary carcinomas without distant metastasis; ## – Data at time of diagnosis of distant metastasis. BChE – butyrylcholinesterase, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, PON1 – paraoxonase 1, SAA – serum amyloid A

Overall survival

Cox regression hazards model was applied to estimate the impact of APP and antioxidants (determined at diagnosis) on overall survival (table 5.7 and figures 5.1-5.4). The multivariate analysis revealed that concentrations of albumin and of BChE at diagnosis were independent predictors of mortality. Queens with decreased serum albumin concentrations at diagnosis presented a longer survival time than queens with serum albumin in the reference range. Moreover, Kaplan-meier curves showed that serum concentrations of BChE at diagnosis lower than 1.15 μmol/ml.min were significantly related to a shorter survival. Diseased queens with serum BCHE < 1.15 μmol/ml.min had a MST of 9.0 months (95% CI: 2.4-15.6 months), while cats with BChE > 1.15 μmol/ml.min presented a MST of 16.9 months (95% CI: 12.7-21.0 months) (figure 5.3). Although the differences were not significant, a tendency for a shorter survival time was also observed in diseased queens with increased SAA and decreased IGF1.

Table 5.7. Cox regression hazard model analysis of survival of the acute phase proteins analyzed

Variable	Hazard ratio (95% CI)	*P*
SAA (μg/ml)	1.02 (0.99-1.03)	0.061
Hp (g/l)	1.22 (0.88-1.71)	0.238
Albumin (g/dl)	20.20 (1.18-344.91)	**0.038**
BChE (μmol/ml·min)	13.11 (2.52-68.31)	**0.002**
IGF1 (ng/ml)	0.99 (0.99-1.00)	0.070
PON1 (IU/ml)	0.78 (0.26-2.34)	0.656

BChE – butyrylcholinesterase, CI – confidence interval, Hp – haptoglobin, IGF1 – insulin-like growth factor 1, PON1 – paraoxonase 1, SAA – serum amyloid A

Figures 5.1-5.4. Kaplan-Meier curves of serum amyloid A (1), albumin (2), butyrylcholinesterase (3) and insulin-like growth factor 1 (4)

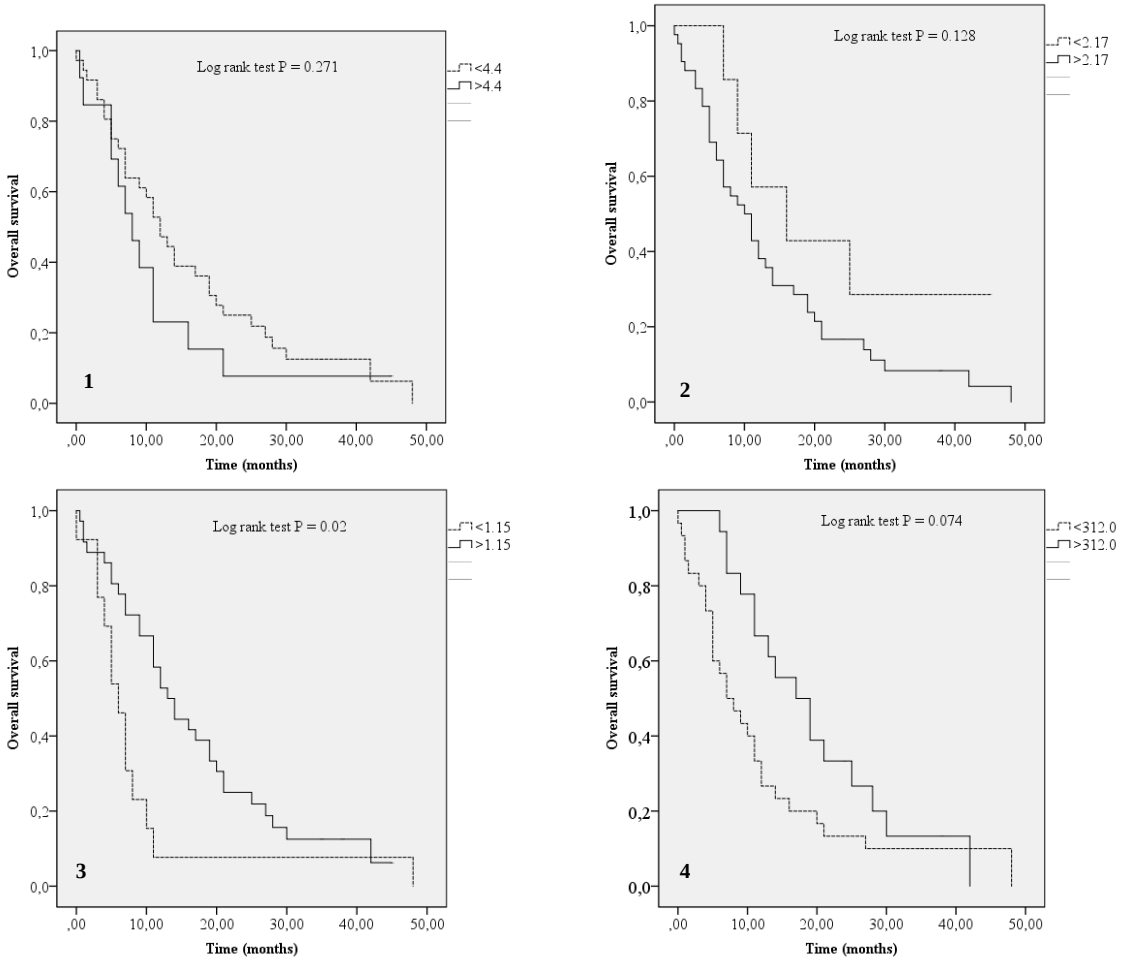

Figure 5.1. Kaplan-Meier curve of serum amyloid A. Diseased queens with serum concentrations of SAA at diagnosis > 4.4 µg/ml had a shorter survival time than cats with SAA < 4.4 µg/ml, but the difference was not significant (*P* = 0.271)

Figure 5.2. Kaplan-Meier curve of albumin. Diseased queens with serum concentrations of albumin at diagnosis > 2.17 g/dl had a shorter survival time than cats with albumin < 2.17 g/dl, but the difference was not significant (*P* = 0.128)

Figure 5.3. Kaplan-Meier curve of butyrylcholinesterase. Diseased queens with serum concentrations of BChE at diagnosis < 1.15 µmol/ml.min had a significantly shorter survival time (MST 9.0 months, 95% CI: 2.4-15.6 months) than cats with BChE > 1.15 µmol/ml.min (MST 16.9 months, 95% CI: 12.7-21.0 months) (*P* = 0.02)

Figure 5.4. Kaplan-Meier curve of insulin-like growth factor 1. Diseased queens with serum concentrations of IGF1 at diagnosis < 312.0 ng/ml had a shorter survival time than cats with IGF1 > 312 ng/ml, but the difference was not significant (*P* = 0.074)

Discussion

Inflammation was proved to be associated with different phases of development of several tumors in humans and animals (Hanahan and Weinberg, 2011; Zappulli et al., 2015; Raposo et al., 2015; Carvalho et al., 2016a). Furthermore, development of an APP response was previously described in human breast cancer and in canine mammary tumors (Tecles et al., 2009; Planellas et al., 2009; Dowling et al., 2012; Bobin-Dubigeon et al., 2015; Wulaningsih et al., 2015; Machado et al., 2015). Acute phase proteins were also proved to be clinically useful biomarkers in feline oncologic diseases (Selting et al., 2000; Tamamoto et al., 2013; Winkel et al., 2015), however, to our best knowledge, this is the first study to comprehensively evaluate the APP response of feline spontaneous malignant mammary tumors.

In the present study, the APP response was evaluated through an APP profile, including a positive major (SAA), a positive moderate (Hp) and four negative (albumin, BChE, IGF1 and PON1) APP. The evaluation of APP profiles that include at least one positive major, one positive moderate and one negative APP are recommended over determination of single APP, in order to better differentiate between pathological states and to obtain information on the evolution of the disease (Gruys et al., 2005; Cerón et al., 2008).

The present study revealed that feline spontaneous malignant mammary tumors are associated with an APP response since significant changes in concentrations of APP were detected in diseased queens when compared with controls.

The overall group of queens with malignant mammary tumors (n = 50) presented, at diagnosis, significantly higher serum concentrations of SAA and Hp, and significantly lower serum albumin and BChE than controls. Significant increases in concentrations of SAA and Hp, and decreases in albumin and BChE were also reported in female dogs with mammary tumors (Tecles et al., 2009; Planellas et al., 2009; Machado et al., 2015). However, canine mammary tumors were considered weak inducers of APP, unless if they were of big

dimensions, of specific histopathological types or were associated with metastasis, ulceration or secondary inflammation (Thougaard et al., 1999; Tecles et al., 2009; Planellas et al., 2009; Yuki et al., 2011). In the present study, the size of the tumor also influenced the development of an APP response, however, significant changes were also observed in smaller tumors, suggesting that mammary tumors may induce a stronger APP response in cats than in dogs. Significant changes in APP were also associated with other tumor characteristics assessed in this study such as presence of ulceration, neoplastic emboli in lymphatic vessels, metastasis in regional lymph nodes and distant organs, histological type and grade, necrosis and higher proliferative activity. In general, the APP response was higher in tumors that presented features associated with a worst prognosis, suggesting that a systemic inflammatory process is related with more severe and complicated cases of feline malignant mammary tumors.

Insulin-like growth factor 1 was also associated with pathogenesis of canine mammary neoplasia, with significantly higher concentrations of serum and tissular IGF1 being described in malignant tumors when compared with benign tumors and healthy controls (Queiroga et al., 2008; Queiroga et al., 2010). Insulin-like growth factor 1 was also proved to be implicated in proliferation of mammary tissues in feline fibroadenomatous change (Ordás et al., 2004). However, in our study, no significant differences in serum concentrations of IGF1 were detected between diseased queens at diagnosis and controls. Moreover, no significant differences in serum IGF1 were detected between the different groups of diseased queens according with the clinical and histopathological parameters evaluated, or between the different groups and controls.

Tumor size, mainly if determined by tumor diameter, is considered as one of the most important prognostic factors of feline malignant mammary tumors (Viste et al., 2002; Morris, 2013; Zappulli et al., 2015), and is a factor considered in the TNM staging system (McNeill et al., 2009). In accordance with our results, size of tumor significantly influenced concentrations of APP of queens with spontaneous mammary cancer. Tumor dimensions also influenced concentrations of APP in female dogs with mammary tumors, however, apparently in a smaller magnitude than in cats. In the study of Tecles et al. (2009), significantly higher concentrations of C-reactive protein (CRP) were detected in dogs with tumors > 5 cm in diameter than in healthy controls animals, however, no significant changes in serum SAA, Hp or albumin were detected in diseased bitches according with tumor size. Moreover, no differences in serum CRP and Hp according with tumor size were detected in the study of

Planellas et al. (2009). In our study, significant positive and negative correlations were detected between size of tumor and positive and negative APP, respectively; and significant differences in concentrations of APP were detected between queens with bigger and smaller tumors, and between queens with bigger tumors and healthy cats. These results suggest that tumor dimensions are related with the systemic inflammatory response associated with malignant mammary tumors in the feline species. Nonetheless, although significant, the correlation coefficients observed between tumor dimensions and concentrations of APP were weak to moderate, which could suggest that despite likely dependent to each other, other factors than tumor size influence serum APP activity.

Tumor ulceration is another factor reported to influence changes in APP in canine mammary tumors (Tecles et al., 2009; Planellas et al., 2009). As in dogs, tumor ulceration was associated with significant changes in concentrations of APP in our study, showing that tumor ulceration is also implicated in the inflammatory response of feline mammary cancer.

Increases in CRP, SAA and Hp have been detected in female dogs with mammary cancer and metastasis (clinical stages IV and V) when compared with dogs without metastasis and with controls in the study of Tecles et al. (2009), however, no significant differences in CRP and Hp were detected between dogs with and without lymph node metastasis in the study of Planellas et al. (2009). In the feline species, an in vitro study showed that SAA promotes invasion of mammary carcinoma (Tamamoto et al., 2014). In the present study, changes in concentrations of APP in accordance with different stages of the metastatic process were assessed, including presence of neoplastic vascular invasion, metastasis in regional lymph nodes and metastasis in distant organs. Serum Hp at diagnosis was higher in cases of neoplasms with vascular invasion, with metastasis in regional lymph nodes and with metastasis in distant organs; and in cases of vascular invasion and in cases of metastasis in distant organs also serum concentrations of albumin were lower than in controls, suggesting these analytes might be clinically useful in assessment of metastization of feline mammary cancer.

Furthermore, despite no significant differences in concentrations of APP were detected between groups of diseased queens according with clinical stage as detected in canine mammary tumors in the study of Tecles et al. (2009), concentrations of SAA and Hp tended to increase, and of albumin and BChE tended to decrease in diseased queens in higher clinical stages. The changes in APP according with the clinical stage reflect the influence of tumor

size and the presence/absence of metastasis in regional lymph nodes and/or in distant organs in concentrations of these analytes. Nevertheless, results concerning queens with metastasis in distant organs (clinical stage IV) should be interpreted with caution, since only 10 queens of the diseased group presented metastasis in thoracic or abdominal organs at diagnosis. Further studies, with a higher number of female cats with mammary cancer with distant metastasis should be performed to clarify the clinical value of these parameters in queens with advanced disease.

Acute phase proteins were proved to be clinical useful biomarkers in monitoring evolution and/or response to treatment of different feline diseases (Webb et al., 2008; Leal et al., 2014; Winkel et al., 2015; Vilhena et al., 2018). However, to the author´s knowledge, the evolution of these analytes during the course of feline malignant mammary tumors has not been studied. According with our results, increases in serum concentrations of SAA during the course of disease in queens with a previous malignant mammary tumor might suggest development of metastasis in distant organs. However, these results should also be interpreted with caution because of the small sample size (n = 11). Although presence of lymphovascular and regional lymph node neoplastic invasion was assessed, the present study was not designed to detect micrometastasis in distant organs. Future studies, with a higher number of animals and with the use of more sensitive imaging diagnostic exams (such as computed tomography and magnetic resonance) to detect early metastasis should be undertaken in order to elucidate the clinical significance of these and others APP in carcinogenesis, and in monitoring evolution and response to treatment of feline malignant mammary tumors.

Significant differences in concentrations of APP between diseased and control animals were detected according with the histological type of the tumor. Significant changes in APP according with specific histopathological types have also been described in canine mammary tumors, including significantly higher CRP in dogs with mammary carcinomas (Planellas et al., 2009) and with complex mammary carcinomas (Yuki et al., 2011) when compared with controls, and a marked increase in serum CRP reported in one female with mammary fibrossarcoma (Planellas et al., 2009). These changes probably reflect differences in the inflammatory response associated with the different histological types, but future studies are necessary in order to better characterize the inflammatory histological features of the different tumor types.

Histological grading of mammary tumors was based on the assessment of three morphological features, namely degree of tubule formation, nuclear pleomorphism, and mitotic counts (Elston and Ellis, 1991). Tumor proliferation activity, assessed by different methods including mitotic counts, mitotic index, proliferation index of Ki-67 and proliferating cell nuclear antigen (PCNA) among others has been considered an important prognostic factor of canine and feline mammary tumors (Hughes and Dobson, 2012; Santos et al., 2013; Mills et al., 2015; Carvalho et al., 2016b). Histological grade has also been described as an important prognostic factor of feline malignant mammary tumors (Seixas et al., 2011; Mills et al., 2015; Zappulli et al., 2015). In our study, significant changes in concentrations of APP were detected in queens with malignant mammary tumors according with the proliferative activity (assessed by determination of mitotic counts) and the histological grade, showing that these features also influence the inflammatory response of feline mammary cancer. To the author´s knowledge, information regarding the influence of tumor proliferative activity and tumor histological grading of canine mammary tumors in concentrations of APP is scarce (Machado et al., 2015).

Tumor necrosis is reported to stimulate a pro-inflammatory tumor microenvironment, which enhances the tumor-promoting potential (Hanahan and Weinberg, 2011). Our results revealed that tumor necrosis was associated with significant changes in concentrations of APP, probably reflecting the changes in tumor microenvironment, and suggesting that the presence of tumor necrosis also influences the inflammatory response. To the authors' knowledge, information concerning the influence of tumor necrosis in concentrations of APP of canine mammary tumors is lacking.

In the present study, queens with mammary adenocarcinomas were significantly older than queens of the control group. This difference is related with the fact that feline mammary tumors are more frequent in middle aged to older queens (Millanta et al., 2012; Islam et al., 2012; Soares et al., 2016), while the control group included queens presented for elective ovariohysterectomy and assessment of the FIV and FeLV infection status, which are frequently performed at younger ages. Increasing age was associated with higher concentrations of SAA (but not of other APP) in felines, which was attributed to the higher incidence of subclinical diseases in geriatric cats (Kann et al., 2012). However, the reported influence of age in concentrations of APP (Kann et al., 2012) is not sufficient to explain the

magnitude of differences in concentrations of APP between diseased and control queens obtained in this study.

Several serum and tissular biomarkers of inflammation, including APP, have been implicated in prognosis of human breast cancer (Lee et al., 2006; Choi et al., 2009; Liu et al., 2015; Yue et al., 2017; Petekkaya et al., 2017; Hwang et al., 2017; Chen et al., 2018). Similar information concerning canine and feline mammary tumors is scarce (Carvalho et al., 2014; Martins et al., 2016). The multivariate analysis of the impact of APP, determined at diagnosis, on overall survival of queens with mammary carcinomas revealed that serum concentrations of albumin and BChE were independent predictors of mortality. Interestingly, results concerning albumin showed that diseased cats with concentrations in the reference range at diagnosis had 20.2 times more chances of having a shorter survival than queens with lower concentrations. Pre-treatment concentrations of albumin have also been implicated in survival of women with breast cancer, however, as expected and in contrast with our results, decreased concentrations were associated with a worst prognosis (Liu et al., 2015; Al Murri et al., 2007). Moreover, in our study, diseased queens with neoplastic vascular invasion and metastasis in distant organs, which are associated with a worst prognosis, had lower albumin than healthy controls cats. Further studies should be performed in order to clarify the prognostic significance of altered concentrations of albumin in feline mammary cancer. Kaplan-Meier curves showed that pre-treatment serum BChE concentrations lower than 1.15 μmol/ml·min were significantly related to a shorter survival time. These results provide important clinical information concerning prognosis, and might be important in treatment decision of feline mammary cancer. Increases in concentrations of SAA and decreases in IGF1 were associated with a worst prognosis in women with breast cancer (Biran et al., 1986; Pierce et al., 2009; Tas et al., 2014), while increased IGF1 was associated with a poor prognosis in dogs with mammary tumors (Queiroga et al., 2010). In our study, a tendency to a shorter survival was also observed in queens with increased SAA and decreased IGF1, however, the results obtained were not significant. Future studies should be performed in order to confirm or contradict these results.

During the study period, only a small number of queens were presented due to presence of mammary hyperplasias, dysplasias and benign mammary tumors, and for that reason were not included in this research. The lack of information concerning the APP response in queens with benign mammary lesions and benign mammary tumors is a limitation of this study, since

detection of serum biomarkers that could aid in differentiation of benign conditions from malignant mammary tumors before mastectomy and histopathology would have a major clinical relevance in practice. Further studies should be performed in order to evaluate the differences in the APP response between queens with mammary gland hyperplasias, dysplasias, benign and malignant mammary tumors.

Conclusions

According with our results, feline spontaneous malignant mammary tumors are associated with an APP response. Different tumor features, including bigger size, ulceration, lymphovascular neoplastic invasion, metastasis in regional lymph nodes and distant organs, advanced clinical stage of the disease, histological type, higher histological grade, necrosis and higher proliferative activity influenced the inflammatory response associated with feline mammary carcinomas. Furthermore, some of these analytes proved to have prognostic value. At diagnosis, increases in serum Hp and decreases in albumin may suggest tumor metastization; and increases in SAA during the course of the disease may suggest development of metastasis in distant organs. Decreased albumin was associated with a longer survival, and serum BChE <1.15 μmol/ml.min was associated with a shorter survival time on multivariate analysis.

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Chapter 6

General Discussion

General Discussion

The APP are being increasingly used in human and in veterinary medicine as biomarkers of general health screening, diagnosis, monitoring the evolution of disease, monitoring the response to treatment and prognosis of different diseases (Eckersall and Bell, 2010: Kann et al., 2012; Parrinello et al., 2015). However, the knowledge related with the APP response and with the clinical application of APP in feline medicine is still scarce.

This investigation had the main objective of contributing to the knowledge of the clinical applications of APP in feline medicine, through the study of the APP response in different feline diseases, including inflammatory, infectious and neoplastic diseases. To the authors' knowledge, these were the first studies to comprehensively evaluate the APP response in cats naturally infected with hemoplasmas, including infections with one agent and co-infections with different species of hemoplasmas, in cats with pyometra and in cats with spontaneous malignant mammary tumors. All these feline conditions induced and APP response, and therefore, APP proved to be clinically useful biomarkers of these infections / diseases.

In these studies, the APP responses were evaluated through an APP profile, including a positive major, a positive moderate and at least one negative APP. The evaluation of APP profiles that include at least one positive major, one positive moderate and one negative APP were recommended over determination of single APP, in order to differentiate the acute from the chronic phase, and to obtain information on the evolution of the diseases (Gruys et al., 2005; Cerón et al., 2008; Korman et al., 2012). In the studies of feline natural infection with hemoplasmas and of feline pyometra, the APP responses were explored through determination of serum concentrations of SAA, Hp and albumin, which are APP evaluated in several feline diseases (Kajikawa et al., 1999; Giordano et al., 2004; Cerón et al., 2005). As expected, SAA and Hp presented a positive major and moderate APP behavior (respectively), and albumin a negative APP activity. In the study of feline spontaneous malignant mammary tumors, besides SAA, Hp and albumin, the APP response was also evaluated through determination of concentrations of serum IGF1, BChE and PON1. The biological role of

BChE and PON1 as negative APP in cats was previously described (Da Silva et al., 2010; Tecles et al., 2015; Vilhena et al., 2017). In our study, only BChE showed a negative APP behavior, with significant lower serum concentrations being detected in diseased queens at diagnosis when compared with controls. Although no significant changes in serum concentrations of IGF1 and PON1 were observed in queens with mammary carcinomas at diagnosis, the results obtained in our study showed that these analytes could also be potentially clinically useful biomarkers of feline spontaneous mammary carcinomas since serum concentrations of PON1 were influenced by tumor dimensions, and a tendency to a shorter survival was observed in diseased queens with lower concentrations of IGF1 at diagnosis. The evaluation of other APP and of other analytes with a possible APP behavior in our studies would have been clinically useful, in order to detect other APP and / or APP patterns that could be used as biomarkers of feline hemoplasmas infection, pyometra and spontaneous malignant mammary tumors.

The influence of age and gender in the APP response in cats is controversial. Older age was associated with higher concentrations of SAA in healthy and sick cats, which was attributed to the higher incidence of subclinical diseases in geriatric animals; however, no significant influence of age was observed in serum AGP, Hp and albumin (Kann et al., 2012). Gender was also suggested to influence concentrations of APP in felines (Kann et al., 2012). However, no significant influence of age or gender in concentrations of APP was detected in other studies (Kajikawa et al., 1999; Vilhena et al., 2017). In our research, the influence of age and gender on the concentrations of APP was evaluated in the hemoplasmas infected cats. Significant positive correlations between age and serum concentrations of Hp were detected in cats infected with hemoplasmas, however, the correlation coefficients were weak, suggesting that other factors also influenced this association. Nonetheless, no significant influence of age was observed in SAA or albumin activity. Furthermore, no significant differences in concentrations of APP were detected between the males and females infected with hemoplasmas. Future studies are needed to clarify the influence of physiological variations, including age and gender, among others, in serum concentrations of APP.

Feline hemoplasmas have a worldwide distribution (Willi et al., 2007; Sykes, 2010). *Mycoplasma haemofelis* is considered the most pathogenic of the hemoplasmas that infect cats, causing hemolytic anemia, which can be severe and life-threatening in some cases; but CMhm and CMt can also cause anemia in infected animals (Willi et al., 2006; Reynolds and Lappin, 2007; Tasker et al., 2009; Baumann et al., 2013; Ghazisaeedi et al., 2014; Weingart et al., 2016). *Candidatus Mycoplasma haematoparvum* and Mhc are considered the species of hemoplasmas that infect dogs (Sykes et al., 2005; Novacco et al., 2010; Roura et al., 2010). Infection by these agents has also been reported in domestic cats, but the clinical significance of these infections in felines is unknown (Sykes et al., 2007; Martínez-Díaz et al., 2013; Bergmann et al., 2017). Treatment with antibiotics is effective in resolution of clinical signs in most cases, however, the elimination of the agent is not always achieved (Tasker et al., 2004; Tasker et al., 2006; Ishak et al., 2008). The reactivation of infection is a risk in the chronic carriers, and alternating periods of symptomatic and asymptomatic infection have been reported (Berent et al., 1998; Foley et al., 1998; Novacco et al., 2011; Weingart et al., 2016). For these reasons, detection of biomarkers of feline infection by hemoplasmas / hemoplasmosis would be clinically useful.

Serum concentrations of APP have proved to be useful biomarkers in several feline infectious diseases (Duthie et al., 1997; TerWee et al., 1998; Kann et al., 2014; Leal et al., 2014; Hazuchova et al., 2017, Silvestre-Ferreira et al., 2017). Acute phase proteins were also described as useful biomarkers in experimental single infections with Mhf and CMhm (Harvey and Gaskin, 1978; Korman et al., 2012). However, to the authors´ knowledge, the APP response in cats naturally infected with hemoplasmas or the APP response in cats co-infected with different species of hemotropic mycoplasmas was not previously investigated.

According with our results, feline natural single and co-infections with hemoplasmas in cats are associated with an APP response, with increases in SAA and Hp concentrations, and decreases in serum albumin activity. The results obtained in SAA concentrations in our study are in accordance with those obtained in the experimental study of Korman et al. (2012), which reported significant increases in SAA concentrations in Mhf and CMhm single infections. Moreover, in our study, significant increases in SAA and decreases in albumin were associated with manifestation of clinical signs of the infection, suggesting that SAA and albumin might be biomarkers of the symptomatic rather than the chronic asymptmatic phase of the infection, as it was also reported for SAA in the experimental study of Korman et al.

(2012). Available data about serum Hp behavior in hemoplasmas infection in cats are contradictory. While increased serum Hp concentrations were detected in cats experimentally infected with Mhf in the study of Harvey and Gaskin (1978), as occurs in our study, no significant changes in Hp activity were detected in cats experimentally infected with Mhf or CMhm, despite some cats manifested increases in Hp values, in the report of Korman et al. (2012). These variations could be attributed to the influence of other factors besides acute phase response, such as hemolysis that produces a decrease in Hp (Kann et al., 2012; Kuleš et al., 2014). Moreover, in the present study, while significant increases in SAA concentrations and significant decreases in albumin values were only observed in symptomatic cats, significant increases in concentrations of Hp were detected in symptomatic cats but also in asymptomatic animals. These results suggest that serum Hp might be useful in detection of subclinical infection with hemoplasmas. A similar Hp behavior was detected in *Hepatozoon felis* and *Babesia vogeli* infected cats, in *Dirofilaria immitis* seropositive cats (Silvestre-Ferreira et al., 2017; Vilhena et al., 2017). Increases in serum Hp were also described in asymptomatic dogs seropositive to *Leishmania infantum* (Martínez-Subiela et al., 2002). Development of an APP response, with transient increases in AGP, SAA and Hp was also described in asymptomatic FCoV exposed and infected cats (Giordano et al., 2004; Paltrinieri et al., 2007a,b), and persistent increases in AGP and SAA, but not of Hp, were described in one asymptomatic FCoV exposed cat before development of FIP (Giordano et al., 2004). Nonetheless, an APP response was not detected in asymptomatic cats experimentally infected with *Chlamydia psitacci*, and in cats with natural *Chlamydiae* subclinical infection (Terwee et al., 1998; Ström Holst et al., 2011). Discovery of biomarkers of the subclinical phase of infection is of major clinical importance, since contribute to detection of chronic carriers, and eventually to prediction of development of the symptomatic phase of infection. Development of APP responses was also previously described in asymptomatic infections in humans and in other animals (Jahoor et al., 1999; Jimenez-Coello et al., 2008; Pomorska-Mól et al., 2014; de Mast et al., 2015), however, lack of changes in serum APP were also described in other asymptomatic infections in human and veterinary medicine (Bern et al., 2007; Sikora et al., 2016; Milanović et al., 2017; Kim et al., 2018). For that reason, as in the symptomatic processes, it is necessary to evaluate which individual APP and which APP patterns are clinically useful in each disease.

Co-infections with two or more hemoplasmas species are frequently described in different studies (Peters et al., 2008; Sykes et al., 2008; Martínez-Díaz et al., 2013; Aquino et al., 2014), however, to the authors´ knowledge, no information regarding the clinical implications of hemoplasmas co-infections in cats are reported in the literature. In our study, concentrations of APP were not significantly different between CMhm infected cats and the groups of co-infected animals. Moreover, despite infection with Mhf was reported to induce a greater SAA response than infection with CMhm, which was attributed to the higher pathogenecity of Mhf when compared with CMhm (Tasker et al., 2009; Korman et al., 2012), in our study, animals co-infected with CMhm and Mhf did not show greater changes in APP than cats single infected with CMhm. These results suggest that the systemic inflammatory response is of similar magnitude in feline single infections and in co-infections with hemoplasmas. However, the APP response in natural (single) infection with Mhf was not evaluated in our study.

A previous study evaluated the influence of FIV in the APP response of cats experimentally infected (single infections) with Mhf and CMhm (Korman et al., 2012). In this study, cats co-infected with Mhf and FIV had significantly higher concentrations of Hp than animals single infected with Mhf (Korman et al., 2012). However, no significant influence of FIV-co-infection was observed in SAA concentrations of both Mhf and CMhm infected cats, nor in Hp serum concentrations of CMhm infected animals. In our study, no significant influence of retrovirus infection, FIV and / or FeLV, was detected in the APP response of hemoplasmas naturally infected cats. Nonetheless, only a small number of cats had their FIV / FeLV infection status determined. Further investigations with a greater number of animals should be performed to better evaluate the influence of retrovirus in the APP response of cats infected with hemotropic mycoplasmas in natural conditions.

The APP have proved to be useful biomarkers in monitoring the evolution of the disease and the response to treatment of different feline diseases, including Mhf and CMhm single infections in experimental conditions (Kajikawa et al., 1999; Tamamoto et al., 2009; Korman et al., 2012; Gil et al., 2014). The APP are probably also useful biomarkers for monitoring the evolution of the infection and the response to treatment of feline hemoplasmas infection in natural conditions, but further studies are required to confirm it since serial measurements of APP were not determined in our study.

The retrospective nature of this study originated several limitations. One of these limitations was the low number of cats included in some groups and subgroups of infected cats, including the lack of a representative group of cats naturally infected (single infection) with Mhf, which is considered the most pathogenic feline hemoplasmas (Foley et al., 1998; Willi et al., 2006; Tasker et al., 2009). The lack of serial measurements of APP in order to evaluate the progression of the infection / disease and the response to treatment is another major limitation of this study. Future studies should be performed in order to clarify this topic.

Pyometra is a frequent reproductive disease in queens, characterized by a suppurative inflammatory process in the endometrium and accumulation of a purulent content in the uterine lumen (Scott et al., 2002; Misirlioglu et al., 2006; Hagman et al., 2014; Hollinshead et al., 2016). The synthesis of pro-inflammatory mediators in the uterus originates an inflammatory reaction, which contributes to the local and systemic clinical signs presented by animals with pyometra (Kenney et al., 1987; Brady et al., 2000; Hagman et al., 2009). Most queens recover successfully after ovariohysterectomy, which is considered the treatment of choice in most cases, however, severe and life-threatning complications can occur in some cases (Brady et al., 2000; Scott et al., 2002; Demirel and Acar, 2012; Majoy et al., 2013). Therefore, the use of biomarkers for clinical evaluation and for monitoring the evolution of the disease and the response to treatment could be clinically useful. Previous studies already described changes in inflammatory markers in queens with pyometra, including fever, increases in the white blood cell counts, in 15-keto-(13,14)-dihydro-PGF2α and in 6-keto-PGF1α, and also development of SIRS (Kenney et al., 1987; Hagman et al., 2009; Jursza-Piotrowska et al., 2016).

Serum concentrations of APP were previously proved to be clinically useful biomarkers in diagnosis of pyometra in different species, including bitches, cows and mares (Børresen and Skrede, 1980; Dabrowski et al., 2009; Brodzki et al., 2015; Dabrowski et al., 2015; El-Bahr and El-Deeb, 2016), and also as useful biomarkers in monitoring the post-ovariohysterectomy period in females dogs with pyometra (Dabrowski et al., 2007; Dabrowski et al., 2009; Yuki et al., 2010; Dabrowski et al., 2015). However, to our knowledge, information about the APP response in queens with pyometra was lacking. An APP response, detected by an APP profile (including one major and one moderate positive APP, and a negative APP) was also detected

in queens with pyometra at diagnosis in our study. Moreover, serum concentrations of SAA, Hp and albumin tended to return to physiologic concentrations after the surgery in the queens that recovered from the pyometra, and remained altered in one queen that developed severe complications (septic shock and multiple organ failure) and died from the disease. These results indicate that in cats, as in other species, APP could be useful in the diagnosis of pyometra, and also in monitoring the evolution of the disease and the response to treatment in the clinical setting.

Moreover, the dynamics observed in concentrations of APP during the time course of the disease in the queens with pyometra that recovered adequately from the disease, and in the queen that developed severe complications and died of the disease, represents a typical APP response. These findings suggest that feline pyometra could be an adequate model to the study of the feline APR.

Nonetheless, a careful interpretation of the individual results obtained in SAA concentrations in queens with pyometra should be made, since in our study six diseased queens presented, at diagnosis, SAA values in the reference range. This lack of increment in SAA concentrations was already described in other feline diseases, including FIP, rhinitis feline lower urinary tract disease and lymphoma, among others (Hansen et al., 2006; Tamamoto et al., 2008). We hypothesized that this lack of increment could be attributed to different causes, namely the method used, which was designed for human SAA, and therefore could present a lower sensitivity with feline samples as previously reported (Hansen et al., 2006; Tamamoto et al., 2008); to the fact that feline SAA could present different isoforms, as described in humans and other animal species (Jacobsen et al., 2006; Soler et al., 2011; Li et al., 2012), that could be responsible for the lack of cross-reactivity of antibodies used in the reagents; or to the fact that the uterus could be less reactive to pathologic stimulus than other organs, since is adapted to the cyclic changes that occur during the consecutives estrous cycles and pregnancies, and consequently a systemic response is only observed in the later stages of the disease, as reported in other biomarkers of inflammation (Kenney et al., 1987; Potter et al., 1991; Hagman, 2018). Preliminary results obtained in proteomics research developed by our group in samples of control queens and cats with pyometra that presented, in this research, physiologic and elevated concentrations of SAA suggest that different isoforms of SAA also exist in cats (data not published). However, further research is necessary to clarify this topic,

and to evaluate these and other factors for this lack of increase in SAA concentrations in some cats with pyometra.

Although the repeated uterine exposure to ovarian hormones or to exogenous synthetic analogues is considered the principal etiologic factor in feline pyometra, other factors, including uterine tumors, have been suggested to be involved in pyometra development in queens (Potter et al., 1991; Miller et al., 2003; Agudelo, 2005; Payan-Carreira et al., 2013; Pires et al., 2016). In our study, a higher number of queens with pyometra (15 out 23 queens, 65%) presented a concomitant endometrial adenocarcinoma. No significant differences in APP concentrations were detected between queens with pyometra with or without a concomitant endometrial adenocarcinoma, suggesting that the inflammatory response induced by feline pyometra is of similar magnitude regardless of origin. However, further studies are required in order to confirm these observations. Interestingly, SAA was reported to be a biomarker of uterine neoplasia in women (Cocco et al., 2009; Cocco et al., 2010), however, to the author´s knowledge, information related to the APP response in queens with uterine tumors is not available.

Inflammation was proved to be associated with carcinogenesis in several tumors in humans and animals (Hanahan and Weinberg; 2011; Zappulli et al., 2015; Raposo et al., 2015; Carvalho et al., 2016). Moreover, APP were proved to be clinically useful biomarkers in several oncologic diseases in human medicine, and in a lesser extent, also in veterinary oncology, including in feline tumors (Falconer et al., 1995; Selting et al., 2000; Chase et al., 2012; Dowling et al., 2012; Winkel et al., 2015; Cooper et al., 2018). Furthermore, APP responses were previously described in human breast cancer and in canine mammary tumors, and these analytes were proved to be clinically useful in diagnosis of human breast cancer and canine mammary tumors, and in prognosis and in monitoring the evolution of the disease and response to treatment of mammary neoplasia in humans (Tecles et al., 2009; Planellas et al., 2009; Dowling et al., 2012; Bobin-Dubigeon et al., 2015; Wulaningsih et al., 2015; Machado et al., 2015; Dieli-Conwright et al., 2016; Mei et al., 2017).

Mammary tumors are among the most frequent neoplasias diagnosed in queens (Vascellari et al., 2009; Egenvall et al., 2010). Most of feline mammary tumors are malignant and present an aggressive behavior (Hayes et al., 1981; Ito et al., 1996; Millanta et al., 2002; Figueira et

al., 2014), however, the reported survival times from diagnosis vary significantly from a few days to several years (Viste et al., 2002; Preziosi et al., 2002; De Campos et al., 2016). Thus, the discovery of biomarkers that could be useful in diagnosis, in prognosis, and in monitoring the evolution of the disease and the response to treatment of feline mammary carcinomas is of clinical interest. Several studies characterized and evaluated different biomarkers of feline mammary tumors, most on clinical, histopathologic and molecular features of these neoplasias (Hayes et al., 1981; Seixas et al., 2011; Hughes and Dobson; 2012; Mills et al., 2015; Zappulli et al., 2015). However, information on serum biomarkers of feline mammary tumors is scarce (Yoshida et al., 2014; Soares et al., 2016; Marques et al., 2018), and to our best knowledge, information concerning the APP response in feline mammary tumors was lacking.

The present study revealed that feline spontaneous malignant mammary tumors are associated with an APP response, with significant increases in SAA and Hp, and decreases in albumin and BChE detected at diagnosis. Moreover, in general, the APP response at diagnosis was of greater magnitude in diseased queens with tumors that presented clinical and histopathological features associated with a worst prognosis, namely bigger size, ulceration, lymphovascular neoplastic invasion, metastasis in regional lymph nodes and distant organs, advanced clinical stage of the disease, higher histological grade, necrosis and higher proliferative activity (Viste et al., 2002; Seixas et al., 2011; Hughes and Dobson; 2012; Morris et al., 2013; Mills et al., 2015; Zappulli et al., 2015), suggesting that a systemic inflammatory process might be related with more severe and complicated cases of feline malignant mammary tumors.

Our results also suggest that feline malignant mammary tumors induce a stronger APP response than the canine counterpart, with significant changes in APP detected in smaller tumors than in dogs. Significant increases in concentrations of SAA and Hp, and decreases in albumin and BChE were also reported in female dogs with mammary tumors at diagnosis (Tecles et al., 2009; Planellas et al., 2009; Machado et al., 2015), however, canine mammary tumors were considered weak inducers of APP, unless if were of big dimensions, of specific histopathological types or associated with metastasis, ulceration or secondary inflammation (Thougaard et al., 1999; Tecles et al., 2009; Planellas et al., 2009; Yuki et al., 2011).

Our results also suggest that APP might be clinically useful in monitoring the evolution of the disease and in prognosis of feline mammary carcinomas. The APP were previously proved to be clinical useful biomarkers in monitoring evolution and response to treatment of different feline diseases (Webb et al., 2008; Leal et al., 2014; Winkel et al., 2015; Vilhena et al., 2018). However, to the author´s knowledge, the evolution of these analytes during the course of the disease in queens with malignant mammary tumors was not studied. According with our results, increases in serum concentrations of SAA during the course of disease in queens with a previous malignant mammary tumor might suggest development of metastasis in distant organs. These results are in accordance with an *in vitro* study that showed that SAA promotes invasion of different feline mammary carcinoma cell lines (Tamamoto et al., 2014). However, our results should be interpreted with caution because this progression was only evaluated in 11 diseased queens. Although presence of lymphovascular and regional lymph node neoplastic invasion was assessed, the present study was not designed to detect micrometastasis in distant organs. Future studies, with a higher number of animals and with the use of more sensitive imaging diagnostic exams to detect early metastasis, such as computed tomography, magnetic resonance, positron emission tomography or scintigraphy, should be undertaken in order to elucidate the clinical significance of these and others acute phase reactants in carcinogenesis, and in monitoring evolution and response to treatment of feline malignant mammary tumors.

Acute phase proteins and other serum and tissular biomarkers of inflammation have been associated with prognosis of human breast cancer (Lee et al., 2006; Choi et al., 2009; Liu et al., 2015; Yue et al., 2017; Petekkaya et al., 2017; Hwang et al., 2017; Chen et al., 2018). Similar information concerning canine and feline mammary tumors is scarce (Carvalho et al., 2014; Martins et al., 2016). In our study, serum concentrations of albumin and BChE at diagnosis were independent predictors of mortality. Kaplan-Meier curves showed that pre-treatment serum concentrations of BChE lower than 1.15 μmol/ml·min were significantly related to a shorter survival time. These results provide important clinical information concerning prognosis, and might be important in treatment decision of feline mammary cancer. Results concerning serum albumin concentrations at diagnosis in queens with malignant mammary tumors observed in this study are somehow contradictory. On the one hand, diseased queens with neoplastic vascular invasion and metastasis in distant organs at diagnosis, which are associated with a worst prognosis, had lower serum albumin than healthy

controls cats; but on the other hand, diseased cats with concentrations of albumin in the reference range at diagnosis had 20.2 times more chances of having a shorter survival than queens with lower concentrations. Pre-treatment concentrations of albumin have also been implicated as prognostic biomarkers of women with breast cancer, however, as expected and in contrast with our results, decreased concentrations have been associated with a worst prognosis (Al Murri et al., 2007; Liu et al., 2015). Further studies should be performed in order to clarify the prognostic significance of altered concentrations of albumin in feline mammary cancer.

In our study, a tendency to a shorter survival was also observed in queens with increased SAA and decreased IGF1 at diagnosis, however, the differences were not significant. Increases in concentrations of SAA and decreases in IGF1 were associated with a worst prognosis in women with breast cancer (Biran et al., 1986; Pierce et al., 2009; Tas et al., 2014). Insulin-like growth factor 1 was also associated with pathogenesis of canine mammary neoplasias, with significantly higher concentrations of serum and tissular IGF1 being described in malignant tumors when compared with benign tumors and healthy controls, and increases in serum and tissular IGF1 associated with a poor prognosis (Queiroga et al., 2008; Queiroga et al., 2010). In feline medicine, IGF1 was mostly studied as a biomarker of endocrine diseases (Niessen et al., 2007; Schaefer et al., 2017; Rochel et al., 2018), however, was also proved to be implicated in proliferation of mammary tissues in feline fibroadenomatous change (Ordás et al., 2004). Nonetheless, in our study, no significant changes in serum IGF1 were detected in diseased queens at diagnosis, and also no significant influence of any of the different clinical and histopathological features of the mammary tumors evaluated was detected on serum concentrations of IGF1 determined at diagnosis. Future studies should be made in order to evaluate the biological role of SAA and IGF1 in feline mammary carcinomas.

Detection of serum biomarkers that could aid in differentiation of benign mammary lesions from malignant mammary tumors before mastectomy and histopathology would have a major clinical relevance in practice. However, the low number of queens with mammary hyperplasias, dysplasias and benign mammary tumors observed during the study period precluded that evaluation. This represents a major limitation of our study. Further studies should be performed in order to evaluate if APP could be clinically useful in discrimination of feline benign mammary lesions from malignant mammary tumors.

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Chapter 7

Conclusions

Conclusions

The results obtained in our studies may contribute to the knowledge of the APP response in feline medicine, and may contribute to their use in clinical practice. According with the results obtained in our reaserch:

. Feline natural infection with one (single infection) or various (co-infections) species of hemotropic mycoplasmas (hemoplasmas) induced an APP response.

. The magnitude of the APP response was greater in symptomatic than in asymptomatic animals infected with hemoplasmas.

. In cats infected with hemoplasmas, increases in concentrations of SAA and decreases in albumin were related with development of an acute infection with manifestation of clinical signs.

. In cats infected with hemoplasmas, increases in serum concentrations of Hp occurred in symptomatic and also in asymptomatic cats. Therefore, Hp could be a clinically useful biomarker in identification of cats with subclinical infection with hemoplasmas.

. An APP profile, including SAA (a positive major APP), Hp (a positive moderate APP) and albumin (a negative APP), could be useful in discriminating the acute symptomatic from the chronic asymptomatic phase of feline infection with hemoplasmas.

. The magnitude of the APP response was similar between single- and co-infections with hemoplasmas.

. Feline pyometra induced an APP response.

. The APP SAA, Hp and albumin showed to be potentially useful biomarkers in diagnosis and in monitoring the post-operative period in feline pyometra.

. Feline pyometra showed to be an adequate model to the study of the feline APR.

. Feline spontaneous malignant mammary tumors induced an APP response.

. The APP SAA, Hp albumin, BChE, IGF1 and PON1 demonstrated to be potentially useful biomarkers in diagnosis and in monitoring the clinical course of feline spontaneous malignant mammary tumors.

. Different clinical and histological features of feline mammary carcinomas, including bigger size, ulceration, lymphovascular neoplastic invasion, metastasis in regional lymph nodes and distant organs, advanced clinical stage of the disease, histological type, higher histological grade, necrosis and higher proliferative activity influenced the inflammatory response.

. Increases in serum concentrations of Hp detected at diagnosis suggested metastization of feline malignant mammary tumors.

. Increases in concentrations of SAA during the course of the disease suggested development of metastasis of mammary carcinomas in distant organs.

. Decreases in albumin detected at diagnosis may suggest metastization of feline malignant mammary tumors. However, decreased albumin concentrations at diagnosis were also associated with a longer survival time of cats with malignant mammary tumors. Further studies are necessary to clarify the clinical significance of changes in serum concentrations of albumin in feline malignant mammary tumors.

. Serum concentrations of BChE < 1.15 μmol/ml.min at diagnosis were associated with a shorter survival time in feline mammary carcinomas.

. Butyrylcholinesterase presented a negative APP behaviour in spontaneous feline malignant mammary tumors.