ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Acute phase proteins in queens with pyometra – Serum Amyloid Alpha

Telma Adriana Ferreira Santos

Coimbra, janeiro 2018

ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

Acute phase proteins in queens with pyometra – Serum Amyloid Alpha

Coimbra, janeiro 2018

Telma Adriana Ferreira Santos
Aluna do Mestrado Integrado em Medicina Veterinária

Constituição do Júri

Presidente do Júri: Profª Liliana Montezinho
Arguente: Profª Ana Cristina Silvestre Ferreira
Orientador: Profª Drª Sofia Duarte

Orientador Interno
Prof.ª Drª Sofia Duarte
Co-orientador Interno
Mestre Hugo Vilhena
Orientador Externo
Profº Drº José Céron
Laboratório Interdisciplinar de Análises Clínicas Interlab-UMU, Universidade de Murcia

Dissertação do Estágio Curricular dos ciclos de estudo conducentes ao Grau de Mestre em Medicina Veterinária da EUVG

Resumo

A resposta de fase aguda traduz uma reação sistémica que implica a ativação de vários mecanismos fisiológicos, como resposta a um estado inflamatório, com o objetivo de restaurar a homeostase. As principais adaptações resultantes deste processo estão relacionadas com a síntese de proteínas de fase aguda. Embora estes mecanismos não estejam profundamente elucidados em felinos, o conhecimento que se obteve até à data suporta a importância da utilização destas proteínas como meios auxiliares de diagnóstico e prognóstico. A proteína sérica amiloide alfa (SAA) é considerada, em várias espécies, a apolipoproteína com maior sensibilidade. É a proteína mais rapidamente sintetizada no fígado, e a sua concentração aumenta drasticamente, atingindo concentrações 100 a 1000 vezes superiores aos valores normais nas primeiras 48 horas após a agressão. É por isso considerada como um potencial biomarcador para diagnóstico, monitorização e prognóstico.

O objetivo deste estudo foi avaliar as possíveis causas que justificassem a ausência de aumento da concentração de SAA em algumas gatas diagnosticadas com piómetra. Para tal foi aplicada a técnica de eletroforese bidimensional de forma a confirmar a presença de SAA em fêmeas diagnosticadas com piómetra. Devido às características da proteína em estudo o método analítico foi otimizado.

Os resultados obtidos mostraram que mesmo as fêmeas doentes com concentrações normais de SAA, apresentavam nitidamente o spot correspondente a esta proteína no gel de eletroforese. Concluiu-se assim, que a ausência de aumento da concentração de SAA em gatas com piómetra deve-se provavelmente à existência de diferentes isoformas desta proteína, o que exige a adequação dos métodos analíticos aplicados.

Palavras-chave*: resposta de fase aguda, proteínas de fase aguda, proteína sérica amiloide alfa, felinos, piómetra, diagnóstico*

Agradecimentos

O desenvolvimento desta dissertação reúne contributos a várias pessoas e instituições, sem os quais esta investigação não teria sido passível de realizar. Posto isto, desejo deixar os meus agradecimentos à Profª Draª Sofia Duarte que sem pensar duas vezes aceitou o compromisso de me acompanhar nesta jornada. Desde a escolha do local de estágio ao desenvolvimento desta dissertação, pela orientação prestada, pela preocupação, pelo incentivo e a disponibilidade constante, fico deveras grata.

Ao coorientador Profº Drº Hugo Vilhena que de igual forma fez questão de acompanhar esta experiência, pela sua disponibilidade e também pelo apoio na elaboração deste projeto, lhe expresso a minha gratidão.

Pretendo também demonstrar o meu apresso a todas as pessoas envolvidas na equipa do Laboratório Interdisciplinar de Análises Clínicas da Universidade de Múrcia, que fizeram a minha estadia um verdadeiro prazer e que me acompanharam desde o primeiro dia, tratando de me integrar e de me fazer sentir em "casa" e de transmitir todo o conhecimento que adquiri nesta etapa. Em especial tenho que agradecer ao Prof.º Dr. º José Madrigal e à Dr.ª Asta Tvarijonaviciute que me motivaram durante todo o projeto e me fumigaram o interesse pelas análises clínicas.

Não poderia deixar de agradecer à minha família, principalmente à mulher que contribuiu para que esta etapa da minha vida fosse possível e que me deu todo o seu apoio. Não poderia ter mais orgulho ou expressar por palavras todo o carinho que sinto. Também aos meus amigos e às pessoas especiais que conheci pelo caminho e que afastaram qualquer solidão que poderia estar inerente a ir para um país diferente.

Table of contents

List of figures

List of tables

List of acronyms

Aa/Bis – Acrylamyde/Bis-Acrylamyde
APR – Acute phase response
APP – Acute phase protein
EDTA - Ethylenediamine tetra acetic acid
DTT - Dithiothreitol
IL – Interleukin
MS – Mass spectrometry
SR-BI – Scavenger receptor class B type I
SAA – Serum α-amyloid
TNF – Tumour necrosis factor
2DGE – Two-dimensional gel electrophoresis

Acute phase proteins in queens with pyometra – Serum Amyloid Alpha

Telma Santos[a*], Lorena Martinez[b], Hugo Vilhena[a,c,d], Asta Tvarijonaviciute[b], José Céron[b], Sofia Duarte[a,e]

a. Center for Investigation Vasco da Gama (CIVG), Department of Veterinary Medicine, Vasco da Gama University School, Av. José R. Sousa Fernandes, 197 Lordemão, 3020-210, Coimbra, Portugal
b. Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, University of Murcia, Campus de Espinardo Ed. 16, 30100 Espinardo, Murcia, Spain
c. Baixo Vouga Veterinary Hospital, EN1, 355, 3750-742, Águeda, Portugal
d. Animal and Veterinary Research Centre (CECAV), University of Trás-os-Montes and Alto Douro, Quinta de Prados, 5000-801, Vila Real, Portugal
e. LAQV-REQUIMTE, Group of Bromatology, Pharmacognosy and Analytical Sciences, Faculty of Pharmacy, University of Coimbra, Coimbra, Portugal

*** Corresponding author:** Telma Santos
e-mail: telma_santos1994@hotmail.com
mail: Escola Universitária Vasco da Gama, Avenida José R. Sousa Fernandes 197 Lordemão, 3020-210 Coimbra, Portugal

Lorena Martinez – lorena.franco2@um.es
Hugo Vilhena - hcrvilhena@hotmail.com
José J Cerón - jjceron@um.es
Asta Tvarijonaviciute - asta@um.es
Sofia Duarte - s.cancela.duarte@gmail.com

Abstract

The acute phase response (APR) is a systemic reaction that implies the activation of physiological mechanisms in response to an inflammatory state, aiming to restore homeostasis. One of the major adaptations is the acute phase proteins (APP) synthesis. Although this mechanism has not been completely understood in felines, the present knowledge supports the use of APP as important diagnostic and prognosis tools. Serum amyloid alpha (SAA) is an apolipoprotein and is considered the most sensitive APP in felines and in several other species. Besides the fact that its concentration increases dramatically after an inflammatory insult, it has also been recognized as the fastest reactant to increase after tissue injury, increasing 100 to 1000-fold in the first 48 hours, therefore being considered a strong potential biomarker in evaluation of pathological conditions, and also in monitoring tissue injury and response to therapy.

The aim of our study was to evaluate the cause behind the lack of SAA increase in some reported cases of cats with pyometra. For this purpose, two-dimensional polyacrylamide gel electrophoresis was applied in order to evaluate the presence of SAA in females diagnosed with pyometra, with different concentrations of the same protein when quantified by an automated analyser. The analytical method was optimized regarding the characteristics of the protein under study.

Our results showed that diseased females in which SAA concentration was normal when measured with an automated analyser, presented an evident SAA spot in the two-dimensional polyacrylamide gel electrophoresis. It was concluded that the low concentration of SAA probably resulted from the existence of different isoforms of this protein, which requires an adaptation of the analytical methods in use.

Key Words: acute phase response, acute phase proteins, serum amyloid alpha, feline, pyometra, diagnosis

1. Introduction

The acute-phase response (APR) is a systemic nonspecific and complex immunological reaction, considered as part of the innate immune system, that is activated in the early stages of aggression (Cerón, Eckersall, & Martínez-Subiela, 2005; Gómez-Laguna, Salguero, Pallarés, & Rodríguez-Gómez, 2010). This mechanism is triggered by tissue injury resulting from inflammatory, infectious, immunologic, neoplastic or traumatic factors, and the main purpose is to restore homeostasis, isolate and neutralize pathogens, minimize tissue damage and promote tissue repair (Giordano, Spagnolo, Colombo, & Paltrinieri, 2004; Niine *et al.*, 2017).

During the APR, due to tissue damage, inflammatory cytokines are produced and released, such as interleukin (IL)-1, IL-6 and tumour necrosis factor (TNF)-α. Both pro-inflammatory cytokines and endogenous glucocorticoids promote hepatic synthesis of plasma proteins, designated acute phase proteins (APP) (Eckersall, 1995; Gómez-Laguna *et al.*, 2010). Although several studies suggests that most tissues can express APP synthesis in variable amounts, even in healthy conditions, hepatocytes are the main target cell and because of that, the liver suffers major metabolic alterations (Eckersall *et al.*, 2001; Gahmberg & Andersson, 1978; Lin *et al.*, 2001; Marques *et al.*, 2017). Thereby, APP can be described as liver-derived molecules whose plasma concentrations increase or decrease during the inflammatory response (Jain, Gautam, & Naseem, 2011). Based on the variation of their serum concentration during the inflammatory process, APP are classified as positive (*e.g.* C-reactive protein, Serum amyloid-α – SAA and Haptoglobin) or negative (*e.g.* Albumin) (Eckersall & Bell, 2010).

Serum amyloid alpha is a small sized glycoprotein (12-14 kDa) from the apolipoprotein family, that can be found circulating bound to the surface of the high density lipoprotein 3 (HDL3) (Lepedda *et al.*, 2013; Tamamoto, Ohno, Ohmi, Goto-koshino, & Tsujimoto, 2008). This protein is classified as a positive major APP, since its concentration can increase more than a thousand-fold after tissue injury (Kisilevsky & Manley, 2012; Tamamoto *et al.*, 2008). Like most positive APP, this is mainly synthesized in the liver and, although in lower proportion, also in other tissues (*e.g.* gastrointestinal tract and integumentary system) (Berg, Thomsen, Andersen, Jensen, & Jacobsen, 2011; Lecchi, Dilda, Sartorelli, & Ceciliani, 2012; Marques *et al.*, 2017).

The specific physiological role of this inflammatory mediator remains to be elucidated (Table 1), but the fact that apolipoproteins have been highly conserved throughout evolution and the fact that there is a severe increase of the levels of SAA in response to potentially life-threatening physiological challenges suggests a protective role in the acute-phase response (Jensen *et al.*, 2017; Uhlar & Whitehead, 1999).

Table 1. Literature reported biological functions of SAA.

	Description	***References***
Inflammation	Chemotactic recruitment of inflammatory cells to inflammation site Direct inhibition of myeloperoxidase release and lymphocyte proliferation Opsonization of Gram-positive and Gram-negative bacteria Stimulation of the response of innate phagocytic cells Indirect control of the inflammatory process	(Cerón *et al.*, 2005) (Soler *et al.*, 2011) (Hari-Dass, Shah, Meyer, & Raynes, 2005)
Lipid metabolism	Inhibition of SR-BI-mediated selective lipid uptake Mediation of lipid transport by shifting cholesterol balance from ester to free cholesterol	(Cai, De Beer, De Beer, & Van Der Westhuyzen, 2005) (Cai *et al.*, 2005)
Protein metabolism	Precursor of protein amyloid A, being involved in the pathogenesis of AA-amyloidosis	(Cai *et al.*, 2005)

(SR-BI, scavenger receptor class B type I)

In cats, SAA has been recognized as a useful inflammatory biomarker; nonetheless, it has been scarcely studied (Cerón *et al.*, 2005; Kajikawa, Furuta, Onishi, Tajima, & Sugii, 1999). Sasaki, Ma, Khatlani, Okuda, & Inokuma (2003) demonstrated that cats with pathologic abnormalities had higher concentration levels of SAA than healthy individuals. In addition, it was demonstrated that in felines, SAA is the fastest APP to increase after an inflammatory stimulus (Kajikawa *et al.*, 1999). Furthermore, Tamamoto *et al.* (2013) emphasized the potential of SAA as a significant and independent prognostic factor for inflammatory states in diseased cats.

Pyometra is a clinical relevant problem that may start as a sub-clinical unrecognized disorder which can rapidly evolve to sepsis (Hagman, Ström, Möller, & Egenvall, 2014; Miert, 1967). Thus, it can result in the death of the animal if not promptly diagnosed and treated. During the early stages of uterine bacterial infection, immune specific T and B lymphocytes, as well as other immune and pro-inflammatory molecules are activated. Moreover, endometrial cells facilitate the release of cytokines, such as TNF-α, involved in the activation of the acute phase response (Jursza-piotrowska & Siemieniuch, 2015). Given the marked inflammatory response, females with pyometra can be considered one of the best animal models to evaluate the APR in felines. It also reinforces the need for additional studies with the aim to identify endocrine biomarkers that facilitate the diagnosis of this life-threatening pathology.

In the recent study of Vilhena et al. (2018), serum concentrations of APP, including SAA, haptoglobin and albumin were determined in queens with pyometra. The previous research suggests that APP could be potential biomarkers for diagnosis and post-surgical prognostic monitoring in feline pyometra. However, and contrary to what would be expected, normal concentrations of this protein were observed in six diseased queens. The same SAA behaviour was previously described by Tamamoto *et al.* (2013) in cats with neoplastic, inflammatory and other diseases.

Given the above mentioned, our study aimed to evaluate the hypothesis of the existence of different isoforms of feline SAA to justify the lack of SAA increase when quantified by the described method.

For that purpose, samples enrolled in the study of *Vilhena et al.* (2018) were submitted to proteomic analysis. By definition, proteomics consists in the characterization of the entire protein complement of an organism, tissue or cell (Anderson & Anderson, 2000; Graves & Haystead, 2002). Two-dimensional gel electrophoresis (2DGE) and mass spectrophotometry (MS) are the current leading techniques used in proteomic analysis, since they allow comparison of proteome patterns between healthy and diseased individuals. Thus, when applied in diagnosis, may allow the identification of potential biomarkers (Adkins et al., 2002; Gundacker et al., 2006; Hu, Huang, Chen, & Yao, 2004; Miller & Gemeiner, 1992; Stoughton & Friend, 2005; Thadikkaran et al., 2005).

2. Materials and methods

2.1. Samples and experimental setup

This study was approved by the Scientific Council of Vasco da Gama University School as complying with the Portuguese legislation for the protection of animals Law no. 92/1995 (A.R., 1995).

Analysed samples were obtained from aliquots of samples entailed in a previous study of our research team, as reported and described by Vilhena *et al.* (20182018). Briefly, whole blood samples were collected by puncture of the jugular vein to plain tubes (Vacuette® Z serum clot activator, Greiner Bio-One International GmbH, Kremsmünster, Austria), left for 15 minutes to clot at room temperature, centrifuged at 2000 x *g* for 10 minutes at 4°C, and serum samples were stored at -20ºC until analyses.

For the present study, a total of eight samples were selected and animal characterization is presented in **Table *2***. Samples were organized in two groups, Group A included five females diagnosed with pyometra and Group B included three healthy females used as controls. Animals of the group A (queens with pyometra) were further divided in two groups according with serum concentrations of SAA determined on the automated analyser: group A1, which included three animals with increased concentration of SAA, and group A2, which included two diseased queens with concentration of SAA in the reference range (**Table 2**). The laboratory reference range for SAA concentration <5 µg/ml (Vilhena et al., 2018) was considered for this sub-division.

Pyometra was diagnosed in all animals based on clinical history, physical examination and results of complementary diagnostic exams, including abdominal ultrasonography and histopathology of the reproductive organs to confirm pyometra on diseased females, and to discard uterine pathology on queens of the control group.

2.2. Total serum protein and SAA quantification

Total serum protein concentration of each sample was measured at the Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, University of Murcia, Spain, using an automated biochemistry analyser (Olympus AU600®, Olympus Europe GmbH, Hamburg, Germany) in order to prepare samples for polyacrylamide gel electrophoresis.

Concentration of SAA in plasma samples was determined by a human turbidimetric immunoassay (LZ-SAA; Eiken Chemical Co.), validated for use in feline samples (Hansen, Schaap, & Kjelgaard-Hansen, 2006), using an automated biochemistry analyser (Olympus AU600®, Olympus Europe GmbH, Hamburg, Germany). A limit of detection of 0.38 µg/ml was considered.

2.3. Two-dimensional polyacrylamide gel electrophoresis

Each pair of samples were suspended in rehydration buffer consisting of 8M Urea, 2% CHAPS, 50 mM 1,4-Ditiotreitol (DTT), 0.5% IPG buffer and bromophenol blue (Bio-Rad, Hercules, CA, USA) for a final concentration of 150 µg/µL. Each individual 11 cm ReadyStrip™ IPG Strip (immobilized pH gradient, pH 7-11 linear, Bio-Rad) was re-hydrated with a final volume of 200 µL. The rehydration occurred overnight, protected from the light and at room temperature.

Horizontal isoelectric focusing was performed in Protean IEF Cell (Bio-Rad). After electrofocuse, the strips were frozen at -20 ºC until further use.

Each strip was equilibrated, during 10 minutes with 6 M Urea, 0.375M Tris pH 6.8, 70% glycerol, 20% sodium dodecyl sulphate and 2% DTT. The same procedure was repeated, per 5 minutes, exchanging DTT for 2.5% iodoacetamide. Secondly, the stripes were fixed on the top of the 1.5 mm thick second-dimensional gels with stacking gel (1% agarose) at 70ºC and prepared for the Protean II XL multi Cell (Bio-Rad).

Gels were prepared in the day before their use, with 45% Acrylamide/Bis-Acrylamide (Aa/Bis 30%) or 50% Aa/Bis30%, tris(hydroxymethyl)aminomethane (tris), miliQ, tetramethylethylenediamine and ammonium persulphate.

Each vertical electrophoresis program was performed using a pair of two samples according to a 600V, 30mA and 9W protocol that lasted five to six hours (Tvarijonaviciute, Gutiérrez, Miller, Razzazi-fazeli, & Tecles, 2012).

2.4. Two-dimensional polyacrylamide gel electrophoresis image analysis

In the end gels were stained with Page Blue protein staining solution (ThermoFisher Scientific, Waltham, MA, USA) based in the colloidal Coomassie blue G250 for later scanning in an ImageScanner II (GE Healthcare Europe GmbH, Uppsala, Sweden) and evaluation, using specific software (Image Master 2D Platinum 7.0, GE Healthcare Europe GmbH, Uppsala, Sweden).

3. Results

3.1. SAA quantification

Characterization of the animals entailed in this study, including total serum protein concentration (g/dl) and concentration of SAA (µg/ml) are presented in **Table 2**.

Table 2. Characterization of the animals entailed in this study, including total serum protein concentration (g/dl) and concentration of SAA (µg/ml)

			Age (years)	Breed	Weight (kg)	Total protein concentration (g/dl)	Concentration of SAA (µg/ml)
GROUP A	A1 - High [SAA]	H1	6	ES	3.5	5.41	63.60
		H2	6	ES	3.3	7.95	92.20
		H3	6	ES	3.8	5.91	93.80
	A2 - Low [SAA]	L1	20	ES	5.0	9.28	0.38
		L2	11	ES	2.8	8.14	0.38
GROUP B	Control Group	C1	14	ES	3.5	6.76	0.38
		C2	15	Persa	4.2	7.63	0.38
		C3	6	ES	3.7	7.33	0.38

(ES, European shorthair)

3.2. Two-dimensional polyacrylamide gel electrophoresis image analysis

Two-dimensional polyacrylamide gel electrophoresis image analysis was used for SAA spot detection, as well as for comparison of results between Groups A and B, and between samples with high and normal SAA concentration.

Due to the small molecular weight (14 to 15 kDa) of SAA, no clear results were obtained in the first gels which were prepared based on a standard protocol for serum proteins separation (Soler et al., 2011). Thereby, new gels were prepared with 50% Aa/Bis30%, and 2DGE was performed at 300V. With this increase in the matrix gel concentration, a better resolution was obtained, since protein separation was more patent. Moreover, the putative SAA spot (orange arrow) was detected in all queens from Group A, either with high or normal concentration of SAA.

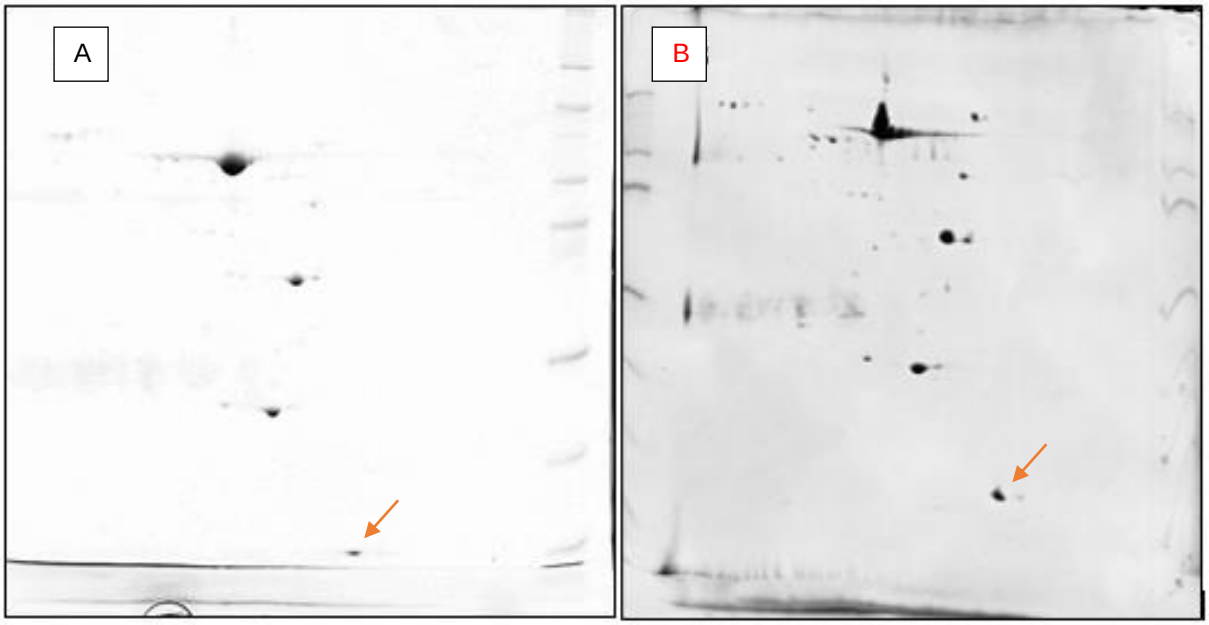

Figure 1. Two-dimensional gel electrophoresis image analysis of sample A2 (normal concentration of SAA) in a gel preparation with 45% Acrylamide/Bis-acrylamide 30% (Image A) and in a gel preparation with 50% Acrylamide/Bis-acrylamide 30% (Image B).

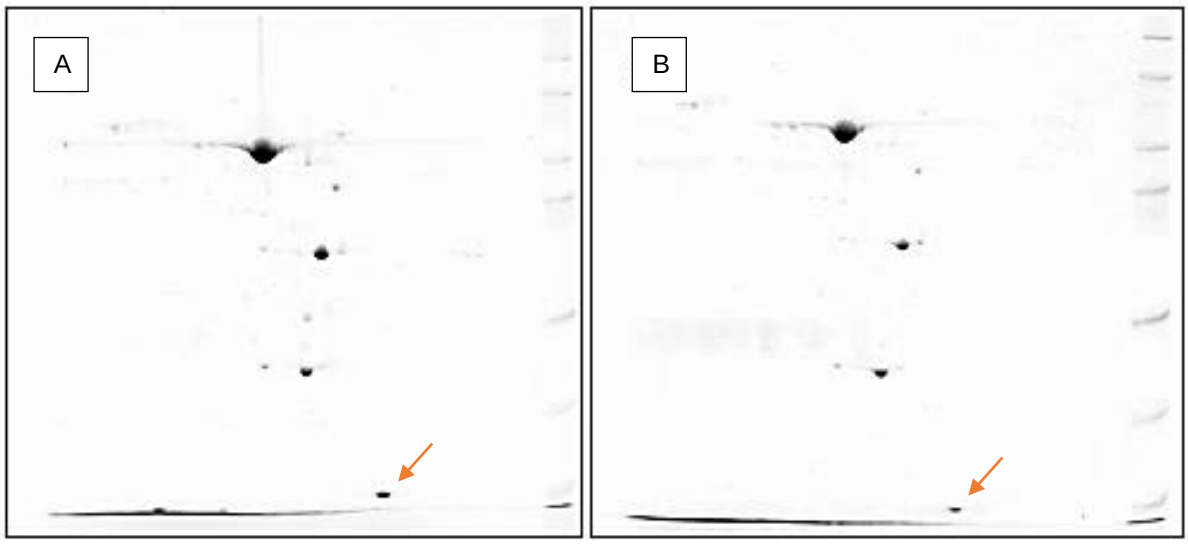

Figure 2 Two-dimensional gel electrophoresis image analysis of a sample from A1 group (high concentration of SAA; Image A) and of a sample from the A2 group (normal concentration of SAA; Image B), both gels prepared with 50% Acrylamide/Bis-acrylamide 30%.

4. Discussion

Protein studies were introduced recently as an essential tool to elucidate pathologic mechanisms (Graves & Haystead, 2002). Alterations of protein concentration, structure or even function are potential useful indicators of pathological abnormalities, what suggests that protein patterns may represent a foremost sensitive and specific way of diagnosis when compared with a single disease marker (Xiao, Prieto, Conrads, Veenstra, & Issaq, 2005). Biomarkers provide a dynamic and powerful

information for disease diagnosis, and also to the understanding of the spectrum of a specific pathology, what translates to other clinical applications such as monitoring therapy response and prognosis (Mayeux, 2004; Xiao *et al.*, 2005).

Although 2DGE and MS represent the leading techniques used in proteomics, due to the high sensitivity and specificity, there are technical issues that prevent their routine application in a clinical or hospital context. The technical difficulty, the time required to perform the protocols, and the numerous steps which result in a higher risk of human error and contamination of samples represent important limitations of this technique, being considered a time-consuming and intense labour process techniques (Graves & Haystead, 2002).

Regarding acute phase proteins profiles, the lack of knowledge on their interpretation is one of the main reasons for their scarce use in clinical practice to date. However, some studies have already shown that the assessment of the health status of an animal that does not include the study of the acute phase response should not be considered optimal (Eckersall, 2004).

Despite the difficulties imposed by the technique and the interpretation of data, the quality of the results allied with the importance of using APP as a method of diagnosis or as a successful way for monitoring treatment and prognosis, justified the importance of evaluating this same parameter in feline models. Although there are similar studies performed to evaluate the APR in queens and bitches with pyometra (Da browski, Kostro, & Szczubiał, 2013; Dabrowski, Kostro, Lisiecka, Szczubiał, & Krakowski, 2009), the mechanism was not thoroughly described in cats (Cerón *et al.*, 2005).

One of the major difficulties in isolating SAA using 2D gel electrophoresis is the molecular size of this inflammatory reactant. Thus, although the first gels were prepared according to protocols already described in other publications, an optimization was required to obtain more satisfactory results. Considering the characteristics of the protein in study, the gels depicted in Figure 1B, 2A and 2B were prepared with 50% Aa/Bis30% and vertical electrophoresis was performed at 300V. From the optimization of the polymerization process, smaller sized pores were obtained which allowed a better separation of the spot of interest.

In the study performed by Kajikawa *et al.* (1999), with the purpose to determine the proteins involved in the acute phase response in felines, SAA was determined to be the fastest reactant, beginning to increase approximately eight hours after the inflammatory/infectious/traumatic stimulus and reaching a maximum concentration between 36 and 48 hours (**Table *3***). Despite that, in the scope of a diagnosis, interpretation should not be based exclusively in SAA concentrations since recent studies evidenced that felines with inflammatory processes can exhibit normal values. Considering the previously described information, there are several factors that may justify the different concentrations of this APP in diseased animals.

Table 3. Time evolution of SAA concentration (mean ± SD) after induced inflammation with and intramuscular injection of Lipopolysaccharides (LPS) or Turpentine oil (Kajikawa *et al.*, 1999)

	SAA concentration (µg/ml)	
	Injection of LPS	**Injection of turpentine oil**
0th hour	44.7 ±12.5	29.1 ± 15.1
8th hour	96.6 ± 33.1	76.5 ± 23.3
24th hour	146.6 ± 20.4	85.5 ± 33.9
36th hour	118.4 ± 30.7	115.2 ± 17.6
48th hour	---	118.4 ± 3.5

Moreover, the uterus is an organ that is cyclically suffering physiological alterations during oestrus and pregnancy, and so, in reproductive disease such as pyometra, the development of an acute phase response can appear later than expected (Hollinshead & Krekeler, 2016; Jursza-piotrowska & Siemieniuch, 2015). Furthermore, biochemical changes resulting from this response may be fainter (Hollinshead, 2015).

In the present study, it was observed that all studied females with pyometra (group A) expressed the potential SAA spot, even females that manifested normal concentration of the same protein when measured by the automated biochemistry analyser (Olympus AU600®, Olympus Europe GmbH, Hamburg, Germany). Thus, the main possible reason for not detecting an SAA increment, as reported by Vilhena *et al*. (2018), may be related to the presence of different isoforms of feline SAA, not detectable by the applied analytical methodology. In this case, the method applied used a latex reagent sensitized with anti-human SAA antibodies, responsible for the passive agglutination process generated when in contact with the antigenic epitope of SAA (De Buck et al., 2016). It is known that minor structural differences between protein isoforms can imply completely different biological properties and lead to distinct biological functions and interactions (Godovac-Zimmermann, Kleiner, Brown, & Drukier, 2005). In addition, the changes of the amino acid code of each isoform may result in a structural modification of the antigenic epitope of SAA that impairs the antibody-antigenic reaction needed for the determination of SAA concentration (De Buck et al., 2016).This was already described in previous studies in pigs, in which the presence of different isoforms of SAA during inflammation was related with the lack of cross-reactivity of the antibodies used by the automated methods (Soler *et al.*, 2011). Thus, suitable analytical methods that distinguish between different isoforms are required.

Considering the previous information, and based on the literature, MS would be the next step in this investigation. This technique has been recognized as the technology of choice for direct protein identification and characterization when associated to 2DGE, since it allows to identify the proteins separated in the gel with high sensitivity and accuracy (Graves & Haystead, 2002). This step would allow the identification of all the spots and determine the exact location of SAA isoforms, by comparing gels between healthy and diseased animals. It would further enable the measurement of SAA

concentration of each isoform and the evaluation of the expression of this protein in different clinical situations.

5. Conclusions

Serum amyloid A behaves as a potential biomarker for diagnosis and monitoring of inflammatory processes in queens. However, based on the cases in which a lack of increase in concentration of this APP occur, the use of this biomarker should be carried out with caution and critical sense, associating other parameters to consolidate the interpretation of the values of SAA. According with our results, the existence of different isoforms of feline SAA may justify the lack of detection of this protein in the automated methods described in the literature. The results of this work underscore the need of further studies to identify and characterize the different isoforms of feline SAA, specifically through tandem mass spectrometry, and to develop new automated methods that are able to detect the different isoforms of feline SAA.

Acknowledgements

The authors express their gratitude to all the members of the Interdisciplinary Laboratory of Clinical Analysis Interlab-UMU, University of Murcia, Spain and to the Center for Investigations Vasco da Gama / Department of Veterinary Medicine (CIVG/DVM), Vasco da Gama University School for the financial support granted.

References

A.R., A. da R. (1995). Decreto-Lei 92/95 de 12 de setembro.

Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., … Pounds, J. G. (2002). Toward a Human Blood Serum Proteome: Analysis By Multidimensional Separation Coupled With Mass Spectrometry. *Molecular & Cellular Proteomics*, *1*(12), 947–955. https://doi.org/10.1074/mcp.M200066-MCP200

Anderson, N. G., & Anderson, N. L. (2000). Twenty years of two-dimensional electrophoresis: Past, present and future. *Electophoresis*, *17*, 443–453.

Assay, L. A. (n.d.). Latex Agglutination Assay for measurement of SAA protein in blood serum.

Berg, L. C., Thomsen, P. D., Andersen, P. H., Jensen, H. E., & Jacobsen, S. (2011). Serum amyloid A is expressed in histologically normal tissues from horses and cattle. *Veterinary Immunology and Immunopathology*, *144*(1–2), 155–159. https://doi.org/10.1016/j.vetimm.2011.06.037

Cai, L., De Beer, M. C., De Beer, F. C., & Van Der Westhuyzen, D. R. (2005). Serum amyloid a is a ligand for scavenger receptor class B type I and inhibits high density lipoprotein binding and selective lipid uptake. *Journal of Biological Chemistry*, *280*(4), 2954–2961. https://doi.org/10.1074/jbc.M411555200

Cerón, J. J., Eckersall, P. D., & Martínez-Subiela, S. (2005). Acute phase proteins in dogs and cats:

Current knowledge and future perspectives. *Veterinary Clinical Pathology*, *34*(2), 85–99. https://doi.org/10.1111/j.1939-165X.2005.tb00019.x

Kajikawa, T., Furuta, A., Onishi, T., Tajima, T., Sugii, S. (1999). Changes in concentrations of serum amyloid a protein, α1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. *Veterinary Immunology and Immunopathology, 68(1),* 91-98.

Da browski, R., Kostro, K., & Szczubiał, M. (2013). Concentrations of C-reactive protein, serum amyloid A, and haptoglobin in uterine arterial and peripheral blood in bitches with pyometra. *Theriogenology*, *80*(5), 494–497. https://doi.org/10.1016/j.theriogenology.2013.05.012

Dabrowski, R., Kostro, K., Lisiecka, U., Szczubiał, M., & Krakowski, L. (2009). Usefulness of C-reactive protein, serum amyloid A component, and haptoglobin determinations in bitches with pyometra for monitoring early post-ovariohysterectomy complications. *Theriogenology*, *72*(4), 471–476. https://doi.org/10.1016/j.theriogenology.2009.03.017

De Buck, M., Gouwy, M., Wang, J. M., Snick, J. Van, Opdenakker, G., Struyf, S., & Van Damme, J. (2016). Structure and Expression of Different Serum Amyloid A (SAA) Variants and their Concentration-Dependent Functions During Host Insults. *Current Medicinal Chemistry*, *23*(17), 1725–55. https://doi.org/10.2174/0929867323666160418114600

Eckersall, P. D. (1995). Acute phase proteins as markers of inflammatory lesions. *Comparative Haematology International*, *5*(2), 93–97. https://doi.org/10.1007/BF00638925

Eckersall, P. D. (2004). The time is right for acute phase protein assays. *Veterinary Journal*, *168*(1), 3–5. https://doi.org/10.1016/j.tvjl.2003.09.003

Eckersall, P. D., & Bell, R. (2010). Acute phase proteins: Biomarkers of infection and inflammation in veterinary medicine. *Veterinary Journal*, *185*(1), 23–27. https://doi.org/10.1016/j.tvjl.2010.04.009

Eckersall, P. D., Young, F. J., McComb, C., Hogarth, C. J., Safi, S., Weber, A., … Fitzpatrick, J. L. (2001). Acute phase proteins in serum and milk from dairy cows with clinical mastitis. *Veterinary Record*, *148*(2), 35–41. https://doi.org/10.1136/vr.148.2.35

Gahmberg, B. Y. C. G., & Andersson, L. C. (1978). Leukocyte surface origin of human α1-acid glycoprotein. *Journal of Experimental Medicine* 507–521.

Giordano, A., Spagnolo, V., Colombo, A., & Paltrinieri, S. (2004). Changes in some acute phase protein and immunoglobulin concentrations in cats affected by feline infectious peritonitis or exposed to feline coronavirus infection. *The Veterinary Journal 167*, 38–44. https://doi.org/10.1016/S1090-0233(03)00055-8

Godovac-Zimmermann, J., Kleiner, O., Brown, L. R., & Drukier, A. K. (2005). Perspectives in spicing up proteomics with splicing. *Proteomics*, *5*(3), 699–709. https://doi.org/10.1002/pmic.200401051

Gómez-laguna, J., Salguero, F. J., Pallarés, F. J., & Rodríguez-gómez, I. M. (2010). Acute Phase Proteins as Biomarkers in Animal Health and Welfare.

Graves, P. R., & Haystead, T. a J. (2002). Molecular Biologist ' s Guide to Proteomics. *Microbiology and Molecular Biology Reviews*, *66*(1), 39–63. https://doi.org/10.1128/MMBR.66.1.39

Gundacker, N., Bayer, E., Traxler, E., Zwickl, H., Kubicek, M., Stöckl, J., & Gerner, C. (2006). Knowledge-based proteome profiling: Considering identified proteins to evaluate separation

efficiency by 2-D PAGE. *Electrophoresis*, *27*(13), 2712–2721. https://doi.org/10.1002/elps.200500964

Hagman, R., Ström, B., Möller, L., & Egenvall, A. (2014). Theriogenology Incidence of pyometra in Swedish insured cats. *Theriogenology*, *82*(1), 114–120. https://doi.org/10.1016/j.theriogenology.2014.03.007

Hansen, A. E., Schaap, M. K., & Kjelgaard-Hansen, M. (2006). Evaluation of a commercially available human serum amyloid A (SAA) turbidimetric immunoassay for determination of feline SAA concentration. *Veterinary Research Communications*, *30*(8), 863–872. https://doi.org/10.1007/s11259-006-3373-6

Hari-Dass, R., Shah, C., Meyer, D. J., & Raynes, J. G. (2005). Serum amyloid A protein binds to outer membrane protein A of gram-negative bacteria. *Journal of Biological Chemistry*, *280*(19), 18562–18567. https://doi.org/10.1074/jbc.M500490200

Hollinshead, F. (2015). Pyometra in the Queen. *Control & Therapy Series* (5457), 27–29.

Hollinshead, F., & Krekeler, N. (2016). Pyometra in the queen To spay or not to spay?. *Control & Therapy Series* 21–33. https://doi.org/10.1177/1098612X15623114

Hu, Y., Huang, X., Chen, G. Y. J., & Yao, S. Q. (2004). Recent advances in gel-based proteome profiling techniques. *Molecular Biotechnology*, *28*(1), 63–76. https://doi.org/10.1385/MB:28:1:63

Jain, S., Gautam, V., & Naseem, S. (2011). Acute-phase proteins: As diagnostic tool. *Journal of Pharmacy and Bioallied Sciences*, *3*(1), 118. https://doi.org/10.4103/0975-7406.76489

Jensen, L. E., Hiney, M. P., Shields, D. C., Uhlar, C. M., Lindsay, A. J., & Whitehead, A. S. (2017). Acute phase proteins in salmonids: evolutionary analyses and acute phase response. *The Journal of Immunology 158,* 384-392. http://www.jimmunol.org/content/158/1/384

Jursza-piotrowska, E., & Siemieniuch, M. J. (2015). Identifying diagnostic endocrine markers and changes in endometrial gene expressions during pyometra in cats. *Phytochemistry Letters*, 2–8. https://doi.org/10.1016/j.repbio.2015.12.003

Kajikawa, T., Furuta, A., Onishi, T., Tajima, T., & Sugii, S. (1999). Changes in concentrations of serum amyloid a protein, α1-acid glycoprotein, haptoglobin, and C-reactive protein in feline sera due to induced inflammation and surgery. *Veterinary Immunology and Immunopathology*, *68*(1), 91–98. https://doi.org/10.1016/S0165-2427(99)00012-4

Kisilevsky, R., & Manley, P. N. (2012). Acute-phase serum amyloid A : Perspectives on its physiological and pathological roles, *Amyloid 19(1)*, 5–14. https://doi.org/10.3109/13506129.2011.654294

Lecchi, C., Dilda, F., Sartorelli, P., & Ceciliani, F. (2012). Widespread expression of SAA and Hp RNA in bovine tissues after evaluation of suitable reference genes. *Veterinary Immunology and Immunopathology*, *145*(1–2), 556–562. https://doi.org/10.1016/j.vetimm.2011.12.017

Lepedda, A. J., Nieddu, G., Zinellu, E., Muro, P. De, Piredda, F., Guarino, A., … Formato, M. (2013). Proteomic analysis of plasma-purified VLDL, LDL and HDL fractions from atherosclerotic patients undergoing carotid endarterectomy: identification of Serum Amyloid A as a potential marker. *Oxidative Medicine and Cellular Longevity, Vol. 2013*. http://dx.doi.org/10.1155/2013/385214

Lin, Y., Rajala, M. W., Berger, J. P., Moller, D. E., Barzilai, N., & Scherer, P. E. (2001). Hyperglycemia-

induced production of acute phase reactants in adipose tissue. *Journal of Biological Chemistry*, *276*(45), 42077–42083. https://doi.org/10.1074/jbc.M107101200

Marques, A. T., Nordio, L., Lecchi, C., Grilli, G., Giudice, C., & Ceciliani, F. (2017). Widespread extrahepatic expression of acute-phase proteins in healthy chicken (Gallus gallus) tissues. *Veterinary Immunology and Immunopathology*, *190*(June), 10–17. https://doi.org/10.1016/j.vetimm.2017.06.006

Mayeux, R. (2004). Biomarkers: potential uses and limitations. *The Journal of the American Society for Experimental NeuroTherapeutics*, *1*(2), 182–188. https://doi.org/10.1602/neurorx.1.2.182

Miert, A., Frens, J. (1967). The Reaction of different animal species to bacterial pyrogens. *Institute of Veterinary Pharmacology and Toxicology, University of Utrecht* (dog 22).

Miller, I., & Gemeiner, M. (1992). Two-dimensional electrophoresis of cat sera: Protein identification by cross reacting antibodies against human serum proteins. *Electrophoresis*, *13*(1), 450–453. https://doi.org/10.1002/elps.1150130193

Niine, T., Peetsalu, K., Nieminen, M., Oksanen, A., Soveri, T., & Orro, T. (2017). Giardia and Cryptosporidium infections in neonatal reindeer calves : Relation to the acute phase response. *Comparative Immunology, Microbiology and Infectious Diseases*, *54*(August), 45–50. https://doi.org/10.1016/j.cimid.2017.08.001

Sasaki, K., Ma, Z., Khatlani, T. S., Okuda, M., & Inokuma, H. (2003). Evaluation of Feline Serum Amyloid A (SAA) as an inflammatory marker. *J. Vet. Med. Sci.* 65(4), 545-548.

Soler, L., Luyten, T., Stinckens, A., Buys, N., Cerón, J. J., & Niewold, T. A. (2011). Serum amyloid A3 (SAA3), not SAA1 appears to be the major acute phase SAA isoform in the pig. *Veterinary Immunology and Immunopathology*, *141*(1–2), 109–115. https://doi.org/10.1016/j.vetimm.2011.02.019

Stoughton, R. B., & Friend, S. H. (2005). How molecular profiling could revolutionize drug discovery. *Nature Reviews. Drug Discovery*, *4*(4), 345–50. https://doi.org/10.1038/nrd1696

Tamamoto, T., Ohno, K., Ohmi, A., Goto-koshino, Y., & Tsujimoto, H. (2008). Verification of Measurement of the Feline Serum Amyloid A (SAA) Concentration by Human SAA Turbidimetric Immunoassay and Its Clinical Application. *J. Vet. Med. Sci 70(11),* 1247-1252.

Thadikkaran, L., Siegenthaler, M. A., Crettaz, D., Queloz, P. A., Schneider, P., & Tissot, J. D. (2005). Recent advances in blood-related proteomics. *Proteomics*, *5*(12), 3019–3034. https://doi.org/10.1002/pmic.200402053

Tvarijonaviciute, A., Gutiérrez, A. M., Miller, I., Razzazi-fazeli, E., & Tecles, F. (2012). A proteomic analysis of serum from dogs before and after a controlled weight-loss program. *DAE*, *43*(4), 271–277. https://doi.org/10.1016/j.domaniend.2012.04.004

Uhlar, C. M., & Whitehead, A. S. (1999). Serum amyloid A , the major vertebrate acute-phase reactant. *Eur. J. Biochem. 265*, 501-523.

Urieli-shoval, S., Linke, R. P., & Matzner, Y. (2000). Expression and function of serum amyloid A , a major acute-phase protein , in normal and disease states. *Current Opinion in Hematology 7, 64-69*.

Vilhena, H., Figueiredo, M., Cerón, J., Pastor, J., Miranda, S., Craveiro, H., … Tvarijonaviciute, A. (2018). Acute phase proteins and antioxidant responses in queens with pyometra. *Theriogenology*, *115*, 30–37. https://doi.org/10.1016/j.theriogenology.2018.04.010

Xiao, Z., Prieto, D., Conrads, T. P., Veenstra, T. D., & Issaq, H. J. (2005). Proteomic patterns: Their potential for disease diagnosis. *Molecular and Cellular Endocrinology*, *230*(1–2), 95–106. https://doi.org/10.1016/j.mce.2004.10.010