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Veterinary Biomarker Discovery: Proteomic Analysis of Acute Phase Proteins

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1. Introduction

Modulation of acute phase protein (APP) expression in biological fluids and tissues during disease has emerged as a primary means of assessing both local and systemic innate immune responses. From a veterinary biomarker perspective, APPs are attractive candidates for the diagnosis of disease in food animals, as well as for differentiating between chronic and acute inflammation and for evaluating response to therapeutics (Horadagoda et al., 1999). Several diseases of economic importance in livestock, most notably respiratory disorders and mastitis in cattle, remain a primary focus of veterinary disease research, due to the lack of efficacious treatment options for the profound inflammation caused by exposure to causative bacterial agents. Additionally, inflammatory diseases are often accompanied by the widespread use of antibiotics for treatment and prevention; an aspect that causes concern for food safety, as well as for the emergence of resistant strains of bacteria. Accordingly, recent veterinary biomarker discovery initiatives have focused on the identification of sensitive and reliable indicators for use in evaluating the efficacy of adjunctive therapies for the treatment of inflammation associated with disease in food animal species. As a result, modulation in several APP, including haptoglobin (HPT), alpha-1-acid glycoprotein (AGP), bovine serum albumin (BSA), inter-alpha-trypsin inhibitor heavy chain-4 (ITIH4), and serum amyloid A (SAA), have been detected in the analyses of bovine milk, plasma, and broncheoalveolar lavage fluid (BALF) during both naturally occurring and experimentally induced disease (Boehmer et al., 2010; Danielsen et al., 2010; Mitchell et al., 2007; Eckersall et al., 2006).

Classic approaches to the characterization of protein changes in complex biological fluids have required the availability of species-specific antibodies for the detection and quantification of a given protein. Use of antibody-based strategies such as enzyme-linked immunosorbent assays (ELISA) in biomarker discovery analyses, however, limits the identification and characterization of novel candidates, as well as the detection of post-translational modifications (PTM) of target proteins. Previous research has established that APPs are subject to modifications, such as glycosylation, in altered physiological states (Gruys et al., 2005; Higai, et al., 2003; Anderssen et al., 2001). While APPs are thought to lack specificity as candidate biomarkers of disease because of their prominence during the innate immune response, there is evidence to suggest that the modification of certain APPs could be disease-specific. Thus, characterization of the post-translational modifications in APPs

during the inflammatory response in different diseases could aid in the establishment of the specificity of APPs as biomarkers.

Proteomics, defined as the identification of all proteins within a cell or tissue, involves the use of analytical methodologies such as liquid chromatography (LC) to separate proteins or peptides, and mass spectrometry (MS), to isolate, identify, and characterize proteins and their associated PTMs. An advantage of proteomics in biomarker discovery is the ability to detect a theoretically unlimited number of proteins in a given sample without the need for antibodies. Bottom-up proteomics including proteolytic digestion of proteins prior to the use of LC to separate peptides coupled with tandem MS (MS/MS) for peptide sequencing, a process commonly referred to as LC-MS/MS, has become the most widely used proteomic approach for the identification of individual proteins in complex mixtures. Additionally, advances in ion fragmentation strategies, including electron transfer dissociation (ETD), have provided superior tools for the characterization of modified peptides. Detection of disease-specific modifications of APP glycopeptides, however, is still an emerging aspect of veterinary biomarker discovery. The following chapter will discuss the role of APP as biomarkers of infection and inflammation in food animals, as well as proteomic strategies and the proteomic analyses of APPs during inflammatory disease in ruminant species. Strategies for characterizing modified glycopeptides will also be introduced, including the potential disease-specific modification of APPs.

2. Acute phase proteins: Biomarkers of infection and inflammation in food animals

Because APPs have demonstrated changes as great as 25% in serum concentrations during altered physiological states, APPs have garnered attention for use as potential biomarkers for diagnosing animal disease, monitoring health status, and evaluating responses to primary and adjunctive veterinary therapies (Eckersall and Bell, 2010). In particular, APPs have been investigated as potential biomarkers for inflammatory diseases in food animals, because the etiological agents are bacterial in origin, and the use of antibiotics for treatment and prevention is common. Antibiotic use in food animals, however, causes concern for food safety, and could increase selective pressures for the emergence of resistant strains of bacteria. Additionally, antibiotics are not always the appropriate treatment for diseases in food animals with affiliated inflammatory responses. Though APPs have been characterized as non-specific responders to pro-inflammatory signals, differences in APP expression during disease across species have been reported, and evidence exists that circulating concentrations of APPs in blood and other biological fluids are a direct indication of disease severity (Eckersall and Bell, 2010; Gruys et al., 2005; Murata et al., 2004). Additionally, more recent data based on the proteomic analyses of APPs during disease suggests that modification of glycoproteins could be disease specific (Wilson et al., 2008). The prospect of using APPs as biomarkers of inflammation and infection for veterinary applications has inspired a significant body of research, including the development of antibodies and other quantitative methods for analyzing APP expression during disease in food animal species (Murata et al., 2004). More recently, interest in APPs as potential veterinary biomarkers has led to the application of proteomic strategies for the evaluation of APPs and measures of the host response in complex biological samples (Bendixen et al., 2011; Boehmer et al., 2010; Danielsen et al., 2010; Smolenski et al., 2007).

2.1 Modulation of acute phase proteins during disease in swine

Alteration in APP expression has been associated with a number of food animal diseases (Table 1), including mastitis, metritis, and amyloidosis in lactating dairy cattle (Chan et al., 2010; Safi et al., 2009; Suojala et al., 2008; Eckersall et al., 2001, 2006; Takahashi et al., 2006; Grönlund et al., 2003, 2005); sepsis, trauma, and respiratory diseases in swine (Amory et al., 2007; Sorensen et al., 2006; Hultén et al., 2003; Heegaard et al., 1998; Eckersall et al., 1996); infectious bronchitis virus in chickens (Nazifi et al., 2011); respiratory diseases in beef cattle (Orro et al., 2011; Heegaard et al., 2000; Godson et al., 1996; Horadagoda et al., 1995); as well as pulmonary damage and caseous lymphadenitis in sheep (Eckersall et al., 2007; Pfeffer and Rogers, 1989).

Protein	Diseases	Species
Haptoglobin	Pneumonia, Sepsis, Inflammation	Swine
Serum amyloid A	Pneumonia, Sepsis	Swine
C-reactive rpotein	Sepsis, Inflammation	Swine
pig-MAP[1]	Sepsis	Swine
α-1-acid glycoprotein	Inflammation	Swine
Ceruloplasmin	Inflammation	Swine
Acid soluble glycoprotein	Inflammation	Swine
Haptoglobin	Infectious bronchitis	Chickens
Serum amyloid A	Infectious bronchitis	Chickens
Lipopolysaccharide binding protein	Mastitis, Respiratory Disease	Cattle
Serum Amyloid A	Mastitis, Respiratory Disease, Amyloidosis	Bovine
Haptoglobin	Mastitis, Respiratory Disease, Amyloidosis	Bovine
α-1-acid glycoprotein	Mastitis, Respiratory Disease	Bovine
Haptoglobin	Caseous lymphadenitis, Pulmonary damage	Sheep
Serum amyloid A	Caseous lymphadenitis	Sheep
α-1-acid glycoprotein	Caseous lymphadenitis	Sheep
Fibrinogen	Pulmonary damage	Sheep
Ceruloplasmin	Pulmonary damage	Sheep
Haptoglobin	Inflammation	Goat
Serum amyloid A	Inflammation	Goat
Fibrinogen	Inflammation	Goat
Acid soluble glycoprotein	Inflammation	Goat

pig-MAP[1] = pig major acute phase protein

Table 1. Acute phase proteins associated with disease in food animals

In swine, increased levels of serum HPT have been associated with pathological indications of *Mycoplasma hyopneumoniae*, a leading cause of porcine enzootic pneumonia (Amory et al., 2007). Similarly, serum concentrations of HPT and SAA were elevated in pigs with experimentally induced *Actinobacillus pleuropneumoniae* pneumonia (Hultén et al., 2003; Heegaard et al., 1998). Peak expression of HPT and SAA in pigs with *A.pleuropneumoniae*

pneumonia was detected approximately 4 days following infection. However, serum HPT and SAA levels were lower in pigs treated with the antimicrobial enrofloxacin following experimental infection with *A.pleuropneumoniae*, which indicated the feasibility of using APPs to monitor response to therapeutic treatment (Hultén et al., 2003).

During experimental induction of sepsis, pigs infected with *Streptococcus suis* exhibited increases in serum concentrations of the APPs CRP, SAA, HPT, and pig-MAP (Sorensen et al., 2006). Both CRP and SAA peaked on day 1 following infection with *S. suis*, and returned to near baseline levels 5-12 days following infection, while HPT and pig-MAP peaked on days 5-8 following infection and remained elevated for the duration of the study (Sorensen et al., 2006). Conversely, serum levels of HPT and CRP peaked just 2 days after the experimental induction of inflammation in pigs, while ceruloplasmin levels did not reach peak concentrations until 4 days after experimental treatment with turpentine (Eckersall et al., 1996). Though the same positive APPs were detected in a variety of different experimental swine disease models, it should be noted that temporal expression patterns and peak serum concentrations of each APP varied for each disease, indicating some level of disease specificity.

2.2 Modulation of acute phase proteins during ruminant disease

Currently, only limited data is available regarding acute phase protein expression in small ruminants during disease. Experimental induction of inflammation in goats revealed increases in serum levels of HPT, SAA, fibrinogen, and acid soluble glycoprotein following injection with turpentine (González et al., 2008), while experimental-induction of caseous lymphadenitis in sheep revealed increases in serum levels of SAA, HPT, and AGP (Eckersall et al., 2007).

Conversely, there have been several investigations into the acute phase response during both naturally-occurring and experimentally-induced bovine diseases. In studies of bovine respiratory disease, peak levels of serum SAA and HPT were reported during week 3 of an outbreak of respiratory disease in calves caused by respiratory syncytial virus (Orro et al., 2011), while experimental infection with respiratory syncytial virus caused maximal increases in serum levels of HPT and SAA in cattle as early as 7-8 days following inoculation (Heegaard et al 2000). Of the bovine diseases studied, however, APP expression has been investigated most extensively during mastitis in lactating dairy animals (Safi et al., 2009; Suojala et al., 2008; Eckersall et al., 2006, 2001; Grönlund et al., 2003; Hirvonen et al., 1999). Mastitis is defined as an inflammation of the mammary gland, and is considered the most dominant and costly of diseases to affect lactating dairy cattle. *Staphylococcus aureus* and *Escherichia coli* are perhaps the most prevalent species of Gram-positive and Gram-negative bacteria, respectively, that cause clinical mastitis. Due to affiliated financial losses and food safety concerns, characterization of the innate immune response during clinical mastitis has remained a primary focus of veterinary research. Studies of the first line of defense against invading pathogens in the bovine mammary gland have provided valuable information on the expression of acute phase proteins during inflammation in dairy cattle, and have revealed differences in temporal expression of APPs dependent on the causative bacterial species (Bannerman et al., 2004). APPs have been the center of numerous studies of both naturally-occurring and experimentally-induced mastitis because of their ability to opsonize and trap microorganisms, activate complement, neutralize enzymes, and modulate the host response to infection (Gruys et al., 2005). Likewise, the bovine mammary gland is an

established source of extra-hepatic APP production; thus the evaluation of APP expression in milk could aid in the early detection of mastitis (Hiss et al., 2004).

Studies directed at the innate immune response in the bovine mammary gland following challenge with either LPS or *Escherichia coli* (*E. coli*) have reported increases in the APPs SAA, HPT, AGP, and lipopolysaccharide binding protein (LBP) in milk and serum from cows during clinical mastitis (Suojala et al., 2008; Ceciliani et al., 2005; Hiss et al., 2004; Eckersall et al., 2001; Hirvonen et al., 1999). Increases in serum concentrations of both SAA and HPT following intra-mammary challenge with *E. coli* were reported 24 hours after experimental infection, with peak expression of both APPs apparent on day 3 after challenge (Suojala et al., 2008; Hirvonen et al., 1999). Intracisternal injection of lipopolysaccharide, however, caused elevations in serum HPT levels in infected cows as early as 9 hours following challenge (Hiss et al., 2004). Reports of serum HPT levels in cows with naturally occurring coliform mastitis were in accord with values detected following experimental challenge (Ohtsuka et al., 2001). LBP appeared to increase more rapidly than HPT and SAA in cows with experimentally-induced coliform mastitis, as peak expression was reported as early as 36 hours following challenge (Suojala et al., 2008). Conversely, reports of AGP indicate that peak expression was not detected in serum from cows with coliform mastitis until 9 days after the onset of clinical symptoms (Ohtsuka et al., 2001). The evaluation of APP expression in milk from cows with experimentally-induced coliform mastitis revealed increases in HPT, SAA, and LBP as early as 12 hours following challenge with peak expression at 44, 60, and 36 hours, respectively (Suojala et al., 2008). Increases in both milk and serum levels of HPT and SAA in cows experimentally infected with *Staphylococcus aureus* (*S. aureus*) have also been reported (Hiss et al., 2007; Eckersall et al., 2006; Grönlund et al., 2003). Evaluation of the acute phase response following experimental challenge with *S. aureus* revealed increases in HPT and the mammary isoform of SAA (M-SAA3) in both bovine serum and milk that were in accord with data from experimental challenge with *E. coli* (Eckersall et al., 2006; Grönlund et al., 2003).

While the primary source of APPs is the liver, local production of SAA, HPT, and AGP in the bovine mammary gland have also been demonstrated, which mark APPs as potential indicators of both systemic and local inflammation in cattle (Larson et al., 2006; Jacobsen et al., 2005; Ceciliani et al., 2005; Hiss et al., 2004). Additionally, aside from both local and hepatic production of AGP, the granules of bovine neutrophils are likewise a reported source of APPs (Rahman et al., 2008). Regardless of source of production, however, ruminant animals differ from other species in terms of APP expression during inflammation, in that HPT is the major APP in bovine innate immunity, whereas in other animal species CRP predominates the acute phase response (Ecksersall and Bell, 2010; Petersen et al., 2004).

2.3 Detection methods for acute phase proteins in biological samples

Most prior analyses of the acute phase response during bovine mastitis have utilized ELISAs, immunodiffusion, haemoglobin binding, or the evaluation of messenger RNA (mRNA) expression to evaluate changes in APP expression during disease (Hiss et al., 2007; Eckersall et al., 2006; 2005; 2001; Jacobsen et al., 2005; Grönlund et al., 2003). Use of such strategies in biomarker discovery analyses, however, limits the identification and characterization of novel protein candidates, as well as the detection of potential PTMs of APPs, due to a reliance on the availability of species-specific antibodies, and the ability to evaluate only one APP per assay. The most crucial element of the ELISA detection strategy

is a highly specific antibody-antigen interaction. Thus, characterization of protein changes in complex biological fluids using ELISA requires the availability of species-specific antibodies for the detection and quantification of a given protein. Unfortunately, a very limited number of antibodies are commercially available for livestock species. Additionally, there is no practical protocol for the development of an immunoassay targeting a modified site if neither the site nor the modification is known.

Genomic methodologies, including gene arrays and the evaluation of mRNA expression can also limit biomarker discovery because of the demonstrated weak correlation that often exists between mRNA levels and actual protein concentration. Thus, quantitative mRNA data is often an inadequate indicator of protein expression (Gygi et al., 1999; Ideker et al., 2001; Griffin et al., 2002; Tian et al., 2004). However, advances in soft ionization techniques in mass spectrometry (MS), including electro-spray ionization (ESI), nano-spray ionization, and matrix-assisted laser desorption/ionization (MALDI), have broadened the applications of mass spectrometry to include the characterization of biopolymers such as intact proteins and peptides, and has given rise to a new field of protein study termed proteomics (reviewed in Mann et al., 2001). Proteomics, defined as a scientific approach used to elucidate all proteins within a cell or tissue (Colantonio and Chan, 2005) boasts a significant advantage over genomic and antibody-based analyses, because proteomics involves the use of analytical methodologies, such as LC and MS, to isolate, identify, and characterize proteins, and is not reliant on the use or availability of antibodies. Additionally, proteomic methodologies can detect a theoretically unlimited number of proteins in a given sample without the need for antibody or reagent development.

3. Proteomic strategies

Protein identification through the use of MS can be divided into two main categories, referred to as top-down and bottom-up. The primary distinguishing features between the two main proteomic approaches is the isolation and fragmentation of intact proteins using MS in a top-down approach, versus proteolytic digestion of mixtures of proteins, and the subsequent separation and fragmentation of peptides, in bottom-up proteomics. Identification of proteins in complex biological mixtures using bottom-up proteomics is reliant upon the measurement of the masses of the peptides that are generated following proteolytic cleavage of the proteins. The mass of a peptide is determined using MS, and is based upon a mass-to-charge ratio (*m/z*). Charged peptides are generated as a result of ionization, or the addition of a proton to the peptide, which results in the conversion of the peptide into an ion. The two most popular forms of ionization used in bottom-up proteomic analyses are ESI and MALDI.

3.1 Two-dimensional gels and MALDI-TOF mass spectrometry

Over the past two decades, two-dimensional gel electrophoresis (2D-GE) followed by MALDI time-of-flight (TOF) MS and LC-MS/MS have become the most widely used proteomic approaches in bottom-up proteomic analyses (Ferguson et al., 2003). Protein profiling by 2D-GE is characterized by a first dimension separation of proteins by charge (isoelectric point), followed by a second dimension separation by molecular weight. Advances in 2D-GE technology including gel strips with immobilized pH gradients have dramatically increased the resolving power of 2D-GE. Additionally, the development of radioactive and fluorescent labeling has improved the ability to visualize proteins in a 2D

gel, as well as the detection of low-abundance and post-translationally modified proteins (Van den Bergh et al., 2005).

In a MALDI-TOF/MS experiment, the protein or peptide(s) of interest is mixed with a suitable energy-absorbing matrix and allowed to co-crystallize by air-drying on a stainless steel plate. Matrices used in MALDI-TOF/MS are typically small aromatic molecules capable of absorbing high levels of UV light at a specific wavelength, such as sinapinic acid (SPA) or alpha-cyano-4-hydroxycinnamic acid (CHCA). The generation of ions in MALDI-TOF/MS is initiated by short pulse irradiation with a laser (Karas and Krüger, 2003), typically nitrogen or neodymium-doped yttrium aluminum garnet (Nd:YAG), and occurs when the matrix becomes electronically excited following absorption of photons from the UV laser. Analytes in the sample accept a proton from the matrix and become singly charged positive ions as they are ejected from the matrix and converted into the gas phase. The ions are then directed into the TOF analyzer where they are separated based on their *m/z* and generate a mass spectrum. Separation of ions is based on the principle that ions with smaller *m/z* "fly" faster than larger ions. The subsequent protein identification is accomplished by peptide mass fingerprinting, which is the comparison of the set of peptide masses generated from a specific protein to a protein database containing theoretically calculated mass fingerprints of all known proteins. A process called post source decay (PSD) can be used on TOF instruments equipped with a reflectron to further enhance resolution of the MALDI peak separation. In this technique, the voltage on the reflectron is modulated during analysis to allow the detection of ion fragments formed during ionization or acceleration down the flight tube (Kaufmann et al., 1994).

3.2 LC-MS/MS

Despite the recent advancements in reproducibility and protein quantification, 2D-GE as a means of protein separation still suffers from issues including isolation of proteins with low abundance, high hydrophobicity, or extreme isolelectric points. Consequently, LC has emerged as the best alternative for the separation of proteins or peptides in solution prior to mass analysis using MS. Recently, the combination of two LC-based separation techniques coupled with MS was introduced, and has profoundly increased the ability to resolve and detect a greater number of peptides in LC-MS/MS-based proteomic experiments. Multidimensional protein identification technology (MudPIT), utilizes the combination of strong cation-exchange chromatography and reverse-phase chromatography followed by ESI-MS/MS for the characterization of proteins in a complex mixture (Washburn et al., 2001). Using the MudPIT approach, proteins are typically digested into peptides using a protease such as trypsin, which cleaves at every arginine and lysine residue, and separated online by 2-dimensional LC prior to introduction into the mass spectrometer for mass analysis. In 2-dimensional LC, peptides are separated in the first dimension by charge using ion exchange chromatography, and are then further separated in the second dimension by hydrophobicity using reversed-phase (RP) chromatography.

In a one-dimensional (1D) LC-MS/MS experiment, peptide mixtures are typically separated only by hydrophobicity by passage over a column packed with non-polar stationary phase. Thus, the number of proteins identified using 1D-LC-MS/MS is directly dependent on the efficiency of peptide separation prior to introduction into the mass spectrometer (Jensen et al., 1999). In LC-MS/MS experiments, ESI is the dominant method of ionization. Ionization occurs in ESI after the peptide solution is dispersed as a fine spray of charged droplets after passage through a heated metal capillary tube to which voltage is applied. The charged

droplets get desolvated by a dry inert gas, and multiply charged ions are produced. Nano-spray ionization (NSI) functions in essentially the same manner as ESI, but flow rates from the LC instrument into the ionization source of the mass spectrometer are much lower with nano-spray than those used for ESI, and ionization efficiency is greatly improved (Wilm et al., 1996). Ions resulting from either ESI or NSI are then directed into the vacuum chamber of the MS instrument, and are resolved according to their *m/z* ratio to produce the first MS spectrum. While the first MS scan generates the mass of all peptides, peptide(s) of interest are subjected to further fragmentation by a process called collision-induced dissociation (CID) in the second MS scan. An inert gas such as argon (Ar) or helium (He) is introduced into the collision cell of the mass spectrometer which results in the production of a tandem or MS/MS spectrum. Peak lists generated from the fragment ion masses in tandem mass spectra are then searched against a protein database to determine the amino acid sequence of the peptides in the complex mixture. The assignment of the sequenced peptides to a given protein is the means by which protein identification is accomplished (Jensen et al., 1999).

3.3 Post-translational modifications

Comparative proteomic analyses are designed to elucidate changes in the relative abundance of proteins among different biological states, most commonly healthy versus diseased. Detection of the same peptides from a given protein is not always possible in comparative studies, however, because post-translational modification of peptides as a result of disease is expected. Characterization of PTMs is crucial for biomarker discovery, because much of the regulation of the biological activity of proteins is mediated by the modification of peptide amino acid residues, including the phosphorylation of serine and threonine, and the glycosylation of asparagine, arginine, or tyrosine. Identification of PTMs is especially useful for the detection and characterization of APPs during disease because APPs are glycoproteins and are subject to modification. Unfortunately characterization of PTM's has been hindered in past experiments due to the fact that modifications are labile and are often lost in a CID experiment. Electron-transfer dissociation (ETD), which is a superior fragmentation strategy for the analysis of PTMs, however, was recently introduced, and shows promise as a strategy for the characterization of APP modification during disease (Syka et al., 2004). The ETD technique uses electrons to promote fragmentation along the peptide backbone, which produces a series of c and z ions, instead of CID fragmentation, which produces a series of b and y ions. The fragmentation of the peptide backbone using ETD allows for amino acid side chains and modifications such as glycosylation and phosphorylation to remain intact, making it possible not only to deduce the amino acid sequence of a peptide, but also to detect any modified residues (Syka et al., 2004). Furthermore, the combination of CID and ETD has proven effective as well in the characterization of isolated glycopeptides, including modified peptides from HPT in human lung cancer patients (Wang et al., 2011).

4. Proteomic analysis of acute pphase proteins during animal disease

Proteomic approaches boast the capability to analyze an unlimited number of protein targets in a single experiment, independent of antibody availability. Proteomics is rapidly gaining popularity in veterinary biomarker studies, especially those aimed at the discovery of biomarkers of disease, productivity, product quality, and animal welfare in cattle and swine (Bendixen et al., 2010). Furthermore, compared to a recent review of the evaluation of

APP expression during disease in companion animals and cattle (Eckersall et al., 2010), a far greater overall number of APPs have been identified in biological fluids from food animals using proteomic strategies (Table 2) than have been detected using more traditional approaches.

The most widely studied biological fluids in proteomic-based biomarker discovery analyses in swine and cattle have been serum, plasma, and milk. To date, protein profiles have been generated for serum and plasma of both healthy cattle and pigs, using 2D-GE (Miller et al., 2009; Talamo et al., 2003; Wait et al., 2002). Comparative proteomic analyses have likewise been conducted using 2D-GE to profile differentially expressed proteins in plasma from pigs with peritonitis-induced sepsis, with findings that the APP inter-alpha trypsin inhibitor-heavy chain-4 (ITIH-4), HPT, hemopexin, alpha-2-HS-glycoprotein, albumin, and apolipoprotein-A1 all exhibited modulated expression levels as a result of disease (Thongboonkerd et al., 2009). Proteomics has also been applied to the study of the gastrointestinal tract in swine (Wang et al., 2009; Danielsen et al., 2007, 2006), as well as diet-induced fatty liver disease in Ossabaw pigs (Bell et al., 2010).

Studies aimed at the elucidation of potential biomarkers of bovine mastitis have specifically dominated veterinary biomarker initiatives in cattle, however, due in large part to affiliated inflammation, economic and food safety concerns, and the lack of efficacious treatment options (Boehmer et al., 2010; 2008; Danielsen et al., 2010; Smolenski et al., 2007; Hogarth et al., 2004). Several comparative proteomic studies have focused on the identification of diagnostic biomarkers of mastitis in bovine milk, though the proteomic analysis of mammary tissue from healthy cows and cows with clinical mastitis has also been conducted (Yang et al., 2009). Likewise, other diseases of economic importance to the cattle industry have been the focus of proteomic-based veterinary biomarkers studies, including the analysis of bronchoalveolar lavage fluid (BALF) from the bovine respiratory tract following administration of dexamethasone, as well as after the stress of transport (Mitchell et al., 2008, 2007).

The earliest comparative proteomic analyses of normal versus mastitic bovine milk were accomplished using 2D-GE followed by MALDI-TOF/MS (Smolenski et al., 2007; Hogarth et al., 2004), or MALDI-TOF/TOF PSD (Boehmer et al., 2008). Despite a limited number of proteins detected, promising discoveries that resulted from the 2D-GE- MALDI-TOF/MS of bovine milk included the identification of the APP α-1-acid glycoprotein (AGP) in both normal and mastitic whey samples, and the apparent higher relative abundance of AGP in mastitic milk as early as 18 hours following challenge (Boehmer et al., 2008). Previously, the analyses of APP expression in milk during bovine mastitis using more traditional quantitative strategies had only identified the APPs SAA, HPT, and LBP (Hiss et al., 2004; Bannerman et al., 2004; Eckersall et al., 2001). Similar to the 2D-GE- MALDI-TOF/MS of bovine milk, the proteomic analysis of changes in the bovine BALF proteome induced by dexamethasone revealed increases in the APPs alpha-2-HS-glycoprotein, alpha-1-antichymotrypsin, alpha-1-antitrypsin, and AGP in treated animals when compared to controls (Mitchell et al., 2007).

To avoid some of the limitations imposed by a 2D-GE-MALDI-TOF/MS proteomic experiment, more recent proteomic analyses of bovine milk have been accomplished through the use of LC-MS/MS (Boehmer et al., 2010; Danielsen et al., 2010; Smolenski et al., 2007). The first attempt to characterize proteins related to host defense in mastitic bovine milk did result in the identification of the APPs serum albumin, fibrinogen, and SAA, but very few biological replicates were used in the analyses, and the objectives were strictly

proteome coverage, not comparisons between the healthy and diseased states (Smolenski et al., 2007). The two most recent comparative proteomic analyses of normal versus mastitic bovine milk, however, have not only identified several APPs in milk, but tracked changes in APPs over the course of infection, and quantified modulation in relative abundance of APPs during disease (Boehmer et al, 2010; Danielsen et al., 2010).

Protein	Accession Number	Species	Fluid	Method of Detection
Inter-α-trypsin inhibitor heavy chain-4	P79263	Swine	Plasma	2D-GE-MALDI-TOF/MS
Haptoglobin	Q8SPS7	Swine	Plasma	2D-GE-MALDI-TOF/MS
α-2-HS-glycoprotein	P29700	Swine	Plasma	2D-GE-MALDI-TOF/MS
Serum albumin	P08835	Swine	Plasma	2D-GE-MALDI-TOF/MS
Apolipoprotein-A1	P18648	Swine	Plasma	2D-GE-MALDI-TOF/MS
Serum Amyloid A	P35541	Bovine	Milk	LC-MS/MS
Haptoglobin	Q2TBU0	Bovine	Milk	LC-MS/MS
α-1-acid glycoprotein	Q3SZR3	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS
		Bovine	BALF[1]	2D-GE MALDI-TOF/MS
Serum albumin	P02769	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS
Serotransferrin	Q29443	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS
α-2-HS-glycoprotein	P12763	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS
		Bovine	BALF[1]	LC-MS/MS
α-2 Macroglobulin	Q7SIH1	Bovine	Milk	LC-MS/MS
Inter-α-trypsin inhibitor heavy chain-4	Q3T052	Bovine	Milk	LC-MS/MS
Apolipoprotein-A1	P15497	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS
α-1- Antitrypsin	P34955	Bovine	Milk	2D-GE MALDI-TOF/MS
		Bovine	BALF[1]	2D-GE MALDI-TOF/MS
α-1- Antichymotrypsin	Q28921	Bovine	BALF[1]	LC-MS/MS
Fibrinogen	P02672	Bovine	Milk	LC-MS/MS, 2D-GE MALDI-TOF/MS

BALF[1] = Bronchoalveolar lavage fluid (Mitchell et al., 2008; 2007)

Table 2. Acute phase proteins identified disease in food animals using proteomic strategies

4.1 Quantification of acute phase proteins in bovine milk using proteomics

Relative and absolute quantification of changes in the abundance of potential biomarkers identified in biological matrices using proteomic strategies is a topic that has garnered significant attention in recent years (Simpson et al., 2009; Mueller et al., 2008; Fenselau, 2007; Roe and Griffin, 2006). Several strategies exist for the quantification of individual proteins in complex mixtures using proteomics; however, quantification methods can be assigned to one of two broad categories: a labeling approach that requires the incorporation of labels into proteins or peptides prior to MS analysis, or the use of a label-free method such as ion intensities or spectral counts (Simpson et al., 2009). The modulation of the APPs HPT and SAA during mastitis have been evaluated in bovine milk using both a labeled (Danielsen et al., 2010) and a label-free approach (Boehmer et al., 2010). Despite different experimental approaches in the *in vivo* challenge portion of the studies, both proteomic analyses of mastitic bovine milk detected changes in the concentration of SAA and HPT in within hours after challenge. Following infection with LPS, nearly 3-fold changes were detected in both SAA and HPT in bovine milk as early as 7 h after induction of disease (Danielsen et al., 2010). Unlike previous ELISA analyses that were limited to the evaluation of only targeted APP, changes were likewise detected in the APPs serum albumin, alpha-2-macroglobulin, alpha-2-HS-glycoprotein, and serotransferrin in bovine milk during clinical mastitis using

the incorporation of isotopic labels and proteomic identification strategies (Danielsen et al., 2010).

In the proteomic analyses of a longitudinal set of bovine milk samples collected over the course of clinical mastitis following intra-mammary infusion with Gram-negative *E. coli*, modulation in the APPs serum albumin, SAA, HPT, alpha-2-HS-glycoprotein, AGP, inter-alpha trypsin inhibitor heavy chain-4 (ITIH4), serotransferrin, apolipoprotein- A1, and the α-, β-, and γ-chains of fibrinogen, (Figure 1) were evaluated using spectral counts. The theory behind spectral counting, or the number of MS/MS spectra that contribute to the identification of a given protein, is that the abundant proteins, when proteolytically digested, will yield numerous copies of the same peptide (Zybailov et al., 2005; Liu et al., 2004). Furthermore, the probability that abundant peptides will trigger multiple MS/MS events is higher than the likelihood of repeatedly sampling a peptide from a lower abundance protein. In previous investigations into the accuracy and linearity of spectral counts, the spectral counts for peptides from proteins spiked into yeast samples at known concentrations exhibited linearity over two orders of magnitude, and were highly correlated to relative protein abundance (Liu et al., 2004).

Spectral counts were used to evaluate the expression of the APPs HPT and SAA in bovine milk over the course of clinical mastitis, and trends revealed by spectral counts were compared to quantification of the APPs using commercially available ELISAs (Figure 2). Though the comparison indicated slight advantages in the sensitivity of the ELISA to detect the presence of APPs in milk at earlier time points than MS, overall trends were similar. Furthermore, the fact that peptides from relatively low abundance acute phase proteins were detected in an extremely biologically complex matrix using LC-MS/MS supported the use of spectral counts to track changes in proteins for which no antibody or ELISA currently exists.

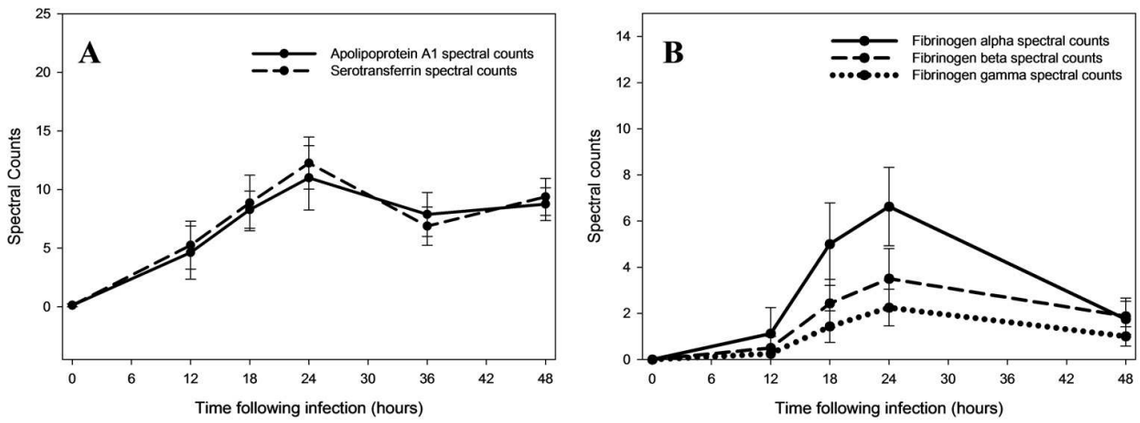

Fig. 1. Boehmer et al., 2010. Temporal expression patterns of proteins involved in the acute phase response following experimental induction of mastitis in bovine milk determined using total spectral counts (mean spectral counts ± standard error) for (A) apolipoprotein-A1 and serontransferrin, and (B) the three chains of the blood coagulation protein fibrinogen.

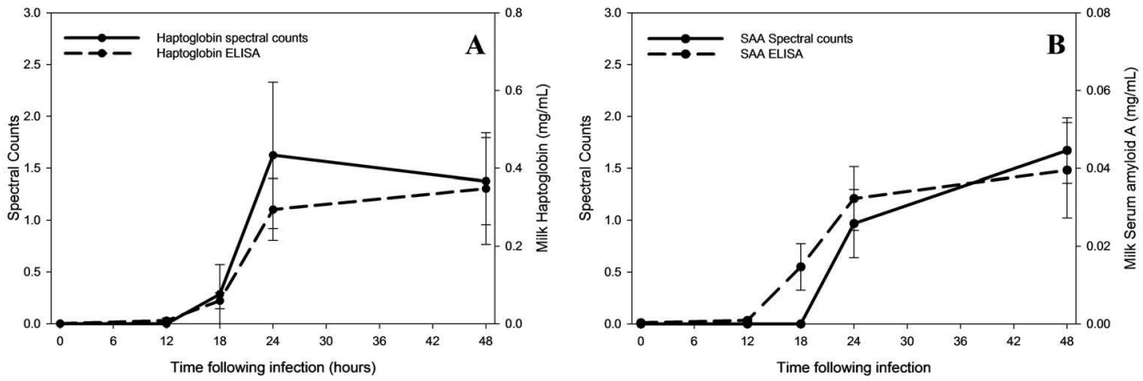

Fig. 2. Boehmer et al., 2010. Comparison of temporal expression patterns of low abundance acute phase proteins determined using ELISA and total spectral counts (mean spectral counts ± standard error) for (A) milk Haptoglobin and (B) milk serum amyloid A. Though sensitivity levels differ, the correspondence of the overall patterns exhibited by the LC-MS/MS data and the ELISA data indicates that spectral counts can be used as a screening tool to profile changes in biologically relevant proteins without a reliance on antibodies.

The added advantage of using proteomics to characterize APP expression during disease was the identification and characterization of the APP ITIH4, a potentially novel biomarker of coliform mastitis. Prior reports of ITIH4 in cattle were limited to isolation of the APP from the serum of heifers with experimentally induced summer mastitis (Pineiro et al., 2004). The association of ITIH4 with innate immunity has, however, been studied in models of acute inflammation in swine (Gonzalez-Ramon et al., 2000), and ITIH4 was recently reported to be a novel marker of acute ischemic stroke in humans (Kashyap et al., 2009). Previous research and similarity to a human homolog led to the classification of ITIH4 as a plasma kallikrein-sensitive glycoprotein, but the exact role and function of ITIH4 in the bovine mammary gland during inflammation associated with coliform mastitis is not yet clear (Nishimura et al., 1995).

As with any analytical method, drawbacks exist regarding the use of proteomic strategies to characterize proteins involved in the acute phase response during disease in livestock species. However, given the fact that the majority of previous reports of APP expression during mastitis and other food animal diseases have used data derived from ELISAs, and that there are only a limited number of commercially available antibodies for livestock species, proteomic strategies afford clear advantages over traditional methods for the detection and characterization of APP during animal disease. Additionally, proteomic strategies offer the opportunity to evaluate the post-translational modification of APP during disease, which could lead to the establishment of specific relationship between the modification of APP and select animal diseases.

5. Detection of the modification of acute phase proteins using proteomics

Modification of the glycosylation patterns of APPs has been implicated in a number of inflammatory diseases in humans and food animals (Gruys et al., 2005). Because post-translational modifications often dictate the biological activity of certain proteins, the characterization of glycosylation patterns of APPs during disease may advance current

knowledge of the mechanisms involved in food animal disease, and aid in the development of new therapeutics. Glycosylation is the most diverse PTM involved in the modulation of protein function (Ohtsubo et al., 2006). Glycosylation is a site specific enzymatic process which covalently binds sugar moieties to proteins in two ways, either through linkage of polysaccharides to the amide nitrogen of asparagine side chains, or to the oxygen atoms of serine, threonin, or tyrosine, which forms N-linked or O-linked glycans, respectively. N-linked glycosylation modulates protein folding and stability through a variety of mechanisms, whereas O-linked glycosylation plays important roles in protein localization, trafficking, and solubility (Spiro 2002).

5.1 Post-translational modification of acute phase proteins during human disease

The majority of published reports detailing the proteomic analysis of the PTM of APPs have focused on the glycosylation pattern of serum proteins associated with human inflammatory diseases (Higai, et al., 2003; Brinkman-van der Linden et al., 1996; Turner et al., 1992), and different forms of cancer (Mazhar et al., 2006; Saldova,et al., 2007; Latif et al., 2002). Modification of the biantennary structures and the α1, 3 fucosylated N-glycan structures of alpha-1-acid-glycoprotein (AGP) have been reported in patients with acute inflammation (Turner et al., 1992), and chronic conditions such as rheumatoid arthritis and diabetes mellitus (Higai, et al., 2003). A remarkable feature of AGP is the microheterogeneity of its sugar moieties and the modification of these sugars during disease. AGP has five N-linked complex type glycans which may be present as bi-, tri-, and tetra-antenary structures (Gornik et al., 2008). Studies have indicated significant enhancement of bi-antennary complex glycans and alpha-1-3fucosylated bi-, tri-, and tetra-antennary glycans, as well as decreases in tri-antennary glycans of AGP, in patients with inflammatory diseases. However, the modifications of AGP in patients with diabetes were not disease-specific (Higai, et al., 2003).

Different forms of human cancer have likewise been associated with alterations in the glycosylation patterns of APPs. The most well characterized N-glycosylation changes in APPs have been studied in AGP. Modulation of glycan branching, which is the number of N-acetylglucosamine (GlcNAc) residues attached to the chitobiose core, and the levels of Sialyl Lewis X (SLex) structures has been reported (Van Dijk et al., 1994; Chandrasekaran et al., 1984,; Katnik et al., 1988). Lung cancer patients were reported to exhibit significant alterations in N-linked glycosylation in total blood serum (Arnold, et al., 2011). In contrast, decreases were observed in core-fucosylated biantennary glycans, with some being detectable as early as Stage I lung cancer. In the same study, the N-linked glycan profile of HPT revealed similar modifications to those detected in the total serum glycome (Arnold, et al., 2011). Specific HPT isoforms have also been evaluated in serum from patients with small cell lung cancer (Shah et al., 2010). The human form of HPT was reported to have only one type of beta subunit, but two different isoforms of its alpha subunit (Shah et al., 2010). Higher circulatory levels of alpha HPT were reported in patients with small cell lung cancer when compared to healthy control subjects. Additionally, a beta chain variant of HPT was discovered that appears to be differentially expressed only in small cell lung cancer patient serum (Shah et al., 2010). Changes in the glycosylation patterns of APPs have also been reported for ovarian cancer (Saldova et al., 2007). Reported findings indicated that the changes in glycosylation patterns of APPs associated with ovarian cancer involved doubling the amount of core fucosylated, galactosyl biantennary glycans and sialyl Lewis X.

5.2 Post-translational modification of acute phase proteins during animal disease

In veterinary research, differential expression and increased abundance of fucosylated peptides have been reported for lymphoma and transitional cell carcinoma (TCC) in dogs (Wilson et al., 2008). The progression of canine lymphoma was monitored during the course of disease, and it was discovered that the same fucosylated peptides that increased during the pre-chemotherapy period were reduced post-chemotherapy treatments. Additionally, the same peptides increased in abundance upon recurrence of the lymphoma. Furthermore, the comparison of all fucosylated peptides detected in lymphoma and TCC, revealed only two common peptides for the two different forms of cancer. The results of the study indicated the power of glycoproteomics in discriminating between fucosylation of specific glycopeptides in two different types of cancer in dogs (Wilson et al., 2008).

Modulation in the glycosylation of the APP HPT in serum from dogs with several diseases including anaemia, inflammation, lymphoma, and chronic progressive hepatitis has also been reported (Anderssen et al., 2001). Following pretreatment of serum with a fucose-specific lectin, abnormal microeterogeiety in the glycosylation pattern of HPT was detected using iso-electric focusing (IEF) and immunoblotting. The major modification of HPT was increased fucosylation, and was predominantly observed in dogs with anaemia, but also to a lesser extent in dogs with inflammation and lymphoma. Conversely, dogs with liver disease exhibited reduction of sialic acid residues in HPT (Andersson et al, 2001).

Similar to the PTM of HPT, glycosylation of AGP is also altered during animal diseases. During inflammation, increases in plasma concentrations of AGP, as well as many structural modifications including the glycoslyation pattern and the degree of branching and fucosylation of AGP have been reported (Ceceliani et al., 2007). The structure of the glycoprotein AGP has been analyzed in several species including cows, sheep and rats, with the results indicating that the glycosylation pattern of AGP was quite variable (Nakano et al., 2004). Using MALDI-TOF MS, it was determined that in sheep the mono- and disialodiantennary carbohydrate chains of AGP were elevated, while the abundance of tri- and tetra-sialo triantennary carbohydrate chains were decreased. Some novel carbohydrate chains containing both N-acetylneuraminic acid and N-glycolylneuraminic acid were observed in bovine AGP. No triantennary carbohydrate chains were detected in bovine AGP, however, elevated abundance of diantennary carbohydrate chains with tri- or tetra-sialyl residues were observed. In rats, a complex mixture of disialo carbohydrate chains of N, O-acetylneuraminic acids were detected on AGP (Nakano et al., 2004). The glycosylation pattern of AGP in cats was investigated in animals with feline immunodeficiency virus and feline leukemia virus. Increased sialylation of AGP was observed in cats with lymphoma; however, decreased sialylation of AGP was detected in animals with feline infectious peritonitis (Pocacqua et al., 2005; Ceciliani et al., 2004).

Due to complexity and the fact that low abundant N-glycosylated proteins or peptides exist in complex mixtures among a large excess of nonglycosylated counterparts, complete analysis of these markers in a clinical laboratory is still not feasible. In the research laboratory setting, enrichment methods including lectin-affinity chromatography or chemical linkage of the carbohydrate to surfaces are used to isolate glycosylated proteins from complex mixtures (Abbott, et al., 2010; Ito et al., 2009). However, peptide enrichment combined with proteomic strategies such as LC-MSMS is the latest technology for large-scale analysis of glycosylated proteins. In particular the use of fragmentation strategies including CID and ETD has shown to dramatically improve detection and characterization of the post-translational modification of glycoproteins (Alley et al., 2009 and Zielinska et al,

2010). All data available to date indicate that in both human and veterinary medical research, identification of APPs and characterization of the alteration in glycosylation patterns could provide valuable clinical information for use as specific biomarkers of many divergent diseases.

6. Conclusion

While results of comparative proteomic analyses conducted on biological samples collected from food animals during naturally-occurring and experimentally-induced disease have revealed promising candidate acute phase protein biomarkers and identified PTMs that are potentially disease specific, inherent roadblocks still exist that have precluded the validation of APPs as biomarkers of food animal disease. Caveats to proteomic strategies include the complexity of biological matrices, both before and during disease, the intense dynamic range of proteins present in most biological fluids, the lack of a universal analyses platform (i.e. the use of different instrument systems), as well as the intrinsic variability apparent across biological replicates during *in vivo* challenge models. Nonetheless, the data generated on APPs during recent comparative proteomic analyses of biological fluids collected from food animals is more comprehensive than information compiled from previous research, due in large part to the fact that LC-MS/MS methodologies allow for peptide identification and subsequent protein discovery without a reliance on antibody availability. Likewise, innovations in MS ion fragmentation strategies, including the use of ETD to elucidate modified amino acid residues, have advanced current capabilities to characterize post-translational modifications using MS, and show promise as a means to determine the specificity of APP as biomarkers of disease. Some attempts have been made to conduct comparative proteomic analyses of disease specific PTMs of APPs and glycopeptides for veterinary applications, but the specificity of APP modifications as biomarkers of disease is still an emerging area of research. Further development of sample preparation strategies designed to isolate glycoproteins and glycopeptides, coupled with advances in the capabilities of both LC and MS instrumentation to separate, detect, and characterize the post-translational modifications of APPs will undoubtedly broaden current knowledge of the role of APPs in inflammation, and aid in the establishment of APPs as specific biomarkers of disease.

7. References

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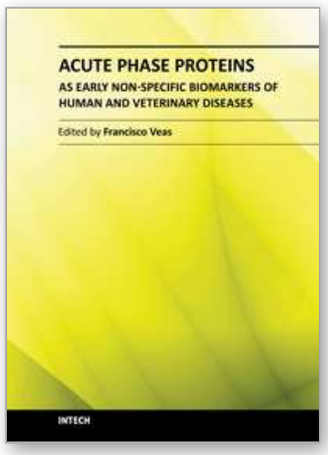

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The two volumes of Acute Phase Proteins book consist of chapters that give a large panel of fundamental and applied knowledge on one of the major elements of the inflammatory process during the acute phase response, i.e., the acute phase proteins expression and functions that regulate homeostasis. We have organized this book in two volumes - the first volume, mainly containing chapters on structure, biology and functions of APP, the second volume discussing different uses of APP as diagnostic tools in human and veterinary medicine.

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