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Identification and the Significance of Selective Proteins in Bile and Plasma of Normal and Health-compromised Chickens

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by

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ABSTRACT

During the last 50 years, animal breeding programs in commercial poultry have made significant progress in the bodyweight gain of broilers but led to several metabolic and skeletal disorders. Lameness associated with proximal femur known as femoral head separation (FHS) or femoral head necrosis (FHN) is one of the major metabolic disorders in poultry industry. In order to select for healthy chickens, markers that can distinguish between healthy and affected birds are required. Biomarkers from blood represent an ideal and rich source of markers which can be obtained using minimally invasive methods. The biomarkers were explored in an experimental model for FHS where samples from spontaneously affected broilers and lipopolysaccharides (LPS) injected broilers were analyzed using matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF-MS) and liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS). In the plasma of glucocorticoid induced FHS, there was an increase in apolipoprotein A-I (Apo-AI), whereas proteins such as vascular endothelial growth factor-C-isoform-1 (VEGF-C-1) and Protocadherin-15 (PCDH-15) were absent in FHS predisposed chickens. The increase in Apo AI may lead to vascular thromboembolism, and the lack of VEGF and PCDH-15 can be associated with vascular insufficiency and cell-adhesion problems, respectively. In spontaneously FHS affected birds, decrease in fetuin, fibrinogen, alpha-1 acid glycoprotein (AGP), and increases in Apo-AI, gallinacin-9, and hemoglobin chains were observed. These proteins could be related to skeletal mineralization disorders, coagulation problems, platelet aggregation, dyslipidemia, hemorrhage and erythrolysis. Due to the involvement of one or more acute phase proteins such as AGP and defensin, we tested the changes in protein profile of chicken plasma in response to LPS. Proteins AGP, cathelicidin-2, heperanase, chemokine CCLI10 were increased in response to LPS, which was different and distinct from FHS affected birds. Hence, the distinct protein profile of FHS affected birds could

be useful as candidate biomarkers for distinguishing healthy birds from susceptible or affected chickens.

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LIST OF PUBLICATIONS (FIRST AUTHOR)

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2. Packialakshmi, B., R. Liyanage, J.O. Lay, R. Okimoto, and N.C. Rath. Prednisolone induced predisposition to femoral head separation and the accompanying plasma protein changes in chickens. Biomarker Insights (under review)

CO-AUTHOURED PUBLICATIONS (NOT SUBMITTED IN THIS DISSERTATION)

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I. Introduction

Ever since the dawn of agriculture, humans have domesticated chickens for meat and egg production, by both artificial and natural selection (Rubin, et al., 2010). During the nineteenth century, owing to the improvements in animal breeding, chickens were subjected not only to intense selection for muscles but also raised under artificial growing conditions in farms. For example, during the past 50 years alone, the bodyweight of chickens almost quadrupled and attain the marketable bodyweight at a very young age (42 days) but with half the original amount of feed (Havenstein, et al., 2003). Broiler chickens were raised under extreme conditions such as, 23 hours photoperiod and at a very high stocking density, where their locomotion was restricted (Bessei, 2006). Such confined animal farming operations (CAFO) were behind the commercial success of poultry industry and white meat supply to the entire United States. The CAFO and intense animal breeding programs have led to several production and metabolic disorders that include ascites and lameness in broiler chickens (Julian, 1998). In a quest towards rapid weight gain, skeletal system became weaker and susceptible to several diseases (Prisby, et al., 2014; Talaty, et al., 2010).

In case of lameness, the proximal aspect of load bearing bones especially femur is affected by problems such as femoral had necrosis (FHN) or femoral head separation (FHS) (Thorp, et al., 1993). These diseases were attributed to several factors ranging from metabolic disorders, nutritional deficiency, microbial etiology and stress (Knowles, et al., 2008). There are two ways to ameliorate these problems; one is to modify the external growth conditions such as providing outdoor access while reducing stocking density or photoperiod (Sørensen, et al., 2000). Second is to improve the skeletal traits via genetic selection, thus enabling chickens to withstand

these artificial growth conditions (Rubin, et al., 2007). However, changing the external growth conditions counters the basic tenet of CAFO and hence the latter option would be more viable.

In order to select the birds against skeletal disorders, markers that can discriminate between the healthy and diseased or even susceptible birds, is required (Durairaj, et al., 2009). The markers can be based on nucleic acids (DNA or RNA) and proteins such as collagens from blood. The advantage of proteins from blood is that, they are obtained by non-invasive methods and they represent an ideal source of biomarkers for skeletal metabolism such as hydroxyproline, collagens, enzymes and lipids (Seibel, 2005). The study of human proteins is feasible because of the availability of antibodies to almost every known human protein or mammalian models such as mice or rat, but in chickens such studies are complicated by the lack of suitable antibodies. Hence we resorted to proteomic methods using matrix assisted laser desorption ionization mass spectrometry and time of flight (MALDI-TOF) and liquid chromatography and tandem mass spectrometry (LC-MS/MS) to study peptides and proteins respectively. Another disadvantage is that, there are very limited models to study the disease in young birds. Thus, we had three major objectives to find biomarkers to the avian FHS

1. Develop and use the prednisolone induced FHS model in younger birds for the identification of biomarkers
2. Apply the methods developed to the spontaneous (natural) FHS and find relevant markers for FHS
3. Test the specificity of FHS markers by checking proteomic responses in an inflammatory model

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II. Review of literature

Avian Femoral Head Separation and Necrosis

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Summary

Femoral head separation (FHS) is an idiopathic, metabolic skeletal disorder in fast growing poultry, which affects the proximal femur and leads to lameness. In the femoral epiphysis, articular cartilage tends to separate from its growth plate, leading to necrosis, infection and eventually osteomyelitis. Although widely attributed to rapid growth rate, the etiology of FHS may be multifactorial encompassing genetic, stress, nutritional, and environmental factors. In this review anatomical, physiological, and biomechanical features behind susceptibility of the femoral epiphysis to FHS as well as some of the experimental models to study this problem are discussed. We also describe the progress in discovery of biomarkers that may facilitate diagnosis and early prognosis of the FHS.

Key words

Femoral head separation; osteonecrosis; poultry lameness; biomarkers; apolipoproteins; bone

List of abbreviations

AC Articular cartilage

BCO Bacterial Chondrosis and Osteonecrosis (BCO)

ECM Extracellular Matrix

GP Growth plate

ON Osteonecrosis

FHS Femoral head separation

FHN Femoral head necrosis

LPS Lipopolysaccharides

MHC Major Histocompatibility complex

TD Tibial Dyschondroplasia

Introduction

In the past 50 years there has been significant progress in commercial poultry production resulting in improvements of feed conversion efficiency and the growth rate to the extent that the market-age weight of poultry has nearly quadrupled (42). However, the selection for intense growth rate has also resulted in some negative metabolic consequences such as pulmonary hypertension, fatty-liver syndrome, and skeletal problems, which largely affect young adolescent birds and raise welfare concerns (21, 57). Leg problems in poultry can be attributed to a wide variety of neuro-muscular-skeletal disorders that can cause pain and discomfort to birds (11). However, most leg problems in the proximal aspects of long bones such as femur, tibia, or tibio-tarsus are associated with a weak skeleton that fail to support the increase in bodyweight (63). Femoral head separation (FHS) and necrosis (FHN) are the leading leg problems in heavy birds where the growth plate (GP) of the proximal femur tends to separate from its articular cartilage (AC) leading to lameness. The FHS problem often remains subclinical without visible signs but can manifest later, and impair the performance of birds. In severe cases the proximal femur separates from its metaphysis resulting in the breakage of bone. Femoral head problems in humans and farm animals have been studied using various mammalian and limited avian models (13). In this review we discuss the pathophysiology of FHS, the risk factors associated with the problems, and the progress to identify biomarkers for the disease.

Terminologies and diagnosis

Among the several terminologies that connote femoral head problems, femoral head necrosis (FHN) is the most generic and popular term used in avian literature, interchangeably, with femoral degeneration (120). The term “necrosis” implies premature death of cells and tissues

caused by several factors such as sterile injury and avascular ischemia. A degenerative disease is caused by the loss of structure and function due to genetic or metabolic disorders resulting from sterile damage, not by pathogens. The damaged GP is thus vulnerable to colonization by opportunistic pathogens under poor hygienic conditions. Such infection can lead to bacterial chondro-osteonecrosis and osteomyelitis (BCO) (77). Thus, FHS stems from structural and functional breach that can progress to FHN with or without systemic infection (99). Human femoral head problems such as "epiphysiolysis" and **"**slipped capital femoral epiphysis (SCFE)" might be similar to avian FHS (85)**.** Osteochondrosis is another skeletal problem associated with the epiphyseal head, has also been described in poultry and other fast growing livestock such as pigs and horses (122, 135). Table 1 shows some of the common terminologies that connote the femoral head problems encountered in poultry. In the following section we describe some of the anatomical and biomechanical aspects of the femoral head that can make the epiphysis of young birds vulnerable to separation under adverse conditions.

Femoral head anatomy, physiology, and biomechanics

The proximal femoral epiphysis of a 6-7 week-old broiler essentially consists of a GP and the AC, which envelops it, and attaches to the hip socket by a capital femoral ligament (31). The AC is a hyaline cartilage that performs supportive function, reduces friction, and provides resistance against deforming stress. The bulk of AC is composed of collagen, proteoglycans, and water and is sparsely populated with chondrocytes estimated to range between 2-7 % of the total tissue volume (113). The orientation of collagen fibrils in the direction of stress is thought to protect AC from excessive deforming stress (6). The friction generated during biomechanical functions of articular joints is reduced by lubrication from hyaluronans and proteoglycans that also constitute parts of synovial fluid (108). The synovial fluid also provides nutrition and immune

protection (8). Additionally, AC is protected from friction by low molecular weight extracellular glycoproteins such as lubricin (100), which are secreted in response to mechanical stimulation (83), and by the action of growth factors such as TGF-β (54). The AC is susceptible to degenerative diseases and the healing of AC remains elusive (5, 138).

Histologically, the AC and GP are two separate tissues and the continuity between the boundaries of these two are maintained by the binding of transmembrane adhesion molecules with the outer extracellular matrix (ECM), and the intracellular cytoskeletal components (68). The continuity of cytoskeleton, adhesion molecules, and extracellular matrix maintain the tissue integrity. Among the several adhesion molecules, the cadherins are calcium dependent adhesion molecules essential for cartilage development (86) while integrins bridge the extracellular matrix (ECM) with the cytoskeletal protein actin maintaining adhesion, integration and signal transduction function (109). The adhesion between the AC and GP is not a constant or static adhesion but a dynamic one that renews during growth of long bones in order to allow proliferation of cells at the proximal zone of GP (102). Cell adhesion is also essential for signal transduction and the strength of the tissue because the signals for adaptive remodeling under loading are received and transmitted by the integrins (96). Therefore, any disruption of adhesion can impair signal transduction processes (40) and affect both structural and functional integrity of the tissues.

In contrast to avascular AC in mammals which obtain nutrition by diffusion, the avian epiphysis are moderately vascularized, the blood supply to the femur is achieved through branches of femoralis, ischiatic and middle femoral nutrient arteries (133). The blood capillaries from the GP and the cartilage canals may be the main portal for nutrients to the AC (Figure 1). The evolutionary significance of epiphyseal vascularity in birds in contrast with mammalian long

bones is not understood. Vascularity of avian epiphysis can also be a disadvantage by making it more susceptible to ischemic damage if the blood supply is interrupted.

In young birds, the femoral epiphysis not only works as a load bearing organ but also grows in length and dimension through endochondral ossification. The leg bones develop proportional to the body mass as shown by Applegate & Lilburn (4) using 1-6 weeks old chickens. Paxton et al., (91, 93) observed differential increases in thigh mass of 6 week-old chickens which was attributed to the increases in their muscle mass. The increases in muscle mass might impose higher loads in the proximal femur which is already constrained by higher bodyweight. Although in humans, the relationship between larger diameter of the femoral head and dislocation is demonstrated (60, 115), such measurements and their relationship with FHS has not been explored. The abnormal growth in thigh muscle mass, along with prolonged rest periods can impinge and contribute to micro damage of epiphysis and lead to degenerative problems.

Etiology and risk factors for FHS

FHS is an idiopathic problem with multifactorial roots, which may include genetic predisposition, disorders of mineral metabolism, inadequate exercise, environmental impositions, chemical exposure, and stress. These factors can independently or interactively produce traumatic changes to affect both the structural and functional integrity of epiphysis. Several researchers have studied the roles played by these factors in the context of poultry leg problems (15, 66). The following discussion provides a brief overview of the roles of some of the factors with respect to FHS.

Genetics, sex and development

The bodyweight, altered stance, gait and displaced center of gravity (23, 92) in commerical broilers may contirbuite to high incidences of femoral head problems (64). However, the incidences show variations among different breeds suggesting the diversity in genotypes may underlie skeletal weakness (59, 65, 66, 127). Genetics determine the skeletal mineralization and hence the structural integrity and strength (118). Similarly, genetics also influences innate immunity and the major histocompatibility complex (MHC), which are involved in recognition of foreign antigens and the broilers with susceptible MHC can succumb to infections leading to BCO (19, 53).

Gender also determines disease susceptibility; the male broilers have higher incidences of femoral head problems possibly because of higher bodyweight and the sire genetics influences the incidences of osteonecrosis (127). This phenomenon is comparable to human osteonecrosis where incidence is higher of ON in males (50) and the Legg-Calvé-Perthes disease that causes femoral problems in humans also affects mostly young, obese, males (95).

In addition to genetics, developmental signals from physical activity can also shape the skeletal strength and hence lack of such cues may predispose the birds to degenerative diseases. Skeletal development starts *in-ovo* and requires an optimal temperature for blood vessel development and skeletal health (90). During the post-natal growth period, the increase in mechanical strength in response to frequent loading and exercise is well known (37). As endochondral bone growth and secondary ossification is still under development during the 6-7 weeks of age, lack of physical activity inside a cage, can affect the skeletal strength and circulation in femoral epiphysis predisposing the birds to lameness (111).

Growth conditions and stress

The artificial growth conditions used in commercial poultry production such as incubation, high stocking density, extended photoperiod, litter conditions, hygiene, and transportation are designed essential for the efficient production but they deviate from nature, inflicting stress (11). High stocking densities provide less opportunity for locomotion (41, 119) that can exacerbate skeletal weakness (107). Similar to chickens, turkeys are also affected by stress. For example, it is customary in the United States to move the poults between 2-3 houses throughout the grow-out period. These transfers may involve catching, handling, and transportation stresses that can impose excessive strain and physical damage that results in leg problems. Such stressors induce the release of glucocorticoids, which have an impact on various organ systems by suppressing the growth factors, which reduces skeletal development (70). The stress related glucocorticoid production induces nutrition diversion and immunosuppression, which can predisposes the birds to diseases such as osteomyelitis (45). The function of immune system is not only to provide defense against pathogens but also to repair the damages. Hence immunosuppression can leave damaged tissues unrepaired, aggravating the underlying skeletal weakness and infection.

The basic stance and walking of chickens is essentially determined by the nature of the flooring materials used in the farms. Normally, the entire body weight is spread over but the use of certain flooring materials, such as a wire floor, can offset the posture and gait. The wire flooring was shown to lead to increase the incidences of lameness, probably because of the excessive strain exerted by the entire weight balanced over a small surface area (128).

Nutrition

Nutrition is the principal requirement of growth and hence genetic inefficiency to utilize feed properly or deficiency may cause metabolic disorders. Several reviews deal with the importance of micronutrients, such as vitamins and minerals that affect bone development and avian lameness (89, 97,109, 124). Vitamin D maintains calcium homeostasis and vitamin C is required for hydroxylation of amino acids and collagen synthesis. Although supplementation with vitamin D (9, 46, 116) and vitamin C (72, 87) are reported to be beneficial for bone health, the poultry diet formulations in general, meet or exceed NRC recommendations. Certain vitamins exhibit paradoxical effects, for example higher doses of vitamins can be harmful (81). Supplements such as probiotics which can modulate immunity, was shown to reduce FHS incidences (128). There is no conclusive evidence to assert nutritional supplementation alone can ameliorate FHS. Taking in account that dyslipidemia has been observed in affected birds (34), and fats are essential for matrix mineralization, and growth plate development (14, 94), the impact of dietary fat on avian bone health needs to be explored (7, 137).

Chemicals and antibiotics

Roxarsone, a growth-promoting antibiotic used to prevent coccidiosis, was reported to increase poultry leg problems (69) but the nature of the leg problem or its effect on the cartilage or bone is not known. However, in a controlled trial, roxarsone did not affect leg health (98). Enrofloxacin, a fluroquinone antibiotic used in poultry management, was reported to cause arthropathies associated with femoral head problems in broilers (51, 75) but the effect could not be replicated in our laboratory experiments (unpublished observation). Similarly, mycotoxins in

feed, which can interact with vitamin D, also affect bone health (47), but their effect on FHS has not been reported.

Infection, osteomyelitis, FHN

FHS is aseptic but makes the epiphysis vulnerable to infections from opportunistic pathogens under conditions such as poor hygiene and low immunity which results in BCO associated with some of the following etiological agents; *Staphyloccous spp* (16, 39), *Enterococcus cecorum* (114), *Escherichia coli* (28), *Staphylococci* (77). Approximately 0.1-0.25% of turkeys succumb to osteomyelitis-related problems which is more prevalent in males (44). *Staphylococci* produce specific proteins that bind to collagen and bone sialoproteins, and help these organisms to infect the musculoskeletal system causing osteomyelitis and soft tissue infections (2, 105).

A viral etiology is also implicated in femoral degeneration (55, 121) and may predispose birds to the bacterial infections associated with femoral head degeneration. For example chicken anemia virus and infectious bursal disease virus (IBDV) can cause immunosuppression and increase the incidence of femoral degeneration when infected with bacterial agents (76)

Apart from virus, intracellular pathogens such as Mycoplasma are reported in lameness (71, 80). Increased predisposition to FHS was observed in chickens experimentally challenged with M*ycoplasma synovie* (32). *Mycoplasma* infection upregulates the apoptotic mechanisms in chondrocytes and leads to cartilage death (35). Apoptosis induced by infection or metabolic disorder is an important factor that can lead to FHS.

Experimental models for FHS

Avian FHS lacks a suitable experimental model to study its pathology, mechanisms under laboratory conditions, explore biomarkers, and develop management strategies. As noted in Table 2, there are different mammalian models of FHN induced by a wide variety of factors such as steroids, endotoxins, heterologous serum, freeze insult, surgery or ethanol many of which have not been explored with avian situations. As with different mammalian models utilizing several quadruped animals (12), the biped chickens have been used as a model to study FHN (27). Unlike adult or older animals used in most of the studies, avian FHS affects younger birds of 5-6 weeks of age when GP is still active and the secondary ossification centers are not established. However, glucocorticoids (GC) such as prednisolone can increase the FHS incidence in 4-5 week old broilers and causes dyslipidemia as with spontaneously occurring problems but the bodyweight is decreased in GC treated birds unlike spontaneous avian FHS (33). Similarly, Dexamethasone another synthetic GC also increases FHN incidence in broilers (129). The major advantage of GC is the ease of administration either via feed, orally, or injected unlike complicated procedures such as surgery or freeze insult. As the bone marrow mesenchymal stem cells are potential progenitors of adipocytes and osteoblasts (26), the GC induces adipocyte formation, hyperlipidemia and apoptosis of osteocytes. Elevated serum lipids also increase the chances of vasoconstriction, emboli or clot formation in the femoral head leading to “avascular” or ischemic conditions (62) that can precipitate FHS. One of the recent innovations in avian leg problem has been the use of a wire-flooring model to induce FHS that can be useful to select the lame birds by keeping food and water on separate ends of floor pens (128).

The naturally occurring spontaneous hypertensive model of FHN hint at the association of circulatory system with FHS. Endogenous inhibitors of angiogenesis such as thrombospondin, γ-

interferon, and TGF-β are known risk factors for avascular necrosis (112). Similarly, LPS or serum sickness models connect the immune system (82), coagulation cascade, thrombosis (25) and hypercoagulability that might lead to immune complex deposition, ischemia or avascular conditions in ON. Sickle cell disease, which can increase blood coagulation can also induce ON (79) because cogulation might induce avascular conditions. The major supporting factor for the lipid induced and/ or avascular ischemia theory is that the administration of statins (27), vasodilators (36) and blood thinning agents (123) can reduce the incidence of the disease. The oxidative model and the reduction in incidence by vitamin E (67) indicate that redox homeostasis might be an accessory factor leading to ON.

Mechanisms of FHS

The principle mechanism for avian FHS proposed here is based on studies in avian models, spontaneously affected birds, and relevant mammalian literature. Based on the steroid induced FHS model, the affected birds show dyslipidemia, deficiency of an isoform of angiogenic factor "VEGF-C" and an adhesive protein "Protocadherin 15" in plasma (manuscript under review). Dyslipidemia alone or in combination with these factors can lead to vascular disruption by emboli or thrombi formation. Deficiency in VEGF and Protocadherin can also compromise angiogenesis and structural weakness respectively. Ischemia can cause nutritional deficiency and reperfusion injuries leading to cell death.

Dyslipidemia is observed in spontaneously affected birds from commercial farms. In addition to dyslipidemia, hypertrophic adipocytes in spontaneous FHS, there are apoptotic chondrocytes present at the proximal GP-AC junction (Figure 2) (34). Apoptosis is associated with several cases of mammalian idiopathic or induced ON (17, 126). Even a focal or limited cell death and

damage in epiphysis can potentially result in a weak femoral head susceptible to tissue separation.

Although the status of cell adhesion molecules in spontaneously affected birds is yet to be determined, some mammalian studies provide several examples linking cell adhesion to skeletal development. Chondrocytes from murine epiphyseal GP shows the expression of cadherin-11, an adhesive protein (74), which is essential for a healthy skeleton. Adhesive interactions between cells to cell and cell to matrix are essential for chondrocyte development (86) and tissue integrity. Hence, deficiency of cell adhesion can affect skeletal development and strength, which can potentially lead to FHS.

Based on the mammalian literature, evidence from avian experimental models and spontaneously affected birds, cell adhesion, dyslipidemia, vascular interruption and the associated cell death may be the mechanisms behind FHS (Figure 3). These factors further interacting with bodyweight, genetics, physical inactivity, metabolic disorders and growth conditions may precipitates the FHS on a poultry farm. Although some of the core concepts of aves and mammals are similar, only further experimental validation can provide further support to our hypothesis and add to the knowledge on mechanisms of FHS.

Markers

Markers are tags for identification, which help to distinguish a healthy physiological state from diseased. The biomarker concept is built on the assumption that long before the manifestation of the disease, there may be molecular signatures that can predict the disease such as DNA mutations or changes in proteins. Markers such as the collagen derived peptides, pyridinium cross links, and enzymes (110) are not only just ‘tags’ but provide valuable insights in to the

overall bone metabolism and pathologies. In avian FHS, there are different approaches to identify a disease; visual selection of lame birds, necropsy, biochemical, and macromolecular (nucleic acid and protein) markers.

In humans, the FHS diagnosis is straightforward because of self-reported pain and sophisticated imaging such as computed tomography, magnetic resonance imaging and radiography, but in farm animals such instrumentation though valuable, are seldom used. Besides, these methods such as Lixiscope imaging which can screen for tibial dyschondroplasia (TD) (65), cannot prognose TD but can identify TD only after the appearance of clinical symptoms.

In poultry, simple visual observation of gait is a popular and an inexpensive way to identify lame birds. The major disadvantage of gait score is that it is not possible to measure subclinical stages and must wait until the birds grow to an ideal bodyweight when the visual changes in gait occurs. There are some other disadvantages with gait scores; they are not reliable indicators, lack accurate predictability, and are subject to bias between different observers (22, 38, 106). Similar to gait scores, the nature of chickens to dislike sitting on water is used in the latency to lie (LTL) test (10, 125).

The FHS/FHN are often evaluated post mortem on the basis of the separation of AC from the GP, loosely referred to as “decapping”. However, the incidences are reported based on the propensity of the AC to separate from GP under an induced pressure, which can be subjective. Application of a force to separate the AC means that these birds are susceptible (predisposed) to FHS, not necessarily affected. Recently, Paxton et al., (93) reported a visual score of BCO incidence in femur and tibia of broiler chickens that spanned between 63-88% in the femur of ‘healthy birds’ but these birds were killed previously. As the muscles stiffen post-mortem, the

joint tissues also behave similarly and therefore, separate easily contributing to higher incidences of bone breakage. As Riddell et al., (101) suggested some of the incidences could be “artifacts during necropsy”. Hence, caution should be applied in the evaluation of FHS based on post mortem analysis. However necropsy is still essential for analysis of incidences, lesion score and for collecting tissues for histology or bacterial culture.

As the methods discussed above suffer from a number of drawbacks, we need some other markers that can prognose FHS using minimally invasive methods such as DNA sequences or protein biomarkers from blood. The genetics being central to many health problems, DNA analysis may reveal loci associated with bone phenotype of the skeletal system (78) and the skeletal traits could be mapped to the chicken genome (131) and used as tags for selecting healthy birds. Not only DNA regions, but sometimes morphological markers such as comb (52), could be linked to bone health. Genotyping the FHS susceptible and resistant lines of chickens can also provide the influence of genetic background involved in the disease based on DNA-QTL analysis or RNA microarray (103, 104). The analysis of nucleic acids can provide information on genes responsible for FHS and facilitate genetic selection against lameness.

Similarly, various metabolites such as proteins, peptides, lipids, hormones, or their degradation products circulating in blood are useful to identify the health status of the animals. It is assumed that before the disease is manifested, the damage-induced changes in the tissue metabolism can leach into the blood which can be used as markers. Based on the dyslipidemia observed in the steroid induced FHS model and in spontaneously affected birds, serum lipids and lipoproteins were proposed as surrogate markers for FHS (33, 34). Subsequently, in the steroid induced FHS model, Apolipoprotein A-I peptides were found to be elevated in the plasma of chickens using mass spectrometry (Manuscript in review). However, further exploration would be required

before using serum or plasma lipoproteins or their degradation products as markers for FHS because, lipids and lipoproteins are implicated in several diseases including coronary heart diseases to osteoarthritis (18).

Although analysis of serum metabolites can provide a marker, only the analysis of the proximal femur by proteomic or genomic methods can expose the changes in the femoral head of susceptible animals. Proteomic analysis of the proximal femoral head, articular cartilage (3) or synovial fluid (24), could also offer insights in to the different proteins or other macromolecules involved in potential pathological mechanisms.

The nucleic acids (DNA or RNA) in blood could act as biomarkers in cases where FHS progress to FHN with a microbial etiology. The 16s rDNA, could also act as a biomarker if the DNA or the infectious agents are in circulation. The microbial agents in the femoral head associated with lesions are characterized using sequencing (136). Recently, Wideman et al., used 16S rRNA-based diagnosis as marker to identify various microbes present in BCO affected femoral head (1).

Management strategies

Understanding the etiology and the mechanisms of FHS/FHN may help develop management strategies to manage the disease. Apart from biomarkers that help the genetic selection of healthy animals by breeding, nutritional mitigation may be one of the best strategies to control the problem in production animals but it needs to be verified by independent studies across different genotype, locations and growth conditions. There are several feed additives ranging from oils (137), organic acids (117), anti-inflammatory lipids (7) and mineral sources (88) which were tested for improving bone health but remain controversial because there have been no follow up

studies about them. Probiotics, which modulate immunity can reduce the incidence of FHS/FHN (128) especially when antibiotics cannot be used in farming.

Paradoxical nature of management strategies

Although several authors suggest reducing growth rate by photoperiod, feed restriction, stocking density, and increasing outdoor access are reported to improve bone health, these strategies counter the basic tenets of commercial poultry. Confined animal farming operations are economically successful only because of shorter time, larger scale and high-density farming. For example, feed restriction might reduce growth rate of broilers and hence live weight at marketable age. Photoperiod must be high enough (23 hours) to stimulate feeding and weight gain even though disturbances of circadian rhythm will eventually impact bone health. Stocking density should also be reasonable to provide current level of economic returns while providing out door acncess is not feasible for large-scale producers. The feed restriction and reducing rapid growth provides only disease escape not resistance, which leads to susceptible birds with subclinical conditions. Hence the best approach would be to understand the mechanisms of the pathology using models and spontaneously affected birds, find markers for selection, and reduce the incidence by animal breeding in combination with management approaches.

Conclusion

In the quest for selection for rapid weight gain, skeletal health was given lesser importance compared to muscles and especially breast muscles. FHS/FHN are significant problems in poultry industry; however, the true incidence of these problems are often overestimated because the accuracy in the current method for identifying the problem. Development of experimental

models of FHS to identify the subclinical disease using either imaging or biochemical techniques may help to select healthy birds and lead to management strategies for FHS/FHN.

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Figure 1. Proximal femoral epiphysis of a 5-week-old chicken showing blood capillaries penetrating the articular cartilage and cartilage canal (orange saffranin staining)

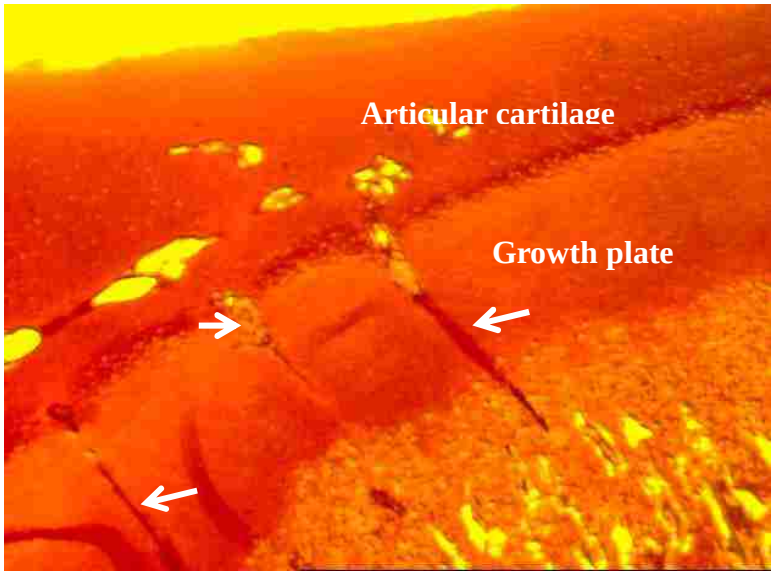

Figure 2. Epiphyseal region of the femoral head of the chicken showing growth plate chondrocytes at the proximal zone showing apoptotic changes identified by TUNEL staining (Reproduced from (34) with permission which is given in appendix, page number 181)

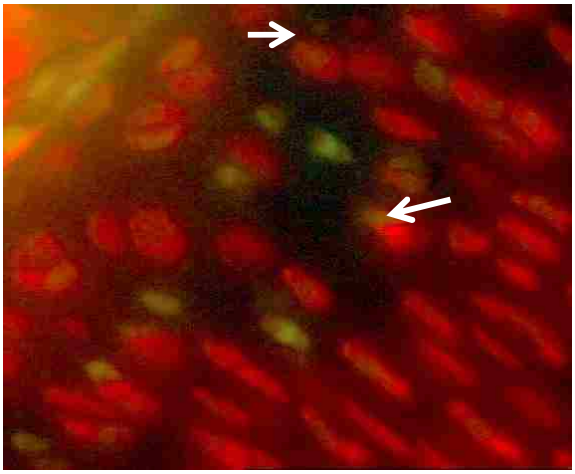

Figure 3. Possible mechanisms leading to avian FHS

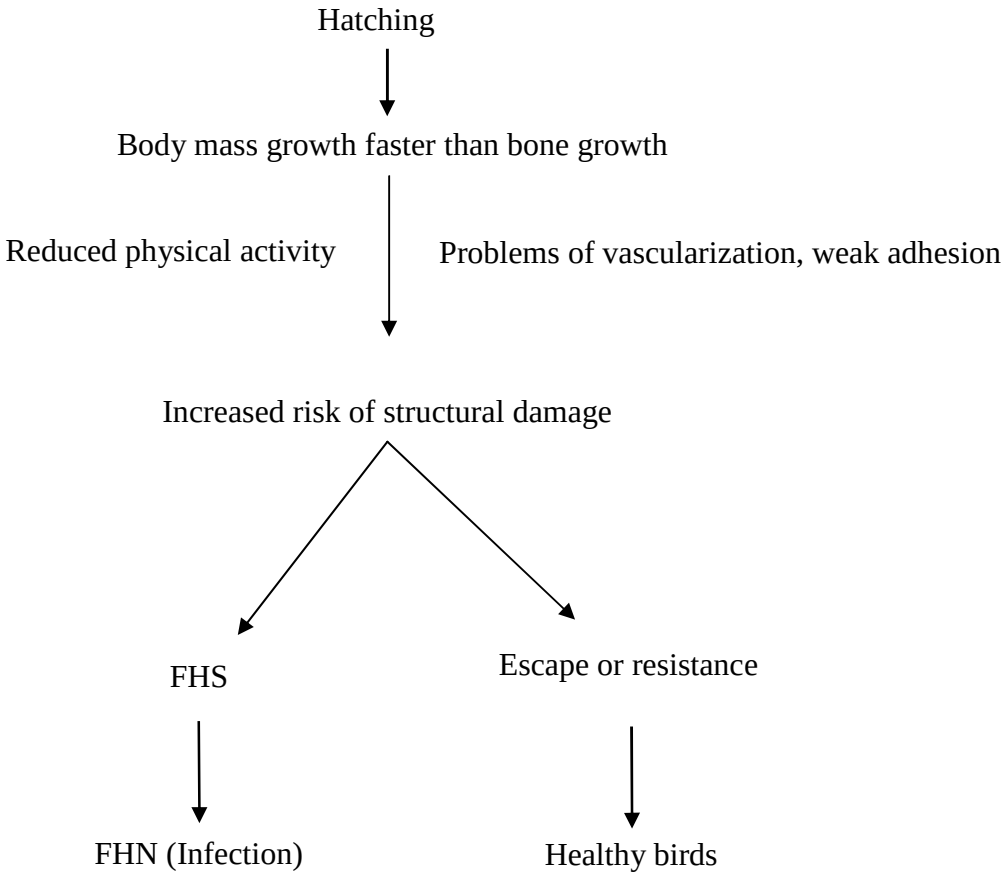

Table 1. Terminologies and connotations of proximal femoral degeneration and FHS

Terminologies	**References**
Femoral head or epiphyseal detachment	(34, 58)
Bacterial chondrosis and osteonecrosis (BCO)	(28, 130)
Osteochondrosis	(56)
Osteomyelitis	(44)
Artifacts happening during necropsy	(101)
Capital femoral epiphyseal infarction	(29)
Proximal femoral degeneration	(120)
Femoral head abnormalities	(30)

Table 2. Models of femoral head osteonecrosis and their relevance to avian FHS

Name	Principle	Model	Insights for aves	Disadvantages	Ref.
Spontaneous hypertension	Spontaneously high blood pressure but side effects causes ON	Rats	Coagulation induced restriction in blood supply	Hypertension model is available in birds but ON is not yet reported	(43)
Lipopolysaccharides (LPS)	Injection of LPS	Rabbits	Thrombi, hyperlipidemia, immune injury, infection	Not explored in birds	(49)
Serum sickness	Herologous horse serum	Rabbits	Immune complex mediated injury, microcirculation problems	Not used in birds	(82)
LPS + Glucocorticoid	LPS+ methyl prednisolone	Rabbits	Innate immunity injury, lipid elevation	Not used in birds	(132)
Surgery	Surgical blood deprivation or damage the tissues	Dogs, rats	Vascular deprivation, damage to ligaments	Complicated and laborious procedure, not used in chickens	(84)

Table 2. Models of femoral head osteonecrosis and their relevance to avian FHS (Cont.)

Ethanol	Injection of ethanol into femoral head	Sheep	Tissue damage	Alcohol induces ON in humans, but not known in chickens	(73)
Temperature	Freezing insult	Emu	Tissue damage	Complex procedure, may not be controllable	(20)
Glucocorticoids	Hypercoagulability and growth suppression	Rats, mice, rabbits, chickens	Dyslipidemia, and avascular ischemia	Bodyweight of chickens is reduced unlike heavy farm birds showing femoral head separation	(61, 79, 134)
Oxidative stress	Depletion of anti-oxidants	Rats	Redox homeostasis	Not used in birds	(48)
Wire flooring	Sustained footing instability	Chicken	Femoral head lesions, infection	Only in birds	(128)

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III. Prednisolone induced predisposition to femoral head separation and the accompanying plasma protein changes in chickens

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Biomarker Insights

ABSTRACT

Femoral head separation (FHS) is an idiopathic bone problem that causes lameness and production losses in commercial poultry. In a model of prednisolone-induced susceptibility to FHS, the changes in plasma proteins and peptides were analyzed to find possible biomarkers. Plasma from control and FHS-susceptible birds were depleted of their high abundant proteins by acetonitrile precipitation then subjected to cation exchange, and reverse phase (RP) fractionations. Analysis with Matrix Assisted Laser Desorption Ionization-Time-of-Flight Mass Spectrometry (MALDI-TOF-MS) showed several differentially expressed peptides two of which were isolated by RP-HPLC and identified as the fragments of Apolipoprotein A-I. The acetonitrile fractionated plasma proteins were subjected to reduction/alkylation, and trypsin digestion followed by liquid chromatography and tandem mass spectrometry which showed the absence of protocadherin 15, vascular endothelial growth factor-C, certain transcription- and ubiquitin mediated proteolytic factors in FHS prone birds. It appears that prednisolone induced dyslipidemia, vascular, and tissue adhesion problems may be consequential to FHS.

KEY WORDS: femoral head separation, glucocorticoids, chickens, mass spectrometry, biomarker, proteomics

List of Abbreviations

LC-MS/MS Liquid Chromatography and tandem mass spectrometry

ACN Acetonitrile

CH Cholesterol

FHN Femoral head necrosis

FHS Femoral head separation

GO Gene Ontology

HCCA α-Cyano-4-hydroxycinnamic acid

HDL High density lipoprotein

FA Formic acid

IDL Intermediate density lipoproteins

LDL Low density lipoprotein

MALDI-TOF-MS Matrix Assisted Laser Desorption Ionization-Time of Flight-Mas Spectrometry

RP-HPLC-ESI-MS Reverse Phase- High Pressure Liquid Chromatography- Electrospray Ionization- Mass Spectrometry

SCX Strong cation exchange

TG Triglycerides

TRIP-12 Thyroid hormone receptor interactor 12

VEGF-C Vascular Endothelial Growth Factor isoform C

VLDL Very Low Density Lipoprotein

Introduction

Femoral head separation (FHS) is an idiopathic bone disease in commercial poultry, which affects proximal femur leading to the separation of articular cartilage from its growth plate, and renders the birds prone to bone infection, femoral head necrosis (FHN), and lameness [1-6]. FHS occurs in rapidly growing broilers and breeders leading to production losses and welfare issues [7]. Dystrophic and degenerative changes in the femoral epiphysis, most likely, predispose the articular and the growth plate cartilage to separate under minimal stress. Early identification of FHS-prone birds using biomarkers can facilitate their elimination from the breeding pool. However, the infrequent occurrence of FHS in a normal population of birds can be a limiting factor for its study that can be circumvented by the use of experimental models of the disease. Glucocorticoids induce avascular necrosis of femoral head in adult mammals and birds that can be the closest model for avian FHS [8-11]. However, the early detection of FHS during its subclinical progression and its pathogenesis has not been addressed in younger animals including poultry. Previously we found that a synthetic glucocorticoid, prednisolone, was able to increase predisposition of chickens to FHS [12, 13]. Similarly, dexamethasone, another synthetic glucocorticoid, was reported to induce lameness in broilers that was attributed to femoral head problems [14]. Serum or plasma metabolites and biomolecules can be a rich source of biomarkers because the disease-associated changes can lead to their qualitative and quantitative changes. Since the proteins constitute both structural and functional basis of the tissues, we hypothesized that the changes in proteins may be useful as biomarkers. Therefore, we used the plasma protein and peptides to find their changes under prednisolone induced predisposition of young broilers to FHS.

Methods

Animals. The animal procedures were approved and carried out in accordance with the University of Arkansas IACUC guidelines. Forty eight Cobb 500 broiler chicks were raised on floor pens at the density of 8 square feet /bird from day 1 through 39, provided diets formulated per National Research Council specifications [15], and *ad libitum* water. The birds were divided into two groups: one received saline and the other prednisolone (MP Biomedicals, OH) suspended in saline, administered by gavage at an approximate dose of 10 mg/kg body weight on days 28 and 34. On day 39, the chickens were bled through the wing vein and blood was collected in K-EDTA Vacutainer tubes (BD Bioscience), and euthanized. At necropsy, the femoral joints were subjected to a mild dorsal pressure at the hip joint to induce femoral head separation (Durairaj, et al., 2009: 2012). Chickens with predisposition to FHS showed the separation of articular cartilage from its growth plate with mild to severe damage whereas the healthy femurs remained intact. Femoral heads from five birds in each group were fixed in formalin for histology.

Clinical chemistry and histology. Blood was centrifuged at 2,000 *g*, for 10 min to separate plasma and stored at -20°C for subsequent clinical chemistry and proteomic analyses. Only the plasma samples from normal chickens with intact femoral heads (CTRL) and those predisposed to FHS induced by prednisolone (FHS) were used for analyses. Albumin, cholesterol (CH), triglycerides, and the high-density lipoprotein (HDL) concentrations in plasma were analyzed using an Express plus automated clinical chemistry analyzer (Ciba-Corning Diagnostics Corp., Medfield, MA). Low density lipoprotein (LDL) concentrations were calculated using the following formula: LDL = TC - HDL - TG/5.0 (mg/dL) [16]. The femoral head tissues were embedded in paraffin and processed for histology. Hematoxylin-eosin stained sections were

examined and photographed using an Olympus IX-70 microscope. Bodyweight (BW), FHS incidence, and serum chemistry were analyzed by GLM procedure with pooled standard error of mean and significant means differentiated using Duncan's multiple range tests using SAS software [17]. Means were considered significant at $p \leq 0.05$.

Plasma peptide and protein analysis. For peptide analyses, we used 3 samples from each CTRL and FHS groups with each sample prepared by pooling equal volumes of plasma from three individual birds. An aliquot of plasma sample was mixed with two volumes of acetonitrile (ACN) containing 0.1% formic acid (FA) and kept at -20°C for 12 h to precipitates high abundant proteins [18, 19]. The precipitates were centrifuged at 10,000 *g* for 15 min at 4°C and the high abundant protein depleted (HAPD) supernatant was transferred to fresh tubes, and dried using a CentriVap vacuum concentrator (Labconco, Kansas City, MO). The dried content in each tube was dissolved with 0.1% formic acid (FA) to the original volume and desalted using reverse phase (RP) C18 Bond Elut tips (Agilent Technologies, CA) per manufactures' protocol with some minor modifications which consisted of the binding, and washing steps repeated 5 times before final elution. For cation exchange separation, the dried ACN supernatants were separately dissolved in 25mM Na acetate buffer pH 5.5 and fractionated using mini SCX columns (Pierce, Rockford, IL). The eluted materials, which contained 0.5 M NaCl were then desalted with Bond Elut C18 tips prior to subsequent steps.

MALDI analysis. The eluted samples from both procedures were spotted (1µL per spot) on a MALDI 384 target dried and overlaid with an equal volume of sinapinic acid (10 mg/mL 0.1% FA in 50% of ACN). The spots were analyzed using Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) in positive-ion linear mode. The instrument was calibrated using a 5-17.5 kDa protein standard (Bruker Daltonics) and the MS data for peptides between 1-

10 kDa range were collected in an automated mode using the Bruker Flex control software with a constant laser power, and 800 laser shots per spot.

ClinProTools analysis. The MS spectra of peptides from both CTRL and FHS samples were compared using ClinProTools software™ (Version 2.2, Bruker Daltonics) [20]. The quick classifier algorithm was used for automatic peak detection and integration, using peaks exhibiting a signal to noise ratio ≥ 10, and a threshold intensity of at least 5% relative to the largest peak [21]. Individual peaks from all 6 samples were aligned and their areas analyzed for statistical differences. Anderson-Darling's test was used to establish the data distribution and the statistical differences were calculated using t- and Wilcoxon tests, respectively. Values with $p \leq 0.05$ were considered significant and the relevant peaks thus detected as differentially expressed, were subjected to reverse phase HPLC (RP-HPLC) to purify, and identify the respective peptides.

Reverse-Phase LC-ESI-MS. HAPD plasma samples of CTRL and FHS groups were dried, dissolved in 0.1% FA, and subjected to RP-HPLC using a Supelco C18 column (15 cm x 4.6 mm, 5µm particle size, 300 Å pore size, Sigma-Aldrich, St. Louis, MO) attached to a Hewlett 110 HPLC system. The fractions were separated at a solvent flow rate of 0.7 mL/min using 0 to 100 % gradient of 0.1% FA (solvent A) and ACN (solvent B) over a period of 150 min. The HPLC was coupled online to a quadrupole ion trap ESI mass spectrometer (ESI-MS; Bruker Esquire 2000, Bruker, Billerica, MA), operated in positive ion mode with a dry gas temperature of 300°C and flow of 12 mL/min, and a nebulizing N2 pressure of 2.1×10^5 kPa. The mass spectrometer was optimized at *m/z* 1000 with low skimmer voltage to avoid ion fragmentation and charge stripping. The fractions corresponding to differentially expressed peptides by

ClinProTools analyses, were collected in several runs, pooled, dried, and reconstituted with 50 mM ammonium bicarbonate prior to further processing for their identification.

MALDI Peptide mass fingerprinting for LC fractions. The pooled fractions of peptides were reduced with 10 mM dithiothreitol (DTT) for 1 h at 60°C and alkylated with 55 mM iodoacetamide (MP Biomedicals, OH) for 1 h in the dark at room temperature. Excess iodoacetamide was neutralized with DTT then the peptides were digested with trypsin (Promega, Madison, WI) at 37°C for 16 h. The tryptic digests were desalted with Bond Elut C18 tips and spotted on a MALDI target plate with an equal volume of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (10 mg/ml of 50% ACN containing 0.1% FA). Mass spectra were obtained in reflector positive ion mode using a Bruker Daltonics Ultraflex II MALDI-TOF/TOF mass spectrometer. The MALDI peptide mass fingerprint (PMF) was subjected to tandem MS/MS using MALDI LIFT-TOF/TOF (Bruker Daltonics). Bruker Biotools 3.1 was used to combine PMF and LIFT-MS/MS data and searched with parameters listed below.

LC-MS/MS. Two samples of HAPD plasma from the control and FHS groups were dried with CentriVap concentrator, reconstituted with 50mM ammonium bicarbonate to 10^{th} volume of starting HAPD plasma, and the protein content of the solutions estimated using the micro BCA method (Pierce, Rockford, IL). One hundred microgram of protein from 2 samples per group, were reduced and alkylated as described earlier, digested with 2 µg of trypsin at 37°C for 48 h, and centrifuged at 21,000 *g* for 10 minutes to remove any insoluble materials. The supernatant was subjected to LC-MS/MS using an Agilent 1200 series capillary C_{18} RP-HPLC coupled to a Bruker Amazon-SL quadrupole ion trap mass spectrometer, capable of performing data-dependent acquisition. Tryptic peptides were separated by reverse-phase liquid chromatography (RP-HPLC) using a Zorbax SB C_{18} column, (150 x 0.3mm, 3.5 µm particle size, 300 Å pore size,

Agilent Technologies), with a solvent flow rate of 6 µL/min, and a gradient of 0 to 40 % consisting of 0.1% FA (solvent A) and ACN (solvent B).

Data analysis. The peaks with intensities ≥ 10,000 counts and S/N >5 in LC-MS/MS chromatogram, were used to obtain MS/MS peak lists and perform data base search. The Proteinscape™ bioinformatics suite from Bruker Daltonics, coupled with the MASCOT 2.1 search engine (Matrix Science), was used to identify peptides in the NCBI *Gallus gallus* protein database with following parameters: single miscleavage, fixed carbamidomethlyation of cysteine, variable methionine oxidation, and parent ion mass tolerance and fragment ion mass tolerance of 0.6 Da. Peptides with fragmentation ion score of 10 or higher were considered for protein identification. MASCOT automatic decoy database search was also performed with LC-MS/MS datasets. Proteins with <1% false discovery rate (FDR) with at least one unique peptide, and a MASCOT score of ≥ 45 were reported. Common proteins from 2 samples in each of the CTRL and FHS groups were selected with online software (http://www.xlcomparator.net). The proteins present in each of the two CTRL and FHS samples were tallied to find all expressed common proteins in both groups which were than matched to find differentially expressed proteins in each group. Gene Ontology (GO) annotations of the proteins were done using the DAVID bioinformatics software (http://david.abcc.ncifcrf.gov/) [22].

Results

Body weight, serum chemistry, and histology. Prednisolone treatment reduced the bodyweight of chickens compared with the saline (1.63 ± 0.22 kg *vs* 2.10 ± 0.14 kg, $p \leq 0.05$, n=24) and increased the FHS incidence by 38%. The plasma levels of albumin, cholesterol, HDL, and LDL were significantly higher in prednisolone treated birds but the triglyceride concentrations were

not statistically different (Table 1). Histology of femoral head segments of the prednisolone treated birds showed increased adipogenesis (Figure 1).

ClinProTool analysis and the identification of the peptides. The peptide profiles of CTRL and FHS samples, obtained by reverse phase and SCX fractionation methods, are shown in Tables S1 and S2 (Appendix). Although several peaks between the two groups were different per ClinProTool analysis, we isolated only two peptides *m/z* 7304 and *m/z* 3203 by RP-HPLC (Figure 2 & 3) both of which showed as fragments of chicken apolipoprotein A-I (APOA1) derived from its C-terminal region. The peptide *m/z* 3203 was internal to *m/z* 7304 sequence as shown by MS and MS/MS results (Figure 4, 5, 6 & 7). *In silico* analysis using PROSPER [23] suggested a probability of the generation of these fragments by the action of cysteine and serine proteases, respectively (Figure 8 a & b).

LC-MS/MS proteomics. The list of top 10 ranked proteins identified in each of the 2 pools of CTRL and FHS samples are provided in supplementary tables (Table S3, S4, S5 and S6 respectively, Appendix). Among these identifications, less than a quarter (~22%) were annotated while the rest belonged to the predicted (~73%) and hypothetical proteins (~5%). A qualitative comparison of proteins expressed in CTRL and FHS groups was done to identify common and differentially expressed proteins in each group (Figure 9). Comparison of proteins using GO annotations showed that protocadherin-15, a protein associated with adult walking behavior, vascular endothelial growth factor-C (VEGF-C), responsible for angiogenesis, and some calcium ion binding proteins were absent in the FHS samples (Table 2).

Discussion

Glucocorticoids, at pharmacological concentrations, exert both anti-anabolic and catabolic effects on skeletal tissues [24-26]. In younger animals such as 4-6 week-old birds, the anti-anabolic effects may be the principal mechanism, which causes the shrinkage and the arrest of growth plate development, which could lead to FHS (Durairaj et al., 2012). The glucocorticoid induced dyslipidemia and bone marrow hyper adipogenesis, noted in our studies, have also been reported by other investigators [27, 28]. However, the current objective of this study was to identify the changes in plasma proteins and peptides that may be relevant in glucocorticoid-induced FHS. Peptide and protein profiles were therefore compared to identify qualitative and quantitative differences in both groups.

We identified two peptides derived from the C-terminal region of apolipoprotein A1 (APO-A1), which is a major component of HDL as well as LDL, VLDL, and IDL [29-31]. Prednisolone raises the blood levels of both HDL and LDL, which may undergo degradation affecting the levels of their peptide fragments. Thus, APOA1 peptide fragments can be formed (i) by random degradation of their parent proteins during extraction procedure or (ii) by the action of specific proteolytic enzyme(s). Since, in our experiment, both CTRL and FHS samples were extracted identically, the differential increase in APOA1 peptide levels in FHS samples most probably, is related to the physiology of the birds rather than to the extraction procedures. Based on PROSPER analysis, it appears that both 7304 and 3203 Da fragments could be generated from APOA1 by the action of certain cysteine and serine proteases. Glucocorticoids, at high concentrations, induce apoptosis in many cells particularly the endothelial cells which can generate microvascular problems and growth factor deficiencies [32, 33]. Apoptotic cell death accompanies the activation of endoproteases such as caspase [34, 35]**.** During endothelial cell

apoptosis, these cysteine proteases can degrade HDL generating the Apo-A1 peptide fragments. However, the mechanism for the generation of Apo A1 peptide fragments is not clear. Hence, apolipoprotein induced thromboembolism or endothelial apoptosis, raises the possibility of vascular and nutritional deprivation in proximal femur. Because, the avian growth plate is relatively more vascular than its mammalian counterpart [36], the integrity of epiphyseal growth plate may be affected leading to its separation from articular cartilage.

Comparison of proteomic data of CTRL and FHS groups showed almost third of total proteins common to both while the remaining were group specific. The proteins present only in CTRL samples may be associated with healthy stage because they were absent in the FHS group. Similarly proteins identified only in FHS group could be associated with the disease. Analyzing these differentially expressed proteins with DAVID showed that the proteins associated with GO such as, angiogenesis, ubiquitin mediated proteolysis, calcium binding, transcription factors, and adult walking behavior was different in FHS group. The proteins reported here however, were selected based on one of the two criteria, (1) the GO was totally absent in CTRL but present in the FHS (eg: VEGF) and (2) the same GO was present in both groups but the proteins classified under that GO were different (e.g. ubiquitin related proteolysis and transcription factors). These differences might be associated with the mechanisms for FHS susceptibility in prednisolone treated birds.

Protocadherin 15 (PCDH15), a protein associated with adult walking behavior [37], and VEGF-C isoform 2 [38, 39], a protein associated with blood vessel development, were conspicuously absent in FHS group. PCDH15 belongs to cadherin family which are calcium dependent cell adhesion protein that are involved in cell signaling and mechanotransduction [40]. The impairment of adhesion can increase the vulnerability of growth plate to detach from its articular

cartilage. It may also impair signal transduction mechanisms involved in the joint function. Similarly the absence of angiogenesis associated proteins VEGF-C isoform 2 and Myosin-9 can contribute to “avascular” conditions, which may predispose the birds to FHS.

The GO ubiquitin mediated proteolysis was present in both CTRL and FHS samples. But the proteins, Cullin 2 [41] and thyroid hormone receptor interactor 12 (TRIP-12) [42] were present only in FHS samples. By contrast, the CTRL contained a different protein namely mitogen activated protein kinase kinase kinase 1, which is also classified under the same GO, ubiquitin mediated proteolysis (http://www.genome.jp/kegg-bin/show_pathway?map04120) [43]. Although direct experimental evidence to correlate these proteins and FHS was not found, they may be involved in FHS susceptibility and healthy conditions respectively. Both CTRL and FHS samples showed the presence of different transcription factors in plasma, among which only Prohibitin 2 was linked to stress [44] while others were not characterized in relation to FHS. However, the significance of these differentially expressed proteins and their association with FHS or glucocorticoid induced changes remain to be understood.

In conclusion, our results suggest that, prednisolone induced dyslipidemia and deficiencies of growth and adhesion factors may cumulatively contribute to the femoral head problems resulting in FHS. Plasma apolipoprotein A-I and its degradation products may be useful as biomarkers for FHS susceptible birds.

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Disclosures and Ethics

Authors declare no conflict of interest.

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Figure 1. Histology showing prednisolone induced bone marrow adipogenesis (arrows indicate adipocytes)

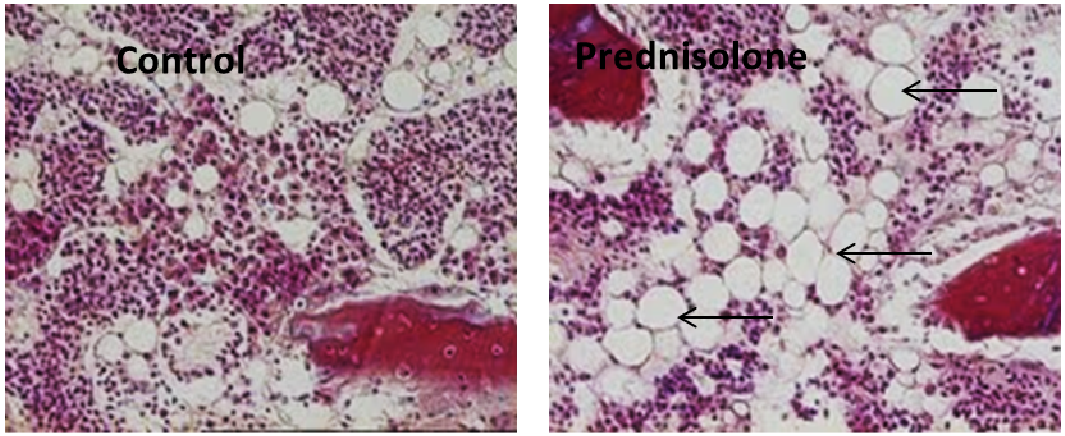

Figure 2. Comparison of MADLI-TOF mass spectra of C18 fractionated HAPD plasma showing the *m/z* 7304 peak analyzed by ClinProTools. The average spectra from CTRL (red) and FHS (green) groups represent the cumulative results of 3 pooled samples from nine birds.

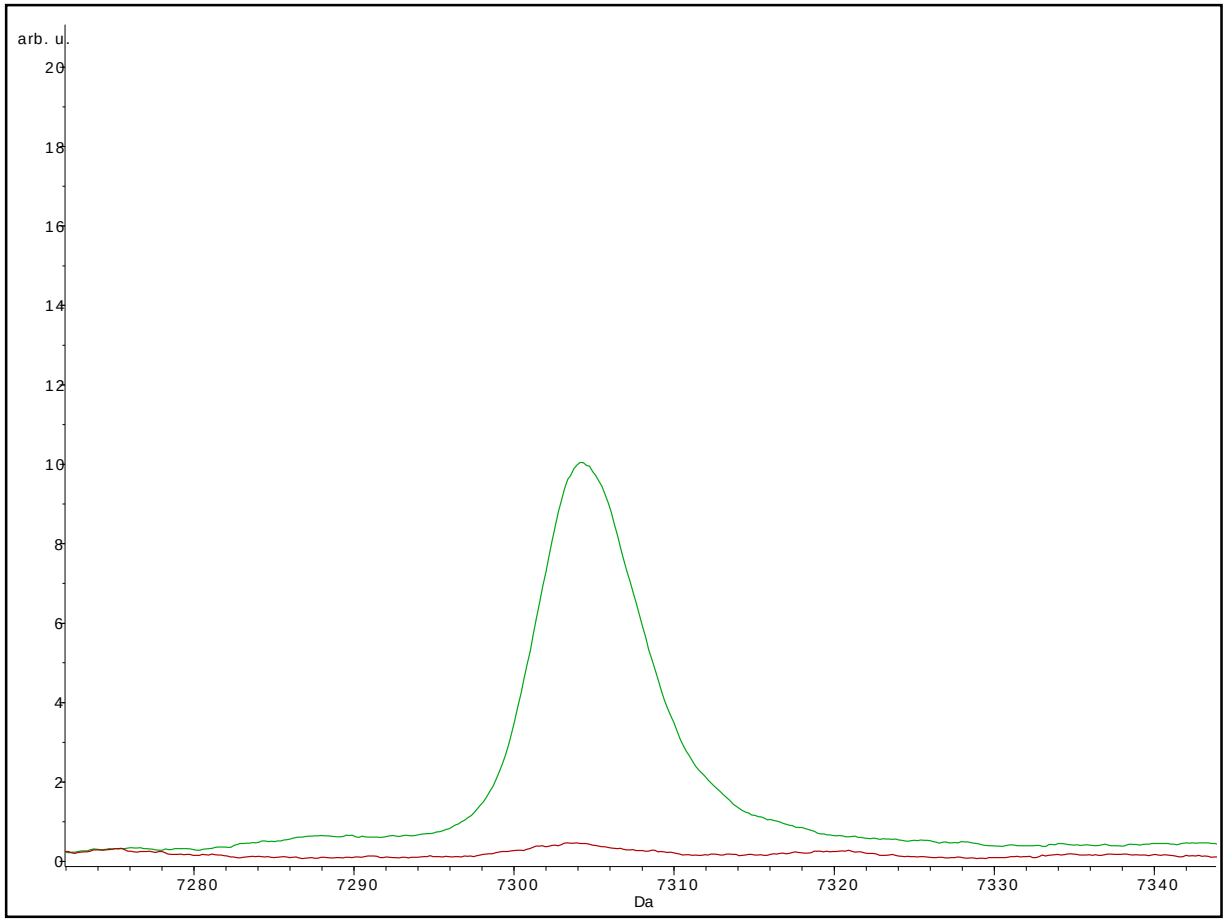

Figure 3. Comparison of MADLI-TOF mass spectra of SCX fractionated HAPD plasma showing *m/z* 3203 region analyzed by ClinProTools. The average spectra from CTRL (red) and FHS (green) groups represent the cumulative results of 3 pooled samples from nine birds.

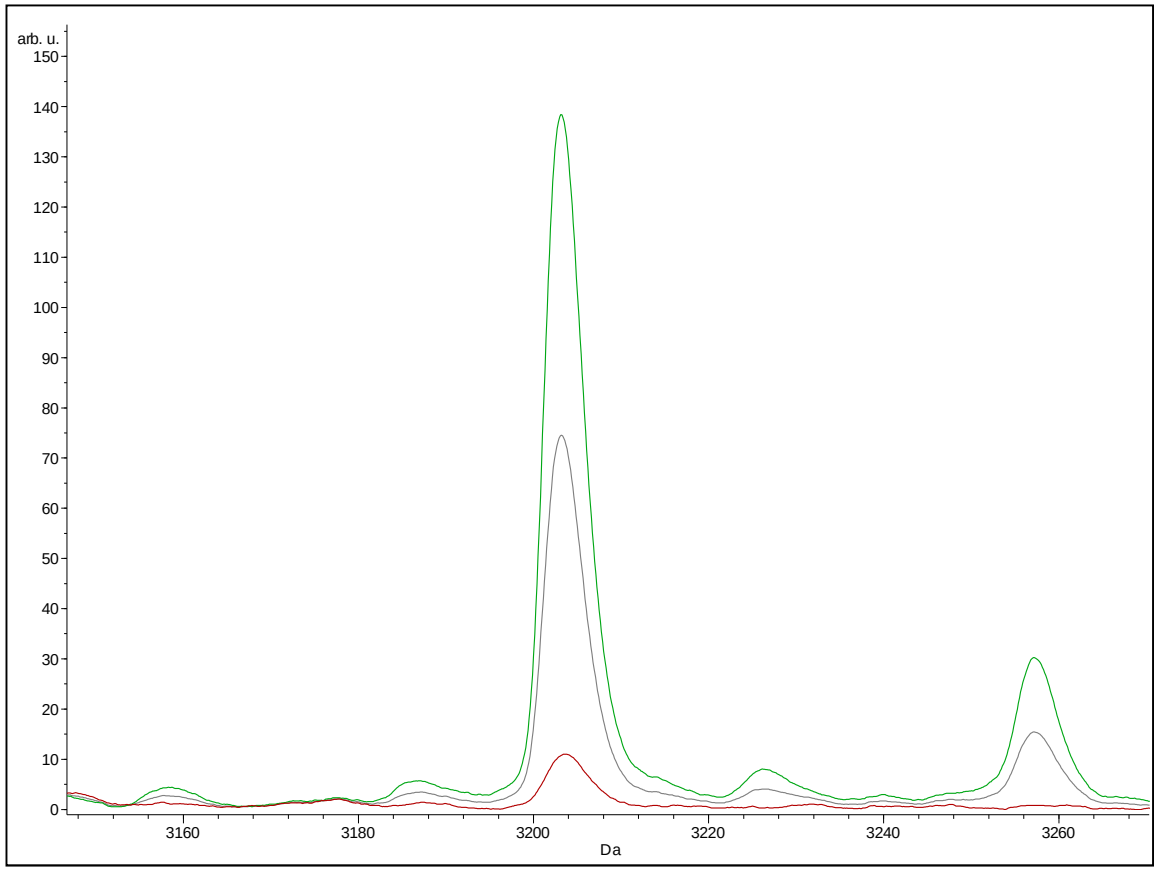

Figure 4. Peptide mass fingerprint (PMF) of 7304 Da peptide

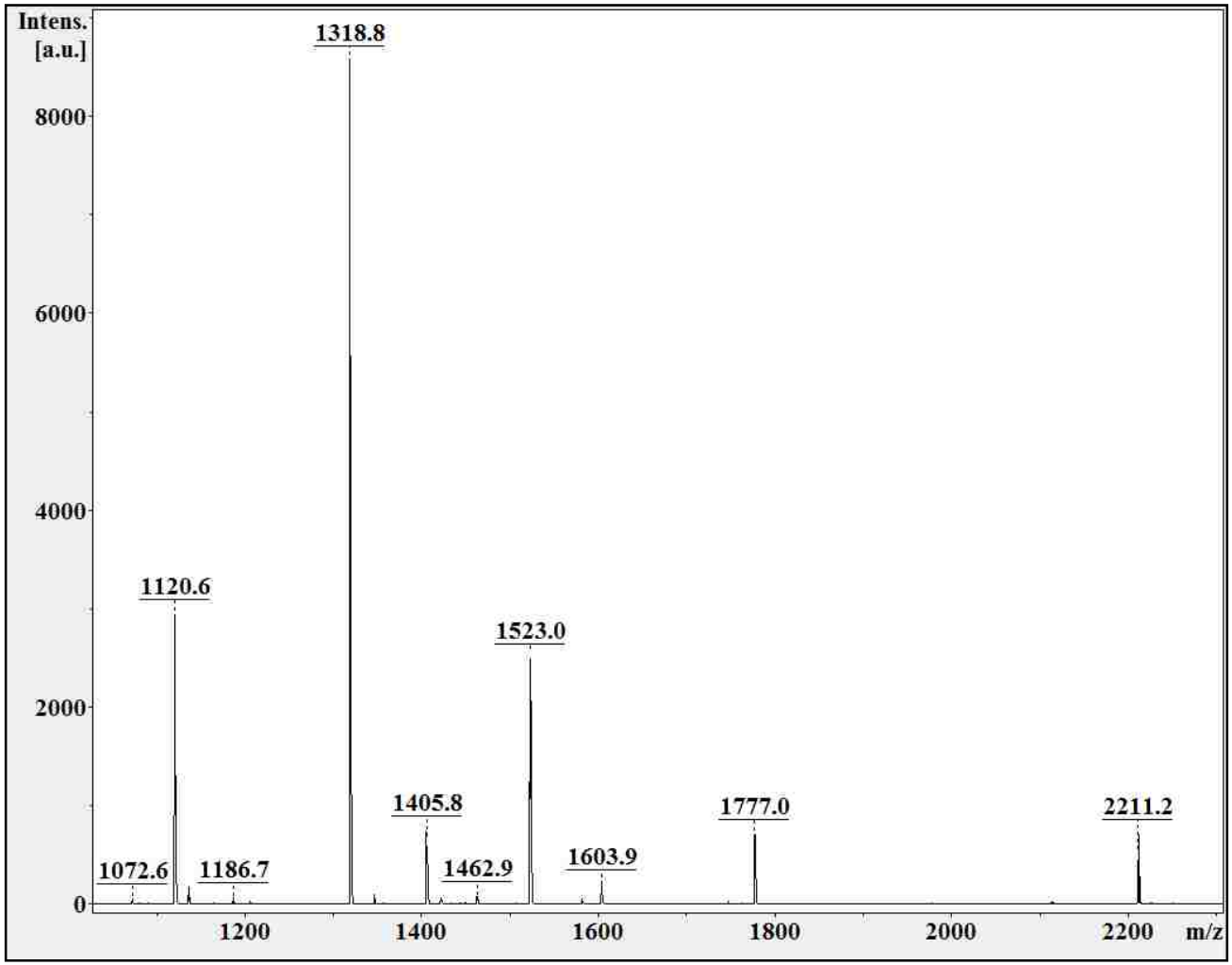

Figure 5. Peptide mass fingerprint (PMF) of 3203 Da peptide

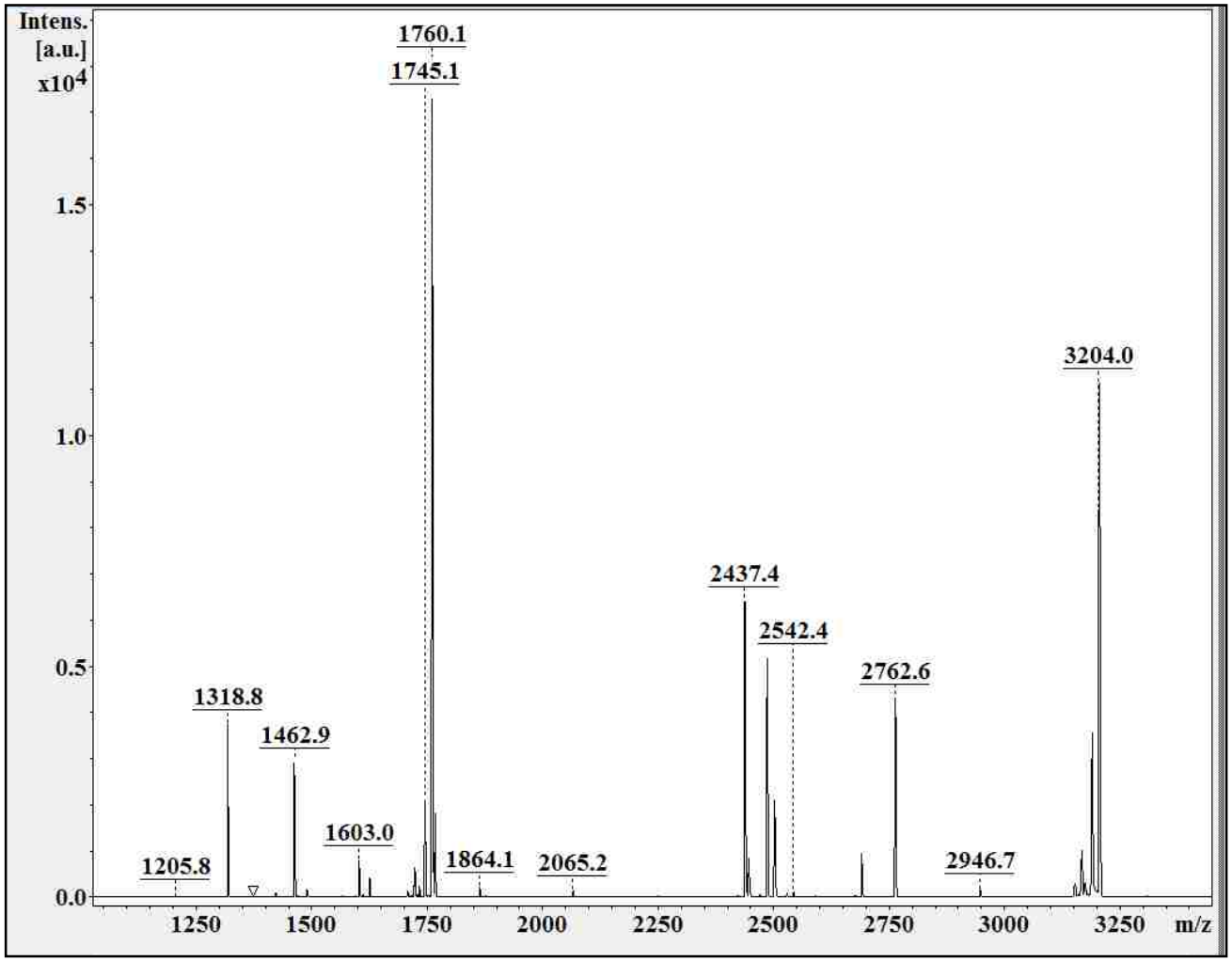

Figure 6. Tandem mass spectrometry (MS/MS) of 1120 Da tryptic fragment derived from 7304 Da peptide

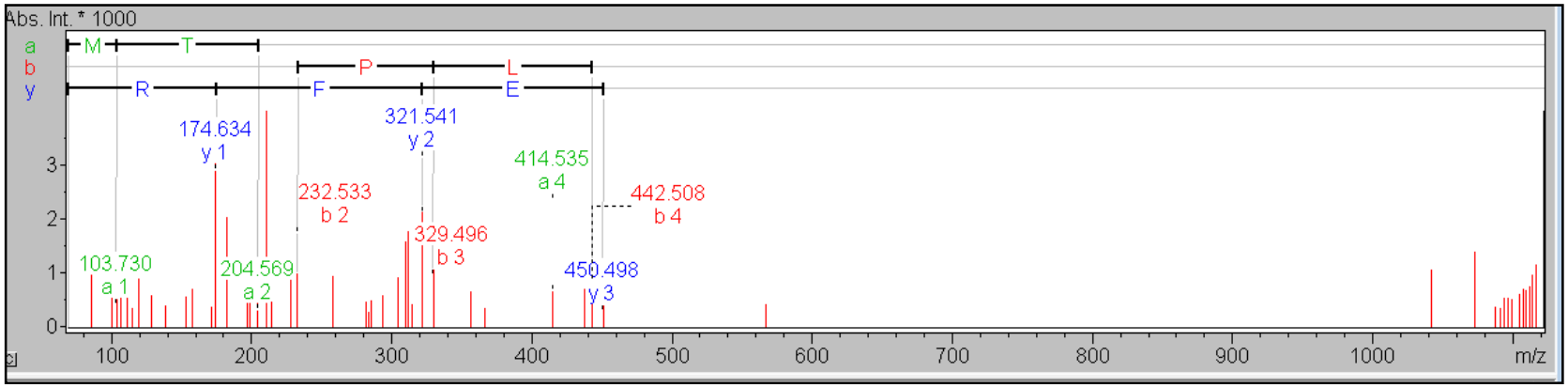

Figure 7. Tandem mass spectrometry (MS/MS) of 1318 Da tryptic fragment derived from 3203Da peptide

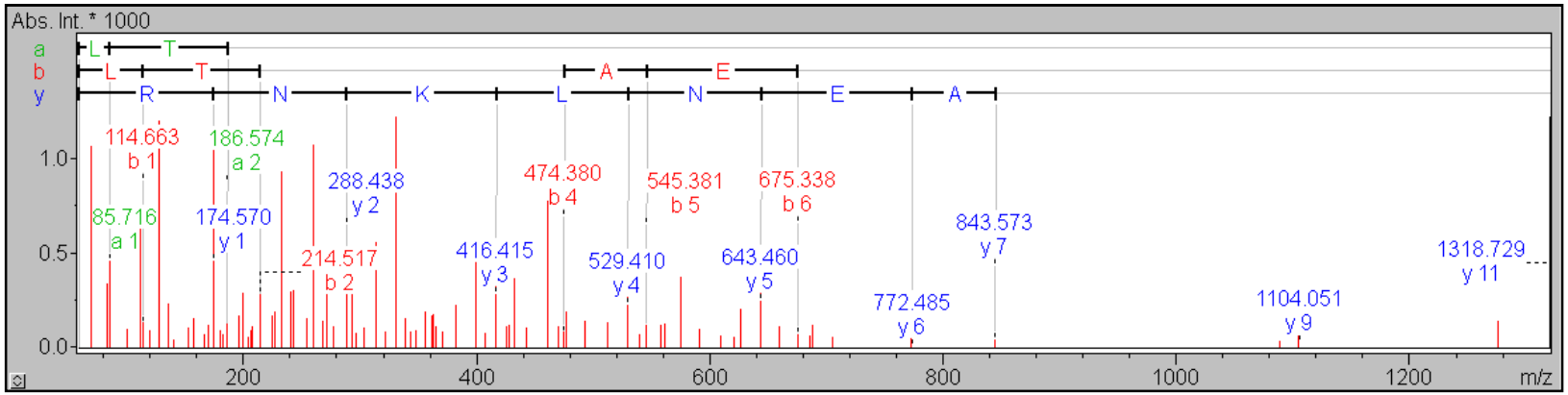

Figure 8. (a) Protein sequence of chicken Apolipoprotein A-I and the corresponding regions representing 7304 Da (underlined) and the 3203 Da peptide shown in bold and (b) The possible cleavage sites which may generate these peptides, predicted by PROSPER online software.

(a) >gi|227016|prf||1613168A apolipoprotein AI

RSFWQHDEPQ TPLDRIRDMV DVYLETVKAS GKDAIAQFES SAVGKQLDLK LADNLDTLSA AAAKLREDMA

PYYKEVREMW LKDTEALRAE LTKDLEEVKE KIRPFLDQFS AKWTEELEQY RQRLTPVAQE LKELTKQKVE

LMQAKLTPVA EEARDRLRGH VEELRKNLAP YSDELRQKLS QKLEEIREKG IPQASEYQAK VMEQLSNIRE

KMTPLVQEF**R ERLTPYAENL KNRLISFLDE LQKSVA**

(b)

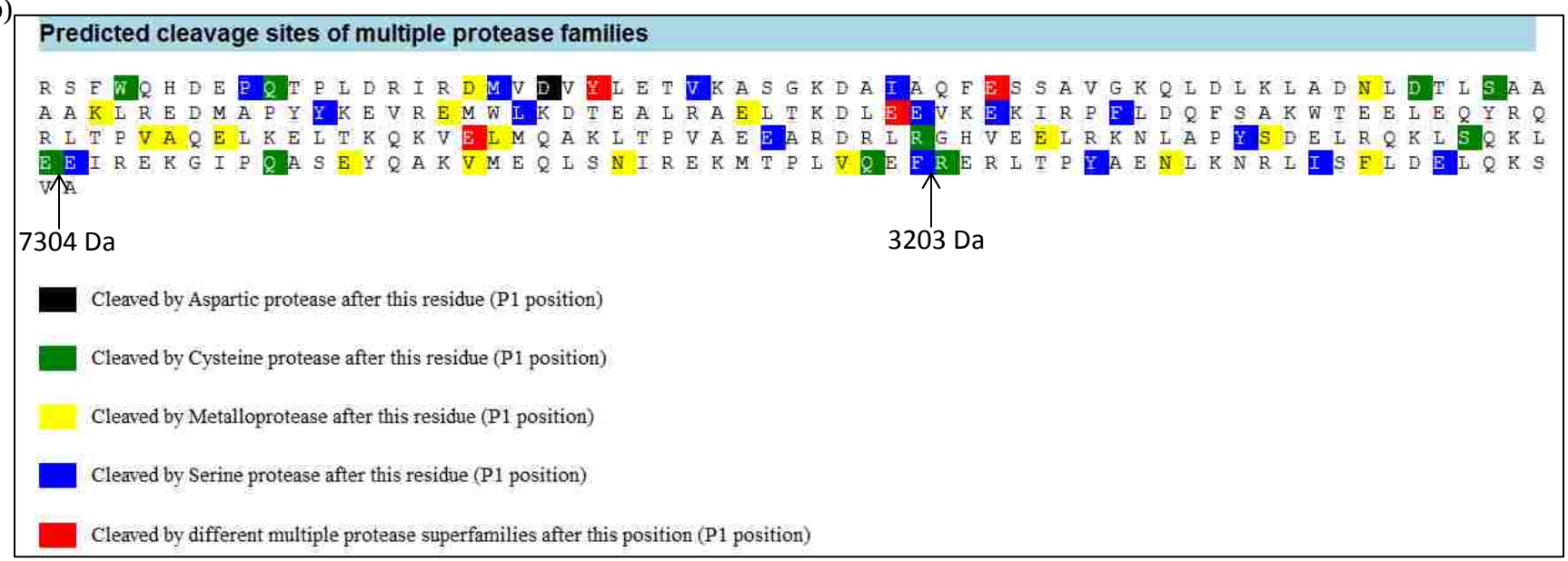

Figure 9. Venn diagram showing the number of proteins identified in CTRL and FHS groups.

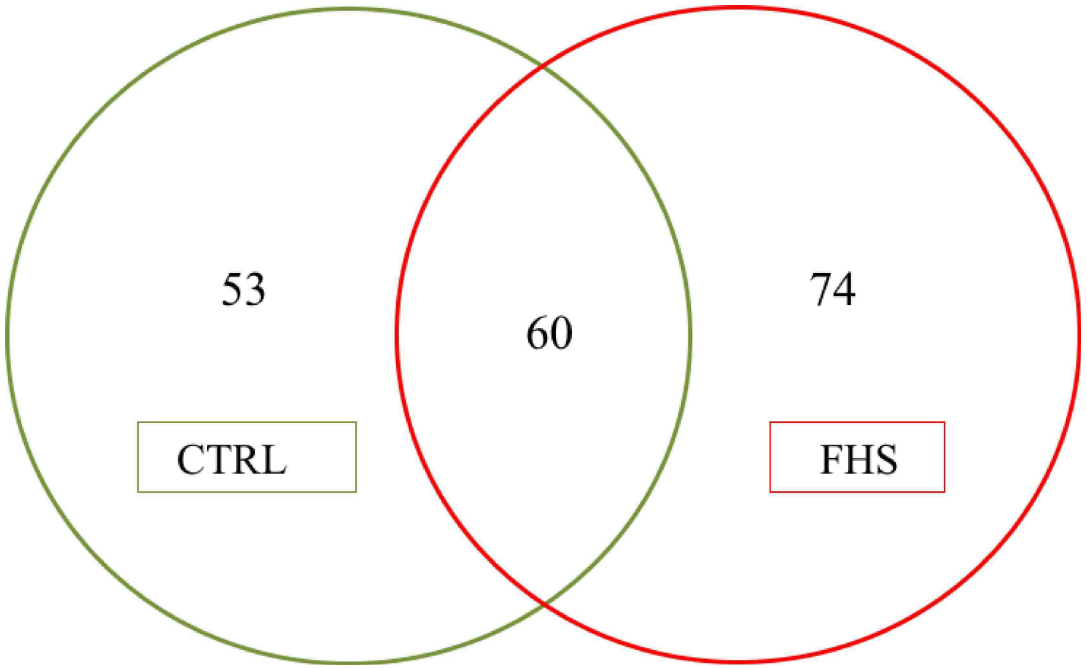

Table 1. Effect of prednisolone on plasma albumin and lipids.

Plasma variables	**CTRL (n=11)**	**FHS (n=9)**
Albumin (mg/dL)	1.31 ± 0.0^{a}	1.5 ± 0.0^{b}
Cholesterol (mg/dL)	122.9 ± 3.5^{a}	138.2 ± 3.9^{b}
High density lipoprotein (HDL) (mg/dL)	36.6 ± 1.1^{a}	42.2 ± 1.2^{b}
Low density lipoprotein (LDL) (mg/dL)	80.8 ± 2.5^{a}	89.5 ± 2.6^{b}
Triglycerides (mg/dL)	27.0 ± 0.5^{a}	32.4 ± 3.2^{a}

* Values are reported as means ± SEM. Values with different superscripts indicate $p \leq 0.05$)

Table 2. List of differentially expressed proteins and their relevant GO annotations

Protein	**CTRL**	**FHS**	**Significance from GO annotation**
Protocadherin 15	+	-	Adult walking behavior and calcium ion binding
Similar to Myosin-9	+	-	Blood vessel development
Vascular endothelial growth factor C (VEGF) isoform-2	+	-	Growth factor activity
Aczonin	+	-	Calcium ion binding
Mitogen-activated protein kinase kinase kinase 1	+	-	Ubiqitin mediated Proteolysis
Cullin 2	-	+	Ubiqitin mediated Proteolysis
Thyroid hormone receptor interactor 12	-	+	Ubiqitin mediated Proteolysis
Zinc finger homeodomain 4	+	-	Regulation of transcription
SET domain containing 1B	+	-	Regulation of transcription
Prohibitin 2	-	+	Regulation of transcription
Zinc finger homeobox 3	-	+	Regulation of transcription
Telomeric repeat binding factor (NIMA-interacting) 1	-	+	Regulation of transcription

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IV. Proteomic changes in plasma associated with spontaneous femoral head separation of broilers

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Abstract

Lameness due to femoral head separation (FHS) is a production and welfare issue in commercial poultry. FHS is an idiopathic disorder, which is attributed to a myriad of factors but in order to improve bone health, broiler breeders must be prognosed for disease susceptibility and selected against FHS using biomarkers. Proteins from plasma of blood, which can be obtained using minimally invasive methods represent an ideal, rich source of biomarkers which might be different in susceptible or affected birds. The peptide and proteins in plasma of healthy (HLTH) and affected birds (FHS) were compared using Matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) and Liquid chromatography and tandem mass spectrometry (LC-MS/MS). The peptide profile of HLTH and FHS were compared using ClinPro tools and the differentially expressed peptides were isolated by Reverse phase liquid chromatography fractionation (C18-RP-HPLC) and identified using peptide mass fingerprinting. Peptides derived from fibrinogen precursor and fetuin were reduced in FHS birds. Based on the proteomic analysis, proteins such as Gallinacin 10, Apolipoprotein A-1 and Hemoglobin chains are elevated in FHS while Alpha 1-acid glycoprotein is reduced in FHS birds. Our study shows that bodyweight, lipid profile and the above mentioned proteins could be useful as a biomarker for improvement of bone health. These proteins indicate that blood lysis, antimicrobial defense and lipid disorder but lack of an inflammatory response might be consequential to FHS.

Key words: Femoral head separation, biomarkers, proteomics, mass spectrometry

List of abbreviation

AC Articular cartilage

ACN Acetonitrile

APR Acute phase response

APP Acute phase proteins

FA Formic acid

FHN Femoral head seperation

FHS Femoral head necrosis

GPM Global Proteome Machine

GP Growth plate

HAPD High abundant proteins depleted

HDL High density lipoprotein

LC-MS/MS Liquid Chromatography and tandem mass spectrometry

MALDI-TOF Matrix assisted laser desorption ionization- time of flight

RP-HPLC Reverse Phase High performance Liquid Chromatography

Introduction

Femoral head separation (FHS) is an idiopathic leg problem that poses production and welfare issues in poultry (Bessei, 2006; Thorp, et al., 1993). FHS affects the proximal femur, characterized by the separation of the growth plate (GP) cartilage from its articular cartilage (AC) leading to femoral head necrosis (FHN) and lameness. The disease is attributed to rapid bodyweight gain (Kestin, et al., 2001), which impinges and impact the femoral epiphysis causing degenerative changes; however, mechanisms of its pathogenesis were not clear. In order to improve bone health by breeding, selection against FHS requires certain biomarkers that can distinguish the healthy from affected birds. Currently, the affected animals are visually identified by poor gait score or total lameness and later confirmed by necropsy (Kestin, et al., 1992). The major disadvantage of the method is that the susceptible animals without a visible symptom will escape selection and the breeder must wait until the visible signs of lameness to appear. Sometimes, these visible scores can fail to identify the truly lame birds (Paxton, et al., 2014). Hence, to prognose the diseased and susceptible birds, biomarkers identified by minimally invasive methods are desirable. Since proteins and peptides are the structural and functional basis of the tissues and their profiles in blood may change under pathology. Hence the objective was to explore protein and peptide biomarkers by comparing plasma samples of healthy (HLTH) and affected (FHS) birds. The Plasma peptides were analyzed using Matrix assisted laser desorption ionization mass spectrometry (MALDI-TOF-MS) and proteins using Liquid chromatography and tandem mass spectrometry (LC-MS/MS). Peptide and protein profile of HLTH and FHS groups were compared using qualitative and label-free quantitative methods. The results of the study are presented here.

Methods

Blood samples Blood samples were collected from 6 weeks old Cobb 500 male broiler by wing vein bleeding and collected in EDTA-coated tubes (BD Vaccutainer®) and the birds were killed thereafter. The chickens were necropsied immediately to identify birds prone to FHS. FHS susceptibility was determined by the facile separation of the AC from GP under a mild pressure applied dorsally (Durairaj, et al., 2009). Based on the necropsy information, the blood from birds was classified in to two groups; HLTH and FHS. The Plasma was separated from blood by centrifugation at 2000 *g* for 10 min at 4°C, pooled in triplicates and stored at -20°C until further analyses.

Clinical chemistry. Plasma from both groups were analyzed for the total cholesterol (TC), triglycerides, and the high density lipoprotein (HDL) concentrations in plasma were analyzed using an Express plus automated clinical chemistry analyzer (Ciba-Corning Diagnostics Corp., Medfield, MA). Low density lipoprotein (LDL) concentrations was calculated using the formula: LDL = TC - HDL - TG/5.0 (mg/dL) (Friedewald, et al., 1972)

Plasma peptide and protein analysis. For peptide analysis, we used 9 samples of plasma from HLTH and FHS groups. An aliquot of each sample was mixed with acetonitrile (ACN) containing 0.1% formic acid (FA) to a final concentration of 57%, sonicated twice for 10 min (Branson 3200, CT, USA) and left at -20°C for 12 h which largely precipitates high abundant proteins (Fernández, et al., 2011; Kay, et al., 2008). The precipitates were centrifuged at 10,000 *g* for 15 min at 4°C and the high abundant protein depleted (HAPD) ACN supernatant was transferred to fresh tubes and dried using a CentriVap vacuum concentrator (Labconco, Kansas

City, MO). The dried content in each tube was dissolved in 0.1% formic acid (FA) for peptide analysis and 50mM ammonium bicarbonate for protein analysis.

Peptide analysis. One hundred microliters of the each group (n=9) in 0.1% FA was desalted using reverse phase (RP) C18 tips (NT1C18, Glygen, MD) per manufactures' protocol with minor modifications, that is the binding and the washing steps were repeated 5 times before final elution with 60% ACN in 0.1% FA. The eluted samples from both procedures were spotted on a MALDI 384 target plate at the volume of 1µL per spot, dried and overlaid with sinapinic acid (10 mg/mL 0.1% FA in 50% of ACN). The spots were analyzed using Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) in positive-ion linear mode. The instrument was calibrated using a 5-17.5 kDa protein standard (Bruker Daltonics) and the MS data for peptides of range 1-10 kDa were collected in an automated mode using Bruker Flex control software with a constant laser power, and 800 laser shots per spot.

ClinPro Tools analysis. The MS spectra of peptides from control and FHS samples were compared using ClinPro Tools (CPT) software™ (Bruker, 2006; Ketterlinus, et al., 2005). The quick classifier algorithm was used for automatic peak detection and integration using peaks exhibiting a signal to noise ratio ≥ 10, and a threshold intensity of at least 5% relative to the largest peak (Bruker, 2006). Individual peaks from all the samples were aligned and analyzed for statistical differences. Anderson- Darling's test was used to establish the data distribution and the statistical differences and the significance calculated using t- and Wilcoxon tests. Values with $p \leq 0.05$ were considered significant and they are considered to be candidate biomarkers.

Reverse-Phase LC-ESI-MS. HAPD plasma samples (50 µL) prepared pooling HLTH and FHS samples were dried, dissolved in 0.1% FA and subjected to reverse-phase liquid chromatography

(RP-HPLC) using a Supelco C_{18} column, (15 cm x 4.6 mm, 5µm particle size, 300 Å pore size, Sigma-Aldrich, St. Louis, MO) attached to a Hewlett 110 HPLC system. The fractions were separated at a solvent flow rate of 0.7 mL/min using 0 to 100 % gradient of 0.1% FA (solvent A) and ACN (solvent B) over a period of 150 min. The HPLC was coupled online to a quadrapole ion trap ESI mass spectrometer (ESI-MS; Bruker Esquire 2000, Bruker, Billerica, MA), operated in a positive ion mode with a dry gas temperature of 300°C and flow of 12 mL/min, and a nebulizing N2 pressure of 2.1 × 105 Pa (30 psi). The mass spectrometer was optimized at *m/z* 1000 with low skimmer voltage to avoid ion fragmentation and charge stripping. The fractions corresponding to the certain peptides in shortlisted *m/z* were collected in several runs, pooled, dried, and reconstituted in 50 mM ammonium bicarbonate prior to further processing for their identification.

MALDI Peptide mass fingerprinting. The fractions corresponding to certain peptides in the CPT list were reduced with 10 mM dithiothreitol (DTT) for 1 h at 60°C, alkylated with 55 mM iodoacetamide (MP Biomedicals, OH) for 1hr in dark at room temperature. Excess iodoacetamide was neutralized with DTT then digested with trypsin (Promega, Madison,WI) at 37°C for 16 h. The tryptic digests were desalted using OMIX C18 tips and the eluted peptides were spotted on MALDI 384 target plate mixed with an equal volume of α-cyano-4-hydroxycinnamic acid (HCCA) matrix (10 mg/ml in 0.1% FA in 50% of ACN). The instrument was calibrated using standard peptide calibrators spotted adjacently.

In-solution digestion. Protein content of the HAPD plasma solution was estimated using BCA method (Pierce, Il) and diluted to 1µg/µL. One hundred microgram equivalent of protein from HLTH and FHS samples (n=3) were reduced with 10 mM dithiothreitol for 1 h at 60°C and alkylated with 55 mM iodoacetamide (MP Biomedicals, OH) for 1 h in the dark at room

temperature. Excess iodoacetamide was neutralized with DTT then the samples were digested with 2µg of trypsin (Promega, Madison, WI) at 37°C for 48 h.

LC-MS/MS. The digested samples were subjected to LC-MS/MS using an Agilent 1200 series capillary C_{18} RP-HPLC coupled to a Bruker Amazon-SL quadrupole ion trap mass spectrometer, capable of performing data-dependent acquisition. Tryptic peptides were separated by reverse-phase liquid chromatography (RP-HPLC) using a Zorbax SB C_{18} column, (150 x 0.3mm, 3.5 µm particle size, 300 Å pore size, Agilent Technologies), with a solvent flow rate of 6 µL/min, and a gradient of 0 to 40 % consisting of 0.1% FA (solvent A) and ACN (solvent B) over a time period of 2000 min.

Data analysis. The peaks with intensities ≥ 10,000 counts and S/N >5 in LC-MS/MS chromatogram, were used to obtain MS/MS peak lists and perform data base search. The mzXML files exported from Data analysis 4.0 (Bruker) were submitted to Global proteome machine (GPM) (http://www.thegpm.org) to search in Chicken genome with following parameters for X!tandem (Bjornson, et al., 2008). Fragment mass error of 0.6 Da, Carbamidomethylation and methionine oxidation as fixed and variable modification, do not search for known post-translational modifications (PTM), trypsin as enzyme, Ion trap (4 Da) as the predefined method and data was not archived in GPM database. Protein identifications were considered true if there is at-least one unique peptide per protein, and up to an acceptable e-value for a false positive rate < 5% as displayed in the corresponding results page. The results were downloaded as *.xml files for skyline software and proteins were downloaded as excel files for qualitative comparison.

Qualitative analysis. The common proteins present in the three biological replicates of HLTH and FHS samples were selected using an online excel comparison program

(www.xlcomparator.net). The list of proteins in both groups were mapped to their Ensembl gene ID using Biomart and analyzed for relative enrichment, clustering, and GO annotations using DAVID software (www. david.abcc.ncifcrf.gov) (Huang, et al., 2009a; Huang, et al., 2009b) with an EASE score of 0.1. The proteins present only in the HLTH groups, not identified in FHS or vice versa, were considered to be candidate biomarkers in addition to quantitative comparison of common proteins.

Label free quantitation of peptides. The spectra files (mzXML) and GPM protein results (xml) files were loaded in skyline software (http://proteome.gs.washington.edu/software/skyline) and MS1 filtering (Schilling, et al., 2012) and label free quantitation was performed using Skyline external tool ”MSstats” (Choi, et al., 2014). The quantitation of proteins was performed with peptide peak area obtained from extracted ion chromatogram with rank 1 peptide for three biological replicates of HLTH and FHS groups. The group comparison function of “MSstats” was used to normalize and generate Volcano plot of differentially expressed proteins.

Statistics. Bodyweight (BW) and serum chemistry were analyzed by GLM procedure with pooled standard error of mean and significant means differentiated using Duncan’s multiple range tests using SAS software (SAS, 2009). Means were considered significant at $p \leq 0.05$. The peptide and protein profile were analyzed using the statistical tools built in CPT and Skyline software respectively.

Results

Bodyweight: The FHS were 102.5g heavier than HLTH birds (Figure 1).

Lipid profile: Total cholesterol (TC) and LDL of FHS group were significantly lesser than HLTH chickens. However, there were no differences in HDL and TG fractions (Table 1).

Peptide analysis: Based on the CPT analysis and comparison, several peptides (Table 2), showed differential expression in FHS group compared to HLTH group. Among them two peptides 3671 Da and 4708 Da (Figure 2 and 3) were isolated by HPLC and subjected to PMF (Figure 4 and 5) and tandem mass spectrometry (Figure 6 and 7), and identified as derived from Alpha-2-HS-glycoprotein (Fetuin) and Fibrinogen beta chain respectively. Fibrinogen beta chain was identified using Biotools to generate a *de novo* tag from MS/MS of 2048 Da ("SDEENDY") and BLAST-P against chicken database.

Proteomic analysis: HLTH and FHS samples had > 300 proteins in each sample of which common and unique proteins in the triplicate samples and between these two groups are shown in Figure 8. Proteins expressed only in FHS but not in any sample of HLTH or vice versa are given in Table 3.

Gene enrichment analysis. The gene enrichment analysis and clusters generated by DAVID for HLTH and FHS is given in table 4 and 5. Fibronectin cluster is present in HLTH but not in FHS while negative regulation of apoptosis is present in FHS samples not in HLTH.

Skyline quantitative proteomics. The volcano plot generated using skyline and the list of proteins differentially expressed were given in figure 8 and table 6 respectively. Gallinacin-9 and Apolipoprotein A-I (Apo-AI) are elevated while Alpha-1-glycoprotein (AGP) and albumin (ALB) are reduced in FHS samples in comparison to HLTH.

Discussion

FHS is an idiopathic skeletal problem in young fast growing broilers, with multifactorial roots such as genetic predisposition and disorders of mineral metabolism (Knowles, et al., 2008; Sørensen, et al., 2000; Talaty, et al., 2010). These factors can interactively produce traumatic

changes to affect structural and functional integrity of the epiphysis. Based on our analysis, BW of the FHS birds were higher conforming the proposition that higher BW may be consequential to leg problems (Kestin, Gordon, Su and Sørensen, 2001; Knowles, Kestin, Haslam, Brown, Green, Butterworth, Pope, Pfeiffer and Nicol, 2008; Paxton, et al., 2013). Although broiler chickens are selected for their rapid growth and their bodyweight nearly quadruples during 4-6 weeks of age, the skeletal system of a higher animal like chicken has a limit on loads imposed and hence, the load bearing bones such as proximal femur would be adversely affected by heavy BW.

Additionally, dyslipidemia characterized by lowered TC and LDL were observed in FHS birds, which are in contrast with our earlier data where the batch of chicken with FHS had higher cholesterol (Durairaj, et al., 2012; Durairaj, Okimoto, Rasaputra, Clark and Rath, 2009). Although based on Skyline software quantitation, the affected birds showed increase in the Apo-AI, a component of LDL and HDL (Roman, et al., 2009), whether the changes in LDL or HDL levels were related to their degradation is not known. But it appears that dyslipidemia may be associated with FHS.

The peptide profile analysis showed that several peaks >50 differentially expressed in FHS of which only two were isolated and identified. The fetuin or Alpha-HS-glycoprotein (Lebreton, et al., 1979; Schäfer, et al., 2003) is secreted by the liver and regulates calcification by binding to Transforming growth factor –β (Mori, et al., 2011). The levels of fetuin rise in blood under ischemic stroke (Weikert, et al., 2008). The down regulation of fetuin could lead to increased protease activity and changes in skeletal mineralization in the FHS affected birds. Although, people normally associate lower levels of fibrinogen with lack of coagulation, the levels could be

low similar to the hyper-coagulation disorder known as disseminated intravascular coagulation (DIC) in humans (Baglin, 1996).

We compared the common proteins of HLTH with common proteins of FHS identified using LC-MS/MS that showed the presence of certain proteins in FHS not in HLTH and vice versa. Two proteins in FHS were related to proteolysis that indicates proteolysis function may have been affected in FHS birds. Although, Tdrd3 (tudor domain containing 3) gene in rats has Quantitative Trait Loci (QTL) associated with BW (Seda, et al., 2005), cholesterol level (Kato, et al., 2000), bone mineral density, and bone structure and strength (Alam, et al., 2006), the relevance of these QTLs in avian genome is not known. The significance of the presence of the myosin, and golgin only in FHS and the presence of one myosin and two transcription factors in HLTH is not clear.

Based on DAVID gene annotation and clustering analysis, proteins associated with Fibronectin type III cluster are enriched in HLTH not in FHS while regulation of apoptosis is present only in FHS. In FHS presence genes associated with apoptosis, shows that apoptosis and cell death could be associated with skeletal degeneration as in case of spontaneous or induced femoral head problems in humans (Calder, et al., 2004; Weinstein, et al., 2000). Previously we also reported the apoptosis in femoral epiphysis of spontaneously FHS affected broilers based on TUNEL staining (Durairaj, Okimoto, Rasaputra, Clark and Rath, 2009). One of the major connection between fibronectin and apoptosis is that cell adhesion to ECM is essential for cell survival and lack of adhesion can lead to apoptosis (Zhang, et al., 1995). Fibronectin type domain is present in several proteins but in the context of tissue architecture, ECM and cell adhesion are important functions of this domain (Pankov and Yamada, 2002; Potts and Campbell, 1996). Cellular adhesion and binding of chondrocyte integrins to fibronectin is essential for tissue integrity and

cartilage development (Aszodi, et al., 2003; Enomoto, et al., 1993), the absence of cell adhesion and apoptosis in FHS could lead to reduced skeletal strength.

Based on the quantitative comparison, five proteins show differential expression in FHS samples. The increase in Apo-AI, which is a component of HDL and LDL was also observed in our previous study using glucocorticoid induced model of avian FHS (manuscript submitted for review). Since, the CH and LDL levels are reduced in plasma of FHS group, the increase in Apo-AI could be related to their degradation may be a consequence of dyslipidemia. The only explanation for the low plasma levels of Apo-AI while increase in peptides lies in the rate of degradation of lipoproteins, which might lead to dyslipidemia or a consequence of dyslipidemia. Increase in free hemoglobin was traditionally associated with erythrolysis and several human diseases (Rother, et al., 2005) but the hemolysis is a non-specific marker to indicate wide range of underlying problems that can lead to hemolysis. Although the increase in Gallinacin-9 can be easily associated with infection because of its antimicrobial activity, this defensin is reportedly produced by the epithelial cells and bone marrow (van Dijk, et al., 2008). The source of the gallinacin-9 and internal hemolysis needs further verification but it appears that certain microvascular disorders could have led to hemorrhage and release of defensin-9.

In contrast to Apo-AI, AGP whose levels increases during acute phase response and inflammation (Takahashi, et al., 1994), was reduced in our current study. As AGP was implicated in prevention of platelet aggregation (Costello, et al., 1979), such decrease in AGP levels could lead to coagulation and vascular occlusion. The decrease in albumin levels popularly known as hypoalbuminemia (Ballmer, 2001) is one of the indicator of nutritional deficiency. As poultry feed exceeds the NRC (NRC, 1994) recommendations, the metabolic disorder not nutritional deficiency might be the factor behind reduced serum albumin. The reduction in

peptidase inhibitor SPINK-7, and its relationship with FHS is not understood. In humans' literature, SPINK were associated with inhibition of progression of cancer cell migration (Cheng, et al., 2008). The disturbance of the delicate balance between proteases and their inhibitors, similar to the proteins associated with proteolysis observed by qualitative comparison could be speculated as a reason for the development of FHS in broilers.

Based on our analysis, we conclude that heavy weight of broilers dyslipidemia, impaired cell adhesion, apoptosis and hemolysis could be associated with avian FHS. Hence, the weight of broilers, blood lipids, apolipoprotein, albumin, defensin and hemoglobin proteins could be useful as candidate biomarkers for selection against FHS.

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Figure 1. Bodyweight of HLTH and FHS birds. Dissimilar alphabets indicates significant differences.

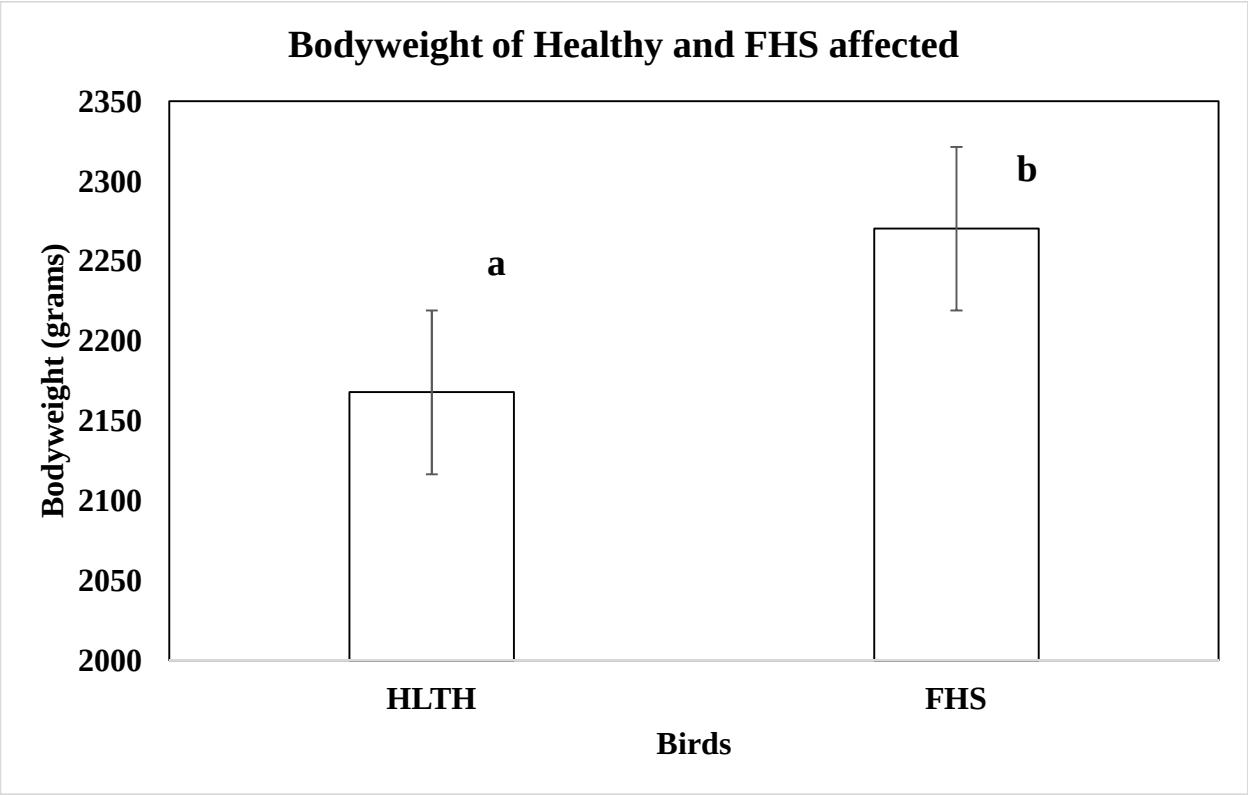

Figure 2. Comparison of peptide 3671 Da in HLTH (red) and FHS (green) group using ClinPro tools analysis

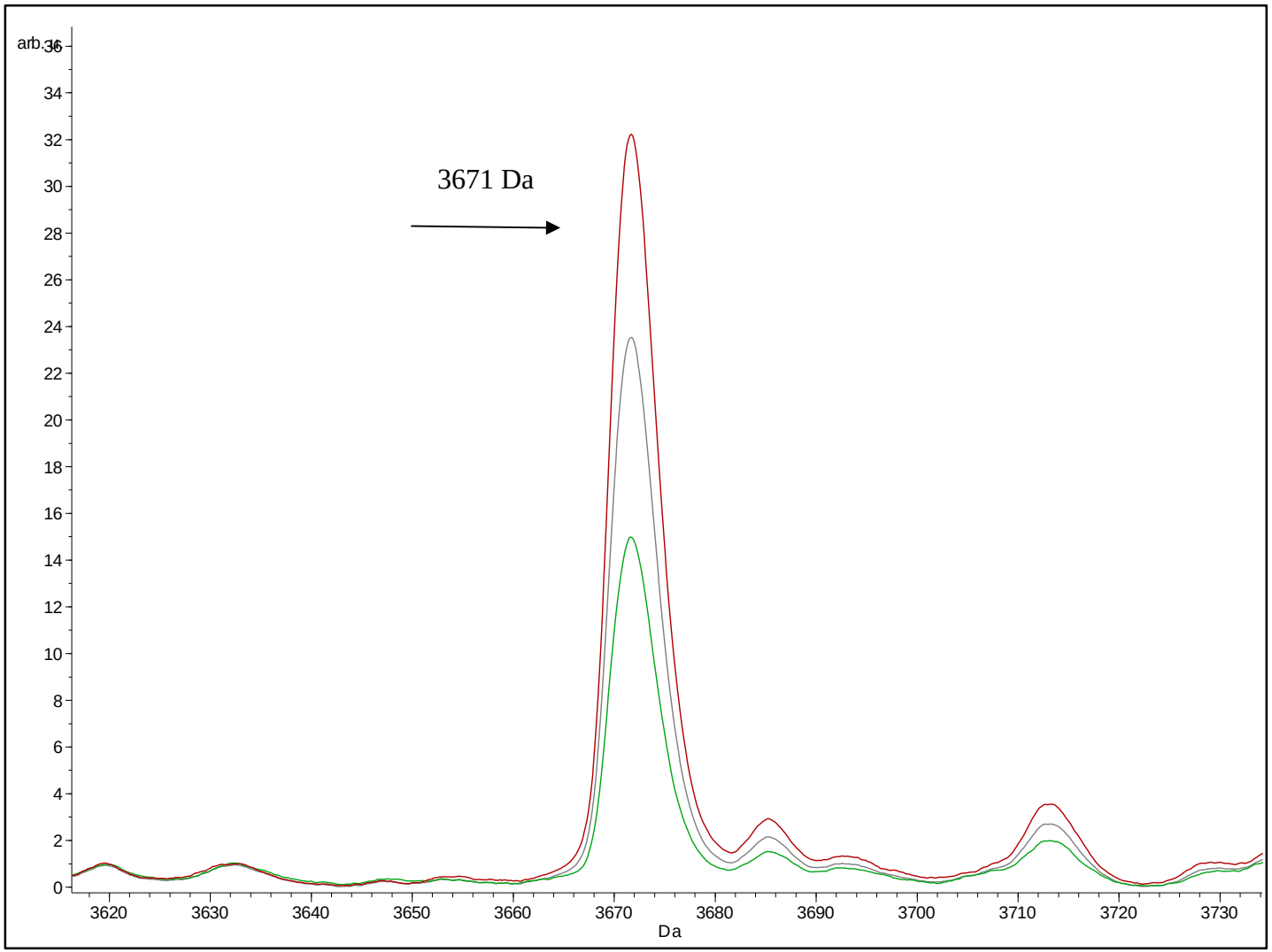

Figure 3. Comparison of peptide 4708 Da in HLTH (red) and FHS (green) group using ClinPro tools analysis

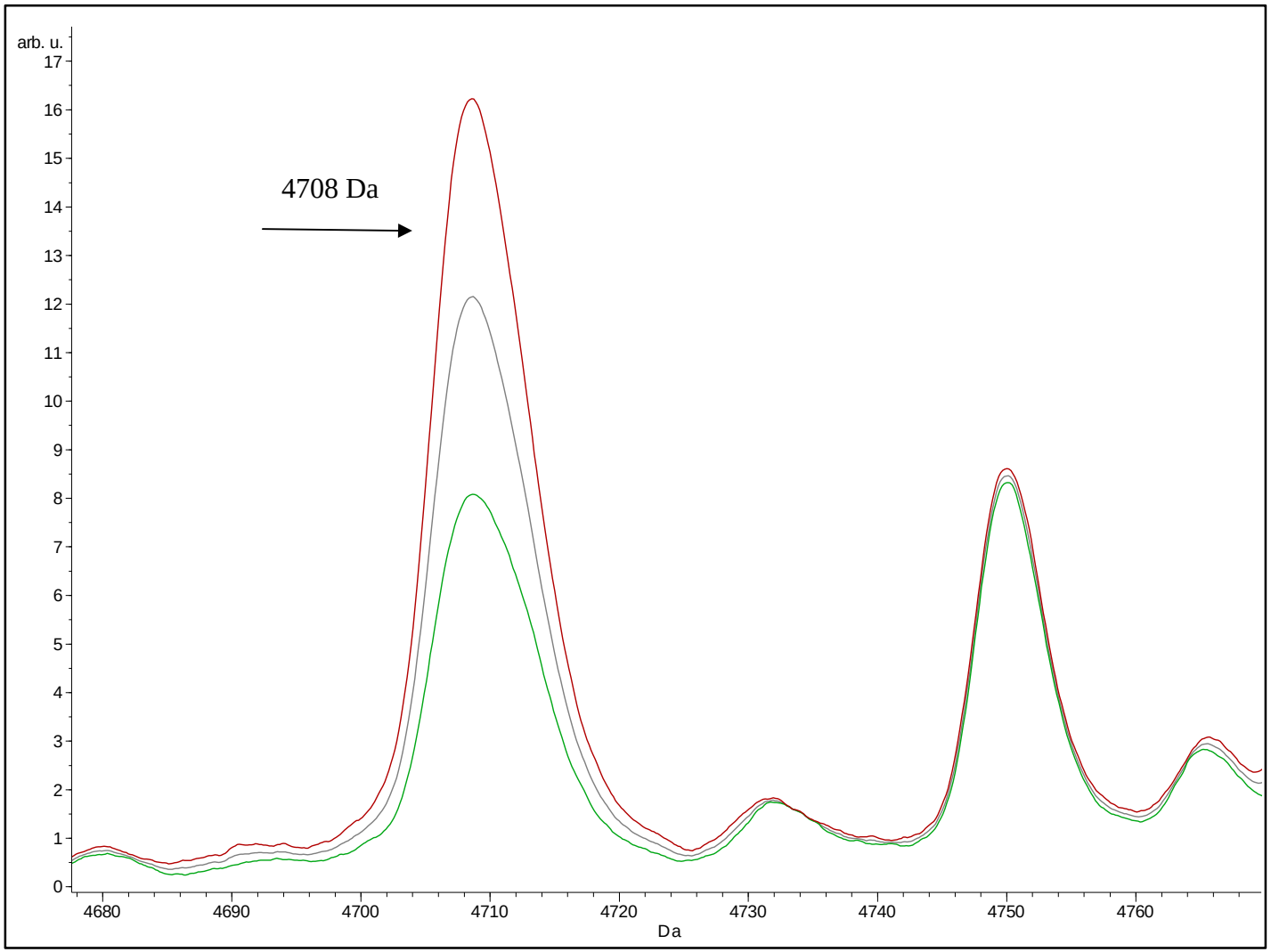

Figure 4. Peptide mass fingerprint (PMF) of peptide 3671 Da

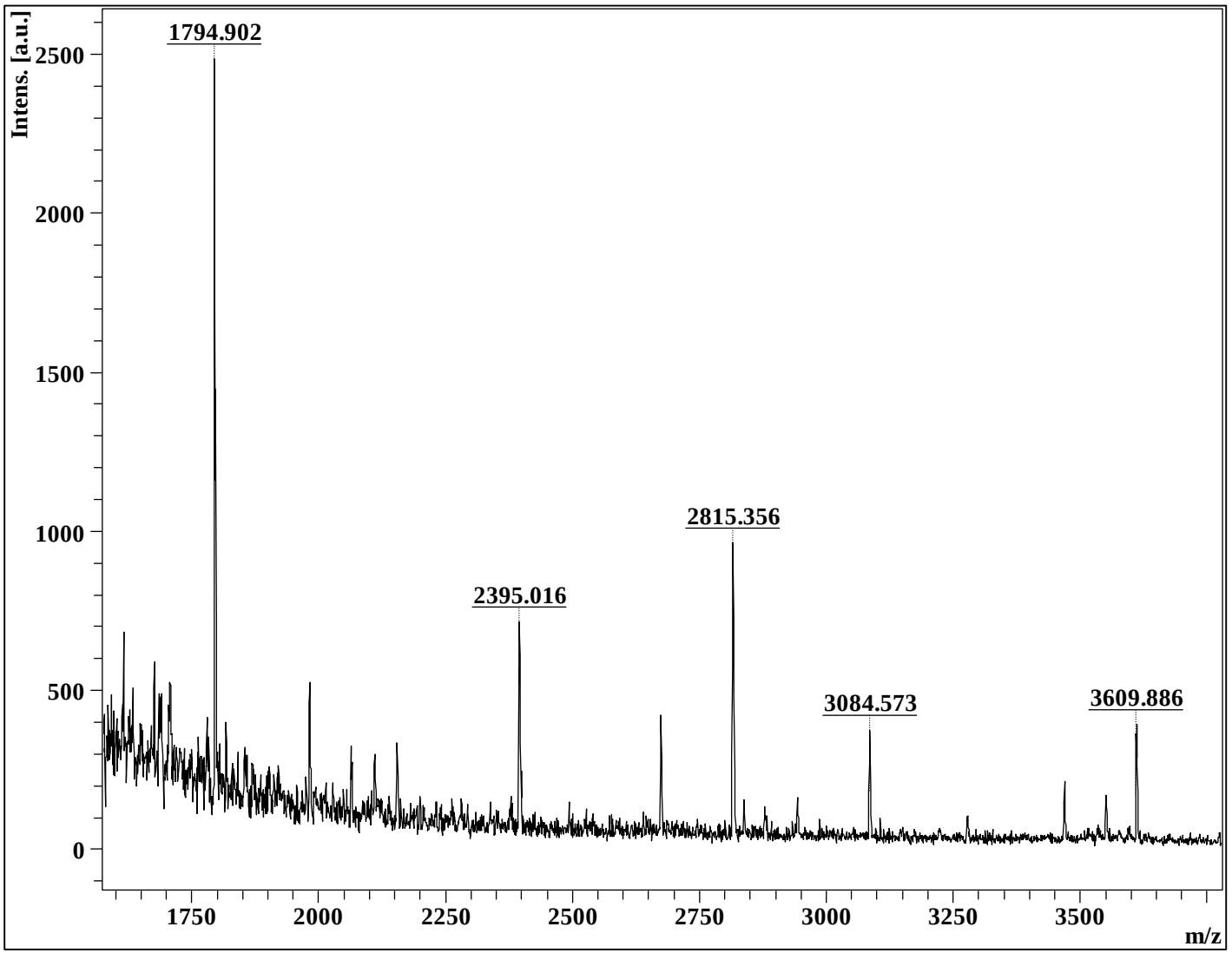

Figure 5. Peptide mass fingerprint (PMF) of peptide 4708 Da

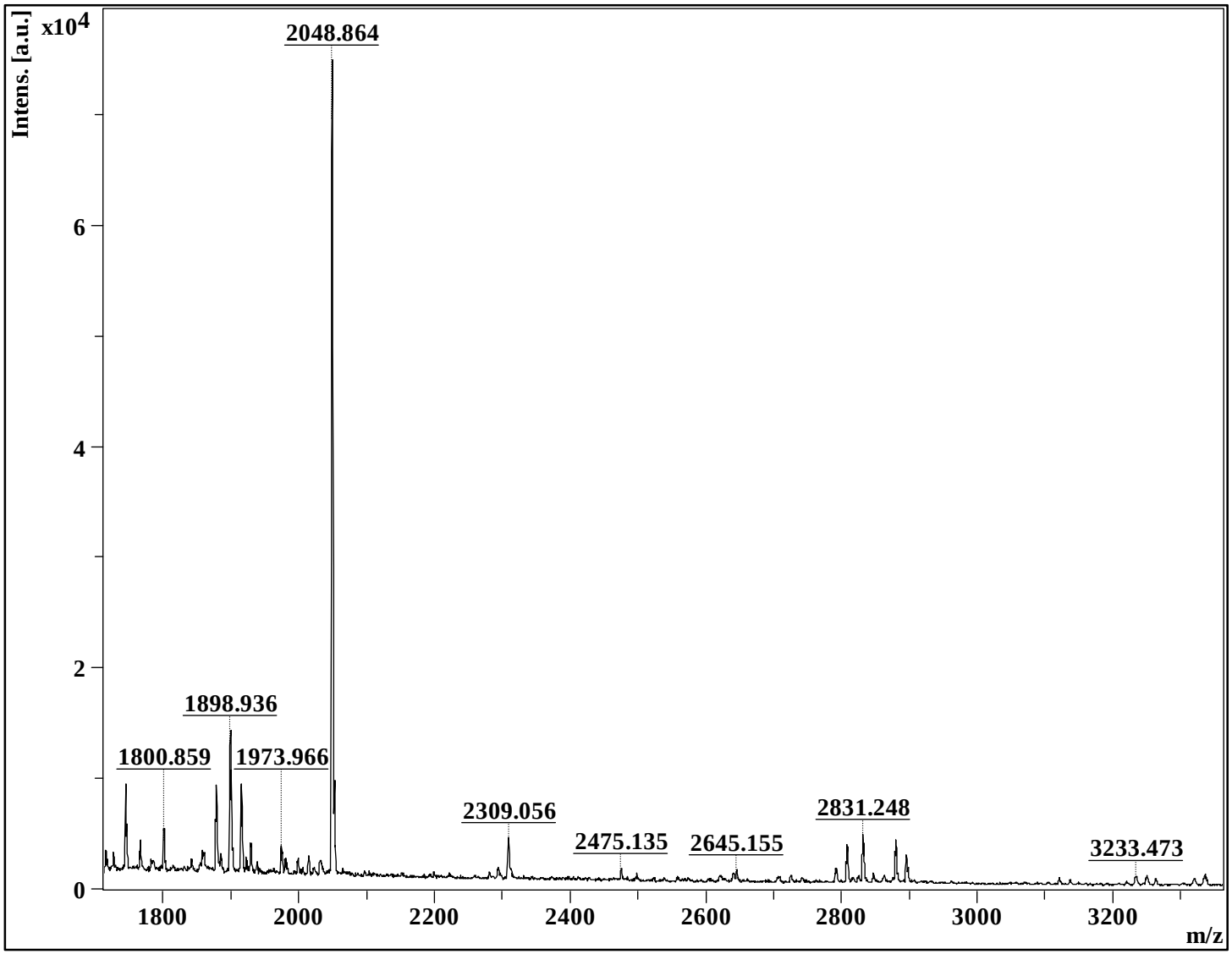

Figure 6. Tandem mass spectrometry of the peak 2815 Da from the peptide 3671 Da

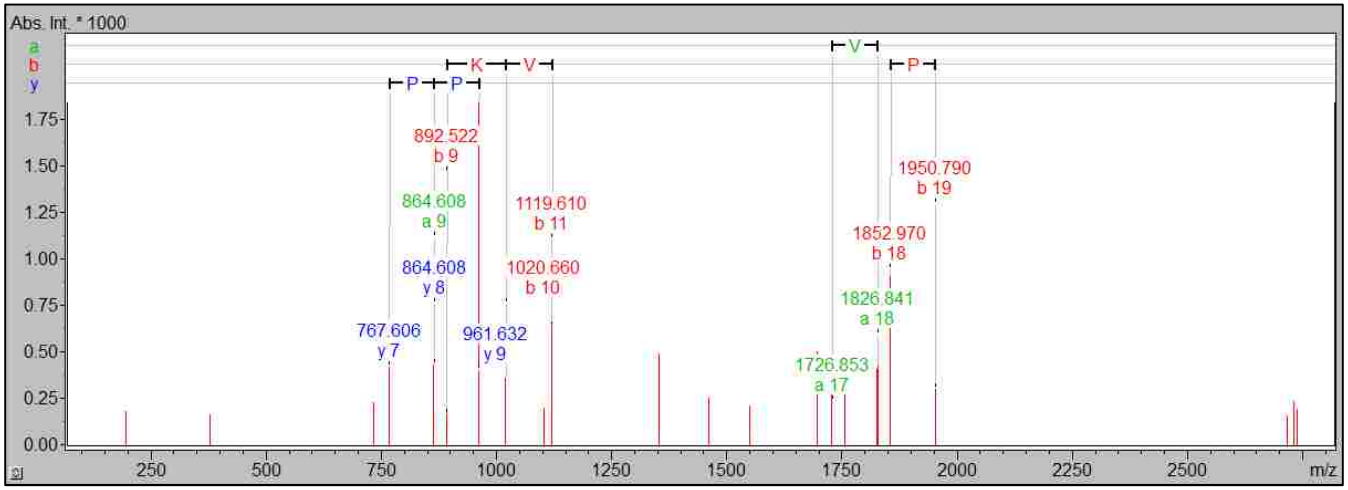

Figure 7. Tandem mass spectrometry of the peak 2048 Da from the peptide 4708 Da

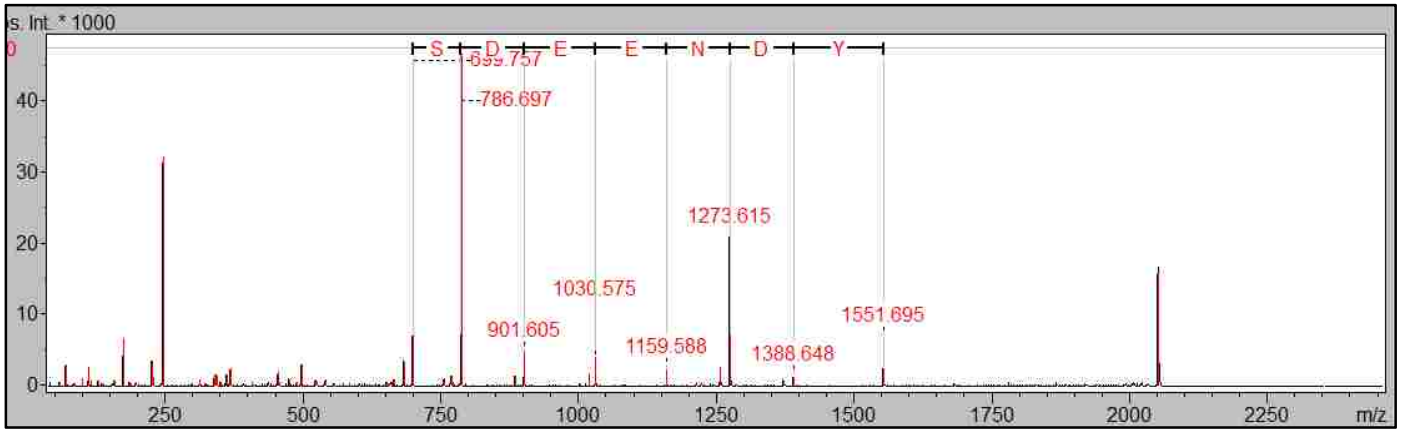

Figure 8. Venn diagram showing the number of proteins identified in each group

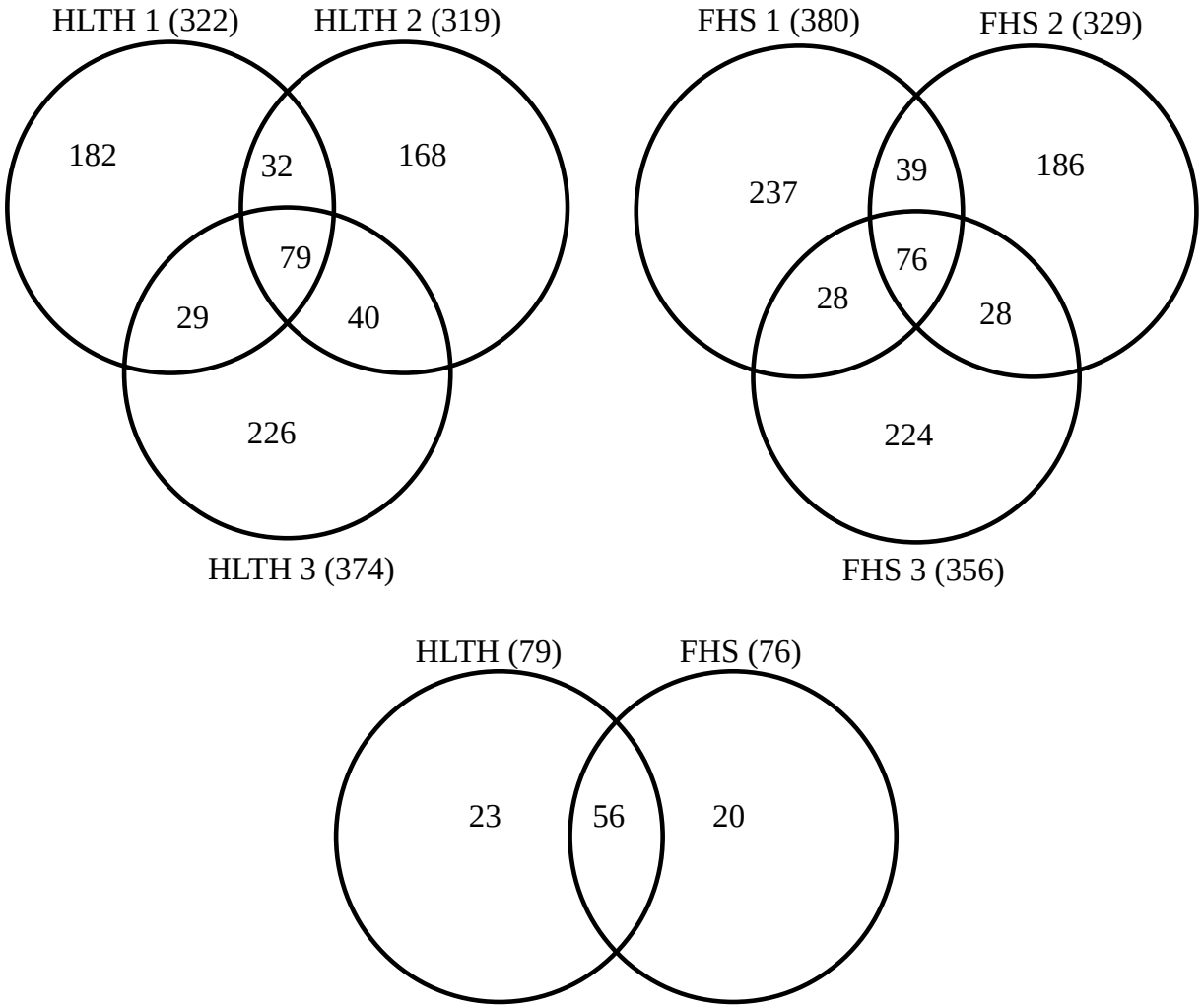

Figure 9 Volcano plot showing differentially expressed proteins in FHS group with respect to HLTH group. Red and blue indicates increase and decrease of proteins.

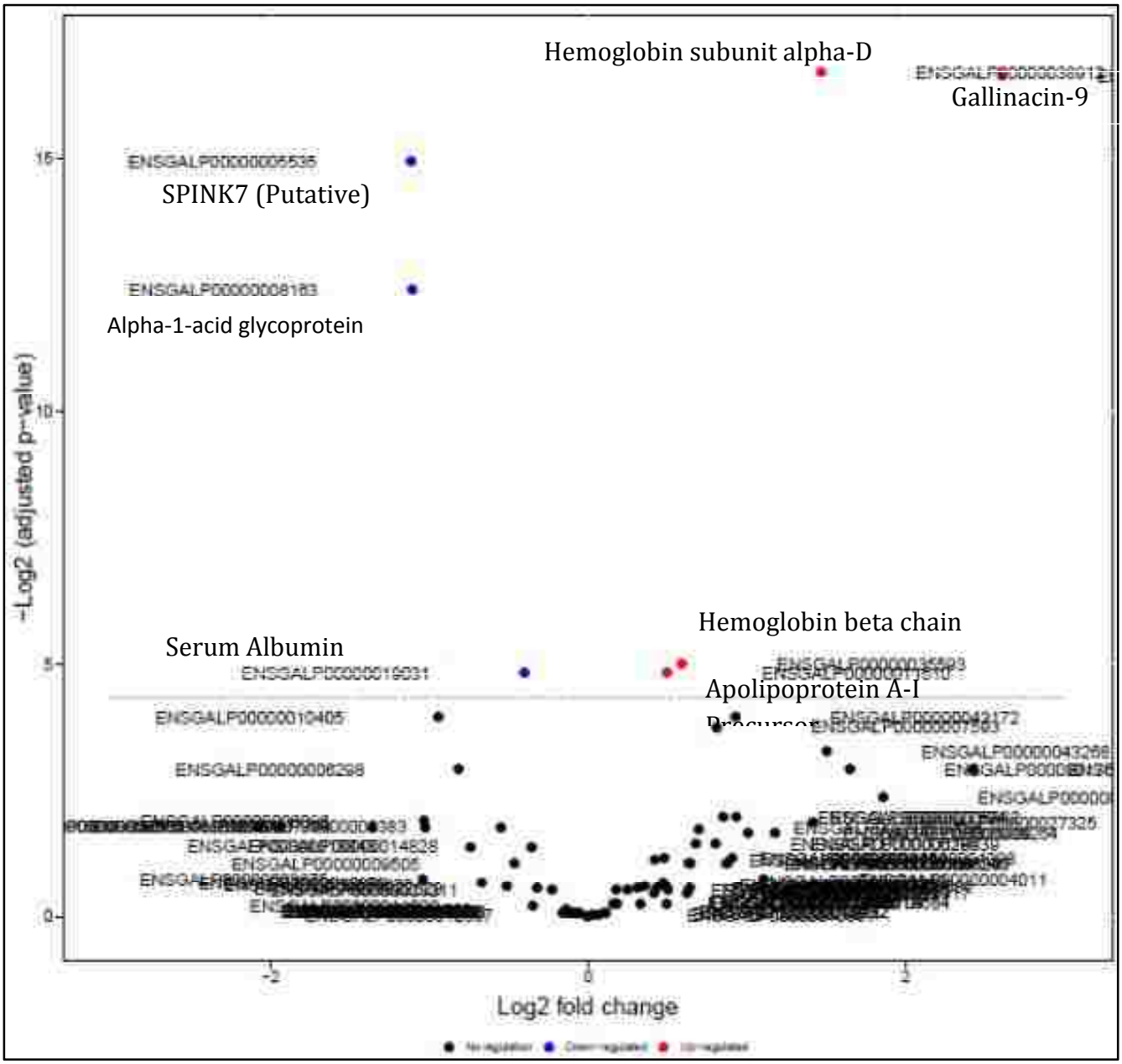

Table 1. Plasma cholesterol and triglycerides in HLTH and FHS samples (n=12). Vales are presented as Mean ± SEM, dissimilar superscript indicates significant differences.

Variables	**HLTH**	**FHS**
Cholesterol	132.2 ± 6.6 [a]	110.5 ± 7.8 [b]
High Density lipoprotein	38.6 ± 2.2 [a]	38.9 ± 2.5 [a]
Triglycerides	24.8 ± 2.0 [a]	25.3 ± 5.1 [a]
Low density lipoprotein	88.6 ± 5.3 [a]	66.6 ± 6.1 [b]

Table 2. List of peptides differentially expressed in HLTH and FHS samples based on ClinPro tools analysis (Only top 14 were given)

Mass	DAve	PTTA	PWKW	PAD	Ave1	Ave2	StdDev1	StdDev2	CV1	CV2
4775.52	4.2	< 0.000001	0	< 0.000001	9.21	5.01	2.55	1.34	27.73	26.73
3855.5	5.52	< 0.000001	0	0.0000127	10.92	5.4	3.4	2.04	31.11	37.8
3671.89	17.69	< 0.000001	0	0.0000762	33.53	15.84	11.41	7.58	34.04	47.84
3529.5	3.61	< 0.000001	0	0.000132	8.69	5.08	2.42	2.08	27.82	40.97
4134.9	0.51	< 0.000001	0	0.0414	2.69	2.18	0.35	0.29	13.18	13.51
3713.16	1.64	< 0.000001	0	0.0012	4.55	2.91	1.21	0.87	26.65	29.97
10220.73	6.07	< 0.000001	0	< 0.000001	12.57	6.49	4.88	2.36	38.81	36.38
3878.07	1	< 0.000001	0	< 0.000001	2.97	1.97	0.82	0.46	27.59	23.21
3761.09	1.28	< 0.000001	0	< 0.000001	3.95	2.67	1.06	0.56	26.91	20.84
4041.53	0.65	< 0.000001	0	0.0445	2.94	2.29	0.51	0.37	17.21	16.36
4362.76	0.86	< 0.000001	< 0.000001	< 0.000001	3.21	2.35	0.71	0.41	22	17.5
4709.09	8.23	< 0.000001	< 0.000001	< 0.000001	17.2	8.97	7.05	4.3	40.97	47.97
8945.73	0.5	< 0.000001	< 0.000001	0.0000156	2.36	2.86	0.35	0.49	14.91	17.06
3685.3	1.42	< 0.000001	< 0.000001	< 0.000001	3.86	2.44	1.43	0.86	37.15	35.07

Table 3. Proteins present only in the HLTH and FHS samples based on qualitative analysis. +/- indicates their presence or absence in respective samples

Protein	Name	Function	HLTH	FHS
ENSGALP00000031021	MyosinVI	cytoskeleton	+	-
ENSGALP00000026627	Additional sex combs like 2 (Drosophila)	Regualtion of transcription	+	-
ENSGALP00000000545	RNA binding motif protein 15	Regulation of transcription	+	-
ENSGALP00000026921	Meprin A, alpha (PABA peptide hydrolase)	Proteolysis	-	+
ENSGALP00000007080	Ubiquitin specific peptidase 34	Ubiqutin mediated proteolysis	-	+
ENSGALP00000001182	Myosin, heavy chain 13, skeletal muscle	Cytoskeleton	-	+
ENSGALP00000035723	Tudor domain containing 3	RNA binding	-	+
ENSGALP00000012641	Not characterized (golgin)	Golgi complex associated	-	+

Table 4. Clustering based on DAVID gene enrichment analysis for HLTH group

Annotation Cluster 1	Enrichment Score: 9.83			Count	P_Value
INTERPRO	Collagen triple helix repeat	RT		11	7.7E-18
GOTERM_CC_FAT	extracellular matrix	RT		17	2.2E-17
GOTERM_CC_FAT	extracellular region part	RT		20	2.8E-17
SP_PIR_KEYWORDS	collagen	RT		11	5.6E-17
GOTERM_CC_FAT	extracellular region	RT		23	8.7E-17
KEGG_PATHWAY	ECM-receptor interaction	RT		12	2.8E-16
GOTERM_MF_FAT	extracellular matrix structural constituent	RT		9	5.8E-15
GOTERM_CC_FAT	collagen	RT		9	1.2E-14
GOTERM_CC_FAT	proteinaceous extracellular matrix	RT		15	1.6E-14
GOTERM_CC_FAT	extracellular matrix part	RT		11	3.2E-14
SP_PIR_KEYWORDS	Secreted	RT		15	1.6E-12
SP_PIR_KEYWORDS	hydroxylation	RT		8	5.9E-12
GOTERM_MF_FAT	structural molecule activity	RT		15	6.1E-12
KEGG_PATHWAY	Focal adhesion	RT		12	6.3E-12
SP_PIR_KEYWORDS	extracellular matrix	RT		9	3.0E-9
SP_PIR_KEYWORDS	signal	RT		16	3.8E-9
GOTERM_BP_FAT	cell adhesion	RT		12	8.2E-9
GOTERM_BP_FAT	biological adhesion	RT		12	8.2E-9
UP_SEQ_FEATURE	region of interest:Triple-helical region	RT		5	1.6E-7
SP_PIR_KEYWORDS	triple helix	RT		5	2.3E-7
UP_SEQ_FEATURE	signal peptide	RT		16	2.4E-7
SP_PIR_KEYWORDS	disulfide bond	RT		11	1.4E-5
SP_PIR_KEYWORDS	hydroxyproline	RT		4	1.7E-5
SP_PIR_KEYWORDS	glycoprotein	RT		10	6.0E-4
SP_PIR_KEYWORDS	coiled coil	RT		7	1.4E-3
GOTERM_CC_FAT	extracellular space	RT		6	2.3E-3
UP_SEQ_FEATURE	disulfide bond	RT		9	4.8E-3
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		10	5.1E-3
Annotation Cluster 2	**Enrichment Score: 5.25**			**Count**	**P_Value**
SP_PIR_KEYWORDS	hydroxylation	RT		8	5.9E-12
SP_PIR_KEYWORDS	triple helix	RT		5	2.3E-7
INTERPRO	Fibrillar collagen, C-terminal	RT		4	1.9E-6
SMART	COLFI	RT		4	3.4E-6
SP_PIR_KEYWORDS	trimer	RT		3	3.7E-4
UP_SEQ_FEATURE	propeptide:C-terminal propeptide	RT		3	4.5E-4
UP_SEQ_FEATURE	domain:Fibrillar collagen NC1	RT		3	4.5E-4
SP_PIR_KEYWORDS	coiled coil	RT		7	1.4E-3
Annotation Cluster 3	**Enrichment Score: 3.88**			**Count**	**P_Value**
SP_PIR_KEYWORDS	hydroxylation	RT		8	5.9E-12
SP_PIR_KEYWORDS	hydroxyproline	RT		4	1.7E-5
UP_SEQ_FEATURE	short sequence motif:Cell attachment site	RT		5	2.5E-5
INTERPRO	von Willebrand factor, type A	RT		4	6.4E-5
PIR_SUPERFAMILY	PIRSF002259:collagen VI	RT		3	8.6E-5
SMART	VWA	RT		4	1.1E-4
SP_PIR_KEYWORDS	hydroxylysine	RT		3	3.7E-4
SP_PIR_KEYWORDS	heterotrimer	RT		3	3.7E-4
UP_SEQ_FEATURE	domain:VWFA 3	RT		3	4.5E-4
SP_PIR_KEYWORDS	cell adhesion	RT		5	1.0E-3
SP_PIR_KEYWORDS	coiled coil	RT		7	1.4E-3
UP_SEQ_FEATURE	domain:VWFA 1	RT		3	1.6E-3
UP_SEQ_FEATURE	domain:VWFA 2	RT		3	1.6E-3
GOTERM_CC_FAT	sarcolemma	RT		3	3.6E-3
SP_PIR_KEYWORDS	alternative splicing	RT		4	5.4E-2
Annotation Cluster 4	**Enrichment Score: 3.5**			**Count**	**P_Value**
INTERPRO	Fibrillar collagen, C-terminal	RT		4	1.9E-6
SMART	COLFI	RT		4	3.4E-6
GOTERM_BP_FAT	collagen fibril organization	RT		3	1.1E-3
GOTERM_BP_FAT	extracellular matrix organization	RT		3	1.5E-2
GOTERM_BP_FAT	extracellular structure organization	RT		3	2.8E-2
Annotation Cluster 5	**Enrichment Score: 1.25**			**Count**	**P_Value**
INTERPRO	Fibronectin, type III	RT		3	4.9E-2
INTERPRO	Fibronectin, type III-like fold	RT		3	5.2E-2
SMART	FN3	RT		3	7.0E-2

Table 5. Clustering based on DAVID gene enrichment analysis for FHS group

Annotation Cluster 1	Enrichment Score: 6.64			Count	P_Value
GOTERM_CC_FAT	extracellular region part	RT		20	9.6E-17
INTERPRO	Collagen triple helix repeat	RT		10	1.4E-15
SP_PIR_KEYWORDS	collagen	RT		10	8.8E-15
GOTERM_CC_FAT	extracellular matrix	RT		15	7.5E-14
GOTERM_MF_FAT	extracellular matrix structural constituent	RT		8	6.1E-13
KEGG_PATHWAY	ECM-receptor interaction	RT		10	2.9E-12
GOTERM_CC_FAT	collagen	RT		8	3.1E-12
GOTERM_CC_FAT	proteinaceous extracellular matrix	RT		13	2.8E-11
GOTERM_CC_FAT	extracellular matrix part	RT		9	1.8E-10
GOTERM_MF_FAT	structural molecule activity	RT		13	7.0E-10
SP_PIR_KEYWORDS	hydroxylation	RT		7	7.0E-10
SP_PIR_KEYWORDS	Secreted	RT		13	8.9E-10
GOTERM_BP_FAT	cell adhesion	RT		12	5.1E-9
GOTERM_BP_FAT	biological adhesion	RT		12	5.1E-9
KEGG_PATHWAY	Focal adhesion	RT		10	8.8E-9
SP_PIR_KEYWORDS	signal	RT		15	5.2E-8
UP_SEQ_FEATURE	signal peptide	RT		15	2.4E-7
SP_PIR_KEYWORDS	extracellular matrix	RT		7	2.3E-6
UP_SEQ_FEATURE	region of interest:Triple-helical region	RT		4	1.5E-5
SP_PIR_KEYWORDS	hydroxyproline	RT		4	1.7E-5
SP_PIR_KEYWORDS	triple helix	RT		4	2.5E-5
INTERPRO	von Willebrand factor, type A	RT		4	6.4E-5
PIR_SUPERFAMILY	PIRSF002259:collagen VI	RT		3	8.6E-5
SMART	VWA	RT		4	9.0E-5
UP_SEQ_FEATURE	domain:VWFA 3	RT		3	3.6E-4
SP_PIR_KEYWORDS	hydroxylysine	RT		3	3.7E-4
SP_PIR_KEYWORDS	heterotrimer	RT		3	3.7E-4
UP_SEQ_FEATURE	short sequence motif:Cell attachment site	RT		4	5.2E-4
SP_PIR_KEYWORDS	disulfide bond	RT		9	6.7E-4
UP_SEQ_FEATURE	domain:VWFA 2	RT		3	1.2E-3
UP_SEQ_FEATURE	domain:VWFA 1	RT		3	1.2E-3
GOTERM_CC_FAT	sarcolemma	RT		3	3.9E-3
SP_PIR_KEYWORDS	coiled coil	RT		6	8.3E-3
SP_PIR_KEYWORDS	cell adhesion	RT		4	1.1E-2
SP_PIR_KEYWORDS	glycoprotein	RT		8	1.2E-2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		8	3.1E-2
UP_SEQ_FEATURE	disulfide bond	RT		7	3.6E-2
Annotation Cluster 2	**Enrichment Score: 5.29**			**Count**	**P_Value**
GOTERM_MF_FAT	extracellular matrix structural constituent	RT		8	6.1E-13
GOTERM_CC_FAT	collagen	RT		8	3.1E-12
INTERPRO	Fibrillar collagen, C-terminal	RT		3	3.3E-4
SMART	COLFI	RT		3	4.3E-4
GOTERM_BP_FAT	collagen fibril organization	RT		3	1.0E-3
GOTERM_BP_FAT	extracellular matrix organization	RT		3	1.4E-2
GOTERM_BP_FAT	extracellular structure organization	RT		3	2.6E-2
Annotation Cluster 3	**Enrichment Score: 1.92**			**Count**	**P_Value**
GOTERM_CC_FAT	extracellular space	RT		8	3.9E-5
GOTERM_BP_FAT	negative regulation of apoptosis	RT		3	8.0E-2
GOTERM_BP_FAT	negative regulation of programmed cell death	RT		3	8.2E-2
GOTERM_BP_FAT	negative regulation of cell death	RT		3	8.2E-2

Table 6. List of proteins differentially expressed where + and – indicates increase and decrease of their levels in FHS samples respectively

Protein	Name	+/-	Possible reason
ENSGALP00000035593	Hemoglobin beta chain	+	Hemolysis
ENSGALP00000038912	Hemoglobin subunit alpha-D	+	Hemolysis
ENSGALP00000035930	Gallinacin-9	+	Immune activation
ENSGALP00000011510	Apolipoprotein A-I Precursor	+	Dyslipidemia
ENSGALP00000008163	Alpha 1-acid glycoprotein	-	Dyslipidemia
ENSGALP00000019031	Albumin	-	Nutritional deficiency
ENSGALP00000005535	(SPINK7) serine peptidase inhibitor, Kazal type	-	Proteolysis

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V. Proteomic changes in chicken plasma induced by lipopolysaccharides

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Abstract

Lipopolysaccharides (LPS) are cell wall components of gram negative bacteria which interact with to produce inflammation and sickness through genetic and proteomic activation. The objective of our study was to identify proteomic changes associated with inflammation and infections in chickens. We used mass spectrometry and bioinformatics to identify the proteomic changes using chickens treated with saline (CTRL) or LPS. Plasma was prepared from blood collected at 24 hours post injection then pooled in equal volumes from 3 birds each, to make triplicate samples in each group for proteomic analyses. The plasma was depleted of high abundant proteins (HAPD) using acetonitrile. Peptides and proteins in HAPD samples of CTRL and LPS-treated birds were analyzed by Matrix assisted laser desorption ionization- time of flight- mass spectrometry (MALDI-TOF-MS) and liquid chromatography–tandem mass spectrometry (LC-MS/MS). Peptides, isolated binding to reverse phase C18 tips were subjected to MALDI-TOF-MS and ClinPro tools analysis. The results showed a fibrinogen peptide increased while Apolipoprotein AII-isoform X-1 decreased in LPS treated birds. The proteome from depleted plasma was reduced, alkylated, trypsin digested, and subjected to LC-MS/MS. The proteins in spectra were identified and compared using Skyline software which showed an elevation in alpha-1 acid glycoprotein, chemokine-CCLI10, and cathelicidin levels while an interferon stimulated gene-12-2 protein decreased in LPS treated birds. These differentially expressed proteins are associated with coagulation cascade, lipid transport, and defense associated functions. Some of these proteins may have potential for use as biomarkers of infection and inflammation in poultry.

Key words: Proteomics, Lipopolysaccharides, innate immunity, acute phase proteins

List of abbreviations

ACN Acetonitrile

APR Acute phase response

APP Acute phase proteins

LPS Lipopolysaccharide

FA Formic acid

LC-MS/MS Liquid Chromatography and tandem mass spectrometry

GO Gene ontology

GPM Global Proteome Machine

HAPD High abundant proteins depleted

HDL High density lipoprotein

LC-MS/MS

MALDI-TOF Matrix assisted laser desorption ionization- time of flight

RP-HPLC Reverse Phase High performance Liquid Chromatography

Introduction

Lipopolysaccharides (LPS) are cell wall components of gram negative bacteria which elicits inflammatory, septic shock and acute phase responses (APR) during infection (Salomao, et al., 2012). The interaction of these endotoxins with host leads to the production of various cytokines that can alter the overall homeostasis, immunity and metabolism which is in turn reflected in the levels of proteins, peptides and lipids in blood (Kemna, et al., 2005; Waage, et al., 1989). The plasma proteins and peptides not only constitute structural entities of tissues but also govern growth, development, nutrient transport and hence, the changes in their levels are expected to play an important role during such disturbances in homeostasis and its eventual restoration. LPS induced humoral changes in serum or plasma have been extensively analyzed in humans (Qian, et al., 2005), mice (Wait, et al., 2005), and some farm animals such as cow (Danielsen, et al., 2010) and sheep (Chemonges, et al., 2014). Although in chickens, changes in plasma proteins and APR were extensively studied using traditional approaches (Cray, et al., 2009; Eckersall and Bell, 2010), proteomic methods were less utilized (Gilbert, et al., 2011). Our objective was to explore the changes in plasma proteins and peptides of chickens in response to LPS challenge using proteomics methods such as matrix assisted laser desorption ionization- time of flight-mass spectrometry (MALDI-TOF-MS), and liquid chromatography and tandem mass spectrometry (LC-MS/MS). These gel free proteomic methods might expand the spectrum of known plasma protein changes during infection and provide novel candidate proteins important for avian immunity and candidate biomarkers for APR such as acute phase proteins. Our study shows that the proteins involved in coagulation, lipid transport, innate immunity and antimicrobial defense are differentially regulated and are consequential to the response to LPS.

Methods.

Chickens, treatment, and blood collection. The animal procedures were approved and carried out in accordance with the University of Arkansas IACUC guidelines. Eighteen Cobb 500 broiler chicks were raised on floor pens at the density of 8 square feet /bird, provided diets formulated per National Research Council specifications (NRC, 1994), and *ad libitum* water. Five week old chickens were divided into 2 groups (n=9); the control (CTRL) received saline and the LPS group received LPS (*Salmonella typhimurium* Sigma-Aldrich, St. Louis, MO) dissolved in saline at the dose of 1mg/kg bodyweight, injected intramuscularly on left thigh. The weight of the birds were taken prior to and 24 h post injection. The chickens were monitored for the sickness behavior upto 6 h following the challenge and until bleeding. Blood was collected through cardiac puncture at 24 h K-EDTA Vacutainer tubes (BD Bioscience), and euthanized.

Plasma preparation. Plasma was seperated from blood by centrifugation at 2,000 *g*, for 10 min at 4°C and stored at -20°C for subsequent analyses. Three samples for each group were prepared pooling equal volumes of plasma from 3 individual birds, and centrifuged at 21,000 *g* for 10 minutes at 4°C to pellet insoluble precipitates. An aliquot of plasma sample was mixed with 2 volumes of 90% acetonitrile (ACN) containing 0.1% formic acid (FA) to reach a final concentration of 60% ACN, mixed, and sonicated twice in an ultrasonic water bath (Branson 3200, CT) for 10 min each and kept at -20°C for 16 h to precipitate high abundant proteins (Fernández, et al., 2011; Kay, et al., 2008). The supernatant containing high abundant proteins depleted (HAPD) plasma was dried in a CentriVap vaccum concentrator (Labconco, Kansas City, MO) and dissolved in 50 mm ammonium bicarbonate to determine the protein concentration by BCA protein method (Pierce, IL). These HAPD samples were used in all subsequent analyses.

Peptide analysis by MALDI-TOF-MS

Seventy five microliters of the CTRL and LPS samples (n=3) were desalted using reverse phase (RP) C18 tips (NT1C18, Glygen, MD) per manufactures' protocol except that the binding and washing steps were repeated 5 times. The eluted samples were spotted on a MALDI 384 target plate, dried and overlaid with an equal volume of sinapinic acid (10 mg/mL 0.1% FA in 30% of ACN), and the spots were analyzed using an Ultraflex II MALDI-TOF/TOF instrument (Bruker Daltonics, Billerica, MA) in the positive-ion linear mode. The instrument was calibrated using a 5-17.5 kDa protein standard (Bruker Daltonics) and the MS data for peptides of range 1-10 kDa were collected in an automated mode using Bruker Flex control software with a constant laser power, and 800 laser shots per spot.

ClinProTools analysis. The MS spectra of peptides from CTRL and LPS samples were compared using ClinProTools software™ (Version 2.2, Bruker Daltonics) (Ketterlinus, et al., 2005). The quick classifier algorithm was used for automatic peak detection, integration with peaks exhibiting a signal to noise ratio ≥ 10, and a threshold intensity of at least 5% relative to the largest peak (ClinProtools user manual 2.1, 2006). Individual peaks from all 6 samples were aligned and the areas under each peak analyzed for statistical differences. Anderson- Darling's test was used to establish the data distribution and the statistical differences and the significance calculated using t- and Wilcoxon tests. Values with $p \leq 0.05$ were considered to be significant.

In-solution digestion. The protein concentrations of HAPD samples from both groups were adjusted to 1µg/µL with 50mM ammonium bicarbonate and 100µL of each sample was reduced with 10 mM dithiothreitol (MP Biomedicals, OH) for 1 h at 60°C and alkylated with 55 mM iodoacetamide (MP Biomedicals, OH) for 1 h at room temperature in dark. Excess

iodoacetamide was neutralized with DTT then the samples were digested with 2µg of trypsin (Promega, Madison, WI) at 37°C for 48 h. The digests were centrifuged at 21,000 *g* for 10 min at 4°C to remove insoluble precipitates and the supernatant were dried using Centrivap concentrator. The dried peptides were dissolved in 5% ACN containing 0.5% formic acid (FA) in water and desalted with C18 Spin Columns (Pierce, IL, USA) per manufacturer's protocol. The eluted peptides were dried and suspended in 0.1% FA for LC-MS/MS.

LC-MS/MS. The digested samples were subjected to LC-MS/MS using an Agilent 1200 series capillary C18-RP-HPLC coupled to a Bruker Amazon-SL quadrupole ion trap mass spectrometer, capable of performing data-dependent acquisition. Tryptic peptides were separated by reverse-phase liquid chromatography (RP-HPLC) using a Zorbax SB C18 column, (150 x 0.3mm, 3.5 µm particle size, 300 Å pore size, Agilent Technologies), with a solvent flow rate of 6 µL/min, and a gradient of 0 to 40 % consisting of 0.1% FA (solvent A) and ACN (solvent B) for 300 min.

Data analysis. The mzXML files exported from Data analysis 4.0 (Bruker) were submitted to Global proteome machine (GPM) (http://www.thegpm.org) to search against Chicken genome with following parameters for X!tandem (Bjornson, et al., 2008); Fragment mass error of 0.6 Da, Carbamidomethylation and methionine oxidation as fixed and variable modification respectively, search for known post-translational modifications (PTM), trypsin enzyme with semi-style cleavage, removal of redundant spectra, allow spectrum synthesis, ion trap (4 Da) as the predefined method and data was not archived in GPM database. Protein identifications were considered true if there is at-least one unique peptide per protein, and only up to an acceptable e-value for a false positive rate < 5% displayed in the corresponding results page. The results were

downloaded as *.xml files for skyline software and list of identified proteins were downloaded as excel files for qualitative analysis.

Qualitative analysis. The common proteins present in the three samples (biological replicates) of each group were selected using an online excel comparison program (www.xlcomparator.net). The proteins present in all the LPS samples and none of the CTRL samples or vice versa were considered to be differentially expressed. The Protein ID were converted to ENSEMBL gene ID using Biomart (Guberman, et al., 2011) and analyzed for enrichment and clustering using DAVID program with an EASE score of 0.1 (www. david.abcc.ncifcrf.gov) (Huang, et al., 2009a; Huang, et al., 2009b). The clusters present only in the LPS group but not in CTRL or vice versa were considered relavent as reponses to LPS. In cases where GO annotations for Protein/Gene IDs were not available in DAVID data base or Uniprot, the sequence was subjected to BLAST-P and relevant matching proteins annotations were provided.

Label free quantitation of peptides. The mass spectra (*.mzXML) and identification (*.xml) files were loaded in Skyline software (http://proteome.gs.washington.edu/software/skyline) to perform MS1 filtering (Schilling, et al., 2012) of both CTRL and LPS groups, followed by the label free quantitation using an external tool ”MSstats” (Choi, et al., 2014). The quantitation of proteins were performed with peptide peak areas obtained from extracted ion chromatogram with default parameters for Precursor mass analyzer- QIT. The group comparison function of “MSstats” was used to generate volcano plots of differentially expressed proteins.

Statistics

Effect of LPS on bodyweight of birds was analyzed by SAS (SAS Institute Inc., Cary, NC) to perform a 1-way ANOVA and Duncan's t-test. A *P*-value of ≤0.05 was considered to be significant.

Results

Effect of LPS on morphological parameters. Although both groups of chickens had similar BW prior to injection, the LPS treated chickens lost weight within 24 h of treatment (Figure 1). The birds expressed sickness behavior indicated by lethargy, sleepiness, and avoidance of feed and water, evident within 3-6 h of treatment. However, at 24 h, they appeared to have recovered.

Peptide analysis. The list of differentially expressed peptides based on ClinPro tools analysis of MALDI-TOF data are given in table 1. Among these 68 peptides, the peptides 4707 Da and 8108 Da (Figure 2 and 3) were derived from Fibrinogen precurosor and Apolipoprotein AII- Isoform X1 (Apo AII-X1) respectively. The idendity were given based on our previous analysis of these peptides from chicken plasma assocaited with a skeletal disorder (data not shown). The identity of other differntially expressed peaks (figure 4 and 5) were not known.

Plasma proteome. Qualitative comparison of the common proteins of CTRL with the common proteins of LPS (Figure 6) showed that only two proteins, Alpha-1 acid glycoprotein (AGP or ORM1) and Chemokine CCLI10 were present only in the LPS group and absent in all of the CTRL samples. Label free quantitation with Skyline software generated a volcano plot showing four differentially expressed proteins in LPS samples (Figure 7). The proteins such as Cathelicidin precursor (CATH-2) and AGP and a heparanase precursor (HPSE) are elevated

while a interferon alpha induced protein downregulated isdownlregulated in LPS group (Table 2).

DAVID enrichment and clustering. Enrichment analysis using DAVID showed that cluseter of proteins involved in lipid binding and extracellular space are enriched in CTRL only while in LPS, the proteins with kinase activity were enriched (Table 3 and 4). Gene-Annotation Association on 2-D View of these proteins are given in Figure 8 and 9.

Discussion

LPS induced activation and changes in cells have been extensively studied at the transcriptomic (Hu, et al., 1997; Lefevre, et al., 2008; Zhang, et al., 2013) and proteomic level (Aulak, et al., 2001; Haglund, et al., 2008; Liu, et al., 2008) using several animal models including chickens (Burgess, 2004). However, there are very limited studies of its effect in biological fluids at the proteomic level particularly with serum and plasma (Chemonges, Tung and Fraser, 2014; Danielsen, Codrea, Ingvartsen, Friggens, Bendixen and Røntved, 2010; Qian, Monroe, Liu, Jacobs, Anderson, Shen, Moore, Anderson, Zhang, Calvano, Lowry, Xiao, Moldawer, Davis, Tompkins, Camp, Smith and Program, 2005; Wait, Chiesa, Parolini, Miller, Begum, Brambilla, Galluccio, Ballerio, Eberini and Gianazza, 2005).

Although the analysis of serum proteome might be a straight forward methodology, there are several constraints at the level of organism, dynamics of the change in proteins and methods. At the level of an organim, chicken is a outbred population in which biological response are variabile in order and magnitude owing to the individual physiology and genetics. During such a complex biological challenge, circulating proteins have different turnover rates that affects their half life in blood. Some of these responses can be protracted lasting for longer duration as in case

of acute phase proteins (APP) (Cray, Zaias and Altman, 2009), or may last for a short duration as in the cytokines (Janský, et al., 2003). The serum proteome is also complex with few high abundant proteins such as albumin and transferrin constituite >90% of the total proteins while the cytokines and other biomarker proteins might be very low in abundance and thus evade detection. Nevertheless, analysis of chicken serum proteome 24 hrs post challenge would reflect the changes in host induced by LPS and reveal certain key proteins mediating these changes.

Treatment of chickens with LPS induced distinct physiological changes evident by sickness behavior, feed avoidance, APR and cachexia which is consistent with our previous observations (Xie, et al., 2000). In order to remove high abundant proteins from plasma, we employed a cost and time effective procedure that involved ACN depletion (Kay, Barton, Ratcliffe, Matharoo-Ball, Brown, Roberts, Teale and Creaser, 2008). Although several strateiges such as proteominer and immunodepletion are avilable for depeletion of these proteins, we used a cheap organic solvet and used HAPD plasma in the protemic anlayses.

In LPS group, over 60 peptides are differentially expressed, which might be due to the changes in activity of the proteases and their inhibitors (Juhan-Vague, et al., 1989; van Vugt, et al., 1986). Based on our previous analysis of chicken plasma for a skeletal disorder, the identity of two peptides, m/z 4708 and 8111 were matched to be derived from fibrinogen precursor and Apo AII-X1, respectively. The fibrinogen is involved in coagulation cascade while the Apo AII is a part of high density lipoprotein (HDL) important for lipid transport.

The comparison of common proteins analyzed by LC-MS-MS showed that two proteins, □1 acid glycoprotein (AGP) and CCL10 present in the plasma of LPS-treated birds. The qualitative comparison method showed these proteins are present only in LPS because their level in CTRL

might be too low for detection while LPS had higher levels of these proteins. AGP (orosomucoid 1) is a well characterized avian protein which has been shown to be elevated in response to inflammation and is regarded as an acute phase protein (Nakamura, et al., 1998; Takahashi, et al., 1994). However, the protein CCLI10 (Kaiser, et al., 2008), an inflammatory chemokine produced by macrophages, was identified analyzing spleen transcriptome after infection with a *Salmonella enteritidis* serovar (Matulova, et al., 2012). It is not known whether this chemokine is common to all types of inflammatory challenges or a specific response to *Salmonella* endotoxins only.

Similarly cathelicidin (CATH), a peptide involved with antimicrobial defense (van Dijk, et al., 2005; Xiao, et al., 2006) was elevated in the plasma of chickens treated with LPS. Heparanase (HPSE) is an enzyme that maintains balance of coagulation cascade (Kozek-Langenecker, et al., 2000) and degrades glycosylated residues outside epithelial cells facilitating the diapedesis of white blood cells (Schmidt, et al., 2012). The interferon alpha stimulated protein (ISG-12-2), which is significantly reduced, still remains as a "putative" protein. Interaction of ISG with interferon and immunity is characterized as a a pro-inflammatroy protein in mammalian models. The knockout of ISG-12 increases survival by lowering inflammatory stimulus (Uhrin, et al., 2013). Thus, in the birds decrease in ISG-12-2 could be a strategy to mitigate the adverse effects of inflammation.

DAVID based gene enrichment provides information about differentially enriched proteins in each group that may not be evident by a quantitative analysis because of their low expression . The CTRL group had a cluster containing proteins involved with lipid transport but absent in LPS because, when the animals avoid feed, lipid transport are likely to be altered. Similarly, the LPS groups showed the presence of a cluster of proteins associated with phosphorous

metabolism and kinase activities. The kinases are involved in might act as signal molecules medidating inflammatory pathways and resistance against LPS. The heat map showed the functions of these clusters of proteins characterized from laboratory studies or predicted from experimetns with other animals.

Based on our analysis, it is evident that the proteins such as fibrinogen, Apo AII-X1, AGP, CATH-2, HSPE and ISG-12-2 are significantly altered in LPS treated chickens. These protien must be consequential to the host response to LPS and have potential as candidate biomarkers in the event of APR, injury, and stress. In comparison to the LPS response, Skeletal disorder femoral head seperation manifests a entirelty different protein biomaker profile characterized by reduction in albumin, AGP, Fetuin, Fibrinogen, Apo AII-X1 and increase in Apolipoprotein A-I, Gallinacin-9 and hemoglobin chains.

List of figures

Figure 1. Effect of LPS on bodyweight of Chickens

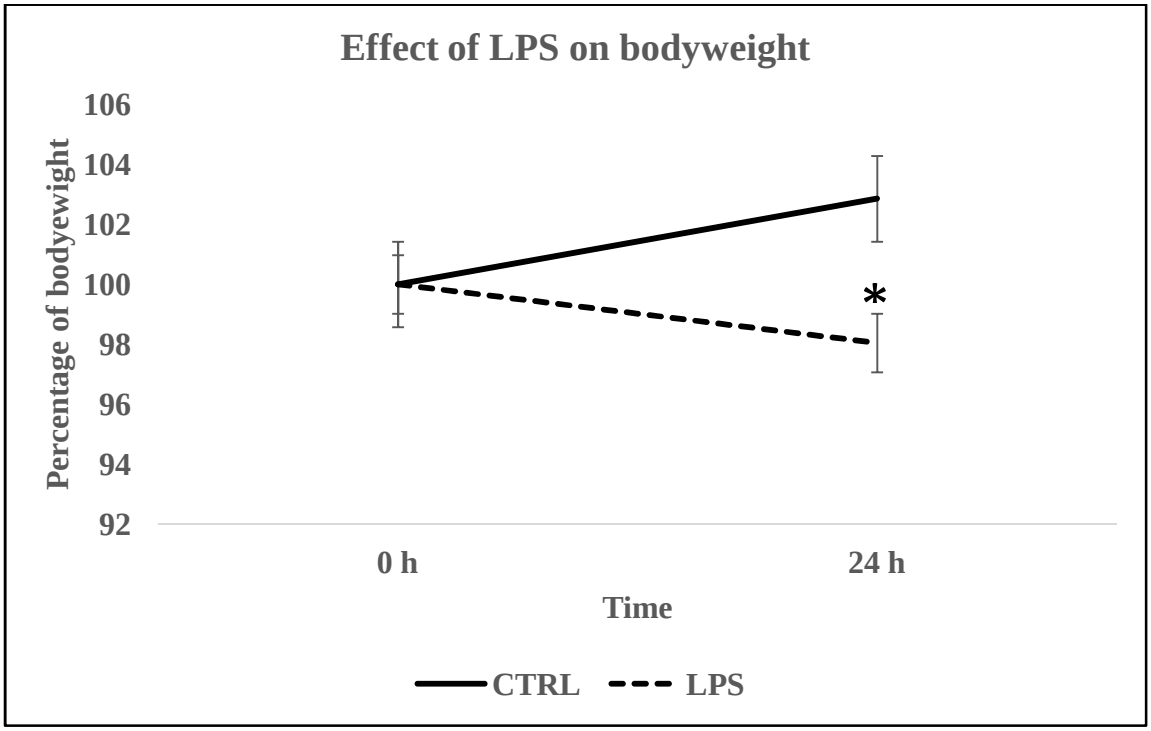

Figure 2. Comparison of MALDI-TOF profile showng difference in peptide 4707 Da. The CTRL, LPS and average spectra were shown in red, green and grey respectively.

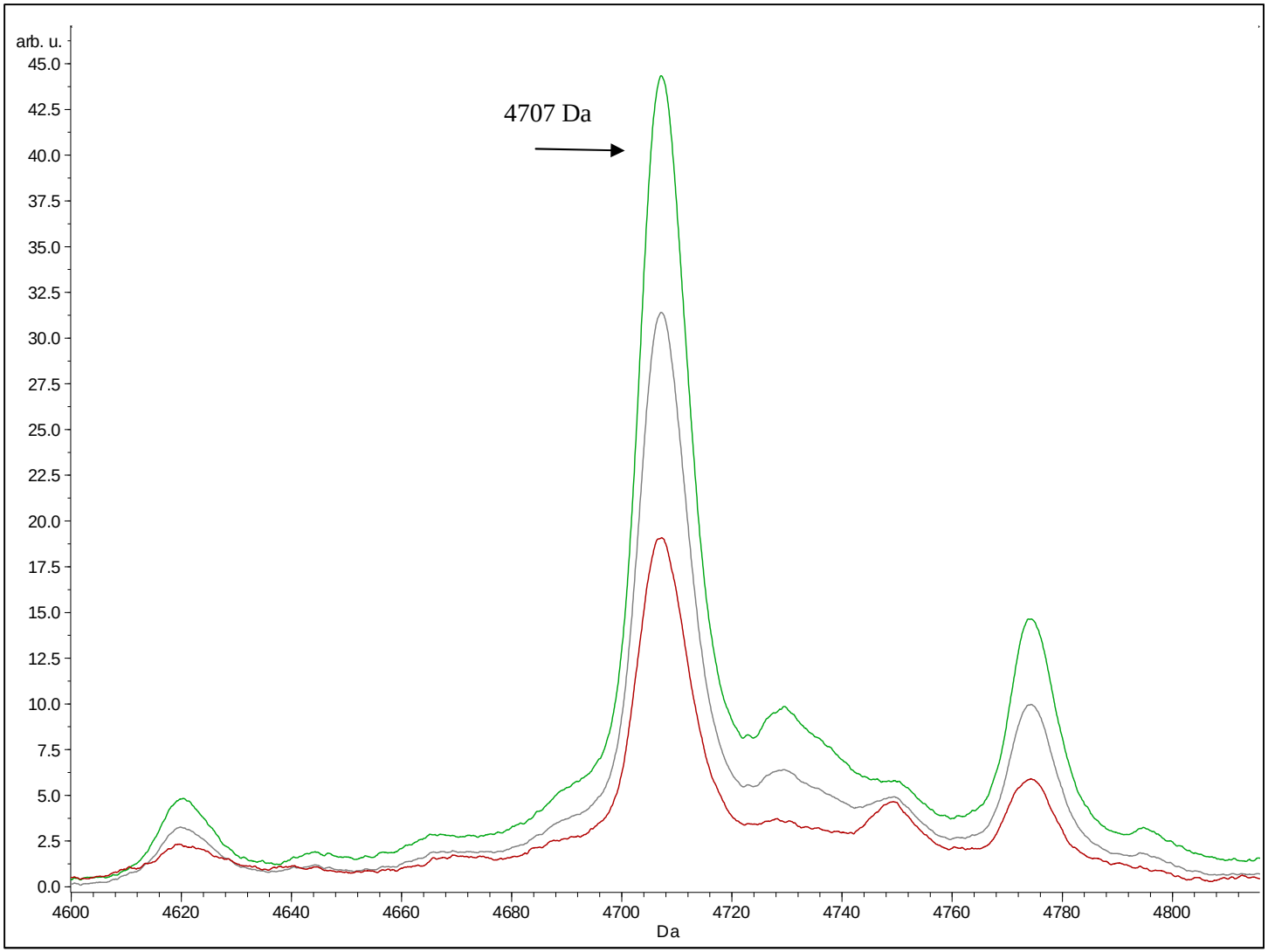

Figure 3. Comparison of MALDI-TOF profile showing difference in peptide 8108 Da. The CTRL, LPS and average spectra were shown in red, green and grey respectively.

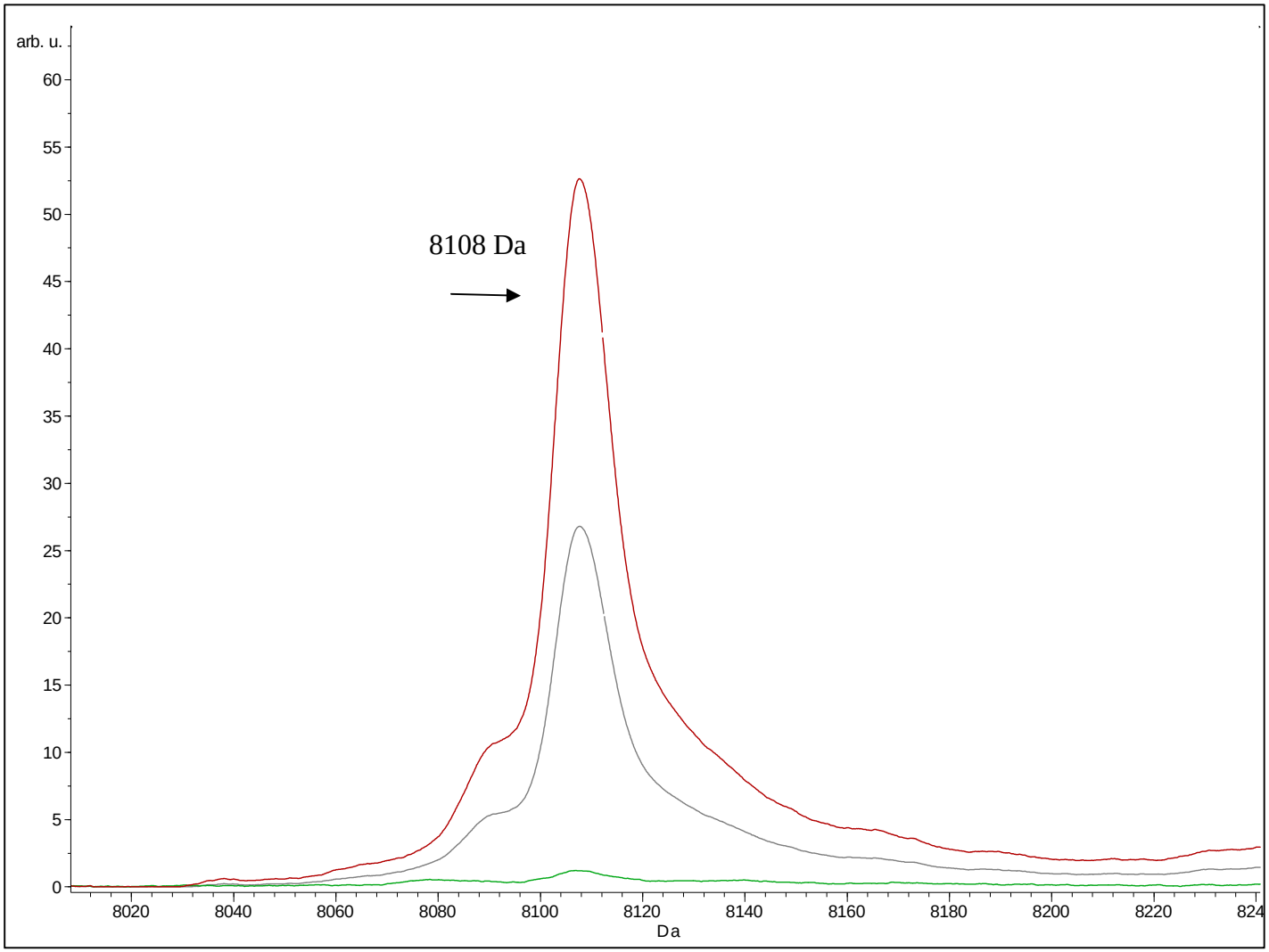

Figure 4. Comparison of MALDI-TOF profile showing difference in peptide MW 3-4 kDa region. The CTRL, LPS and average spectra were shown in red, green and grey respectively.

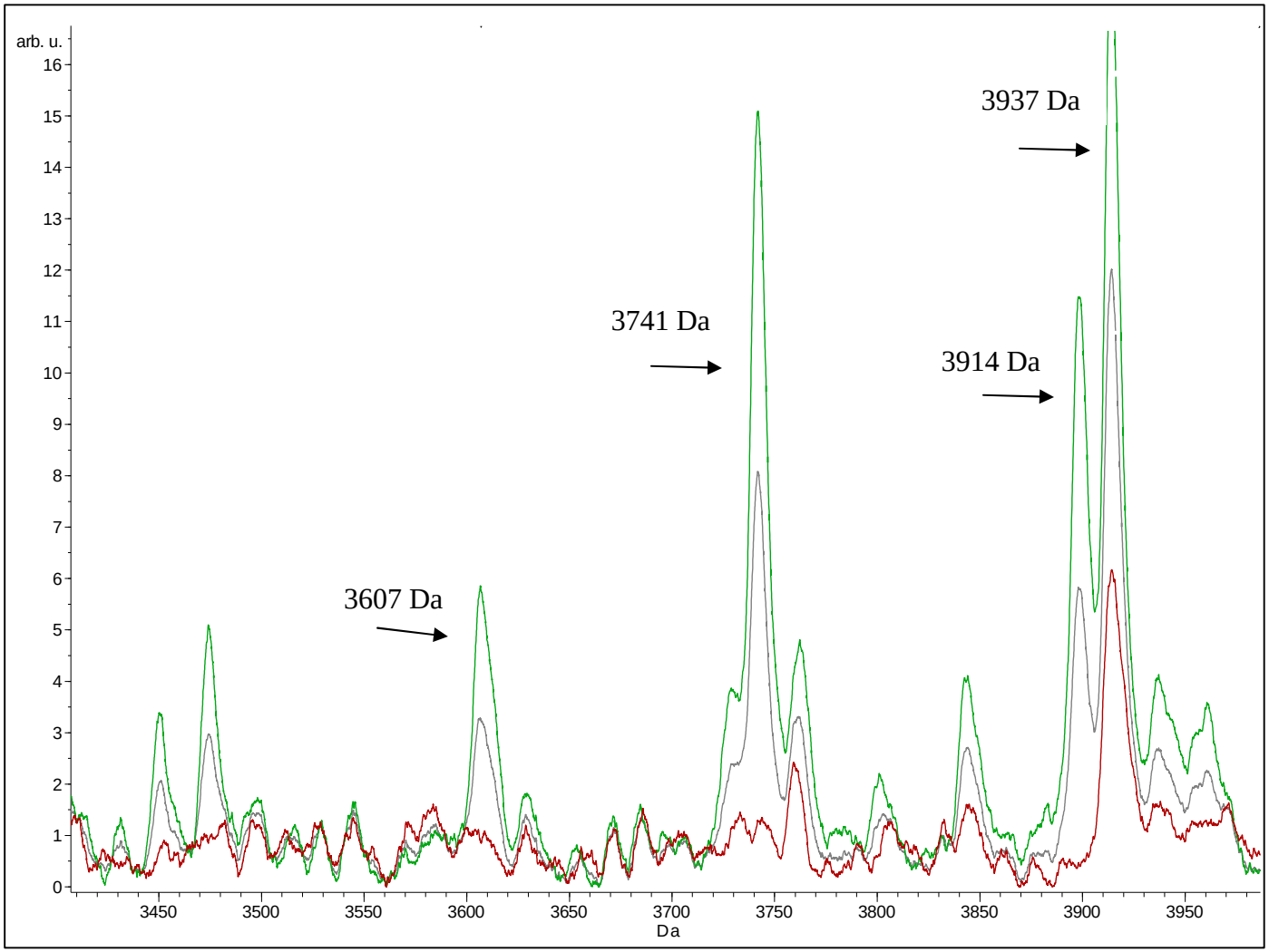

Figure 5. Comparison of MALDI-TOF profile showing difference in peptide MW 8-9 kDa region. The CTRL, LPS and average spectra were shown in red, green and grey respectively.

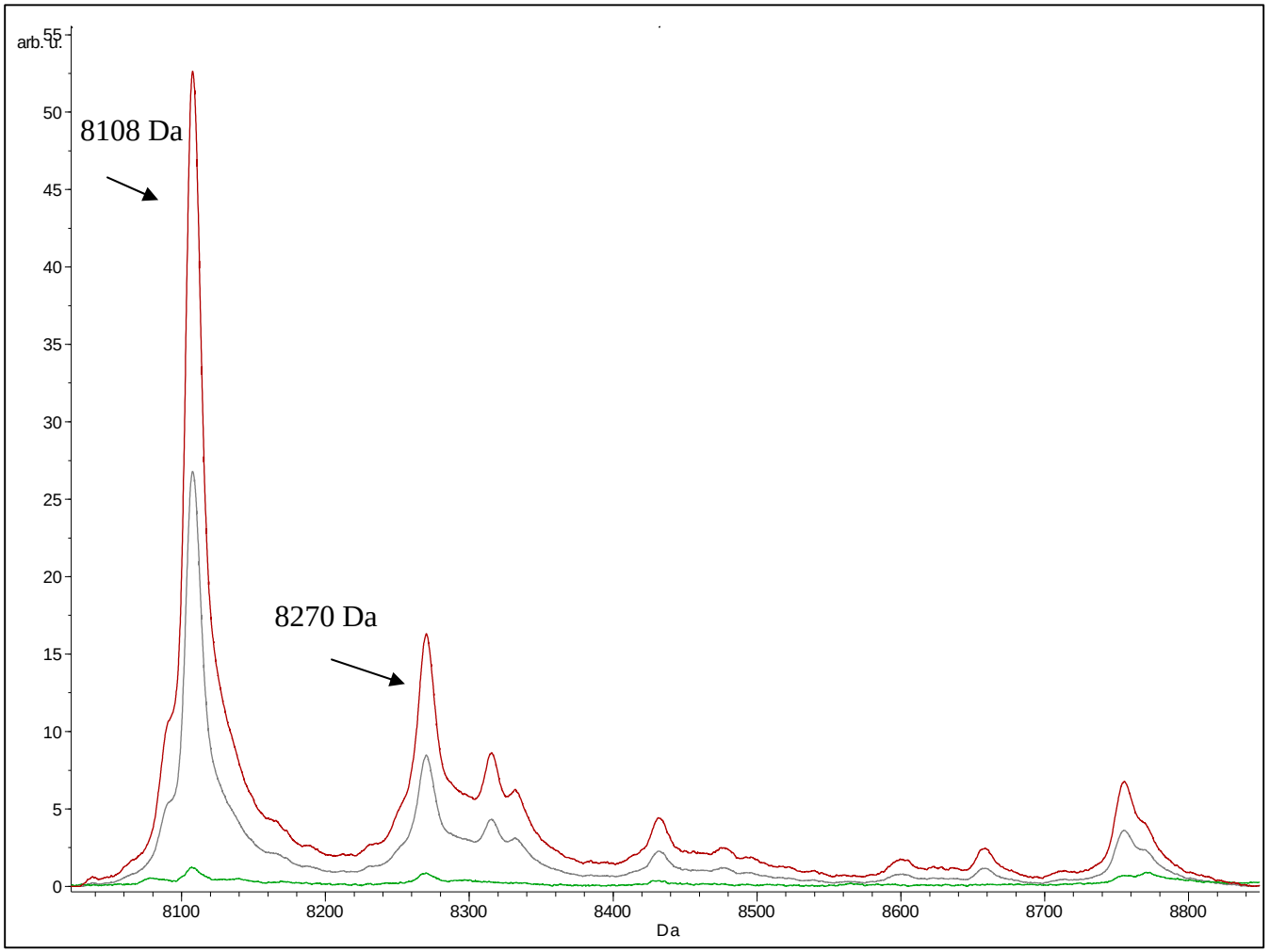

Figure 6. Venn diagram showing number of common and unique proteins in CTRL and LPS samples

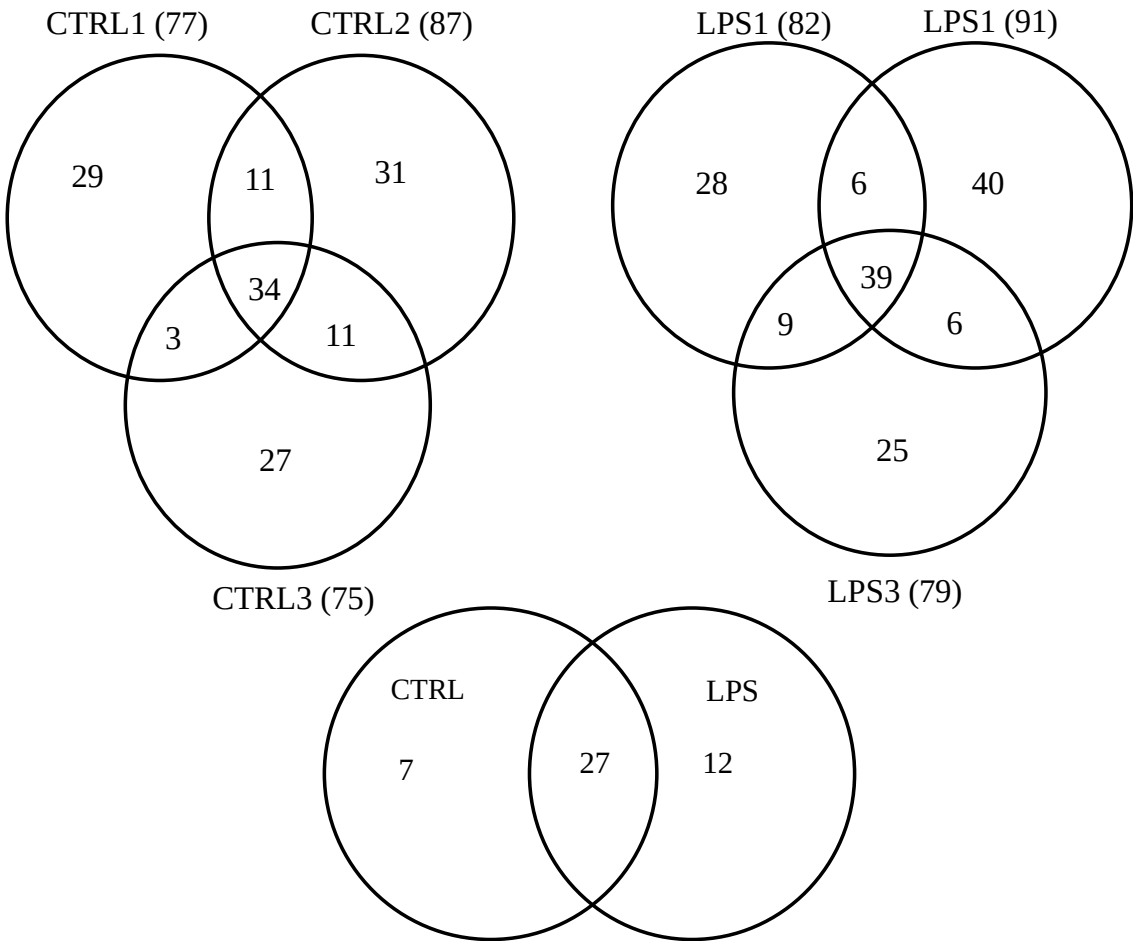

Figure 7. Volcano plot showing the differentially expressed proteins in LPS with respect to CTRL group based on Skyline-MSstat analysis.

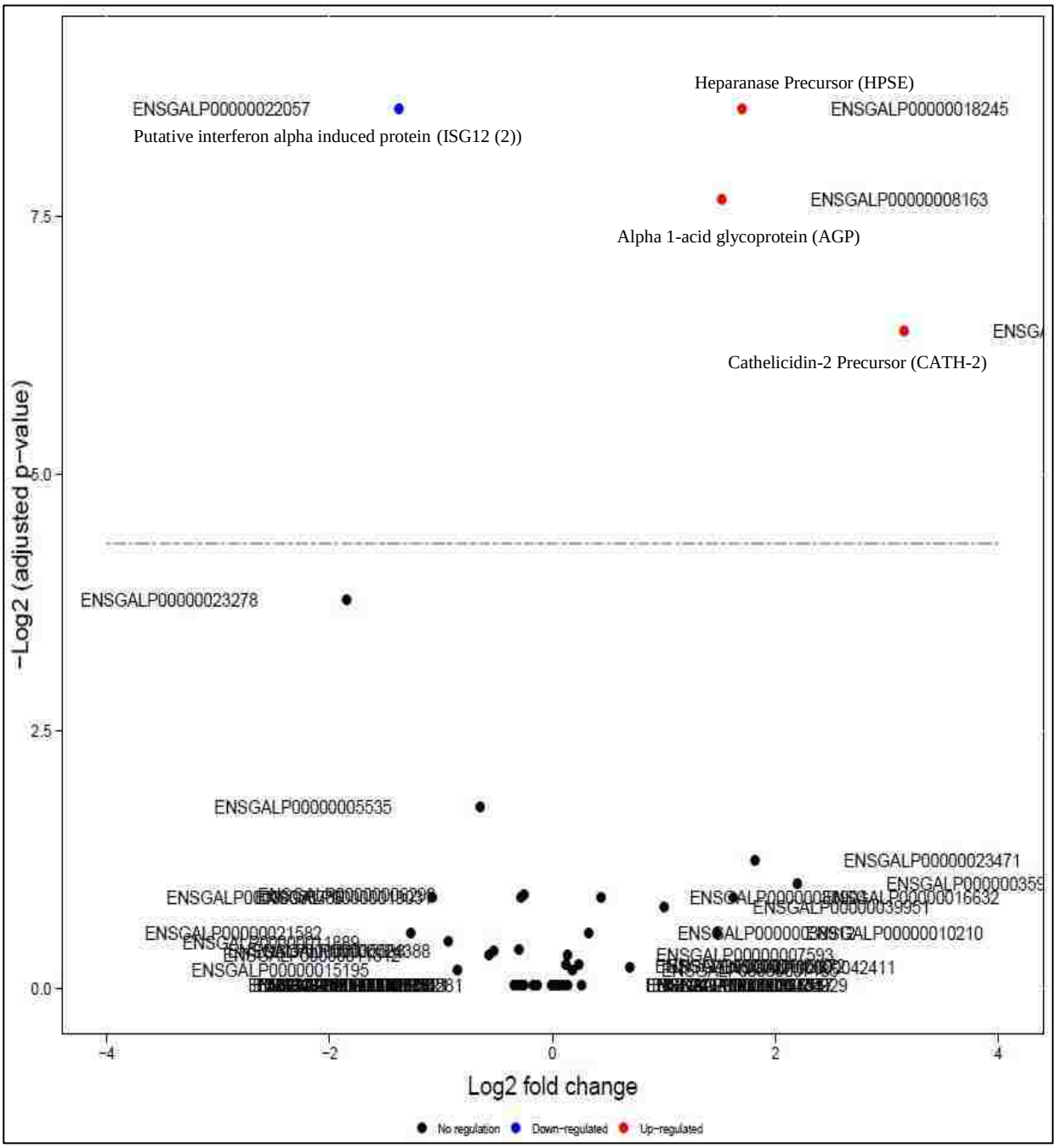

Figure 8. DAVID based protein-function association of cluster 3 (Table 3) in CTRL Green and black represents gene-funtion assocation positively reported and not reported yet respectively

Figure 9. DAVID based protein-function association of cluster 4 (Table 4) in LPS . Green and black represents gene-funtion assocation positively reported and not reported yet respectively

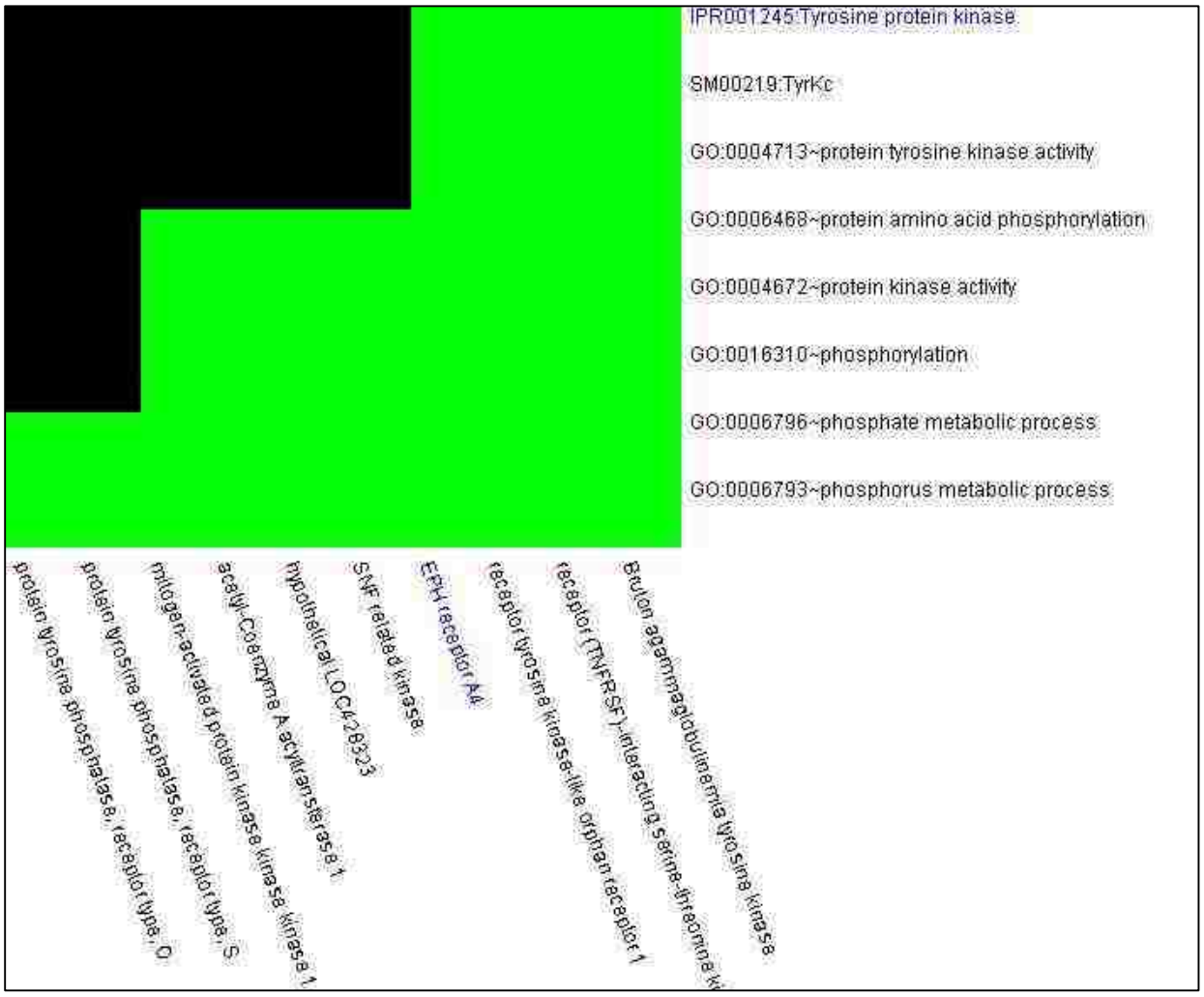

Table 1. List of peptides differntially expressed in CTRL and LPS based on ClinPro tools analysis

Mass	DAve	PTTA	PWKW	PAD	Ave1	Ave2	StdDev1	StdDev2	CV1	CV2
2494.62	1.05	0.129	0.259	0.125	9.38	8.33	2.92	2.22	31.13	26.61
2503.63	1.33	0.018	0.0236	0.016	8.65	7.32	2.28	1.95	26.33	26.62
2517.16	0.57	0.37	0.28	0.0825	8.95	8.38	2.49	2.37	27.79	28.31
2531.24	0.85	0.15	0.103	0.00333	7.83	6.98	2.27	2.22	28.96	31.85
2538.62	0.45	0.474	0.259	0.0803	6.55	7	2.76	2.09	42.11	29.86
2548.08	0.71	0.15	0.0625	0.00333	6.34	7.05	1.88	1.85	29.64	26.27
3389.8	0.58	0.175	0.259	0.418	4.75	5.33	1.75	1.51	36.78	28.27
3474.37	3.69	< 0.000001	< 0.000001	4.05E-06	2.64	6.33	0.51	1.13	19.16	17.85
3607	4.53	< 0.000001	< 0.000001	< 0.000001	2.57	7.1	0.55	0.99	21.52	13.95
3741.96	13.56	< 0.000001	< 0.000001	< 0.000001	2.83	16.39	0.41	3.12	14.66	19.02
3761.19	2.63	< 0.000001	< 0.000001	0.00838	3.48	6.11	0.78	0.85	22.53	14
3843.9	2.58	< 0.000001	< 0.000001	1.46E-05	2.92	5.5	0.59	1.39	20.09	25.38
3898.61	10.66	< 0.000001	< 0.000001	< 0.000001	2.02	12.68	0.51	2.49	24.99	19.65
3914.18	12.05	< 0.000001	< 0.000001	2.69E-06	7.26	19.31	2.37	6.58	32.61	34.09

3937.05	2.53	< 0.000001	< 0.000001	< 0.000001	2.86	5.39	0.5	1.75	17.51	32.47
3960.56	2.1	< 0.000001	< 0.000001	0.0607	2.87	4.97	0.77	1.05	26.68	21.14
4075.85	0.85	0.000012	1.31E-05	0.0613	2.91	3.76	0.48	0.82	16.35	21.91
4141.15	2.64	< 0.000001	< 0.000001	0.00223	3.28	5.92	0.54	1.16	16.49	19.67
4620.45	2.62	< 0.000001	< 0.000001	0.022	3.18	5.8	1.26	1.29	39.61	22.21
4669.71	1.58	< 0.000001	< 0.000001	0.0544	2.47	4.06	0.9	0.87	36.57	21.54
4707.33	25.48	< 0.000001	< 0.000001	0.00684	19.85	45.32	11.94	10.85	60.19	23.94
4729.03	5.88	< 0.000001	< 0.000001	0.0908	5.03	10.91	1.4	2.34	27.92	21.44
4748.23	1.85	7.1E-06	0.000029	0.0607	5.46	7.32	1.54	1.38	28.28	18.82
4774.34	8.88	< 0.000001	< 0.000001	0.11	6.77	15.65	3.19	3.53	47.1	22.58
4949.52	1.61	7.12E-05	0.00058	0.0153	3.21	4.81	1.35	1.57	42.25	32.59
4962.19	3.64	< 0.000001	< 0.000001	0.242	4.06	7.7	2.36	1.94	58.13	25.24
5109.12	4.2	< 0.000001	< 0.000001	< 0.000001	4.42	8.62	0.78	2.87	17.55	33.36
5122.15	2.27	< 0.000001	< 0.000001	0.0279	3.38	5.65	1.26	0.93	37.27	16.37
5168	0.64	2.02E-05	0.000156	0.0141	2.41	3.05	0.31	0.66	12.97	21.56
5188.11	0.25	0.019	0.0408	0.125	2.43	2.18	0.48	0.31	19.69	14.24

5210.72	0.68	< 0.000001	< 0.000001	0.0476	3.06	2.38	0.36	0.51	11.69	21.47
5236.29	1.33	2.02E-05	0.00127	< 0.000001	2.31	3.64	0.36	1.41	15.42	38.86
5265.21	1.01	0.000051	0.000282	0.000015	2.67	3.68	0.55	1.11	20.58	30.04
5308.55	1.24	< 0.000001	1.54E-06	0.178	3.91	5.15	0.78	0.85	19.96	16.47
5352.38	8.06	< 0.000001	< 0.000001	< 0.000001	6.17	14.23	2.18	6.72	35.3	47.26
5458.51	2.4	< 0.000001	1.62E-06	< 0.000001	1.39	3.79	0.23	2.06	16.87	54.25
5528.3	3.16	< 0.000001	< 0.000001	< 0.000001	1.19	4.35	0.33	2.28	27.89	52.39
5656.76	0.85	< 0.000001	< 0.000001	0.00361	2.22	1.37	0.53	0.25	23.82	17.92
6027.21	0.99	< 0.000001	< 0.000001	0.376	1.45	2.45	0.4	0.52	27.58	21.26
6041.54	0.85	< 0.000001	< 0.000001	0.00116	1.31	2.16	0.4	0.58	30.1	27.05
6073.99	2.01	3.76E-06	< 0.000001	< 0.000001	2.19	4.2	0.47	1.91	21.65	45.53
6096.59	0.13	0.452	0.0625	0.00459	2.36	2.22	0.38	0.87	16.26	39.31
6256.39	0.73	< 0.000001	< 0.000001	0.0166	1.87	1.14	0.34	0.23	18.09	20.55
7402.11	0.97	1.17E-06	< 0.000001	< 0.000001	1.93	0.96	0.88	0.16	45.76	16.94
7529.46	0.46	< 0.000001	< 0.000001	< 0.000001	1.32	0.87	0.39	0.14	29.9	15.62
8108.2	51.5	3.81E-05	< 0.000001	< 0.000001	53.28	1.78	59.37	0.73	111.42	40.92

8212.72	2.07	1.09E-05	< 0.000001	< 0.000001	2.77	0.7	2.17	0.17	78.58	24.47
8270.29	15.45	< 0.000001	< 0.000001	< 0.000001	16.85	1.4	9.19	0.55	54.5	39.55
8315.33	8.29	5.59E-06	< 0.000001	< 0.000001	9.21	0.92	8.32	0.23	90.36	24.79
8432.17	4.08	< 0.000001	< 0.000001	< 0.000001	5.02	0.95	0.66	0.18	13.14	19.35
8475.99	2.38	< 0.000001	< 0.000001	< 0.000001	3.1	0.73	1.07	0.15	34.53	21.22
8600.31	1.73	< 0.000001	< 0.000001	< 0.000001	2.5	0.77	0.79	0.17	31.39	21.78
8623.55	1.23	< 0.000001	< 0.000001	< 0.000001	1.91	0.67	0.76	0.15	39.81	22.69
8658.35	2.31	0.000017	< 0.000001	< 0.000001	3.08	0.77	2.51	0.15	81.36	19.99
8715.51	0.96	< 0.000001	< 0.000001	< 0.000001	1.7	0.74	0.68	0.14	40.27	19.01
8756.04	5.91	0.000115	< 0.000001	< 0.000001	7.41	1.51	7.42	0.44	100.08	29.24
8913.13	0.49	0.000728	0.00926	< 0.000001	1.37	0.88	0.72	0.16	52.3	18.54
9074.49	0.57	< 0.000001	< 0.000001	< 0.000001	1.3	0.72	0.41	0.12	31.71	16.69
9463.51	0.31	< 0.000001	< 0.000001	0.152	0.95	0.64	0.17	0.14	18.18	21.84
9965.23	0.27	0.000922	0.00906	3.88E-06	0.92	1.18	0.36	0.22	38.88	18.38
10219.52	9.42	0.0163	0.247	< 0.000001	27.93	37.34	18.8	8.6	67.3	23.03
10316.93	0.14	0.754	0.272	0.00684	6.67	6.54	2.2	1.15	32.92	17.6

10382.72	1.26	0.179	0.149	0.245	12.62	11.36	4.53	2.32	35.92	20.4
10427.18	6.78	< 0.000001	< 0.000001	0.218	16.3	9.52	2.87	1.85	17.61	19.4
10526.81	3.38	< 0.000001	< 0.000001	0.00361	7.1	3.72	0.91	0.71	12.88	19.19
10567.23	1.02	0.00981	0.0192	0.251	5.68	4.65	1.8	1.06	31.75	22.84
10771.37	0.64	< 0.000001	< 0.000001	0.0107	1.99	1.35	0.4	0.22	20.08	16.02
11047.91	0.3	6.93E-06	2.24E-06	1.58E-06	0.81	0.51	0.3	0.12	36.85	22.72

Mass – *m/z* value.

DAve – Difference between the maximal and the minimal average peak area/ intensity of all classes.

PTTA -- P-value of t-test OR ANOVA

PWKW -- P-value of Wilcoxon test OR Kruskal-Wallis test

PAD -- P-value of Anderson-Darling test

Ave*N* -- Peak area/intensity average of class *N* Where 1=CTRL and 2= LPS.

StdDev*N* -- Standard deviation of the peak area/intensity average of class *N*.

Table 2. List of differentially expressed proteins and their possible relevance in APR

Name (common short name)	**Uniport ID**	**Change**	**Physiological function**	**Roles in APR**
Cathelicidin-2 Precursor (CATH-2)	Q2IAL7	+	Immune response	Antimicrobial peptide
Alpha 1-acid glycoprotein (AGP)	A7UEB0	+	Lipid carrier	Carrier, biomarker of APR
Heparanase Precursor (HPSE)	F1NYI9	+	Angiogenesis, wound healing	Repair function
Interferon alpha induced protein (ISG12 (2))	Q6IEC5	-	(Uncharacterized) possibly interferon signaling	Immune system regulation

Table 3. DAVID enrichment analysis of all the protein present in CTRL samples

Annotation Cluster 1	Enrichment Score: 7.74			Count	P_Value
GOTERM_MF_FAT	extracellular matrix structural constituent	RT		8	3.0E-11
GOTERM_CC_FAT	collagen	RT		8	8.6E-11
GOTERM_CC_FAT	extracellular matrix part	RT		10	2.6E-10
GOTERM_CC_FAT	extracellular region	RT		23	5.9E-10
KEGG_PATHWAY	ECM-receptor interaction	RT		10	6.4E-10
INTERPRO	Collagen triple helix repeat	RT		8	3.4E-9
SP_PIR_KEYWORDS	collagen	RT		8	6.7E-9
KEGG_PATHWAY	Focal adhesion	RT		12	7.7E-9
GOTERM_CC_FAT	extracellular region part	RT		17	8.1E-9
GOTERM_CC_FAT	proteinaceous extracellular matrix	RT		13	1.0E-8
GOTERM_CC_FAT	extracellular matrix	RT		13	1.9E-8
GOTERM_MF_FAT	structural molecule activity	RT		12	4.2E-6
SMART	COLFI	RT		3	1.2E-3
INTERPRO	Fibrillar collagen, C-terminal	RT		3	1.2E-3
Annotation Cluster 2	**Enrichment Score: 3.57**			**Count**	**P_Value**
INTERPRO	Collagen triple helix repeat	RT		8	3.4E-9
SP_PIR_KEYWORDS	collagen	RT		8	6.7E-9
SP_PIR_KEYWORDS	hydroxylation	RT		6	1.1E-6
SP_PIR_KEYWORDS	Secreted	RT		13	1.6E-6
UP_SEQ_FEATURE	region of interest:Triple-helical region	RT		4	3.8E-5
UP_SEQ_FEATURE	signal peptide	RT		15	7.4E-5
SP_PIR_KEYWORDS	triple helix	RT		4	1.3E-4
SP_PIR_KEYWORDS	signal	RT		15	2.1E-4
SP_PIR_KEYWORDS	extracellular matrix	RT		6	6.8E-4
GOTERM_BP_FAT	cell adhesion	RT		7	3.1E-3
GOTERM_BP_FAT	biological adhesion	RT		7	3.1E-3
UP_SEQ_FEATURE	short sequence motif:Cell attachment site	RT		3	2.1E-2
SP_PIR_KEYWORDS	disulfide bond	RT		9	2.6E-2
UP_SEQ_FEATURE	disulfide bond	RT		8	5.2E-2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		9	5.9E-2
SP_PIR_KEYWORDS	coiled coil	RT		6	7.2E-2
SP_PIR_KEYWORDS	glycoprotein	RT		9	8.0E-2
Annotation Cluster 3	**Enrichment Score: 2.28**			**Count**	**P_Value**
GOTERM_MF_FAT	lipid binding	RT		7	2.3E-4
GOTERM_CC_FAT	extracellular space	RT		6	1.8E-2
GOTERM_BP_FAT	steroid metabolic process	RT		3	3.6E-2
Annotation Cluster 4	**Enrichment Score: 1.86**			**Count**	**P_Value**
INTERPRO	Beta defensin	RT		3	5.2E-3
SP_PIR_KEYWORDS	defensin	RT		3	8.1E-3
GOTERM_BP_FAT	defense response to bacterium	RT		3	9.4E-3
SP_PIR_KEYWORDS	antibiotic	RT		3	1.2E-2
GOTERM_BP_FAT	defense response	RT		4	1.4E-2
SP_PIR_KEYWORDS	Antimicrobial	RT		3	1.5E-2
GOTERM_BP_FAT	response to bacterium	RT		3	2.6E-2
UP_SEQ_FEATURE	disulfide bond	RT		8	5.2E-2
Annotation Cluster 5	**Enrichment Score: 1.51**			**Count**	**P_Value**
SMART	FN3	RT		4	2.9E-2
INTERPRO	Fibronectin, type III	RT		4	3.0E-2
INTERPRO	Fibronectin, type III-like fold	RT		4	3.3E-2

Table 4. DAVID enrichment analysis of all the protein present in LPS samples

Annotation Cluster 1	Enrichment Score: 6.05			Count	P_Value
GOTERM_CC_FAT	collagen	RT		10	6.4E-15
GOTERM_MF_FAT	extracellular matrix structural constituent	RT		10	1.3E-14
INTERPRO	Collagen triple helix repeat	RT		11	7.1E-14
GOTERM_CC_FAT	extracellular matrix part	RT		12	1.7E-13
SP_PIR_KEYWORDS	collagen	RT		11	2.1E-13
KEGG_PATHWAY	ECM-receptor interaction	RT		12	2.5E-13
GOTERM_CC_FAT	extracellular region part	RT		21	5.9E-13
GOTERM_CC_FAT	extracellular region	RT		26	7.0E-13
GOTERM_CC_FAT	proteinaceous extracellular matrix	RT		16	3.2E-12
GOTERM_CC_FAT	extracellular matrix	RT		16	7.2E-12
SP_PIR_KEYWORDS	hydroxylation	RT		8	1.3E-9
KEGG_PATHWAY	Focal adhesion	RT		12	4.4E-9
SP_PIR_KEYWORDS	triple helix	RT		6	5.7E-8
INTERPRO	Fibrillar collagen, C-terminal	RT		5	1.6E-7
SP_PIR_KEYWORDS	Secreted	RT		15	2.0E-7
SMART	COLFI	RT		5	2.8E-7
SP_PIR_KEYWORDS	extracellular matrix	RT		9	1.3E-6
GOTERM_BP_FAT	cell adhesion	RT		12	2.4E-6
GOTERM_BP_FAT	biological adhesion	RT		12	2.4E-6
UP_SEQ_FEATURE	signal peptide	RT		19	4.4E-6
GOTERM_MF_FAT	structural molecule activity	RT		13	4.5E-6
SP_PIR_KEYWORDS	signal	RT		19	5.4E-6
GOTERM_BP_FAT	collagen fibril organization	RT		4	6.5E-5
UP_SEQ_FEATURE	region of interest:Triple-helical region	RT		4	7.8E-5
SP_PIR_KEYWORDS	hydroxyproline	RT		4	1.4E-4
GOTERM_CC_FAT	fibrillar collagen	RT		3	6.4E-4
SP_PIR_KEYWORDS	disulfide bond	RT		13	6.8E-4
UP_SEQ_FEATURE	domain:Fibrillar collagen NC1	RT		3	1.1E-3
UP_SEQ_FEATURE	propeptide:C-terminal propeptide	RT		3	1.1E-3
SP_PIR_KEYWORDS	heterotrimer	RT		3	1.5E-3
SP_PIR_KEYWORDS	trimer	RT		3	1.5E-3
UP_SEQ_FEATURE	short sequence motif:Cell attachment site	RT		4	2.6E-3
GOTERM_BP_FAT	extracellular matrix organization	RT		4	3.3E-3
GOTERM_BP_FAT	extracellular structure organization	RT		4	8.5E-3
UP_SEQ_FEATURE	disulfide bond	RT		11	8.7E-3
SP_PIR_KEYWORDS	glycoprotein	RT		12	1.4E-2
SP_PIR_KEYWORDS	coiled coil	RT		8	1.4E-2
GOTERM_BP_FAT	cell-cell adhesion	RT		5	1.5E-2
GOTERM_BP_FAT	heart morphogenesis	RT		3	2.6E-2
UP_SEQ_FEATURE	glycosylation site:N-linked (GlcNAc...)	RT		11	3.9E-2
GOTERM_BP_FAT	skeletal system development	RT		4	6.3E-2
SP_PIR_KEYWORDS	cell adhesion	RT		4	6.6E-2
Annotation Cluster 2	**Enrichment Score: 2.44**			**Count**	**P_Value**
GOTERM_BP_FAT	defense response	RT		6	5.4E-4
GOTERM_BP_FAT	defense response to bacterium	RT		4	9.4E-4
SP_PIR_KEYWORDS	Antimicrobial	RT		4	1.3E-3
GOTERM_BP_FAT	response to bacterium	RT		4	4.3E-3
INTERPRO	Beta defensin	RT		3	6.7E-3
UP_SEQ_FEATURE	disulfide bond	RT		11	8.7E-3
SP_PIR_KEYWORDS	defensin	RT		3	1.1E-2
SP_PIR_KEYWORDS	antibiotic	RT		3	1.6E-2
Annotation Cluster 3	**Enrichment Score: 2.05**			**Count**	**P_Value**
INTERPRO	Fibronectin, type III	RT		5	7.3E-3
INTERPRO	Fibronectin, type III-like fold	RT		5	8.4E-3
SMART	FN3	RT		5	1.2E-2
Annotation Cluster 4	**Enrichment Score: 1.52**			**Count**	**P_Value**
GOTERM_BP_FAT	phosphorus metabolic process	RT		10	1.5E-2
GOTERM_BP_FAT	phosphate metabolic process	RT		10	1.5E-2
GOTERM_BP_FAT	protein amino acid phosphorylation	RT		8	2.3E-2
GOTERM_MF_FAT	protein kinase activity	RT		8	2.8E-2
INTERPRO	Tyrosine protein kinase	RT		4	3.5E-2
GOTERM_BP_FAT	phosphorylation	RT		8	4.7E-2
SMART	TyrKc	RT		4	5.2E-2
GOTERM_MF_FAT	protein tyrosine kinase activity	RT		4	5.6E-2

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VI. Isolation and characterization of chicken bile matrix metalloproteinase

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ABSTRACT

Avian bile is rich in matrix metalloproteinases **(MMP)**, the enzymes that cleave extracellular matrix **(ECM)** proteins such as collagens and proteoglycans. Changes in bile MMP expression have been correlated with hepatic and gall bladder pathologies but the significance of their expression in normal, healthy bile is not understood. We hypothesized that the MMP in bile may aid the digestion of native collagens that are resistant to conventional gastric proteases. Hence, the objective of this study was to characterize the bile MMP and check its regulation in association with dietary factors. We used substrate zymography, azocoll protease assay, and gelatin affinity chromatography to identify, and purify the MMP from chicken bile. Using zymography and SDS PAGE, 5 bands at 70, 64, 58, 50, and 42 kDa were detected. The bands corresponding to 64, 50 and 42 kDa were identified as MMP2 using trypsin in-gel digestion and matrix-assisted laser desorption time-of-flight mass spectrometry **(MALDI-TOF-MS)** and peptide mass fingerprinting. Chickens fed diets containing gelatin supplements showed higher levels of MMP expression in the bile by both azocoll assay and zymography. We conclude that the bile MMP may be associated with the digestion of collagens and other ECM proteins in avian diets.

Key words: bile; collagen digestion; matrix metalloproteinase-2; mass spectrometry

INTRODUCTION

Bile is the hepatic fluid consisting of a complex mixture of bile acids, cholesterol derivatives, heme derived pigments, mucins, enzymes, and protein breakdown products (Trauner and Boyer, 2003). It is also responsible for the emulsification and digestion of fat in the intestine (Hornbuckle, 1997; Tuchweber, et al., 1996). Previously we showed that normal turkey bile contained substantial gelatinolytic activities, which belonged to the matrix metalloproteinase (**MMP**) family of enzymes (Rath, et al., 2001). The MMP are zinc dependent endopeptidases which degrade extracellular matrix (**ECM**) and non-ECM proteins during tissue remodeling (Iyer, et al., 2012; Nagase, et al., 2006). The elevation of bile MMP has been generally linked to hepatic pathologies involving fibrosis, cancer, bile duct, and gall bladder diseases in humans, and other mammalian models (Consolo, et al., 2009; Hirashita, et al., 2012; Kirimlioğlu, et al., 2009; Okada, et al., 2001; Syed, et al., 2012). A recent study in fish showed that the elevation of their bile MMP levels was associated with aquatic pollution (Hauser-Davis, et al., 2012). The significance of MMP in bile fluid is not fully understood. We hypothesized that the bile MMP may serve to denature and digest native ECM proteins such as collagens. The ECM proteins constitute major parts of animal connective tissue that are part of the natural diets of omnivorous birds (Duke, 1997; Hauser-Davis, et al., 2012; Klasing, 1998). Because, the native interstitial collagens can be resistant to degradation by conventional digestive proteases, the MMP in bile could likely aid their denaturation and subsequent digestion (Bornstein, et al., 1966; Chung, et al., 2004; Etherington, 1977). However, the exact nature of avian bile MMP is not known, because in aves as in mammals, there are several classes of MMP with different substrate specificities (Nagase and Visse, 2009; Sekhon, 2010; Snoek-van Beurden and Von den Hoff,

2005). Therefore, the objective of this study was to isolate and characterize the bile MMP and find whether certain feed additives can modulate their activities and digestive function.

MATERIALS AND METHODS

Chemical and reagents

Azocoll™ (mesh >50, EMD Millipore), BCA protein assay reagent (Pierce, IL), calf skin soluble type I collagen (Worthington Biochemical Corporation, NJ), dithiothreitol (**DTT**) (Omnipur, Canada), GM 6001 (Ilomastat, collagenase inhibitor) and E-64 (N-[N-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine, a cysteine protease inhibitor) (Calbiochem,CA), iodoacetamide (ICN Biomedicals, Inc., OH), Omix Tips C18 (Varian, CA), Page ruler™ protein molecular weight markers (ThermoScientific, IL), Spectra/Por® Molecular Weight Cut-off (**MWCO**) 3500 dialysis membrane (Spectrum Medical Industries Inc., CA), gelatin sepharose™ 4B (gehealthcare.com), SilverQuest™ silver staining kit (Invitrogen, Carlsbad, CA), trypsin gold, mass spectrometry grade (Promega, Madison, WI), chromatography mini column (VWR.com), Ultrafree®-MC 10000 NMWL filter unit (Millipore, MA) were purchased from their respective vendors. The poultry diets were made with NRC specifications (NRC, 1994). The feed supplements were obtained from local suppliers except for the beef gelatin which was purchased from luckyvitamin.com. All other chemicals and reagents including porcine skin gelatin type A, 4-amino-phenyl-mercuric acetate (**APMA**), phenylmethyl sulfonyl fluoride (**PMSF**), leupeptin, and **HCCA** (α-cyano-4-hydroxycinnamic acid) were obtained from Sigma-Aldrich chemical Company (St. Louis, MO).

Bile collection

The animal protocols were approved by the Institutional Animal Usage and Care Committee (IACUC), University of Arkansas. Bile was obtained from euthanized chickens using sterile syringes, centrifuged at 21,000*g* for 20 minutes at 4°C, and the supernatant stored at -20°C until further analyses. Protein content was determined by micro BCA method and A_{280} as necessary.

Gelatin and collagen zymography

Zymography was performed using 10% polyacrylamide gels (**PAGE**) containing 0.1% of porcine gelatin or 0.08% type I skin collagen as described previously (Rath, et al., 2001). Intact bile or the gelatin-sepharose affinity purified bile proteins were mixed with non-reducing Laemmli sample buffer and electrophoresed at constant voltage of 100 V in a Novex gel apparatus (Invitrogen, CA). The gels were washed twice with 2.5% triton X-100 for 15 minutes each, and incubated in a buffer consisting of 50 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, and 0.05% Brij-35, pH 7.6 (incubation buffer, **IB**) for 5-8 h at 37°C. The gels were stained with Coomassie brilliant blue R250 to visualize gelatinolytic and collagenlytic bands. **SDS-PAGE** gels were stained with SilverQuest^{TM} silver staining kit and documented using Gel Logic GL2200 (Carestream Health, Inc. NY). The approximate molecular weight (**MW**) of the bands, and their intensities were determined using the Gel Logic system. The MMP activities of samples were expressed as sum of their band intensities per microgram of protein. To test for the activation of enzyme, the bile samples were incubated with 1mM APMA for 30-60 min at 37°C.

Azocoll assay

MMP activity was determined using an azocoll method of Jiang et al., (2007) with modifications. Ten µL of bile sample containing 5-10 µg equivalent protein were added to 190 µL of azocoll

suspension (3 mg/ml of **IB**) and incubated with or without any inhibitor at 37°C for 15 h. The blanks consisted of azocoll reaction mixture to which bile was added after incubation. The tubes were spun for 5 min at 2,000 *g*, transferred to 96 well microtiterplates, and the color was read at 520 nm. Each sample was assayed in triplicate. Different protease inhibitors, 20 mM EDTA (divalent ion chelator and MMP inhibitor), 1 mM PMSF (serine protease inhibitor), 20 µM E64 (cysteine protease inhibitor), 10 µM leupeptin (cysteine, serine, threonine protease inhibitor), and 20 µM GM 6001 (MMP inhibitor), were used to identify the nature of the bile proteases. The percentages of enzyme activities were calculated with respect to controls.

Affinity purification of bile MMP

Pooled samples of chicken bile were dialyzed against IB using 3500 MWCO Sectra/por membranes with 3 successive changes. Affinity purification was performed on a gelatin-sepharose column following a procedure of Zhang and Gottschall (1997). Briefly, one ml of gelatin sepharose beads was loaded in a mini column, equilibrated with IB twice and the dialyzed bile was added to cover the bed volume, and incubated for 4 h at 4°C. Unbound materials were eliminated with three successive washing with IB. The bound materials were eluted with 1ml of IB containing 10% dimethyl sulfoxide. The eluants were concentrated using 10,000 MWCO Ultrafree-MC Millipore filter. The protein content of the retentate was determined by A_{280} and subjected to PAGE and gelatin zymography. The zymogram gels were incubated for 3 h.

In-gel digestion

The silver stained protein bands were excised using a spot picker (The Gel Company, CA), transferred to sterile tubes, destained and washed with water twice for 15 minutes. The gel pieces were then washed for 1 h sequentially with 25 mM ammonium bicarbonate **(NH_4HCO_3)** and

50% acetonitrile (**ACN**) in 50 mM, and vacuum dried. The dried gel pieces were subjected to reduction with 10 mM DTT at 56°C for 1h followed by alkylation with 55 mM iodoacetamide for 1 h in dark at room temperature. Trypsin (20 ng/µL) was added in volume enough to hydrate the gel pieces in ice for 20 minutes followed by the addition of 50 µL of 25 mM NH_4HCO_3 before overnight incubation at 37°C. Gel pieces without any protein were treated identically as control to subtract artifact associated peaks. The digested peptides were eluted from gels with 5% formic acid (**FA**) in 50% ACN for 15 minutes twice in an ultrasonic water batch (Branson 3200, Bransonic® ultrasonic cleaner). The extracted peptides were concentrated using a Speedvac vacuum concentrator and desalted using OMIX C18 tips. Aliquots of desalted solutions were mixed with equal volumes of saturated HCCA prepared in 1:1 ratio of water and ACN containing 0.1% FA then spotted on a Bruker MTP384 target plate to identify the peptides using MALDI-TOF-MS (Hellman, et al., 1995; Rosenfeld, et al., 1992).

Mass spectrometry

Mass spectra were obtained in reflector positive ion mode using a Bruker Daltonics Ultraflex II MALDI-TOF/TOF mass spectrometer. The background peaks present in trypsin treated control gel pieces were removed and the MALDI peptide mass fingerprint (**PMF**) was subjected to tandem mass spectrometry (MS/MS) using MALDI LIFT-TOF/TOF. Bruker Biotools 3.1 was used to combine PMF and LIFT-MS/MS data and searched against NCBI non-redundant *Gallus gallus* database using the MASCOT 2.2 search engine to identify the protein(s). Single missed cleavage, fixed carbamidomethylation of cysteine, variable methionine oxidation, 100 ppm error at MS level, and 0.5 Da error at MS/MS level were used during the data base search.

Effect of dietary additives on bile MMP

Fifty male broiler chickens from a local hatchery were randomly assigned to five groups and received feed according to NRC specifications (NRC, 1994) with or without specified supplements, and *ad libitum* water. The control birds received normal diet while the rest of the groups received supplements consisting 4% of either, beef gelatin, skimmed milk powder, rice powder, or lard from day 1 through 43. Feed was withdrawn overnight before euthanasia to retain bile in the gall bladder. The birds were weighed before killing by carbon dioxide asphyxiation, and the bile was collected from the gall bladder as described earlier. The protein content of bile was determined by micro BCA method. The MMP activity was estimated by azocoll assay and zymogram densitometry. The azocoll assay was performed in triplicate using 5 bile samples per group, incubated for 10 h. The groups were compared using the optical density of the dye released per µg of protein. In densitometry, the activities were calculated as gelatinolytic intensities per µg of protein.

Cathepsin activity of bile

To demonstrate that the gelatinolytic activities were not related to cathepsin, the bile samples were electrophoresed in duplicate using a gelatin containing gel and divided into 2 halves to develop zymogram. One half of the gel was incubated in MMP IB and the other half in a cathepsin incubation buffer **(CIB)** (100mM Na phosphate, 1mM EDTA, and 2mM DTT, pH 5.5). Gels were equilibrated with CIB for 30 minutes, replaced with fresh buffer, and incubated for 5 h at 37°C (Wilder, et al., 2011). The cathepsin and MMP zymogram were visually compared.

Statistics

The results from quantitative assays such as the effect of various inhibitors on azocoll protease activity, and the densitometry were presented as mean ± standard error (SEM). SAS software (SAS Institute Inc. NC) was used to perform a one-way ANOVA and Duncan's t test. A *P* value of ≤ 0.05 was considered to be significant.

RESULTS

Zymography

Gelatin zymography showed 5 gelatinolytic bands corresponding to approximate MW of 70, 64, 58, 50, and 42 kDa respectively (Figure 1a) whereas the collagen zymography showed only 4 bands. Due to differential mobility of MW standards in collagen zymography, an approximate alignment with gelatin gel, showed only 4 bands corresponding to 70, 64, 58, and 42 kDa respectively (Figure 1b). Incubation with APMA for 30 or 60 minutes resulted in similar profiles showing 2 major bands corresponding to 64 and 42 kDa (Figure 2).

Effect of inhibitors on Azocoll protease activity

Azocoll proteolysis was inhibited by both MMP inhibitors, EDTA and GM 6001 but not by serine/cysteine protease inhibitors such as E64, leupeptin, and PMSF (Figure 3). Cathepsin activity was not evident in the samples analyzed (data not shown).

Affinity purification and molecular characterization of MMP

The bands corresponding to 64, 50 and 42 kDa, (Figure 4a, b) were all identified as type IV collagenase preproprotein (NCBI reference sequence NP_989751.1) based on their PMF as listed in Table 1. The band at 64 kDa was identified using 20 peaks in the PMF (Figure 5) and LIFT-

TOF/TOF (MS/MS) data corresponding to five peptide peaks at *m/z* 1071, 1563, 1579, 1670, and 2107, respectively. MASCOT search of the combined PMF (MS) and LIFT-TOF/TOF (MS/MS) data showed a significant protein hit ($P \geq 0.05$, MASCOT score 253) (Matrixscience, 2013) with a sequence coverage of 29%, and matching peptides distributed from 1- 646 of the amino acid sequence. The peak at *m/*z 1670 (Figure 6) had the highest MASCOT ion score of 82 compared with the peaks at 2107, 1580, 1564, 1071 with ion scores of 57, 28, 26, and 10 respectively. The band at 50 kDa was identified using 17 peaks (Table 1) including the same five peptides with LIFT-TOF/TOF (MS/MS) data. This identification was based on a lower MASCOT score (250), with a 25% sequence coverage, peptide hits covering protein sequence from positions 1-590 (Table 1). Similarly the band at 42 kDa was identified based on a lower MASCOT score of 220, sequence coverage 26% and peptide hits distributed from 1- 492 of the amino acid sequence.

Effect of feed supplements on bile MMP activity

Chickens fed diets containing 4% gelatin showed a statistically significant increase in MMP activities based on azocoll assay (Figure 7). Both gelatin and milk powder supplements increased gelationolytic activities determined by zymogram densitometry (Figure 8).

DISCUSSION

Bile plays an important role in digestive physiology especially in the emulsification and digestion of fat (Tuchweber, et al., 1996). We hypothesized that biliary MMP perhaps aids in the digestion of native collagenous proteins, which constitute a significant fraction of body proteins of both vertebrates and invertebrates. Although collagens are assumed to be digested by gastric enzymes at low pH, the fibrous interstitial collagens are generally resistant to proteases such as pepsin, chymotrypsin, and trypsin unless denatured (Bornstein, Kang and Piez, 1966; Harkness,

et al., 1978). Cysteine cathepsins, that can digest collagens under low pH, are not known to occur in normal gastric secretions other than in some pathological conditions (Nagy, et al., 1997; Reiser, et al., 2010). The mammalian MMPs such as MMP1 and 3 but not MMP2 are capable of denaturing and cleaving interstitial, fibrillar collagens under neutral pH conditions (Fields, 2013). Our results showed that MMP protein bands in avian bile belonged to 72 kDa type IV collagenase also known as MMP2, or gelatinase A (Aimes and Quigley, 1995; Hahn-Dantona, et al., 2000; Murphy and Nagase, 2008). The lower molecular weight bands (50 and 42 kDa) appear to be the truncated version of the same MMP2 because they lacked sequence coverage in the C-terminal domain compared with 64 kDa band (Table 1). In MMP2, the signal, pro-peptide and catalytic domains are located near the N-terminus, and the hemopexin domain (HX) at the C terminus that binds substrates and tissue inhibitors of MMP (TIMP) (Morgunova, et al., 2002; Patterson, et al., 2001; Piccard, et al., 2007). Chicken MMP2 has collagenase activity because it can denature and cleave triple helical collagens to produce characteristic ¾ and ¼ fragments (Aimes and Quigley, 1995; Hahn-Dantona, Aimes and Quigley, 2000; Patterson, Atkinson, Knauper and Murphy, 2001). Hence, it is capable of denaturing and digesting fibrillar collagens.

Next we asked if MMP was critical to the digestive process of the birds, then it posits that it may be modulated by their dietary factors resulting in an increase of their digestive activities. Diet-induced adaptive modulation of digestive enzymes has been observed in birds (Brzęk, et al., 2012; Karasov and Hume, 1997; Karasov, et al., 2011). Although direct correlation of dietary protein content and digestive proteases were demonstrated in mammals, (Hara, et al., 2000) such correlation has not been established in birds. Our results with azocoll protease activity assay and densitometry of gelatinolytic bands showed that the gelatin supplemented diets increased bile MMP levels although a modest increase by milk was observed. Nonetheless, the gelatin induced

elevation of bile MMP suggests that food types may regulate adaptive modulation of their function.

Apart from collagen digestion we cannot exclude the possibility of other gastrointestinal functions of MMP such as their abilities to activate of growth factors, antimicrobial proteins, other proteases including MMPs, and several receptor proteins, which need to be explored in the context of digestive physiology (Amadesi and Bunnett, 2004; Nagase and Visse, 2009; Sengupta and MacDonald, 2007). In conclusion, our results show that chicken bile constitutively secretes type IV collagenase (MMP2) that the can be modulated by their dietary constituents, and these enzymes possibly help in the digestion of ECM proteins in their diets.

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List of abbreviations

ACN Acetonitrile

APMA: Amino phenyl mercuric acid

DTT: Dithiothreitol

E64: N-[N-(L-3-trans-carboxyirane-2-carbonyl)-L-leucyl]-agmatine

ECM: Extra cellular matrix

EDTA: Ethylenediaminetetraacetate

FA: Formic acid, GM6001 Galardin or ilomastat

HCCA: α-Cyano-4-hydroxycinnamic acid

MMP: Matrix metalloproteinase

MALDI-TOF: Matrix-Assisted Laser Desorption/Ionization-Time of Flight

PAGE: Polyacrylamide gel electrophoresis

PMF Peptide Mass Fingerprinting

PMSF: Phenylmethanesulfonyl fluoride

Figure legends

Figure 1. Substrate zymography of chicken bile using (a) gelatin and (b) collagen.

Figure 2. Gelatinolytic profiles of bile MMP with or without APMA activation. Lane 1- bile incubated without APMA for 60 minutes. Lane 2, 3, 4- Bile incubated for 0, 30 and 60 minutes with APMA.

Figure 3. Effects of different protease inhibitors on azocoll protease activity * indicates $P \leq 0.05$, (n=3).

Figure 4. Gelatin zymogram (a) and silver stained protein bands (b) of gelatin-sepharose affinity purified bile.

Figure 5. Peptide mass fingerprint (PMF) of the 64 kDa protein band, * indicates the peptide peaks were subjected to MS/MS fragmentation. Values in parenthesis correspond to the amino acid sequences of chicken MMP2.

Figure 6. Tandem mass spectrometry of *m/z* 1670.66 peptide showing *b* and *y* ions with corresponding sequence information.

Figure 7. Effect of different feed supplements on bile protease activity indicated by optical density of azocoll hydrolysis associated dye release (n=5). Dissimilar letters indicate significant statistical differences, $P \leq 0.05$.

Figure 8. Effect of different feed supplements on bile gelatinolytic activity analyzed by densitometry. Dissimilar alphabets over bars indicate significant differences ($P \leq 0.05$)

Table 1. List of peptides, observed in 64, 50 and 42 kDa bands and expected *m/z* of peptides from MMP2.

Figure 1

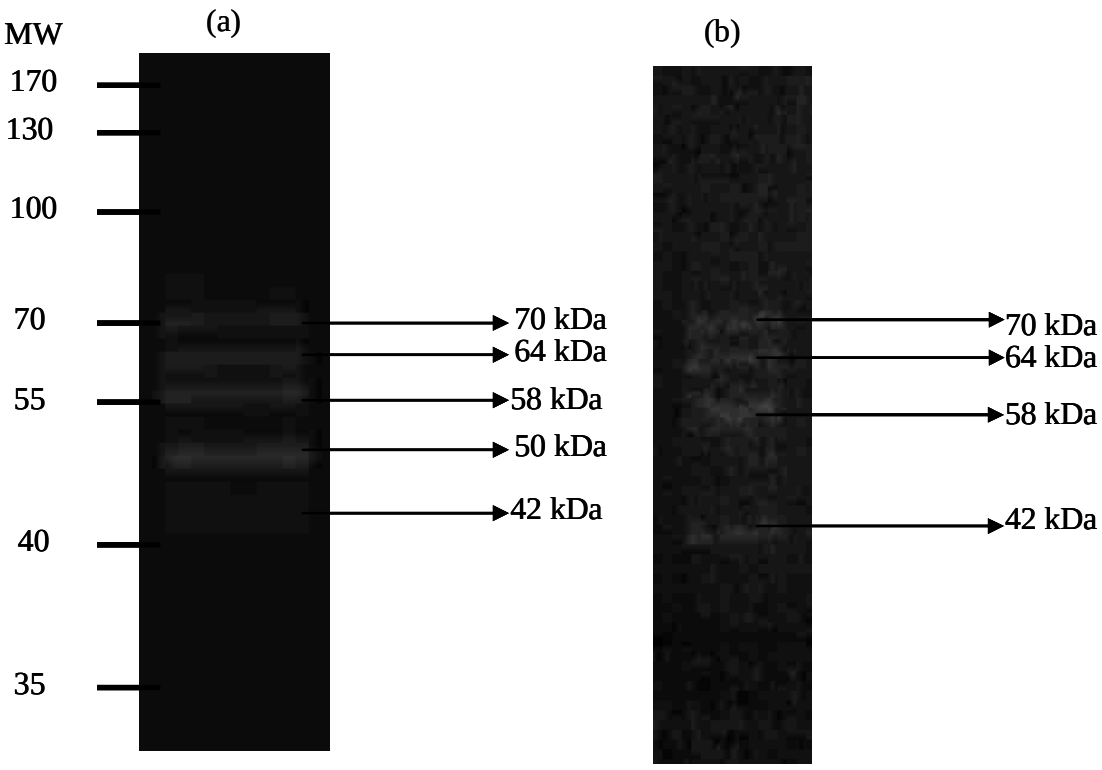
MW
(a)
(b)
170
130
100
70
55
40
35
70 kDa
64 kDa
58 kDa
50 kDa
42 kDa
70 kDa
64 kDa
58 kDa
42 kDa

Figure 2

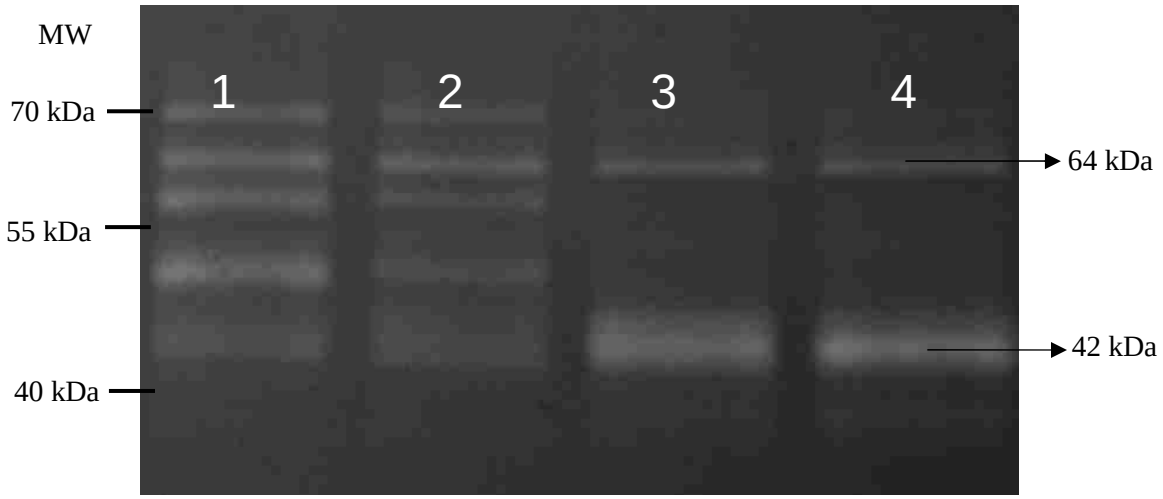

Figure 3

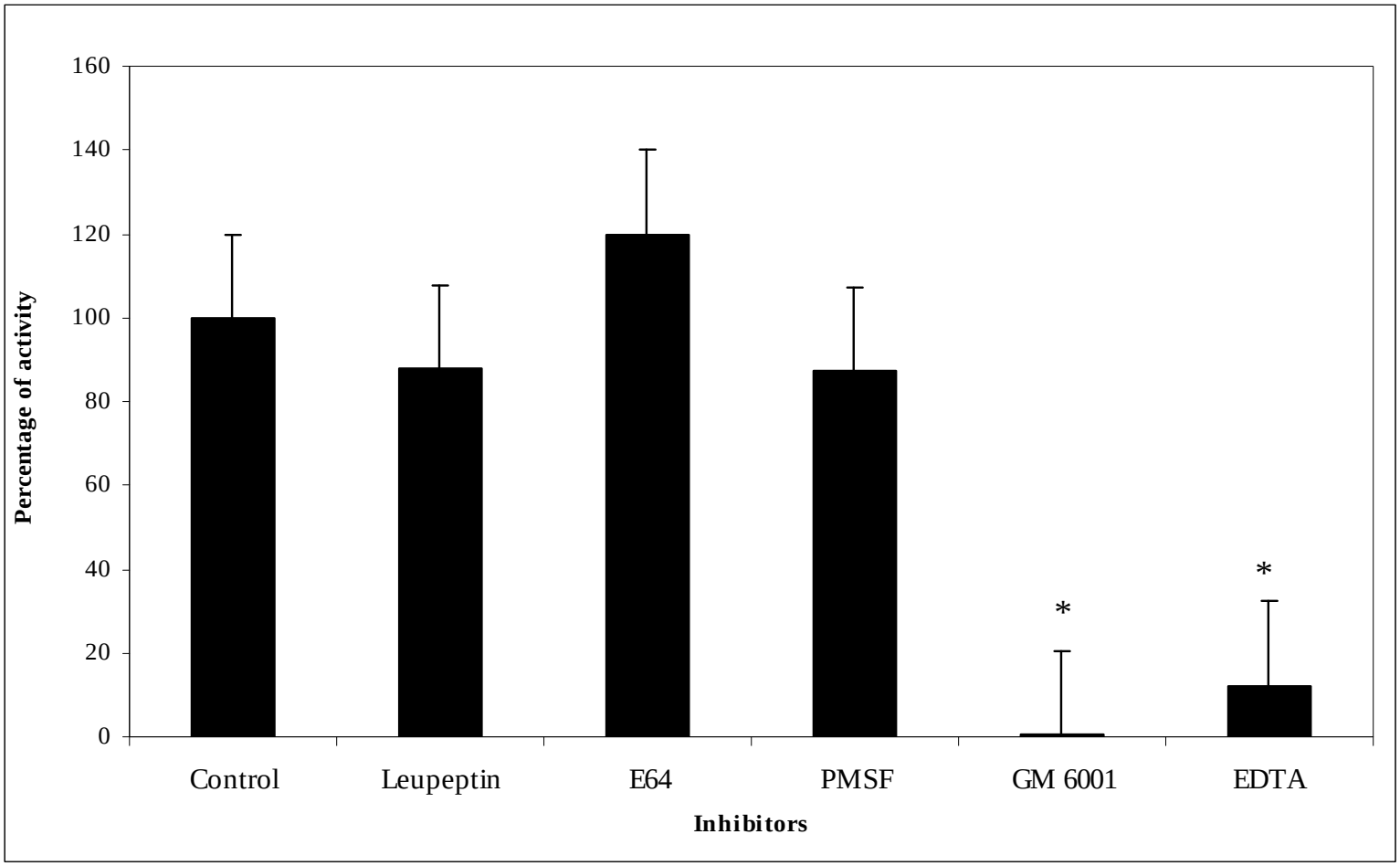
160
140
120
100
80
60
40
20
0
Percentage of activity
*
*
Control
Leupeptin
E64
PMSF
GM 6001
EDTA
Inhibitors

Figure 4

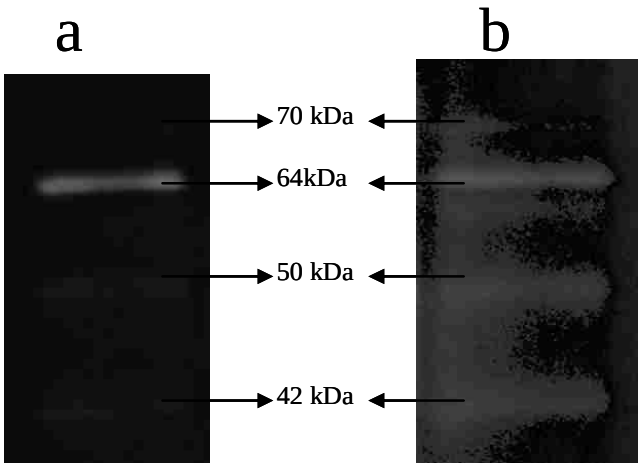

Figure 5

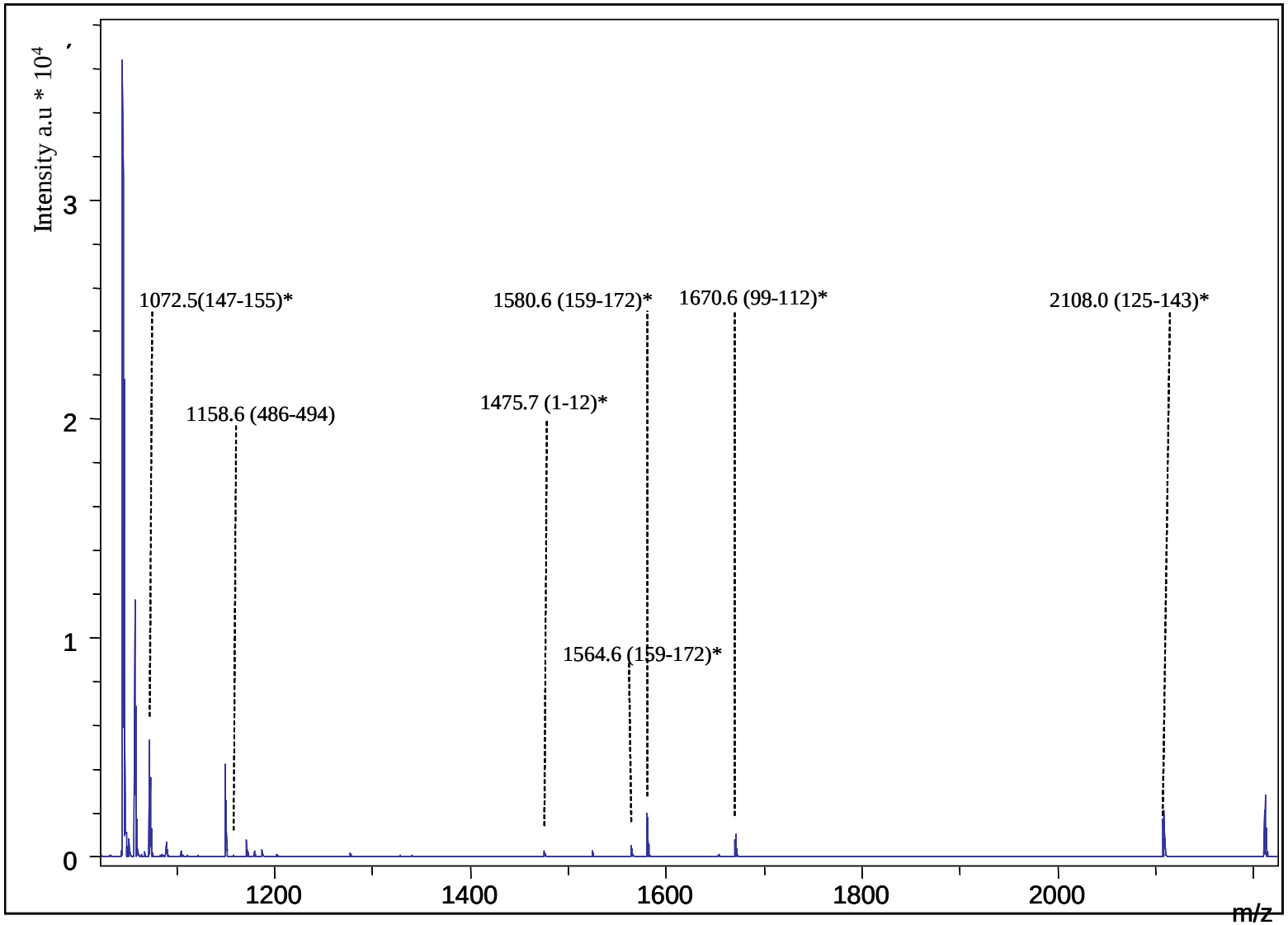
Intensity a.u * 10^4
3
2
1
0
1072.5(147-155)*
1158.6 (486-494)
1475.7 (1-12)*
1580.6 (159-172)*
1564.6 (159-172)*
1670.6 (99-112)*
2108.0 (125-143)*
1200
1400
1600
1800
2000
m/z

Figure 6

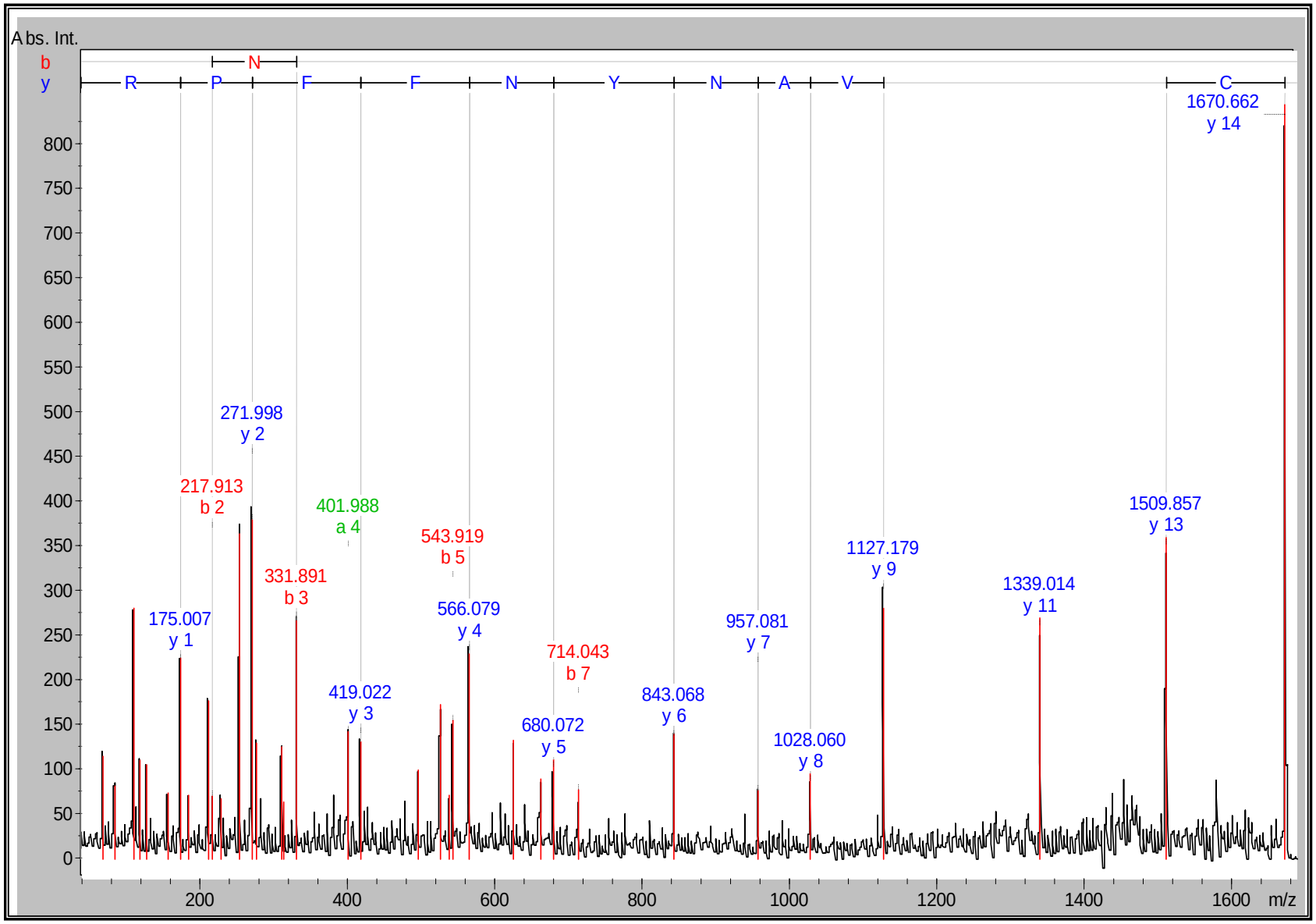
Abs. Int.
b
y
N
R P F F N Y N A V C
1670.662
y 14
271.998
y 2
217.913
b 2
401.988
a 4
543.919
b 5
331.891
b 3
1509.857
y 13
1127.179
y 9
1339.014
y 11
175.007
y 1
566.079
y 4
957.081
y 7
714.043
b 7
419.022
y 3
843.068
y 6
680.072
y 5
1028.060
y 8
800
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
200
400
600
800
1000
1200
1400
1600
m/z

Figure 7

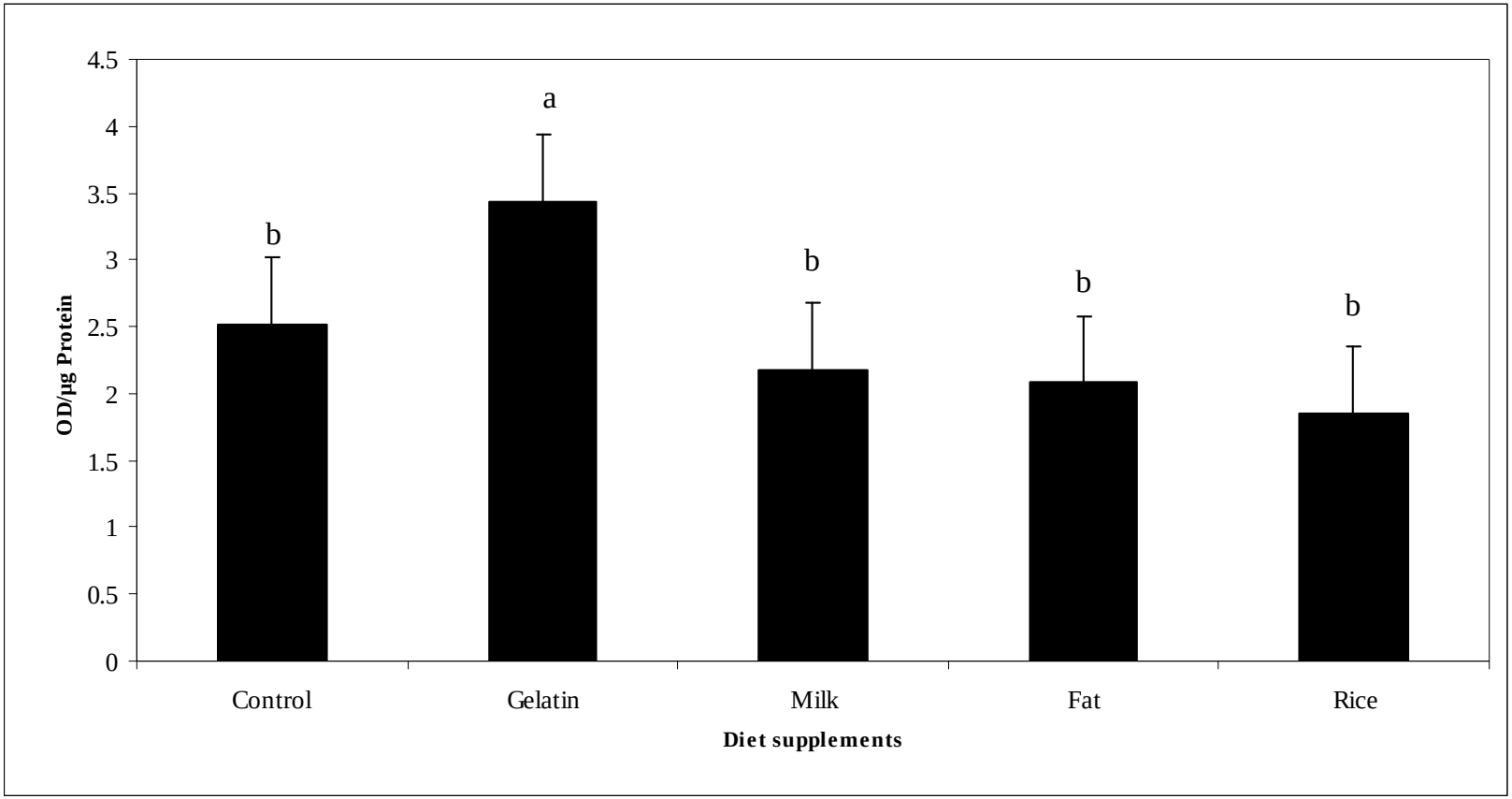
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
OD/µg Protein
a
b
b
b
b
Control
Gelatin
Milk
Fat
Rice
Diet supplements

Figure 8

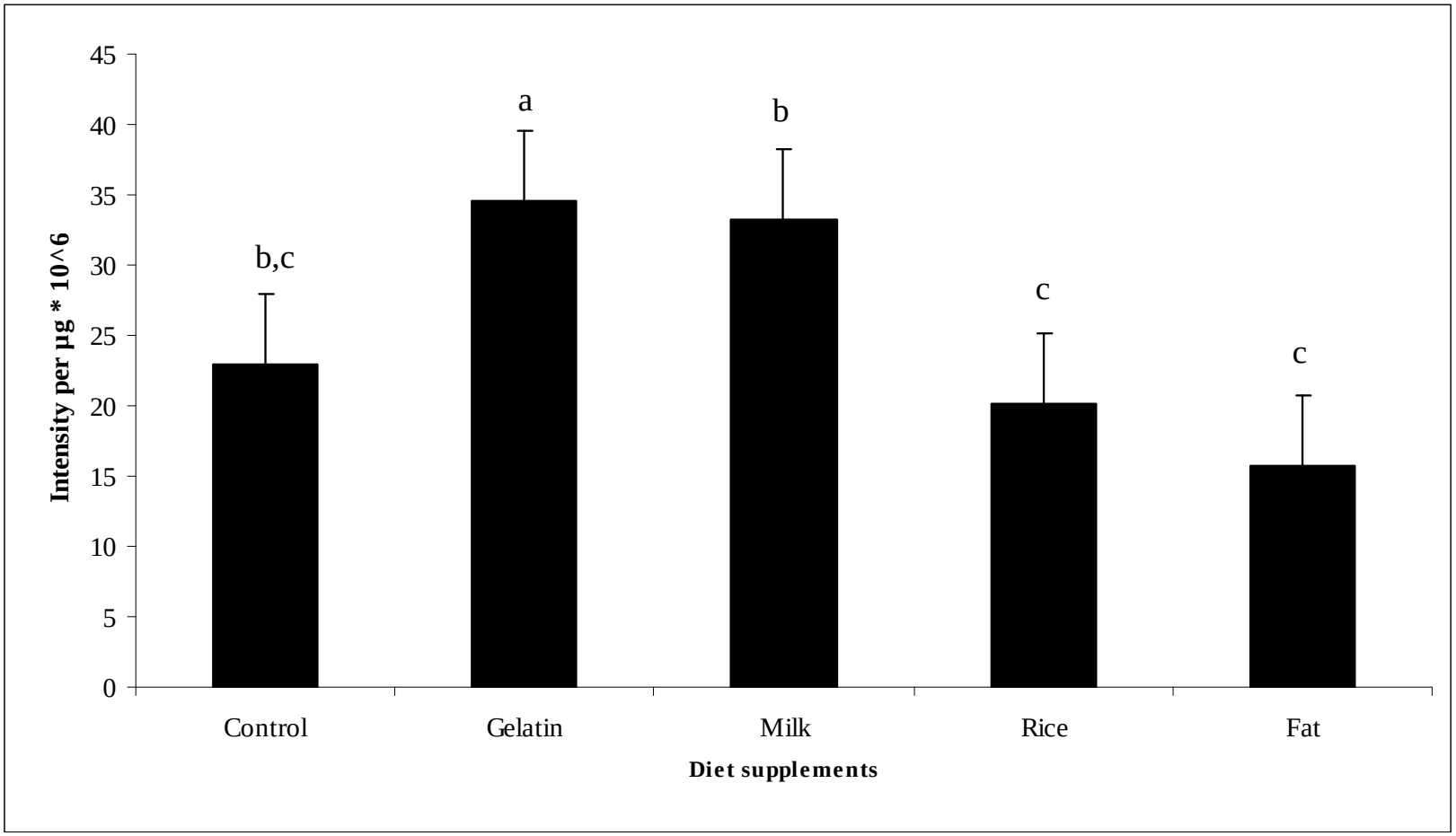
45
40
35
30
25
20
15
10
5
0
Intensity per µg * 10^6
b,c
a
b
c
c
Control
Gelatin
Milk
Rice
Fat
Diet supplements

Table 1.

Position	Sequence	Observed peptides in 64 kDa	Observed peptides in 50 kDa	Observed peptides in 42 kDa	Expected (m/z) (MH+)
1 - 12	MKTHSVFGFFFK	1475.7	1475.7	1475.7	1475.7
99 - 112	CGNPDVANYNFFPR	1670.7	1670.7	1670.8	1670.7
125 - 143	IIGYTPDLDPETVDDAFAR	2108.0	2108.0	2108.1	2108.0
144 - 155	AFKVWSDVTPLR	-	1418.7	-	1418.7
147 - 155	VWSDVTPLR	1072.5	1072.5	1072.4	1072.5
159 - 172	INDGEADIMINFGR	1564.7	1564.7	1564.7	1564.7
159-172	INDGEADIMINFGR oxidation(M)	1580.7	1580.7	1580.7	1580.7
173 - 184	WEHGDGYPFDGK	1407.6	1407.6	1407.6	1407.6
250 - 260	NDGFLWCSTTK	1328.6	1328.6	-	1328.6
261 - 289	DFDADGKYGFCPHESLFTMGGNGDGQP CK oxidation (M)	-	-	3223.6	3223.6
294 - 307	FQGQSYDQCTTEGR	1676.7	1676.7	1676.7	1676.6
313 - 322	WCGTTEDYDR	1302.6	-	1302.5	1302.5
370-382	LWCASTSSYDDDR	1575.6	1575.7	-	1575.6
370 - 383	LWCASTSSYDDDRK	1703.7	1703.7	1703.7	1703.7
474 - 485	HDIVFDGVAQIR	1369.6	1369.8	-	1369.6
474 - 492	HDIVFDGVAQIRGEIFFFK	-	-	2238.2	2238.2
486 - 494	GEIFFFKDR	1158.6	1158.6	-	1158.5
523 - 534	IDAVYESPQDEK	1393.6	1393.7	-	1393.6
558 - 570	KLTSLGLPPDVQR	1423.8	-	-	1423.7
559 - 570	LTSLGLPPDVQR	1295.7	1295.6	-	1295.7
583 - 590	TYIFSGDR	958.4	958.5	-	958.4
637 – 646	DQYYLQMEDK	1332.5	-	-	1332.5

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VII. Conclusion

The review of literature both mammalian and avian pointed to certain vascular deficiency, adhesion problems in growth plate and metabolic disorders associated with femoral head problems. The experimental model not only provided a surrogate marker Apoliporotein A-I (Apo-AI) but also offered insights about the vascular insufficiency, dyslipidemia and adhesion problems that could potentially lead to the disease. Then we applied the method developed in the model to analyze proteins and peptides in the spontaneously FHS affected birds and compared their blood with healthy chickens and found that heavy bodyweight, dyslipidemia, mild immune activation, changes in lipid binding proteins and hemolysis were associated with the affected birds. These parameters could be potentially useful as biomarkers to distinguish between the healthy and diseased birds. Since mild hemolysis has been associated with FHS and several pathogens have been isolated from FHN lesions, we tested the protein changes in plasma in response to a bacterial cell wall component, lipopolysachharides (LPS). LPS induced the changes in one classical acute phase protein, an antimicrobial peptide, heparanse and a chemokine. The changes in FHS affected plasma were distinct from LPS injected birds and hence we suggest that the protein profile of FHS affected birds could be useful to select for the affected birds.

VIII. Appendices

A. Supplementary tables for Chapter III

Table S1. List of peptide peaks different in the CTRL and FHS group shown by ClinPro tools software (C18)

Mass	DAve	PTTA	PWKW	PAD	Ave1	Ave2	StdDev1	StdDev2
8101.33	248.78	0.00199	0.00645	0.501	480.75	729.53	265.1	248.64
7431.42	174.26	0.00199	3.1E-06	< 0.000001	14.35	188.61	5.42	226.12
7447.38	170.54	0.00199	4E-06	< 0.000001	30.87	201.41	22.22	219.5
3199.56	129.13	0.178	0.703	< 0.000001	230.3	359.43	150.8	350.27
8748.14	124.45	0.00588	0.0312	0.0803	252.66	377.1	167.53	124.67
4872.77	115.84	0.00157	0.162	< 0.000001	153.31	37.47	161.48	7.88
8763.51	111.66	0.525	0.895	0.0108	725.92	837.59	464.67	614.03
8666.4	62.74	0.218	0.794	0.00219	183.79	246.53	122.5	187.68
7304.11	59.43	0.000131	1E-06	< 0.000001	5.81	65.25	1.42	57.87
7952.27	58.82	0.19	0.31	0.00000388	277.17	218.34	155.88	111.9

Table S2. List of peptide peaks different in the CTRL and FHS group shown by ClinPro tools software (SCX)

Mass	**DAve**	**PTTA**	**PWKW**	**PAD**	**Ave1**	**Ave2**	**StdDev1**	**StdDev2**
3203.1	500.4	0.102	0.837	< 0.000001	62.75	563.2	42	857.31
4708.88	225.7	0.0000847	0.00086	0.0597	340.79	115.12	102.38	116.86
3257.21	146.9	0.102	0.468	< 0.000001	16.5	163.4	4.89	242.14
4775.99	103.5	0.353	0.29	0.0165	380.39	276.44	154.8	276.85
2482.66	96.67	0.373	0.281	0.000934	421.05	324.39	255.67	212.1
4238.91	63.92	0.0001	0.00086	0.191	107.57	43.64	32.2	33.53
4754.81	50.54	0.0005	0.00117	0.527	113.44	62.9	35.28	24.87
2498.66	49.46	0.687	0.356	0.0141	571.97	621.43	148.35	373.64
3123.84	49.09	0.242	0.118	0.0000111	132.94	83.85	84.88	94.26
4852	47.66	0.0953	0.0386	0.154	125.19	77.53	53.47	62.25

Mass – *m/z* value.

DAve – Difference between the maximal and the minimal average peak area/ intensity of all classes.

PTTA -- P-value of t-test OR ANOVA

PWKW -- P-value of Wilcoxon test OR Kruskal-Wallis test

PAD -- P-value of Anderson-Darling test

Ave*N* -- Peak area/intensity average of class *N*. (1= CTRL, 2= FHS)

StdDev*N* -- Standard deviation of the peak area/intensity average of class *N*.

Table S3. List of proteins identified in CTRL (pool 1)

Accession	Protein	MW [kDa]	pI	#Alt. Proteins	Scores	#Peptides	SC [%]	RMS90 [ppm]
gi|227016	Apolipoprotein AI	28.8	5.5	4	2186.5	50	98.0	209.92
gi|3645997	Apolipoprotein AIV	40.8	4.8	1	895.5	19	63.9	255.09
gi|513193913	Titin isoform X2	3652.0	6.1	11	547.0	39	1.4	364.24
gi|513222341	Apolipoprotein C-III	10.3	8.8	1	393.5	7	52.7	76.05
gi|63748	preproalbumin (serum albumin)	69.9	5.5	1	286.0	6	12.8	429.68
gi|363735454	Beta-microseminoprotein-like isoform X1	12.4	8.3	1	265.9	6	43.6	187.66
gi|363745920	Apolipoprotein A-II isoform X1	11.1	10.0	1	245.1	4	51.5	466.01
gi|63413	Beta-globin	16.5	8.8	7	227.9	4	29.9	305.07
gi|513189629	Nesprin-2 isoform X1	803.8	5.1	2	209.9	16	2.3	233.77

gi|1842051	Myosin heavy chain	223.0	5.6	16	162.0	12	6.6	642.30
gi|363739654	Periplakin	206.0	5.6	2	148.8	9	4.5	756.45

Table S4. List of proteins identified in CTRL (pool 2)

Accession	Protein	MW [kDa]	pI	#Alt. Proteins	Scores	#Peptides	SC [%]	RMS90 [ppm]
gi|227016	Apolipoprotein AI	28.8	5.5	4	2207.4	50	95.9	219.96
gi|3645997	Apolipoprotein AIV	40.8	4.8	2	997.8	22	55.5	272.41
gi|513193913	Titin isoform X2	3652.0	6.1	13	463.2	34	1.2	450.21
gi|513222341	Apolipoprotein C-III	10.3	8.8	1	364.5	9	67.7	163.54
gi|363735454	Beta-microseminoprotein-like isoform X1	12.4	8.3	1	300.4	7	41.8	291.17
gi|63748	Preproalbumin	69.9	5.5	1	293.6	9	16.6	180.38
gi|4699641	Chain B, Hemoglobin D	16.3	8.9	6	253.7	4	30.1	257.24
gi|363745920	Apolipoprotein A-II isoform X1	11.1	10.0	1	252.0	5	67.0	407.27
gi|513189629	Nesprin-2 isoform X1	803.8	5.1	2	224.6	16	2.2	142.67
gi|513176503	Nesprin-1 isoform X6	1010.5	5.4	5	187.4	12	1.4	263.36

Table S5. List of proteins identified in FHS (Pool 1)

Accession	Protein	MW [kDa]	pI	#Alt. Proteins	Scores	#Peptides	SC [%]	RMS90 [ppm]
gi|227016	Apolipoprotein AI	28.8	5.5	3	2199.1	50	89.8	173.12
gi|211146	Apolipoprotein A-I precursor	30.7	6.0	1	2112.2	49	82.2	175.10
gi|3645997	Apolipoprotein AIV	40.8	4.8	1	518.3	11	38.5	293.21
gi|513193910	Titin isoform X3	3745.1	6.0	15	515.0	38	1.1	515.87
gi|63748	preproalbumin (serum albumin)	69.9	5.5	1	428.0	11	22.3	342.60
gi|513222341	Apolipoprotein C-III	10.3	8.8	1	415.8	8	65.6	271.67
gi|363735454	Beta-microseminoprotein-like isoform X1	12.4	8.3	1	290.1	7	43.6	258.21
gi|4699641	Chain B, R-State Form Of Chicken Hemoglobin D	16.3	8.9	7	260.4	5	37.0	273.08
gi|363745920	Apolipoprotein A-II isoform X1	11.1	10.0	1	256.3	5	56.7	329.38
gi|513189629	Nesprin-2 isoform X1	803.8	5.1	2	207.3	16	2.6	106.18

Table S6. List of proteins identified in FHS (Pool 2)

Accession	Protein	MW [kDa]	pI	#Alt. Proteins	Scores	#Peptides	SC [%]	RMS90 [ppm]
gi|227016	Apolipoprotein AI	28.8	5.5	4	2295.7	49	95.9	236.24
gi|3645997	Apolipoprotein AIV	40.8	4.8	1	554.2	15	54.9	320.43
gi|63748	Preproalbumin (serum albumin)	69.9	5.5	1	524.5	13	26.7	258.51
gi|513193910	Titin isoform X3	3745.1	6.0	14	499.8	36	1.3	444.12
gi|513222341	Apolipoprotein C-III	10.3	8.8	1	457.7	9	60.2	101.31
gi|363745920	Apolipoprotein A-II isoform X1	11.1	10.0	1	296.7	6	61.9	464.91
gi|363735454	Beta-microseminoprotein-like isoform X1	12.4	8.3	1	281.7	6	43.6	290.81
gi|4699641	Chain B, Hemoglobin D	16.3	8.9	10	274.2	6	42.5	182.92
gi|513189629	Predicted nesprin-2 isoform X1	803.8	5.1	1	173.4	14	2.5	289.50
gi|513176503	Nesprin-1 isoform X6	1010.5	5.4	5	160.0	10	1.1	414.60

Accession - Database accession number

Protein – Common name of the protein

MW – Molecular weight in Daltons

pI – Isoelectric point

#Alt. Proteins – Number of similar proteins (subset matches) that can be found in the Alternative Proteins View

Scores – Protein score in the format score (M: Mascot score).

#peptides – Number of peptides identified.

SC% -Sequence coverage

RMS90 (ppm) - Deviation from predicted mass (root mean square value / root mean square 90% confidence value).

RANK- Ranking based on number of identified peptides.

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Signed and agreed by:

Digitally signed by Narayan C. Rath
DN: cn=Narayan C. Rath, o=USDA, ou=ARS, email=narayan.rath@ars.usda.gov, c=US
Date: 2014.10.14 15:12:57 -05'00'

Requestor - Signature

10/14/2014
AAAP - Signature and Effective Date

Narayan C. Rath
Requestor - Printed Name

Bob Bevans-Kerr, AAAP Executive Director

C. IACUC protocol approval

2013 - 2015

MEMORANDUM

TO: Narayan Rath

FROM: Craig N. Coon, Chairman
Institutional Animal Care
And Use Committee

DATE: January 3, 2013

SUBJECT: IACUC Protocol **APPROVAL**
Expiration date : **December 30, 2015**

The Institutional Animal Care and Use Committee (IACUC) has **APPROVED** Protocol **#13025 - "Experimentally-induced leg problems and proteomic changes in chickens"**. You may begin this study immediately.

The IACUC encourages you to make sure that you are also in compliance with other UAF committees such as Biosafety, Toxic Substances and/or Radiation Safety if your project has components that fall under their purview.

In granting its approval, the IACUC has approved only the protocol provided. Should there be any changes to the protocol during the research, please notify the IACUC in writing [via the Modification Request form] **prior** to initiating the changes. If the study period is expected to extend beyond **12-30-2015**, you must submit a new protocol. By policy the IACUC cannot approve a study for more than 3 years at atime.

The IACUC appreciates your cooperation in complying with University and Federal guidelines for research involving animal subjects.

cnc/car

cc: Animal Welfare Veterinarian

Administration Building 210 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4572
Fax: 479-575-3846 • http://vpred.uark.edu/199

Animal Use Protocol
University of Arkansas, Fayetteville
Coversheet

Submitted 12/14/12

IACUC use only:			
Protocol number:	_____	Category(s) of animal use:	
Date Received:	_____	☐ Agricultural	
Approval Date:	_____	☐ Biomedical	
Start Date:	_____	☐ Field	
End Date:	_____	LATA Training Verified	☐ Yes ☐ No

Instructions:

- *This is a MicroSoft Word (MSWord) "form". Use MSWord to fill in the information asked for in either the blanks ("____"), or the box ("☐") provided. You can put as much information in the blanks or boxes as you need to. (Note -- It may cause minor complications to use the "Tab" key to move from box to box since the boxes are a cell in a table [consisting of one cell]. Therefore, it should cause less problems to avoid using the tab key. However, if you need to use the Tab key in the cell, you will need to use the Ctrl-Tab combination.)*
- *Submit an electronic copy of your completed protocol to crodlun@uark.edu and be sure to sign (with a scanned signature) the appropriate form(s). If you cannot send a signed electronic copy, then also send a signed paper copy of the completed protocol to **Carol Rodlun; CLAF, A-42 ANSC.***
- *Failure to follow these instructions and adequately fill out the required information may result in the protocol being returned.*
- *The deadline for getting this form to Carol Rodlun, is 12:00 Noon on Monday of the week of the IACUC meeting when it will be acted upon.*

Project Title: Experimentally-induced leg problems and proteomic changes in chickens

Project length (3 years maximum): _____

Start date: December 2012 End date: December 2015

	Principal Investigator:	**Co-Investigator(s)** *(if applicable):*	
Name:	Narayan C. Rath	B. Packialakshmi	_____
Department/Division:	USDA/ ARS; Poultry Science	Sarbjeet Makkar	_____
Campus Mail Address:	nrath@uark.edu	_____	_____
Telephone:	479-575-6189	_____	_____
Fax:	479-575-4202	_____	_____
E-mail:	narayan.rath@ars.usda.gov	_____	_____

Individual(s) responsible for animal care:

Name:	Wally McDonner	Scott Zornes	Narayan C. Rath
Office address:	Bg 235, Poultry Farm	O-317, POSC	O-307, POSC
Office City, State, Zip:	Fayetteville, Ar 72701	Fayetteville, AR 72701	_____
Office phone:	479-575-3122, 479-575-7517	479-575-4304	_____
Home address:	_____	_____	_____
Home City, State, Zip:	_____	_____	_____
Home phone:	_____	_____	_____

Individual(s) responsible for euthanasia:

Name:	Wally McDonner	Scott Zornes	Narayan C. Rath
Office address:	Bg 235, Poultry Farm	O-317, POSC	O-307, POSC