2022

Identifying Bisphosphonate Protein Biomarkers in Equine Sera Using Mass Spectrometry Methods

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Digital Object Identifier: https://doi.org/10.13023/etd.2022.044

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IDENTIFYING BISPHOSPHONATE PROTEIN BIOMARKERS IN EQUINE SERA USING MASS SPECTROMETRY METHODS

THESIS

A thesis submitted in partial fulfillment of the
requirements for the degree of Master of Science in the
College of Agriculture, Food and Environment
at the University of Kentucky

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2022

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ABSTRACT OF THESIS

IDENTIFYING BISPHOSPHONATE PROTEIN BIOMARKERS IN EQUINE SERA USING MASS SPECTROMETRY METHODS

Bone resorptive diseases affect humans and horses, alike. Examples of these diseases include osteoporosis, Paget's disease, and osteogenesis imperfecta in humans, and navicular disease in the horse (Mitchell et al., 2019; Suva et al., 2021). Bisphosphonates are used to treat these diseases, and may remain in the bone for several years after administration, demonstrating the need for newer drug testing methods.

The purpose of the discovery phase of this study was to use blood samples of in-training horses which had been given an initial administration of the bisphosphonate tiludronate and identify protein biomarkers that changed in response to this class of drugs. In the targeted phase, the objective was to validate the previously identified protein biomarkers that can be added into a biomarker library. This library is meant to be referenced when drug testing racehorses and can lengthen the detection window of prohibited or restricted substances.

Fractionation, digestion, and de-salting were performed on sera samples from 19 equine subjects in-training. These samples were injected in an Orbitrap™ Exploris 480[d] coupled with an Ultimate 3000 RSLCnano[j]. Label-free quantitation of proteins was performed in both phases. Four key protein biomarkers were identified in the discovery phase and validated in the targeted phase.

KEYWORDS: Bisphosphonates, Protein Biomarkers, Mass Spectrometry, Bone Remodeling, Equine

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03/11/2022

Date

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DEDICATION

To my mom and the memory of my beloved dog, Sam.

ACKNOWLEDGMENTS

The following thesis would not have been possible without the guidance, mentorship, and contributions of several people. First, I want to thank my advisor, Dr. Scott Stanley, for allowing me to continue my education in his laboratory and for constantly guiding me and supporting me throughout my endeavors. I am especially thankful to have him as my advisor and mentor.

Next, I would like to thank Dr. Abigail Burrows for helping grow my knowledge in several different areas, for always supporting my educational and research goals, and for being a friend (and source of laughter) always. I am so grateful we ended up in the same lab at the same time.

I want to thank my committee members, Dr. Scott Stanley, Dr. Daniel Howe, and Dr. Allen Page for guidance, support, and recommendations while writing my thesis.

Thank you to the veterinarians, Dr. John Cummins and Associates, who collected the samples that I have had the opportunity to do my research on. Without them, this project would not have been possible.

I also would like to thank the Thermo Fisher Scientific Team for their guidance throughout this project during analysis, as well as for providing the instruments on which this work was completed.

Special thanks to Stonestreet Farm for providing the baseline, normal horse samples that were used as a control for the experimental data.

A huge thank you to my mom, without whom I would not have been able to make it this far. She has been a source of steadfast love and support and never fails to remind

me that I can do anything. Thank you for nurturing my love of education and never letting me fail alone.

A special thanks to my sister, Mynah, who never fails to make me laugh when I need it most and is always there to pick me up if I fall. Writing this thesis would have been a lot less fun without you.

Finally, thank you to my wonderful partner, Grant, and our two dogs, Ruth and Daisy, for being a constant source of love and support no matter what. Your encouragement throughout this entire process has made this journey smooth sailing. You fill my life with love and laughter, and I am so grateful for the three of you always.

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CHAPTER 1. LITERATURE REVIEW

1.1 Introduction

Bisphosphonates are a class of drugs that are used therapeutically in humans for the treatment of several diseases, including osteoporosis, Paget's disease, and bone metastases (Drake et al., 2008; Suva et al., 2021). There are currently two subgroups of bisphosphonates: those that contain nitrogen within their side chain and those that do not (Kamm et al., 2008). Those that contain nitrogen and those that do not have different mechanisms of action with the common goal of decreasing osteoclast-mediated resorption of bone during the remodeling process (Drake et al., 2008; Mitchell et al., 2019). In equids, they are used less frequently, but there are two FDA-approved bisphosphonates for the treatment of navicular disease: tiludronate and clodronate (Suva et al., 2021). The FDA approves a final product form of the drug, known as Tildren® or Osphos®, but has not approved the drug itself before modifications, such as tiludronic acid or clodronic acid. Tiludronate and clodronate do not contain nitrogen as a part of their side chain. They have several off-label uses, as well; although, these may prove to be problematic if the off-label use is in a juvenile horse or an actively training horse (Richbourg et al., 2018). Bisphosphonates are only approved for use in horses over the age of four because this is the age when bone remodeling naturally slows (Mitchell et al., 2019). Because these drugs accumulate in the bone and can remain there for years after administration, caution should be taken when giving them to juvenile horses due to their potential adverse effects on the maturation of the epiphyseal plate (Gilday et al., 2020). Furthermore, adult racehorses are at a higher risk of microcracks and microdamage due to their rigorous training regimens (Holmes et al., 2014). When bisphosphonates are administered, this can interfere with the

bone's natural remodeling ability, thereby perpetuating microcracks and increasing the risk of fatigue fractures (Gilday et al., 2020).

In the form of anti-doping control, drug testing in equids is of utmost importance in today's world because of the production of new designer drugs, as well as drugs that have rapid elimination times and may not be detected through normal drug tests (Mori et al., 2015; Wong et al., 2020). These types of substances allow individuals to alter the performance of a racehorse, while concealing the use of the drug, and ultimately put the integrity of the breeding and racing industry at risk. There remains a distinction between prohibited substances and therapeutic substances. Prohibited substances are banned based on their performance enhancing properties. Therapeutic substances are those which are prescribed for horse welfare but must be restricted in use to ensure they do not impact performance on race day. (Wong and Wan, 2014). Bisphosphonates fall into the latter category, as they are approved for use in the horse for treatment of navicular disease and are considered controlled substances according to the International Federation for Equestrian Sports (FEI, 2022). Controlled substances are those which are considered to have therapeutic use in the horse, but still may affect racing performance, and therefore must be prohibited (FEI, 2021).

Due to the rather rapid metabolism and excretion of some drugs, protein biomarkers provide an alternative way to test for the presence of the substance over a longer period. To assess proteins that may be upregulated or downregulated in response to prohibited substances, single re-up proteomics using mass spectrometry is a valid technique that provides this insight (Parker and Borchers, 2014). Mass spectrometry is an excellent way to perform both discovery-based experiments and targeted analytical methods, alike, in

order to identify and validate biomarkers of prohibited substances (Rauniyar and Keck, 2015). There has been a shift from analyzing metabolites to analyzing protein effects in order to increase the window of time that a prohibited substance can be detected (Barton et al., 2009).

1.2 Bisphosphonates

Bisphosphonates are a class of drugs aimed at inhibiting osteoclast-mediated bone resorption. (Mitchell et al., 2019). They were first developed in the 1960s as analogues of inorganic pyrophosphate—a chemical aimed at inhibiting calcification of blood vessels (Kamm et al., 2008). Bisphosphonates' high specificity for hydroxyapatite crystals and preference for binding to bone is part of what makes them effective inhibitors of bone resorption (Soto and Chiappe Barbará, 2014). Bisphosphonates are classified into two groups based on the contents of their side chain: nitrogen-containing and non-nitrogen containing. The nitrogen-containing bisphosphonates have higher potencies and overall increased functionalities (Kamm et al., 2008). In humans, these drugs are used in the treatment of a variety of diseases, including osteoporosis, Paget's disease, and metastatic bone tumor pain (Drake et al., 2008). While there are several bisphosphonates that are widely used in humans, only two drugs in the non-nitrogen containing class are approved for use in horses: tiludronate and clodronate.

1.2.1 Nitrogen-Containing Bisphosphonates

Nitrogen-containing bisphosphonates are more potent inhibitors of bone resorption than non-nitrogen containing bisphosphonates because of their greater binding affinity to bone and hydroxyapatite crystals (Bigi and Boanini, 2018). Drugs in this class include

risedronate, zoledronate, alendronate, pamidronate, and ibandronate (Soto and Chiappe Barbará, 2014). These are known as second and third generation bisphosphonates and are aimed at inducing osteoclast apoptosis (Drake et al., 2008). To do this, the nitrogen-containing bisphosphonates target the mevalonate pathway—a pathway that results in the production of cholesterol and other sterols (Russell, 2007). The drug inhibits the action of farnesyl diphosphate synthase, which downregulates the production of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (Reszka and Rodan, 2003). These two proteins are responsible for isoprenylation of GTPases that occurs post-translationally (Drake et al., 2008). The GTPases include those within the Rab, Ras, and Rho families and are essential for osteoclast activity (Coxon et al., 2006). Osteoclast apoptosis is induced after the cell becomes unable to perform certain processes, such as formation of the ruffled border (Mitchell et al., 2019). Other processes that are essential for osteoclast function and become downregulated due to the inhibition of small GTPases are cytoskeletal arrangement and vesicular trafficking (Kimmel, 2007).

While the mechanism of action of the nitrogen-containing bisphosphonates is the same overall, the individual drugs' relative efficacies differ depending on the nature of their R-group, whether it contains nitrogen or does not contain nitrogen. There are both alkyl and heterocyclic R-groups represented in the individual drugs; this can cause at least a 500-fold difference in the efficacy in which they inhibit farnesyl diphosphate synthase (Kimmel, 2007).

1.2.2 Non-Nitrogen Containing Bisphosphonates

Non-nitrogen containing bisphosphonates are referred to as first generation bisphosphonates (Drake et al., 2008). Drugs in this class include clodronate, tiludronate,

and etidronate (Soto and Chiappe Barbará, 2014). These substances are also aimed at inducing osteoclast apoptosis, albeit through a different mechanism (Coxon et al., 2006). The drugs within this generation are first taken up by the osteoclast and become incorporated into molecules of ATP through an aminoacyl-transfer RNA synthetase enzyme, resulting in the production of cytotoxic ATP (Soto and Chiappe Barbará, 2014). This cytotoxic ATP is non-hydrolyzable and accumulates inside the osteoclast, inhibiting pathways that are ATP-dependent (Mitchell et al., 2019). It has been observed that the cytotoxic ATP generated by clodronate inhibits mitochondrial ADP or ATP translocase and this mechanism leads to apoptosis of the osteoclast (Reszka and Rodan, 2003). There is a series of events resulting from the mitochondria losing its function that lead up to introduction of apoptosis. Tiludronate and etidronate have shown similar mechanisms, although the cytotoxic ATP generated is a lesser amount than that which clodronate produces (Reszka and Rodan, 2003). They are integrated into molecules of ATP due to the similarity of their structure with inorganic pyrophosphate (Drake et al., 2008).

1.2.3 Pharmacology

Pharmacology of bisphosphonates has been well studied, including both the pharmacokinetics (PK) and pharmacodynamics (PD) of the drugs (Cremers and Papapoulos, 2011). Bisphosphonates are artificial compounds that originate from inorganic pyrophosphate with the geminal oxygen being replaced by a carbon to create a phencyclidine bond. These drugs are more stable than endogenous pyrophosphate because the phencyclidine bond is resistant to heat and enzymatic hydrolysis (Reszka and Rodan, 2003). One of the most important aspects of bisphosphonates' pharmacology is their bioavailability depending on the route of transmission (Drake et al., 2008). However,

according to Soto, their main pharmacological effect is their ability to strengthen bone through various mechanisms (Soto and Chiappe Barbará, 2014).

Pharmacokinetics and pharmacodynamics encompass several different aspects of bisphosphonates. Pharmacokinetics assesses how the drug moves throughout the body and includes absorption, distribution, and skeletal elimination (Lin, 1996). Pharmacodynamics discusses the effects of the drug on the body and include the drug's mechanisms of action (Cremers and Papapoulos, 2011).

1.2.3.1 Pharmacokinetics of Bisphosphonates

Bisphosphonates, when ingested orally, have low bioavailability (Lin, 1996). They are taken up in different portions of the gastrointestinal tract and into the blood stream through varying mechanisms of transport, including active and paracellular transport (Cremers et al., 2019). Absorption of bisphosphonates in the gastrointestinal tract has been shown to change as stomach pH increases/decreases (Cremers and Papapoulos, 2011). Studies have shown the oral bioavailability in rats can decrease as much as 6- to 7-fold when given with food (Porras et al.). Their low oral bioavailability is thought to be due to their low lipophilicity. This characteristic prevents transcellular transport—a type of transport where the drug is taken up into the bloodstream through epithelial cells (Lin, 1996). Drugs in the bisphosphonate class tend to be characterized pharmacokinetically due to their similar absorption rates. Due to their poor oral bioavailability in humans, bisphosphonates are administered intravenously or intramuscularly in horses (Soares et al., 2016).

Once in the bloodstream, bisphosphonates tend to bind to plasma and serum proteins. Binding varies depending on the bisphosphonate but has been shown as lower in the plasma

due to endogenous displacers (Cremers et al., 2019). The drug does not typically undergo first or second phase metabolism, with the exception of the incorporation of non-nitrogen containing bisphosphonates into ATP to create cytotoxic ATP analogues (Cremers et al., 2019). Bisphosphonates can be found in the liver, spleen, and other soft tissues after distribution occurs (Cremers and Papapoulos, 2011). Because bisphosphonates cannot easily penetrate cell membranes, their exposure to non-target tissues is decreased (Reszka and Rodan, 2003). The amount of drug which is not found in these tissues will either be excreted through the kidneys or taken up into the bone (Cremers and Papapoulos, 2011). If the bisphosphonate is taken up into the bone, they tend to congregate in areas of high bone turnover, causing their distribution in bone to be highly heterogeneous (Soares et al., 2016). Due to this heterogenous distribution pattern, bisphosphonates tend to be endocytosed into osteoclasts. Once inside the osteoclast, the individual bisphosphonates follow their mechanisms of action in order to decrease bone resorption rates, whether that is by accumulation of cytotoxic ATP analogues or disruption of the mevalonate pathway (Soares et al., 2016).

Bisphosphonates have a relatively slow elimination from the skeleton, which may be due to the drug becoming embedded in the bone and only being released after future resorption (Cremers and Papapoulos, 2011). They exhibit a multiple phase elimination process (Porras et al.). The amount of drug that becomes immersed in bone after ossification is likely inactive until it is released again following resorption (Cremers et al., 2019). As a consequence of their slow elimination time, several studies have shown bisphosphonates in the urine many years after administration (Riggs et al., 2001; Soares et al., 2016).

Excretion may occur either through bile or urine, depending on the drug and its properties. For bisphosphonates, the primary route of excretion is renally, with bile excretion being relatively insignificant (Lin, 1996). Alendronate, when given orally, was detected at a very low percentage in the feces (<0.2%), which suggests that excretion through the bile is minimal (Porras et al.). Alendronate has a terminal half-life in humans estimated at ten years. Pamidronate has been detected in urine up to eight years post-administration. Both findings suggest a slow elimination of bisphosphonates from the skeleton (Baroncelli and Bertelloni, 2014).

1.2.3.2 Pharmacodynamics of Bisphosphonates

The main pharmacodynamic effect of bisphosphonates on the body is their ability to significantly reduce bone resorption (Soto and Chiappe Barbará, 2014). During treatment of Paget's disease in humans, collected data showed an initial decrease in bone resorption which led to a slowed decrease in bone formation because these two processes are coupled within bone remodeling (Cremers and Papapoulos, 2011). Biochemically, the nitrogen-containing bisphosphonates work by disrupting the mevalonate pathway and preventing prenylation of signaling proteins, leading to loss of osteoclast functionality (Cremers et al., 2019). On the other hand, the non-nitrogen containing bisphosphonates act by creating cytotoxic ATP after they are metabolized in the osteoclast, thereby inhibiting ATP-dependent intracellular pathways necessary for osteoclast function and survival (Soto and Chiappe Barbará, 2014).

Not only do bisphosphonates work to inhibit osteoclast activity, but they have also been shown to increase viability of bone-forming cells, such as osteoblasts and osteocytes (Cremers et al., 2019). Because the mode of action of bisphosphonates on osteoblasts and osteocytes happens at concentrations lower than that of their effects on osteoclasts, it is likely that they are acting on these cells through different mechanisms (Soto and Chiappe Barbará, 2014).

1.2.4 Reason for Use in Veterinary Medicine

Bisphosphonates were originally developed for use in human medicine to treat diseases such as osteoporosis, Paget's disease, multiple myeloma, bone metastases, and several childhood skeletal disorders including osteogenesis imperfecta (Cremers et al., 2019). Studies involving bisphosphonate use in veterinary medicine are limited but have grown in the last 10-20 years (Suva et al., 2021).

These drugs are not only used in equids, but have implications in small animal medicine, as well. Bisphosphonates are popularly used in veterinary oncology for palliative care of bone tumors in dogs (Suva et al., 2021). These tumors often cause excruciating pain leading to a decreased quality of life, warranting amputation of the affected limb (Tomlin et al., 2000). However, amputation may not always be possible due to other factors, which is why bisphosphonates have been incorporated as an analgesic mechanism. Dogs being treated for bone tumor pain are frequently prescribed the nitrogen-containing bisphosphonates, pamidronate and zoledronate, which are the two most common bisphosphonates administered for this disease (Suva et al., 2021).

Feline hypercalcemia may also be treated with bisphosphonates such as pamidronate and alendronate (Suva et al., 2021).

Within equine veterinary medicine, tiludronate and clodronate are the only FDA-approved bisphosphonates. They are intended for the treatment of navicular disease and controlling symptoms associated with disease onset in horses aged four or older (Mitchell et al., 2019). Navicular disease is an inflammatory condition that affects any of the components of the trochlear apparatus of the foot, including the navicular bone (Soto and Chiappe Barbará, 2014). Symptoms that arise with navicular disease include pain and lameness. Several studies have shown that clodronate (Osphos™) is effective at reducing lameness in horses who have navicular disease (Markell et al., 2020). However, both tiludronate and clodronate are effective at controlling lameness symptoms of navicular disease (Richbourg et al., 2018). Any administration of bisphosphonates that is not specifically for navicular disease is considered off-label use and has not been FDA-approved due to lack of available studies (Mitchell et al., 2019).

Bisphosphonates have also been effective in off-label use for treatment of distal tarsal osteoarthritis (bone spavin) and chronic back pain (Suva et al., 2021). Currently, two studies have assessed the efficacy of tiludronate in the treatment of bone spavin. Both studies assessed lameness clinical score and showed optimal improvement in lameness two months after administration (Gough et al., 2010; Soto and Chiappe Barbará, 2014). In the second study, compared to placebo, horses treated with tiludronate for lameness caused by bone spavin had a significant improvement in their lameness score at 60 days. If horses were treated again on day 60 with tiludronate, they showed further improvement at 120 days compared to placebo horses (Gough et al., 2010)

1.3 Mass Spectrometry

Mass spectrometry represents several different mass analyzing, detection, and ionization methods (Zubarev and Makarov, 2013). The goal of mass spectrometry is to measure the mass-to-charge ratios of ions in the gas phase (Lane, 2005). It is widely used in molecular research, including proteomics experiments (Savaryn et al., 2016). While proteomics may be connotative to a discovery-based experiment, certain mass spectrometers, such as a triple quadrupole or Orbitraps[d], can perform targeted experiments using single reaction monitoring (SRM), multiple reaction monitoring (MRM), or parallel reaction monitoring (PRM) methods (Addona et al., 2009; Doerr, 2012; Rauniyar and Keck, 2015). Utilizing either bottom-up or top-down techniques, the mass spectrometer is able to control and use the fragmentation data that is generated efficiently for identification of specific mass-to-charge ratio (*m/z)* ions (Mann and Kelleher, 2008).

1.3.1 Discovery Methods

Proteomics is the study of the proteome, which is the complete set of proteins expressed by the genome (Amiri-Dashatan et al., 2018; Dupree et al., 2020). This branch of science aims to identify, characterize, and quantify the proteins which constitute a proteome (Lane, 2005).

In proteomics, biomarkers can be identified beginning with a discovery phase, which is performed using non-targeted techniques. One goal of protein biomarker selection is to determine whether specific proteins have been upregulated or downregulated (Parker and Borchers, 2014). These proteins are found to be contrastingly expressed between a normal and diseased state(s) (Rifai et al., 2006). Proteins detected in the discovery phase are known

as candidate proteins and/or candidate biomarkers and must be verified through a targeted method (Ronsein et al., 2015).

The discovery phase typically utilizes shotgun proteomics, also known as bottom-up proteomics (Parker and Borchers, 2014). In bottom-up proteomics, the protein is digested with an enzyme such as trypsin, and the peptides are then analyzed in the mass spectrometer using either electrospray ionization (ESI) or matrix-assisted laser desorption ionization (MALDI) techniques (Chait, 2006). However, when using trypsin, due to its high efficiency, up to 56% of the peptides that are produced may be too small to be detected by mass spectrometry. Most data analysis tools and software are optimized for tryptic properties, so bottom-up techniques may lead to a restricted view of the proteome (Dupree et al., 2020). Even still, bottom-up proteomics has been used by scientists for several years for quantifying proteins, protein modifications, and profiling the proteome (Zhang et al., 2013). Proteins are monitored by peptides because of their efficient ionization and predictable fragmentation patterns (Dupree et al., 2020).

Serum in both humans and equids is a complex matrix due to the high abundance of endogenous proteins, like albumin, which are proteins that can negatively affect mass spectrometric assays (Liu et al., 2014). During the discovery phase, there are many challenges that must be faced. One challenge is that serum proteins in this phase tend to make samples highly complex and variable. This can be dealt with by depleting proteins which are of high abundance or through fractionation of a sub-proteome (Gillet et al., 2016). Depending on the nature of the sample, it may be essential to perform a depletion to reduce the complexity. This is especially true of serum, which is made up of 50% albumin (Dupree et al., 2020).

1.3.2 Targeted Methods

Once the discovery method has identified candidate biomarkers, these proteins need to be qualified, verified, and validated using a targeted method (Parker and Borchers, 2014). The qualification step ensures that the differential expression of candidate biomarkers seen in the discovery step can also be seen using a targeted method (Rifai et al., 2006). Verification is used to confirm that the expression of candidate biomarkers can be seen in a greater number of samples than what was used in the discovery method (Rifai et al., 2006). Validation of biomarkers requires an even larger number of samples—many more than the qualification and verification (Zhang et al., 2013).

Many different methods can be utilized to perform targeted acquisitions. These methodologies include single reaction monitoring (SRM); multiple reaction monitoring (MRM), which is also known as selected reaction monitoring, or SRM; and parallel reaction monitoring (PRM). The triple quadrupole mass spectrometer is the optimal instrument for MRM experiments (Gillet et al., 2016; Liebler and Zimmerman, 2013).

In MRM, a target peptide's mass is identified and selected in the first quadrupole (Q1), fragmented in the second quadrupole (Q2), and generated fragments are then measured in the third quadrupole (Q3) (Picotti and Aebersold, 2012). The goal of an MRM experiment is to analyze a specific product ion derived from a precursor ion, termed Q3 and Q1, respectively. This allows for high sensitivity in the presence of a busy background (Sherman et al., 2009). By utilizing a triple quadrupole instrument, MRM is able to analyze complex mixtures of proteins and identify peptides which may be less abundant (Savaryn et al., 2016). In order to monitor analyte ions and their corresponding product ions, MRM utilizes the triple quadrupole instrument's ability to help develop assays, otherwise known

as MRM transitions, which must be optimized to verify optimal performance (Doerr, 2012). Three types of information must first be identified to complete an MRM analysis: (1) proteins that make up the target protein set, (2) peptides with good mass spectrometric responses and which discern the identified target proteins, and (3) the fragments which discriminate the peptides from other components in the sample (Lange et al., 2008). One of the challenges accompanying MRM proteomic analyses is the background and the analytes are both composed of peptides, which can sometimes lead to a limit of detection (LOD) (Gallien et al., 2013). However, MRM methods, when performed on a triple quadrupole mass spectrometer, have become the "gold standard" for targeted proteomics (Peterson et al., 2012). This is due to the predetermined scanning events and *m/z* ions associated with a target peptide that allow the triple quadrupole mass spectrometer to readily identify targeted ions in complex matrices.

On the other hand, PRM can be performed on quadrupole-Orbitrap mass spectrometers, where the Orbitrap instrument takes the place of Q3 (Rauniyar and Keck, 2015). Orbitrap mass spectrometers have the benefit of allowing one to perform both discovery and targeted methods on the same instrument (Dupree et al., 2020). PRM has been shown to generate high resolution data and act as a powerful method of targeted analysis (Liebler and Zimmerman, 2013). In contrast to MRM methods where selected transitions are monitored within an experiment, PRM methods allow use of a single analysis to monitor all fragmented ions, subtracting the need for tedious optimizations (Doerr, 2012). PRM is highly specific because all potential product ions, rather than just three to five selected transitions, are analyzed and used to validate peptide identity (Peterson et al., 2012). This makes PRM more selective than MRM due to better

differentiation of the targeted peptide from any background or interferences that may elute at the same time (Shi et al., 2016).

In a study comparing MRM's and PRM's ability to quantify proteins in a lipid nanoparticle, the results showed that they were comparable in their precision and repeatability as well as their linearity and dynamic range (Ronsein et al., 2015). PRM provides an efficient alternative route in the absence of MRM (Gallien et al., 2013).

1.4 Equine Bone Remodeling

Bone remodeling in humans is the process by which bone cells couple resorption and ossification in order to repair bone tissue (Suva et al., 2005). This same process occurs in horses. Bisphosphonates, such as tiludronate and clodronate, are aimed at inhibiting the resorption process that is central to bone remodeling in equids (Richbourg et al., 2018). Bone remodeling also has implications in current controversies in the horse racing world. Off-label use of bisphosphonates may occur in an attempt to improve the radiographs of young Thoroughbreds who are affected by sesamoiditis and are entered into a sale. This use is assumed to decrease bone resorption and potentially increase bone ossification, resulting in improved visuality on the radiograph; however, there is no direct evidence of this phenomenon (Suva et al., 2021). It has also been suggested, in racehorses, that bisphosphonates may be useful to prevent or treat several different diseases, including osteoarthritis and stress fracture risk (McLellan, 2017).

1.4.1 Racehorses

Thoroughbred racehorses are continually going through bone remodeling processes due to their rigorous training (McLellan, 2017). They often suffer from bone fatigue when

undergoing intense exercise, something that can increase the amount of microdamage and microcracks (Holmes et al., 2014). Microcracks can spread further if bisphosphonates have been administered and remodeling has not initiated due to the inhibition of osteoclastic resorption (McLellan, 2017). Fatigue injuries occur in "high load environments" and result from the fast accumulation of microcracks that cannot be repaired quickly due to the reduction in osteoclasts (Holmes et al., 2014; Smith et al., 2016). Fatigue fractures that occur in young Thoroughbred racehorses are located in the third metacarpal bone 70% of the time and are seen within the first year of training (Nunamaker et al., 1990). Many horses are able to work through these fractures with a brief respite from training (R Carpenter 2022, personal communication, 13 April). There have been studies that show more fractures occur during training than in racing, and this is likely due to the fact that horses train each week but only race every three to four weeks (Bailey et al., 1999).

In studies assessing the differences in bone remodeling in horses that are training versus those that are resting, it has been observed that actively training horses have lower levels of bone resorption and higher levels of bone formation due to an increase in habitual loading on certain bones (Holmes et al., 2014). In the racehorse, various diseases may occur due to inappropriate bone remodeling activity, which results from failing to adapt to high rates of strain placed on the affected bone (McLellan, 2017). These diseases include palmar/plantar osteochondral disease, sclerosis of the third carpal bone, and stress fractures of the tibia and pelvis (McLellan, 2017).

During fracture repair in mice, osteoclasts are a vital component to the efficiency of the restoration (Lin and O'Connor, 2017). Clodronate, when used during fracture healing,

has the potential to reduce normal bone healing in equids due to its inhibition of osteoclast recruitment (Richbourg et al., 2018).

Because bisphosphonates have the potential to interfere with normal bone development in juvenile racehorses and may increase fatigue fracture risk in adult racehorses, these drugs should be administered with caution when treating various orthopedic conditions in order to protect the welfare of the horse (Gilday et al., 2020; McLellan, 2017).

1.4.2 Radiograph Clearings of Young Horses

Thoroughbred yearlings are subjected to an examination before they are purchased at auctions. These examinations place a lot of importance on radiographic evidence of anything that may impair future racing (Spike-Pierce and Bramlage, 2003). At the time of these yearling auctions, sesamoiditis has been reported as the most common diagnosis from a radiograph (Mclellan and Plevin, 2013).

Equine sesamoid bones are susceptible to poor healing due to the lack of blood supply, as well as lack of musculature around them (Suva et al., 2021). Because of their location, sesamoid bones are vulnerable to increased force, tension, and fracture risk. These risks are intensified in young racehorses (Suva et al., 2021). It has been seen that some veterinarians may utilize bisphosphonates in the treatment of sesamoiditis of young (e.g., yearling) Thoroughbreds due to the assumption that these drugs heal the disease, at least visually on a radiograph (McLellan, 2017). This use of bisphosphonates is not supported by research. Bisphosphonates, when administered to young racehorses in this manner, may interfere with normal bone development, such as maturation of the epiphyseal plate (Gilday

et al., 2020). Because these drugs can have harmful effects on bone processes in horses under the age of four, caution should be taken when administering them to juvenile racehorses.

1.5 Biomarker Detection

Drug testing in performance horses, particularly those that compete in races, is of utmost importance to verify that races are won based on a horse's athletic ability, to avoid putting at risk the ethics of the horse breeding industry, and to ensure the welfare of horses and their riders (Fragkaki et al., 2017). There must be a strict balance between allowing therapeutic drugs for the welfare of the horse, while also protecting the integrity of the industry by ensuring those drugs are no longer affecting the horse when it comes time to race (Wong and Wan, 2014).

Drugs are metabolized quickly in the horse. The purpose of analyzing biomarkers is to be able to detect drug use across a longer sampling time period. The use of biomarkers allows for the possible detection of prohibited substances by discovery of compounds that persist in the biological system well after the original drug has been excreted (Cawley and Keledjian, 2017). Assessing the effects that drugs have on various proteins within the horse provides a wider time frame for drug detection and allows for observations of changes that may not be detectable through metabolites (Cawley and Keledjian, 2017). Equine doping has recently changed from small molecules to substances like designer drugs—a change that requires a shift of focus to analyzing the effects of doping, or what biological changes are generated through these agents (Barton et al., 2009).

1.5.1 Equine Drug Metabolism and Protein Biomarkers

Biomarkers are defined as "any measurable parameter altered as a result of a challenge to an individual's system" (Cawley and Keledjian, 2017). Drug metabolism in equids is not a well-studied area. Compared to human metabolism data, there is not as much drug metabolism data for horses because the veterinary pharmaceutical industry does not require as much information regarding metabolism for the approval of drugs (Scarth et al., 2011). Drug metabolism studies in equids often require the use of *in vivo* experiments, which can be difficult to obtain approval for due to the ethical aspects of animal experimentation (Scarth et al., 2010). In order to utilize metabolites in the detection of prohibited substances, the drug's metabolism must be understood, which can prove to be ethically problematic with recently developed drugs, such as designer steroids, due to lack of toxicology data (Fragkaki et al., 2017).

1.5.2 The Shift from Equine Metabolite Biomarkers to Equine Protein Biomarkers

Metabolites are often used in identification and/or detection of drugs which are highly metabolized and may only be detected by discovery of their metabolites (Waller and McLeod, 2017). These can be useful even if no initial substance is detected (Waraksa et al., 2019). However, when metabolism studies are performed *in vivo*, metabolites in urine are often quite complicated to detect due to various reasons. These reasons include interferences that complicate identification, long experimental timescales, and the difficulty of determining certain enzymes that play a role in the metabolism of the drug (Scarth et al., 2010). In response to this, drug metabolism studies can be carried out *in vitro*,

which can be more advantageous than *in vivo* studies, although there are still limitations that come with these types of analyses (Scarth et al., 2010).

In recent years, new drugs have been developed that are not as detectable as drugs that have been used in the past. This is where biomarkers become useful because they can be utilized to identify the effect that a drug has on a biological system without requiring detection of the drug itself (Wong et al., 2020). There are also certain drugs, like anabolic steroids, that have rapid elimination times, making them difficult to detect in normal doping tests. This leads to the need for different ways to identify these types of substances, such as looking for upregulation of various proteins that is an indirect result of the drug (Mori et al., 2015).

For these types of doping tests, equine plasma is a fitting medium as it is routinely sampled and it tends to be abundant in biomarkers (Barton et al., 2009). Compared to urine, plasma acts as a better indicator of the effect of the drug, with few exceptions (Toutain, 2010). However, plasma also contains proteins such as albumin that can complicate purification of target biomarkers (Bailly-Chouriberry et al., 2008).

Overall, using metabolites to detect drug use in horses is decreasing in favor of protein biomarkers that are changed in response to the prohibited substance, thereby increasing detection windows (Ho et al., 2015).

1.6 Summary

Bisphosphonates are becoming more relevant for use in the racehorse due to assumptions that this class of drugs may increase bone strength, decrease pain associated with disease, or visually clear radiographic evidence of sesamoiditis when Thoroughbreds

go up for auction as yearlings. Because bisphosphonates have a rapid elimination time, it is difficult to analyze any metabolites.

With the introduction of new drugs that are being designed to conceal substance use in racehorses, there is a need for a better detection method that goes farther than analyzing metabolites. Bottom-up proteomics techniques performed on Orbitrap instruments allow for the shift to occur to analyzing drug effects on proteins within the biological system while increasing the window in which the drug can be detected.

Biomarkers are an important aspect of drug testing in performance horses. These permit the detection of prohibited substances by analyzing proteins that may be upregulated or downregulated in comparison with the same proteins in a normal, untreated horse.

In order to protect the welfare of racing Thoroughbreds, as well as the integrity of the breeding and racing industry, there must be better drug testing implemented that can assess the use of performance drugs and therapeutics, such as bisphosphonates. This can be accomplished by utilizing biomarker detection and proteomics methods, both of which can increase the window of time in which a prohibited substance can be detected.

CHAPTER 2. IDENTIFICATION OF BISPHOSPHONATE PROTEIN BIOMARKERS USING A MASS SPECTROMETRY DISCOVERY METHOD

2.1 Introduction

Equine drug testing has become more important over the last several years due to various factors, such as the development of designer drugs—drugs that are rapidly eliminated from the biological system and make it nearly impossible to detect through normal drug testing techniques. These types of substances put at risk the integrity of the breeding industry, as well as the welfare of the horses that are administered these drugs (Fragkaki et al., 2017). Because of this, there must be new techniques developed for drug testing to increase the window of time in which prohibited substances can be detected in the performance horse. Protein biomarkers, which have changed in response to the administration of some substance, such as a bisphosphonate, allow for increased detection windows of banned substances.

Bisphosphonates are a therapeutic class of drugs that are used to inhibit bone resorption in humans, small animals, and equids for various reasons (Suva et al., 2021). Nitrogen-containing bisphosphonates can be up to 10,000 times more potent than non-nitrogen containing bisphosphonates (Kamm et al., 2008). In humans, they are used to treat bone resorptive diseases such as osteoporosis; Paget's disease of bone; metastatic bone tumor pain; and childhood skeletal disorders, such as osteogenesis imperfecta (Soares et al., 2016). In small animals, they can treat feline hypercalcemia, as well as bone tumor pain in dogs (Suva et al., 2021; Tomlin et al., 2000). Currently, there are two FDA-approved non-nitrogen containing bisphosphonates for the treatment of navicular disease in horses: tiludronate and clodronate.

Bisphosphonates inhibit bone resorption by targeting the bone resorptive cells, osteoclasts, for apoptosis. Non-nitrogen containing bisphosphonates create cytotoxic analogues of ATP, which in turn inhibits ATP-dependent pathways that the osteoclast relies on for survival (Mitchell et al., 2019; Soto and Chiappe Barbará, 2014). Osteoclasts depend heavily on ATP generation, which is why they are full of mitochondria. The non-nitrogen containing bisphosphonates are thought to inhibit the ADP/ATP mitochondrial translocase inside the osteoclast, leading to a series of events that cause apoptosis (Reszka and Rodan, 2003). Nitrogen-containing bisphosphonates target the mevalonate pathway, which is responsible for the production of cholesterol and other sterols (Russell, 2007). This pathway also results in the isoprenylation of proteins, such as small GTPases that are necessary for osteoclast function and survival (Coxon et al., 2006). By inhibiting the enzyme farnesyl diphosphate synthase, nitrogen-containing bisphosphonates inhibit this isoprenylation and further block the formation of the osteoclastic ruffled border, vesicular trafficking, and cytoskeletal arrangement resulting in apoptosis of the osteoclast (Kimmel, 2007; Mitchell et al., 2019).

Bisphosphonates have low oral bioavailability, which is why they are frequently administered in the horse either intravenously or intramuscularly (Cremers et al., 2019; Soares et al., 2016). Uniquely, bisphosphonates do not undergo phase one or two metabolism. These drugs are taken up into bone or excreted renally (Cremers and Papapoulos, 2011). If taken up into bone, they tend to congregate in areas of high bone turnover, making their distribution highly heterogeneous. Their skeletal elimination is very slow, and studies have found evidence of bisphosphonates in the urine years after administration (Riggs et al., 2001; Soares et al., 2016).

Equine athletes experience continuous bone remodeling due to their rigorous training modules (McLellan, 2017). This training can also cause racehorses to undergo bone fatigue, a phenomenon that increases the risk of microcracks and microdamage (Holmes et al., 2014). When bisphosphonates are administered and bone remodeling is impaired due to the lack of osteoclast-mediated resorption, microcracks may spread further because they are unable to be repaired by normal remodeling processes (McLellan, 2017). The perpetuation of the microcracks can increase the risk of fatigue injury in the horse's high loading environments (Holmes et al., 2014; Smith et al., 2016). For this reason, bisphosphonates should be administered with caution in adult Thoroughbred racehorses.

In some instances, juvenile racehorses may be administered bisphosphonates to treat sesamoiditis. This occurs because of the assumption that bisphosphonates visually clear signs of the disease on a radiograph and may make these horses more attractive as yearlings when they go to auction (McLellan, 2017; Suva et al., 2021). This use of bisphosphonates is not supported by research. Bisphosphonates are only approved for use in horses over the age of four because this is when bone remodeling naturally slows (Mitchell et al., 2019). Administration of this class of drug in horses under the age of four may interfere with normal remodeling processes and have harmful effects on the bone development of the juvenile horse. Caution should be taken when giving bisphosphonates to young horses for this reason.

Bisphosphonates are drugs that are rapidly excreted from the urine and make it difficult to use normal drug testing techniques to confirm their administration. This is true for many of the new designer drugs that are hard to detect metabolically, which can increase their risk for abuse in equine athletes (Barton et al., 2009). To lengthen the window of time

in which these drugs can be detected in the horse, a study was conducted in which equine sera samples were analyzed for proteins that may be upregulated or downregulated in response to the administration of bisphosphonates.

Utilizing a bottom-up proteomics method for analysis of equine sera samples and identification of biomarkers requires that the proteins in the samples be digested into their subsequent peptides. Trypsin, the gold standard of enzymatic digestion of proteins, was chosen to digest these samples due to its efficiency, inexpensiveness, and resistance to autolysis (Gillet et al., 2016; Switzar et al., 2013). In bottom-up proteomics, digestion is an essential step to ensure high quality identification of proteins (Switzar et al., 2013). Trypsin may not always be the optimal choice for enzymatic digestion due to a number of factors, so proteases such as pepsin or endoproteinases like Arg-C and Asp-N may be used instead (Switzar et al., 2013). In addition to enzymatic digestion techniques, nonenzymatic cleavage may also be used in bottom-up proteomics to break down the protein. Electrochemical oxidation is a method which occurs at the amino acids tyrosine, tryptophan, histidine, cysteine, and methionine resulting in specific cleavage of the peptide bond that is C-terminal to tyrosine (Permentier and Bruins, 2004).

After digestion of proteins, the peptides must be de-salted and freed of contaminants before injection into the Orbitrap™ mass spectrometer[d]. Any excess salts or detergents can interfere with the mass spectrometry analysis and may contribute to unwanted background noise, so a clean-up step is frequently employed (Gundry et al., 2009). Solid phase extraction (SPE) is a method that is widely utilized in proteomics, which is why Strata-X SPE C^{18} columns were used for the purposes of cleaning up equine sera samples in both phases of this study (Tubaon et al., 2017).

Mass spectrometry is the optimal method for proteomics, which includes analyzing complex protein samples (Aebersold and Mann, 2003). To analyze the proteins that have changed in response to bisphosphonate administration, an Orbitrap™ mass spectrometer[d] was used to conduct discovery-based and targeted experiments. A bottom-up proteomics technique was used, where the protein is digested with trypsin and the resulting peptides are analyzed in an Orbitrap™ mass spectrometer[d] (Chait, 2006). The Orbitrap™ Exploris 480[d] used in this phase was the optimal instrument due to its high resolving power of 480,000 full width at half maximum (FWHM) resolution. Analysis of complex proteomes, such as the ones being examined in this study, is frequently best completed using high resolution technologies, which is why samples were analyzed using the Orbitrap™ Exploris 480[d] (Mann and Kelleher, 2008). This instrument also employs nanoflow technology. In order to detect low abundance endogenous proteins, methodologies must be highly sensitive, a characteristic not available by conventional liquid chromatography. Nanoflow is a methodology developed to utilize low flow rates that result in higher analytical sensitivity for detection of peptides, making nanoflow an optimal technique for the identification of low abundance biomarkers (Cutillas, 2005). Data generated on the Orbitrap™ mass spectrometer[d] is analyzed using Proteome Discoverer™ (PD) Version 2.4[e]. This software has evolved to address several different types of proteomic workflows, including the label-free quantitation that is used in this study. Advantages of PD[e] that factored into its use for the discovery phase include flexible data input from several different sources, direct access to the MS/MS spectra aiding in identification, and the ease of exporting data into an inclusion list format that can be used on the Orbitrap™ mass

spectrometer[d] for verification of identified biomarkers in the targeted phase (Orsburn, 2021).

Biomarkers can be identified in the discovery phase, but must be validated using a targeted method, such as PRM. PRM is a way to generate higher resolution data while subtracting the need for time-consuming optimizations (Doerr, 2012; Liebler and Zimmerman, 2013). It allows for the simultaneous detection of transitions, which is more efficient than SRM or MRM.

The goal of this chapter is to analyze the effects of bisphosphonates on different proteins and identify those which are relevant and can be used in the future as biomarkers of bisphosphonate administration.

2.2 Materials and Methods

2.2.1 Administration Studies

A total of 19 horses (n = 19) were given a single administration of Tildren®, manufactured by Bimeda, at 1.0 mg/kg intravenously over a 90-minute period. These horses were all anonymous, so no information regarding age or sex was given. Serum was collected over a monitored time course, described in **Table 2-1**. Horses were in-training across the time course. The samples were collected at the convenience of the veterinarian, which is why not all time points are represented.

2.2.2 Sample Preparation

All reagents and chemicals used are Optima® or ReagentPlus® LC/MS grade and are manufactured by Fisher Chemical or Sigma-Aldrich. Master horse sera samples were

analyzed in conjunction with the 19 horse samples as a baseline control. The master horse sera samples were a pool of normal horses. They are different horses than those focused on in this study and were not sampled on the same days as the 19 equine subjects. These horses are presumed to be normal and were not administered any substance prior to sampling. They were prepared for LC-MS/MS injection the same way as the 19 equine subjects in this study.

2.2.2.1 Organic Solvent Fractionation

In order to effectively analyze proteins in equine sera through LC-MS/MS techniques, abundant endogenous proteins must be considered and dealt with accordingly. These proteins can interfere with LC-MS/MS analysis and must be removed to increase the sensitivity and ruggedness of assays. The most abundant endogenous protein in sera is albumin, which makes up about 50% of the total protein. There have been several techniques developed to remove this abundant protein, but the most suitable for this study was an isopropanol/trichloroacetic acid (IPA/TCA) fractionation (Liu et al., 2014). This solvent fractionation method is able to remove 95% of the albumin and recover almost 100% of target proteins (Liu et al., 2014). The protocol used for this experiment is based on the IPA/TCA fractionation optimized by Liu et al. (2014).

A solution of 1% TCA in IPA was prepared in a volume of 5000 µL. Equine serum (20 µL) was aliquoted into 1.5 mL Eppendorf tubes. Into the tubes, 200 µL of IPA/TCA solution was added, followed by two minutes of vortexing[l] to mix. The tubes were placed in the centrifuge[i] at 5ºC and spun at 1500 x g for five minutes. The albumin-containing supernatant of IPA/TCA was removed and saved for later analysis. The pellet was washed with 200 µL of methanol, followed by 30 seconds of vortexing[l] to mix. The tubes were

placed back into the centrifuge[i] at 5ºC and spun at 1500 x g for 2 minutes. The supernatant containing methanol was removed and saved for later analysis. The pellet, containing precipitated serum proteins, was placed in a -20ºC freezer until digestion.

2.2.2.2 Tryptic Digestion

For the purposes of analyzing proteins that may have been changed in response to bisphosphonate administration, a bottom-up proteomics technique was used. This method involves breaking down the protein into individual peptides, analysis of peptides using LC-MS/MS technology, and piecing together the resulting information to reveal the protein characteristics (Gundry et al., 2009). To break down the protein into peptides, enzymatic digestion is a popular route to follow. For the purposes of this study, the enzyme used was trypsin. Trypsin is a favored enzyme because of its inexpensiveness, robustness, as well as its ability to generate certain size peptides that are optimal for chromatographic analysis (Gillet et al., 2016).

Several solutions were prepared for this step, including 75 mM ammonium bicarbonate (ABC), 200 mM dithiothreitol (DTT), iodoacetamide (IAA), and 1 µg/µL trypsin. Double-distilled water[b] was added to both ABC and DTT after appropriate amounts were weighed. IAA was weighed out and the appropriate amount of ABC to add was calculated and adjusted if needed due to the light sensitivity of IAA. ABC was not added to the IAA until it was needed for digestion. Stock trypsin was diluted to an active concentration of 1 µg/µL in 75 mM ABC during the last step of digestion.

Previously fractionated protein pellet samples were resuspended in 1000 µL of 75 mM ABC and vortexed[l] rapidly to ensure adequate resuspension. For each sample, 6.25

µL was deposited into a 1.5 mL Eppendorf tube for digestion. To these tubes, 50 µL of 75 mM ABC, 7 µL of 100% acetonitrile (ACN), and 3.5 µL of 200 mM DTT were added. The tubes were vortexed[l] to mix, and pulse centrifuged[h] to ensure removal of components from the side of the tube. The tubes were then placed on a heating block at 37ºC for one hour. After this incubation, the 200 mM IAA was brought to its calculated volume with ABC. Nine µL of prepared 200 mM IAA were added to each tube, followed by vortexing[l] to mix, and pulse centrifugation[h]. The tubes were then placed in a drawer to incubate in the dark for 30 minutes at room temperature. After this incubation, stock trypsin was diluted with ABC to its active concentration (100 ng/µL). The samples were diluted with 50 µL of 75 mM ABC. Two µL of active trypsin was added to each tube, followed by vortexing[l] to mix, and pulse centrifugation[h]. The samples were placed on the heating block at 37ºC for overnight incubation. After this incubation, the samples were placed in the freezer to stop the digestion.

2.2.2.3 Strata Column Clean-Up

To optimize the data generated on the LC-MS/MS, the samples needed to have contaminants removed while retaining target proteins at the best recovery rate. For this, Strata-X SPE columns were used. The manufacturer's protocol was modified for the equine sera samples.

Solutions prepared included 5% ACN/0.1% formic acid/water[b] (equilibration/wash solution), 70% methanol/0.1% formic acid/water[b] (elution solution), 10% formic acid, and 1% formic acid. 100% formic acid was serially diluted to achieve required concentrations. The equilibration/wash solution and elution solution were then prepared in the appropriate volumes. Falcon tubes were placed into a vacuum manifold[k]. The columns were

conditioned twice with 1 mL of methanol, utilizing the vacuum to pull the methanol through each time. Next, the columns were equilibrated once with 1 mL of equilibration/wash solution, which was pulled through using the vacuum. Samples were thawed, centrifuged[h], and the total volume was applied to the column, and pulled through into the Falcon tube using the vacuum. The column was then washed twice with 1 mL of equilibration/wash solution, pulling the contents through the column each time. Digests were eluted from columns by applying 500 mL of elution solution two times, pulling the solution through each time. Falcon tubes containing eluted digests were briefly spun down to collect eluant. The eluants were then transferred to a 1.5 mL Eppendorf tube. The samples were dried down under centrifugal evaporation in the Savant SpeedVac SPD 1030 Integrated Vacuum Chamber[f].

2.2.3 Mass Spectrometry Injection

For LC-MS/MS injection, the resulting protein in the samples must be resuspended. This resuspension allows for a total of 10 µg of protein in digest. The resuspension buffer was 5% ACN, 0.1% formic acid, and double-distilled water[b]. The dried down samples were resuspended in 100 µL of buffer and vortexed[l] thoroughly to mix. They were then pulse centrifuged[h] to ensure all the components were removed from the side of the tube. SureStop MS injection vials were labeled, and glass inserts were placed inside. Thirty µL of the reconstituted digest was transferred into the glass insert and these were then placed in the LC-MS/MS instrument for duplicate 10 µL injections. Digested peptides were chromatographically separated using the UltiMate 3000RSLC nano liquid chromatography system[j]. This system is equipped with an Easy Spray PepMap™ RSLC C18 column[c] (3 µm, 100Å, 75 µm x 15 cm) and an Acclaim™ PepMap™ trap column[a] (3 µm, 0.075 mm

x 20 mm) set at 35ºC. There were two mobile phases, A and B. Mobile phase A contained 0.1% (v/v) formic acid in water[b] while mobile phase B contained 0.1% (v/v) formic acid in acetonitrile. A linear gradient of 5-31% mobile phase B over 85 minutes at a flow rate of 0.3 µL/minute was used for peptide separation. Data-dependent acquisitions (DDA) were performed on a Orbitrap™ Exploris 480 mass spectrometer[d]. The instrument was operated in positive ion mode using a top-20 method.

To acquire survey scan mass spectra (350-1200 *m/z*), a nominal resolution of 60,000 FWHM and a normalized target of 300% were utilized. Acquisition of fragmentation spectra was completed using a nominal resolution of 15,000 FWHM with a normalized collision energy of 30% and dynamic exclusion of 20 seconds, normalized automatic gain control (AGC) target of 50%, maximum injection time of 50 ms, and isolation window of 1.4 *m/z*. The electrospray ionization settings included a spray voltage of 2500 V, capillary temperature of 275ºC, and an RF lens value set at 40%. No sheath, auxiliary, or sweep gas were applied.

2.2.4 Label-Free Quantitation and Data Analysis

Once the samples were finished running on the Orbitrap™ mass spectrometer[d], the acquired mass spectra data were analyzed using PD 2.4[e]. Protein identification and label-free quantitation was performed against the *Equus callabus* database, downloaded from Uniprot (17 March 2021).

Using custom built processing and consensus workflows, the acquired data were analyzed. Processing workflows included two iterative searches using SequestHT, Percolator validation, and Minora Feature Detector. Basic search settings utilized in the

first iterative search included static modification of carbamidomethylation (+57.021 Da); a precursor mass tolerance of 10 ppm; a fragment mass tolerance of 0.02 Da; a maximum of two miscleavages; a dynamic modification of oxidation (+15.999 Da); protein N-terminal modifications of acetylation (+42.011 Da), methionine loss (-131.010 Da), and methionine loss plus acetylation (-89.030 Da). The second iteration searched for any additional peptide modifications.

The role of the consensus workflow was to filter for high confidence peptides with enhanced protein and peptide annotations. The confidence thresholds were determined by target false discovery rates (FDRs) of 0.01 (strict) and 0.05 (relaxed). An FDR is the ratio between false peptide spectra matches (PSM) to the total number of PSMs above the score threshold, or the measure of the incorrect PSMs among all accepted PSMs. The strict FDR is set for highly confident PSMs, while the relaxed FDR is for PSMs with medium to low confidence. Quantitation was performed using unique and razor peptide classification(s). The protein grouping strict parsimony principle was set to true.

The individual horse and its corresponding time points were analyzed as biological replicates to identify any changes (upregulation or downregulation) to protein abundances during the sampled time course. This was repeated for all 19 horses. The workflow executed analyses on these changes and allowed for the visualization of the data through graphs and tables generated in Microsoft 365 Excel (Version 2201) and SigmaPlot (Version 14.5). PD 2.4[e] data was used to produce a heat map that portrayed the changes in the four identified biomarkers across the full-time course. This software and the data it generated also allowed for the determination of percent differences in abundances of the

four proteins compared to their abundance at day 0. Abundance at day 0 was calculated by averaging the five horses who were sampled at day 0 for each protein.

2.3 Results

Through label-free quantitation (LFQ), a total of 19 proteins were identified that were postulated as relevant to bisphosphonate administration (**Table 2-2**). Out of these, four key proteins were focused on: actin (cytoplasmic), carbonic anhydrase, fibrinogen, and fibronectin. Each of these proteins had abundance patterns that were easily identified after performing custom-built workflows. While no significance tests were performed, clear, measurable trends were observed in each of the four protein biomarkers and will be discussed in the following paragraphs. All upregulation and downregulation patterns are those in comparison to previous days' abundances. For example, upregulation at day 7 indicates an increase in abundance when compared to day 3, and a downregulation at day 18 indicates a decrease in abundance when compared to day 7.

Actin (cytoplasmic) exhibited an abundance pattern of upregulation at day 7, downregulation at day 18, and upregulation at the next time point, depending on the horse and whether it was sampled on these particular days. This pattern was observed in five horses with relevant time points. For four other horses, there was an abundance pattern of upregulation at day 49. These patterns can be seen in **Figure 2-1A**, **Figure 2-2A**, and **Figure 2-3**. By using custom built workflows, PD 2.4[e] was able to find high confidence peptides for actin. Fragmentation spectra for two of these high confidence peptides can be found in **Figure 2-4A**. These are both exemplar spectra for this protein and are

representative of MS2 fragmentation patterns. They are from the same time point in the same horse.

Carbonic anhydrase exhibited an abundance pattern similar to that of actin. Five horses showed upregulation at day 7, downregulation at day 18, and upregulation at the next time point. Three horses had an upregulation of carbonic anhydrase at day 49. These patterns can be seen in **Figure 2-1B**, **Figure 2-2B**, and **Figure 2-3**. PD 2.4[e] refined peptides to those with high confidence that identified carbonic anhydrase. Two high confidence peptides' fragmentation spectra can be found in **Figure 2-4B**. These spectra were chosen based on how well they represent MS2 fragmentation. They are from the same time point in the same horse.

Fibrinogen presented the same abundance pattern as the previous two proteins. Five horses showed an upregulation at day 7, downregulation at day 18, and an upregulation at the next time point. Three horses exhibited an upregulation day 49. Abundance patterns are presented in **Figure 2-1C**, **Figure 2-2C**, and **Figure 2-3**. Fibrinogen's high confidence peptides were chosen in PD 2.4[e] using iterative searches and custom-built workflows. In **Figure 2-4C**, fragmentation spectra of two of these peptides are depicted and are representative of MS2 fragmentation. Both spectra are from the same time point in the same horse.

Fibronectin, the final protein identified, displayed an opposite trend compared to the first three identified proteins. Six horses showed a downregulation at day 7 and five of those horses showed a subsequent upregulation at day 18 (due to relevant time points). These patterns are shown in **Figure 2-1D**, **Figure 2-2D**, and **Figure 2-3**. **Figure 2-3** is meant to compare the abundances at each time point to the abundance at day 0. For

fibronectin, this table shows a downregulation at day 0, and this was taken into consideration when analyzing the heat map. It still depicts a downregulation at day 7 and day 21 in comparison to the upregulation seen at day 18, however, the downregulations are not depicted in red due to day 0 showing a downregulation. Day 7 and day 21 show a slight upregulation in comparison to day 0 but are still downregulated in comparison with day 18. Fibronectin fragmentation spectra are shown in **Figure 2-4D**. These spectra represent two of the several high confidence peptides that were found in PD 2.4[e]. They are characteristic of MS2 fragmentation data and are exemplar spectra for this protein.

Average abundances of the four proteins in the master horse can be found in **Table 2-3**. These abundances, for the most part, are higher than those found in **Figure 2-1 A-D** and are represented by the dotted line present on each graph. Carbonic anhydrase is the only protein where the majority of abundances are upregulated compared to the average abundance of carbonic anhydrase in the master horse sera.

2.4 Discussion

The four identified proteins and their individual abundance patterns are postulated to have some relevance to the administration of bisphosphonates. Actin and carbonic anhydrase are both found in osteoclasts and aid in their ability to resorb bone. There is evidence that shows fibrinogen is located on the surface of osteoclasts (Athanasou et al., 1988). Fibronectin is deposited from osteoblasts for the purposes of ossification. These proteins were changed early in comparison to the known maximum efficacy of bisphosphonates at sixty days. However, because the bisphosphonate was administered to

the equine subjects intravenously rather than orally, it is possible that the maximum resorption suppression took place earlier (Drake et al., 2008).

The type of actin identified is beta-actin, which can be found in several different types of cells within the horse. Beta-actin is important in cell motility and migration in all cells of the body (Bunnell et al., 2011). As osteoclasts begin to resorb bone, they must degrade the bone matrix. To do this, they form a complex that consists of a ruffled border and actin rings; the actin rings will form podosomes that evolve into the osteoclast sealing zone, facilitating bone resorption (Han et al., 2019). Podosomes are found to have a dense core of actin, while also consisting of other proteins like integrins (Destaing et al., 2003). The actin rings are unique to osteoclasts, since most other cells arrange their fibrillar actin into stress fibers (Teitelbaum, 2007). Actin's abundance pattern shows an initial upregulation at day 7. This increase is postulated to be the result of an osteoclast receiving a signal to begin resorbing bone. The osteoclasts, in order to facilitate resorption, must utilize actin to create podosomes and, in turn, the sealing zone that is integral to making contact with the bone matrix. This would, in theory, cause an upregulation in the concentration of actin. The goal of bisphosphonates is to reduce bone resorption by targeting osteoclasts. At day 18, there is a downregulation in the concentration of actin. Bisphosphonates accumulate in the bone and become active when bone resorption occurs, thereby releasing them (Lin, 1996). At day 7, when the osteoclast received the signal to begin bone resorption, it increased its actin to facilitate the degradation of the bone matrix, which resulted in the release of the bisphosphonates that had been previously administered. The bisphosphonate, once released, becomes active and causes apoptosis of the osteoclast by blocking its ATP-dependent pathways, resulting in a decrease in actin abundance seen at day 18. It is

important to keep in mind that because actin is located inside the osteoclast, apoptosis, and therefore lysis of the cell, may cause transitory changes in the abundance of actin.

Furthermore, bone resorption requires acidification of the bone matrix. This is enabled by carbonic anhydrase II (CA II), an enzyme that produces protons from the conversion of carbon dioxide into bicarbonate (Riihonen et al., 2007). CA II can be found in erythrocytes, as well as the large intestine in horses (Sasaki et al., 1993). It has been shown *in vivo* that mutation of the CA II gene can lead to osteopetrosis, a disease that results from the inhibition of bone resorption, meaning that this enzyme is also found in bone (Lehenkari et al., 1998). This enzyme, therefore, is likely upregulated when osteoclasts begin resorbing bone since its activity is necessary for the initial degradation of the bone matrix. It is postulated that the initial upregulation in carbonic anhydrase abundance is due to the activation of osteoclastic activity. Again, since bisphosphonates are released from the bone and subsequently activated after osteoclasts begin resorption, it is likely that the apoptosis of the osteoclast resulted in a downregulation of carbonic anhydrase—an enzyme that would not be needed if osteoclastic resorption is not actively occurring.

Fibrinogen is a protein that is typically involved in blood coagulation and is cleaved by thrombin resulting in fibrin protein (Almeida et al., 2020). This protein has also been observed to increase somewhat within 72 hours following inflammation (Copas et al., 2013). There has been evidence that shows fibrinogen binding to the surface of osteoclasts, although the exact reason for this is unknown (Athanasou et al., 1988). Through a study assessing the main function of plasmin as it relates to bone biology, it was demonstrated that fibrinogen is an exogenous activator of the bone remodeling unit, namely bone

resorption (Cole et al., 2014). This protein presented an abundance pattern of upregulation at day 7 and downregulation at day 18. Since the osteoclast likely received a signal at day 7 to begin resorbing bone, its activity presented as an increase in fibrinogen, while the apoptosis that followed was conveyed as a decrease in fibrinogen at day 18.

The final protein identified, fibronectin, displayed an abundance pattern that was the opposite of the previous three proteins, as previously mentioned. Fibronectin is a protein that is found in the bone matrix, but it also acts as the master organizer of extracellular matrix assembly in all cell types (Halper and Kjaer, 2014). Studies have shown that the presence of fibronectin is necessary for the initial assembly of the collagen matrix in bone, but it is also needed continuously to ensure the integrity of the bone matrix (Bentmann et al., 2010). This protein is the earliest protein synthesized by osteoblasts for the bone matrix (Tang et al., 2004). Bone remodeling requires a tightly regulated balance between osteogenesis (performed by osteoblasts) and osteolysis (performed by osteoclasts) to ensure tissue homeostasis (Cole et al., 2014). It was previously postulated that the upregulation of actin, carbonic anhydrase, and fibrinogen at day 7 and downregulation at day 18 was the result of initial osteoclast-mediated bone resorption followed by a bisphosphonate-mediated decrease in osteolysis activity. Because bone cells and their subsequent actions are tightly regulated, an osteoblast would be turned off when osteoclasts are signaled to resorb bone, and then turned on once the osteoclast completes resorption or goes through apoptosis. At day 7, it can be suggested that the osteoclastic activity suppressed the activity of the osteoblast, meaning no fibronectin was being deposited to begin generation of new bone, resulting in a downregulation of fibronectin abundance at this time point. Once the bisphosphonate blocked osteoclast ATP-dependent pathways and

caused apoptosis of the bone resorbing cell, the osteoblast likely received a signal to begin laying down new bone. This process would have caused an increase in fibronectin, at least initially (Bentmann et al., 2010). An upregulation of fibronectin is seen at day 18.

The master horse represents a control in that it depicts basal levels of the four key protein biomarkers in a normal horse. The overall downregulation in each protein compared to the average abundance in the master horse sera can be attributed to the fact that the 19 equine subjects in this study were experiencing a symptomatic disease that required some intervention. The master horse sera are representative of horses that are presumed to be normal and not suffering from any type of disease. Day 0 of the blood samples represented a control, also, since no drug had been administered at that point. Ratios of the identified biomarker abundances at each time point with the abundance at day 0 can be found in **Table 2-3** and **Figure 2-2 A-D**.

Because these proteins are not specific to the bone remodeling process, they can be found in other areas of the biological system. Variability of these biomarkers and their different locations in the horse are discussed in Chapter 3 Section 3.5. Limitations of the discovery and targeted based portions of this study are located at the end of Chapter 3 in Section 3.6.

2.5 Conclusion

The discovery phase of this study identified four protein biomarkers that were hypothesized to change in response to bisphosphonate administration. The natural variation of time points made it somewhat difficult to compare all 19 horses, but there were distinct abundance patterns found in each of the four proteins in almost all subjects. Actin, a protein

found in the podosomes of osteoclasts once they begin resorbing bone, conveyed changes in concentration at day 7, day 18, day 21, day 28, and day 49. These changes were likely due to normal remodeling processes being interrupted by the administration of the anti-resorptive agent, tiludronate. Carbonic anhydrase, an enzyme responsible for the acidification of the bone matrix during remodeling, experienced distinct abundance pattern alterations at day 7, day 18, day 21, day 28, and day 49. Because this enzyme is crucial to osteoclastic activity, those upregulations and downregulations were likely due to the bisphosphonate that was initially administered. Fibrinogen, a protein postulated to be an exogenous activator of bone resorption and found on the surface of osteoclasts, exhibited changes in abundance at day 7, day 18, day 21, day 28, and day 49. Because fibrinogen is found on osteoclasts, it may have been upregulated and downregulated at the same time as the osteoclast. The goal of the bisphosphonate is to cause apoptosis of the osteoclast, which, in turn, causes changes in the concentrations of fibrinogen. Finally, fibronectin is a protein found in the bone matrix and synthesized by osteoblasts, had an abundance pattern opposite of the previous three proteins, with changes being exhibited at day 7, day 18, day 21, day 28, and day 49. Because this protein's goal is opposite of the osteoclast, it was likely upregulated when osteoclastic proteins were downregulated, and vice versa.

Each of these protein biomarkers can be traced back to bone remodeling and bisphosphonate administration in some way. The equine subjects were in training at the time tiludronate was given and when blood samples were taken across the various time points. Because horses in training experience continuous remodeling, especially in high load environments, the initial upregulations in abundance of the first three proteins that are seen at day 7 may have been due to their rigorous training regimens. However,

bisphosphonates' goal is to suppress normal remodeling by inhibiting resorption and increasing the viability of osteoblasts to complete ossification. So, the subsequent downregulation seen in osteoclastic proteins is likely a result of bisphosphonate action on the osteoclast. The upregulation of fibronectin, the osteoblastic protein, was presented at the same time as the downregulation of the proteins in the osteoclast, which may be a display of increased viability of the osteoblast due to the administration of tiludronate.

Further confirmation that these proteins are changed in response to bisphosphonate administration and have some significance to bone remodeling can be seen by the differences in abundance between the 19 equine subjects and the master horse sera. The proteins' abundances are decreased in the equine subjects in this study compared to their average abundance in a master horse serum sample, seen in **Table 2-3** and **Figure 2-1 A-D**. The horses in this study were affected by some disease and were administered bisphosphonates as treatment. It is likely that the abundances of the four key protein biomarkers will be changed in a treated horse in comparison with a normal horse.

These biomarkers, after further validation in the targeted phase, will be integrated into the Equine Biological Passport to create a way to identify the restricted use of bisphosphonates during a race across a longer detection window. The four key proteins identified represent a way to help protect the integrity of the racing industry, as well as the welfare of horses.

TABLES

Table 2-1: Monitored Time Courses of 19 Horses Following Tildren® Administration[1]

		TIME POINTS SAMPLED												TOTAL TIME POINTS
		0d	1d	3d	7d	18d	21d	28d	31d	34d	42d	49d	57d	62d	
HORSE	A												■	■	2
	B										■				1
	C			■	■				■						3
	D										■	■	■	■	4
	E	■	■	■	■	■	■	■		■	■				9
	F	■	■	■	■	■						■			6
	G			■	■				■	■					4
	H									■	■	■	■	■	5
	I			■		■		■		■					4
	J							■		■	■				3
	K						■	■		■	■				4
	L									■	■	■	■	■	5
	M	■	■	■	■	■									5
	N	■	■	■	■	■					■		■	■	8
	O	■	■	■	■	■		■		■	■				8
	P									■	■	■			3
	R								■						1
	S												■	■	2
	T						■								1

[1]19 equine subjects and what days blood samples were taken from them.

Table 2-2: 19 Proteins Identified Through Label-Free Quantitation[2]

Protein	**Accession No.**
Actin beta like 2	F7AHF3
Actin gamma 2, smooth muscle	A0A3Q2HBP5; A0A3Q2KTQ9
Actin, cytoplasmic 1	A0A3Q2KTQ9
Carbonic anhydrase	A0A3Q2HMG5; F6ZBG0
EGF containing fibulin extracellular matrix protein 1	F6PVG3
Elongation factor 1-alpha	F6UME7
Extracellular matrix protein 1	F6QYS3
Fibrinogen	A0A3Q2GS38
Fibrinogen alpha chain	A0A3Q2HTG2;
Fibrinogen gamma chain	A0A5F5PPB8
Fibrinogen beta chain	F6PH38
Fibronectin	A0A5F5PTE1; F7CN11; Q28377
Fibulin 1	F7ABC9
Intercellular adhesion molecule 3	F6SIE2
Interleukin 1 receptor accessory protein	A0A5F5PI46
Matrix metallopeptidase 19	F7C6S0
Talin 1	F6QIZ4
Transforming growth factor-beta-induced protein ig-h2	F6UMQ4
Vitronectin	F6V881

[2]The 19 proteins that were identified from the data through LFQ that showed some significant abundance pattern. Highlighted are the proteins and accessions of the four key proteins identified in the discovery phase.

Table 2-3: Master Horse Sera Average Abundance of Four Key Protein Biomarkers[3]

Accession	Description	Average Abundance	Std Deviation	Std Error
A0A3Q2KTQ9	Actin, cytoplasmic 1 OS=Equus caballus OX=9796 GN=ACTB PE=3 SV=2	**1.03E+07**	**2.46E+06**	**9.30E+05**
F6ZBG0	Carbonic anhydrase OS=Equus caballus OX=9796 GN=CA1 PE=3 SV=1	**1.47E+06**	**5.66E+05**	**2.14E+05**
A0A3Q2HTG2	Fibrinogen alpha chain OS=Equus caballus OX=9796 GN=FGA PE=4 SV=2	**3.62E+06**	**6.58E+05**	**2.49E+05**
F7CN11	Fibronectin OS=Equus caballus OX=9796 GN=FN1 PE=4 SV=2	**3.21E+07**	**7.12E+06**	**2.69E+06**

[3] The average abundances of actin, carbonic anhydrase, fibrinogen, and fibronectin in a master horse sera sample

FIGURES

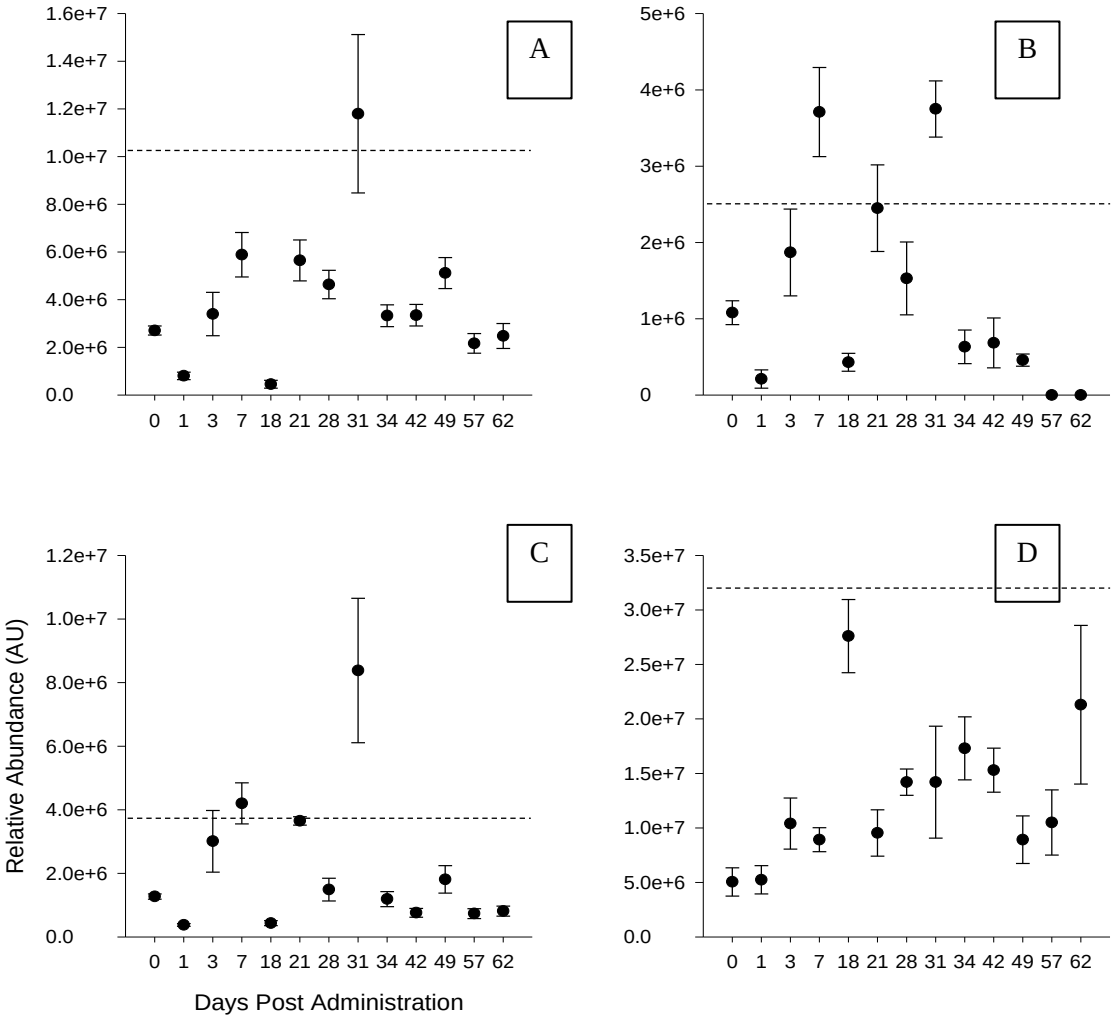

Figure 2-1 A-D: Global Discovery Figure of Relative Abundances of Four Key Proteins Identified with Master Horse Sera Average Abundance.

Figure 2-1: Master horse sera average abundance is represented as a dotted line. (A) The abundance patterns of the protein actin from day 0 to day 62. This constitutes each horse and the standard error are shown. The dotted line represents average abundance of actin observed in master horse sera. (B) The abundance patterns of carbonic anhydrase of each horse that is represented at each time point, as well as the standard error. Master horse sera average abundance of carbonic anhydrase is represented by the dotted line. (C) The abundance patterns of fibrinogen from day 0 to day 62. This constitutes each horse and the standard error is shown. The dotted line represents average abundance of fibrinogen observed in master horse sera. (D) The abundance patterns of fibronectin of each horse that is represented at each time point, as well as the standard error. Master horse sera average abundance of fibronectin is represented by the dotted line.

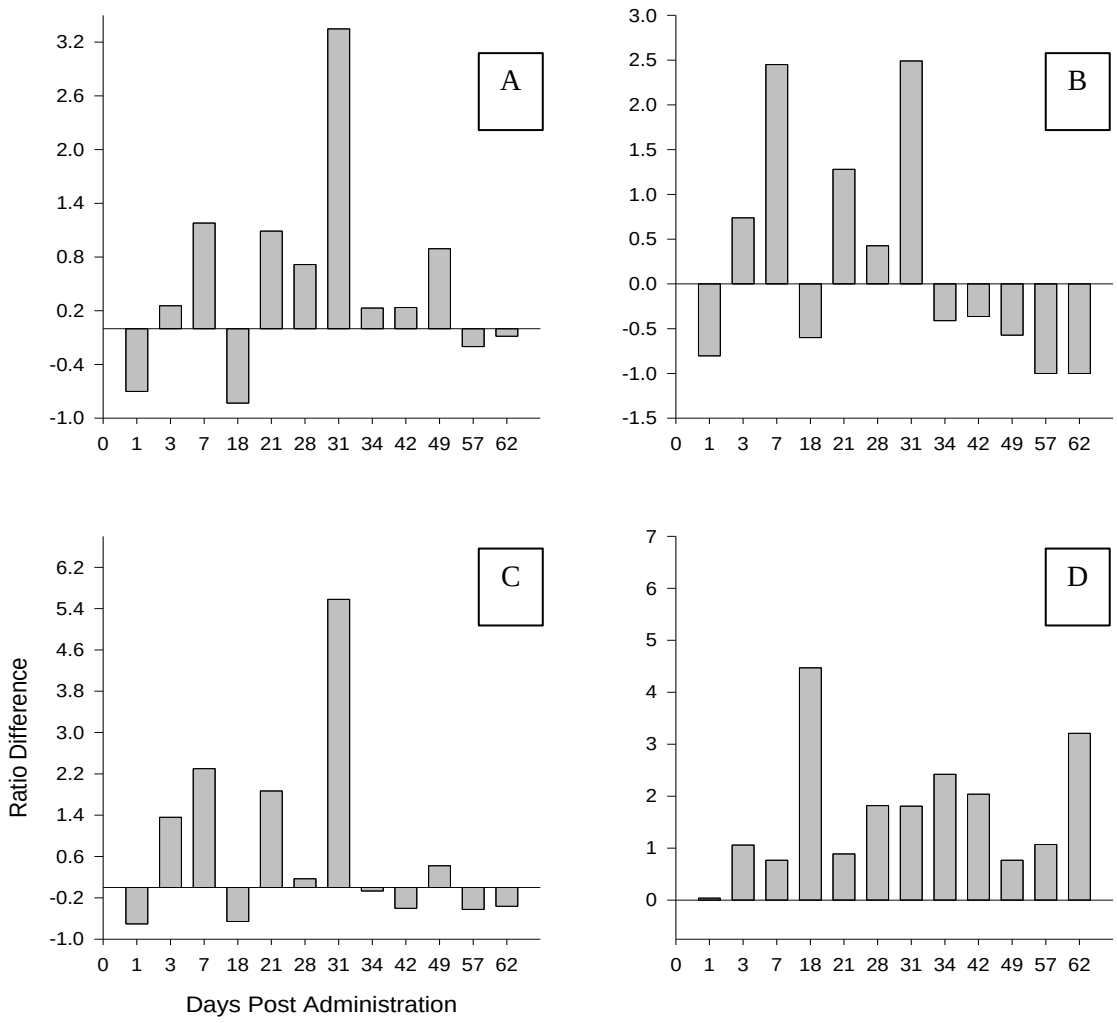

Figure 2-2 A-D: Ratio Differences of Four Key Proteins Compared to Day 0 Abundance.

Figure 2-2: (A) This graph is meant to compare the abundance pattern of actin at each time point with the abundance at day 0. The bars that fall below the central line represent downregulation in comparison to day 0 abundance and the bars that fall above the central line represent upregulation in comparison to day 0 abundance. (B) The abundance pattern of carbonic anhydrase compared to the abundance at day 0. (C) Fibrinogen's abundance pattern in comparison to the abundance at day 0. (D) The abundance pattern of fibronectin as compared to day 0 abundance.

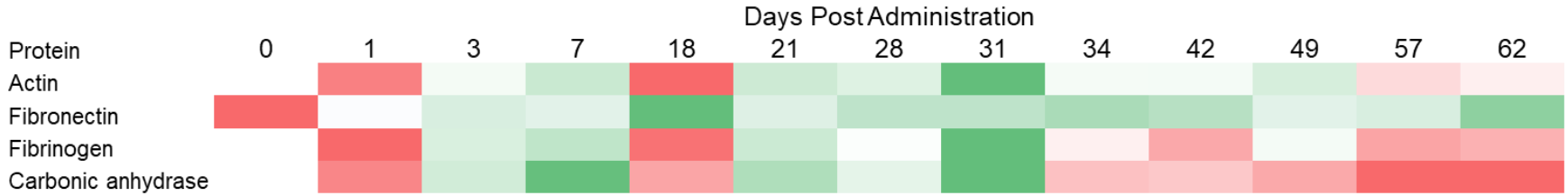

Figure 2-3: Heat Map of Abundance Patterns Compared to Day 0.

Figure 2-3: A heat map of the four key protein biomarkers from day 0 to day 62. Red represents downregulation in comparison to the abundance at day 0 and green represents upregulation in comparison to the abundance at day 0. Actin, carbonic anhydrase, and fibrinogen all exhibit an upregulation at day 7 and day 21, and a downregulation at day 18. Fibronectin, similar to **Figure 2-2D,** shows no downregulation except at day 0. However, the abundances at day 7 and day 21, where there should be downregulation, show a color that is closer to white, which represents a downregulation from the abundance seen at day 18.

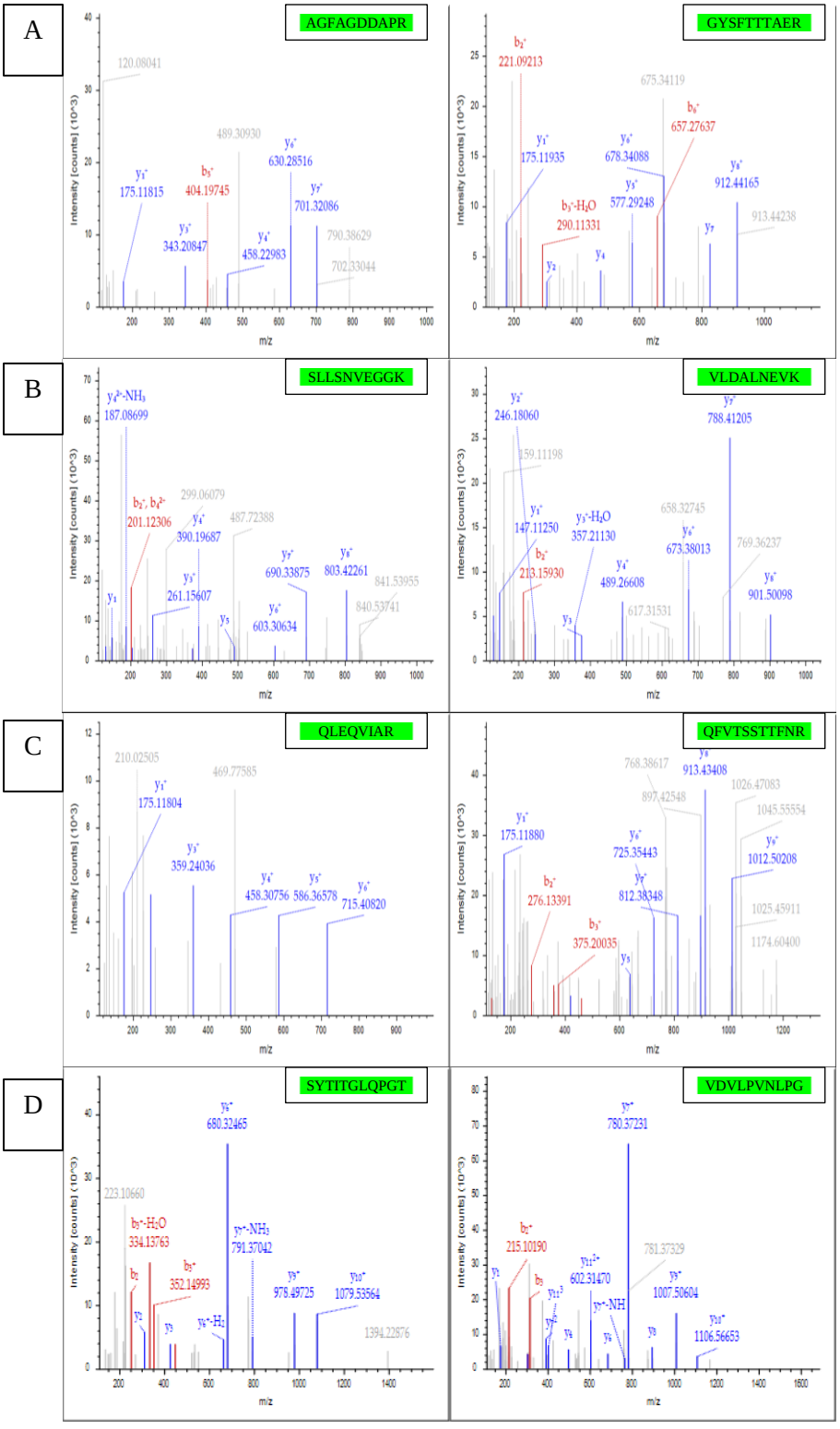

Figure 2-4 A-D: Peptide Fragmentation Spectra.

Figure 2-4: (A) Peptide fragmentation spectra of actin. Each of these spectra are from the same time point in the same horse and were chosen because they are high confidence peptides. These spectra also represent ms2 fragmentation well. This figure depicts good fragmentation patterns for the primary accession of actin. Included in this figure is the peptide that is represented in the fragmentation spectra. (B) Peptide fragmentation spectra of carbonic anhydrase. Two high confidence peptides are represented, and each spectrum is from the same time point in the same horse. The spectra portray quality patterns of fragmentation for carbonic anhydrase's primary accession. Included in this figure is the peptide that is represented in the fragmentation spectra. (C) Peptide fragmentation spectra of fibrinogen. Each spectrum was chosen based on acceptable fragmentation patterns of the primary accession of fibrinogen, as well as good ms2 fragmentation representation. The spectra are from the same time point in the same horse. Included in this figure is the peptide that is represented in the fragmentation spectra. (D) Peptide fragmentation spectra of fibronectin. Two high confidence peptides were chosen based on how well they represented ms2 fragmentation and the quality of their fragmentation patterns. The spectra are from the same time point in the same horse. Included in this figure is the peptide that is represented in the fragmentation spectra.

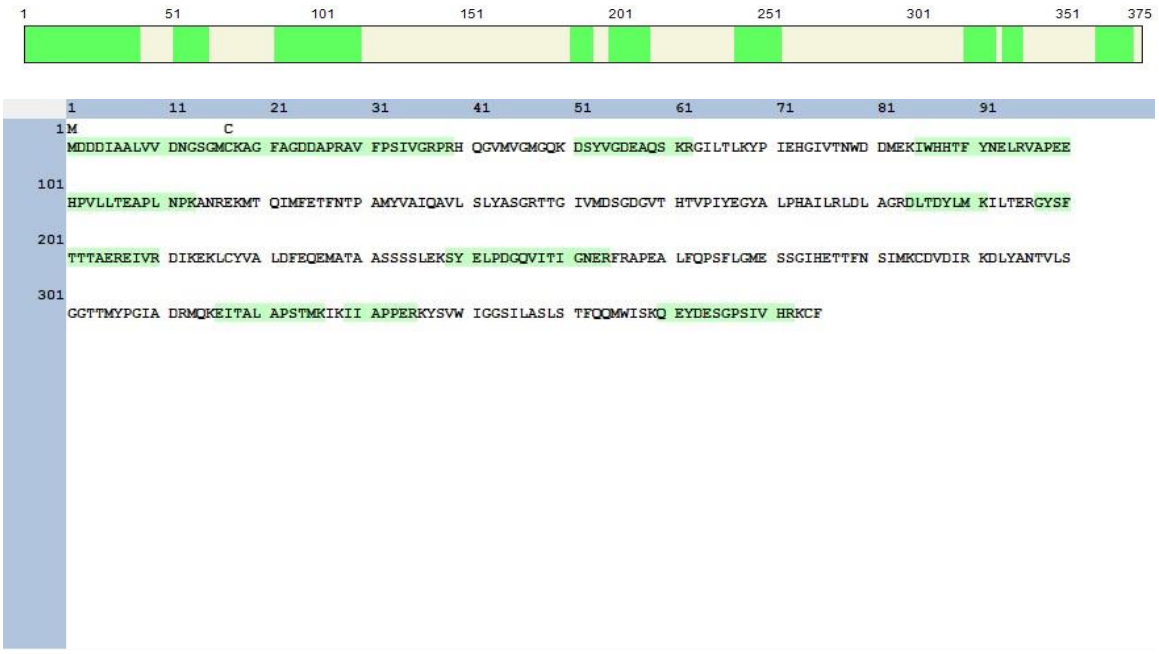

Figure 2-5: Actin Protein Sequence.

Figure 2-5: The protein sequence of the primary accession of actin. The amino acid sequences that are highlighted within the main sequence are the peptides used by PD 2.4[e] for LFQ.

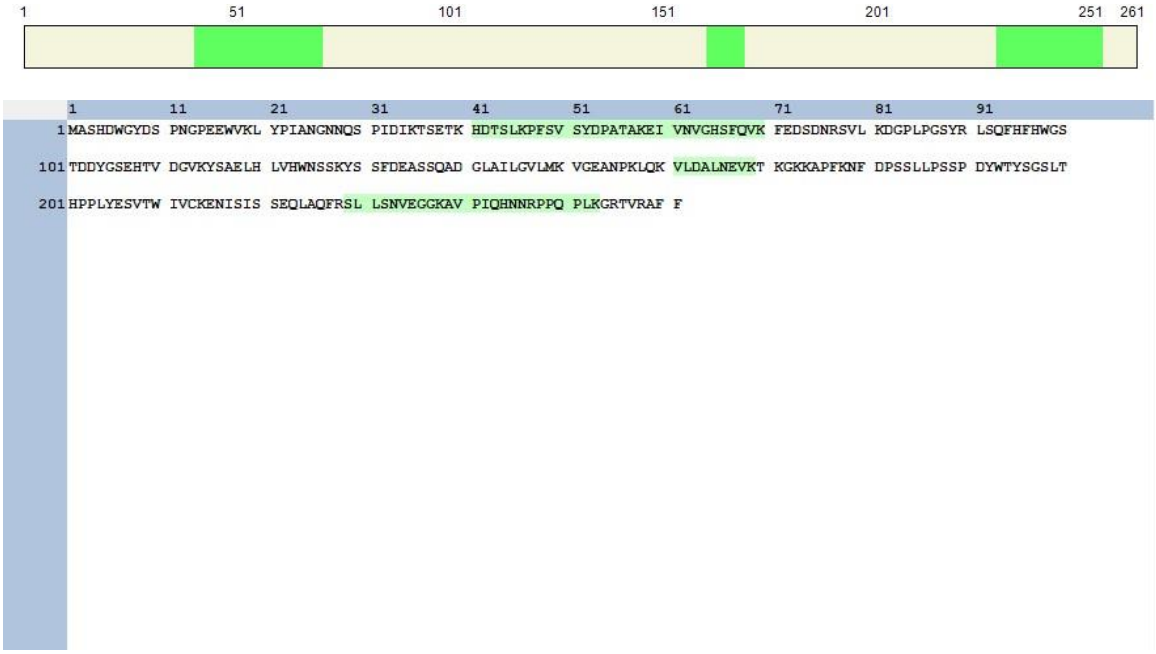

Figure 2-6: Carbonic Anhydrase Protein Sequence.

Figure 2-6: The protein sequence of carbonic anhydrase. This sequence represents the main accession used for data analysis. The highlighted portions are those used by PD 2.4^{e} for LFQ.

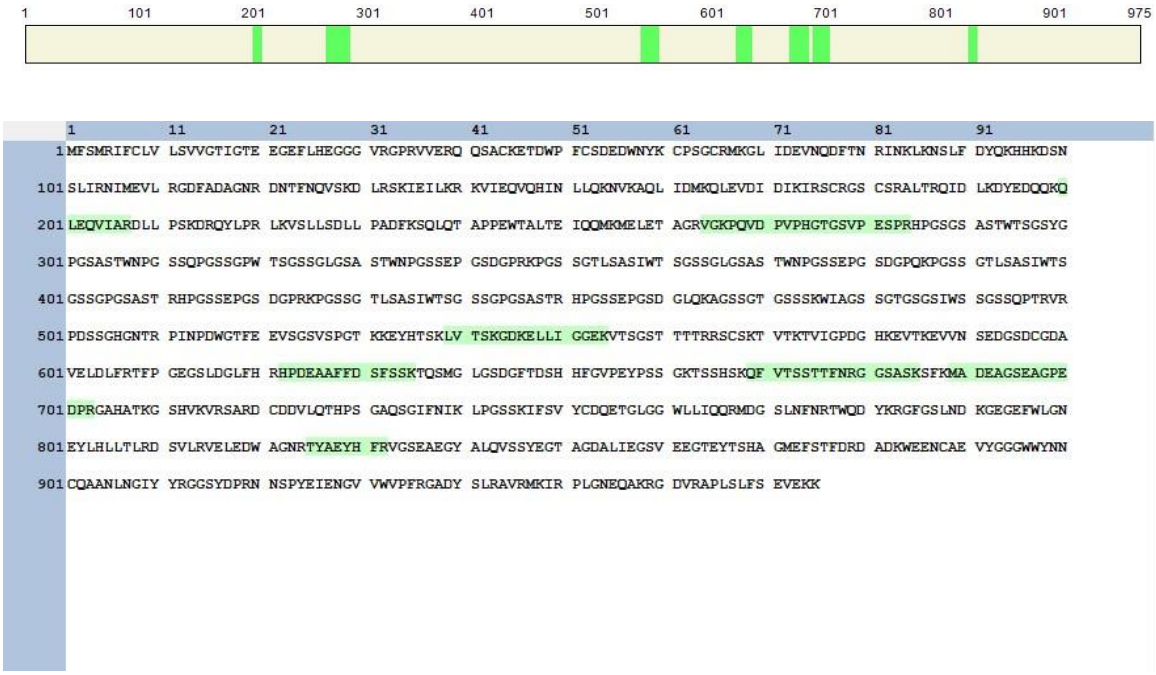

Figure 2-7: Fibrinogen Protein Sequence.

Figure 2-7: The main accession protein sequence of fibrinogen. The small sequences that are highlighted represent the peptides used by PD 2.4^{e} for LFQ.

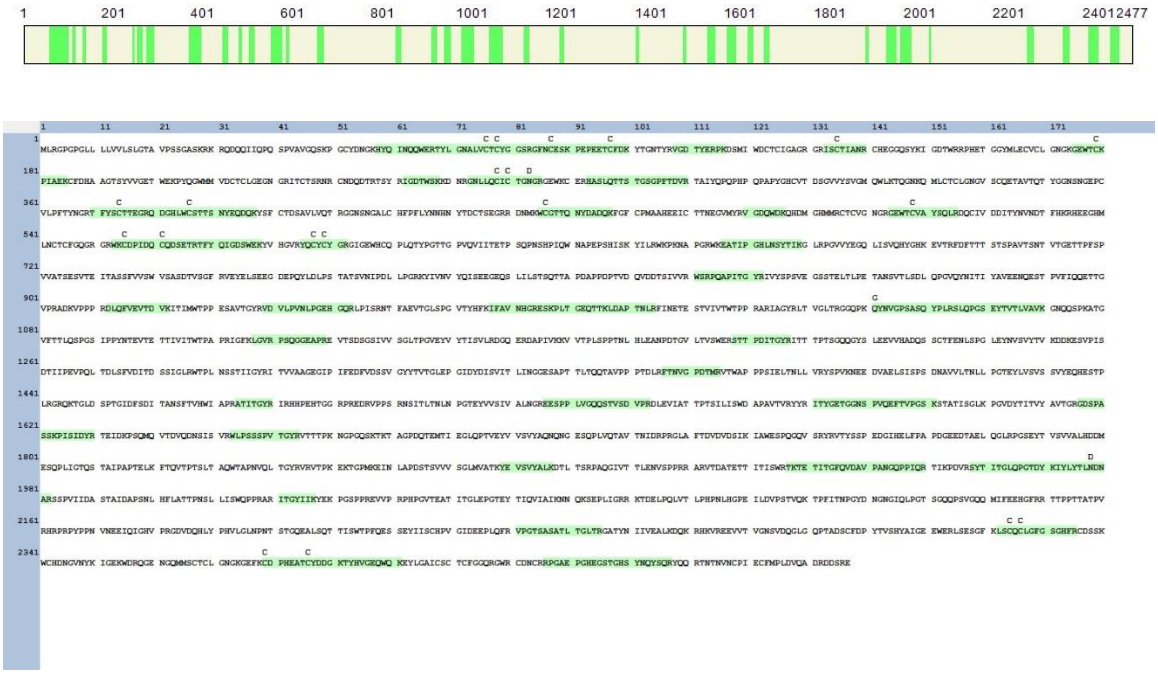

Figure 2-8: Fibronectin Protein Sequence.

Figure 2-8: Fibronectin's main accession protein sequence is depicted in this figure. The highlighted sequences are peptides that were used for LFQ within PD 2.4[e].

CHAPTER 3. VERIFICATION OF IDENTIFIED BISPHOSPHONATE PROTEIN BIOMARKERS USING A MASS SPECTROMETRY TARGETED METHOD

3.1 Introduction

Bisphosphonates are anti-resorptive drugs that are used for treatment of diseases in humans and animals. Once administered either intravenously or intramuscularly into the horse, the drug travels through the bloodstream and deposits into areas of the skeleton that have the highest turnover rates, resulting in a heterogenous deposition (Soares et al., 2016). The proportion of drug that is not incorporated into bone is rapidly excreted through the urine without being metabolized (Reszka and Rodan, 2003). Because of this, a targeted method must be developed to identify and validate any proteins that may be changed in response to the administration of bisphosphonates. These proteins will be considered biomarkers that can aid in the identification of prohibited substances during horse racing.

There are several different targeted techniques that can be used to validate the biomarkers that were identified in the discovery phase. SRM, MRM, and PRM are targeted methods that are widely used in proteomics. SRM is a technique that was used in the beginning of targeted proteomics that only allowed the monitoring of one transition at a time. MRM is a technique that gained popularity when the capacity of instruments to perform workflows increased. This targeted method, sometimes referred to as selected reaction monitoring, is utilized for the quantification of analytes that are predetermined and have known fragmentation properties (Abbatiello et al., 2010; Lange et al., 2008). Used on its optimal instrument, a triple quadrupole mass spectrometer, which can act as a mass filter, MRM can monitor specific analytes and their subsequent fragment ions; the pair that consists of analyte and fragment ion is known as a transition (Picotti and Aebersold, 2012).

This targeted technique can quickly measure the transitions and allow for simultaneous analysis of analytes—a step up from the single transition that SRM could monitor (Picotti and Aebersold, 2012). PRM, the targeted technique used in this study, has been shown to result in a wider range of quantitative data than MRM against a complex background (Peterson et al., 2012). Unlike MRM, PRM does not require pre-selection of transitions, making it easier to develop a method (Rauniyar and Keck, 2015). Another advantage that PRM has over MRM is the availability of all product ions in a single analysis to confirm the peptide's identity, rather than only three to five selected transitions that can be monitored using MRM (Peterson et al., 2012). There have been studies that show PRM and MRM exhibit similar linearity, precision, and repeatability of protein quantification in high density lipoprotein; however, because PRM requires less method development and has the potential to be more specific than MRM, PRM is the preferred technique of a targeted phase (Ronsein et al., 2015). PRM data was analyzed using Skyline[g] software. This software is an application popularly used in targeted proteomics and was selected for this study due to its simplicity of method development, sharing ability, and the way it displays results clearly and efficiently (MacLean et al., 2010).

The four proteins identified in the discovery phase—actin, carbonic anhydrase, fibrinogen, and fibronectin—were validated using PRM for the targeted phase of this study. While these proteins are involved in bone remodeling, they are not specific to the process. However, their abundance changes at the same time points in several of the horses suggest a link to bisphosphonate administration.

3.2 Materials and Methods

3.2.1 Sample Preparation

Samples from each horse and each time point (n = 79) were fractionated, enzymatically digested, and de-salted in duplicate, as previously described in Chapter 2. The duplicate biological samples were injected into the Orbitrap™ mass spectrometer[d] in duplicate.

3.2.1.1 Organic Solvent Fractionation

The goal of organic solvent fractionation is to remove the abundant and complex protein albumin from the samples to increase detection of relevant peptides on the mass spectrometer[d]. The protocol for IPA-TCA fractionation developed and optimized by Liu et al. which was used in the discovery phase of this study, seen in the Materials and Methods section of Chapter 2, is also used in the targeted phase (Liu et al., 2014).

3.2.1.2 Tryptic Digestion

To assess the peptides of the four biomarkers through a PRM method, trypsin was used as it was in the discovery phase to digest the proteins into peptides. These can be reconstructed after mass spectrometric injection to analyze the proteins of interest. Trypsin is a popular enzyme for this step in discovery and targeted phases due to its inexpensiveness and effectiveness when digesting proteins (Gillet et al., 2016). The protocol found in the Materials and Methods section of Chapter 2 is unchanged in the targeted phase.

3.2.1.3 Strata Column Clean-Up

The final step before instrument injection is clean-up. This procedure is important to ensure the quality of peptides being injected and analyzed. Its role is to remove contaminants, like natural salts, that may be present on the peptide, while ensuring that the peptide itself is eluted from the C^{18} size Strata-X SPE column. The protocol from the manufacturer of Strata-X SPE columns (Phenomenex) was modified for the equine sera samples in the Chapter 2 Materials and Methods section and is used in this phase, as well.

3.2.2 Mass Spectrometry Injection

LC-MS/MS injection requires the digested protein to be resuspended in 5% ACN, 0.1% formic acid, and double-distilled water[b] for a total of 10 µg of protein in digest. The resuspension buffer was prepared the same as the buffer in the discovery phase, for which the protocol can be found in the Chapter 2 Materials and Methods section. Samples were resuspended in 100 µL of buffer and SureStop MS injection vials were utilized in this phase, as well. Once samples were prepared and placed in the Orbitrap™ mass spectrometer[d], they were queued for duplicate 10 µL injections for analysis. A PRM method was developed for the targeted phase based on the untargeted data where four proteins of interest were identified. This method was used to analyze digested proteins on an Orbitrap™ Exploris 480 mass spectrometer[d] coupled with an UltiMate 3000 RSLCnano[j].

Specifications for chromatographic separation in the targeted phase were similar to those used in the discovery phase. Digested peptides were chromatographically separated using the UltiMate 3000 RSLCnano liquid chromatography system[j]. The specifications for

the Easy Spray PepMap™ RSLC C18 trap column[c] are unchanged from the Materials and Methods section of Chapter 2. There were two mobile phases: mobile phases A and B. The column was equilibrated for 7 minutes at 2% mobile phase B. The gradient was ramped to 10% mobile phase B from 7-10 minutes. A linear gradient from 10% to 25% mobile phase B, occurred over 25 minutes. A second linear gradient from 25% to 55% mobile phase B over 4 minutes, followed by 98% mobile phase B over one minute. Mobile phase B was maintained at 98% for 5 minutes followed by re-equilibration at 2% mobile phase B for 5 minutes. The total chromatographic run time was 50 minutes.

The instrument was operated in positive ion (+) mode using a targeted mass spectra (tMS2) methodology. To obtain tMS2, an inclusion list of relevant peptides selected from the discovery data acquisitions, described previously in chapter 2, were utilized. The inclusion list consisted of peptide sequence, retention times, *m/z* values, and peptides charge state. A nominal Exploris 480™ resolution of 60,000 FWHM and a normalized target of 300% were utilized. The normalized higher energy C-trap dissociation (HCD) collision energy was 30% and dynamic exclusion was 20 seconds. A standard AGC target, maximum injection time of 200 ms, and isolation window of 1.6 *m/z* were utilized. The electrospray ionization settings for spray voltage, capillary temperature, and RF lens value are unchanged from Chapter 2. As in the discovery phase in Chapter 2, no sheath, auxiliary, or sweep gas were applied.

3.2.3 Label-Free Quantitation and Data Analysis

Data obtained from the Exploris 480™ mass spectrometer[d] were analyzed using Skyline[g] software. For each of the four identified protein biomarkers, the optimal peptides and transitions were selected based on data gathered initially in the discovery and targeted

phases. Using Skyline[g] software and iterative targeted PRM acquisitions to obtain the most favorable peptides and transitions, 103 peptides were refined down to 23 unique peptides based on retention time, fragment ion ppm tolerance (±10 ppm), and number of detectable transitions. These unique peptides were also chosen based on their peak area and proteotypic properties (i.e., being truly tryptic). The refinement process removed peptides that had poor peak shape and area and were undetectable from the background matrix. Detection of ions and peptides is greatly affected by the complexity of the sera background. The 23 unique peptides, as well as their *m/z* ratios and retention times, can be seen in **Table 3-1**.

Semi-quantitative analyses that were performed using PRM methodology and the top three most abundant peptides for each identified biomarker were determined. The percent differences from day 0 were determined for each time point from day 1 to day 62.

3.3 Results

Refinement of the 103 initial peptides resulted in 23 unique peptides for all four proteins. Actin had four unique peptides, carbonic anhydrase had three unique peptides, fibrinogen had five, and fibronectin had eleven. The top five to six transitions for each peptide were included in the data analysis. In Skyline[g], each protein exhibited the same trends that were seen in the discovery phase of this study in both replicates that were analyzed. In the following paragraphs, observations made based on targeted PRM data for each replicate analyzed will be discussed. All upregulation and downregulation patterns are observed the same way as they were in the discovery phase. An upregulation on a certain day indicates an abundance increase when compared to the previous time point,

while downregulation is indicative of an abundance decrease compared to the previous time point. No significance tests were performed, and observed trends are from clear, measurable patterns.

Actin was upregulated at day 7, downregulated at day 18, and upregulated at day 21, day 28, day 42, or day 49 depending on the horse and what days it was sampled. This was true for all four peptides in both replicate one and replicate two. For horses with the relevant time points, the abundance of actin was also upregulated at day 57 and downregulated at day 62 for all four peptides in both replicates. The average of the top three most abundant peptides for actin was used to determine the percent difference from day 0 for each time point. Days 1, 3, 18, 34, 42, 57, and 62 were all downregulated in comparison with day 0. Day 18 was downregulated at a 164% difference from day 0. Day 62 was downregulated at 131%. Day 7 exhibited an upregulation of 37% compared to day 0. These patterns can be seen in **Figure 3-1A**. The abundance patterns of actin are also represented in **Figure 3-2A**. Upregulation is exhibited on day 7, while day 18 is downregulated. There is also a clear upregulation on day 21. The standard error (SE) is also presented on this graph but is not visible because of its very small variance, indicating that the abundances are precise in representing the population. Actin's abundance patterns can also be viewed in the peak area graph created in Skyline software, shown in **Figure 3-3A**. This graph only shows one replicate, but duplicate samples are shown for each time point.

In replicate one, carbonic anhydrase exhibited upregulation at day 7, downregulation at day 18, and, depending on the horse, upregulation at day 21, day 28, day 42, or day 49 in two of the three unique peptides. In the third peptide, the protein still

showed an upregulation at day 7 and downregulation at day 18, but days 21, 28, 42, and 49 were at too low of an abundance to determine upregulation or downregulation. In replicate two, carbonic anhydrase was upregulated at day 7, downregulated at day 18, and upregulated on days 21, 28, 42, and 49 for all three peptides. In replicates one and two, this biomarker was upregulated at day 57 and downregulated at day 62 in all six horses with relevant time points in peptide one. In peptide two, the abundances were too low to confirm a pattern at these time points in any of the six relevant horses. In replicate one, peptide three showed this abundance pattern in two of the six relevant horses. In replicate two, peptide three showed this abundance pattern in all six relevant horses. Carbonic anhydrase, in comparison to day 0, exhibited downregulation on days 1, 3, 18, 34, 42, 49, 57, and 62. Day 1 was downregulated at 157% compared to day 0. Day 18 was downregulated at 110%, day 57 was downregulated at 128%, and day 62 was downregulated at 159% when compared with day 0. Carbonic anhydrase displayed an upregulation in abundance compared with day 0 on days 7, 21, 28, and 31. Day 7 was upregulated at 96%. This can be seen in **Figure 3-1B**. In **Figure 3-2B**, carbonic anhydrase upregulation and downregulation is shown on days 7 and 18. Days 28, 31, 34, 42, and 49 are all upregulated in comparison to day 18, as well. The SE was also calculated for the abundance data at each time point and is shown here, however, it is not visible due to its small variance, indicating precision in representation of the population. Carbonic anhydrase's distinct abundance patterns can be seen in the peak area graph created in Skyline in **Figure 3-3B**.

In replicate one, fibrinogen displayed an abundance pattern of upregulation at day 7 and downregulation at day 18 in four of the five selected peptides. In peptides two and three, three out of five horses with these time points exhibited this pattern. In peptides four

and five, four of the five horses with relevant time points showed this abundance pattern. None of the five relevant horses for peptide one displayed this pattern. In replicate two, fibrinogen exhibited upregulation at day 7 and downregulation at day 18 in all five selected peptides. Peptide one contained one horse out of the five relevant horses that displayed this pattern. Peptides two and three had three out of the five horses with these time points that exhibited this abundance pattern. Peptides four and five showed this pattern in four out of the five relevant horses. Fibrinogen, in comparison with day 0, exhibited downregulation on days 1, 3, 18, 34, 42, 49, 57, and 62. Day 18 showed a 102% downregulation compared to day 0. This biomarker also conveyed an upregulation in abundance compared to day 0 on days 7, 21, and 31. Day 7 exhibited a 67% upregulation difference. **Figure 3-1C** conveys these abundance patterns. Fibrinogen, in **Figure 3-2C**, is upregulated on day 7 and downregulated on day 18. Days 21, 28, 31, 34, 42, and 49 are upregulated, as well, in comparison to day 18. The SE for abundance at each time point was calculated and is represented in this graph. Because of the extremely small variance of SE, it cannot be seen at any time point. Fibrinogen's abundance patterns on days 7 and 18 can also be seen in the peak area graph created in Skyline in **Figure 3-3C**.

Fibronectin was identified in the discovery phase to have the opposite pattern as the other three biomarkers of downregulation at day 7 and upregulation at day 18. In replicate one, one of the eleven peptides only had one horse that displayed this pattern, out of the five that were sampled on days 7 and 18. Three of the eleven peptides contained three horses out of the five that exhibited this pattern. Seven of the eleven peptides showed four of the five relevant horses that displayed this abundance pattern. In replicate two, five of the eleven peptides had three horses that exhibited downregulation at day 7 and

upregulation at day 18. Three of the eleven peptides contained four horses out of the relevant five that displayed this pattern of abundance for fibronectin. Three of the eleven peptides showed all five relevant horses exhibiting this abundance pattern. When percent differences from day 0 abundance were determined, fibronectin displayed an opposite pattern to the previous three biomarkers. This protein exhibited upregulation at all 12 time points, from day 1 to day 62. The days that showed a distinct upregulation were days 7, 18, 21, 28, 31, 34, 57, and 62 with their percent differences above 100. Day 18 exhibited an upregulation higher than that of day 7 at 147% compared to 105. Day 28 displayed a 138% percent difference and day 34 exhibited a 133% percent difference. This can be seen in **Figure 3-1D**. Fibronectin displayed downregulation on day 7 and upregulation on day 18 and these patterns are portrayed in **Figure 3-2D**. As shown in **Figure 3-1D**, no time points were downregulated in comparison with day 0. This is true for **Figure 3-2D**, as well. This graph shows day 0 abundance at below 1e9, while all subsequent time points are above 1e9. SE was calculated for each time point and was presented in this figure but is not prominent due to its small variance. Fibronectin's patterns of abundance that are opposite to the other three key protein biomarkers are also displayed in the peak area graph in **Figure 3-3D**.

3.4 Discussion

Patterns exhibited by the four biomarkers that were identified in the discovery phase were replicated in the targeted phase with greater intensity. The main pattern of actin, carbonic anhydrase, and fibrinogen was upregulation at day 7 and downregulation at day 18; while the main pattern of fibronectin was downregulation at day 7 and upregulation at day 18. The first three biomarkers also exhibited upregulation at the next relevant time

point after day 18, and fibronectin displayed downregulation at the next time point after day 18. While these abundance changes were distinct in the discovery phase of this project, they were much clearer when targeted methodologies were employed.

Protein patterns were seen with greater intensity in this phase because PRM moves away from the global scan performed with discovery. This technique searches for specific peptides that were identified previously and results in clearer patterns. In the targeted phase, equine sera samples were prepared in duplicate and injected in duplicate. There is clear variability between replicates one and two and this may be due to a variety of factors. While each replicate had duplicate samples which were prepared at the same time, replicates one and two were prepared on different days and were ran separately on the Orbitrap mass spectrometer[d]. This may have contributed to the variability seen in the replicates. Peptide variability may be due to pipetting error, calibration variableness when injecting the samples, or process variation during the tryptic digestion. However, the patterns established from the discovery phase were still evident in the targeted phase: upregulation at day 7 and downregulation at day 18 for actin, carbonic anhydrase, and fibrinogen; and downregulation on day 7 and upregulation on day 18 for fibronectin. Replicate variability is important to consider while analyzing this data, and indicates the need for more sample runs to further confirm the patterns that were observed.

Figure 3-2A-D represents the relative abundances of the top three peptides of each protein along with each time point's SE. This graph mirrors **Figure 2-1A-D**; however, clear differences can be seen. The SE bars in the discovery figure are extremely evident and are meant to show how accurately the samples represent the population. The standard error bars in the targeted figure are not visible at all. This is because the targeted methodology

chooses the most optimal peptides out of all those displayed in discovery. The targeted figure also displays abundances that are, at maximum, a 2-log increase compared to discovery abundances. This is due to PRM creating more distinct patterns since it is no longer performing a global scan. Abundances are greater for the targeted phase where the top three peptides were summed together than it is for the discovery phase where the abundances were calculated by determining the mean of abundances for all horses represented at each time point.

The abundance at each time point was measured as compared to the abundance at day 0 for each of the four biomarkers. This can be found in **Figure 3-1 A-D**. Actin, carbonic anhydrase, and fibrinogen all showed upregulation and downregulation in abundance compared to day 0. The patterns established from the discovery methodology for these three proteins, seen in **Figure 2-2 A-D**, are much more obvious through the PRM method. Fibronectin exhibited a downregulation in abundance on day 7 and an upregulation on day 18. The figure for fibronectin's percent difference in abundance compared to day 0 has no bars that fall below the central line, indicating no downregulation in this protein. However, on day 7, fibronectin is 105% upregulated from day 0, and day 18 is 147% upregulated from day 0. While there may be no visible downregulation in comparison with day 0, these percent differences indicate that day 18 is still upregulated compared to day 7. Furthermore, according to the patterns established in the discovery phase, the next relevant time point should be downregulated compared to day 18. Day 21 shows a 103% upregulation compared to day 0, which is less than the upregulation seen on day 18, indicating downregulation.

All four biomarkers that were identified in the discovery phase and validated in the targeted phase have relevance to bone remodeling and some possible association to bisphosphonate administration. When bone remodeling begins, osteoclasts are activated to resorb bone. They do this by adhering to the bone surface through the formation of podosomes and, eventually, a sealing zone (Han et al., 2019). To resorb the bone, they must acidify the matrix. This is accomplished with the protons that are produced from the reaction that converts carbon dioxide into bicarbonate (Riihonen et al., 2007). Once bone resorption is finished, the bone remodeling unit initiates bone ossification with the help of osteoblasts. Bisphosphonates, once administered, can inhibit the bone resorption process, and may increase bone ossification by improving the viability of osteoblasts.

Actin is a protein found in the podosomes of osteoclasts and is likely upregulated when osteoclasts are activated (Destaing et al., 2003). Since bisphosphonates cause apoptosis of osteoclasts in order to disrupt resorption, actin would expectedly be downregulated when these drugs are administered. This upregulation followed by downregulation was exhibited in the discovery phase and is seen with greater intensity in this phase on days 7 and 18, respectively.

Carbonic anhydrase is the enzyme responsible for the catalysis of carbon dioxide to bicarbonate, where the byproduct is protons (Riihonen et al., 2007). These protons, as previously stated, are needed for the acidification of the bone matrix and, subsequently, the resorption process. So, this biomarker is likely upregulated with the activation of osteoclasts and downregulated with the apoptosis of osteoclasts that is caused by administration of bisphosphonates. Actin and carbonic anhydrase both display these changes in abundance on days 7 and 18 and this pattern is repeated in the targeted phase.

Fibrinogen is a protein that is found on the surface of osteoclasts and may be an exogenous activator of the bone-resorbing cell (Athanasou et al., 1988; Cole et al., 2014). This biomarker would be expected to increase in abundance when osteoclasts are activated and decrease when osteoclasts go through apoptosis due to bisphosphonate action. Fibrinogen is upregulated on day 7 and downregulated on day 18 in both phases of this study.

Fibronectin, the final biomarker identified and validated, is the earliest protein synthesized by osteoblasts for ossification and production of the bone matrix (Tang et al., 2004). Osteoblasts are responsible for the part of bone remodeling that is not inhibited by bisphosphonates and may be increased due to improved activity of these cells. This protein showed a trend opposite to the previous three proteins: downregulation on day 7 and upregulation on day 18. This pattern is shown clearly in the targeted phase of this study, repeated from the discovery phase. The downregulation is likely due to inactivity of osteoblasts while osteoclasts are resorbing bone. The upregulation occurred when bisphosphonates caused apoptosis of the osteoclast and may have increased the viability of the osteoblast to ossify bone.

Overall, the trends established in the discovery phase were repeated in the targeted phase with greater intensities. PRM allowed for a more specific scan of the initially identified peptides and the establishment of repeatability for the abundance patterns seen in discovery. These biomarkers, because of their involvement in bone remodeling units, may be good candidates for the detection of prohibited bisphosphonate use when drug testing racehorses.

3.5 Other Possible Causes of Protein Changes

These four proteins, as discussed in Chapter 2, can be found in other areas of the body. Actin, for example, is important in the cell mobility and migration of all cells. The changes observed in actin could be due to other cells preparing for migration within the biological system. Because actin is located inside the osteoclast, apoptosis and lysis of the cell may have caused transitory changes in the abundance of actin that was observed.

Carbonic anhydrase, as discussed in Chapter 2, is found in erythrocytes and large intestine cells. Changes observed in this protein may be due to physical activity, stressors, or other factors that affect red blood cells or large intestine parameters.

Fibrinogen is a protein that is affected by inflammation, as well as changes to red blood cells. Changes observed in this protein may also be due to physical activity, stressors, or other factors affecting inflammation or erythrocytes.

The final biomarker, fibronectin, is a protein which is found in the extracellular matrix of all cells, including bone. The changes in this protein may have been due to normal cellular turnover that occurs throughout the biological system, as it does in bone cells.

Although these proteins are not isolated to bone cells or the bone matrix, their similar abundance patterns following bisphosphonate administration and their association with the bone matrix in some form indicate that their changes may still be associated with bisphosphonate use. Their role in other cells of the biological system indicate a need to increase the specificity at which they are associated with the bone matrix. This may be accomplished in future work.

3.6 Limitations of the Study

A limitation of this study is the lack of a negative control. A negative control would have been a horse or horses that were not administered any substance, including bisphosphonates, and were assumed to be normal. Blood samples would have been taken at the same time points as the 19 equine subjects in this study to act as a comparator. This would allow for more confirmatory identification and validation of the four biomarkers that are postulated to change at certain time points as a result of bisphosphonates being administered. Because these horses had an underlying need for bisphosphonate treatment, it would be beneficial to have a group of horses which did not need this anti-resorptive drug in order to observe how the protein changes are affected.

These horses were not sampled at common time points throughout the study. Having a group of horses that are administered bisphosphonates and have blood samples collected on the same days would aid in confirming any trends that were observed. Another limitation is the lack of several time points for each horse. The horse with the most samples taken was sampled nine times. If there were more time points collected, clearer trends may be seen in the horse.

Because the horses were anonymous, this limits the ability to attribute trends in proteins to factors like age or sex. Though these protein changes may not be due to these factors, having that information would allow for more confirmation that these biomarkers are optimal candidates for bisphosphonate administration. None of these proteins are specific to the bone remodeling process and are often found elsewhere in biological systems. However, the repeatability of their patterns from the discovery phase to the targeted phase and their association with bone turnover, is important and may indicate their

response to bisphosphonates affecting the bone remodeling process. There is also a need to identify more biomarkers that can confirm the administration of bisphosphonates and are more specific to bone remodeling processes.

The depletion methods in this study could also be improved so that background noise in the mass spectrometer[d] is reduced, and more relevant proteins can be identified and validated. These samples could also be analyzed on a different instrument, such as a triple quadrupole mass spectrometer, to compare the data that is generated and further confirm the results from the discovery and targeted phases of this study.

3.7 Future Work

This study represents an excellent starting point at which to identify biomarkers for bisphosphonate administration. However, more work remains to be done in order to verify that the biomarkers which were changed were changed in response to bisphosphonates and are specific to bone turnover. There is also a need to complete the same project with the other non-nitrogen containing bisphosphonate, clodronate. By administering clodronate to a group of horses and assessing the protein effects in the same way, it would allow for more confirmation that these proteins changed in response to a non-nitrogen containing bisphosphonate.

Because there are two different classes of bisphosphonates, it would be worthwhile to assess off-label use of nitrogen-containing bisphosphonates on the horse. This class has a different mechanism of action to induce osteoclast apoptosis and may result in different protein changes than that of the non-nitrogen containing bisphosphonates.

Since these biomarkers will eventually be integrated into the Equine Biological Passport, following more confirmatory studies, it is important that this same procedure be applied to other drugs, as well. These drugs may include growth promoters or respiratory drugs that alter the performance of the racehorse and affect certain proteins within the biological system.

Future work remains in order to further confirm these four protein biomarkers, identify more proteins that are changed in response to bisphosphonates, and assess the protein effects of other classes of drugs with the overarching goal of protecting the welfare of the horse and the integrity of the breeding industry.

3.8 Conclusion

Patterns identified in the discovery phase for the four biomarkers were repeated in the targeted phase in all replicates with greater intensities. While not every horse was sampled on days that showed the most distinct patterns (e.g., day 7 and day 18), those with the relevant time points exhibited clear trends of upregulation and downregulation on these days.

Actin showed upregulation on day 7, downregulation on day 18, and upregulation at the next time point, whether that was day 21, 28, 42, or 49. This trend was exhibited in the actin's top four peptides for both replicates. Its significance is its presence in podosomes of osteoclasts, which are directly affected by bisphosphonates. While actin is not a protein that is specific to bone remodeling, the repeatability of its pattern is indicative of a relationship to the administration of bisphosphonates.

Carbonic anhydrase was upregulated on day 7, downregulated on day 18, and upregulated on days 21, 28, 42, and 49, depending on what day the horse was sampled. This was true in both replicates. Carbonic anhydrase, because of its catalytic activity in the production of protons to aid the osteoclast in resorbing the bone matrix, is likely a good indicator of bisphosphonate administration. This protein also exhibited repeatability that further confirms its association in these processes.

The third biomarker that was identified and validated, fibrinogen, exhibited the same pattern of upregulation on day 7 and downregulation on day 18 in both replicates. While its association with the bone remodeling process is not as definitive as actin or carbonic anhydrase, its presence on the surface of osteoclasts and because it is postulated to be an exogenous activator of bone resorption, the upregulation and downregulation seen on these days is indicative of a connection to bisphosphonate administration.

The fourth and final biomarker that exhibited an opposite trend from the first three proteins is fibronectin. Its downregulation on day 7 and upregulation on day 18 seen in both replicates suggests an association with bone remodeling and, therefore, bisphosphonate administration. Because of its relationship to osteoblasts, a cell type that executes the opposite action of osteoclasts, and its opposite trend compared to the three biomarkers associated with osteoclasts, fibronectin is likely a good biomarker of bisphosphonate use.

TABLES

Table 3-1: Four Key Proteins and Their 23 Unique Peptides[1]

Protein	Peptide	*m/z*	Retention Time
(A) Actin	**AGFAGDDAPR**	488.7277	16.1
	DLYANTVLSGGTTMYPGIADR	1108.0386	34.7
	GYSFTTTAER	566.7671	18
	HQGVMVGMGQK	391.1952	15.7
(B) Carbonic Anhydrase	**AVQQPDGLAVVGVFLK**	820.9721	37.3
	SLLSNVEGGK	502.2745	20.6
	YSAELHLVHWNSSK	557.6143	20.4
(C) Fibrinogen	**DNCCILDER**	597.7475	19.3
	FLQDIYNSNNQK	742.3624	21.7
	HPGSSEPGSDGLQK	465.8881	14.9
	IAIGEGQQFHLGAAK	513.9474	21.5
	TFPGEGSLDGLFHR	511.5879	30.5
(D) Fibronectin	**ATITGYR**	391.2137	15.9
	GATYNIIVEALK	646.3664	32.3
	IFAVNHGR	457.2537	15.4
	ISCTIANR	467.7423	15.7
	ITIMWTPPESAVTGYR	911.4638	33.3
	ITYGETGGNSPVQEFTVPGSK	723.3550	27
	LDAPTNLR	450.2508	17.1
	NTFAEVTGLSPGVTYHFK	656.6669	28.8
	SYTITGLQPGTDYK	772.3855	23.7
	TYHVGEQWQK	425.8754	16.3
	VDVLPVNLPGEHGQR	543.9617	23.9

[1] The 23 unique peptides that were chosen based on peak area, fragment ion ppm tolerance, retention time, and number of detectable transitions. Included in this table are the peptides' *m/z* as well as their retention times found in Skyline[g] software.

FIGURES

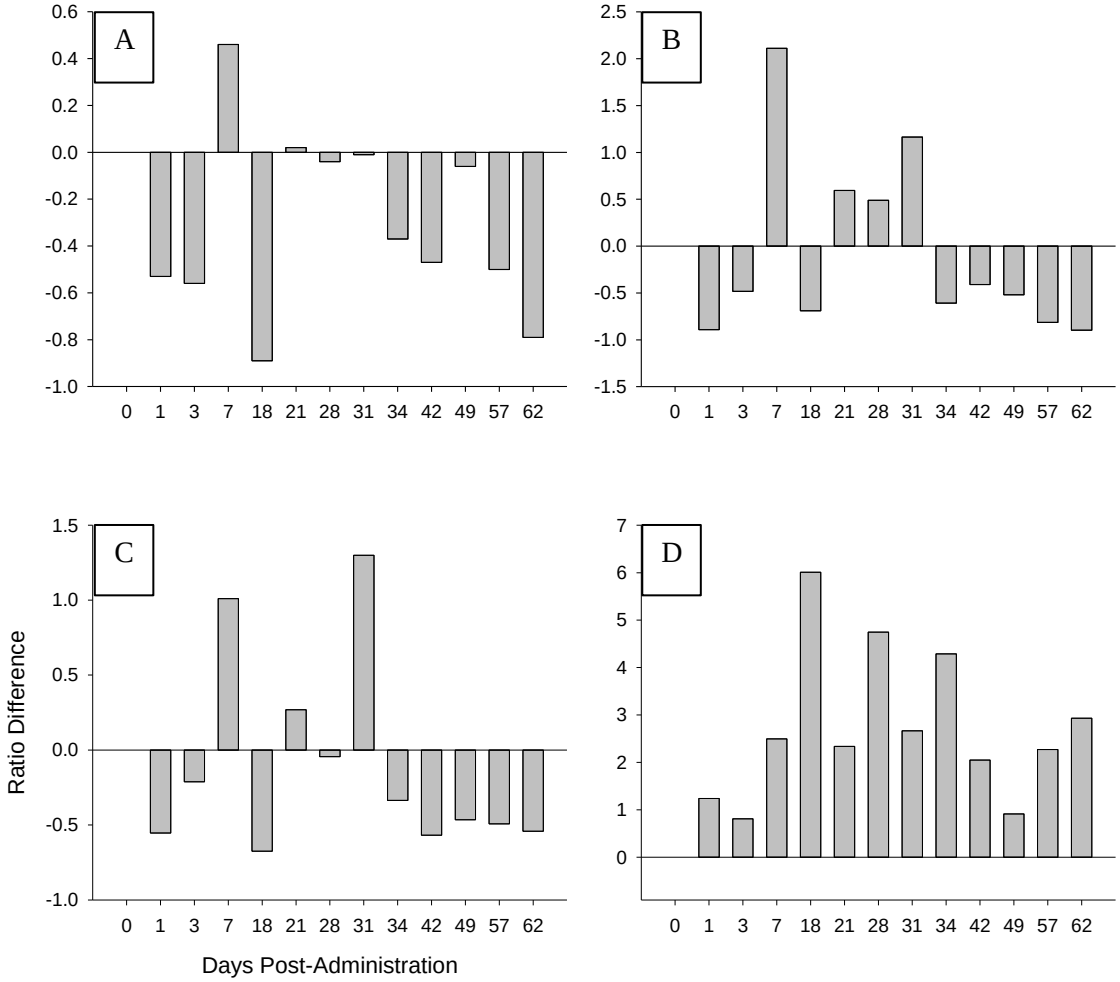

Figure 3-1 A-D: Percent Difference of Identified Biomarker Abundance with Day 0 Abundance.

Figure 3-1: (A) The abundance patterns of actin when compared with the abundance at day 0. (B) Carbonic anhydrase abundance patterns in comparison with the abundance at day 0. (C) The abundance patterns of fibrinogen compared to the abundance of fibrinogen at day 0. (D) Fibronectin abundance patterns compared to day 0 abundance.

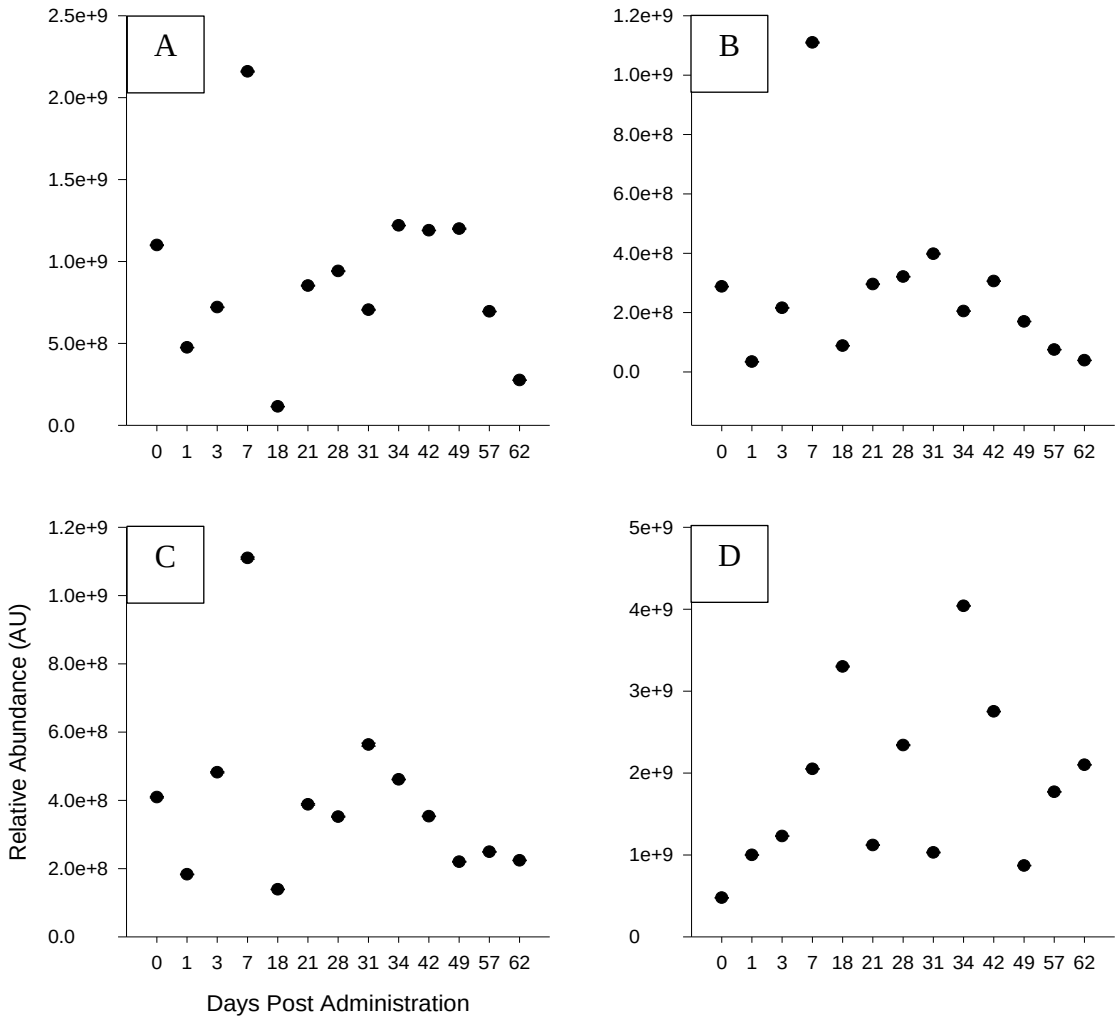

Figure 3-2 A-D: Global PRM Figure of Top Three Peptides Per Protein Summed for Abundance and Standard Error of Population.

Figure 3-2: For each protein, the top three peptides were summed to calculate abundance and standard error. (A) The abundance of actin at each time point, representing all 19 horses, along with the standard error of population. (B) The abundance of carbonic anhydrase from day 0 to day 62 along with the standard error of population. (C) Fibrinogen abundance patterns and standard error. (D) The abundance and standard error of fibronectin at each time point, from day 0 to day 62.

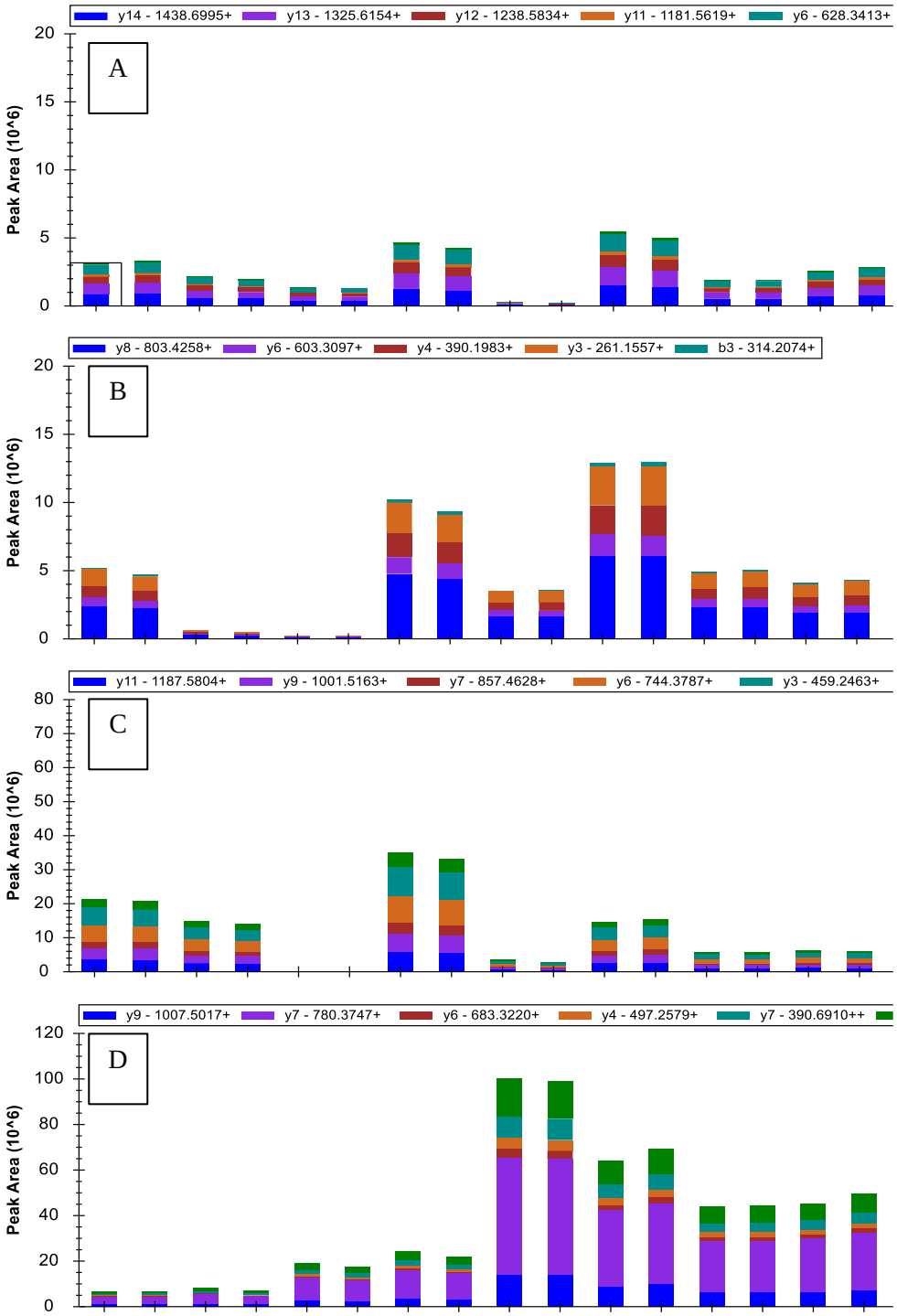

Figure 3-3 A-D: Peak Areas of Four Key Proteins Across Eight Time Points.

Figure 3-3: (A) The peak area replicate comparison graph for actin created in Skyline[g] software. (B) The Skyline[g] software graph for carbonic anhydrase peak areas. (C) The peak areas of fibrinogen for the same horse represented in the previous two graphs, also created in Skyline[g]. (D) The peak areas of fibronectin created in Skyline[g].

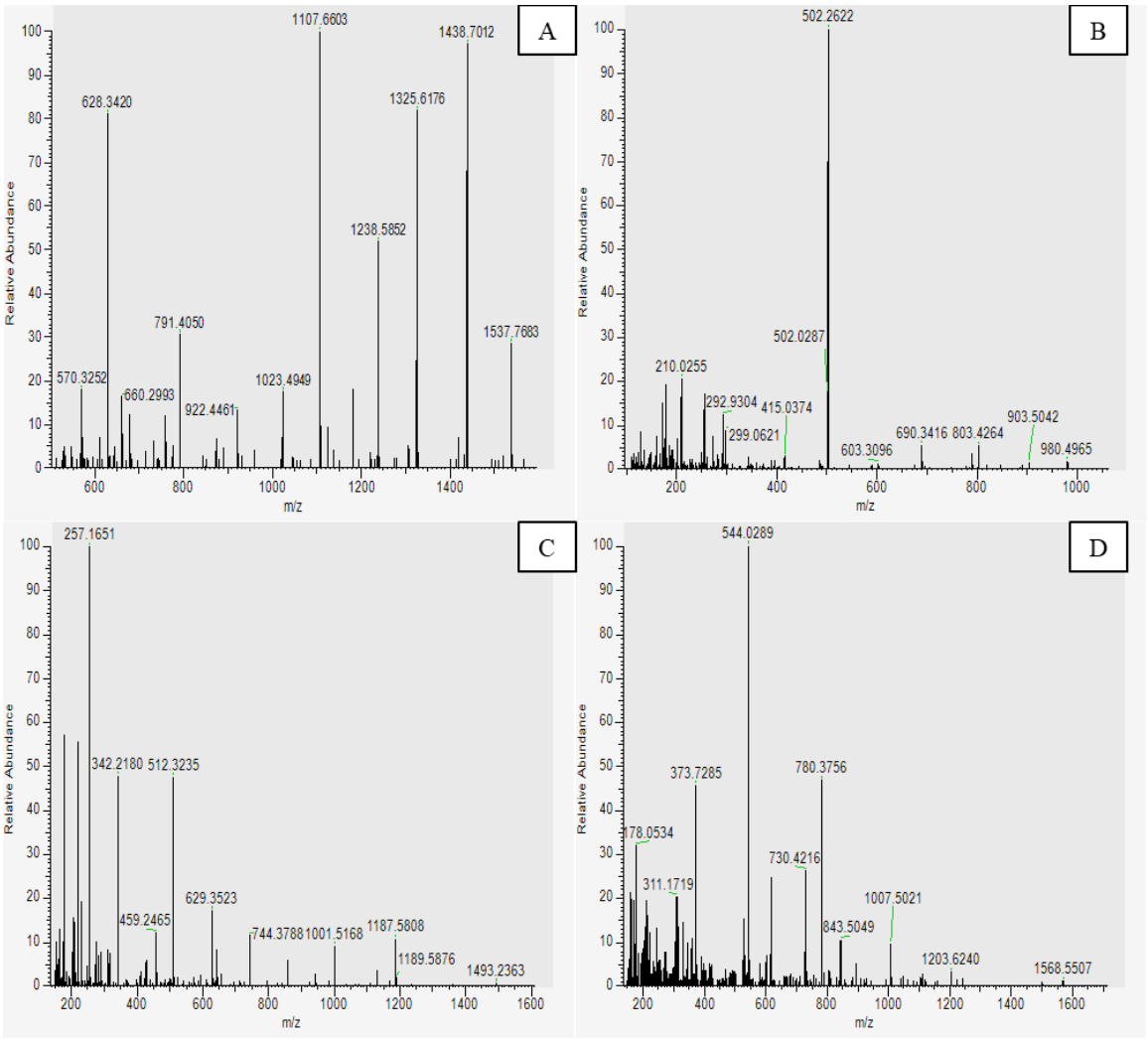

Figure 3-4 A-D: MS2 Fragmentation Spectra for Four Key Protein Biomarkers.

Figure 3-4: The peptides used in this figure are the same peptides used for **Figure 3-3 A-D.** (A) The MS2 fragment spectrum for the protein actin. (B) This is a depiction of the MS2 fragment spectrum for carbonic anhydrase. (C) This is the MS2 fragment spectrum for the third key protein biomarker, fibrinogen. (D) The MS2 fragment spectrum of the protein fibronectin.

APPENDICES

APPENDIX I: GLOSSARY

Abbreviation	Explanation
AGC	Automatic Gain Control
DDA	Data-Dependent Acquisition
ESI	Electrospray Ionization
FDR	False Discovery Rates
FWHM	Full Width at Half Maximum
HCD	Higher Energy C-trap Dissociation
LC-MS/MS	Liquid Chromatography with Tandem Mass Spectrometry
LFQ	Label-Free Quantitation
LOD	Limit of Detection
MALDI	Matrix-Assisted Laser Desorption Ionization
MRM	Multiple Reaction Monitoring
m/z	Mass-to-Charge Ratio
PRM	Parallel Reaction Monitoring
PSM	Peptide Spectra Match
Q1	First quadrupole mass filter

Q2	Quadrupole collision cell
Q3	Second quadrupole mass filter
SRM	Single Reaction Monitoring
tMS2	Targeted MS^2

APPENDIX II: VENDORS

a. Acclaim™ PepMap™ Trap Column: Thermo Fisher Scientific™

b. Double-distilled water: MilliPore Milli-Q Water

c. Easy Spray PepMap™ RSLC C18 Column: Thermo Fisher Scientific™

d. Orbitrap™ Exploris 480 Mass Spectrometer: Thermo Fisher Scientific™

e. Proteome Discoverer™ Version 2.4: Thermo Fisher Scientific™

f. Savant SpeedVac SPD1030 Integrated Vacuum Concentrator: Thermo Fisher Scientific™

g. Skyline Version 21.2.0.425: MacCoss Lab, Department of Genome Sciences, University of Washington

h. Sorvall Legend Micro 21 Centrifuge: Thermo Fisher Scientific™

i. Sorvall X Pro Series Refrigerated Centrifuge: Thermo Fisher Scientific™

j. Ultimate 3000 RSLCnano: Thermo Fisher Scientific™

k. Vacuum Manifold: Alltech

l. Vortex-Genie 2: Scientific Industries

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