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Effects of High-Speed Training on Messenger RNA Expression in Two-Year-Old Thoroughbred Racehorses

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Effects of High-Speed Training on Messenger RNA Expression in Two-Year-Old Thoroughbred Racehorses

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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

By

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ABSTRACT OF THESIS

Effects of High-Speed Training on Messenger RNA Expression in Two-Year-Old Thoroughbred Racehorses

Accumulating high-speed exercise has been identified as a significant risk factor for catastrophic injuries in racing Thoroughbreds. Injuries, regardless of severity, are a main cause of withdrawal from the racing industry, raising animal welfare concerns and resulting in significant economic losses. While most of the current literature focuses on catastrophic injuries incurred during racing rather than training, the present study aims to help fill this gap as well as discuss the associated risk factors. The evaluation of messenger RNA (mRNA) expression changes provides an efficient and straightforward approach to identifying horses at risk for catastrophic injury. While alternative injury risk assessment methods, such as Positron Emission Tomography and other advanced imaging techniques, have been investigated, they present accessibility concerns when evaluating cost, availability, and complexity. As such, peripheral blood was collected weekly, prior to exercise or administration of medication, from eighteen, two-year-old Thoroughbreds throughout their first season of race training. Messenger RNA was isolated from these samples and used to analyze the expression of 34 genes via RT-qPCR. Statistical analysis of the non-injured horses (n=6) showed that 13 genes were significantly associated with increasing average weekly furlong performance while *CXCL1, IGFBP3,* and *MPO* had negative correlations with cumulative high-speed furlongs and week of training for both injured and non-injured groups. Comparison of both groups identified opposing correlations between an anti-inflammatory composite index (*IL1RN, IL-10*, and *PTGS1*) and average weekly furlong performance. Furthermore, evaluation of training effects on mRNA expression during the weeks surrounding injury identified differences between groups in *IL-13* and *MMP9* at -3 and -2 weeks prior to injury. While some previously reported relationships between exercise adaptation and mRNA expression were not noted in this study, this may have been due to the small sample size. Several novel correlations, however, were identified and warrant further investigation as markers of exercise adaptation or potential risk for injury.

KEYWORDS: Horse, mRNA, Exercise, Injury, Inflammation

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1.1 Introduction

Regardless of the cause, injury is the leading reason for Thoroughbreds to leave the racing industry, subsequently leading to the loss of equine athletes, social support for racing, and economic investments. As such, assuring that ample research and clinically relevant information on these topics is available to all within the industry is of utmost importance to help improve safety and welfare. Over the last 20 years, there were 30% fewer publications on *PubMed* related to Thoroughbred training injuries as compared to those incurred during racing. This disparity raises concern as available literature has reported fatal musculoskeletal injuries are three times more likely to occur during training than racing [1]. Additionally, 62% of two-year-old Thoroughbreds have been reported to incur an injury during training, with musculoskeletal injuries comprising 89% of recorded cases [2]. Addressing this imbalance by further investigating the mechanisms underlying injury risk in the equine athlete, particularly during training, is a crucial first step to preserving and improving Thoroughbred safety and welfare.

1.2 Training

1.2.1 Exercise Intensity and Frequency

A typical Thoroughbred racing prospect begins training at 2 years of age and will often begin racing in the same year. These horses spend months gradually working their way up to high-speed exercise once broke to ride. They perfect slow- and mid-speed works like the trot (45 s/furlong, 16 km/h), canter (30 s/furlong, 24 km/h), and gallop (15

s/furlong, 30km/h) works before attempting short breeze, or race-speed (12-13 s/furlong, 55 - 60 km/h) works. Distance is then continually added as the horse progresses through training [3].

Initial research in the area of Thoroughbred training practices has shown only one high-speed exercise per week is necessary to develop sufficient bone strength to minimize fracture risk while additional high-speed work may contribute to an increased risk for injury [4]. While this work was done using cattle as a model for young horses, subsequent work in racing Thoroughbreds has supported the hypothesis that increased frequency of high-intensity training is detrimental to horse health and increases the risk of injury [5-10]. Researchers at the University of Pennsylvania investigated bucked shins, or 'fatigue injury of the third metacarpal', amongst 226 training Thoroughbreds, finding that adding an extra high-speed furlong per week (15-16 m/s) to their training program (1.2 ± 1.0 furlongs/wk.) resulted in a 59% decreased risk of bucked shins [10], while the addition of 1 cantering furlong (11 m/s) to their training program (35.8 ± 12.2 furlongs/wk.) resulted in a 20% increased risk [10]. Overall, these findings suggested horses with bucked shins performed increased amounts of low-intensity exercise (5-11 m/s) compared to those without injury. Although this work did not investigate the effects of increased high-speed training frequency, the investigators do warn against the potentially harmful effects [10]. Conversely, a 2-year observational study of nearly 650 Thoroughbreds suggested that the addition of 4 cantering furlongs per day reduces risk of metacarpophalangeal or metatarsophalangeal joint injury by 30% [9]. Further, injury risk increased by 50% for

every 45 furlongs of cumulative high-speed exercise, or an extra 1.87 furlongs per month of the 2-year study [9].

1.2.2 Training Losses Due to Injury

A 2008 evaluation of 182 two-year-old Thoroughbreds reported that of the 26.8% of training days lost, nearly 82% of those were due to lameness [11]. Lameness caused by fracture (21.6%), the carpal joint (16.3%), and undiagnosed causes (38.1%) were the leading pathologies amongst these young Thoroughbreds [11]. While safety and welfare concerns must remain a top priority with regards to racing injuries, it is impossible to not also evaluate the economic toll associated with these injuries. A survey published in 2006 observed 537 two-year-old and 456 three-year old Thoroughbreds competing in British flat races; investigators reported that training costs were not recovered for 95% and 83% of horses, respectively [2]. With an assumed training cost of £10,000 GBP ($12,411 USD in 2006, $19,034 USD in 2023) [12] and a mere 34% of horses remaining in training as four-year-olds, annual economic losses from young Thoroughbreds leaving the racing industry total over £4.55 million GBP ($5.65 million USD in 2006, $8.52 million USD in 2023) [12], based on this study [2]. In 2011, a similar study of Australian Thoroughbreds reported a recovery rate of only 11.2% with an assumed training rate of A$40,000 ($26,688 USD in 2011, $36,856 USD in 2023) [12, 13]. Further, a more recent 2020 study reported less than 50% of transactions in the Thoroughbred industry end up being profitable [14]. With these striking figures it is important to further evaluate the safety of training Thoroughbreds, to ensure minimal economic losses.

1.3 Exercise-Induced Inflammation

As discussed below, specific proteins or mRNA targets, in addition to combinations of multiple genes, can offer invaluable insights into the pathology of exercise related injury and inflammation. Current research has investigated varying Thoroughbred training practices and their subsequent effects on inflammatory mediators, such as mRNA, and injury risk. While some are observational studies of large horse groups and more representative of real-world training practices, others are small, controlled exercise tests. Although there are benefits and drawbacks of each, there is a level of concordance between findings. Several investigators have reported the use of peripheral blood mRNA and RT-qPCR to evaluate the probability of injury for equine athletes, with many agreeing conclusions. The minimally, efficient, cost-effective nature of mRNA isolation from peripheral blood coupled with the flexibility of RT-qPCR analysis allows for a logistically feasible method for injury screening. By using a panel of mRNA targets, being able to identify Thoroughbred racehorses at risk for injury could become accessible and manageable, assisting in the prevention of injury.

1.3.1 mRNA Expression and Inflammation

In a 2012 observational study conducted by the Royal Veterinary College, investigators found that horses were more at risk for injury to the metacarpophalangeal and metatarsophalangeal joints with each cumulative high-speed furlong ($\leq$ 15.0 m/s) performed in training. Conversely, they reported each furlong of cantering exercise per day decreased the risk for injury [9]. Interestingly, a 2017 study reported horses training at high-speeds only once per week displayed a decreased expression of pro-inflammatory

mRNA, as opposed to horses training twice per week who experienced a significant increase in their inflammatory mRNA expression [3]. Put together, these findings suggest that low-intensity, or less frequent high-intensity, work is more beneficial to the horse than repeated high-speed training.

Observational racing studies have reported concordant findings; a 2013 article indicated immediate post-race increases in *CEBP*β*, CXCL2,* and *IL-8* amongst the 9 horses included in the controlled study [15]. Further, in a large-scale observational study of Thoroughbred racehorses across the United States investigators evaluated nearly 700 peripheral blood samples for expression differences amongst horses with and without catastrophic injuries [16]. The article reported significant increases in *IGF1* and *MMP2*, as well as a significant decrease in *IL1RN* between groups [16].

While long-term effects of training cannot be elucidated from smaller controlled studies, they do offer comparable insights into the inflammatory status of horses exercising at high-speeds. Previous research has shown horses exhibit an increase in in *IFN*-γ and *IL-1*β expression immediately and two hours following exhaustive exercise on a treadmill, respectively [17]. This study also reported delayed *TNF*-α expression increases at six hours post-exercise [17]. The increase of these pro-inflammatory cytokines is indicative of exercise-related systemic inflammation, leading to the recruitment of leukocytes, such as monocytes and lymphocytes. Similarly, an exercise study including a 2-minute gallop, once a week, revealed an increase of lymphocyte and basophil concentrations in peripheral blood [18]. To this point, increased expression of *IL-2, IL-4,* and *IL-8,* which function to attract leukocytes to the site of inflammation or damage, as

well as decreased expression of the anti-inflammatory *TGF-β1*, were reported. Following a 4-minute gallop exercise in training study, the same increases were noted, in addition to increased *IL-1*β and *IL-3,* and decreased *IL-5* expression [18]. The chemotactic properties of IL-1β and IL-3 likely increased the concentration of monocytes detected at this timepoint. IL-5 is mainly responsible for eosinophil recruitment, and, therefore, is not expected to be a crucial player in musculoskeletal inflammation. However, a 2007 article also noted IL-5 stimulates production of mature progenitor cells in bone [19], suggesting a potential role in bone healing and regeneration. Similar to the findings of observational studies, this supports the perception that high-intensity exercise promotes a pro-inflammatory status, increased risk for injury, and decreased healing functionality.

Not surprisingly, broader inflammation-based studies, including non-Thoroughbred horses, have reported similar findings regarding injury-related mRNA changes. In 2019, one study reported on expression changes associated with endurance rides, where 77 horses were evaluated over a 160km course [20]. Increased expression of *IL-8* and *MMP1* were reported during and after the ride, while *ALOX5AP, CD14, IL-10,* and *TLR4* were shown to increase at all timepoints compared to pre-ride expression [20]. Additionally, induced inflammation models have been utilized to observe mRNA changes representing inflammation associated with joint injuries. A 2020 study used *E.coli* derived lipopolysaccharide (LPS; an endotoxin) as an intra-articular antagonist that mimicked a septic joint. The study reported increased expression of *ALOX5AP, CD14, IL-1*β, *IL1RN, IL-8, IL-10, MMP1, MMP9,* and *TLR4* through 12 hours post injection in comparison to pre-injection expression levels [21]. The mRNA expression changes of both studies mentioned

mimic those seen in Thoroughbred training and racing research, further suggesting their roles in exercise-induced inflammation.

1.4 Injuries

Thoroughbred racing and training injuries can be sorted into two main categories: catastrophic and non-catastrophic. Catastrophic injuries (CI) are commonly defined as musculoskeletal injuries that result in euthanasia within 72 hours of the incident and/or the immediate end of the horse's racing career [22], though that timing can vary by study. The non-catastrophic injury category accounts for cases that fall outside of these criteria, such as soft tissue injuries. Due to the imminent welfare concerns for an equine athlete's risk of fracture or sudden death during a race, these events comprise the focus of most research. However, this should not discount the burden of non-catastrophic injuries that can significantly impact the welfare of equine athletes and contribute to the loss of over 19% of training days amongst two and three-year-old Thoroughbreds [11].

1.4.1 Pathobiology of the Distal Equine Limb

There are many anatomic and pathologic factors that influence the susceptibility of the equine limb to injury. First, the minimal vasculature and venous drainage pathways within the limb makes it increasingly difficult to respond to injury and inflammation [23]. Further, the lack of musculature in the most distal portion of the forelimb not only contributes to the lack of vasculature, but also to the lack of physical protection to bones, ligaments, tendons, and joint capsules [23]. Additionally, a 2019 study reported that the ground reaction force of Thoroughbred racehorses at high-speeds (9-13 m/s) can peak at

1.7 times body weight [24]. All considered, the distal limb is subject to immense strain and is asked to perform at the highest caliber, all while being one of the most fragile parts of the equine anatomy.

Today, catastrophic fractures are known to typically occur in areas of pathologic change and are referred to as 'fatigue fractures' [25]. The accumulation of underlying pathologies (as detailed below), as well as micro-damage and cyclic damage applied to bone, cartilage, and soft tissue structures, can lead to the eventual disruption of bone repair processes [25]. There are two processes in which bone adapts to stress: 'modeling' and 'remodeling'. 'Modeling' of a bone occurs when stress, or exercise related loading forces, require the attention of osteoblasts in order to build bigger or more dense bone structures [25]. This process can also occur in reverse, utilizing osteoclasts to cause resorption of bone structures not undergoing loading forces, such as during lay-up periods due to injury [26]. This finding reaffirms the need for gradual reintegration to training after extended periods of rest. In contrast, the 'remodeling' process utilizes both osteoclasts and osteoblasts simultaneously to remove damaged bone and replace it with new bone [25]. Once activation of these cells is initiated, the process is then followed by osteoclastic resorption of fatigued bone, and finally, formation of new bone by osteoblasts [25]. Together, these processes fall under the rule of Wolff's Law, by adding bone when and where it is under significant stress and loading force, and removing it when and where it is not [27].

As discussed above, proteins play a crucial role in the remediation of injury and pathology inflammation. While not an exhaustive list, some cytokines such as BMP-2, FOS,

IGFBP3, MMPs, Osteoprotegerin, RANKL, and TGF-β are involved in the regulation of bone remodeling and healing [28-31].

1.4.2 Common Injuries

Catastrophic injuries occur most commonly in the distal limb, with studies reporting the left front limb to be most prevalent [16, 32, 33]. Approximately 85% of fatal musculoskeletal injuries are due to fracture, while only 10% are due to soft-tissue injuries alone [33]. A 2019 study reported 55% of observed cases had fractures to the proximal sesamoid bone of the forelimb, the majority of which affected the left leg [32]. The 2020 study evaluating over 100 CI's during racing showed that the proximal sesamoid bone was involved in over 70% of fractures, with nearly 50% in the left front limb [16]. Research evaluating training-related injuries have found other fracture types to be most frequent, including a study of 217 English Thoroughbreds which reported a 21% fracture rate of the tibia, followed by fractures to the proximal phalanx, carpus, and pelvis (11-15%) [34].

1.4.3 Catastrophic-Injury Associated Risk Factors

The considerable attention that catastrophic injuries bring to the equine industry places them at the forefront of equine research. As such, research on catastrophic injuries and associated risk factors has taken more focus than risk factors associated with non-catastrophic injuries. Studies investigating CIs have identified roughly 300 factors associated with an increased risk for injury [35]. Interestingly, studies have reported a number of risk factors that are static, or unable to be mitigated, such as sex, jurisdiction, dam parity, purse size of race, and time of year [2, 36]. While these factors may offer an interesting perspective on populations of Thoroughbred racehorses that are more prone

to sustain an injury, more important are those factors that can be avoided or otherwise changed. Such factors, individually and in combination with one another, include time off before racing, cumulative high-speed work, race and track type, jockey, age, number of previous injuries, and appearance on the vet's list before racing [5, 6, 8, 10, 16, 36-38].

Analysis of the Jockey Club's *Equine Injury Database* from 2009 through 2013 reports that simply being placed on the vet's list prior to a race increased the rate for CI by 76.6% [36]. Similarly, odds of sustaining a CI increase by 55% with every previous injury incurred [36]. The same study also reported that horses are 60% more likely to sustain a CI when racing on a muddy, or slow, dirt track as compared to synthetic surfaces [36]. Likewise, an evaluation of Australian flat racing reported that horses were 3 times more likely to incur a CI on a slow track [38] while a 2020 North American study of over 100 catastrophically injured Thoroughbreds found that the majority of injuries occurred during claiming or maiden races [16]. Claiming races are typically fielded by older horses and horses that have not yet raced. This supports other studies that found a negative association between increased age and likelihood of injury [16, 36, 38, 39].

Interestingly, a 2013 study evaluated the effects of corticosteroid injections for the treatment of training and race related injuries [40]. Results of the survey suggested horses receiving just one corticosteroid injection were 3.2 times more likely to sustain another musculoskeletal injury compared to those not receiving injections of any kind [40]. Additionally, horses receiving 2 or more injections were nearly 6.5 times more at risk [40]. Conversely, while a report published in 2018 revealed a large percentage of both

injured horses and control horses (64% and 77%, respectively) receive medication, there was no significant difference in injury risk reported [41].

The large number and heterogeneity of known risk factors has led to confusion as to the best way to decrease injuries, especially when multiple studies do not come to the same conclusions. This is especially true with regards to cumulative high-speed furlong accumulation before racing. Due to the many definitions of 'high-speed', studies have reported on both the deleterious [6, 9, 41] and protective effects [10, 37] of the accumulation of high-speed work with respect to injuries.

1.5 Trackside and Clinical Applications

As research on Thoroughbred training and racing injuries becomes more expansive, alternative screening modalities have surfaced. In 2023, the Equine Injury Database reported the lowest rate of catastrophic injuries amongst Thoroughbreds (1.25 per 1000 race starts) since the initiative began [22]. Further, this was the fourth consecutive year presenting a downward trend, with two-year-old horses assuming the least amount of risk (0.98 per 1000 starts) [22]. While this is considerable improvement, and represents an overall 37.5% decrease in race related fatalities since 2009 [22], there remains ample room for improvement. As such, providing the industry with an injury screening and prediction method that is flexible and able to evolve, yet stable and reliable, should be the main focus of current research.

1.5.1 Alternative Injury Risk Assessment Methodologies

Advancements in biotechnology have allowed for use of alternative, non-invasive, screening methods to evaluate a horse's risk for an injury. Imaging technology such as positron emission tomography (PET) [42], computed tomography (CT) [43], magnetic resonance imaging (MRI) [43], thermographic imaging [44, 45], and ultrasonography [46] screening methods have been investigated, but the complex machinery, associated costs, and use of sedatives or anesthesia make widespread availability throughout the equine community arduous. Due to limited availability and associated costs, research on newer technology such as PET scans, is also limited. While these methods offer interesting insights to how bone, cartilage, and muscle react and remodel after an injury, how they would perform as predictive procedures has yet to be determined in literature.

Other biomarkers indicative of horse health have also been investigated, including analyses of serum protein levels in relation to both degenerative [47, 48] and exercise-related injuries [49, 50]. A 2010 study of 2- and 3-year-old Thoroughbreds (n=238, 59 injured horses) evaluated serum protein changes in the 9 weeks leading up to injury [49]. An array of 7 protein markers was analyzed and significant differences were noted for aggrecan synthesis (CS846), type II collagen synthesis (CPII), and Glycosaminoglycan (GAG), between injured and non-injured horses, on average throughout the months leading to injury [49]. The researchers proposed a link between GAG and osteocalcin (OC), and an appropriate response to exercise, as control horses displayed linear expression changes, unlike their injured counterparts [49].

1.5.2 Regulatory Changes

Over the last few decades, the lack of a central racing authority in the United States has raised concern. With each state having their own racing board or commission, there were disagreements surrounding medication bans and other general safety practices. While there are groups that advocate for the welfare and safety of the horse, there was a lack of an overarching regulatory authority until the creation of the Horseracing Integrity and Safety Authority (HISA) in 2020. Their first major regulatory change was the implementation of the Racetrack Safety Program in July 2022, and was followed by the Anti-Doping and Medication Control (ADMC) program in Spring of 2023 [51]. These programs outline regulations regarding crop use, veterinary oversight, medication use, and jockey concussion reporting, allowing for consistent enforcement at all racetracks and further protecting welfare and equity of equine athletes [51].

While the implementation of HISA has caused concern and infighting within the racing community, there are likely long-term safety and welfare benefits that will accompany this program. Indeed, it was recently reported that the initial 6 months under HISA safety regulations were the safest on record in the sport's history [52].

1.6 Overall Objectives

Thoroughbred racing and training practices are under tremendous pressure to dramatically improve safety and welfare of the sport. As such, the present study aimed to utilize whole-blood from two-year-old Thoroughbred racehorses in their first season of training in order to evaluate how systemic mRNA expression fluctuates in response to

high-speed exercise and injury. This work, utilizing a panel of mRNA targets, may ultimately contribute to the development of a narrow panel of markers that are able to indicate risk for impending injury of any severity. Critically, this approach will expand the accessibility of individualized medicine and training programs for the equine athlete while improving the safety of Thoroughbred racing. Accordingly, the primary objective of this project was to analyze whole blood mRNA obtained from a group of two-year-old Thoroughbreds throughout their first training and racing season. Based on previous works and current research, the hypothesis was that certain mRNA target expressions would differ between injured and non-injured horse groups, as well as in relation to exercise variables throughout their two-year-old training year.

CHAPTER 2. EFFECTS OF HIGH-SPEED TRAINING ON MESSENGER RNA EXPRESSION IN TWO-YEAR-OLD THOROUGHBRED RACEHORSES

2.1 Introduction

With welfare and safety at the forefront of Thoroughbred racing, the identification of horses at risk for injury is a top priority. In recent years, risk factors for injuries have been examined in great detail [5, 6, 16, 32, 34, 35, 37, 38], but are typically related to race type, track surface and conditions, anatomic conformation, and physiology, among others, leading to difficulty with identifying risk at the individual horse level. This is critical given 55% of two-year-old Thoroughbred racehorses were reported to have incurred a musculoskeletal condition that necessitated veterinary attention during the training season [2].

While the availability of current injury screening technology, such as positron emission tomography (PET), computed tomography (CT), and magnetic resonance imaging (MRI) scans, is increasing, it remains expensive and complex, and therefore inaccessible [42, 43]. A simple, cost-effective method to screen and identify at risk horses is a great need and a step towards individualized medicine in the industry. In 2020, a study published by our group evaluated mRNA changes associated with catastrophic injury risk and identified *IGF-1, IL1RN,* and *MMP2* as promising targets for further investigation [16]. Additionally, progress has been made in the area of injury risk factor identification through analysis of changes in mRNA expression [5, 6, 16, 32, 34, 35, 37, 38]. Less work, however, has been directed at investigating potential variations in mRNA expression in response to high-speed training.

Literature that does focus on the response of mRNA response to training and exercise comes to comparable conclusions. A 2010 study by Liburt *et.al.* reported post exercise increases in *IFN*-γ*, IL-1*β, *IL-6,* and *TNF*-α in equine peripheral blood [17]. Work by our group demonstrated similar post exercise increases in *IFN*-γ and *IL-6,* but noted conflicting results in respect to decreased levels of *TNF*-α and *IL-1*β [53]. An observational study involving 2 groups of young Thoroughbreds exercising at different intensities, reported increased *IL-6* expression and a decreased 'Inflammatory Score' amongst the group with a less strenuous training regimen [53]. This progression to an anti-inflammatory state throughout training suggested an appropriate adaptation to race training and thus presents an opportunity for further research into the effects of exercise on young Thoroughbred racehorses.

As such, the goal of this project was to utilize serial whole-blood mRNA expression analysis in a group of two-year-old Thoroughbreds throughout their two-year-old racing season. Further, using this analysis to determine if mRNA expression could be used to identify those horses at risk for injury, as well as whether there are predictable patterns of mRNA expression changes indicative of successful adaptation to exercise. The hypothesis of this study was that mRNA target expressions would differ in relation to exercise intensity and injury.

2.2 Materials and Methods

2.2.1 Horses and Inclusion Criteria

A cohort of eighteen, two-year-old Thoroughbred colts (n=12) and fillies (n=6) managed by the same trainer within the same facility were utilized in this study. This study was approved by the University of Kentucky's Institute of Animal Care and Use Committee. Detailed training, racing, and injury records were kept by the farm staff and attending veterinarians.

To be included in the study, horses must have accumulated sufficient high-speed exercise, which was defined as galloping at speeds of $\leq$ 15 seconds/furlong. Non-injured horses were required to complete $\geq$ 20 cumulative high-speed furlongs (CHSF) throughout the training season to be included. Due to time off incurred from injury, injured horses were included as long as they performed $\geq$ 5 CHSF during the study period. Summarized inclusion criteria and data is displayed in *Table 2.1*. To evaluate the effects of high-speed training on young Thoroughbreds, the horses were subsequently categorized as injured (n=12) and non-injured (n=6). While injury type was not included in the data analysis, injury types were categorized and quantified (*Table 2.2*).

Table 2.1 Overview of horse groups and associated inclusion criteria.

Horse Group	**n**	**Colts** (n)	**Fillies** (n)	**Inclusion Criteria**
All horses	18	12	6	-
Non-Injured Horses	6	4	2	≥ 20 CHSF
Injured Horses	12	8	4	≥ 5 CHSF

Table 2.2 Summary of lameness related injuries sustained throughout the study.

Injured Horses	Injury Type	n	% of injuries
n = 12 Horses	All Injuries	18	-
	Orthopedic	10	56%
	Soft-tissue	5	28%
	Other	3	17%

2.2.2 Training and Racing

There were no modifications to normal exercise or management practices due to the study. Prior to their import, 11 horses were cantering or breezing regularly, while the remaining 7 horses were broke to ride but were not engaging in high-speed exercise. Horses were evaluated as one group, regardless of prior exercise, due to the lack of race experience and the significant deconditioning period between importation and first high-speed exercise in Dubai (101.8 ± 27.8 days). The average CHSF, as well as the average number of works and races are detailed in *Table 2.3*.

Table 2.3 Summary of exercise performed by study horses.
All values are presented as mean ± standard deviation.

Exercise Variable	**Horses** (n)	**n**	**Pace** (sec./furlong)
Cumulative High-Speed Furlongs	18	50.8 ± 29.5 /season	12.5 ± 0.4
Weekly High-Speed Furlongs	18	2.8 ± 0.7 /week	12.5 ± 0.4
Number of Race Starts	14	3.5 ± 1.8 /season	12.3 ± 0.2

2.2.3 Sample Collection

Peripheral blood samples were collected by veterinarians into Tempus™ Blood RNA Tubes (Applied Biosystems) at approximately weekly intervals commencing at the start of training through the end of the racing season. Samples were collected prior to exercise or administration of any medications. Collection timing in relation to feeding is

not available. Additional samples were taken in cases of suspected injury or illness. Blood was stored according to the manufacturer's recommendations until being shipped to the University of Kentucky's Maxwell H. Gluck Equine Research Center in Lexington, KY, USA for storage and RNA isolation.

2.2.4 Sample Processing

Total RNA was obtained from all Tempus™ Blood RNA Tubes and processed for RT-qPCR, as previously described [20, 21]. Briefly, using the MagMax Core kit (Applied Biosystems™) and KingFisher Flex (ThermoFisher Scientific™), RNA was isolated from all Tempus™ tubes per manufacturer recommendations. The only deviation from these recommendations was the omission of DNase and resuspension of pelleted nucleic acids with 600 µL of Purelink Viral Lysis Buffer (Invitrogen™). RNA was then converted to cDNA using the Maxima H-minus RT kit, from ThermoFisher Scientific™, and stored at -20°C until qPCR using TaqMan™ primers/probes (ThermoFisher Scientific™). β-glucuronidase (β-*GUS*) was used as the endogenous control and a group of sedentary Thoroughbred horses were used as a standardized calibrator for calculation of ΔΔCT [16, 54, 55]. Thirty-four genes were evaluated in duplicate with this method. When possible, commercially available primers and probes (ThermoFisher Scientific™) were used (*Table 2.4*). For those genes without pre-designed assays, custom sets of primers and probes were designed by ThermoFisher Scientific™ based on EqCab 3.0 and the custom assay ID provided in *Table 2.4*. A brief summary of each gene and its primary functions, in relation to this research, can be found in *Supplemental Table S.1.*

Table 2.4 Summary of commercial and custom gene assays used in the study. (ThermoFisher Scientific™)

Gene	Assay ID
ALOX5AP, Arachidonate 5-Lipoxygenase Activating Protein	Ec03470747_m1
AZU1, Azurocidin 1	ARRWHAN
β-GUS, β-glucuronidase	Ec03470630_m1
BMP-2, Bone Morphogenic Protein 2	Ec06974239_m1
CAV1, Caveolin 1	Ec03469261_m1
CAVIN1, Caveolae Associated Protein 1	AR2XCA4
CCL8, Chemokine Ligand 8, C-C Motif	Ec03469486_s1
CXCL1, Chemokine Ligand 1, C-X-C motif	Ec04952640_gH
CTSG, Cathepsin G	ARH6DE4
CD14, Cluster of Differentiation 14	Ec04260516_gH
EGR1, Early Growth Response 1	AREPVPA
ELANE, Elastase, Neutrophil Expressed	ARPRNPU
FOS, Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	ARCE7JN
IGF-1, Insulin-like Growth Factor 1	Ec03468689_m1
IGFBP3, Insulin-like Growth Factor Binding Protein 3	ARPRNPR
IL-1β, Interleukin 1β	Ec04260298_s1
IL1RN, IL-1 Receptor Antagonist	Ec03468814_m1
IL-6, Interleukin 6	Ec03468678_m1
IL-8, Interleukin 8	Ec03468860_m1
IL-10, Interleukin 10	Ec03468647_m1
IL-13, Interleukin 13	Ec03470543_m1
MMP1, Matrix Metalloproteinase 1	Ec03468020_m1
MMP2, Matrix Metalloproteinase 2	Ec03469995_m1
MMP9, Matrix Metalloproteinase 9	Ec03469193_m1
MPO, Myeloperoxidase	AR324V4
Osteoprotegerin, Osteoclastogenesis inhibitory factor	Ec07007303_m1
PLS3, Plastin 3	Ec07011956_m1
PRDM16, PR/SET Domain 16	AR47YFW
PTGS1, Prostaglandin-Endoperoxide Synthase 1	Ec03469511_m1
PTGS2, Prostaglandin-Endoperoxide Synthase 2	Ec03467558_m1
RANKL, Receptor Activator of NFκB Ligand	Ec06625532_m1
TLR-4, Toll-Like Receptor 4	Ec03468994_m1
TNF-α, Tumor Necrosis Factor α	Ec03467871_m1
TNNC2, Troponin C2, Fast Skeletal Type	ARCE7JF
VEGFA, Vascular Endothelial Growth Factor A	Ec03467879_m1

2.2.5 Data Analysis

While qPCR expression of all genes was analyzed individually, the average expression of multiple, specific genes were also averaged into single values, herein referred to an 'index', to represent the horse's inflammatory (*IL-1*β, *IL-6, IL-8, PTGS2,* and *TNF*-α), anti-inflammatory (*IL-10, IL1RN,* and *PTGS1*), and orthopedic (CAV-1, CAVIN-1, IGF-1, IL1RN, MMP2, and PRDM16) status based on both prior [3, 16] and unpublished data. Relative quantities (RQs) of mRNA expression were subsequently calculated and transformed by the natural log (ln(RQ)) to achieve the normality, when possible and as previously described [56]. All statistical analyses were performed using SigmaPlot 15.0 (Systat Software, San Jose, CA). Average weekly gene expression of individual genes, as well as the inflammatory, anti-inflammatory, and orthopedic indices were calculated for all non-injured horses. For injured horses, injury weeks (Weeks -4, -3, -2, -1, 0, +1, and +2) were standardized for analysis such that Week 0 of injury was the most recent sample taken prior to injury (4.4 ± 1.2 days before injury). To allow for a 1:1 comparison, all non-injured horses were averaged together to form an 'average non-injured horse' data set for the entirety of the project. This data was then used to provide a comparable dataset for comparison with the injury weeks of each injured horse. This process was repeated for all 12 injured horses using the same pool of 'average non-injured horse' data. Pearson Correlation tests were used separately to evaluate gene expression associations between injured or non-injured horse groups and various aspects of high-speed exercise. The exercise variables included: cumulative high-speed furlong (CHSF) performance, average weekly high-speed furlong performance, and week of training. CHSF was calculated for

each horse by summating high-speed furlongs performed in race or training throughout the season according to week of training. Likewise, average weekly high-speed furlong performance was calculated for individual horses based on the number of high-speed race or training furlongs performed during each week of training. For week of training analysis, injury weeks -4 through +2 were used in the injured horse analysis, while all weeks of training (1-31) were used in the non-injured horse analysis. Only correlation coefficients $\leq$ 0.30 and $\geq$ -0.30 are reported. To directly analyze differences in gene expression between injured and non-injured horse populations, a two-way repeated measures ANOVA (analysis of variance) was used and both groups were evaluated from weeks -4 through +2. All statistical analyses were considered significant at $p < 0.05$.

2.3 Results and Discussion

The use of PCR examine the pathology of injured horses has increased significantly, with nearly 40% of related publications occurring between 2018 and 2022 [57]. Most commonly, peripheral whole blood or muscle samples have been utilized in large observational racing-based studies or small controlled exercise tests [3, 16-18, 37, 53, 58, 59]. Given the stability of RNA collected using Tempus™ Blood RNA Tubes, developing a panel of whole-blood mRNA targets that would identify horses at risk of injury or those not adapting properly to their training regimen could be a beneficial addition to racing and training programs.

2.3.1 Non-Injured Horse Group

Within the non-injured horse group, 13 genes and 2 indices were significantly correlated with increasing average weekly high-speed furlong performance (*Table 2.5*). Of the 8 genes and 2 indices positively associated with average weekly furlongs, *IL-8, TLR-4*, and *TNF*-α are all considered to be pro-inflammatory cytokines. They are associated with chemotaxis of inflammatory mediators such as monocytes, macrophages, and neutrophils. [17, 21, 60-62] Interestingly, *PTGS1*, the gene that encodes cyclooxygenase-1 (*COX-1*), also increased with average weekly furlongs [21, 60]. These findings, especially in the context of *TNF*-α, were unexpected, as previous data from young Thoroughbreds demonstrated a decrease in inflammatory gene expression over multiple weeks of training [3, 17, 53]. The CAV-1/VEGF pathway works to increase angiogenesis, which, in the case of *VEGFA*, increases in times of hypoxia. High-intensity exercise can promote anaerobic energy metabolism due to the increased oxygen demand by peripheral tissues. These two genes work together to increase systemic and localized blood flow through the formation of new blood vessels at the site of inflammation to increase oxygen availability and injury healing [63-68]. *MMP2* has been implicated as a key player in acute joint inflammation as well as chronic, long-lasting, joint diseases [69]. Fibroblasts and chondrocytes, cells that comprise the articular matrix of a joint, secrete MMP2, where it works to degrade collagen and maintain cartilage homeostasis [69-71]. The upregulation of this gene may suggest that these horses are addressing low grade inflammation due to exercise. *Osteoprotegerin* functions to reduce bone loss by inhibiting RANKL, and consequently, preventing osteoclast maturation. Given that osteoclasts are the main

mediator of bone resorption and degradation, particularly in the subchondral bone of joints, [28] this could suggest that in times of inflammation, there is an amplified decrease in osteoclast production, allowing osteoblasts to rebuild injured bone.

Of the 5 genes negatively correlated with average weekly furlong performance in non-injured horses, *CTSG, ELANE,* and *MPO* are all proteolytic enzymes involved with degradation of collagen and inflamed tissues, and the formation of hypochlorous acid. Hypochlorous acid is a natural compound that degrades hyaluronic acid, found in elevated concentrations in the synovial fluid of horses with osteoarthritis [68, 72-82]. The downregulation of these genes may prevent the destruction of protective articular collagen and hyaluronic acid in the joint of the exercising horse, allowing these beneficial components to protect against joint injury. Further, *AZU1* and *CCL8* function to attract leukocytes to the site of inflammation, particularly monocytes in the case of *AZU1,* to induce phagocytosis. Decreased expression of these genes could suggest that these horses are not incurring the significant inflammation that necessitates the production of these cytokines to clean up damaged tissues following injury and inflammation [83-87].

In the non-injured horses, there was a negative correlation between 6 genes (*CCL8, CXCL1, ELANE, IGFBP3, MPO,* and *TNNC2*) and CHSF. Similarly, a negative correlation was seen between *CXCL1, IGFBP3, MPO,* and *TNNC2* expression and week of training (*Table 2.5*). Given that CHSF would increase as week of training increased, these similarities are not unexpected. As the non-injured horses progressed through the training season and accumulated more high-speed furlongs, expression of inflammatory cytokines subsequently decreased, perhaps demonstrating an adaptation to exercise.

Table 2.5 Pearson Correlation summary of the non-injured horse group.
Only genes with correlation coefficients ≥ 0.30 or ≤ -0.30 (top value) AND p-values < 0.05 (bottom value) are reported. Values in bold represent significant values for each exercise category.

Non-injured Group		***AZU1***	***CAV-1***	***CCL8***	***CTSG***	***CXCL1***	***ELANE***	***IGFBP3***	***IL-8***	***MMP2***
Week of Training	*cc*	-0.0827	0.252	-0.258	-0.0932	**-0.451**	-0.253	**-0.391**	0.0848	0.159
	p	0.321	0.00197	0.00153	0.268	**1.01E-08**	0.00199	**9.84E-07**	0.305	0.054
Cumulative	*cc*	-0.192	0.247	**-0.304**	-0.209	**-0.456**	**-0.346**	**-0.404**	0.175	0.182
High-Speed Furlongs	*p*	0.0205	0.00243	**0.000172**	0.0123	**6.37E-09**	**1.79E-05**	**3.95E-07**	0.0332	0.0266
Average Weekly	*cc*	**-0.413**	**0.3**	**-0.324**	**-0.38**	-0.262	**-0.493**	-0.259	**0.565**	**0.412**
High-Speed Furlongs	*p*	**2.23E-07**	**2.11E-04**	**5.76E-05**	**2.93E-06**	1.32E-03	**2.17E-10**	1.54E-03	**7.84E-14**	**1.90E-07**
		MPO	***Osteoprotegerin***	***PTGS1***	***TLR4***	***TNF-α***	***TNNC2***	***VEGFA***	**Anti-Inflammation Index**	**Orthopedic Index**
Week of Training	*cc*	**-0.397**	0.0308	0.0627	0.224	0.0381	**-0.353**	0.0844	0.121	0.118
	p	**6.32E-07**	0.71	0.449	0.00622	0.646	**1.11E-05**	0.308	0.143	0.152
Cumulative	*cc*	**-0.468**	0.0609	0.0878	0.19	0.0785	**-0.325**	0.0743	0.106	0.155
High-Speed Furlongs	*p*	**2.23E-09**	0.462	0.289	0.0205	0.343	**5.56E-05**	0.369	0.2	0.0597
Average Weekly	*cc*	**-0.426**	**0.429**	**0.32**	**0.311**	**0.453**	-0.0907	**0.405**	**0.33**	**0.34**
High-Speed Furlongs	*p*	**7.39E-08**	**5.35E-08**	**7.22E-05**	**1.21E-04**	**7.29E-09**	0.273	**3.36E-07**	**4.16E-05**	**2.32E-05**

CCL8, CXCL1, and ELANE are involved in the chemotaxis and activation of leukocytes, which aim to address inflammation [80-82, 85-90]. Interestingly, IGFBP3 inhibits RANKL and, therefore, osteoclast production. Coupled with the decreasing expression of *ELANE* and *MPO*, this may suggest a decreased need for tissue degradation due to appropriate adaptation to exercise in this group of horses [72-78, 80-82, 91]. *TNNC2*, the gene responsible for fast skeletal muscle contractions and calcium signaling pathways [92], was also downregulated and may further suggest an adaptation to high-intensity training.

2.3.2 Injured Horse Group

Amongst the injured horse group, 6 genes were significantly correlated with an increase in average weekly furlongs (*Table 2.6*). Of these, *EGR1, MMP9,* and *RANKL* had positive correlations with average weekly furlong performance. These genes serve various functions such as angiogenesis, gelatin and collagen degradation, and osteoclast regulation. These genes are also frequently implicated in the pathology of equine osteoarthritis and could suggest a relationship with other types of skeletal inflammation [21, 28, 70, 71, 93, 94]. Additionally, *ALOX5AP, IL1RN,* and *MMP1* had a negative correlation with average weekly furlong performance amongst the injured horses. The downregulation of these cytokines could be indicative of low-grade inflammation secondary to exercise maladaptation and injury as these cytokines work to recruit pro-inflammatory leukocytes (ALOX5AP), inhibit *IL-1*β synthesis (IL1RN), and degrade tissue, (MMP1) [17, 20, 21, 67, 70, 95, 96].

Table 2.6 Pearson Correlation summary of the injured horse group.
Only genes with correlation coefficients ≥ 0.30 or ≤ -0.30 (top value) AND p-values < 0.05 (bottom value) are reported. Values in bold represent significant values for each exercise category.

Injured Group		***ALOX5AP***	***AZU1***	***CXCL1***	***EGR1***	***IGFBP3***	***IL-13***	***IL1RN***
Week of Training	*cc*	-0.0855	**-0.304**	**-0.538**	0.106	**-0.514**	-0.264	-0.059
	p	0.359	**0.000848**	**4.02E-10**	0.256	**3.17E-09**	0.00409	0.527
Cumulative High-Speed Furlongs	*cc*	0.0733	**-0.356**	**-0.535**	0.00842	**-0.338**	**-0.437**	-0.177
	p	0.432	**8.37E-05**	**5.32E-10**	0.928	**0.000196**	**8.54E-07**	0.0559
Average Weekly High-Speed Furlongs	*cc*	**-0.32**	0.277	-0.0635	**0.354**	-0.0432	0.158	**-0.367**
	p	**0.000444**	0.00254	0.496	**8.89E-05**	0.643	0.0898	**4.66E-05**
		MMP1	***MMP9***	***MPO***	***PRDM16***	***RANKL***	**Inflammation Index**	**Anti-Inflammation Index**
Week of Training	*cc*	-0.298	0.0382	**-0.593**	**-0.346**	-0.279	-0.162	-0.0764
	p	0.0011	0.683	**1.86E-12**	**0.000131**	0.0023	0.0808	0.413
Cumulative High-Speed Furlongs	*cc*	-0.259	0.113	**-0.504**	**-0.375**	-0.222	**-0.3**	-0.207
	p	0.00473	0.226	**6.68E-09**	**3.08E-05**	0.0159	**0.000999**	0.0252
Average Weekly High-Speed Furlongs	*cc*	**-0.471**	**0.325**	0.171	0.175	**0.323**	-0.108	**-0.332**
	p	**8.10E-08**	**0.000351**	0.0656	0.059	**0.00039**	0.245	**0.000256**

Increasing cumulative high-speed furlong performance within the injured horse group revealed significant negative correlations with 6 genes (*AZU1, CXCL1, IGFBP3, IL-13, MPO,* and *PRDM16*), as well as the inflammatory index. Significant negative correlations of the same genes, with the exception of *IL-13*, were seen when evaluating effect of week of training on expression. The observed downregulation of these anti-inflammatory cytokines correlates with the concurrent increase of CHSF within the injured horse group, likely due to their maladaptation to training at high speeds. *AZU1* and *CXCL1* function to attract and activate leukocytes, promote angiogenesis, and phagocytize cellular debris at the site of inflammation to promote healing. *IGFBP3, IL-13,* and *MPO* function to induce TNF-α (a pro-inflammatory cytokine), induce fibrosis, and regulate the oxygen dependent mechanism of phagocytosis, respectively [72-78, 82-84, 88-91, 97]. While determining whether any of these cytokines play an active role in injury predilection or prediction cannot be directly elucidated from this data, further work to determine this possibility is warranted.

2.3.3 Group Comparisons

Interestingly, both injured and non-injured horse groups had a negative correlation between 3 genes (*CXCL1, IGFBP3,* and *MPO*) and both increasing CHSF and week of training (*Figure 2.1*). While the main goal of this study was to evaluate differences between groups, this similarity suggests that downregulation of these genes is directly related to an increase of high-speed exercise, regardless of injury status. Although all 3 of these genes play different and important inflammatory roles, this finding suggests that they could be used as markers of fitness in racehorses.

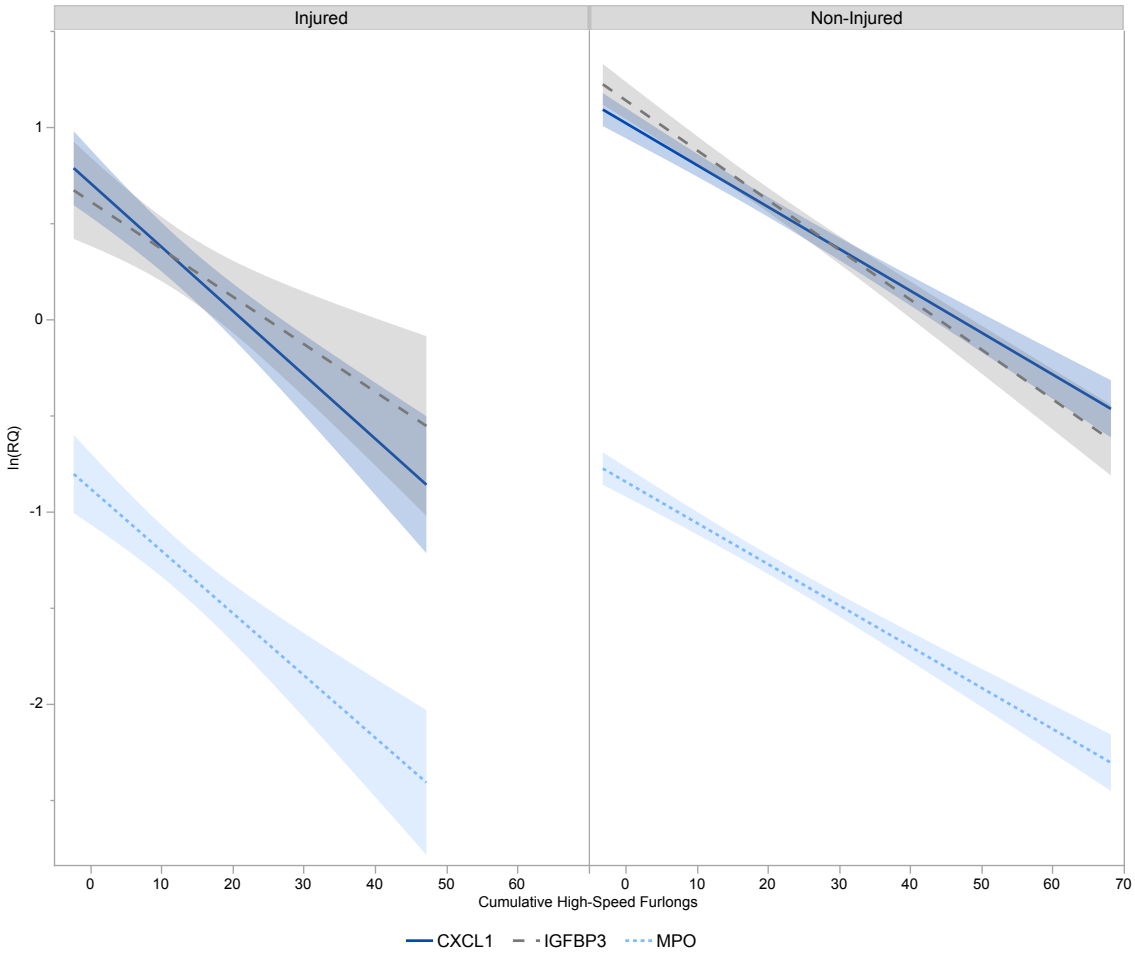

Figure 2.1 mRNA expression similarities of CXCL1, IGFBP3, and MPO.
Line of fit showing mRNA expression values as the natural log of relative quantities amongst the injured and non-injured horse groups in relation to cumulative high-speed furlongs. Figure created with JMP Pro 17.

Perhaps one of the more interesting results from this analysis are the divergent results between the injured and non-injured horse anti-inflammatory index and average weekly furlongs (*Figure 2.2*)**.** The anti-inflammatory index is composed of *IL-10, IL1RN,* and *PTGS1*, which are all commonly referred to as anti-inflammatory cytokines**.** IL-10 and IL1RN inhibit IL-1β production and subsequent inflammatory byproducts such as lymphocytes and monocytes; PTGS1 leads to downstream production of thromboxanes, eicosanoids, and prostaglandins. Certain prostaglandins, such as PGE_2 can reduce swelling by interfering with histamine-related mast cell inflammation pathologies, such as

edema [98]. This trio works together to combat inflammation by addressing fever, pain, inflammatory cytokine production, and tissue repair [21, 60, 67, 99]. Prior work by our group examining differences between two groups of two-year-old Thoroughbreds determined that differences in training regimens can have a profound effect on pro-inflammatory mRNA expression [3]. Further, the study suggested properly trained Thoroughbreds will exhibit an "anti-inflammatory state", which appears to be confirmed here by the increased anti-inflammation index of the non-injured horses (*Figure 2.2*).

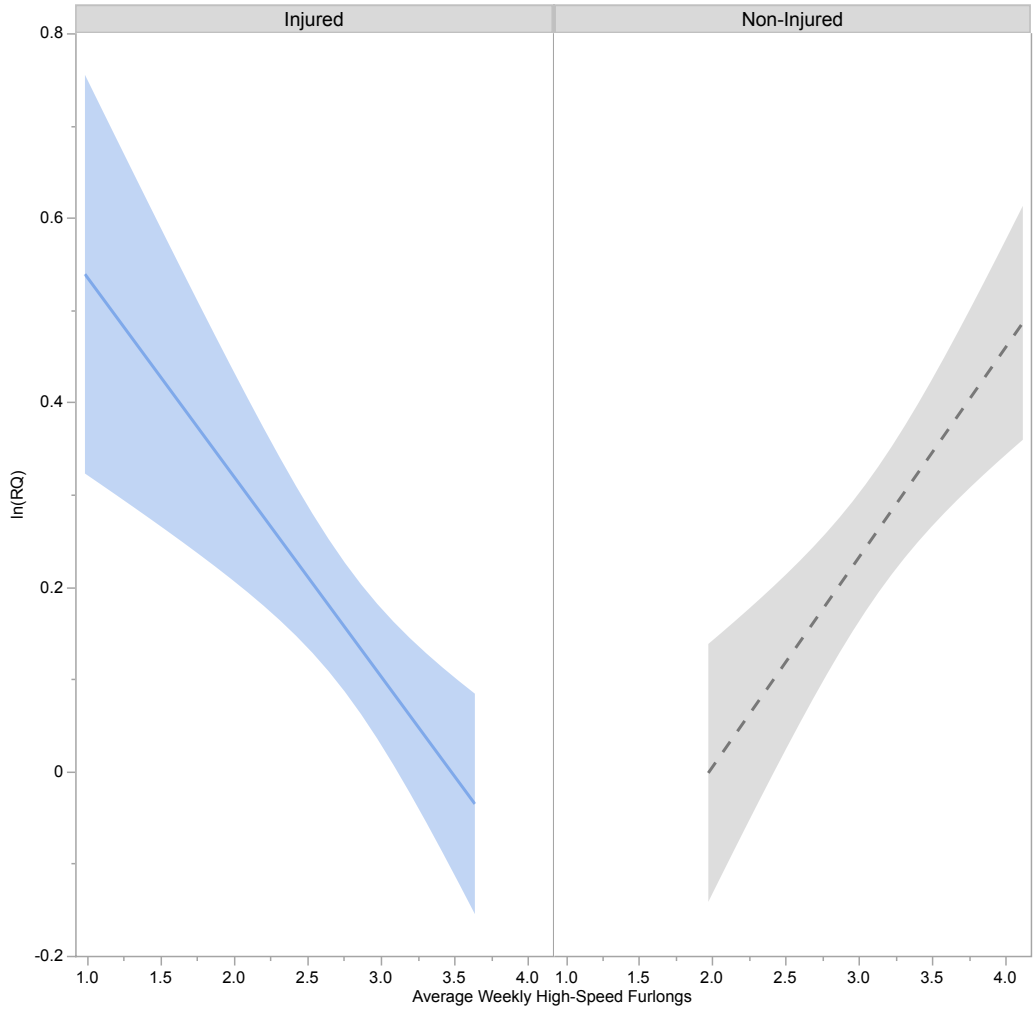

Figure 2.2 mRNA expression differences of the anti-inflammation index.

Line of fit showing mRNA expression values as the natural log of relative quantities amongst the injured and non-injured horse groups in relation to average weekly high-speed furlongs. Figure created with JMP Pro 17.

Assessing the effects of injury on mRNA expression within this population of horses was an important goal of this study, as was determining whether mRNA expression could be used as an early indicator for increased injury risk. Overall, there were 7 genes with significant differences in average expression between the injured horses and the corresponding 'average non-injured horse' encompassing the period from 4 weeks prior to injury through 2 weeks post injury (*Figure 2.3*).

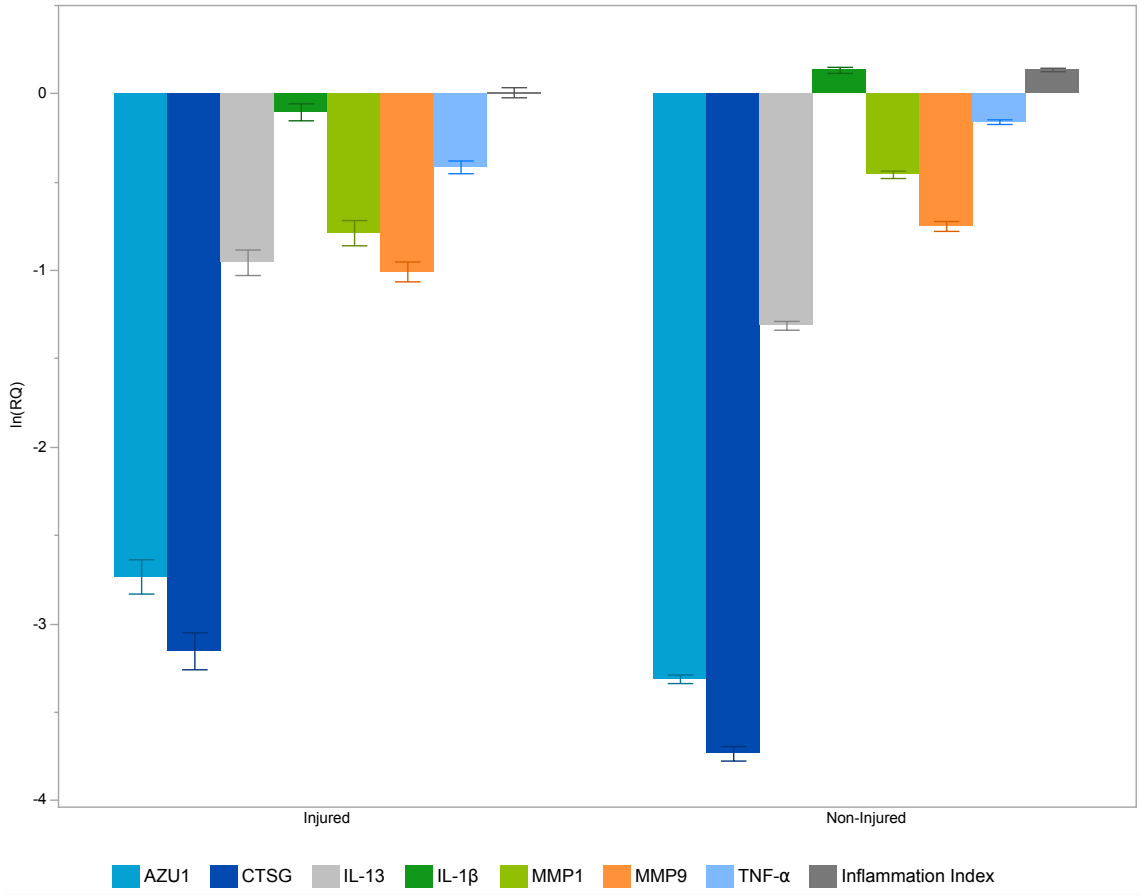

Figure 2.3 Overall mRNA expression differences between injured and non-injured horses shown as mean ± standard error. Expressions, shown as the natural log of relative quantities, were significantly different for all provided genes ($p < 0.05$). Figure created in JMP Pro 17.

Additionally, 9 genes (*AZU1, CAV-1, CTSG, IL-13*, *IL-1*β*, MMP1*, *MMP9*, *PRDM16*, and *TNF*-α) and the inflammatory index had significant expression changes during the week of, or surrounding, injury. *Figure 2.4* shows the significant expression differences between injured and non-injured horse groups. The majority of these pro-inflammatory cytokines are different during Weeks -1, 0, and +1, showing changes in the injured horse group due to their immediate injury response. Further, Week -3 (*AZU1, IL-13, MMP9)* and Week -2 (*IL-13, MMP1, MMP9)* each had 3 genes with significant differences between groups, suggesting that they may play an important role in injury prediction through mRNA analysis.

2.3.4 Limitations

There were several limitations to this study, the first of which was the small sample size. While the study horses were managed and trained as normal, this was a field study and sources of variability can be difficult to control. Another limitation was that the regular administration of medications may have had potential effects on mRNA expression. However, since the horses were treated with similar frequency and this study sought to examine mRNA expression under real-world conditions, medications were not factored into statistical analysis. In addition, samples were collected prior to medication administration and unpublished data from our group suggests mRNA expression returns to steady-state following the administration of most anti-inflammatory medications within 24 hours. To address the aims of the present study, data analysis of these 34 genes was kept to a descriptive and diagnostic level, as opposed to the use of any predictive modeling. With the extent of novel correlations and data supporting previous works,

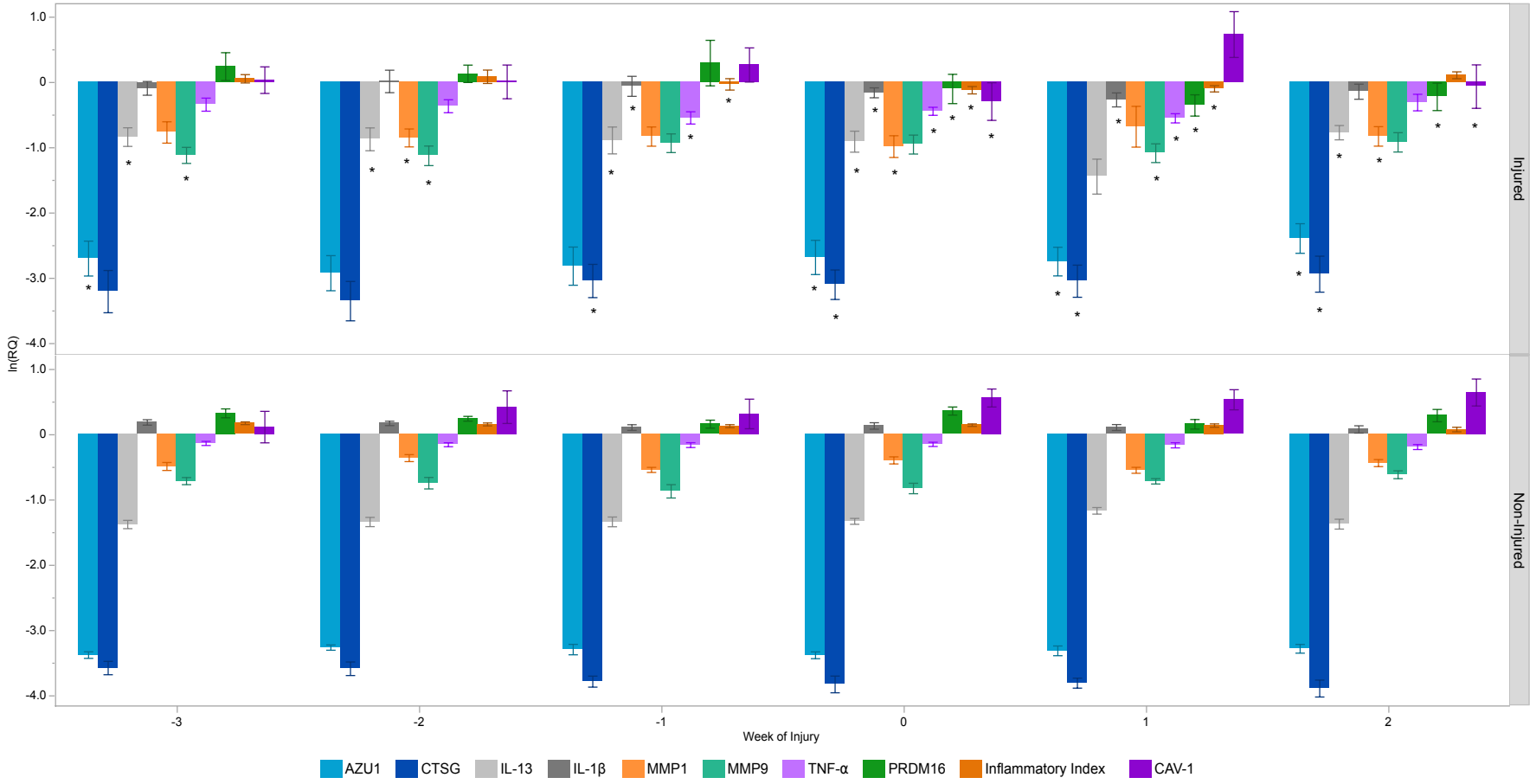

Figure 2.4 mRNA expression values of injured and non-injured horses between individual weeks of injury.
Expression differences between injured and non-injured horse groups at a specific week of injury are denoted with an asterisk (*) when significant ($p < 0.05$). Values shown as the natural log of relative quantities of mean expressions ± standard error. Figure created in JMP Pro 17.

future studies utilizing predictive models of combined mRNA panels may be warranted. Additionally, given the sample size and scope of the project, other previously reported injury risk factors were not evaluated as a part of this project [5, 6, 16, 32, 34, 35, 37, 38]. Lastly, it is important to note that the translation of peripheral blood mRNA into serum protein expression is not equitable. Therefore, we are unable to compare functional protein changes to the noted mRNA expression changes.

2.4 Conclusion

Herein, both significant and novel relationships between Thoroughbred training habits and mRNA expression were identified. Interestingly, the amount of high-speed work per week was the most influential factor for mRNA expression changes between injury groups. Consistent decreases in *CXCL1*, *IGFBP3*, and *MPO* expression amongst all horses suggests that these three genes may be markers of exercise adaptation in the equine athlete. Further, the inverse expression of the anti-inflammatory index (*IL-10, IL1RN,* and *PTGS1*) between injured and non-injured horses, in association with weekly furlongs, implies these genes could be indicators of proper exercise adaptation. Further, the analysis of mRNA expression in the weeks leading to injury indicated that *IL-13* and *MMP9* may serve as markers for injury prediction. All together, these genes appear to be viable targets of interest in further work addressing Thoroughbred injuries and exercise adaptation.

CHAPTER 3. REFLECTIONS AND FUTURE DIRECTIONS

3.1 Project Reflections

If given an opportunity to address the aforementioned limitations in a future study, there are a few, yet substantial, changes I would make, logistics not considered. Foremost, I would aim to address the issues that arise with a small sample size. While a comprehensive study of 18 horses is considerable given this preliminary work, a larger study population, spanning multiple farms under similar management and training conditions, would allow for intra-farm and inter-farm comparisons. This would diminish the possibility that the training and injury-related mRNA changes seen here are isolated to a specific trainer or farm.

The next issue I would address with the current study design in a new, prospective study, would be the addition of a group of horses without non-essential medications. Medication use in the Thoroughbred racing industry is common, and thus not including it as a factor in statistical analysis allowed for a realistic view of how an mRNA panel would function in regular use. The addition of a 'minimal-medication' group would allow for analysis of cytokines between horses regularly receiving medication and those that are not. Further, this would allow for the identification and elimination of any mRNA targets affected by drug interactions through inter-group comparisons.

Lastly and briefly, though Thoroughbred racing and training injuries are a global issue, I think a comparative study amongst US-based racehorses would be beneficial due to the size and scale of the industry.

3.2 Future Directions

In the near future, I hope to see prospective research further evaluating mRNA expression as an indicator and identifier of injury-risk amongst Thoroughbred racehorses. Due to the plasticity of mRNA target panels and the similarity of horse breeds, I foresee this application benefiting all equine athletes, across all disciplines. Further, more RNA-sequencing analysis of catastrophically injured horses, with varying pathologies, from training and racing conditions, should be performed to evaluate mRNA targets for concordance and to continue developing a strong risk assessment panel.

In the less-near future, the production of a stable-side test allowing for accurate and immediate mRNA expression analysis would be an ideal addition to pre-race veterinary screening as well as regular monitoring of equine athletes performing high-speed exercise. Real-time analysis of at-risk horses would provide a quantifiable injury risk mitigation, adding an objective element to current injury risk assessment protocols. While such a product is far-off and idealistic, it is within the realm of possibility as other stable-side tests (such as SAA kits, glucometers, and fecal blood tests) are already in use throughout the industry. Considering the countless bright minds in the equine research industry, my doubts regarding this possibility are slim.

3.3 Concluding Thoughts

I am endlessly grateful for the opportunities and challenges that were presented to me throughout my master's program. I have been asked to think in ways a scientist would, even if I do not yet consider myself a scientist. I have been challenged to take on

projects and tasks outside of my comfort zone, subsequently expanding that range exponentially. The visible, unyielding trust my mentors have had in me has given me confidence that I will, someday, be the scientist they taught me to think as. For now, I will take the wisdom, life-lessons, wit, and knowledge they have instilled in me over the past two years and utilize it in my future careers in industry research. Appreciation and gratitude are not strong enough to express how thankful I am for my experience at UK, the Gluck Center, and with my mentors.

SUPPLEMENTAL TABLE

Table S.1 Supplemental Table: *Summary of investigated genes and their functions.*

Gene	Assay ID	Associated Functions	Sources
ALOX5AP Arachidonate 5-Lipoxygenase Activating Protein	Ec03470747_m1	Synthesis of pro-inflammatory leukotrienes through the arachidonic acid pathway, associated with increased risk of stroke and atherosclerosis	[20, 95, 96]
AZU1 Azurocidin 1	ARRWHAN	Targets inflammatory cells in blood, endothelium, and extravascular space; preferentially binds monocytes and enhances phagocytosis	[83, 84]
β-*GUS* β-glucuronidase	Ec03470630_m1	Housekeeping/reference gene	[100-102]
BMP-2 Bone Morphogenic Protein 2	Ec06974239_m1	Stimulates osteogenesis, cartilage synthesis, and chondrogenesis of SMPCs; part of *TGF*-β ligand superfamily	[103]
CAV1 Caveolin 1	Ec03469261_m1	Caveolae structural component; exercise induces angiogenesis and neurogenesis (CAV1/VEGF pathway), impaired injury repair (downregulation), *TGF*-β signaling	[63, 64]
CAVIN1 Caveolae Associated Protein 1	AR2XCA4	Caveolae structural component; downregulation causes macrophage and protease influx, impairs lipid metabolism and stress-related remodeling	[104]
CCL8 Chemokine Ligand 8, C-C Motif	Ec03469486_s1	Produced by fibroblasts and mononuclear cells, leukocyte chemotaxis and activation, inhibits CD4 mediated entry of HIV	[85-87]
CD14 Cluster of Differentiation 14	Ec04260516_gH	Found in increased concentrations in OA joints, macrophage recruitment and activation, LPS receptor, production of inflammatory cytokines	[105, 106]
CTSG Cathepsin G	ARH6DE4	Serine protease released by neutrophils and monocytes; connective tissue remodeling, increases blood pressure (angiotensin I - II conversion), chemo/cytokine stimulation	[68, 79-81]
CXCL1 Chemokine Ligand 1, C-X-C motif	Ec04952640_gH	Neutrophil chemoattractant, can be released by mast cells, chemotaxis and activation of leukocytes, angiogenesis, atherosclerosis	[82, 88-90]
EGR1 Early Growth Response 1	AREPVPA	Associated with muscle contractility, DNA transcription factor, mitochondria synthesis, angiogenesis	[107]
ELANE Elastase, Neutrophil Expressed	ARPRNPU	Serine protease released by neutrophils; cytotoxic functions, degrades tissue, activates lymphocytes at inflammation site	[80-82]
FOS Fos Proto-Oncogene, AP-1 Transcription Factor Subunit	ARCE7JN	Controls RANKL signaling, involved in osteoblast differentiation and proliferation, differentiates Th1/Th2 cells, AP-1 transcription factor links inflammation to osteoclast/blast signaling pathways	[31]
IGF-1 Insulin-like Growth Factor 1	Ec03468689_m1	Upregulated in OA; stimulates cell growth, fibroblast/collagen proliferation, injury remediation	[67, 70, 105]
IGFBP3 Insulin-like Growth Factor Binding Protein 3	ARPRNPR	Anti-inflammatory, inhibits NF-κB cascade, induces apoptosis and *TNF*-α, prevents bone resorption via inhibition of RANKL/osteoclastogenesis	[91]
IL-10 Interleukin 10	Ec03468647_m1	Stimulates *IL-1*β, immunosuppressive activity, increase Th2 effector functions, deficiency can cause fibrosis	[21, 97]
IL-13 Interleukin 13	Ec03470543_m1	Inhibits pro-inflammatory cytokines, promotes IgE, regulates ECM, involved lung inflammation, mucus secretion, induces fibrosis, activates collagen production	[97]
***IL-1*β** Interleukin 1β	Ec04260298_s1	Pro-inflammatory cytokine; attracts lymphocytes, neutrophils, and monocytes/macrophages, impairs tissue repair	[17, 67]
IL-6 Interleukin 6	Ec03468678_m1	Myokine; releases *IL1RN* and *IL-10*, muscle glucose homeostasis	[15, 17]
IL-8 Interleukin 8	Ec03468860_m1	Produced by macrophages; chemotaxis of neutrophils	[21, 59]
IL1RN IL-1 Receptor Antagonist	Ec03468814_m1	Downregulates inflammatory response, inhibits *IL-1*β binding, promotes injury remediation	[21, 67]
MMP1 Matrix Metalloproteinase 1	Ec03468020_m1	Upregulated in synovitis; extracellular matrix organization, tissue remodeling and degradation	[21, 70]
MMP2 Matrix Metalloproteinase 2	Ec03469995_m1	Upregulated in OA; degrade collagen, secreted by fibroblasts and chondrocytes	[69-71]
MMP9 Matrix Metalloproteinase 9	Ec03469193_m1	Upregulated in OA; degrade gelatin, ECM organization, produced by monocytes, PMNs, and chondrocytes	[21, 69-71]
MPO Myeloperoxidase	AR324V4	Biosynthesis of Hypochlorous acid (degrades articular cartilage), released by neutrophils, involvement in oxygen dependent mechanism of phagocytosis, abundance of neutrophil recruitment can result in capillary blockages	[72-78]
Osteoprotegerin,*'TNFRSF11B',* Osteoclastogenesis inhibitory factor, TNF receptor superfamily member 11B	Ec07007303_m1	Released by osteoclasts, decoy receptor for RANKL (prevents osteoclast maturation), preserves cartilage, inflammation increases concentration in chondrocytes, shown to decrease with Dexamethasone treatment, reduce bone loss	[31, 94]
PLS3 Plastin 3	Ec07011956_m1	Binds F-actin, involved in osteoblast/clast functions, interacts with NKRF, reported relation to osteoporosis/decreased bone density	[108, 109]

PRDM16 PR/SET Domain 16	AR47YFW	Transcription regulator for Brown/Beige Adipose Tissue (BAT, associated with 'lean and healthy' phenotypes, increases energy spending without dysregulating other cells), knockout causes skeletal muscle proliferation	[110]
PTGS1 Prostaglandin-Endoperoxide Synthase 1	Ec03469511_m1	Encodes cyclooxygenase-1 (COX-1); involved in arachidonic acid pathway, regulate fever, pain, and inflammatory cytokine production, tissue homeostasis	[60, 99]
PTGS2 Prostaglandin-Endoperoxide Synthase 2	Ec03467558_m1	Encodes Cyclooxygenase-2 (COX-2); inhibit cell proliferation, involved in arachidonic acid pathway, production of prostaglandins/thromboxane in macrophages	[21, 60, 111]
RANKL, '*TNFSF11*' Receptor Activator of Nuclear Factor Kappa-B Ligand, TNF Superfamily Member 11	Ec06625532_m1	Upregulated in OA; controls osteoclast function (differentiation, bone resorption, survival), inhibits osteoprotegerin, also produced by osteoblasts and chondrocytes, degrades collagen	[28, 94]
TLR-4 Toll-Like Receptor 4	Ec03468994_m1	LPS receptor, production of pro-inflammatory cytokines, response to tissue injury	[61, 62]
TNF-α Tumor Necrosis Factor α	Ec03467871_m1	Shown to increase with intense exercise, pro-inflammatory cytokine; muscle glucose uptake, *IL-6* stimulation	[17, 21]
TNNC2 Troponin C2, Fast Skeletal Type	ARCE7JF	Responsible for skeletal muscle contractions, involved in calcium signaling pathway	[92]
VEGFA Vascular Endothelial Growth Factor A	Ec03467879_m1	Initiates angiogenesis in smooth muscles and leukocytes, hypoxia increases production, involved in vessel damage pathogenesis, vasodilation, increased vascular permeability	[65-68]

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- 2014 – 2019, *Retail Associate*, Pemberton Pharmacy & Gift, Salisbury, MD
- 2016 – 2018*, Sales Associate,* Oakley's Farm Markey, Hebron, MD

III. Professional publications

First-Author

1. "Effect of High-Speed Training in 2-year-old Thoroughbreds on Select mRNA", JEVS July 2023
    - Equine Science Society Symposium presentation, June 2023

Co-Author

1. "The Effect of Inhaled Ciclesonide Treatment on Systemic Markers of Immune Function in Horses", *JEVS expected mid 2023*, Allen Page, DVM, PhD
2. "Residual Effects of Intra-Articular Betamethasone and Triamcinolone Acetonide in an Equine Acute Synovitis Model", EVJ 2022, Emma Partridge, MS
3. "Development and Evaluation of a Muscle Atrophy Scoring System (MASS) for Horses", JEVS 2022, Alisa Herbst, PhD