

**PLASMA CITRULINE LEVELS IN HORSES  
AT RISK OF ACUTE LAMINITIS**

An Undergraduate Research Scholars Thesis

by

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## **ABSTRACT**

Plasma Citrulline Levels in Horses at Risk of Acute Laminitis. (May 2013)

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Laminitis is a painful and irreversible disease in horses in which the soft tissue structures of the foot, called the laminae (connecting the coffin bone to the hoof wall), lose blood flow and deteriorate. Without the support of these laminae the coffin bone rotates downward, applying pressure to the sole of the foot and crushing the underlying structures, resulting in severe pain. Laminitis typically progresses through three stages: the early developmental stage is treatable yet undetectable, while the later acute and chronic stages are symptomatic but irreversible. Therefore, the identification of a diagnostic marker capable of detecting the developmental stage would allow earlier and more effective treatment.

Laminitis is often triggered by unrelated events occurring elsewhere in the body such as gastrointestinal (GI) upset episodes, typically called “colic”, which involve intestinal epithelial cell death. Human studies have concluded that intestinal epithelium health can be measured using plasma citrulline concentrations. Citrulline is an  $\alpha$ -amino acid circulated in the plasma that is produced mainly by intestinal epithelial cells. We hypothesized that horses in the

developmental stage of laminitis would have reduced plasma citrulline concentrations resulting from intestinal epithelial cell death occurring from a GI upset episode.

In this study, blood samples were collected from control horses (n=23) and horses at risk for developing laminitis (n=20). Plasma citrulline concentration was measured using chromatography based amino acid analysis. The normal range was then calculated from the control group and compared to the concentrations from horses that did or did not develop laminitis. Five of the 20 cases developed laminitis symptoms and also had reduced plasma citrulline concentrations. If decreased citrulline levels correlate with laminitis onset across a large set of samples, a simple and affordable blood test could be developed in the future to predict the likelihood of the disease progression to the acute and chronic (irreversible) stages. This would allow veterinarians to begin treatments that could significantly reduce the chance of the horse developing the condition, greatly improving their prognosis.

## ACKNOWLEDGEMENTS

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I would also like to thank my mom for her continued support, encouragement, and funding in this project as well as everything else I decide to do during my time here at Texas A&M University.

This study also would not be possible without the help from the wonderful veterinarians and technicians who provided my samples including Dr. James Schulze from Equine Veterinary Associates as well as Dr. Cliff Honnas and Evan Scott from Texas Equine Hospital. I would also like to thank Virginia Johnson from the Texas A&M Protein Chemistry Laboratory for performing the amino acid analysis.

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# CHAPTER I

## INTRODUCTION

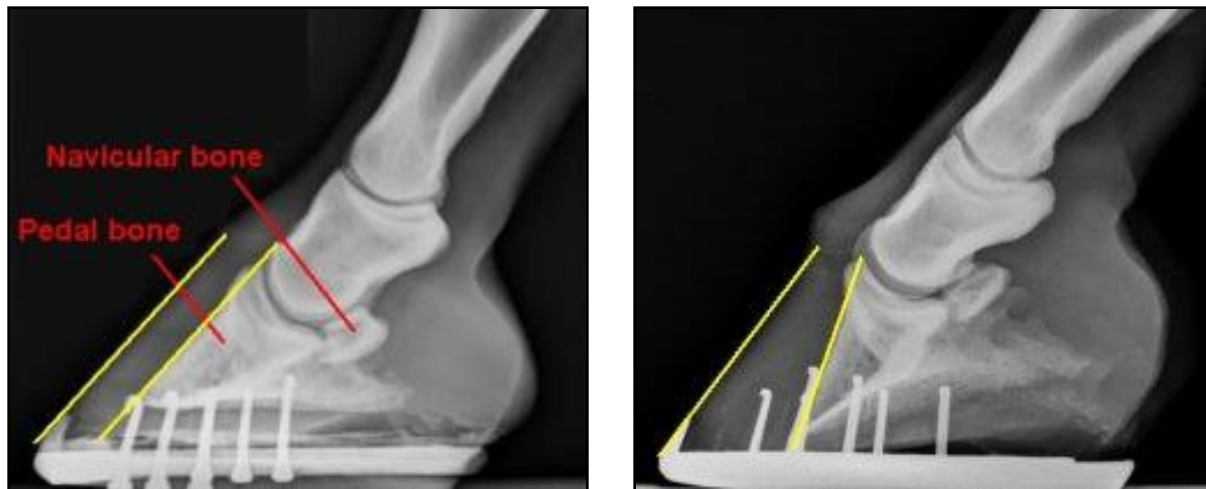
### Background

Laminitis, the same disease that claimed the lives of the famous racehorses Secretariat and Barbaro, afflicts many horses worldwide at some point in their lifetime and is often career ending and life threatening to those afflicted (Moyer, 2004).

During laminitis, the soft tissue structures called the laminae, which lie between the third phalanx (also called the coffin bone or P<sub>3</sub>) and the hoof wall, lose blood flow and deteriorate (Hood, 1993). These laminae act as Velcro attaching the bone to the hoof wall and without their support the bone begins to rotate downward, applying pressure to the sole of the foot and crushing the underlying structures, resulting in severe pain.

Laminitis typically progresses through three phases. The first is the developmental phase at which no clinical signs are present, making it impossible to begin treatment. Signs such as foot pain and increased digital pulse occur during the onset of the second stage, known as acute laminitis. This typically begins around 24 to 48 hours after a trigger event and usually lasts several days. The third and often irreversible phase is called chronic laminitis. In this phase, lamellar damage has become so great that the coffin bone shifts downward (Figure 1.), (Pollitt, 2002). Treatments such as NSAID pain relievers and cryotherapy can prevent or lessen the degree of laminitis if begun in the developmental phase before symptoms occur (Van Epps, 2004); however, once horses enter the acute stage there is little effective treatment for this

disease. Therefore if a diagnostic marker could be identified for the developmental stage, veterinarians could begin treatment early enough to ameliorate the pain and tissue damage caused by laminitis.



**Figure 1. Rotation of the proximal phalanx.**

Radiographs of a normal front foot (left) compared to a laminitic front foot (right). The normal foot radiograph shows the wall of the proximal phalanx (coffin bone) at an angle parallel to the hoof wall, while the coffin bone of the laminitic foot has rotated downward (Thompson, 2011).

### **Laminitis and Citrulline**

Laminitis onset is often triggered by unrelated events occurring elsewhere in the body, such as gastrointestinal (GI) system upset episodes (De La Rebière de Pouyade, 2009). Although the mechanism linking the GI tract to the foot is unknown, there is evidence that intestinal epithelial cell death allows the escape of bacteria into the bloodstream, and those bacterial proteins or

immune system factors travel to the foot where they trigger laminitis (Kyaw-Tanner, 2001). Interestingly, the health of the intestinal epithelium can be measured using plasma citrulline concentrations (Crenn, 2008). Citrulline is an  $\alpha$ -amino acid circulated in the plasma of most mammals and is produced mainly by intestinal epithelial cells. Studies in humans have shown reduced plasma citrulline concentrations in individuals with short bowel syndrome, villous atrophy, and Crohn's disease; all of which involve malabsorption and dysfunction of segments of bowel (Crenn, 2000). This particular study concluded that reduced plasma citrulline levels are an innovative and quantitative biomarker of intestinal epithelial cell death and loss of intestinal barrier function and can be used to predict the health and function of the intestine.

Although there are currently no published data regarding citrulline levels in horses with acute gastrointestinal diseases, our laboratory has recently found significantly decreased plasma levels of citrulline in horses during the developmental stage of experimentally-induced laminitis, suggesting that citrulline could also function as a biomarker to predict the onset of symptomatic laminitis.

Citrulline has been long identified as an intermediate in the urea cycle (Cox, 2000), and is mainly produced by enterocytes of the small intestine in humans (Crenn, 2008). No previous study clearly identifies the location in the equine GI tract at which citrulline is produced, however. Because colic and other GI diseases can effect one or multiple locations in the equine GI tract, localizing the origin of citrulline production will be beneficial to the validity and rationale of the study. For example, if citrulline is only produced in the small intestine, an explanation will be needed as to how a large colon impaction can potentially effect citrulline production.



## **Potential Outcomes and Study Objectives**

If citrulline is indeed prognostic for the onset of acute laminitis, a simple and affordable blood test could be developed to predict a horse's chance of developing the disease before it progresses too far to treat. This would allow earlier and more effective treatment to significantly reduce the chance of the horse developing the condition, greatly improving their prognosis. This study will also contribute to our understanding of the interaction between intestinal epithelial cell function and laminitis. Another possible outcome is the use of citrulline as a less invasive and more effective diagnostic indicator of intestinal death during colic than currently used methods.

Two currently used indicators of intestinal damage in humans are serum IgA (DeMeo, 2002), and D-Lactate (Murray, 1994). These tests measure intestinal health by analyzing the presence of bacterial by-products and immune factors present in the animal's blood. High levels indicate intestinal barrier function is compromised, thus allowing the escape of microorganisms and by-products into the animal's bloodstream. Blood and abdominal fluid lactate concentrations are also used in horses as an indicator of intestinal damage and surgical prognosis (Van Den Boom, 2010). The performance of citrulline as both an indicator of GI health as well as a predictor of laminitis onset must be comparable or greater than that of currently used tests to prove useful.

In this study we therefore examined citrulline levels in a clinical population of horses at risk of laminitis development. Citrulline concentrations were compared between healthy horses, horses at risk of laminitis that did not develop laminitis, and horses at risk of laminitis that did develop laminitis. We also measured plasma IgA and D-lactate concentrations and compared the effectiveness of these tests to that of citrulline in regards to the ability to predict laminitis onset.

In order to determine the location of citrulline synthesis, we also analyzed tissues from different locations in the equine GI tract for presence of citrulline synthesis enzymes.

## **CHAPTER II**

### **METHODS**

#### **Sample Collection**

Blood samples were collected from client animals at two local veterinary clinics as detailed in Animal Use Protocol 2012-115. Briefly, 10 mL of blood was collected from the jugular vein into a Vacutainer™ heparin tube by either a licensed veterinarian or a veterinary technician. Blood was sampled from healthy control horses (n=23) and horses at risk of laminitis (n=20). Blood samples were stored at 4°C from time of collection until centrifugation. Samples were centrifuged for 5 minutes at 2000 g to separate the red and white blood cells from the plasma, which was then stored in 500 µl aliquots at -20°C until analysis.

The veterinarians assisting with this study were encouraged to use their professional knowledge of the disease to choose cases which they felt were at risk of developing laminitis based on hospital admission for the GI condition being treated, such as severe colic, diarrhea, or grain overload. The veterinary clinic or horse owner was contacted 3 to 5 days post blood collection to determine if the horse developed signs of laminitis or other adverse clinical outcomes.

#### **Citrulline Assay and Statistical Analysis**

Plasma amino acid concentrations were analyzed using a chromatography-based amino acid analysis by the TAMU Protein Chemistry Laboratory. This method uses automated precolumn derivatization of amino acids from plasma with o-phthalaldehyde and reversed-phase chromatography for separation of the derivatives. Fluorescence detection is used to measure

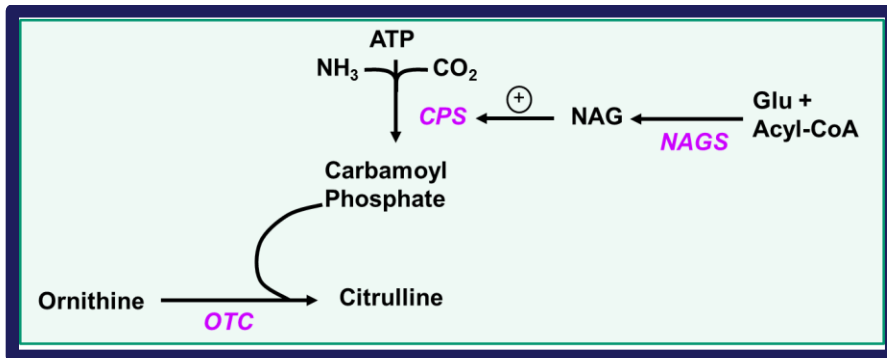
quantification of amino acids (Teerlink, 1994). Citrulline levels are presented as a percentage of total amino acids detected in each sample in order to normalize for inter-individual variation in the total concentration of amino acids. The mean citrulline concentration and 95% confidence interval of the control group was calculated based on the percent composition citrulline and used to determine the normal range of plasma citrulline.

The GI disease group of horses was further divided into two subgroups consisting of horses that experienced adverse outcomes including laminitis or death, and horses that did not. The percent citrulline composition of the two test groups were compared to the normal citrulline range derived from the control group. An ANOVA was used to compare citrulline levels in control horses, those that did not develop laminitis, and those that did develop laminitis. The ANOVA was also performed for all amino acids detected in the amino acid analysis to allow us to recognize changes between the three groups of horses for each amino acid.

### **Localization of Citrulline Production**

Archived equine tissue samples from the stomach, small intestine, cecum, and large intestine, were used to identify the location in the digestive system at which citrulline is produced. RNA was extracted from each of the tissue samples with Qiazol cell lysis buffer followed by RNA cleanup (RNeasy RNA Isolation Kit, Qiagen) and then reverse transcribed to cDNA (Superscript VILO cDNA Synthesis Kit, Invitrogen). Polymerase chain reaction (PCR) primers were designed using the equine nucleotide sequences obtained from the NCBI database for carbamoyl phosphate synthetase 1 (CPS1), N-acetylglutamate synthase (NAGS), and ornithine transcarbamylase (OTC), which collaboratively produce citrulline (Figure 2.). cDNA from tissue

samples was paired with nucleotide sequences from each enzyme and amplified using PCR. Each reaction consisted of 0.1  $\mu\text{l}$  dTTP, 100 mM Solution (BP2564-1D Fischer Scientific); 1  $\mu\text{l}$  10 X PCR Buffer (P2192-1VL SIGMA Life Science); 0.25  $\mu\text{l}$  REDTaq DNA Polymerase (D5684-1KU SIGMA Life Science); 0.3  $\mu\text{l}$  each forward and reverse primer stock (10  $\mu\text{M}$ ); 7.05  $\mu\text{l}$  double-distilled H<sub>2</sub>O; and 25 ng cDNA sample. The PCRs were performed in a thermocycler machine which initially incubated the reactions at 95°C for one minute, then proceeded to cycle through the following temperatures 31 times, holding the reactions at each temperature for 30 seconds; 94°C, 58°C, 72°C. After completion of the final cycle the reactions were incubated at 72°C for five minutes. PCR products were then separated by agarose gel electrophoresis to visualize the expression of these enzymes in each of the digestive tract locations.



**Figure 2. Horse enzymatic citrulline synthesis pathway.**

Figure 2. illustrates the need for the enzymes NAGS, CPS, and OTC, to synthesize citrulline.

## **IgA and D-Lactate**

Two other methods of assessing GI tract damage commonly used in humans are the analysis of plasma IgA and D-Lactate. Both of these tests were performed on plasma samples as a comparison to citrulline for effectiveness in differentiating between the three groups of horses: control, GI disease with adverse outcome, and GI disease without adverse outcomes.

IgA was analyzed using an ELISA. For the ELISA, Horse IgA antibody unconjugated (Bethyl Laboratories) was diluted 1:100 with PBS coating buffer and 100  $\mu$ l was added to each well of a 96 well flat bottom culture plate and incubated overnight at 4 °C. Wells were then washed 5 times using PBST wash buffer. Wells were blocked using 300  $\mu$ l Superblock (Thermo-Pierce) per well, then washed 5 more times using PBST. Next, samples were diluted 1:4000 with 10% Superblock PBS assay buffer, and a standard curve was prepared with doubling dilutions of reference serum (1.5 mg/ml IgA, Bethyl Laboratories) from 1:250 to 1:16,000. Samples, standards, and controls were added at 100  $\mu$ l per well in duplicate and incubated for 90 minutes at 37 °C, then washed 5 times with PBST. One hundred microliters of Horse IgA antibody (HRP conjugated, Bethyl Laboratories) was added to wells at a dilution of 1:150,000 and the plate was incubated for two hours at room temperature and then washed 7 times with PBST. Next, SIGMAFAST OPD tablets (SIGMA Life Science) were dissolved in 20 ml water and added to the wells at 200  $\mu$ l per well, then incubated for 30 minutes in the dark. Finally, 50  $\mu$ l 3M HCl was added to stop the reaction and the plate was read at 492 nm using a microplate reader.

D-Lactate was measured using a D-Lactate Assay Kit (Abcam) according to the manufacturer's protocol and the absorbance was measured at 450 nm using a microplate reader.

**Approval**

All animal procedures were approved by the Institutional Animal Care and Use Committee as well as the Texas A&M University College of Veterinary Medicine's Clinical Research Review Committee (AUP 2012-115).

## **CHAPTER III**

### **RESULTS**

#### **Clinical Outcome**

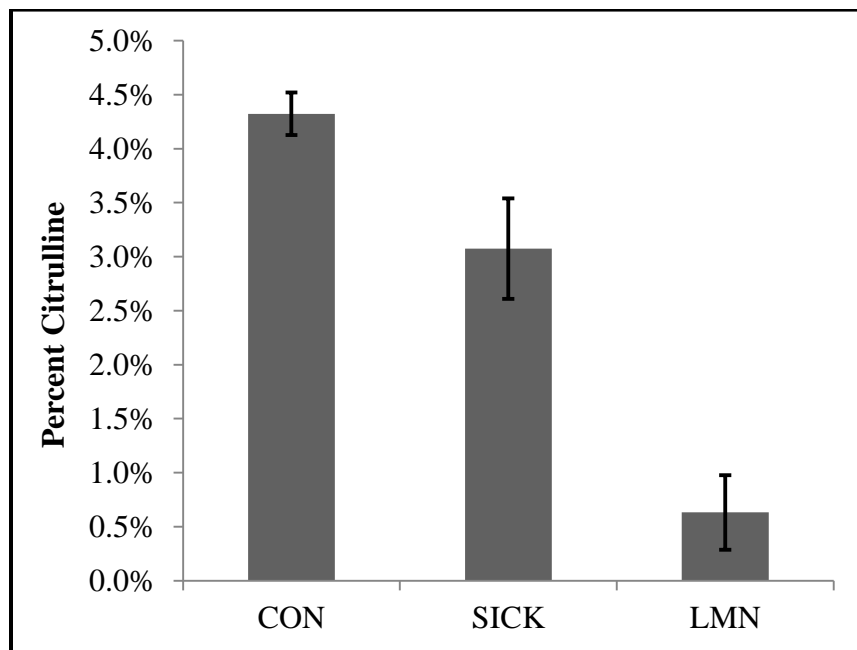
All 23 control horses were healthy at the time of blood collection. Of the 19 horses presenting with acute GI disease or other condition known to trigger laminitis onset, six developed adverse outcomes within 72 hours after blood collection. Five developed symptoms of laminitis and one was euthanized prior to the time symptoms would be observed. Of these six, one was diagnosed with abortion and colic, three were diagnosed with colitis, one with colic, and one with pneumonia and lipemia. Of the 13 horses that recovered successfully, seven were diagnosed with routine, non-surgical colic, two were diagnosed with colitis, and three were diagnosed with slight grain overload. One of the horses was diagnosed with acute unilateral laminitis with no known trigger event.

#### **Citrulline Concentrations**

The mean amino acid percent composition from the control group was 4.29% citrulline with a standard deviation of 1.01%, giving a 95% confidence interval of 2.27% to 6.31%, (Figure 3.). The mean percent composition citrulline from the six cases resulting in laminitis symptoms or death was 1.69% with values ranging from 0.29% to 2.22% leaving the mean and all samples outside the 95% confidence interval. The mean percent composition citrulline from the 13 cases not resulting in laminitis or death was 3.07%, with values ranging from 1.19% to 5.28% citrulline. Six horses from this group also fell below the normal range for citrulline concentrations. All horses clinically diagnosed with colitis exhibited citrulline concentrations



below the normal range. Horses with GI symptoms that included severe diarrhea also fell below the normal range of citrulline concentrations. Among the control horses, no correlation was found between breed, age, or sex with regards to citrulline concentration.



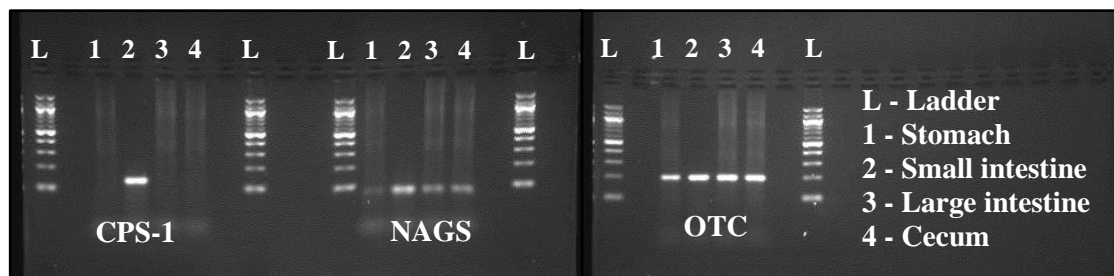
**Figure 3. Plasma citrulline in a clinical population of horses.**

Mean plasma concentrations of citrulline normalized to total free amino acid content are shown for healthy controls (CON), horses with colic (SICK), and horses with colic that went on to develop laminitis (LMN). Horizontal bars represent the mean  $\pm$  SEM.

### **Expression of Citrulline Synthesis Enzymes in Equine GI Tract**

PCR performed on tissue samples from different locations of the equine digestive tract indicated that the enzyme OTC is highly expressed throughout the GI tract (Figure 4.). The enzyme NAGS

was found to be highly expressed in the small intestine and expressed to a lesser degree in the stomach, large intestine, and cecum. The enzyme CPS-1 was exclusively expressed in the small intestine. Because citrulline synthesis requires all three enzymes (Cox, 2000), this data indicates citrulline is primarily synthesized in the small intestine.



**Figure 4. Electrophoresis gel visualization of PCR products.**

This figure indicates the expression of the three citrulline synthesis enzymes in tissue samples from four different locations of the GI tract. White bands show the expression of the indicated enzyme at the corresponding location of the GI tract.

### Concentrations of Other Amino Acids

Citrulline performed better than any other amino acid detected at differentiating the horses into the groups: control (CON), GI upset with no laminitis (SICK), and GI upset with laminitis (LMN). Trends were noticeable via ANOVA, however, among the amino acids serine, glycine, alanine, phenylalanine, valine, leucine, and proline, in addition to citrulline (Table 1.). Serine, alanine, and leucine concentrations were elevated among the SICK and LMN horses, glycine concentrations were elevated in the LMN group only, citrulline concentrations were decreased among SICK and LMN horses, valine and phenylalanine concentrations rose only in the SICK group, and proline concentrations were found to be decreased in the LMN horses only.

	CON	SICK	LMN
ASP	0.54 ± 0.30 <sup>a</sup>	0.68 ± 0.44 <sup>a</sup>	0.49 ± 0.32 <sup>a</sup>
GLU	2.85 ± 1.21 <sup>a</sup>	4.09 ± 2.66 <sup>a</sup>	2.83 ± 0.96 <sup>a</sup>
ASN	1.91 ± 1.18 <sup>a</sup>	1.03 ± 0.43 <sup>a</sup>	1.05 ± 0.64 <sup>a</sup>
SER	9.38 ± 2.25 <sup>a</sup>	6.65 ± 1.76 <sup>b</sup>	6.38 ± 2.10 <sup>b</sup>
GLN	14.67 ± 2.50 <sup>a</sup>	14.71 ± 4.41 <sup>a</sup>	14.50 ± 3.84 <sup>a</sup>
HIS	3.35 ± 0.61 <sup>a</sup>	3.71 ± 0.64 <sup>a</sup>	3.14 ± 0.73 <sup>a</sup>
GLY	19.22 ± 2.83 <sup>a</sup>	17.84 ± 3.89 <sup>a</sup>	21.92 ± 3.93 <sup>b</sup>
THR	4.00 ± 1.13 <sup>a</sup>	3.92 ± 1.05 <sup>a</sup>	3.34 ± 0.75 <sup>a</sup>
CIT	4.48 ± 1.17 <sup>a</sup>	3.02 ± 1.65 <sup>b</sup>	1.60 ± 1.21 <sup>b</sup>
ALA	7.39 ± 2.13 <sup>a</sup>	9.93 ± 2.69 <sup>b</sup>	11.78 ± 4.11 <sup>b</sup>
ARG	4.64 ± 1.56 <sup>a</sup>	3.53 ± 1.79 <sup>a</sup>	3.63 ± 2.41 <sup>a</sup>
TYR	2.35 ± 0.47 <sup>a</sup>	2.29 ± 0.41 <sup>a</sup>	1.94 ± 0.38 <sup>a</sup>
VAL	7.22 ± 0.87 <sup>a</sup>	8.72 ± 1.37 <sup>b</sup>	8.50 ± 2.96 <sup>a</sup>
MET	1.55 ± 0.45 <sup>a</sup>	1.45 ± 0.30 <sup>a</sup>	1.26 ± 0.33 <sup>a</sup>
PHE	2.54 ± 0.41 <sup>a</sup>	3.36 ± 0.95 <sup>b</sup>	2.84 ± 0.51 <sup>a</sup>
ILE	2.37 ± 0.33 <sup>a</sup>	2.54 ± 0.73 <sup>a</sup>	2.48 ± 1.07 <sup>a</sup>
LEU	4.47 ± 0.62 <sup>a</sup>	5.76 ± 0.88 <sup>b</sup>	5.72 ± 1.60 <sup>b</sup>
LYS	3.22 ± 0.64 <sup>a</sup>	3.21 ± 1.19 <sup>a</sup>	3.61 ± 1.52 <sup>a</sup>
PRO	3.87 ± 0.88 <sup>a</sup>	3.56 ± 0.83 <sup>a</sup>	2.98 ± 0.43 <sup>b</sup>

<sup>1</sup> Means within a row without a common letter differ, P < 0.05

**Table 1. Amino acid ANOVA results.**

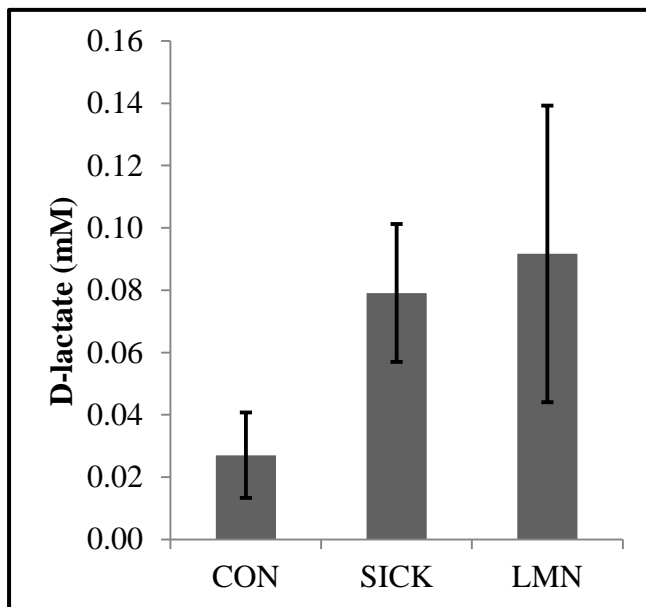
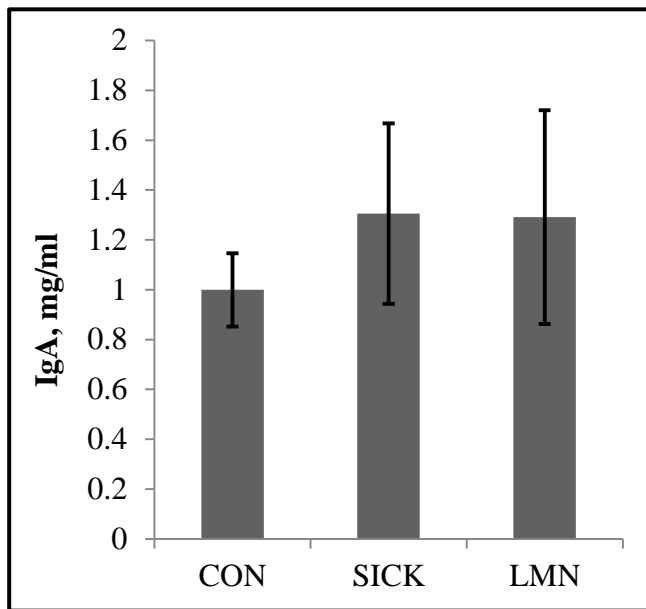
Plasma amino acid concentrations, presented as percent of total free amino acids in clinically normal horses (CON, n = 23), horses with colic or sepsis (SICK, n = 13), and horses with colic or sepsis that experienced adverse outcomes, including laminitis or death (LMN, n = 6)<sup>1</sup>.

## **IgA**

The mean plasma concentration of IgA for the control group was 0.999 mg/ml with a standard deviation of 0.622 producing a 95% confidence interval range of -0.245 to 2.243 mg/ml. No CON horses had IgA concentrations outside the normal range, two SICK horses fell outside the normal range, and the one horse from the LMN group which was euthanized fell outside the normal range. All horses with concentrations outside the normal range had elevated IgA concentrations. The mean IgA concentration of the SICK group was 1.305 mg/ml and the mean of the LMN group was 1.291 mg/ml, both lying inside the 95% confidence interval (Figures 5 and 6.). The ANOVA test also did not detect differences in IgA concentrations between the three groups of horses.

## **D-Lactate**

The mean plasma concentration of D-lactate for the control group was 0.021 mM with a standard deviation of 0.052 producing a 95% confidence interval range of -0.084 to 0.125 mM. One CON horse had D-lactate concentrations outside the normal range, two SICK horses fell outside the normal range, and two LMN horses fell outside the normal range. All horses with concentrations outside the normal range had increased D-lactate concentrations. The mean D-lactate concentration of the SICK group was 0.079 mM and the mean of the LMN group was 0.092 mM, both inside the 95% confidence range. The ANOVA test also did not show differences in D-lactate concentrations between the three groups of horses.



**Figures 5 and 6. Plasma D-lactate and IgA concentrations among three groups of horses.**

Mean D-lactate (bottom) and IgA (top) concentrations are shown for healthy control horses (CON), horses with colic (SICK), and horses with colic that proceeded to develop laminitis (LMN). Horizontal bars represent the mean  $\pm$  SEM.

## **CHAPTER IV**

### **CONCLUSIONS**

#### **Discussion**

Because our results showed that all horses that developed laminitis also had plasma citrulline concentrations less than two standard deviations below the mean for normal horses, a future test would most likely give a 'positive' reading for all colicing horses that would proceed to develop laminitis. This test would most likely also give a 'positive' reading for some horses that would not develop laminitis, since 40% of horses from this study which did not develop laminitis had low citrulline levels. We can therefore conclude that a potential citrulline test would be highly sensitive, but have low specificity. This type of test would still be useful for identifying all horses at high risk of laminitis development during a colic or other GI upset episode. Fortunately cryotherapy is an effective preventative measure for laminitis during the developmental phase that is not costly or harmful to horses, therefore treating all horses that tested 'positive' would not cause negative outcomes (Van Epps, 2004).

Our data suggests that citrulline performed better at discriminating between the three groups of horses than IgA or D-lactate. Because the mean concentrations of both IgA and D-lactate for the different groups of horses do not fall outside the confidence interval for the normal range and did not fall into different groupings via ANOVA, IgA and D-lactate do not predict adverse outcomes as well as citrulline does in horses.

Immunologic responses act as one of many protective mechanisms to the gastrointestinal system. In humans, IgA is the primary antibody secreted into the gut and acts to bind foreign antigens (DeMeo, 2002). Therefore increased concentrations of IgA can signify bacterial infection and loss of intestinal barrier function (DeMeo, 2002). D-lactate is a byproduct of bacterial metabolism and is also used in humans as an indicator for many gastrointestinal diseases (Murray, 1994). Increased D-lactate concentrations in humans indicate bacterial infection or loss of intestinal barrier function (Smith, 1986). Because our results did not show significant changes in IgA or D-lactate among the three groups of horses, these findings contrast the results of IgA and D-lactate in humans. This could result from the numerous differences between the GI systems of humans and horses such as diet, location of bacterial fermentation, bacterial flora, and many anatomical and physiological differences (Dyce, 2010) .

Similar biomarkers such as lactate concentrations are commonly and accurately used in horses to identify sepsis and measure intestinal health (Van Den Boom, 2010). The most common cause of hyperlactaemia is hypervolemia and the resulting decrease in tissue perfusion. Lactate concentrations can therefore be measured and compared between the blood and the abdominal fluid. An abdominal fluid lactate concentration higher than that of the blood lactate concentration is considered evidence of intestinal strangulation and sepsis (Moore, 1976). There is no published data regarding the use of lactate as an indicator of laminitis, and there are not any commercially available tests capable of predicting laminitis onset or published studies regarding potential biomarkers for laminitis.

Concentrations of amino acids have been studied as an indicator of sepsis in humans, and these findings differ from the data of our three groups of horses (Freund, 1979). All the aromatic and sulfur containing amino acids were found to increase during sepsis in humans, while only phenylalanine from this group was found to be elevated in our data from GI upset horses. Valine, leucine, arginine, and isoleucine were found to decrease during sepsis in humans. Our data showed increased concentrations of valine and leucine, and normal concentrations for arginine and isoleucine in GI upset horses. Also, serine, alanine, glycine, and proline were found to remain normal in humans, yet showed changes among the different groups of horses in our data.

Our PCR results showed the expression of all three citrulline synthesis enzymes only in the small intestine, concluding the location of citrulline synthesis in the GI tract. This would imply that all cases resulting in decreased citrulline levels must have suffered a compromised small intestine. Interestingly, many of the horses displaying decreased citrulline levels presented with colitis or large colon or cecal impactions/displacements, rather than small intestinal issues. A possible explanation for this could be that due to the hindgut blockage; the small intestine was secondarily compromised, thus decreasing its citrulline production. There does not appear to be published data regarding the effects of hindgut colic or colitis on the small intestine, however small intestinal motility and barrier function decreases during diarrhea, and small intestinal blood flow and barrier function decreases during endotoxemia (Clark, 1990). Because all of the horses suffering diarrhea and endotoxemia due to severe colic or colitis in our study had reduced citrulline levels, we can suggest the decrease resulted from small colon loss of functionality resulting from these events.



## **Clinical Implications**

If further studies conclude that citrulline levels correlate with laminitis onset across a large set of samples, a commercially available blood test could be developed in the future to identify horses at high risk of developing laminitis before it progresses too far to treat. Veterinarians could then begin treatments on horses at high risk of laminitis to reduce the chance of the horse developing the condition and greatly improve their prognosis. Further studies will also contribute to our understanding of the interaction between intestinal epithelial cell function and laminitis. Because citrulline levels were reduced in the most severe GI upset cases including diarrhea, colitis, endotoxemia, and displacement, another possible outcome of this study is the use of citrulline as a less invasive diagnostic indicator of intestinal death during colic than the currently used abdominocentesis procedure for obtaining abdominal fluid.

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