UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA ANIMAL: EQUINOS

TESE DE DOUTORADO

Marcadores moleculares como preditores de desempenho atlético em cavalos de hipismo

(Molecular markers as performance predictors in horses)

Autora: Priscila Beatriz da Silva Serpa

Porto Alegre/RS

2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA ANIMAL: EQUINOS

MARCADORES MOLECULARES COMO PREDITORES DE DESEMPENHO ATLÉTICO EM CAVALOS DE HIPISMO

(Molecular markers as performance predictors in horses)

Autora: Priscila Beatriz da Silva Serpa

Tese defendida junto à Faculdade de Veterinária como requisito parcial para obtenção de grau de Doutora em Medicina Animal: Equinos.

Orientadora: Petra Garbade

Co-orientador: André Chen Shih

PORTO ALEGRE – RS - BRASIL

2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL

FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM MEDICINA ANIMAL: EQUINOS

TÍTULO:

Marcadores moleculares como preditores de desempenho atlético em cavalos de hipismo

Molecular markers as performance predictors in horses

APROVADO EM: 09 de Novembro de 2015

AUTORA:

Priscila Beatriz da Silva Serpa

ORIENTADORA:

Prof.ª Dr.ª Petra Garbade

CO-ORIENTADOR NO EXTERIOR

Dr. André Chen Shih

PRESIDENTE DA BANCA EXAMINADORA:

Prof.ª Dr.ª Petra Garbade

BANCA EXAMINADORA:

Prof. Dr. Eduardo Malschitzky

Prof. Dr. Fernando Silvério Ferreira da Cruz

Prof. Dr. Gustavo Henrique Zimmermann Winter

If you don't make mistakes, you're not working on hard enough problems. And that's a mistake.

(Frank Anthony Wilczek, Nobel Prize in Physics in 2004)

Se você não erra, provavelmente não está trabalhando em problemas suficientemente desafiadores.

E isto sim é um grande erro.

(Frank Anthony Wilczek, Prêmio Nobel de Física em 2004)

RESUMO

Vários estudos procuram associar genética e fenótipo positivo para o desempenho atlético em equinos. Neste contexto, dois estudos foram desenvolvidos com o objetivo de investigar a base molecular de uma possível melhora nas variáveis fisiológicas utilizando a suplementação oral de substâncias ergogênicas e utilizar estes parâmetros como marcadores moleculares de desempenho. Para isso, no primeiro estudo, uma técnica de *high resolution melting* do SNP g.66493737C>T no gene da miostatina (MSTN) utilizando sondas não marcadas foi desenvolvida como forma de triagem genotípica. Vinte e três cavalos de salto foram genotipados com uma sonda não marcada com um C3-*spacer*. Sete animais apresentaram curvas de *melting* inconsistentes, enquanto os demais 16 animais foram genotipados com sucesso com um nível médio de confiança de 84,4%. A frequência de T/T foi de 63,6%, de T/C foi de 31,8%, e de C/C foi de 4,5%. Em seguida, dezesseis cavalos de salto foram suplementados durante oito semanas com placebo (n = 8) ou creatina (n = 8, 1ª semana = 0,3 g/kg, 2ª-8ª semana = 0,05 g/kg), para avaliação dos efeitos da creatina sobre os níveis de miostatina plasmática. Peso corporal, creatina, miostatina, creatinina e creatina quinase (CK) foram avaliadas em 0, 4 e 8 semanas de suplementação. A suplementação com creatina não alterou o peso corporal, creatinina, CK, e miostatina. A creatina sérica foi significativamente maior na 4ª semana, mas não na 8ª semana de suplementação. A estratificação dos dados por sexo dentro de cada grupo não afetou a análise estatística; a estratificação pelo genótipo não foi possível. No segundo estudo, os efeitos da suplementação oral com ácido linoleico conjugado (CLA) sobre o peso corporal, o influxo de lactato nos eritrócitos através do transportadores de monocarboxilato (MCTs) e na expressão dos gene MCT1, CD147 e PPRA-α na medula óssea foram avaliados. Doze pôneis receberam CLA (n = 6, 0,01% PV) ou óleo de milho (n = 6, dieta isocalórica). Os dados foram coletados em 0, 30 e 60 dias. CLA não afetou o peso corporal e o influxo de lactato. Embora não seja significativo, houve um aumento na expressão de PPRA-α no grupo CLA e do influxo de lactato eritrocitário. Por outro lado, os animais do grupo controle apresentaram uma diminuição de expressão dos três genes avaliados, bem como do influxo de lactato eritrocitário, embora de forma não significativa. Tomados em conjunto, estes resultados indicam que o uso de substâncias ergogênicas com o objetivo de modificar a expressão e compreender a regulação de genes, como o MSTN e MCT1/CD147, pode ajudar na escolha do melhor genótipo e fenótipo para a prática esportiva, e torná-los marcadores genéticos e moleculares de desempenho, não só em cavalos de salto, mas em outras raças e esportes.

Palavras-chave: miostatina, creatina, HRM, CLA, MCT, PPRA-α, eritrócito.

ABSTRACT

Several studies aim to make an association between genetic background and positive phenotype for sports practice in horses. In this context, two studies were developed with the aim of investigate the molecular basis of a possible improvement in physiological variables using oral supplementation with well-known ergogenic aids in order to use these parameters as molecular markers of performance. For that, in the first study, a high resolution melting analysis to identify the SNP g.66493737C>T in the MSTN gene using unlabeled probes was developed and used as a rapid genotype screening. Twenty three jump horses were genotyped with a C3-spacer unlabeled probe. Seven animals revealed inconsistent melting curves, but the remaining 16 animals were successfully genotyped with a mean level of confidence of 84.4%. Frequency of T/T was 63.6%, T/C was 31.8%, and C/C was 4.5%. Hereafter, sixteen jump horses were supplemented during 60 days with placebo (n = 8) or creatine monohydrate (n = 8, 1st week = 0.3 g/kg, 2-8th week = 0.05 g/kg), in order to evaluate the effects of creatine on plasma myostatin levels. Body weight, creatine, myostatin, creatinine and CK were evaluated in blood samples at 0, 4 and 8 weeks of supplementation. Creatine monohydrate did not change body weight, creatinine, creatine kinase (CK), and myostatin. Serum creatine was significantly increased at week 4, but not at week 8 of creatine supplementation. Stratification of data by sex within each group did not affect the statistical analysis; stratification by genotype was not possible. In the second study, the effects of oral supplementation with CLA on body weight, lactate influx into RBCs through the monocarboxylate transporter (MCT), and in the gene expression of MCT1, CD147 and PPRA-α in bone marrow was evaluated. Twelve ponies received CLA (n = 6, 0.01% BW) or corn oil (n = 6, isocaloric). Data were collected at 0, 30 and 60 days. CLA did not affect body weight, and lactate influx into erythrocytes. Although not significant, there was an increase in PPRA-α expression and in RBC lactate influx in CLA group. In the other hand, control animals presented decreased gene expression of all genes and erythrocytes lactate influx along the period of supplementation, but not significantly. Taken together, those results indicate that the use of ergogenic aids with the purpose of modify the expression and understand the regulation of genes, such as MSTN gene and MCT1/CD147, can help to select the best genotype and phenotype for sport practice, and make those genes candidates of genetic and molecular markers of performance, not only in jump horses, but in other breeds and sports.

Key words: myostatin, creatine, HRM, CLA, MCT, PPRA-α, erythrocyte.

LIST OF FIGURES

LIST OF TABLES

TABLE OF CONTENTS

INTRODUCTION

Given the high financial investment in the husbandry and preparation of sport horses, the selection of horses with improved phenotypic traits, such as a lower propensity to fatigue and better muscle fitness, reduces the chances to be investing in an animal with inferior physiological characteristics and increases the likelihood of positive results in competition. Several studies seek to make an association between genetic background and positive phenotype for sports practice. Studies have revealed a large number of genes involved in the performance of elite athletes (Schröder, Klostermann e Distl, 2011). In horses, many physiological and metabolic aspects related to sports have been analyzed; however few studies address the scope of the molecular genetic analysis. Two examples of genes correlated with sports practice and with great applicability are the myostatin hormone associated with muscle development, and monocarboxylate transporters associated with the transport of intra and intercellular lactate, since the selection of animals with greater musculature needs to be followed by metabolic adaptations, such as greater lactate clearance.

Myostatin is an autocrine/paracrine hormone responsible for the inhibition of muscle growth by decreasing protein synthesis and cell differentiation, acting both on myocytes and myoblasts during embryonic development and throughout adulthood. Its deficiency or absence produces both muscle hypertrophy (increase in cell size) and hyperplasia (increased number of cells) in varying degrees. The study of this hormone also enables the elucidation of a number of human and animal diseases, such as muscle atrophy, obesity, diabetes, insulin resistance, and HIV infection (Huang, Chen e Chen, 2011). Recently, the inhibition of myostatin appears to be a novel action of creatine (Saremi *et al.*, 2010). Creatine is a nonprotein amino acid produced in the liver and used as energy storage by muscle. Creatine can also be orally supplemented in sport athletes as an ergogenic aid, including men, dogs and horses (Feldman, 1999; Harris e Harris, 2005). To date, the effects of creatine on myostatin hormone in horses were still not evaluated.

Other important aspect for performance, besides muscle development, is the muscle activity during exercise. Lactate removal during and after exercise is crucial for the cellular balance and muscle activity (Kitaoka, Hoshino e Hatta, 2012). Several researches on sports medicine have showed that lactate is an useful tool to evaluate performance and physical preparation because it allows the assessment of both aerobic and anaerobic metabolism in horses (Evans, 2008). The main membrane transporter of lactate is the monocarboxylate transporters (MCTs), responsible for lactate influx and efflux in different cell types, including erythrocytes. Since erythrocytes play an important role in lactate clearance (Koho, Hyyppä e

Pösö, 2006), the study of lactate transporters in red blood cells (RBCs) and its influence on performance is valuable. It was found a unique bimodal distribution of lactate transport activity among horses (Väihkönen e Pösö, 1998). These animals present different amount of protein expression (MCT1 and its ancillary protein CD147) and they can present high or low capacity of lactate transport by MCTs in RBCs, but no genetic cause was found to date (Koho *et al.*, 2012). Horses with high transport capacity would eliminate blood lactate more efficiently, favoring the post-exercise recovery.

Recently, it was found a common regulatory element between MCT1–which is the main lactate transporter in erythrocytes–and CD147: PPAR-α, peroxisome proliferator-activated receptor type alpha. This transcription factor is believed to work as a nutritional status sensor in mammals (König *et al.*, 2008). PPAR-α have demonstrated to be involved in the up-regulation of MCT1 and CD147 expression in liver tissue of pigs and rats after supplementation with conjugate linoleic acid (CLA) or other natural sources of PPAR-α agonists (König *et al.*, 2010). Conjugated linoleic acid (CLA) is a class of polyunsaturated fatty acid with biological properties (Pariza, Park e Cook, 2001; Gholami e Khosravi-Darani, 2014). The mechanism which CLA and other PPRA-α agonist interfere in MCT1 synthesis is still unknown. The effects of CLA supplementation on the expression of MCT1 and CD147 in other cells, such as RBCs or its precursors, were not investigated yet, especially in horses. Moreover, PPRA-α mRNA concentration, as well as possible mutations in the PPRA-α gene may be responsible for bimodal distribution of lactate influx into red blood cells (RBCs) in horses.

In this context, two studies were developed with the aim of investigate the molecular basis of a possible improvement in physiological variables using oral supplementation of well-known ergogenic aids. If this improvement would be confirmed, they may be used as molecular markers of equine performance and may be used in the future as a tool of genetic selection for sports practice. For that, in the first study, the objectives were to develop the identification of the single nucleotide polymorphism (SNP) g.66493737C>T in the MSTN gene by high-resolution melting analysis for rapid screening of the genotype, and to evaluate the effects of oral creatine supplementation on plasma concentration of myostatin, as well as to stratify the samples by sex and MSTN genotype. In the second study, the effects of oral supplementation with conjugated linoleic acid on lactate influx in erythrocytes through the monocarboxylate transporter 1 (MCT1) and in the gene expression of MCT1, CD147 and PPAR-α in the bone marrow was evaluated.

The realization of this thesis project is justified by the economic importance of the horse on both national and international scenarios. With the use of molecular markers of performance suggested in this project, we intend to predict whether an animal will present a superior phenotype based in its genotype compared to others, promoting a previous selection of horses before the high investment in training.

CHAPTER I

High-resolution melting analysis to detect a single nucleotide polymorphism in the MSTN gene and effects of oral supplementation with creatine on serum myostatin in jump horses

1 LITERATURE REVIEW

1.1 Myostatin

Myostatin, previously known as growth differentiation factor 8 (GDF-8), is a peptide hormone which belongs to the family of the transforming growth factor beta (TGF-β), responsible for negative regulation of skeletal muscle growth (Mcpherron, Lawler e Lee, 1997). It is found in serum and skeletal muscle tissue in its active or inactive (propeptide) forms (Zimmers *et al.*, 2002; Dominique e Gérard, 2006). Discovered in 1997, myostatin is synthesized as a precursor molecule of 376 amino acids (aa). This precursor (**Figure 1.1**) consists of a signal sequence located at N-terminal, an N-terminal propeptide domain (27,640 Da), and a C-terminal domain (12,400 Da). For activation, the myostatin precursor needs to suffer two cleavages. The first cleavage is by a furin family enzyme that removes the signal sequence of 24 aa. Therefore, two precursor molecules undergo dimerization through disulfide bonds in the C-terminal domain, whereupon the second enzyme, a metalloproteinase BMP1 (bone morphogenetic protein type 1) separates the two N-terminal propeptide domains from the C-terminal domains. The mature myostatin is formed of two dimerized C-terminal domains, and is highly conserved among different species (Hosoyama *et al.*, 2002; Huang, Chen e Chen, 2011).
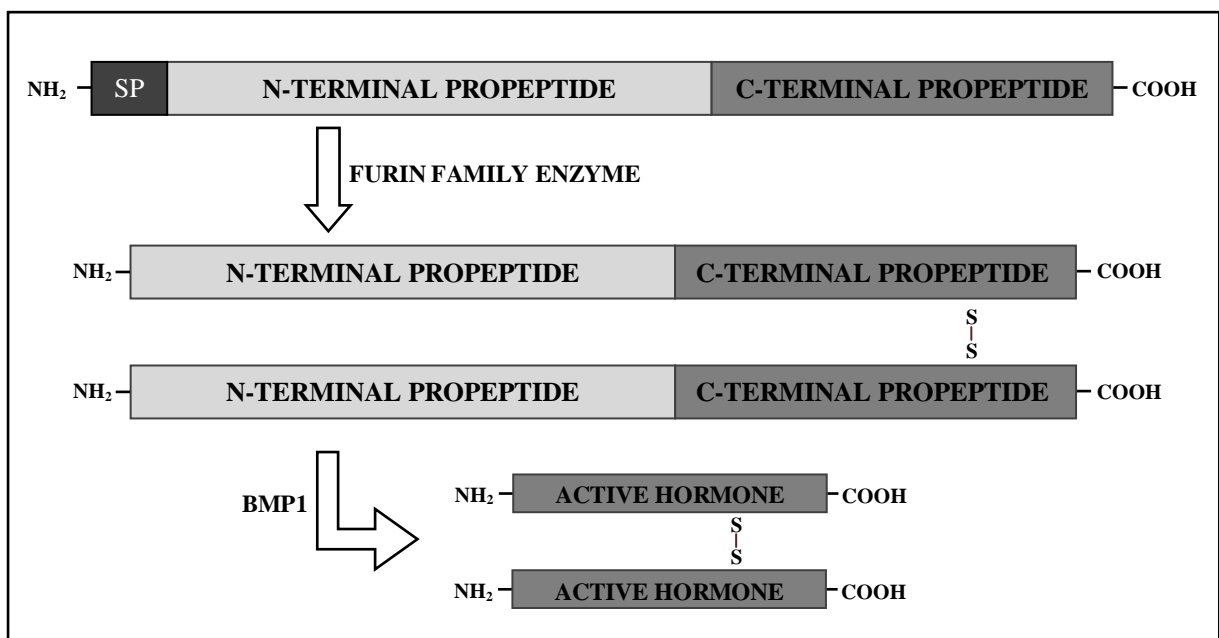
Figure 1.1: Myostatin protein activation. The first step is the cleavage of the signal peptide (SP) at the N-terminal by furin family enzymes. Secondly, two myostatin propeptide suffer dimerization at the C-terminal propeptide. Finally, the metalloproteinase BMP1 (bone morphogenetic protein type 1) cleaves the complex to form the active hormone.

Myostatin is produced by developing myotome compartment, and later in all skeletal muscle in the body, especially in fast fibers (Mcpherron, Lawler e Lee, 1997; Dominique e Gérard, 2006). Its action is through binding to activin receptor IIb (ActRIIb) (Lee e Mcpherron, 2001). After binding to the receptor, a second receptor-ActRIb or ALK4-is recruited and phosphorylated to form a heterotetramer complex, which promotes the activation of Smads. Smad1, Smad2, Smad3, Smad4, Smad5, and Smad8 participate in the regulation of the expression of different inhibitory genes. Other Smad-nondependent signaling cascades appear to be involved in the myostatin regulatory signal transduction, however these cascades have not been fully elucidated yet (Dominique e Gérard, 2006; Huang, Chen e Chen, 2011).

During embryogenesis, myostatin expression occurs restricted to the myotome compartment of developing mesodermal somites. In mice, myostatin can be detected 9.5 days post-coitum. In this phase, myostatin inhibits myoblast proliferation and differentiation (Mcpherron, Lawler e Lee, 1997). In adults, their target cells are satellite cells, the mononuclear progenitors of the muscle lineage present within muscle fibers, keeping them in a quiescent state through the mechanisms described above (Mcfarlane *et al.*, 2008).

Myostatin gene (MSTN) is a well conserved gene composed of three exons and two introns (Dall'olio *et al.*, 2010), present in different chromosomes (**Table 1.1**) according to the species.

Table 1.1: Chromosomal location of myostatin gene in different species.

Species	Chromosome
Cattle	2
Dog	37
Horse	18
Man	2
Mouse	1
Rat	9
Sheep	2

Source: http://www.ncbi.nlm.nih.gov/gene/?term=mstn

Naturally occurring mutations in MSTN gene have already been identified in horses, dogs, sheep, cattle, mice, and humans. Those mutations can decrease or interrupt myostatin production, leading to the development of greater muscle mass compared to individuals

without the mutation, and also promote effects on growth, reproduction, performance and carcass quality traits (Dall'olio *et al.*, 2010).

In dogs and mice, mutations in myostatin are deletions. Mosher *et al.* (2007) found that racing whippet dogs that have heterozygous genotype to a myostatin mutation have better results in races compared to homozygous wild animals and homozygous mutants. In whippet dogs, the mutation is two-base-pair deletion in the third of three exons, causing a premature stop codon, removing 63 amino acids from the predicted 375 aa of the normal myostatin. In mice, the deletion of 12 base pairs removing five aa (224 to 228 aa) in the sequence and creates a phenylalanine residue (Varga *et al.*, 1997; Szabó *et al.*, 1998).

In cattle, the phenotype known as "double-muscling" occurs in different European breeds and is due to mutations in myostatin as well. In Belgian Blue cattle breed, an 11-base-pair mutation in the third exon, removing four aa (274 to 277 aa), results in changing of the remaining 102 aa in the sequence, exactly the active portion of the peptide (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; Mcpherron e Lee, 1997). In other cattle breeds, such as Piedmontese and Marchigiana, there are polymorphisms in MSTN. Marchigiana has a G>T transversion in the third exon that changes a glutamic acid into a stop codon (Marchitelli *et al.*, 2003). Piedmontese has a G>A transition in the first exon resulting in a cysteine to tyrosine substitution (313 aa) modifying the tertiary structure of the protein (Mcpherron e Lee, 1997). Other four mutations were already found in other seven double-muscle breeds (Grobet *et al.*, 1998).

Clop *et al.* (2006) found that Texel sheep has a G to A transition in the 3'UTR (untranslated region) that creates a target to the action of microRNAs that inhibits MSTN and contributes to the muscular hypertrophy seen in this breed. A deletion in the 320th aa was also reported in Norwegian White sheep causing a disrupted reading frame until a premature stop codon in the 359 aa (Boman *et al.*, 2009).

In 2000, a German boy was born with extraordinary muscular mass, with protruding muscle in thighs, leg and arms. The mother and four other relatives were reported to be unusually strong. The boy was homozygous to a G>A transversion in intron 1, while his mother were heterozygous. No detectable amount of myostatin was found in the boy's serum (Schuelke *et al.*, 2004).

In horses, Hill, Gu, *et al.* (2010) found a C>T transition within intron 1 in MSTN gene in Thoroughbreds. Besides, they documented a high correlation between the three genotypes of this SNP (single nucleotide polymorphism) and the optimal race distance performance. The results of this work bring some interesting and relevant information. First, dividing horses in

short (≤ 1400 m) and middle-long distance racers (> 1600 m), allele C was twice as frequent in short distance than in middle and long distance winners (P = 1.88e-5). Secondly, the means of best racing distance for each genotype was determined as 1,247 m for C/C, 1,831 m for C/T, and 2,113 m for T/T (P = 4.85e-8). Moreover, the frequency of each genotype was considered different for non-Thoroughbred horses evaluated. In Egyptian Arabian horses, the frequency of T/T genotype was 90%, while the frequency of C/C in quarter horses was 83%. At last, the relationship of mass (kg) to height at withers (cm) ratio with the g.66493737C>T mutation was also significant. The means for each genotype was determined as 2.94 kg/cm for C/C, 2.88 kg/cm for C/T, and 2.83 kg/cm for T/T (P = 0.0147), which corroborates that sprinters have more developed muscles, smaller bodies and more robust aspect compared to endurance animals and stayers. These results are in agreement with Tozaki *et al.* (2010); Tozaki *et al.* (2011); and Tozaki *et al.* (2012).

In a genome-wide SNP-association study, the same group found an important association (P < 0.001) between the SNP g.66493737C>T reported before and another SNP close to the NGFI-A binding protein 1 gene (NAB1 gene). NAB1 is expressed in cardiac muscle and has been reported to be a transcriptional regulator of cardiac growth. The study used Equine SNP50 Genotyping BeadChip, which contained only three of 12 known SNPs in NAB1 gene. The statistical analysis rejected an association between race performance and these three SNPs, but the authors precociously disregarded this candidate gene as a potential contributor to optimum racing distance (Hill, Mcgivney, *et al.*, 2010). Heart size is highly related to maximum stroke volume, cardiac output, and hence aerobic capacity and performance (Young, Rogers e Wood, 2005; Poole e Erickson, 2008), thus an important trait to study in sport horses.

Since low levels or absence of myostatin is correlated with increased muscle mass, alternatives to inhibit myostatin may be useful to study muscular metabolism and associated diseases and conditions, such as caquexy. Different proteins are able to bind myostatin and inhibit its activity. Growth and differentiation factor-associated serum protein 1 (GASP-1) and follistatin are two examples. GASP-1 binds to both inactive and mature myostatin and can regulate the activation of the hormone. Follistatin is another protein known to direct antagonize myostatin by inhibiting its ability to bind to the ActRIIb receptor, and to drastic increase skeletal muscle mass in mice, similar to knocked-out MSTN -/- or activin receptor IIb dominant-negative mice (Lee e Mcpherron, 2001; Dominique e Gérard, 2006).

Beyond the possible biomedical application of myostatin to enhance muscle growth, or maybe treat obesity, the genetic selection of animals with positive phenotypes for agricultural

purposes, such as meat and carcass quality, may play a important role in the near future (Rodgers e Garikipati, 2008). In addition, specifically for equine industry, genotyping horses early in their sport carriers can optimize training, and direct animals in competitions with foremost likelihood of success.

1.2 Creatine

Creatine (**Figure 1.2**) is a nonprotein amino acid formed from methionine, arginine and glycine in the liver, kidneys (cortex, proximal tubule), and pancreas (islet alpha cells) (Feldman, 1999). Creatine is used mainly in muscle and in the brain to form phosphocreatine (creatine phosphate), an energy storage compound. Synthesis of creatine requires the incorporation of a methyl group (from methionine) and an amidine group (from arginine) to an entire glycine. The amidine group is transferred from arginine to glycine by the enzyme glycine amidinotransferase (AGAT) to form guanidinoacetate. Methionine has a nucleoside incorporated by methionine adenosyltransferase (MAT) to form s-adenosylmethionine. Guanidinoacetate is finally methylated from s-adenosylmethionine to form creatine by guanidinoacetate methyltransferase (GAMT), whereupon is transported to muscle and nervous cells (Brosnan, Da Silva e Brosnan, 2011). Inside nervous and muscle cell, creatine kinase mediates the conversion of creatine to phosphocreatine, a phosphate donor essential for synthesis of ATP. Creatine is spontaneously degraded to creatinine by the loss of water, which is solely excreted from the body by renal route (Feldman, 1999). Approximately 95% of total body creatine is in skeletal muscle, while 60-67% is in the phosphorylated form in resting muscle (Feldman, 1999; Snow e Murphy, 2001).
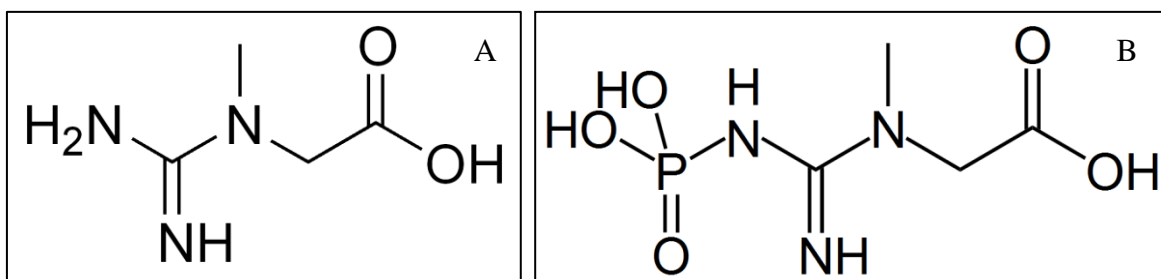
Figure 1.2: Structural formula of creatine monohydrate (A), and creatine phosphate (B).

There is a membrane creatine transporter (CreaT) responsible for cellular uptake of creatine from the blood stream. CreaT consists of a 635 aa protein with 12 transmembrane domains, and has been classified as a member of the Na^+-dependent neurotransmitter transporter family, closely related with the GABA transporter family. CreaT is present in

different tissues, like skeletal muscle (predominantly in I and IIa red fibers in rats), kidney, heart, brain, and liver. Many factors control the activity of CreaT, such as substrate concentration, Na^+ gradient, various hormones (catecholamines via β_2 adrenoceptors, insulin, insulin growth factor I, and others) (Snow e Murphy, 2001). In addition, the presence of a CreaT in rat jejunal brush border enterocyte demonstrated the existence of a Na^+ and Cl^--dependent carrier-mediated mechanism for creatine absorption at intestinal level (Tosco *et al.*, 2004).

A down-regulation of CreaT activity (approximately 50%) was observed when cells are chronically exposed to high levels of extracellular creatine (Snow e Murphy, 2001). Loike *et al.* (1988) prevented this down-regulation *in vitro* with cycloheximidine, a protein biosynthesis inhibitor produced by *Streptomyces griseus.*

The proposed mechanisms of action of creatine are not yet fully elucidated. There is strong evidence that oral creatine supplementation increases muscle glycogen concentrations, IGF-1, IGF-2, number of satellite cells, myosin synthesis, and increases cell volume by water attraction (Olsen *et al.*, 2006; Rawson e Persky, 2007; Cooper *et al.*, 2012). Creatine can also modify the expression of several genes (Deldicque, Atherton*, et al.*, 2008). Inhibition of myostatin appears to be a novel mechanism of creatine (Saremi *et al.*, 2010).

Creatine was first identified in meat extract in 1835, while phosphocreatine was identified only in the 1920's. The first studies reporting supplementation in humans date from 1910 (Feldman, 1999). However, the popularization of creatine as a performance aid was shortly after divulgation that creatine pushed British athletes to gold medals in Barcelona Olympic Games 1992 (Bird, 2003).

In humans, the degradation rate of creatine is 2 g/day, thus 2 g of creatine needs to be ingested or synthesized daily (Snow e Murphy, 2001). Oral supplementation with creatine results in a number of benefits, such as improvement of short-duration and high-intensity exercise, increase in lean body mass, increase in strength during resistance training, acceleration of skeletal muscle recovery after prolonged immobilization, slowing down of muscle wasting in certain myopathies, and improvement of cognitive performance (Deldicque, Decombaz*, et al.*, 2008; Cooper *et al.*, 2012).

In horses, creatine does not present a regular absorption rate as seen in men and dogs (Harris e Harris, 2005). Sewell e Harris (1995) studied the oral absorption of creatine monohydrate in two studied. In the first one, the dose of 0.05 g/kg was administered diluted in 2 L of tap water by nasogastric intubation and resulted in a modest raise in plasma levels in comparison with the same dose in man. In the second study, 0.15 g/kg/day was divided in

three equal doses diluted in 4 L of drinking water during 13 days. The authors did not report how long the horses took to drink the whole bucket of water, and inconstant intake of creatine may have occurred. Plasma and muscle creatine concentrations were evaluated. Apparently, two of the four horses did not absorb creatine, as the creatine plasma concentration was constant and low during the 13 days. No difference was observed in muscle creatine content.

Schuback, Essen-Gustavsson e Persson (2000) aimed to investigate the effect of oral creatine on a maximal treadmill exercise test. They used six horses in a crossover study, in which each animal performed the test three times: without supplementation (baseline), after 25 g of creatine (n=3) or lactose (n=3) twice a day dissolved in lukewarm water and spread over food for 6.5 days, and, at last, after 2 weeks of washout, treatment were switched between animals and repeated in the same dose and period. This was the same experimental design used by Essen-Gustavsson, Lindholm e Persson (1994), but with half the dose (50 g twice a day was used in this previous study). Both studies could not show any influence of creatine supplementation on muscle or plasma creatine concentrations, and both highlighted the fact that some horses did not absorb creatine.

Ferraz *et al.* (2006) evaluated longer periods of creatine supplementation and used the VLa4 as parameter to comparison instead of exhaustion at treadmill. They used Arabian horses, instead of Standardbred (Schuback, Essen-Gustavsson e Persson, 2000), and a dose of approximately 0.2 g/kg/day (same dose used by Essen-Gustavsson, Lindholm e Persson (1994)), during 90 days. Horses underwent treadmill exercise at days 30, 60 and 90 of supplementation, and there was significant increase in VLa4 at days 60 and 90 in comparison to baseline. No serum or muscle creatine levels were evaluated. In these same horses, it was observed no effect of creatine on cross sectional area of each fiber type in muscle biopsy samples of gluteus medius muscle, neither on relative frequency of each fiber type (D'angelis *et al.*, 2005).

In humans, Saremi *et al.* (2010) compared the effects of the association of creatine oral supplementation plus resistance training, resistance training plus oral placebo, with a control group (placebo and no resistance training) during eight weeks. They evaluated body composition, muscle strength and serum levels of myostatin and GASP-1, an inhibitor of myostatin. The results indicated a significant effect of creatine plus resistance training to increase lean body mass, and increase muscle strength. GASP-1 increased along the study, but did not differ in subjects that received or not creatine, but myostatin significantly decreased more in the group receiving creatine than in the group only submitted to resistance training.

These findings arise a new and important mechanism of action of creatine that was not previously studied in horses, and that requires further investigation.

2 SCIENTIFIC ARTICLE NUMBER 1

2.1 Title

High-resolution melting analysis for detection of a sequence polymorphism in myostatin gene in horses

2.2 Authors and filiations

Priscila Beatriz da Silva Serpa[a], Petra Garbade[a], Cláudio Corrêa Natalini[a,b], Ananda da Rocha Pires[c], Tainor de Mesquita Tisotti[c]

[a] Programa de Pós-Graduação em Medicina Animal: Equinos, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000, Brazil; [b] School of Animal and Veterinary, The University of Adelaide, Roseworthy, SA, 5371, Australia; [c] Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000 Brazil.

2.3 Abstract

The objective of this study was to develop a test that could allow rapid and reliable detection of the polymorphism g.66493737C>T in the MSTN gene in horses. For that, genomic DNA was extracted from 23 venous blood samples of clinically healthy jump horses (Brazilian Sport Horse, Selle Français, Holsteiner, and Hanoverian). DNA samples of three horses (C/C genotype from a Quarter Horse, T/T genotype from an Arabian Horse, C/T genotype from a Thoroughbred) not correlated with this study were sequenced and used as positive controls of the three genotypes. Three different protocols were tested in one high-resolution melting analysis equipment: a standard protocol, a high-salt concentration (1 M KCl and 0.5 M Tris-HCl pH 8) before the first cycle of melting, and the addition of an unlabeled probe with the 3' end blocked by the addition of a 3-carbon spacer. All samples were run in triplicate. The use of the primers alone (standard protocol) or with high salt did not result in consistent melting curves. Unlabeled probes resulted in satisfactory repeatability of the shape of the curves between the triplicates. Seven animals revealed inconsistent melting curves, resulting in impossibility to genotype when the cutoff level of confidence was set at 70%. From the remaining samples, the mean level of confidence was 84.4%. In this sample of jump horses, the frequency of the genotype T/T were 63.6%, T/C were 31.8%, and C/C were 4.5%. The frequency of the alleles T and C was 79.5% and 20.5% respectively. In conclusion, HRM directly discriminates the heterozygotes and homozygotes, proving to be a rapid and

cost-effective alternative method for mutation screening. In the remaining samples, sequencing must be performed to genotype the animals and to identify the source of aberrant melting curves.

2.4 Introduction

Polymerase chain reaction (PCR) amplification of nucleic acids represented an enormous advance in the study of diseases and physiological cellular processes. The method was typically separated in two sequential phases of amplification by automated equipment and detection of the final product (amplicon). The usual detection involved agarose or polyacrylamide electrophoresis gels for size separation and visualization with fluorescent dyes (Erlich, Gelfand e Sninsky, 1991; Ririe, Rasmussen e Wittwer, 1997). Today, amplification and detection can occur simultaneously thanks to double-stranded-specific DNA intercalating dyes, like ethidium bromide, SYBR Green I, LC Green, SYBR Gold, PicoGreen, TOTO-1, Syto9, EvaGreen and YOYO-1 (Wittwer *et al.*, 2003; Wittwer, 2009). Added to the PCR reaction, dyes generate fluorescence proportional to the amount of dsDNA present in the reaction tube, but also undesirable detection, such as that due to primer dimerization, occurs. Since the dye fluorescence is only emitted when it is intercalated to dsDNA, the denaturation of the strands extinguishes the fluorescence signal. Plotting fluorescence by temperature generates a melting curve of the product, which is dependent of the GC content, length and sequence of the amplicon (Ririe, Rasmussen e Wittwer, 1997).

High-resolution melting (HRM) analysis is a recent closed-tube method based in fluorescence detection for genotyping and mutation scanning developed by the University of Utah and Idaho Technology in 2002 (Reed, Kent e Wittwer, 2007). HRM is a simple, rapid and inexpensive technique, but depend on good PCR design, specific instrument for detection and dyes. The procedure consists of a conventional PCR followed by a melting step, and it is an excellent technique for detection of single nucleotide polymorphisms (SNPs), but it is not as good for insertions and deletions (Van Der Stoep *et al.*, 2009; Wittwer, 2009).

Myostatin, also known as growth differentiation factor 8 (GDF-8), is a peptide hormone, responsible for negative regulation of skeletal muscle growth (Mcpherron, Lawler e Lee, 1997). It is in all skeletal muscle and has as target the satellite cells, the mononuclear progenitor of the muscle lineage present within muscle fibers, keeping them in a quiescent state (Mcfarlane *et al.*, 2008). Naturally occurring mutations in the myostatin gene (MSTN) have already been identified in horses, dogs, sheep, cattle, mice, and humans (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; Mcpherron e Lee, 1997; Varga *et al.*, 1997; Grobet *et al.*, 1998;

Szabó *et al.*, 1998; Marchitelli *et al.*, 2003; Schuelke *et al.*, 2004; Clop *et al.*, 2006; Mosher *et al.*, 2007; Boman *et al.*, 2009). Those mutations can decrease or interrupt myostatin production, leading to the development of greater muscle mass compared to individuals without the mutation, and also promote effects on growth, reproduction, performance and carcass quality traits (Dall'olio *et al.*, 2010). In horses, Hill, Gu*, et al.* (2010) found a C>T transition within intron 1 in MSTN gene in Thoroughbreds (g.66493737C>T). Besides, they documented a high correlation between the three genotypes of this SNP and the optimal race distance performance, with allele C being twice as frequent in short distance than in middle and long distance winners. Moreover, the frequency of each genotype was considered different for non-Thoroughbred horses evaluated. In Egyptian Arabian horses, the frequency of T/T genotype was 90%, while the frequency of C/C in quarter horses was 83%.

The SNP reported by Hill, Gu*, et al.* (2010) can be used as molecular markers of equine performance, and may be used in the future as a tool of genetic selection for sports practice. The objective of this study was to develop a minimum time-consuming, reliable and cost-effective diagnosis of g.66493737C>T polymorphism in the MSTN gene in horses. For that, three different protocols were tested in one high-resolution melting analysis equipment in 23 samples.

2.5 Material and Methods

2.5.1 Animals and samples

Study design and methodology were approved by the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (UFRGS, Nº 24556), Porto Alegre, Brazil, and adhered to the principles for the humane treatment of animals in veterinary clinical investigations.

Venous blood samples were withdrawn in vacuum tubes with EDTA as anticoagulant from 23 clinically healthy jump horses in athletic activity of different breeds (Brazilian Sport Horse, Selle Français, Holsteiner, and Hanoverian), 13 males and 10 females, aged between 4 and 17 years with the consent of the owners.

Genomic DNA was extracted and purified using commercial kits with silica columns (PureLink® Genomic DNA Mini Kit, Invitrogen®, Carlsbad, CA, USA). The amount of DNA extracted was measured by fluorimetry (Qubit® 2.0 Fluorometer, Invitrogen®, Carlsbad, CA, USA) with a specific kit (Qubit® dsDNA BR Assay Kit, Invitrogen®, Carlsbad, CA, USA). DNA samples of three horses (C/C genotype from a Quarter Horse, T/T genotype from an

Arabian Horse, C/T genotype from a Thoroughbred), from the DNA database of the Laboratory of Animal Farmacogentics (*Laboratório de Farmacogenética Animal*, UFRGS, Porto Alegre, RS, Brazil) not correlated with this study were used as positive controls of the three genotypes.

2.5.2 Polymerase chain reaction and direct sequencing

For detection of g.66493737C>T in the MSTN gene, a new pair of primers was designed with the goal of generating a product with less than 150 base pairs for further analysis by HRM. The primer design tool of the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih.gov) was used (EquCab 2.0, chromosome 18, MSTN, NC_009161.2). Primers were synthesized by Integrated DNA Technologies IDT, Inc. (Coralville, IA, USA).

The PCR for detection of the mutation was carried in a total volume of 25 µL under the following conditions: approximately 20 ng of DNA, 1X PCR buffer, magnesium chloride concentrations ranging from 0.75 to 3 mM, 0.5 U Taq polymerase (Recombinant Taq DNA Polymerase, Invitrogen®, Carlsbad, CA, USA), 0.1 mM of each dideoxynucleotide (dNTP) and 10 pmol of each primer (forward 5'-GAC ACA ACA GTT TCA AAA TAT TGT TCT CCT T-3' and reverse 5'-CCA GGA CTA TTT GAT AGC AGA GTC A-3'), designed to obtain a 98 bp amplicon. The cycles in a thermocycler (Veriti® 96-Well Thermal Cycler, Applied Biosystems®, Foster City, CA, USA) were adjusted to the following conditions: initial denaturation at 94°C for 3 minutes, 35 cycles of 94°C for 30 seconds, annealing temperature ranging from 55°C, 57°C or 59°C for 30 seconds, and extension at 72°C for 1 minute, and final extension cycle at 72°C for 10 minutes. An aliquot of the amplification product was visualized on a native polyacrylamide 12% gel after electrophoresis at 100 V for 60 minutes and silver staining according to Sanguinetti, Neto e Simpson (1994). The amount of DNA present after PCR was measured by fluorimetry (Qubit® 2.0 Fluorometer, Invitrogen®, Carlsbad, CA, USA) with a specific kit (Qubit® dsDNA HS Assay Kit, Invitrogen®, Carlsbad, CA, USA).

PCR products were purified (Wizard® SV Gel and PCR Clean-Up System, Promega, Madison, WI, USA) before sequencing. Samples were sequenced at the Center of Experimental Research (*Centro de Pesquisa Experimental*, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil) using ABI 3500 Genetic Analyzer with 50 cm capillaries and POP7 polymer (Applied Biosystems®, Foster City, CA, USA). Five nanograms of PCR product were labeled with 3.2 pmol of the forward primer and 1 µL of BigDye® Terminator

v3.1 Cycle Sequencing Kit (Applied Biosystems®, Foster City, CA, USA) in a final volume of 10 µL. Labeling reactions were performed in a thermocycler (Veriti® 96-Well Thermal Cycler, Applied Biosystems®, Foster City, CA, USA) with an initial denaturing step of 96ºC for 1 minute followed by 35 cycles of 96ºC for 15 sec, 50ºC for 15 seconds and 60ºC for 4 minutes. Labeled samples were purified using BigDye® XTerminator Purification Kit (Applied Biosystems®, Foster City, CA, USA) and electroinjected in the automatic sequencer.

2.5.3 High-resolution melting analysis

As recommended by the manufacturer (Type-it® HRM PCR Kit, Qiagen®, Hilden, Germany), the reaction was prepared in 25 µL with 2x PCR Master Mix, which contains the HotStarTaq® Plus DNA Polymerase, dNTPs, and Q-Solution® PCR buffer with EvaGreen® as fluorescence marker, 0.7 µM of each primer previously tested, 20 ng of DNA and water qsp. The thermocycler (Rotor-Gene® Q, Qiagen®, Hilden, Germany) was programmed for an initial enzyme activation step of 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 10 seconds. Immediately after the amplification step HRM was programmed to ramp from 65 to 95°C with 0.1°C increments every 2 seconds for data acquisition and determination of the T_m (melting temperature). A second protocol by adding 1 µL of high salt concentration (1 M KCl and 0.5 M Tris-HCl pH 8) before the first cycle of melting was tested. A third protocol was assessed by the addition of a probe with the 3' end blocked by the addition of a 3-carbon spacer (C3 probe unlabeled spacer, Integrated DNA Technologies IDT, Inc., Coralville, IA, USA) - 5'-CAG GTT ATA ATG CAC CAA ATA ATT TTC/3SpC3/-3' in a 5:4:1 ratio (reverse primer: probe: forward primer). Different protocols were tested with the purpose of obtain a satisfactory repeatability of the method. Each protocol was run once with all samples in triplicate.

2.5.4 Statistical analysis

The software Rotor Gene Q version 2.3.1 was used to calculate the R values and to provide the level of confidence for each curve in comparison with the controls. Fluorescence threshold was manually determined. Fluorescence intensity values were normalized between 0% and 100% by first defining linear baselines before and after the melting transition of each sample.

2.6 Results

In the PCR design, optimal annealing temperature and MgCl$_2$ concentration was set as 55ºC and 1.5 mM respectively. With these same primers, sequencing was successfully obtained in 20 of the 23 samples (except H21, H22, H23).

In the HRM analysis, the use of the designed primers alone or with high-salt did not result in consistent melting curves. In the other hand, the use of unlabeled probes with the 3' end blocked by the addition of a 3-carbon spacer did result in consistent melting curves, with repeatability of the shape of the curve between the triplicates. The results below are derived from the unlabeled probes.

The raw data generated by the melting was normalized for correction of nonspecific signal and plotted against temperature (**Figure 2.1**). Threshold was set in 0.04192. Cycles before 10 were eliminated from the analysis. The difference graph is shown in **Figure 2.2** considering the genotype T/T (red) as reference.
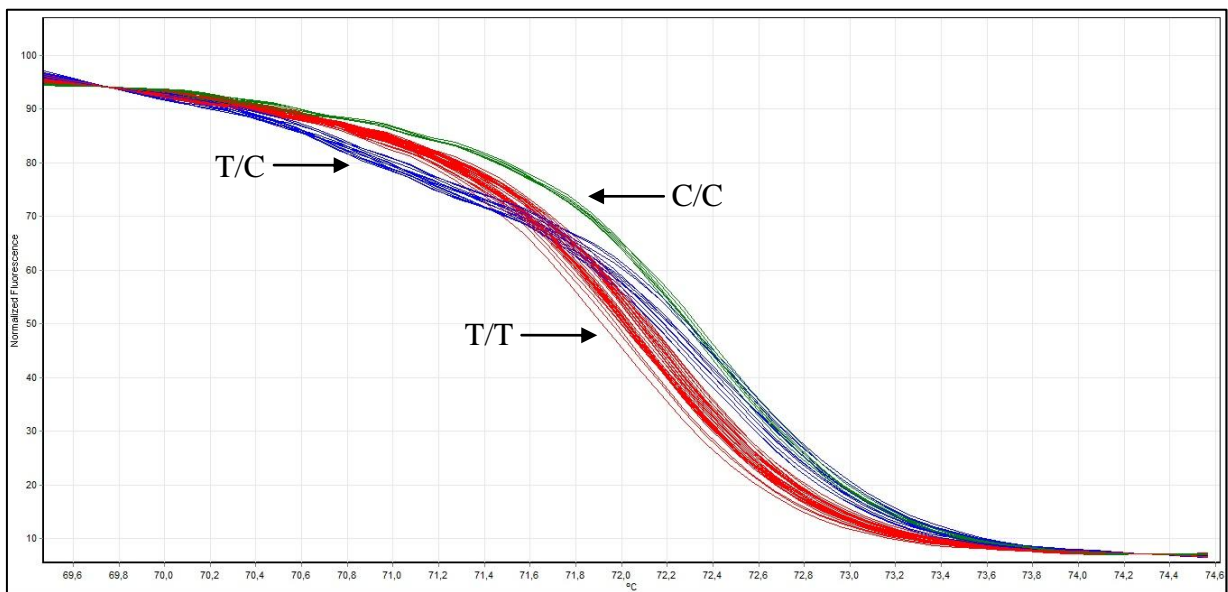
Figure 2.1: Normalized graph of high-resolution melting analysis of the g.66493737C>T polymorphism in the MSTN gene indicating the three genotypes: homozygous T/T (red), heterozygous T/C (blue), and homozygous C/C (green).
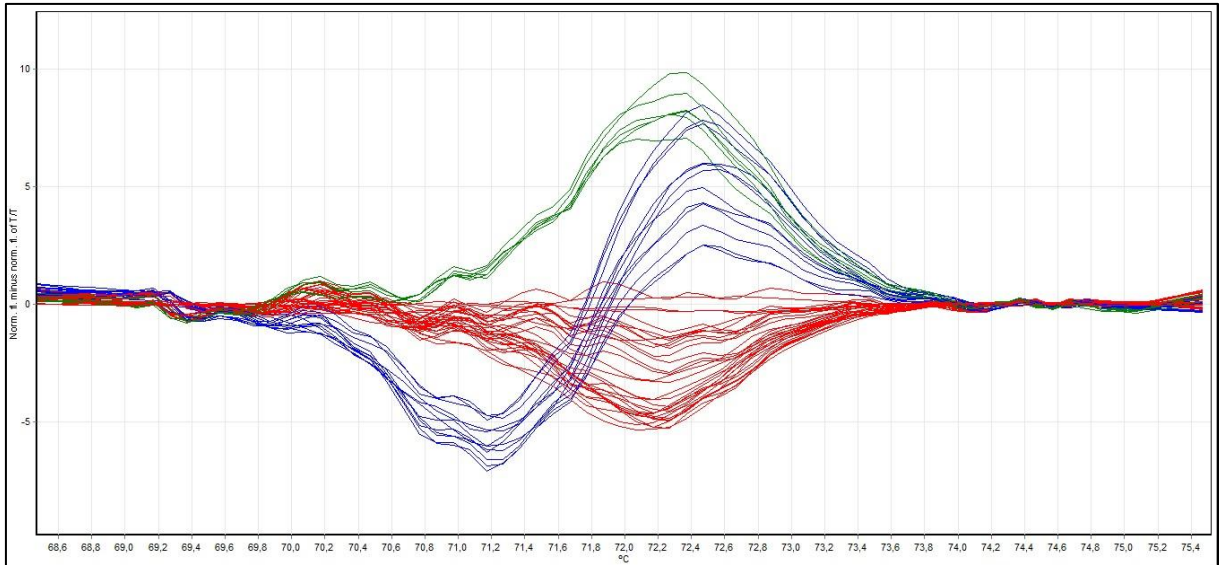
Figure 2.2: Difference graph of high-resolution melting analysis of the g.66493737C>T polymorphism in the MSTN gene indicating the three genotypes: homozygous T/T (red), heterozygous T/C (blue), and homozygous C/C (green).

The results of HRM analysis of 16 animals followed the same pattern of one of each control samples, with the curves showing a visible pattern as seen in both figures above. On the other hand, seven animals (H3, H6, H10, H15, H19, H20 and H21) revealed inconsistent melting curves, resulting in impossibility to genotype when the cutoff level of confidence was set at 70% (those curves were not shown in the figures above). The HRM analysis of these samples was repeated and they remained ungenotypable with this method (**Table 2.1**). All samples genotyped presented 100% concordance with the sequencing results. Between the seven ungenotypable samples, three were T/T and three were T/C by sequencing analysis. From the remaining samples, the mean level of confidence was 84.4%. In this sample of jump horses, the frequency of the genotype T/T were 63.6%, T/C were 31.8%, and C/C were 4,5%. The frequency of the alleles T and C was 79.5% and 20.5% respectively.

Table 2.1: High-resolution melting genotyping and sequencing results of 23 samples analyzed in triplicates with mean C_t (threshold cycle) values (± SD, standard deviation), genotypes and mean level of confidence (%) of each sample.

Sample	Mean C_t (± SD)	Genotyping	Mean Confidence %	Sequencing
H1	25.34 (0.08)	T/T	87.3	T/T
H2	25 (0.7)	T/T	78.4	T/T
H3	-	Variation	-	T/T
H4	23.74 (0.24)	T/C	88.5	T/C
H5	25.29 (0.63)	T/T	80.3	T/T
H6	25.61 (0.70)	Variation	-	T/T
H7	23.9 (0.08)	T/T	96.8	T/T
H8	24.6 (0.22)	T/T	88.3	T/T
H9	24.25 (0.64)	T/T	76.5	T/T
H10	23.93 (0.39)	Variation	-	T/T
H11	24.7 (0.21)	T/T	89.8	T/T
H12	23.97 (0.22)	T/T	85.6	T/T
H13	23.96 (0.58)	T/C	79.2	T/C
H14	24.5 (0.46)	C/C	96.7	C/C
H15	24.14 (0.21)	Variation	-	T/C
H16	25.29 (0.47)	T/C	80.1	T/C
H17	24.27 (0.36)	T/T	80.3	T/T
H18	24.53 (0.17)	T/T	74.0	T/T
H19	25.37 (0.37)	Variation	-	T/C
H20	24.11 (0.32)	Variation	-	T/C
H21	24.58 (14.59)	Variation	-	Variation
H22	26.15 (3.98)	T/T	80.2	Variation
H23	22.99 (1.55)	T/C	87.4	Variation
Control T/C	24.59 (0.67)	T/C	98.7	T/C
Control C/C	22.93 (0.25)	C/C	98.4	C/C
Control T/T	23.99 (0.11)	T/T	99.4	T/T

2.7 Discussion

This was the first time that a prevalence of each genotype for the SNP g.66493737C>T was determined in Warmblood horses by a HRM analysis. The technique developed was able to discriminate the genotype of 70% of samples screened with a mean confidence level of 84.4%.

The PCR in this study was delineated to have an amplicon as small as possible (98 bp), since PCR products of 300 bp or less is more sensitive and more specific in the HRM analysis. Moreover, longer amplicons melt in two stages, which generates complex melting curves (Reed e Wittwer, 2004). The designed primers originated an amplicon visualized in polyacrylamide gel accordingly to the predicted size, and after that was tested to perform the HRM analysis.

The use of the designed primers alone was not successful in the HRM analysis. We evaluated and discarded the most common reported sources of variation in the HRM, such as inadequate product length, ionic strength of buffer solution, variation in the DNA extraction method, and instrument quality of data acquisition (Reed, Kent e Wittwer, 2007; Vossen *et al.*, 2009), because all samples was subjected to the same variables and it does not explain the inconsistency of melting curves. Use of high-salt concentration was also not successful in the HRM analysis. Although the results are unpredictable, the addition of high-salt buffer in the reaction before a second melting can promote the sharpening of the curves and increase the resolution and clustering (Vossen *et al.*, 2009).

In the other hand, the use of an unlabeled probe in this study was effective. Employing sequence-specific probes with labeled substances as FAM, HEX and others, is highly efficient, but with added cost in comparison with nonlabeled oligonucleotides (Zhou *et al.*, 2004; Liew *et al.*, 2007). In unlabeled probes, the 3' extremity is blocked to prevent polymerase extension, which creates two amplicons per tube (one in the designed size and a smaller one) and an extra asymmetric curve to help to differentiate the genotypes. The blocking can be accomplished with 3'-phosphorylation, 2'3'-dideoxynucleotide, 3'-deoxynucleotide, 3'-3' linkage, inverted dT, amino-modified C6, C3 spacer or mismatching the last two 3' bases. We opted to use a 3' C3 spacer due to its reported stability (Zhou *et al.*, 2004; Dames *et al.*, 2007; Erali, Voelkerding e Wittwer, 2008). Instead of a visible additional curve in the temperature range of data acquisition, the unlabeled probe helped to generate curves with more distinguishable temperatures between each other, which in our knowledge were not reported before. The probe T_m was not approximately 10ºC above primers T_m neither above 56ºC as recommended due to the low GC content within this sequence, which ideally must be 40-60% for HRM genotyping (Dieffenbach, Lowe e Dveksler, 1993; Rozen e Skaletsky, 1999; Taylor, 2009; Van Der Stoep *et al.*, 2009).

We expected to have a few or no C/C animals due to the physical conformation of jump horses, and this belief was confirmed. The inconsistency in the melting curves of the seven animals could be due to polymorphisms within the amplicon, which modifies the melting temperature. However, sequencing of these samples did not reveal any polymorphism, and the origin of the aberrant melting behavior could not be addressed. We also believed to have an equal or close number of T/C and T/T in the animals studied due to the association of desirable characteristics in a jump horse, such as weightlessness, but in the same time power for propelling. However, almost two thirds (63.6%) of the genotyped animals were T/T, below the frequency reported in Egyptian Arabian horses (Hill, Gu, *et al.*, 2010).

In conclusion, in the majority of samples, HRM directly discriminates the heterozygotes and homozygotes, proving to be a fast and cost-effective alternative method for mutation screening of the SNP g.66493737C>T. The fact of being a closed-tube technique decreases the operational risks associated with the manipulation of biohazard material, such as ethidium bromide or polyacrylamide. The modification of the amplicon size and the T_m of the unlabeled probe may improve the efficacy of this technique in the future.

2.8 Acknowledgements

2.9 Conflicts of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

3 SCIENTIFIC ARTICLE NUMBER 2

3.1 Title

Effects of oral supplementation with creatine monohydrate in plasma myostatin concentration in jump horses

3.2 Authors and filiations

Priscila Beatriz da Silva Serpa[a], Petra Garbade[a], Cláudio Natalini[a,b], Joaquim Borges Neto[a], Ananda da Rocha Pires[c], Tainor de Mesquita Tisotti[c]

[a] Programa de Pós-Graduação em Medicina Animal: Equinos, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000, Brazil; [b] School of Animal and Veterinary, The University of Adelaide, Roseworthy, SA, 5371, Australia; [c] Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000 Brazil.

3.3 Abstract

Myostatin is a peptide hormone responsible for negative regulation of skeletal muscle growth. Creatine is a nonprotein amino acid commonly used as ergogenic aid. There is evidence indicating a possible effect of creatine inhibiting circulating levels of myostatin, which can increase muscle mass and improve performance. The aim of this study was to evaluate the effect of oral supplementation with creatine during 8 weeks in serum creatine and plasma myostatin in horses and to evaluate if there is difference responses accordingly to the genotype for the SNP g.66493737C>T. Sixteen clinically healthy jump horses (male = 10, female = 6) were equally and randomly distributed into groups control (PLA, placebo) and treatment (CRE, creatine). Treatment consisted of 8 weeks of oral supplementation with creatine monohydrate (1st week = 0.3 g/kg, 2-8th week = 0.05 g/kg). Doses were compounded into an oral paste to ensure individuality and precisely dosage. PLA received the same compounded pastes, but without creatine. Venous blood samples were collected on the day before starting supplementation, and after 4 and 8 weeks of supplementation to determination of creatine, myostatin, creatinine and creatine kinase (CK). Body weight was also assessed in the three time points. Genomic DNA was used to detection of the polymorphism g.66493737C>T in the MSTN gene by high-resolution melting (HRM) analysis. There was no difference between PLA and CRE in body weight, creatinine, CK, and plasma myostatin. Serum creatine was significant increased in CRE at week 4. Ten horses were T/T (PLA = 5,

CRE = 5), five were heterozygous (PLA = 3, CRE = 2), and none were C/C. One animal could not be genotyped by HRM. Stratification of data by sex within each group did not affect the statistical analysis, and stratification by genotype was not possible. In conclusion, oral supplementation with creatine during 8 weeks with a loading dose of 0.3 g/kg/day during 1 week and a maintenance dose of 0.05 g/kg/day during 7 weeks increases serum creatine in the first 4 weeks of supplementation, but does not changed creatinine, CK and myostatin concentrations in jump horses.

3.4 Introduction

Myostatin, also known as growth differentiation factor 8 (GDF-8), is a peptide hormone responsible for negative regulation of skeletal muscle growth (Mcpherron, Lawler e Lee, 1997). It is found in serum and skeletal muscle tissue in its active or inactive (propeptide) forms (Zimmers *et al.*, 2002; Dominique e Gérard, 2006). Myostatin is produced by developing myotome compartment, and later in all skeletal muscle in the body (Mcpherron, Lawler e Lee, 1997; Dominique e Gérard, 2006). In adults, their target cells are satellite cells, the mononuclear progenitor of the muscle lineage present within muscle fibers, keeping them in a quiescent state (Mcfarlane *et al.*, 2008). Its action is through binding to activin receptor IIb (ActRIIb) (Lee e Mcpherron, 2001). After binding to the receptor, a second receptor-ActRIb or ALK4-is recruited and phosphorylated to form a heterotetramer complex, which promotes the activation of Smads, and whereupon regulates the expression of different inhibitory genes. Other Smad-nondependent signaling cascades appear to be involved in the myostatin regulatory signal transduction, however these cascades have not been fully elucidated yet (Dominique e Gérard, 2006; Huang, Chen e Chen, 2011).

Naturally occurring mutations in the myostatin gene (MSTN) have already been identified in horses, dogs, sheep, cattle, mice, and humans (Grobet *et al.*, 1997; Kambadur *et al.*, 1997; Mcpherron e Lee, 1997; Varga *et al.*, 1997; Grobet *et al.*, 1998; Szabó *et al.*, 1998; Marchitelli *et al.*, 2003; Schuelke *et al.*, 2004; Clop *et al.*, 2006; Mosher *et al.*, 2007; Boman *et al.*, 2009). These mutations can decrease or interrupt myostatin production, leading to the development of greater muscle mass compared to individuals without the mutation, and also promote effects on growth, reproduction, performance and carcass quality traits (Dall'olio *et al.*, 2010).

In horses, Hill, Gu*, et al.* (2010) found a C>T transition within intron 1 in MSTN gene in Thoroughbreds (g.66493737C>T). Besides, they documented a high correlation between the three genotypes of this SNP (single nucleotide polymorphism) and the optimal race distance

performance, with allele C being twice as frequent in short distance than in middle and long distance winners.

Saremi *et al.* (2010) found a significant effect of creatine on serum levels of myostatin in human subjects submitted to eight weeks of oral supplementation. These findings arise a new and important mechanism of action of creatine. Creatine is a nonprotein amino acid formed from methionine, arginine and glycine in the liver, kidneys, and pancreas (Feldman, 1999), and is used mainly in muscle and in the brain to form phosphocreatine, an energy storage compound. Creatine is spontaneously degraded to creatinine by the loss of water, which is solely excreted from the body by renal route (Feldman, 1999). Approximately 95% of total body creatine is in skeletal muscle, while 60-67% is in the phosphorylated form in resting muscle (Feldman, 1999; Snow e Murphy, 2001). The proposed mechanisms of action of creatine are not yet fully elucidated. There is strong evidence that oral creatine supplementation increases muscle glycogen concentrations, IGF-1, IGF-2, number of satellite cells, myosin synthesis, and increases cell volume by water attraction (Olsen *et al.*, 2006; Rawson e Persky, 2007; Cooper *et al.*, 2012).

Oral supplementation with creatine results in a number of benefits, such as improvement of short-duration and high-intensity exercise, increase in lean body mass, increase in strength during resistance training, acceleration of skeletal muscle recovery after prolonged immobilization, slowing down of muscle wasting in certain myopathies, and improvement of cognitive performance (Deldicque, Decombaz*, et al.*, 2008; Cooper *et al.*, 2012).

In horses, creatine does not present a regular absorption rate as seen in men (Harris e Harris, 2005). Oral doses from 0.04 g/kg to 0.2 g/kg were already tested in different ways of administration (nasogastric intubation, drinking water, spread on food), during different periods (from 6.5 to 90 days), and studies disagree about absorption, muscle incorporation, and effects on performance (Essen-Gustavsson, Lindholm e Persson, 1994; Sewell e Harris, 1995; Schuback, Essen-Gustavsson e Persson, 2000; D'angelis *et al.*, 2005; Ferraz *et al.*, 2006).

To our knowledge, the effect of creatine on serum myostatin was not studied in horses yet. The aim of this study was to evaluate the effect of oral supplementation with creatine during 8 weeks in serum creatine and plasma myostatin in horses and to evaluate if there is difference responses accordingly to the genotype for the SNP g.66493737C>T.

3.5 Material and Methods

3.5.1 Ethics

Study design and methodology were approved by the Institutional Animal Care and Use Committee of Federal University of Rio Grande do Sul (UFRGS, Nº 24556), and adhered to the principles for the human treatment of animals in veterinary clinical investigations.

3.5.2 Animals

Sixteen clinically healthy jump horses (Brazilian Sport Horse - BH, Holsteiner, and Hanoverian), ten males and six females, were equally and randomly distributed into groups control (PLA, placebo) and treatment (CRE, creatine). Sample size was calculated considering a difference of 5 ng/mL of miostatin between control and treatment (α=0.05, β=0.20). The mean $\pm$ standard deviation of weight and age were 568.4 $\pm$ 46.6 kg and 8.6 $\pm$ 4.1 years respectively. All animals were in regular training for athletic performance in equestrian events of up to 1.30 m. The horses were housed individually in a training center in Porto Alegre, RS, Brazil, eating a balanced diet according to their body weight (BW) and activity level, consisting primarily of a concentrated feed and alfafa hay divided into two daily meals, and water *ad libitum.*

3.5.3 Oral supplementation

The animals studied were submitted to eight weeks of oral supplementation with creatine monohydrate administered based on a previous study in human subjects (Saremi *et al.*, 2010). During the first week, the animals received a single daily loading dose of 0.3 g/kg of creatine paste or placebo paste. In the remaining seven weeks, the animals were given a single daily maintenance dose of 0.05 g/kg of creatine paste or placebo paste.

The doses were compounded into an oral paste with 0.375 g/kg of a commercial available 80% creatine monohydrate powder (Creatina Vetnil, Vetnil® Saúde Animal, Louveira, SP, Brazil), 100 mg/kg of carboxymethylcellulose 2% (CMC FG 3000-4000, Hexus Food Ingredients, Portão, RS, Brazil), 10 µL/kg of artificial flavoring (Pineapple Flavor, Iceberg Ind. Com. Ltda., Jandira, SP, Brazil), and 50 µL/kg of water during the loading period. In the maintenance period, the formulation was 0.0625 g/kg of commercial creatine, 20 mg/kg of carboxymethylcellulose 2%, 3 µL/kg of artificial flavoring, and 50 µL/kg of water. Group PLA received the same compounded pastes but without creatine.

3.5.4 Blood samples

Venous blood samples were withdrawn from jugular vein in vaccum tubes at three time: on the day before starting supplementation (Week 0); after 29 days, i.e. the next day after 4 weeks of supplementation (Week 4), and after 57 days, i.e. the next day after 8 weeks of supplementation (Week 8). Serum was used for determination of serum creatine (Creatine Assay Kit, Cat # MAK079, Sigma-Aldrich®, St. Louis, MO, USA) by spectrophotometry. Myostatin plasma levels were measured in EDTA blood samples by ELISA (Horse Growth/differentiation factor 8 ELISA kit, Cat # MBS940744, MyBiosource, San Diego, CA, USA). Both creatine and myostatin were determined using a microplate reader (Biochrom Zenyth 200rt UV-Vis, Anthos Labtec Instruments, Wals, Austria). Creatinine and creatine kinase were measured in heparinized blood (Reflotron® Plus, Roche Diagnostics Ltd., Basel, Switzerland).

3.5.5 Analysis of MSTN genotype

Venous blood samples were withdrawn in vacuum tubes with EDTA as anticoagulant and used for genomic DNA extraction and purification using commercial kits with silica columns (PureLink® Genomic DNA Mini Kit, Invitrogen®, Carlsbad, CA, USA). For detection of g.66493737C>T in the MSTN gene, the high-resolution melting (HRM) analysis was performed (Rotor-Gene® Q, Qiagen®, Hilden, Germany) as previously described (**Section 2**) with the addition of a probe with the 3'-end blocked by the addition of a 3-carbon spacer (Integrated DNA Technologies IDT, Inc., Coralville, IA, USA).

3.5.6 Statistical analysis

Data were submitted to two-way analysis of variance (ANOVA) of repeated measures within each group with a 95% confidence level. Tukey's test was used as post hoc to compare times within each group. Multiple t test using Holm-Sidak method ($P < 0.05$) was used to compare groups within each time. The values were represented as mean ± standard deviation. Calibration curves were obtained by linear regression to define the coefficient of determination (R^2). The programs used for statistical analysis was GraphPad Prism 5 (GraphPad Software Inc., La Jolla, CA, USA) and Microsoft® Excel version 2007. The melting curves were normalized and the level of confidence was determined by software Rotor-Q Gene® software (Qiagen®, Hilden, Germany).

3.6 Results

The genotypic analysis by HRM revealed five T/T animals in each group, while there were three T/C animals in PLA and two in CRE. One animal in CRE group could not be genotyped.

A variety of breeds of horses were used in this study, but there was no statistical difference between treatments (P = 0.35) in body weight, although there was time effect (**Table 3.1**). Values of blood CK presented significant treatment effect, while blood creatinine and serum creatine presented significant time effect in the analysis of variance. Plasma myostatin concentration showed no difference between PLA and CRE groups. In the CRE group, three animals presented no increase in creatine serum levels after 8 weeks of oral supplementation

Table 3.1: Values of blood creatinine and creatine kinase (CK), serum creatine and plasma myostatin between groups control (PLA) and treatment (CRE) along the times studied in 16 jump horses. Data are expressed as mean values ± standard deviation.

	PLA	CRE	P values	
Weight (kg)				
Week 0	584.4 ± 56.4^{aA}	553.1 ± 37.3^{aA}	Interaction	P = 0.346
Week 4	562.9 ± 70.2^{aA}	546.9 ± 34.3^{aA}	Time effect	P = 0.026*
Week 8	583.3 ± 55.5^{aA}	558.1 ± 38.7^{aA}	Treatment effect	P = 0.442
Creatine kinase (U/L)				
Week 0	28.24 ± 8.29^{aA}	34.48 ± 21.35^{aA}	Interaction	P = 0.890
Week 4	24.93 ± 6.07^{aA}	35.52 ± 8.59^{aA}	Time effect	P = 0.987
Week 8	23.58 ± 9.23^{aA}	38.56 ± 18.84^{bA}	Treatment effect	P = 0.028*
Creatinine (mg/dL)				
Week 0	1.60 ± 0.13^{aAB}	1.48 ± 0.16^{aA}	Interaction	P = 0.640
Week 4	1.68 ± 0.11^{aA}	1.44 ± 0.34^{bA}	Time effect	P = 0.015*
Week 8	1.43 ± 0.21^{aB}	1.31 ± 0.17^{aA}	Treatment effect	P = 0.060
Creatine (ng/µL)				
Week 0	4.18 ± 1.19^{aA}	4.15 ± 1.76^{aA}	Interaction	P = 0.572
Week 4	5.61 ± 1.69^{aA}	6.77 ± 3.79^{aB}	Time effect	P = 0.002**
Week 8	3.27 ± 1.87^{aA}	4.16 ± 1.45^{aA}	Treatment effect	P = 0.433
Myostatin (ng/mL)				
Week 0	3.23 ± 2.76^{aA}	2.95 ± 2.45^{aA}	Interaction	P = 0.069
Week 4	0.58 ± 0.57^{aA}	3.81 ± 2.48^{bA}	Time effect	P = 0.464
Week 8	1.05 ± 0.71^{aA}	3.66 ± 2.65^{bA}	Treatment effect	P = 0.075

* P < 0.05 **P < 0.01. Different lower case letters within the same row indicate statistical difference (P < 0.05) between groups. Different upper case letters within the same column indicate statistical difference (P < 0.05) between times.

Stratification of data by sex within each group did not affect the statistical analysis, which shows $P < 0.05$ in the same effects and variables. It was not possible to stratify the data by genotype, since there were only two T/C animals in group CRE.

3.7 Discussion

In this study, we used a loading dose of 0.3 g/kg for one week followed by a maintenance dose of 0.05 g/kg. The loading dose used here is the highest dose reported to date in horses, but in agreement with doses in men (Saremi *et al.*, 2010). The concept of using a loading dose is justified based in the maximum capacity of muscle to incorporate creatine and in the saturation theory (Hultman *et al.*, 1996; Cooper *et al.*, 2012). In this first week, each horse required three to four 60 mL syringes in order to get the whole dose of the compounded creatine. There were no problems with acceptance of the formula. We believed that the oral paste is a good alternative to administration of creatine, since nasogastric intubation would be impracticable for long periods, and administration through water or food would create biases (loss of creatine powder inside buckets and feeders, different times of feeding and drinking due to different training routine of the horses). In the remaining seven weeks of supplementation, the volume of paste decreased to one syringe only. Although there were some animals that did not increased the serum level of creatine, as previously reported in the literature, in the majority of them (5 out of 8), the serum concentration increased from 4.15 at baseline to 6.77 ng/µL in the fourth week. Even in men, that generally have a good absorption, there is variance in response to creatine supplementation (Cooper *et al.*, 2012). Interestingly, the concentration of serum creatine returned to baseline in the eighth week of supplementation. The longest supplementation applied to date was 90 days by Ferraz *et al.* (2006), that reported a crescent improvement in VLa4 in Arabian horses supplemented with creatine, which may indicate a continuing absorption, although there was no measure of serum creatine. A down-regulation of creatine transporter activity (approximately 50%) was observed when cells were chronically exposed to high levels of extracellular creatine (Snow e Murphy, 2001). Three possibilities arise from these findings: (1) horses could have responded to the chronic exposure to creatine between the fourth and eighth week of supplementation, (2) a higher maintenance dose may be required in horses, or (3) a second loading dose in the fifth week could be beneficial in case of those horses may have depleted their muscle reserves of creatine.

In concordance with D'angelis *et al.* (2005), there was no difference in body weight promoted by creatine supplementation. In humans, an increase in body weight is reported,

probably due to the increase lean body mass and to water retention (Bird, 2003). This effect would be difficult to see in horses due to their high body mass, thus a considerable increase in body weight would happen in order to cause significant statistical increase. Also, there was no effect of treatments in blood creatinine, which contradicts and does not follow the increased levels of creatine in CRE. In men, the excretion of creatinine seems to be proportional to creatine intake (Feldman, 1999). Schuback, Essen-Gustavsson e Persson (2000) also did not find difference in creatinine levels in horses receiving 25 g of creatine twice a day during 6.5 days.

Horses were randomly assigned to each group and the genotype evaluation was performed only after the supplementation period. We expected to have a few or no C/C animals due to the physical conformation of jump horses, and this belief was confirmed. We also believed to have an equal or close number of C/T and T/T in the animals studied due to the association of desirable characteristics in a jump horse, such as weightlessness, but power for propelling. However, 63% of the genotyped animals were T/T and the remaining 31% were C/T. In Egyptian Arabian horses, the frequency of T/T genotype was 90%, while the frequency of C/C in quarter horses was 83% (Hill, Gu, *et al.*, 2010). A study with a larger number of jump horses must be performed to determine the prevalence of each genotype and the correlation of genotype to performance in championships.

Creatine kinase presented a treatment effect at the analysis of variance. However, the horses in the creatine group aleatory higher levels of CK in comparison with placebo group, and this fact may bias the statistical analysis, since the baseline are included in ANOVA. In accordance with our observation, the multiple comparison test did not indicate difference within each time between groups.

The supplementation with creatine did not significantly change the plasma levels of myostatin in our study. How creatine interferes in human myostatin is still not clear (Saremi *et al.*, 2010), but creatine *per se* can interfere in the expression of multiple genes. An increase in mRNA concentration of collagen type I, myosin heavy chain I, and GLUT-4, but not myostatin in muscles samples in untrained men after 5 days of creatine supplementation was observed (Deldicque, Atherton, *et al.*, 2008), in agreement with our study. In an attempt to remove variances due to different food intake or levels of training, a new study with animals at rest may elucidate a possible effect of creatine on MSTN gene expression and correlation with circulating levels of this hormone.

In conclusion, oral supplementation with creatine during eight weeks with an initial one-week 0.3 g/kg/day dose and a seven-week 0.05 g/kg/day dose increases serum creatine in the

first four weeks of supplementation, but does not changed creatinine, CK and myostatin concentrations in jump horses.

3.8 Acknowledgements

3.9 Conflicts of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

CHAPTER II

Conjugated linoleic acid and lactate influx through monocarboxylate transporters 1 in erythrocytes

4 LITERATURE REVIEW

4.1 Monocarboxylate transporters

The monocarboxylate transporters (MCTs) are a 14-member family of transmembrane proteins also known as SLC16 solute carriers. Each isoform has different tissue-specific pharmacological and kinetics properties (Halestrap e Price, 1999; Halestrap, 2012). The MCTs catalyze the transport in and out the plasma membrane of L-lactate, D-lactate, pyruvate, short-chain fatty acids, ketone bodies, and other molecules (Poole e Halestrap, 1993). Four of these transporters (MCT1-4) were confirmed as proton-linked monocarboxylate cotransporters. MCT8 transports thyroid hormones, while MCT10, originally called TAT1 (T-type amino acid transporter 1), transports aromatic amino acids. The remaining eight MCTs have not been fully elucidated yet. All family members are predicted to have 12 transmembrane domains with the N- and C-termini located intracellularly (Halestrap, 2012).

The MCTs are present in different cells and tissues, such as pancreas, kidney, liver, erythrocytes, leukocytes, intestines, skeletal and cardiac muscle, smooth muscle, corneal and retinal epithelia, thymocytes, brain, placenta, ovaries, testes, spermatozoa, basolateral and parietal cells of the stomach, blood-brain barrier, mitochondrial membrane, and tumor cells. Therefore, the basic functions of the MCTs allow a broad biological activity, contributing with acid-base balance and homeostasis, as well as intercellular communication and energy metabolism (Poole e Halestrap, 1993; Halestrap e Meredith, 2004).

Monocarboxylate transporters 1 to 4 are the most studied isoforms. They require ancillary glycosylated proteins to carry out their function and to undergo translocation from cytoplasm to the membrane, following a ratio of one MCT to each two ancillary proteins. MCT1, MCT3, and MCT4 are associated with cluster of differentiation 147 (CD147), also called basigin, neurotelin or EMMPRIN (extracellular matrix metalloproteinase inducer), while MCT2 is associated to embigin (Halestrap, 2012). The CD147 is a glycoprotein with a transmembrane domain, an intracellular portion containing the C-terminus domain, and an extracellular portion containing both the N-terminus and two immunoglobulin-like domains (Halestrap e Meredith, 2004; Mykkänen *et al.*, 2011). Embigin, also called Gp 70, is highly correlated with CD147, sharing 28% overall amino acid sequence identity and 50% identity in the transmembrane domains, being a member of the same subset of the immunoglobulin superfamily as CD147 (Tachikui *et al.*, 1999).

MCT1 and MCT4 isoforms are present in skeletal muscles of horses and most of the studied species. In humans and horses, MCT1 is predominantly present in oxidative fibers (red fibers), whereas MCT4 is present in glycolytic fibers (white fibers), showing an uptake and an extrusion activity of lactate in myocytes, respectively (Halestrap e Price, 1999; Mykkänen, Hyyppä, *et al.*, 2010). MCT1 and MCT2 isoforms are present in equine erythrocytes (Koho, Väihkönen e Pösö, 2002). MCT2 is a transporter with different levels of expression in different tissues and in different species, which may arise the possibility of post-transcriptional regulation and alternative splicing (Jackson *et al.*, 1997).

There is evidence of adaptive responses in MCT concentration to physiological and pathological stimuli in cells (Thomas *et al.*, 2012). Regulation of the amount of MCTs in the body can occur via transcriptional, translational, or post-transcriptional mechanisms, which may vary according to the individual's age and type of tissue (König *et al.*, 2010), although very little is known about specific promoters or inhibitors. In infants, for example, MCT1 and MCT2 have been identified in different cells of the nervous tissue (Halestrap, 2012). Leino and colleagues (1999) observed 25 times more gold particles of gold-labeled MCT1 per μm of cortex cell membranes in lactating rats compared to adults in a quantitative electron microscopic immunogold study. Similar results were found by another study that showed lower levels of MCT1 mRNA in adult mice compared to newborns, demonstrating the existence of a transcriptional regulation of MCTs, besides an adaptive response of the central nervous tissue under development to maintain adequate energy intake (Pellerin *et al.*, 1998). An example of post-transcriptional regulation of MCTs were observed by Bickham *et al.* (2006). They observed an increase in MCT1 concentration in muscle biopsies of endurance-trained runners after short-term sprint training but no increase in mRNA levels.

In addition to extern stimulus, such as exercise or growth, the expression of CD147 seems to be associated with MCT expression. Without the chaperone protein CD147, MCT1 and MCT4 are retained in a perinuclear compartment. The close association between MCTs and its ancillary protein appears to be responsible for the translocation to the plasma membrane and/or its correct localization of both proteins, as already demonstrated in cultured cells (Kirk *et al.*, 2000). Interestingly, the knocking down of both MCT4 in a model of breast cancer cells and MCT1 in intestinal epithelial cells resulted in intracellular accumulation of CD147 as well, demonstrating that not only MCT depend on CD147, but *vice versa*. (Bai *et al.*, 2014).

4.1.1 MCTs in muscle tissue

Since 1996, the content of MCT in muscle cells has been studied in different species. MCT1 and MCT4 may increase or decrease in muscles varying differently according to the age, intensity, duration and volume of exercise (Kitaoka *et al.*, 2011; Thomas *et al.*, 2012). Since there is a limited number of studies with large sample variability, such as species, sex, different types of exercise, and different levels of training, some results are difficult to reconcile. Low or high intensity exercise for long periods, strenuous exercise for short periods, and acute and chronic electrical stimulation have been evaluated *in vitro* and *in vivo*. In general, these studies show an increase of both isoforms independently of the previous level of training, but disagreements occur in relation to the duration and intensity of exercise, although the expression of MCT1 appears to be more sensitive to these variations compared to MCT4 (Thomas *et al.*, 2012).

Baker, Mccullagh e Bonen (1998) showed that both levels of training–moderate *versus* high-intensity–produce increased levels of MCT1 in the heart of rats. High-intensity exercise is also able to increase the concentrations of MCT1 in oxidative skeletal muscles, but not in glycolytic muscles. Under these conditions, the *in vitro* uptake of lactate in the same muscles is also increased. In horses, Kitaoka, Wakasugi*, et al.* (2010) evaluated the effect of high-intensity training in Thoroughbred horses on concentrations of MCT1 and MCT4 in the gluteus medius muscle. Five animals underwent treadmill exercise until exhaustion before training and it was repeated after seven weeks of training. After training, the animals showed increased concentrations of both isoforms, although not significantly, and increased maximal oxygen uptake ($\dot{V}O_{2max}$) and time spent on the treadmill. However, there was a positive correlation (r = 0.88) between MCT1 protein expression and the duration of the test after training, as well as positive correlation (r = 0.85) between the percentage of change in pre and post training MCT4 with the increment of time in the treadmill. There was no correlation between $\dot{V}O_{2max}$ and duration of exercise. One of the conclusions of the authors is that the increase in the capacity to sustain exercise for longer periods due to training is more likely to be a greater capacity to transport lactate than by increasing the capacity of oxygen consumption by the cells. Revold *et al.* (2010) observed the same increase in MCT1 and also its ancillary protein CD147 in muscle fibers of cold-blooded horses undergoing high-intensity training.

The same group (Kitaoka, Masuda*, et al.*, 2010) evaluated the effects of detraining in MCT1 and MCT4 concentrations in Thoroughbreds. They submitted twelve horses to 18 weeks of high-intensity training and compared the MCT levels in the skeletal muscle after six

weeks of moderate-intensity training or completely training cessation. Each horse was subjected to an incremental all-out treadmill test until exhaustion before training, after the 18-week training, and after the 6-week detraining period. MCT1 and MCT4 significantly increased after high-intensity training in comparison with pretraining values. MCT1 returned to pretraining values in the total rest group but not in the moderate-intensity training group, while MCT4 returned to pretraining values in both groups. Interestingly, although horses had significantly decreased the plasma lactate concentration after the high-intensity training, moderate-intensity training maintained the lactate at low levels while the cessation did not, but horses were not able to keep the same peak speed and the duration of the test in the treadmill in both groups after detraining, returning to pretraining values. The authors suggested that MCT4 might be more important for maximal exercise performance and training adaptation than MCT1, probably because of its lactate efflux activity. It is also noticeable that the MCT1 were more easily inducible, while the MCT4 required longer and more constant stimulus to increase its number, although a single high-intensity training also increases both levels of MCT1 and MCT4 in gluteus medius muscle six hours after exercise in Thoroughbred horses (Kitaoka *et al.*, 2013).

Chronic stimulation of skeletal muscle, e.g. endurance training, also seems to be able to stimulate the synthesis or expression of MCT. Mccullagh *et al.* (1997) used electrical stimulation of the fibular nerve for 24 hours during seven days in mice to mimic chronic muscle activity without extracellular variables related to the exercise. The results indicated increased MCT1 concentration in glycolytic fibers, when usually these fibers express small amounts of this transporter. Although the chronic stimulus used in this study can be considered exaggerated (since in normal conditions an animal will not have a 24/7 muscle activity neither in training nor in competition), it demonstrated a possible pattern of MCT1 response to exercise. One study with endurance horses in regular training would be necessary to confirm this effect on glycolytic fibers. MCT4 concentration was not assessed in both studies cited above.

A pathological metabolic state, such as diabetes mellitus, also seems to be able to alter the concentrations of MCTs. Juel, Holten e Dela (2004) compared the concentrations of MCT1 and MCT4 in healthy and type 2 diabetic humans who underwent four-week unilateral strength training. MCT4 levels were similar in both groups before training, but MCT1 were higher in healthy men. However, after strength training, type 2 diabetic subjects have a lower increase in MCT1 compared with normal subjects (+48% and +5% respectively) and statistically irrelevant increase in MCT4 (+6% against +32% in healthy subjects). In addition

to what Juel, Holten e Dela (2004) reported in men, streptozotocin-induced diabetic rats–an experimental model of type 1 diabetes–exhibit reduced expression of MCT1 and MCT4 in heart and skeletal muscle (Enoki *et al.*, 2003), and reduced MCT1 in adipose tissue (Hajduch *et al.*, 2000). Py *et al.* (2002) have not observed this difference in muscle and heart tissue, possibly due to shorter experimental diabetes–15 days against 21 days of Juel, Holten e Dela (2004). The same researchers believe that the changing in MCT concentration is not due to insulin deficiency. To date, there is no evidence of hormonal influence on the expression of MCT1 and MCT4 in muscles (Halestrap e Meredith, 2004).

4.1.2 Lactate transport into erythrocytes

At physiological pH, due to its pK of 3.86, lactic acid produced in the body is 99% dissociated in H^+ and lactate (La^-). The lactate formation occurs primarily in skeletal muscle, myocardium and erythrocytes through anaerobic glycolysis (Gladden, 2004). Lactate main functions are to recycle the nicotinamide adenine dinucleotide (NAD^+) consumed in the process of transformation of glucose into pyruvate, and to provide energy through the Cori cycle (Philp, Macdonald e Watt, 2005). The preference for the lactate route (anaerobic glycolysis) generates less energy to muscle cells; however it is a shortcut to the rapid formation of ATP and maintenance of the contractile activity. In cells with low or no oxidative capacity, such as leukocytes and erythrocytes, respectively, anaerobic glycolysis is the only possible route for the generation of ATP. When the glycolytic activity is very intense (as in muscles during intense exercise), there is no time to the pyruvate formed by the breakdown of glucose to enter the entire sequence of reactions of the Krebs cycle and oxidative phosphorylation in order to provide energy. Consequently, rapidly concentrations of ATP and NAD^+ decreases within the cell, while the concentrations of ADP and NADH increases. The lack of NAD^+ tends to inhibit the activity of pyruvate dehydrogenase–enzyme that converts pyruvate to acetyl CoA–and it forces the reaction toward the formation of lactate by lactate dehydrogenase (LDH). Since intracellular NAD^+ concentrations are limited, glycolysis would stop without the recycling of NAD^+ promoted by the lactate production (Champe e Harvey, 1997).

Primarily, the red blood cell (RBC) exists mainly to transport respiratory gases (Ness e Stengle, 1974), but today other important functions have been elucidated. Red blood cells are not only lactate producers but they also exert a central role removing lactate from plasma, especially when the concentration of plasma lactate is high, e.g. during intense exercise. Up to 50% of blood lactate is actually inside the RBCs. It appears to help the lactate outflow from

muscles, carrying this metabolite for tissues that can further metabolize it, such as liver, heart and other muscles, allowing the continuing muscle activity (Pösö, Lampinen e Räsänen, 1995; Pösö, 2002). During exercise, catecholamines cause splenic contraction elevating the amount of circulating erythrocytes. In horses, the hematocrit can reach up to 65% (Kingston, 2008). This fact consequently increases the removal capacity of lactate from muscles to blood due to the temporary increase in erythrocytes (Mykkänen, Pösö, *et al.*, 2010).

Lactate can be transported in and out the RBC through three distinct mechanisms: nonionic diffusion, exchange with inorganic anions (HCO_3^- and Cl^-), and MCTs. The exchange of lactate by bicarbonate and chloride ions is realized by a carrier-type antiporter called band 3 protein (Poole e Halestrap, 1993). This is the main route for lactate transport in sedentary species, such as goats and cattle, in which there is a negligible expression of MCT or its ancillary CD147 in erythrocyte membranes (Skelton *et al.*, 1995). In horses, dogs, rabbits, rats and humans, the transport through MCTs accounts for up to 90% of lactate absorption into erythrocytes (Skelton *et al.*, 1995), although Väihkönen, Hyyppä e Pösö (1999) observed in their study with 20 times more horses that the transport through band 3 protein represented 20 to 40% of the total lactate influx into RBC.

The process of ion transport by MTCs follows an ordered sequence. First, the proton is bounded, immediately followed by the lactate anion. Subsequently, both are driven through the cell membrane and released from the carrier. This is a reversible process, whose equilibrium will occur once the following condition occurs intra and extracellularly: internal [lactate]/external [lactate] = external [H^+]/internal [H^+] (Halestrap e Price, 1999).

In horses, it was observed a large interindividual variation in the RBCs lactate concentration after submaximal and maximal exercise in Standardbred, Finnhorse trotters, and Thoroughbreds (Pösö, Lampinen e Räsänen, 1995; Mykkänen, Pösö, *et al.*, 2010). This difference was also observed *in vitro* by Väihkönen e Pösö (1998) which studied 89 Standardbred horses from two weeks to nine years old. They clearly observed a bimodal distribution in the ability of these animals to use MCT to absorb lactate into RBCs, forming two distinct classes of horses: horses with high transport activity of lactate (HTA), and horses with low transport activity of lactate (LTA). HTA horses have a rate of lactate influx of >4 nmol of lactate per mg protein/min and >1 nmol of lactate per mg protein/min at an *in vitro* lactate concentration of 30 and 10 mM respectively (Väihkönen e Pösö, 1998; Väihkönen, Ojala e Pösö, 2002). About 70% of the animals studied were considered HTA, with the MCTs being the main route of absorption, while in LTA horses, band 3 and nonionic diffusion seem to play the most important role (Väihkönen e Pösö, 1998; Väihkönen *et al.*, 2001). Also,

animals that were born with LTA do not change for a HTA or *vice versa* (Väihkönen, Ojala e Pösö, 2002). So far, horses are the only species with this bimodal distribution (Skelton *et al.*, 1995; Väihkönen *et al.*, 2001).

The influence of MCT activity in RBCs on performance was previously investigated, although a definitive conclusion cannot be taken. Räsänen, Lampinen e Pösö (1995) demonstrated that Standardbred horses with high *in vivo* RBC lactate influx have improved athletic performance than those whose absorption capacity was low. They found a positive correlation of both blood and cell lactate concentration but not plasma lactate with performance indexes. However, these results were not reproduced in other study with 55 Standardbred trotters, probably due to the fact that there was little variation among the animals used–all with performance indexes higher than average. In the other hand, it was evidenced that the *in vitro* difference between HTA and LTA is correlated with the *in vivo* values (Väihkönen, Hyyppä e Pösö, 1999), notwithstanding that the *in vivo* measures of lactate do not allow a clearly separation of horses into the two groups, highlighting the importance of the *in vitro* measurement. Mykkänen, Pösö*, et al.* (2010) did not find correlation between MCT1, MCT2 and CD147 concentrations in RBCs with performance indexes (racing post rating, official rating, top speed and carrier prize money) in 77 Thoroughbred horses studied as well.

Different than muscles, training does not improve the RBC transport activity in horses, as showed when comparing 19 sedentary Standardbred with eight endurance and 13 Standardbred trotter horses (Väihkönen *et al.*, 2001). Corroborating with this findings, sucking foals present similar lactate influx activity than adult racing horses (Väihkönen e Pösö, 1998).

Correlations between RBC levels of MCT1, MCT2, and CD147 were investigated previously. The same concentrations of MCT1 and MCT2 are found in both HTA and LTA horses, although the amount of CD147 in HTA horses are almost 10-fold higher than in LTA (Koho, Väihkönen e Pösö, 2002; Koho, Hyyppä e Pösö, 2006), and can also be used to separate horses between HTA and LTA (Mykkänen, Pösö*, et al.*, 2010). The concentration of CD147 in RBCs was significantly correlated with the amount of this chaperone protein in muscle, however with a correlation coefficient of 0.62 (Koho, Hyyppä e Pösö, 2006).

There is a lack of studies with a larger number of horses with a standardized level of training and age. This would be necessary for a final conclusion of the real influence of concentration and activity of MCT1, MCT2, and CD147 in the influx of lactate in erythrocytes to determine whether it would be a good performance marker in horses. Recent

investigations (Reeben *et al.*, 2006; Mykkänen *et al.*, 2011; Koho *et al.*, 2012) about possible mutations in those traits do not lead to any conclusion about genetic causes of the bimodal distribution in horses, though a hereditability can be observed (Väihkönen, Ojala e Pösö, 2002).

4.2 Peroxisome proliferator activated-receptor

Recently, CD147 and MCT1 have shown a regulatory element in common: PPAR-α, peroxisome proliferator-activated receptor type alpha. The PPRAs are nuclear receptors, members of the steroid hormone nuclear receptor family, whose function is to serve as transcription factor. PPRAs contain one ligand-binding domain and one DNA-binding domain that will interact in or around promoter regions (PPRE) of target genes. It forms a heterodimer complex with another nuclear receptor, the retinoid X receptor (RXR), to support the binding and release of other accessory molecules that can be even co-repressor or co-activator factors. The interaction of PPRA/RXR/accessory molecules and phosphorylation determine the activity of this transcriptional complex (Ziouzenkova *et al.*, 2002).

Currently, there are three main types of PPRAs known, all of them somewhat involved in energy metabolism: α, γ, and δ. PPRA-α plays a central role in the fatty acid oxidation, and HDL production, as well as the regulation of inflammation, through the inhibition of both proinflammatory cytokines and expression of endothelial adhesion molecules. PPRA-γ is directly involved in the adipogenesis and adipocyte differentiation, although it is also present in skeletal muscle, liver, heart, intestine, vascular system and immune system, and seems to be involved in glucose homeostasis and also exerts anti-inflammatory effects. Probably, PPRA-γ is involved in other important mechanisms and cell signaling, because PPRA-γ-null mice do not survive. PPRA-δ is the most widely distributed in the tissues, but also the receptor with its role less known. It appears to be involved in membrane lipid synthesis and turn over, oxidation of fatty acids in muscle, and fatty acids catabolism (Ahmed *et al.*, 2007).

PPRA-α is known to be expressed in liver, kidney, intestine, pancreas, heart, skeletal muscle, vasculature, immune cells and in other tissues in humans (Ahmed *et al.*, 2007). PPRA-α can modulate the expression of target genes in response to fasting, when an increase uptake and oxidation of fatty acids are required by the liver (Kersten *et al.*, 1999). Until now, approximately 100 genes have shown to be affected by PPRA-α activation (Ahmed *et al.*, 2007). Today, it also has been investigated the potential benefit of PPRA-α agonists in the treatment/prevention of atherosclerosis and other heart diseases (Lefebvre *et al.*, 2006). PPRA-α has as main ligands some dietary polyunsaturated fatty acids – as linoleic acid, and

arachidonic acid – and eicosanoids – as 8(S)-hydroxyeicosatetraenoic acid (HETE) - but also fibrates, such as ciprofibrate and clofibrate (Forman, Chen e Evans, 1997), WY 14,643 (pirinixic acid) and GW 7647 (Lefebvre *et al.*, 2006).

König and colleagues (2008) studied the effects of PPRA-α agonists *in vitro* and *in vivo*. Since MCT1 facilitates the transport of ketone bodies, and PPRA-α is believed to work as a nutritional status sensor in mammals, mediating the adaptive response to fasting, an interaction between these two proteins may occur. They conducted four experiments. In the first, the effects of fasting for 24 hours were evaluated in rats. In the second, clofibrate, an agonist of PPRA-α, was fed to rats during four days. In the third, PPRA-α-null mice received WY 14,643, another PPRA-α agonist, per gavage during four days. In the fourth, fao rat hepatoma cells were incubated with WY 14,643 or troglitazone, an agonist of PPRA-γ. Rats treated with clofibrate had increased mRNA concentration of MCT1 and mitochondrial 3-hydroxy-3-methylglutaryl-CoA (mHMG-CoA) synthase, one target gene of PPRA-α. Fasting also significantly increased MCT1 and mHMG-CoA synthase mRNA, but in a less intense manner. The same effect was observed in the cultured cells in the presence of PPRA-α agonists, but not in the presence of troglitazone. PPRA-α-null mice failed to respond to WY 14,643, while control mice presented the same pattern of response than rats and hepatocyte cells: increased mRNA of MCT1 and mHMG-CoA synthase, showing that, in conclusion, the MCT1 does respond to PPRA-α agonists.

Latter, the same group (König *et al.*, 2010) studied whether MCT1 and CD147 are up regulated by different natural PPRA-α agonists in liver tissue of rats and pigs. Rats were fed for 6 days with sunflower oil (control), oxidized sunflower oil, conjugated linoleic acid (CLA) or 250 mg/kg of clofibrate. Pigs were fed for 28 days with fresh fat (control, 93:7 sunflower oil: palm oil), oxidized sunflower oil or 5 g/kg of clofibrate. All treatments resulted in increased expression of both MCT1 and CD147 compared with control in rats. In pigs, only the clofibrate increased the expression of CD147, but all treatments increased MCT1 mRNA concentration in liver. The conclusion of the authors are that natural PPRA-α agonists up-regulated the expression of MCT1 and its chaperone CD147.

The mechanism which CLA and other PPRA-α agonist interfere in MCT1 synthesis is still unknown. However, König *et al.* (2010) also made an *in silico* analysis of possible PPREs within mouse MCT1 promoter and intronic regions. They found two putative regions in the first intron with high homology to the consensus PPRE (AGGTCAAAGGTCA), yet they were not functional and probably PPRA-α do not up-regulate MCT1 expression through direct binding, but possible for other post-transcriptional mechanisms.

4.3 Conjugated linoleic acid

Conjugated linoleic acid (CLA) is the term used to refer to a class of 28 eighteen-carbon polyunsaturated positional and geometric isomers of linoleic acid (*cis*-9, 12 18:2). For each positional isomer, there are four geometric isomers–*cis-trans*, *trans-cis*, *cis-cis*, and *trans-trans* (Pariza, Park e Cook, 2001; Gholami e Khosravi-Darani, 2014). The double bounds are located between positions 8 and 11 (first bound) and between 10 and 13 (second bond) (Churruca, Fernandez-Quintela e Puy Portillo, 2009). The most important and biologically active isomers are *cis*-9, *trans*-11 (which is also the most abundant in nature) and *trans*-10, *cis*-12 (Pariza, Park e Cook, 2001; Andrade *et al.*, 2012) (**Figure 4.1**).
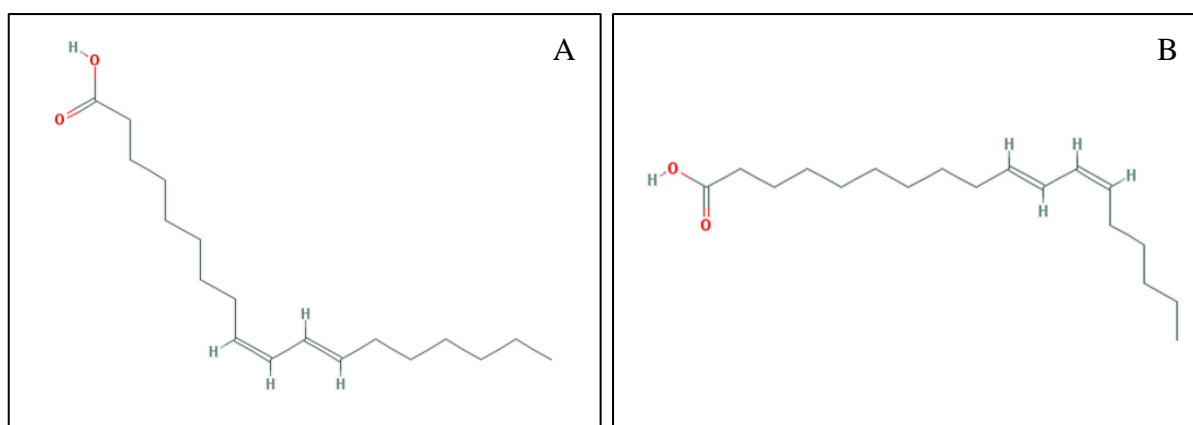
Figure 4.1: (A) CLA *cis*-9, *trans*-11, an omega 7 oil, and (B) CLA *trans*-10, *cis*-12, an omega 6 oil. Source: https://pubchem.ncbi.nlm.nih.gov/.

Conjugated linoleic acid has recognized positive physiological effects in human health, including anticarcinogenic, antioxidant, antiatherosclerogenic, antidiabetic, and antiosteoporotic activity, reduction of fat tissue leading to increased lean body mass, and immunomodulation (Dilzer e Park, 2012; Koba e Yanagita, 2014). These fatty acids can be obtained from dietary sources or synthetic supplementation. The main sources of dietary CLA are from dairy products, beef, pork, and chicken meat, egg yolk, seafood and some vegetable oils (Koba e Yanagita, 2014), but the amounts are not enough to produce the desirable biological effects cited above (Andrade *et al.*, 2012).

The biosynthesis of CLA occurs through the activity of bacteria present in the rumen–mainly *Butyrivibrio fibrosolvens*, but also *Propionibacterium freudenreiche*, *Bifidobacterium spp.*, *Lactobacillus spp.* and others–which isomerizes the vegetable sources of linoleic acid producing *cis*-9, *trans*-11, also called rumenic acid. The *cis*-9, *trans*-11 is absorbed and is integrated into other tissues. Part of the rumenic acid can be biohydrogenated in the rumen

into vaccenic acid (*trans*-11 18:1), which is also absorbed and can be further retransformed to rumenic acid in adipose tissue and mammary glands by the enzyme Δ^9-desaturase (Churruca, Fernandez-Quintela e Puy Portillo, 2009; Andrade *et al.*, 2012; Wang e Lee, 2015).

The industrial production of CLA is acquired by alkaline isomerization at high temperature of linoleic acid from vegetable oils (like safflower and soybean oil), which approximately equal amounts of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 are obtained (Salamon *et al.*, 2012; Koba e Yanagita, 2014).

Researchers have isolated CLA from fetal calf, newborn calf, cow and horse sera. In horses, an unexpected high amount of *trans*-10, *cis*-12 was found. However, the equine fermentation chambers are the cecum and colon, where the absorption of long-chain fatty acids is minimal, raising the possibility of other tissues and other enzymes being present in horses to produce CLA (Park e Pariza, 1998; Pariza, Park e Cook, 2001). Some studies have evaluated the content of CLA in horse milk (Jahreis *et al.*, 1999; Doreau e Martuzzi, 2006), adipose tissue and meat (He, Ishikawa e Hidari, 2005; Mamani-Linares e Gallo, 2013), which is extremely low in comparison with cow milk, fat and meat. These evidences raise the fact that in horses the main biosynthesis of CLA occurs probably in the tissues and not by gut flora.

The only study evaluating the effects of CLA supplementation in horses was done by Headley *et al.* (2012). They supplemented twelve horses with corn oil or CLA at a rate of 0.0055% of body weight during a 6-week period in a crossover study in order to investigate whether CLA change body fat distribution, body composition and plasma fatty acids profile. CLA supplementation did not have an effect on neither body weight nor body composition scores, but did promote increased plasma levels of *cis*-9, *trans*-11, *trans*-10, *cis*-12, and *trans*-9, *tras*-11 in comparison with corn oil. Moya-Camarena, Vanden Heuvel e Belury (1999) showed that CLA also failed to promote changes in body composition of rats, but increased the liver concentration of the CLA isomers. The use of CLA to specifically interfere in MCT-1 activity in horses has not been tested to date.

The mechanisms of action of CLA are still obscure. Many different genes presented up and down regulation effects with the use of CLA, as well as variation of protein synthesis in *in vitro*, animal and human models was already observed (Mccrorie *et al.*, 2011). The effect in body composition appears to be related to the action of CLA in both adipose tissue (site of fat storage), through the inhibition of stearoyl-CoA desaturase and lipoprotein lipase activity, and in muscle tissue (site of fat consumption), through the enhancing of carnitine palmitoyltransferase activity (Pariza, Park e Cook, 2001). CLA can also displace arachidonic

acid from the membrane phospholipids, and inhibit or antagonize cycloxygenase-1 and 2, decreasing the availability of those substrates to the production of eicosanoids, leading to anti-inflammatory and immunomodulatory effects (Belury, 2002). Other effects such as anticarcinogenic and antiatherosclerotic are still unrolved.

5 SCIENTIFIC ARTICLE NUMBER 3

5.1 Title

Oral supplementation with conjugated linoleic acid and the effects in lactate influx through monocarboxylate transporters in equine erythrocytes

5.2 Authors and filiations

Priscila Beatriz da Silva Serpa[a], Petra Garbade[a], Andre Chen Shih[b], Cláudio Corrêa Natalini[a,c], Victoria Ashley Obert[b], Ruben Lundgren Cavalcanti[a], Ananda da Rocha Pires[d], Tainor de Mesquita Tisotti[d]

[a] Programa de Pós-Graduação em Medicina Animal: Equinos, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000, Brazil; [b] Department of Large Animal Clinical Science, College of Veterinary Medicine, University of Florida, Gainesville, FL, 32608, USA; [c] School of Animal and Veterinary, The University of Adelaide, Roseworthy, SA, 5371, Australia; [d] Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, 91540-000, Brazil.

5.3 Abstract

Red blood cells (RBCs) are important characters in the clearance of lactate during exercise. Monocarboxylate transporters 1 (MCT1), associated with its ancillary protein cluster of differentiation 147 (CD147), are important membrane transporters present in erythrocytes responsible for catalyze the in and out transport of lactate. Recently, CD147 and MCT1 have been shown a regulatory element in common: PPAR-α (peroxisome proliferator-activated receptor type alpha). Since MCT1 and PPRA-α are believed to interact with each other, we aimed to investigate if oral supplementation with conjugated linoleic acid (CLA), a known PPRA-α agonist, would increase the expression of MCT1, CD147, and PPRA-α and, in turn, increase the *in vitro* influx capacity of lactate into equine RBCs, as an aid to improve performance in horses. Twelve clinically healthy female ponies received conjugated linoleic acid (n=6, 0.01% BW, CL) or corn oil (n=6, isocaloric, CO). Body weight, *in vitro* RBC lactate influx and mRNA expression of MCT1, CD147 and PPRA-α in bone marrow by qPCR were evaluated in days 0 (D0), 30 (D30), and 60 (D60) of oral supplementation. There was no statistical difference between CL and CO in body weight, and lactate influx into RBCs with or without inhibitors (CHC and DIDS). The percentage of inhibition by CHC and DIDS was higher in HTA animals, while in LTA animals both inhibitors presented no *in vitro* inhibition.

Although not significant, there was an increase in PPRA-α expression in CL group, which also maintained constant levels of MCT1 and CD147 expression in bone marrow samples, which was followed by an increase in total lactate influx, MCT and band 3 influxes in those animals. In the other hand, CO animals presented decreased expression of MCT1, CD147, PPRA-α, decreased total lactate influx, MCT and band 3 influx along the period of supplementation, but not significant. Taken together, those results indicate that the oral supplementation with CLA for 60 days in a dose of 0.0056% BW did not promoted significant difference in lactate influx. Prolonged supplementation with a higher dose must be attempted before exclusion of a possible effect of CLA in horses.

5.4 Introduction

Red blood cells (RBCs) are not only lactate producers but they also exert a central role removing lactate from the plasma, especially when the concentration of plasma lactate is high, e.g. during intense exercise. Up to 50% of blood lactate is actually inside the RBCs. RBCs help the lactate outflow from muscles, carrying this metabolite to tissues that can further metabolizes it, such as liver, heart and other muscles, allowing the continuing muscle activity (Pösö, Lampinen e Räsänen, 1995; Pösö, 2002). Lactate can be transported in and out the RBCs through three distinct mechanisms: nonionic diffusion, exchange with inorganic anions (HCO_3^- and Cl^-, through the band 3 protein), and monocarboxylate transporters (MCTs) (Poole e Halestrap, 1993).

The monocarboxylate transporters (MCTs) are a 14-member family of transmembrane proteins present in different cells and tissues, whose function is to catalyze the transport in and out the plasma membrane of L-lactate, D-lactate, pyruvate, short-chain fatty acids, ketone bodies, and other molecules. Therefore, the basic functions of the MCTs allow a broad biological activity, contributing with acid-base balance and homeostasis, as well as intercellular communication and energy metabolism (Poole e Halestrap, 1993; Halestrap e Price, 1999; Halestrap e Meredith, 2004; Halestrap, 2012). MCT1 and MCT2 isoforms are the ones present in equine erythrocytes (Koho, Väihkönen e Pösö, 2002) and require ancillary glycosylated proteins to carry out their function and to undergo translocation from the cytoplasm to the membrane. MCT1 is associated with cluster of differentiation 147 (CD147), while MCT2 is associated to embigin (Halestrap, 2012).

In horses, it was observed a large inter individual variation in RBC lactate concentration after submaximal and maximal exercise in Standardbred, Finnhorse trotters, and Thoroughbreds (Pösö, Lampinen e Räsänen, 1995; Mykkänen, Pösö, *et al.*, 2010), with a clear

bimodal distribution in the ability of these animals to use MCT to absorb lactate into RBCs, forming two distinct classes of horses: horses with high transport activity of lactate (HTA), and horses with low transport activity of lactate (LTA). HTA horses have a rate of lactate influx of > 4 nmol of lactate per mg protein/min and >1 nmol of lactate per mg protein/min at an *in vitro* lactate concentration of 30 and 10 mM respectively. About 70% of the animals studied were considered HTA, with the MCTs being the main route of absorption, while in LTA horses, band 3 and nonionic diffusion seem to play the most important role (Väihkönen e Pösö, 1998; Väihkönen *et al.*, 2001; Väihkönen, Ojala e Pösö, 2002). Recent investigations (Reeben *et al.*, 2006; Mykkänen *et al.*, 2011; Koho *et al.*, 2012) about possible mutations in those traits do not lead to any conclusion about genetic causes of the bimodal distribution in horses, though a hereditability can be observed (Väihkönen, Ojala e Pösö, 2002).

The influence of MCT activity in RBCs in equine performance was previously investigated, although a definitive conclusion cannot be taken (Räsänen, Lampinen e Pösö, 1995; Väihkönen, Hyyppä e Pösö, 1999; Mykkänen, Pösö*, et al.*, 2010). Different than in muscle, training do not improve the RBC transport activity in horses (Väihkönen *et al.*, 2001). In addition, the same concentrations of MCT1 and MCT2 are found in both HTA and LTA horses, although the amount of CD147 in HTA horses are almost 10-fold higher than in LTA (Koho, Väihkönen e Pösö, 2002; Koho, Hyyppä e Pösö, 2006), and can also be used to separate horses between HTA and LTA (Mykkänen, Pösö*, et al.*, 2010).

Recently, CD147 and MCT1 have shown a regulatory element in common: PPAR-α, peroxisome proliferator-activated receptor type alpha. PPRA-α is one of three nuclear receptors, member of the steroid hormone nuclear receptor family, expressed in liver, kidney, intestine, pancreas, heart, skeletal muscle, vasculature, immune cells and in other tissues in humans, that works as transcription factor (Ziouzenkova *et al.*, 2002; Ahmed *et al.*, 2007). PPRA-α has as main ligands some dietary polyunsaturated fatty acids–such as conjugated linoleic acid–and eicosanoids, but also fibrates, such as ciprofibrate and clofibrate (Forman, Chen e Evans, 1997). Since MCT1 facilitates the transport of ketone bodies, and PPRA-α is believed to work as a nutritional status sensor in mammals, mediating the adaptive response to fasting, it is believed in an interaction between these two proteins. Indeed, MCT1 and CD147 are up regulated by different natural PPRA-α agonists in liver tissue of rats and pigs, and showed that natural PPRA-α agonists up-regulated the expression of MCT1 and its chaperone CD147 in both species (König *et al.*, 2008; König *et al.*, 2010).

Conjugated linoleic acid (CLA) is the term used to refer to a class of 28 eighteen-carbon polyunsaturated positional and geometric isomers of linoleic acid (Pariza, Park e Cook, 2001;

Gholami e Khosravi-Darani, 2014). The most important and biologically active isomers are *cis*-9, *trans*-11 (which is also the most abundant in nature) and *trans*-10, *cis*-12 (Pariza, Park e Cook, 2001; Andrade *et al.*, 2012). These fatty acids can be obtained from dietary sources, although the amounts are not enough to produce a desirable biological effects, or synthetic supplementation (Andrade *et al.*, 2012). Researchers have isolated CLA from horse sera and an unexpected high amount of *trans*-10, *cis*-12 was found. However, the equine fermentation chambers are the cecum and colon, where the absorption of long-chain fatty acids is minimal, different than in ruminants, whose ruminal flora is responsible for CLA production, raising the possibility of other tissues and other enzymes being present in horses to produce CLA (Park e Pariza, 1998; Pariza, Park e Cook, 2001). CLA oral supplementation in horses at a rate of 0.0055% of body weight during a 6-week period do promote increased plasma levels of *cis*-9, *trans*-11, *trans*-10, *cis*-12, and *trans*-9, *tras*-11 in comparison with corn oil (Headley *et al.*, 2012).

Based in the evidence that there are adaptive responses in MCT concentration to external stimulus (Thomas *et al.*, 2012), and that animals that were born with LTA to not change for a HTA or vice versa (Väihkönen, Ojala e Pösö, 2002), we hypothesized that oral supplementation with CLA would increase the concentration of MCT1 in RBCs through the stimulation of PPRA-α and, in turn, increase the *in vitro* influx capacity of lactate into equine erythrocytes, as an aid to improve performance in horses. To support the findings, the expression of MCT1, CD147 and PPRA- α in bone marrow was investigated in a tentative to address the increase in RBC influx to its precursor cells.

5.5 Material and Methods

5.5.1 Ethics

Study design and methodology were approved by the Institutional Animal Care and Use Committee of University of Florida (Nº 201408290), and adhered to the principles for the human treatment of animals in veterinary clinical investigations.

5.5.2 Animals and sampling

Twelve clinically healthy female mixed breed ponies were equally and randomly distributed into groups control (CO) and treatment (CL). Sample size was calculated considering a difference of 2 nmol x mg^{-1} x min^{-1} in lactate influx between control and treatment (α=0.05, β=0.20). Age in horses studied was 13.9 ± 2.4 years. The horses were

divided into two different pastures at the College of Veterinary Medicine, University of Florida, Gainesville, FL, with free access to water. They were fed twice a day with hay and a concentrated feed (Blue Ribbon 12, Seminole Feed®, Ocala, FL, USA). Once a day, the concentrated feed were topped with corn oil (isocaloric diet) or conjugated linoleic acid oil (Lutalin®, BASF Corporation, Florham Park, NJ, USA) at a dose of 0.01% of body weight according to Headley *et al.* (2012) and offered individually. As the source of CLA provides 28% of each of the two major CLA isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12), total supplementation was 0.0056% BW.

Data were collected in days 0 (D0), 30 (D30), and 60 (D60) of oral supplementation. Body weight was assessed at D0, D30 and D60. Twelve milliliters of venous blood were withdrawn from jugular vein into EDTA tubes at D30 and D60 for lactate influx assay. Four milliliters of bone marrow were collected from sternum after sedation with intravenous detomidine hydrochloride 20 µg/kg (Dormosedan®, Zoetis Animal Health, Florham Park, NJ, USA) and local anesthesia with 5-10 mL of lidocaine hydrochloride 2% (Vedco, St. Joseph, MO, USA) at D0 and D60 into EDTA tubes for RNA extraction. All blood and bone marrow samples were immediately put on ice after withdraw and processed within 24h.

5.5.3 Lactate influx

To measure the lactate influx in erythrocytes, the method of Skelton *et al.* (1995) was modified as follows. One milliliter of blood was used to perform hemoglobin (Hb) and packed cell volume (PCV, pre-PCV). The remaining sample was centrifuged (3,000 rpm, 15 minutes, room temperature) to removal of plasma and buffy coat. Two milliliters of RBCs were transferred to a 50 mL conical tube and mixed with 40 mL of chloride buffer (150 mM NaCl, 10 mM sodium tricine, pH 8.0 at 37°C), and incubated in a water bath for 30 minutes at 37°C. This step was performed to remove intracellular lactate. The cells were then washed three times with 8 mL of the same buffer. After the final wash, RBCs were suspended into a volume of HEPES buffer (90 mM NaCl and 50 mM HEPES, pH 7.4 at 37°C) equivalent to a 30% cell suspension with different solutions to form three stock cells. PCV was performed to ensure dilution (post-PCV). For total influx measurement, the first portion contained no inhibitor. Second portion contained 5mM alpha-cyano-4-hydroxycinnamic acid (CHC, Cat # 145505, Sigma-Aldrich®, St. Louis, MO, USA), which inhibits both the MCT and the band 3 protein (Poole e Halestrap, 1993). The last portion contained 0.2 mM 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS, Cat # D3514, Sigma-Aldrich®, St. Louis, MO, USA), which inhibits the band 3 protein (Poole e Halestrap, 1993). One aliquot was used

to measure lactate and confirm that lactate was depleted. To measure the lactate influx, 25 µL of each stock cell were added to a test tube containing 75 µL of 13.33 mM lactate HEPES buffer, to obtain a final concentration of 10 mM. RBCs were incubated for 20 seconds at 37°C. The incubation was stopped with 5 ml of ice-cold stop solution (150 mM NaCl, 10 mM 2-(N-morpholino)ethane-sulfonic acid, pH 6.5 at 4°C). The cells were collected by centrifugation for 15 minutes at 3,000 rpm and 4°C, and were washed twice with the same volume of ice cold stop buffer. After the final centrifugation, the RBCs were hemolyzed with 1 mL of erythrocyte lysis solution (1.5 M ammonium chloride, 100 mM sodium bicarbonate, 10mM disodium EDTA, pH 7.4 at 37°C). Lactate and Hb were measured by a laboratory analyzer (Stat Profile Critical Care Xpress, Nova Biomedical, Waltham, MA, USA). All measurements were made in duplicate.

The results are expressed as nanomoles of lactate per milligram of protein per minute. The amount of lactate measured after 20 seconds of incubation was multiplied by 3 to obtain the concentration of lactate in one minute. To estimate the amount of protein, the pre-PCV, post-PCV and Hb were used (Skelton *et al.*, 1995). To obtain the *in vitro* transport activity of MCT and band 3 protein, the value of influx with 5 mM CHC and 0.2 mM DIDS was subtracted by the total influx value respectively. Since the CHC do not inhibit the flux through MCTs only, this influx is somewhat overestimated (Skelton *et al.*, 1995). The percentage of both MCT and band 3 obtained with CHC and DIDS respectively was calculated dividing the amount of lactate influx of each assay by the total influx (without inhibitors).

5.5.4 Quantitative real-time PCR (qPCR)

Total RNA was extracted from bone marrow samples and purified using commercial kits with silica columns (NucleoSpin® RNA, Macherey-Nagel Invitrogen®, Düren, Germany). Total RNA were converted into cDNA with GoScript® Reverse Transcription System (Promega, Madison, WI, USA). Quantitative real-time PCR was performed using a Rotor-Gene® Q (Qiagen®, Hilden, Germany).

The qPCR conditions were 50°C for 2 minutes, 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute in a Rotor-Gene® Q thermocycler (Qiagen®, Hilden, Germany). The master mix used was TaqMan® Universal Master Mix II with UNG (Life Technologies Corporation, Foster City, CA, USA). The cDNA input was 2.5 ng. All primers were used at 300nm and all probes were used at 250nm. The PCR reaction volumes was 20µL. Probes and primers were designed with the aid of the primer design tool

of the National Center for Biotechnology Information (NCBI, available at http://www.ncbi.nlm.nih.gov) using the database of the equine species and were synthesized by Integrated DNA Technologies IDT, Inc. (Coralville, IA, USA). Equine beta-2-microglobulin (B2M) was used as housekeeper gene (**Table 5.1**). A comparative quantification analysis was performed based in the amplification efficiency of the normalized results of each target gene in comparison with the housekeeper gene.

Table 5.1: Sequence of forward (Fwd) and reverse (Rev) primer sets and probes used to perform quantitative PCR in the monocarboxylate transporter 1 (MCT1), cluster of differentiation 147 (CD147), peroxisome proliferator-activated receptor type alpha (PPRA-α) and beta-2-microglobulin (B2M, housekeeping gene). Melting temperatures (T_m) of each sequence and amplicon size in base pairs (bp), as well as accession number in the Genbank (EquCab2.0 NCBI–National Center for Biotechnology Information) are provided.

Gene		Sequence (5' to 3')	T_m (°C)	Amplicon	NCBI Accession
MCT1				94	AY457175.1
	Fwd	CTTGTGTATCGGTGTCATTGG	53.6		
	Rev	GGGTCGCCTCTTGTAGAAATA	54.1		
	Probe	/56-FAM/TCCGGCTCT/ZEN/GACCATGATTGGCAA/3IABkFQ/	62.8		
CD147				137	EF564280.1
	Fwd	GCCAGAGCATGGAGTATGA	54.3		
	Rev	CTTCTTGACGGCCTTAATGTG	53.9		
	Probe	/56-FAM/AGTACGTGT/ZEN/GCATCTTCCTCCCGGA/3IABkFQ/	63.4		
PPRA-α				112	NM_001242553.1
	Fwd	GGAACTGGACGACAGTGATATT	54.4		
	Rev	CACGATACCCTCCTGCATTT	54.8		
	Probe	/56-FAM/TCGTCCTGG/ZEN/CCTTCTAAACGTGGGA/3IABkFQ/	63.6		
B2M				101	NM_001082502.3
	Fwd	GTGTACAGCACTCTACTCTCAA	53.7		
	Rev	GTCAAATCCAAATGAGGAATCT	53.1		
	Probe	/5HEX/AGTGGGATC/ZEN/GAGACCTCTAACCAGCA/3IABkFQ/	62.6		

5.5.5 Statistical analysis

Data were submitted to two-way repeated measures analysis of variance to compare the effect of treatment and time on lactate influx, body weight, and comparative quantification. Bonferroni's test was used for multiple comparisons. Pearson's correlation was also calculated. The *P* value was set as 0.05 for all analysis. The software utilized was GraphPad Prism 6. Values are expressed as mean ± standard deviation (SD). The software Rotor Gene Q version 2.3.1 was used to provide the take-off points (TOP). QGene Software (Joehanes e

Nelson, 2008) was used to determine the amplification efficiency of each gene and to normalize the expression against the housekeeper gene using TOP.

5.6 Results

5.6.1 Body weight

In body weight, there was a statistical difference along times studied (P = 0.0033), but it cannot be explained by the treatments (P = 0.69). The values in CO varied from 123.5 ± 18.6 kg to 127 ± 20.5 kg at D30, and to 130.2 ± 21.5 kg at D60, while in CL body weight ranged from 129.5 ± 26.8 kg to 131.5 ± 24.1 kg at D30, and 135.5 ± 22.7 kg at D60 (**Figure 5.1**).
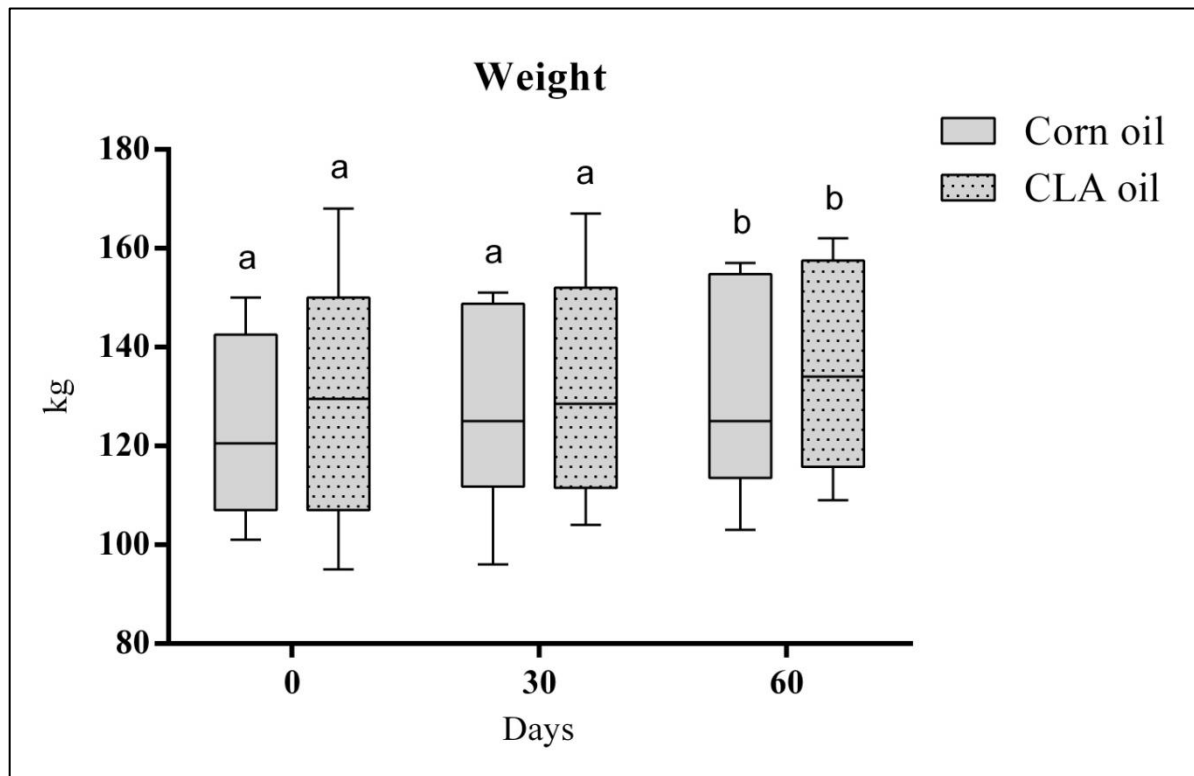
Figure 5.1: Difference in body weight (kg) along the 60 days of study. Values expressed as mean and standard deviation. Different letters indicate statistical significance (P < 0.05) in comparison with day 0 of each group.

5.6.2 Lactate influx

The total lactate influx assay into RBCs is reported in **Figure 5.2**. There was no difference in lactate influx into RBCs between days (P = 0.923) or between CO and CL (P = 0.819), neither any interactions between time and treatment (P = 0.501). In group CO, five

animals were considered HTA at D30, although only two presented values consistently far from the border line of 1 nmol x mg^{-1} x min^{-1} that separates LTA from HTA animals. In group CL, although three animals were considered HTA at D30, only one was consistently far from the borderline, while the other two presented values too close to 1 nmol x mg^{-1} x min^{-1}. It could also be observed that in the second moment of analysis (D60), the number of animals considered HTA in both CO and CL groups changed to two and one respectively. Individually taking into account each animal, from the two animals considered HTA at D60 in CO, one were considered LTA in the first assay (lactate influx changed from 0.76 to 1.47 nmol x mg^{-1} x min^{-1}). All the other four HTA animals in CO decreased their influx activity after 30 days receiving corn oil. In group CL, from the three HTA animals, two decreased (and turned LTA) and one kept its HTA status at D60.
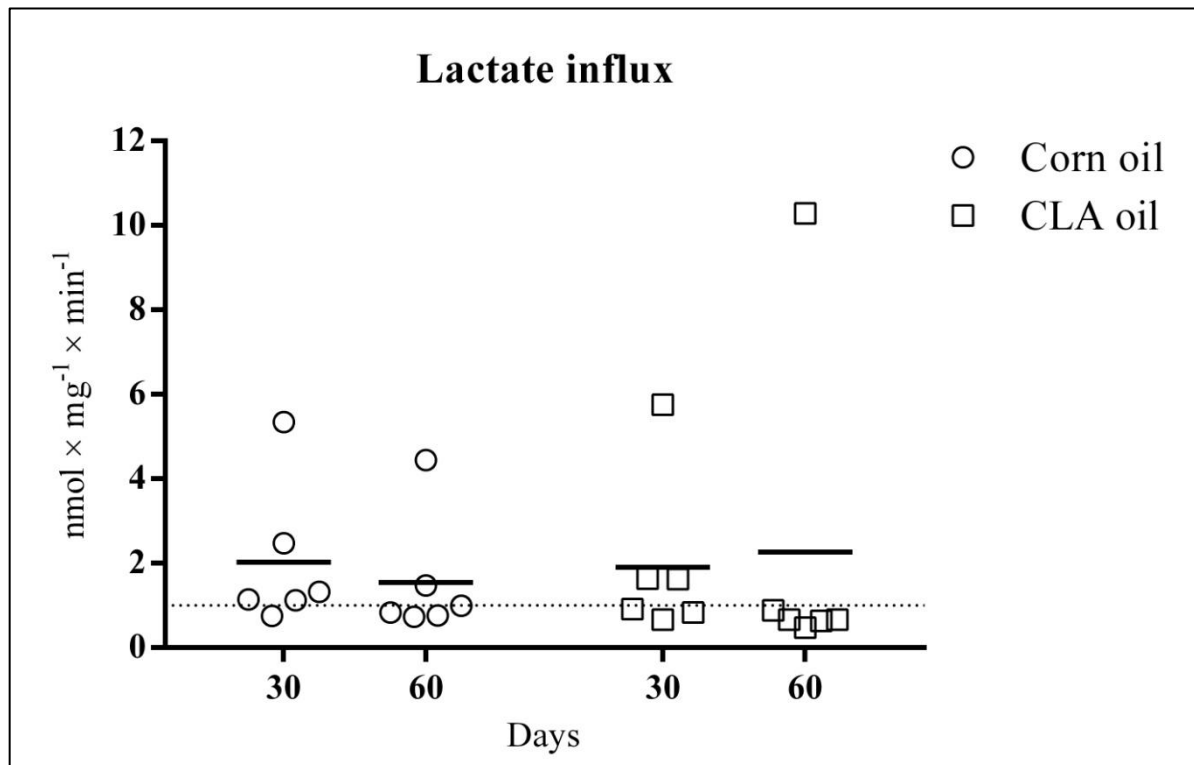
Figure 5.2: Total lactate influx (nmol x mg^{-1} x min^{-1}) in erythrocytes of horses at 30 and 60 days of oral supplementation with conjugated linoleic acid (CLA) or corn oil. The dotted line indicates 1 nmol x mg^{-1} x min^{-1}, the borderline between low transport activity (LTA), below line, and high transport activity (HTA) horses, above line. The solid lines represent the means.

The *in vitro* lactate influx into RBCs without any inhibitor of membrane transporters, with CHC, and with DIDS (considering the whole group, only HTA or only LTA animals) are present in **Table 5.2**. There was no difference between CL and CO in relation to times or treatments in the three *in vitro* assays, as well as in the percentages of inhibition of each

pathway. Individually analyzing the animals studied, it was observed that some ponies did not present *in vitro* inhibition of DIDS or CHC. There was no relation of this absence of inhibition with influx activity (regarding if animals were HTA or LTA) or with treatment applied.

On the subject of calculated influx through membrane transporters, the MCT influx represented 0.83 ± 1.74 and 0.68 ± 1.54 nmol x mg^{-1} protein x min^{-1} in CO and 0.88 ± 1.61 and 1.42 ± 3.30 nmol x mg^{-1} protein x min^{-1} in CL at days D30 and D60 respectively. In turn, band 3 represented 0.72 ± 1.34 and 0.61 ± 1.38 nmol x mg^{-1} protein x min^{-1} in CO and 0.59 ± 1.33 and 1.33 ± 3.18 nmol x mg^{-1} protein x min^{-1} in CL at days D30 and D60 respectively. This decrease in MCT and band 3 activities in CO, as well as the increase in MCT and band 3 activities in CL along the study did not shown statistical significance.

Table 5.2: *In vitro* values of lactate influx (mean ± standard deviation) into erythrocytes of horses at 30 and 60 days of oral supplementation with conjugated linoleic acid (CL) or corn oil (CO) without inhibitor (control), with CHC (alpha-cyano-4-hydroxycinnamic acid), and with DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid). The mean percentage of inhibition of each pathway is indicated. HTA (high transport activity), LTA (low transport activity).

			Lactate influx nmol x mg^{-1} protein x min^{-1}					
	n	**Control**	**CHC**	**DIDS**	**n**	**Control**	**CHC**	**DIDS**
			CO - HTA$^{#}$				*CL - HTA**	
Day 30	5	2.28 ± 1.80	1.62 ± 0.51	1.44 ± 0.53	3	3.01 ± 2.38	1.27 ± 0.35	1.53 ± 0.87
Inhibition, %			-4.74 ± 60.43	22.54 ± 28.46			46.47 ± 22.94	42.37 ± 23.06
Day 60	2	2.96 ± 2.11	1.42 ± 1.10	1.22 ± 0.26	1	10.29	2.14	2.49
Inhibition, %			17.75 ± 95.82	40.49 ± 51.37			79.19	75.84
			CO - LTA*				*CL - LTA$^{#}$*	
Day 30	1	0.76	0.63	0.82	3	0.80 ± 0.13	0.88 ± 0.28	1.03 ± 0.32
Inhibition, %			16.67	-8.33			-8.54 ± 18.64	-28.89 ± 34.21
Day 60	4	0.83 ± 0.11	1.21 ± 0.59	0.99 ± 0.33	5	0.67 ± 0.15	0.87 ± 0.49	1.66 ± 2.40
Inhibition, %			-48.93 ± 84.26	-19.05 ± 38.44			-44.1 ± 116.64	-110.41 ± 254.00
			CO - HTA + LTA				*CL - HTA + LTA*	
Day 30	6	2.03 ± 1.72	1.46 ± 0.61	1.34 ± 0.53	6	1.90 ± 1.93	1.08 ± 0.35	1.28 ± 0.65
Inhibition, %			-1.18 ± 54.75	17.39 ± 28.41			18.97 ± 35.46	6.74 ± 46.95
Day 60	6	1.54 ± 1.45	1.28 ± 0.68	1.06 ± 0.31	6	2.27 ± 3.93	1.08 ± 0.68	1.80 ± 2.17
Inhibition, %			-26.70 ± 85.34	0.80 ± 48.58			-23.56 ± 115.83	-79.37 ± 239.57

* Not possible to perform analysis of variance. $^{#}$ Unpaired test performed due to different number of animals in each time.

5.6.3 qPCR

All primers and probes performed well. Amplification efficiency varied between 85.54% and 107.95%, and standard curves presented R^2 between 0.947 and 0.994.

There were no differences in the expression of mRNA of MCT1, CD147 nor PPRA-α in the animals supplemented for 60 days between CO and CL (**Figure 5.3**). Stratification of data by lactate transport activity within each group did not affect the statistical analysis, and no difference was observed in the expression of the genes studied.
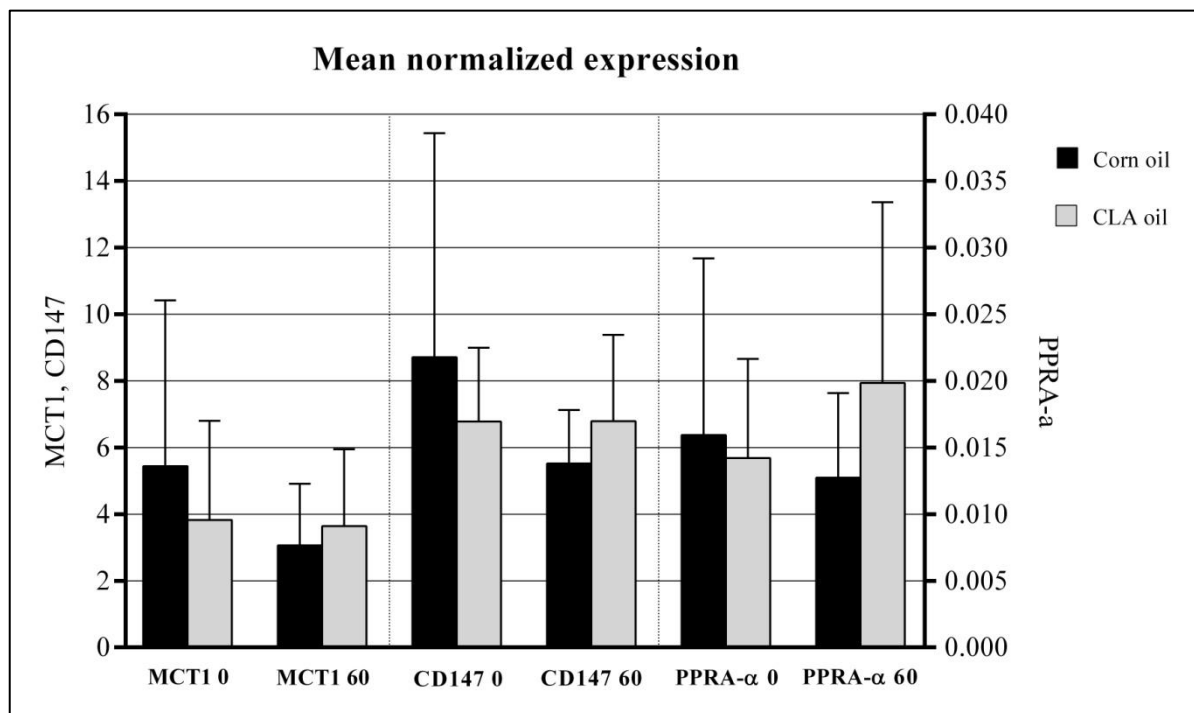
Figure 5.3: Mean normalized expression of monocarboxylate transporter 1 (MCT1), cluster of differentiation 147 (CD147), and peroxisome proliferator-activated receptor type alpha (PPRA-α) in equine bone marrow samples at day 0 and day 60 of oral supplementation with corn oil or conjugated linoleic acid (CLA) oil. Arbitrary units. Data are expressed as mean ± standard deviation.

It was not observed correlation between expression of MCT with CD147 or PPRA-α, or any correlation with those genes and lactate influx in CO. In CL, there was a positive correlation between MCT and CD147 expression (P = 0.014, r = 0.900)

5.7 Discussion

In our knowledge, this was the first study to try to modify the influx activity in horses by the application of a treatment. Up to date, the assessment of lactate influx into RBCs was only performed considering different training levels, training protocols, species, age or sex. In

humans, the use of recombinant human erythropoietin three times a week for four weeks increased the lactate transport and number of MCT1 in RBCs of nine athletes (Connes *et al.*, 2004). In sport horses the use of erythropoietin is considered doping, although its medical use in critical care patients would be of value to decrease lactic acidosis and could be further investigated in horses. Since LTA horses can perform in the same fashion as HTA horses (Väihkönen, Hyyppä e Pösö, 1999), using the oral supplementation with CLA would improve the results of animals with average or submaximal performance.

As far as we are aware, this was the first experiment using ponies as a model to study lactate influx. Handling and sampling were not different from horses, and housing and feeding was cost-effective. In addition, eight of the 12 ponies in this study were HTA, a percentage within what has been previously reported (Väihkönen e Pösö, 1998), slightly lower in comparison with hot-blooded horses (Mykkänen, Pösö, *et al.*, 2010).

The usual response of supplementation with CLA is a decrease in body weight by reducing body fat gain and enhancing lean body mass gain. This effect is currently associated by a reduction in lipid uptake by adipocytes due to CLA direct action on stearoyl-CoA desaturase and lipoprotein lipase activity (Mccrorie *et al.*, 2011). It was already observed in rodents (Peters *et al.*, 2001), chickens, pigs (Mccrorie *et al.*, 2011), and humans (Van Wijlen, 2011). The ponies in this study presented increased body weight, probably due to better feeding conditions, and not by the CLA supplementation, as indicated by the statistical analysis. Others already reported no difference in body weight, body condition score and rump fat thickness after 42 days of oral supplementation of CLA in mares (Headley *et al.*, 2012).

In this study, lactate influx was not measured with labeled ^{14}C-lactate as described by other authors (Deuticke, Beyer e Forst, 1982; De Bruijne, Vreeburg e Van Steveninck, 1983; Deuticke, 1989; Skelton *et al.*, 1995). None of those papers properly describe the validation methods of the assays with labeled radioactive compounds. In addition, the use of radiolabeled compounds and the production of radioactive waste must be avoided and alternative methods must be used in place of those in our opinion. We opted to use a laboratory analyzer. The detection limit of the equipment used was 0.3 mmol/L. After calculations to convert the lactate detected to the amount of protein in RBCs, 0.3 mmol/L of lactate is equivalent to approximately 0.4 nmol x mg^{-1} protein x min^{-1}. Since Väihkönen, Hyyppä e Pösö (1999) detected LTA horses with influx varied between 0.44 and 0.60 nmol x mg^{-1} protein x min^{-1}, we believed that the analyzer would fit to our purposes.

The total lactate influx into RBCs did not differ between control and treatment groups, although a trend to decreasing was observed along times in CO, as well as a tendency to increase in CL. The influx through MCT and band 3 also decreased in CO and increased on CL along times studied. The values of total lactate influx and influx with DIDS were slightly below the ones reported in the literature, while the values of influx with CHC were in concordance (Väihkönen e Pösö, 1998; Väihkönen, Hyyppä e Pösö, 1999; Väihkönen, Ojala e Pösö, 2002), which means that ponies may have less MCTs or less CD147, since DIDS blocks the influx through band 3 proteins, and the result of the influx in those assays is due to MCT transport and nonionic diffusion. These differences may be caused by genetic and metabolic variance between sport horses like Standardbreds used in those studies in comparison with mixed breed ponies. Because the concentration of MCT in the RBC membrane was not evaluated in this study, it is not possible to determine if the ponies had a lower amount of MCT and CD147 in comparison with horses.

The inhibition of a carrier is used to estimate the percentage of participation of each one of the three pathways (MCTs, band 3 protein and nonionic diffusion) which lactate can use to enter RBCs. Väihkönen, Ojala e Pösö (2002) observed that HTA horses presented 63-70% of inhibition by CHC, i.e. the contribution of MCTs to the influx, and 0-28% of inhibition by DIDS, i.e. the contribution of band 3 protein to the influx. LTA horses showed 0-30% of inhibition by DIDS but no inhibition by CHC. In our study, the percentage of inhibition of both CHC and DIDS presented a highly interindividual variation. Some animals did not present *in vitro* inhibition, while other presented grades of inhibition close to the ones reported previously, and this behavior was present in both HTA and LTA animals. Individually, in the animals that presented inhibition, it was 4-77% with DIDS and 10-86% with CHC. In HTA horses, means of inhibition were 23-76% and 18-79% with DIDS and CHC respectively; while in LTA horses the inhibition of both was negligible. We observed an inconsistency in the action of inhibitors that was already reported before (Väihkönen e Pösö, 1998; Väihkönen, Ojala e Pösö, 2002). Other authors believe that the absence of inhibition of CHC may be due to low concentrations of MCT, and the main carrier in LTA animals would be band 3 and nonionic diffusion. However, Koho, Väihkönen e Pösö (2002) showed that the difference in HTA and LTA horses was due to difference in concentrations of CD147 and not in MCT amounts in RBC membrane. HTA and LTA horses have the same amounts of MCT1 and MCT2 in their RBC membranes, but the amounts of CD147 is significant lower in LTA (Koho, Väihkönen e Pösö, 2002; Mykkänen, Pösö, *et al.*, 2010). In addition, the K_m value of band 3 protein (300 mM), MCT1 (3.5 mM) and MCT2 (0.74 mM) leads to believe that in the

physiological concentration of lactate (< 10 mM), band 3 is the least important carrier (Poole e Halestrap, 1993; Halestrap e Price, 1999). Likewise, we disagree that animals with low in vitro inhibition of both DIDS and CHC may have nonionic diffusion as the most important mechanism of transport (Väihkönen e Pösö, 1998) due to the lactate pK of 3.86, which means that at physiological pH, lactic acid is 99% dissociated in H^+ and lactate (La^-) (Gladden, 2004). In LTA animals, MCT1 may have compromised functions caused by the low concentration of CD147 which may inhibit CHC binding to the carriers-MCT1, MCT2 and band 3 (this last with less affinity), according to Halestrap e Meredith (2004). The ideal *in vitro* inhibitor would be pMCBS (p-chloromercuribenzenesulfonate), because it inhibits MCT1 by binding to CD147, which is not present in MCT2 (Halestrap, 2012).

We observed that some animals changed its lactate influx condition (high or low activity) during the 60 days of supplementation with both corn and CLA oils. This result is in contrast with other study that evaluated the same horses from 2 weeks to 4 years of age and found that animals do not change their influx activity along their lives (Väihkönen, Ojala e Pösö, 2002). However, those animals were in growth phase during the study and were training or racing in three of five data collection points. In Thoroughbred horses evaluated from 2 to 24 months of age, there was an increase the content of MCT1, as well as the oxidative capacity in muscle samples associated to the growth itself (Kitaoka *et al.*, 2011). Also, training is known to increase MCT1 in skeletal muscle (Kitaoka, Masuda, *et al.*, 2010), although studies disagree about the effect of training in RBC lactate influx (Väihkönen *et al.*, 2001; Mykkänen, Pösö, *et al.*, 2010). Those two studies with RBC lactate influx did not compared the same animals before and after training, which may obscure the results due to the high interindividual variance found in horses (Väihkönen e Pösö, 1998). The ponies in our study were adult sedentary animals, and a possible influence of training and growth can be eliminated as variables in our evaluation.

CLA is natural PPRA-α agonists known to up-regulate the expression of MCT1 and its chaperone CD147 in rats. The mechanism which CLA and other PPRA-α agonist interfere in MCT1 synthesis is still unknown. PPRA-α do not up-regulate MCT1 expression through direct binding, but possible for other post-transcriptional mechanisms (König *et al.*, 2008; König *et al.*, 2010). In bone marrow samples of mixed breed pony mares, the levels of PPRA-α mRNA did not differ between CO and CL group, neither in HTA animals analyzed separately. However, it was noticed a clearly tendency in animals fed CLA to increase the expression of this gene. A possible effect in mRNA levels of MCT1 and CD147 was not statistical significant, but CO showed decreased values after 60 days of supplementation and

CL kept its level constant. If the experimental animals would be in training for athletic performance, maybe the effects of CLA in addition to the exercise could be marked.

Before affirming that CLA has no effect in MCT in horses, the dose and period of supplementation must be evaluated. The dose of 0.01% BW used in this study was the same as reported previously (Headley *et al.*, 2012), when there was significant increased plasma levels of *cis*-9, *trans*-11, *trans*-10, *cis*-12, and *trans*-9, *tras*-11 in comparison with corn oil. In rats, supplementation of 1.7% BW did increase the expression of MCT1 and CD147 mRNA in liver samples, but serum concentration was not assessed (König *et al.*, 2010). Diets of approximately 0,12% BW, considered high-fat diet for horses, was already tested for up to 6 months with no apparent adverse effect (Harris *et al.*, 1999), as well as soybean oil supplementation at up to 0.3% BW during 82 days (Godoi *et al.*, 2010). Obviously, amounts as much as 1% BW would not be possible to offer to horses, since it would represent almost 5 L of oil per day and adverse effects could occur. Regarding the period of supplementation, we considered that horse erythrocytes have a life span of 145 days, the daily production of new cells is approximately 50,000 cells/µL/day (Carter *et al.*, 1974; Radin, Eubank e Weiser, 1986), and the time necessary to differentiate one erythroid precursor cell into a new erythrocyte is around three weeks (Olver, 2011). We chose to supplement the animal for 60 days, because at least two batches of new cells would be under influence of CLA, as well as all the cells which already have nucleus and all genetic machinery until the metarubricyte phase. To completely eliminate all RBCs from the bloodstream, it would be necessary around 6 months of supplementation. In summary, we suggest a 10-fold increase in dose and 3-fold increase in period of supplementation with CLA then the ones utilized in this study in order to investigate the effects of this oil in lactate influx into RBCs in horses.

In conclusion, we found that the oral supplementation with conjugated linoleic acid during 60 days in healthy adult sedentary pony mares did not affect significantly the expression of MCT1, CD147, and PPRA-α, neither a difference in RBC lactate influx, although tendency of improvement with CLA could be observed. There was no statistical correlation between the lactate influx and the mRNA quantification in the animals studied.

5.8 Acknowledgements

5.9 Conflicts of interest

None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

INTEGRATED DISCUSSION

This study aimed to find evidences of how molecular markers can be used to improve performance and select animals prior to investment, especially in jump horses, in which performance is harder to be evaluated, i.e. how to determine if the degree of contribution of the results is due to the horse or to the rider, and, in addition, there is no direct measure (e.g. race horses can be evaluated in the ratio of distance by time).

Compared to other mammals (30-40%), horses have a high muscle mass rate in relation to its total weight. A Thoroughbred horse can achieve 55% of total muscle mass of its body weight (Gunn, 1986). The study of the equine genome revealed the selection by strength, speed and endurance traits (Hill, Katz e Machugh, 2013). Genes responsible for fatty acid oxidation, increased insulin sensitivity and muscle strength in the Thoroughbred was found as positively selected loci (Gu *et al.*, 2009). The heritability of best distance, for example, was determined to be of 0.94 ± 0.03% in race Thoroughbreds (Williamson e Beilharz, 1998) before the studies of Hill, Gu, *et al.* (2010), and is one indicative of a good trait to select. Another study with 744 horses of 33 diverse breeds identified signatures of selection in chromosome 18 in performance breeds, in chromosome 23 in gaited breeds, and in chromosome 11 in draft and miniature breeds (Petersen *et al.*, 2013).

The use of ergogenic aids, such as conjugated linoleic acid and creatine was not proposed as doping, with the purpose of artificially and in an unfair manner increase the performance, but as a tool to try to modify the expression and understand the regulation of two genes-myostatin gene and MCT1/CD147. With the results of this study, the future perspectives are, using a larger sample of animals and with dose and duration adjustments, repeat the evaluation of a possible myostatin inhibition by creatine and a possible improvement of lactate clearance by CLA. In addition, a study must be performed including a larger sample of the main breeds of jump horses to determine the prevalence of the SNP g.66493737C>T in the MSTN gene and if the bimodal distribution of lactate influx activity in erythrocytes influence or not the performance in this sport. With theses answers, it will be possible to evaluate which is the best genotype and phenotype of these two genes to be selected in jump horses, in order to be possible to make the best reproductive decisions, selection and training of animals for sport purpose, and finally maximize the genetic potential of a horse, reducing operational costs and providing better returns on investments. Finally, with modern diagnostic tools, it will be possible to use in the future this two genes or the clearance of plasma lactate as genetic and molecular markers of performance, not only in jump horses, but in other breeds and sports.

REFERERENCES

AHMED, W. et al. PPARs and their metabolic modulation: new mechanisms for transcriptional regulation? **Journal of Internal Medicine,** v. 262, n. 2, p. 184-198, Aug 2007. ISSN 0954-6820.

ANDRADE, J. C. et al. Production of conjugated linoleic acid by food-grade bacteria: A review. **International Journal of Dairy Technology,** v. 65, n. 4, p. 467-481, Nov 2012. ISSN 1364-727X.

BAI, Y. et al. Importance of N-Glycosylation on CD147 for Its Biological Functions. **International Journal of Molecular Sciences,** v. 15, n. 4, p. 6356-6377, Apr 2014. ISSN 1422-0067.

BAKER, S. K.; MCCULLAGH, K. J. A.; BONEN, A. Training intensity-dependent and tissue-specific increases in lactate uptake and MCT-1 in heart and muscle. **Journal of Applied Physiology,** v. 84, n. 3, p. 987-994, 1998. ISSN 8750-7587.

BELURY, M. A. Dietary conjugated linoleic acid in health: Physiological effects and mechanisms of action 1. **Annual Review of Nutrition,** v. 22, n. 1, p. 505-531, 2002. ISSN 0199-9885.

BICKHAM, D. C. et al. The effects of short-term sprint training on MCT expression in moderately endurance-trained runners. **European Journal of Applied Physiology,** v. 96, n. 6, p. 636-643, 2006. ISSN 1439-6319.

BIRD, S. P. Creatine Supplementation and Exercise Performance: A Brief Review. **Journal of Sports Science & Medicine,** v. 2, n. 4, p. 123-132, 2003. ISSN 1303-2968.

BOMAN, I. A. et al. A frameshift mutation in the coding region of the myostatin gene (MSTN) affects carcass conformation and fatness in Norwegian White Sheep (Ovis aries). **Animal Genetics,** v. 40, n. 4, p. 418-422, Aug 2009. ISSN 0268-9146.

BROSNAN, J. T.; DA SILVA, R. P.; BROSNAN, M. E. The metabolic burden of creatine synthesis. **Amino Acids,** v. 40, n. 5, p. 1325-1331, May 2011. ISSN 0939-4451.

CARTER, E. et al. The kinetics of hematopoiesis in the light horse I. The lifespan of peripheral blood cells in the normal horse. **Canadian Journal of Comparative Medicine,** v. 38, n. 3, p. 303, 1974.

CHAMPE, P. C.; HARVEY, R. A. Introduction to Metabolism and Glycolysis. In: (Ed.). **Bioquímica Ilustrada**. 2nd. Porto Alegre: Artes Médicas, 1997. cap. 8,

CHURRUCA, I.; FERNANDEZ-QUINTELA, A.; PUY PORTILLO, M. Conjugated linoleic acid isomers: Differences in metabolism and biological effects. **Biofactors,** v. 35, n. 1, p. 105-111, Jan-Feb 2009. ISSN 0951-6433.

CLOP, A. et al. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. **Nature Genetics,** v. 38, n. 7, p. 813-818, Jul 2006. ISSN 1061-4036.

CONNES, P. et al. Injections of recombinant human erythropoietin increases lactate influx into erythrocytes. **Journal of Applied Physiology,** v. 97, n. 1, p. 326-332, Jul 2004. ISSN 8750-7587.

COOPER, R. et al. Creatine supplementation with specific view to exercise/sports performance: an update. **Journal of the International Society of Sports Nutrition,** v. 9, Jul 20 2012. ISSN 1550-2783.

D'ANGELIS, F. H. F. et al. Aerobic training, but not creatine supplementation, alters the gluteus medius muscle. **Journal of Animal Science,** v. 83, n. 3, p. 579-585, Mar 2005. ISSN 0021-8812.

DALL'OLIO, S. et al. Analysis of Horse Myostatin Gene and Identification of Single Nucleotide Polymorphisms in Breeds of Different Morphological Types. **Journal of Biomedicine and Biotechnology,** v. 2010, p. 11, 2010. ISSN 1110-7243.

DAMES, S. et al. Characterization of aberrant melting peaks in unlabeled probe assays. **Journal of Molecular Diagnostics,** v. 9, n. 3, p. 290-296, Jul 2007. ISSN 1525-1578.

DE BRUIJNE, A. W.; VREEBURG, H.; VAN STEVENINCK, J. Kinetic analysis of l-lactate transport in human erythrocytes via the monocarboxylate-specific carrier system. **Biochimica et Biophysica Acta (BBA) - Biomembranes,** v. 732, n. 3, p. 562-568, 1983. ISSN 0005-2736.

DELDICQUE, L. et al. Effects of resistance exercise with and without creatine supplementation on gene expression and cell signaling in human skeletal muscle. **Journal of Applied Physiology,** v. 104, n. 2, p. 371-378, Feb 2008. ISSN 8750-7587.

DELDICQUE, L. et al. Kinetics of creatine ingested as a food ingredient. **European Journal of Applied Physiology,** v. 102, n. 2, p. 133-143, Jan 2008. ISSN 1439-6319.

DEUTICKE, B. Monocarboxylate transport in red blood cells: Kinetics and chemical modification. In: SIDNEY FLEISCHER, B. F. (Ed.). **Methods in Enzymology**: Academic Press, v.173, 1989. cap. 18, p.300-329. ISBN 0076-6879.

DEUTICKE, B.; BEYER, E.; FORST, B. Discrimination of 3 parallel pathways of lactate transport in the human-erythrocyte membrane by inhibitors and kinetic properties **Biochimica Et Biophysica Acta,** v. 684, n. 1, p. 96-110, 1982. ISSN 0006-3002.

DIEFFENBACH, C.; LOWE, T.; DVEKSLER, G. General concepts for PCR primer design. **PCR Methods Appl,** v. 3, n. 3, p. S30-S37, 1993. ISSN 1054-9803.

DILZER, A.; PARK, Y. Implication of Conjugated Linoleic Acid (CLA) in Human Health. **Critical Reviews in Food Science and Nutrition,** v. 52, n. 6, p. 488-513, 2012. ISSN 1040-8398.

DOMINIQUE, J.-E.; GÉRARD, C. Myostatin regulation of muscle development: molecular basis, natural mutations, physiopathological aspects. **Experimental cell research,** v. 312, n. 13, p. 2401-2414, 2006. ISSN 0014-4827.

DOREAU, M.; MARTUZZI, F. Fat content and composition of mare's milk. In: MIRAGLIA, N. e MARTIN-ROSSET, W., 3rd European Workshop on Equine Nutrition, 2006, Campobasso, Italy. Wageningen Academic Publishers, 20-22 June. p.77-87.

ENOKI, T. et al. Exercise training alleviates MCT1 and MCT4 reductions in heart and skeletal muscles of STZ-induced diabetic rats. **Journal of Applied Physiology,** v. 94, n. 6, p. 2433-2438, 2003. ISSN 8750-7587.

ERALI, M.; VOELKERDING, K. V.; WITTWER, C. T. High resolution melting applications for clinical laboratory medicine. **Experimental and Molecular Pathology,** v. 85, n. 1, p. 50-58, Aug 2008. ISSN 0014-4800.

ERLICH, H. A.; GELFAND, D.; SNINSKY, J. J. Recent advances in the polymerase chain reaction. **Science,** v. 252, n. 5013, p. 1643-1651, 1991. ISSN 0036-8075.

ESSEN-GUSTAVSSON, B.; LINDHOLM, A.; PERSSON, S. G. B. Circulatory and muscle metabolic responses in the horse to a standardised exercise test with and without creatine supplementation. In: KALLINGS, P.;BONDESSON, U.*, et al*, Proceedings of the 10th International Conference of Racing Analysts and Veterinarians, Stockholm, Sweden, 1994, 1994. p.85-89.

EVANS, D. Exercise testing in the field. In: KANEPS, K. W.;HINCHCLIFF, R. J.*, et al* (Ed.). **Equine Exercise Physiology**. Edinburgh: W.B. Saunders, 2008. cap. 1.2, p.12-27. ISBN 978-0-7020-2857-1.

FELDMAN, E. B. Creatine: A dietary supplement and ergogenic aid. **Nutrition Reviews,** v. 57, n. 2, p. 45-50, Feb 1999. ISSN 0029-6643.

FERRAZ, G. D. C. et al. Long-term creatine supplementation improves the aerobic capacity of horses. **Ciencia Rural,** v. 36, n. 2, p. 514-519, Mar-Apr 2006. ISSN 0103-8478.

FORMAN, B. M.; CHEN, J.; EVANS, R. M. Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors alpha and delta. **Proceedings of the National Academy of Sciences USA,** v. 94, n. 9, p. 4312-7, Apr 1997. ISSN 0027-8424.

GHOLAMI, Z.; KHOSRAVI-DARANI, K. An Overview of Conjugated Linoleic Acid: Microbial Production and Application. **Mini-Reviews in Medicinal Chemistry,** v. 14, n. 9, p. 734-746, 2014. ISSN 1389-5575.

GLADDEN, L. B. Lactate metabolism: a new paradigm for the third millennium. **Journal of Physiology,** v. 558, n. 1, p. 5-30, 2004. ISSN 0022-3751.

GODOI, F. N. D. et al. Performance of eventing horses fed high fat diet. **Revista Brasileira de Zootecnia,** v. 39, p. 335-343, 2010. ISSN 1516-3598.

GROBET, L. et al. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. **Nature Genetics,** v. 17, n. 1, p. 71-74, Sep 1997. ISSN 1061-4036.

GROBET, L. et al. Molecular definition of an allelic series of mutations disrupting the myostatin function and causing double-muscling in cattle. **Mammalian Genome,** v. 9, n. 3, p. 210-213, Mar 1998. ISSN 0938-8990.

GU, J. et al. A Genome Scan for Positive Selection in Thoroughbred Horses. **PLOS One,** v. 4, n. 6, Jun 2009. ISSN 1932-6203.

GUNN, H. M. Muscle, bone and fat proportions and muscle distribution of Thoroughbreds and other horses. In: GILLESPIE, J. R. e ROBINSON, N. E., Equine Exercise Physiology 2: Proceedings of the Second International Conference on Equine Exercise Physiology, 1986, San Diego, CA. ICEEP Publications, August 7-11. p.253-264.

HAJDUCH, E. et al. Lactate transport in rat adipocytes: identification of monocarboxylate transporter 1 (MCT1) and its modulation during streptozotocin-induced diabetes. **Febs Letters,** v. 479, n. 3, p. 89-92, 2000. ISSN 0014-5793.

HALESTRAP, A. P. The monocarboxylate transporter family-Structure and functional characterization. **IUBMB Life,** v. 64, n. 1, p. 1-9, 2012. ISSN 1521-6543.

HALESTRAP, A. P.; MEREDITH, D. The SLC16 gene family - from monocarboxylate transporters (MCTs) to aromatic amino acid transporters and beyond. **Pflugers Archiv-European Journal of Physiology,** v. 447, n. 5, p. 619-628, Feb 2004. ISSN 0031-6768.

HALESTRAP, A. P.; PRICE, N. T. The proton-linked monocarboxylate transporter (MCT) family: structure, function and regulation. **Biochemical Journal,** v. 343, p. 281-299, Oct 1999. ISSN 0264-6021.

HARRIS, P. A.; HARRIS, R. C. Ergogenic potential of nutritional strategies and substances in the horse. **Livestock Production Science,** v. 92, n. 2, p. 147-165, Feb 2005. ISSN 0301-6226.

HARRIS, P. A. et al. Effect of feeding thoroughbred horses a high unsaturated or saturated vegetable oil supplemented diet for 6 months following a 10 month fat acclimation. **Equine Veterinary Journal,** v. 31, n. S30, p. 468-474, 1999. ISSN 2042-3306.

HE, M. L.; ISHIKAWA, S.; HIDARI, H. Fatty acid profiles of various muscles and adipose tissues from fattening horses in comparison with beef cattle and pigs. **Asian-Australasian Journal of Animal Sciences,** v. 18, n. 11, p. 1655-1661, Nov 2005. ISSN 1011-2367.

HEADLEY, S. et al. Dietary supplementation of conjugated linoleic acid in horses increases plasma conjugated linoleic acid and decreases plasma arachidonic acid but does not alter body fat. **Journal of Animal Science,** v. 90, n. 13, p. 4876-4882, Dec 2012. ISSN 0021-8812.

HILL, E. W. et al. A Sequence Polymorphism in MSTN Predicts Sprinting Ability and Racing Stamina in Thoroughbred Horses. **PLOS One,** v. 5, n. 1, Jan 2010. ISSN 1932-6203.

HILL, E. W.; KATZ, L. M.; MACHUGH, D. E. Genomics of performance. In: CHOWDHARY, B. P. (Ed.). **Equine genomics**. Oxford: Wiley-Blackwell, 2013. cap. 17, p.265-283. ISBN 9781118522158.

HILL, E. W. et al. A genome-wide SNP-association study confirms a sequence variant (g.66493737C > T) in the equine myostatin (MSTN) gene as the most powerful predictor of optimum racing distance for Thoroughbred racehorses. **BMC Genomics,** v. 11, n. 552, Oct 2010. ISSN 1471-2164.

HOSOYAMA, T. et al. Molecular Cloning of Equine (Thoroughbred) Myostatin cDNA and Detection of Myostatin Precursor Proteins in the Serum. **Journal of Reproduction and Development,** v. 48, n. 4, p. 335-342, 2002. ISSN 0916-8818.

HUANG, Z.; CHEN, X.; CHEN, D. Myostatin: A novel insight into its role in metabolism, signal pathways, and expression regulation. **Cellular Signalling,** v. 23, n. 9, p. 1441-1446, Sep 2011. ISSN 0898-6568.

HULTMAN, E. et al. Muscle creatine loading in men. **Journal of Applied Physiology,** v. 81, n. 1, p. 232-237, Jul 1996. ISSN 8750-7587.

JACKSON, V. N. et al. Cloning of the monocarboxylate transporter isoform MCT2 from rat testis provides evidence that expression in tissues is species-specific and may involve post-transcriptional regulation. **Biochemichal Journal,** v. 324, n. Pt 2, p. 447-53, Jun 1997. ISSN 0264-6021.

JAHREIS, G. et al. The potential anticarcinogenic conjugated linoleic acid, cis-9,trans-11 C18 : 2, in milk of different species: Cow, goat, ewe, sow, mare, woman. **Nutrition Research,** v. 19, n. 10, p. 1541-1549, Oct 1999. ISSN 0271-5317.

JOEHANES, R.; NELSON, J. C. QGene 4.0, an extensible Java QTL-analysis platform. **Bioinformatics,** v. 24, n. 23, p. 2788-2789, 2008. ISSN 1367-4803.

JUEL, C.; HOLTEN, M. K.; DELA, F. Effects of strength training on muscle lactate release and Mct1 and MCT4 content in healthy and type 2 diabetic humans. **Journal of Physiology-London,** v. 556, n. 1, p. 297-304, Apr 2004. ISSN 0022-3751.

KAMBADUR, R. et al. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. **Genome research,** v. 7, n. 9, p. 910-915, 1997. ISSN 1088-9051.

KERSTEN, S. et al. Peroxisome proliferator–activated receptor α mediates the adaptive response to fasting. **Journal of Clinical Investigation,** v. 103, n. 11, p. 1489, 1999.

KINGSTON, J. K. Hematologic and serum biochemical responses to exercise and training. In: HINCHCLIFF, K. W.;GEOR, R. J., *et al* (Ed.). **Equine Exercise Physiology**. New York: Saunders Elsevier, 2008. cap. 7.1, p.398-422.

KIRK, P. et al. CD147 is tightly associated with lactate transporters MCT1 and MCT4 and facilitates their cell surface expression. **Embo Journal,** v. 19, n. 15, p. 3896-3904, Aug 2000. ISSN 0261-4189.

KITAOKA, Y. et al. Effect of acute exercise on monocarboxylate transporters 1 and 4 in untrained and trained Thoroughbreds. **American Journal of Veterinary Research,** v. 74, n. 4, p. 642-647, Apr 2013. ISSN 0002-9645.

KITAOKA, Y.; HOSHINO, D.; HATTA, H. Monocarboxylate transporter and lactate metabolism. **The Journal of Physical Fitness and Sports Medicine,** v. 1, n. 2, p. 247-252, 2012. ISSN 2186-8131.

KITAOKA, Y. et al. Effect of growth on monocarboxylate transporters and indicators of energy metabolism in the gluteus medius muscle of Thoroughbreds. **American Journal of Veterinary Research,** v. 72, n. 8, p. 1107-1111, Aug 2011. ISSN 0002-9645.

KITAOKA, Y. et al. Effect of training and detraining on monocarboxylate transporter (MCT) 1 and MCT4 in Thoroughbred horses. **Experimental Physiology,** v. 96, n. 3, p. 348-355, Mar 2010. ISSN 0958-0670.

KITAOKA, Y. et al. Effects of high-intensity training on monocarboxylate transporters in Thoroughbred horses. **Comparative Exercise Physiology,** v. 6, n. 4, p. 171-175, 2010. ISSN 1755-2540.

KOBA, K.; YANAGITA, T. Health benefits of conjugated linoleic acid (CLA). **Obesity Research & Clinical Practice,** v. 8, n. 6, p. E525-E532, Nov-Dec 2014. ISSN 1871-403X.

KOHO, N.; VÄIHKÖNEN, L.; PÖSÖ, A. Lactate transport in red blood cells by monocarboxylate transporters. **Equine Veterinary Journal,** v. 34, n. S34, p. 555-559, 2002. ISSN 2042-3306.

KOHO, N. M.; HYYPPÄ, S.; PÖSÖ, A. R. Monocarboxylate transporters (MCT) as lactate carriers in equine muscle and red blood cells. **Equine Veterinary Journal. Supplement**, n. 36, p. 354-8, Aug 2006.

KOHO, N. M. et al. Sequence variations and two levels of MCT1 and CD147 expression in red blood cells and gluteus muscle of horses. **Gene,** v. 491, n. 1, p. 65-70, Jan 2012. ISSN 0378-1119.

KÖNIG, B. et al. Monocarboxylate transporter 1 and CD147 are up-regulated by natural and synthetic peroxisome proliferator-activated receptor α agonists in livers of rodents and pigs. **Molecular nutrition & food research,** v. 54, n. 9, p. 1248-1256, 2010. ISSN 1613-4133.

KÖNIG, B. et al. Monocarboxylate transporter (MCT)-1 is up-regulated by PPARα. **Biochimica et Biophysica Acta (BBA)-General Subjects,** v. 1780, n. 6, p. 899-904, 2008. ISSN 0304-4165.

LEE, S. J.; MCPHERRON, A. C. Regulation of myostatin activity and muscle growth. **Proceedings of the National Academy of Sciences of the United States of America,** v. 98, n. 16, p. 9306-9311, Jul 2001. ISSN 0027-8424.

LEFEBVRE, P. et al. Sorting out the roles of PPARα in energy metabolism and vascular homeostasis. **Journal of Clinical Investigation,** v. 116, n. 3, p. 571-580, 2006. ISSN 0021-9738.

LEINO, R. L.; GERHART, D. Z.; DREWES, L. R. Monocarboxylate transporter (MCT1) abundance in brains of suckling and adult rats: a quantitative electron microscopic

immunogold study. **Developmental Brain Research,** v. 113, n. 1-2, p. 47-54, Mar 1999. ISSN 0165-3806.

LIEW, M. et al. Closed-tube SNP genotyping without labeled probes a comparison between unlabeled probe and amplicon melting. **American Journal of Clinical Pathology,** v. 127, n. 3, p. 341-348, Mar 2007. ISSN 0002-9173.

LOIKE, J. D. et al. Extracellular creatine regulates creatine transport in rat and human muscle cells. **Proceedings of the National Academy of Sciences,** v. 85, n. 3, p. 807-811, 1988. ISSN 0027-8424.

MAMANI-LINARES, L. W.; GALLO, C. Fatty acid profile of sheep and horse meat under an extensive production system (Perfil de acidos grasos de carne de ovino y caballo criados bajo un sistema de produccion extensiva). **Revista de Investigaciones Veterinarias del Peru (RIVEP),** v. 24, n. 3, p. 257-263, 2013. ISSN 1682-3419.

MARCHITELLI, C. et al. Double muscling in Marchigiana beef breed is caused by a stop codon in the third exon of myostatin gene. **Mammalian Genome,** v. 14, n. 6, p. 392-395, Jun 2003. ISSN 0938-8990.

MCCRORIE, T. A. et al. Human health effects of conjugated linoleic acid from milk and supplements. **Nutrition Research Reviews,** v. 24, n. 2, p. 206-227, Dec 2011. ISSN 0954-4224.

MCCULLAGH, K. J. A. et al. Chronic electrical stimulation increases MCT1 and lactate uptake in red and white skeletal muscle. **American Journal of Physiology-Endocrinology and Metabolism,** v. 273, n. 2, p. E239-E246, Aug 1997. ISSN 0193-1849.

MCFARLANE, C. et al. Myostatin signals through Pax7 to regulate satellite cell self-renewal. **Experimental Cell Research,** v. 314, n. 2, p. 317-329, Jan 2008. ISSN 0014-4827.

MCPHERRON, A. C.; LAWLER, A. M.; LEE, S. J. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. **Nature,** v. 387, n. 6628, p. 83-90, May 1997. ISSN 0028-0836.

MCPHERRON, A. C.; LEE, S. J. Double muscling in cattle due to mutations in the myostatin gene. **Proceedings of the National Academy of Sciences of the United States of America,** v. 94, n. 23, p. 12457-12461, Nov 1997. ISSN 0027-8424.

MOSHER, D. S. et al. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. **PLOS Genetics,** v. 3, n. 5, p. 779-786, May 2007. ISSN 1553-7390.

MOYA-CAMARENA, S. Y.; VANDEN HEUVEL, J. P.; BELURY, M. A. Conjugated linoleic acid activates peroxisome proliferator-activated receptor a and B subtypes but does not induce hepatic peroxisome proliferation in Sprague-Dawley rats. **Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,** v. 1436, n. 3, p. 331-342, Jan 1999. ISSN 1388-1981.

MYKKÄNEN, A. K. et al. Immunohistochemical analysis of MCT1 and CD147 in equine skeletal muscle fibres. **Research in Veterinary Science,** v. 89, n. 3, p. 432-437, Dec 2010. ISSN 0034-5288.

MYKKÄNEN, A. K. et al. MCT1, MCT4 and CD147 gene polymorphisms in healthy horses and horses with myopathy. **Research in Veterinary Science,** v. 91, n. 3, p. 473-477, Dec 2011. ISSN 0034-5288.

MYKKÄNEN, A. K. et al. Expression of lactate transporters MCT1, MCT2 and CD147 in the red blood cells of three horse breeds: Finnhorse, Standardbred and Thoroughbred. **Equine Veterinary Journal,** v. 42, p. 161-166, Nov 2010. ISSN 0425-1644.

NESS, P. M.; STENGLE, J. M. Historical Introduction. In: SURGENOR, D. M. (Ed.). **The Red Blood Cell**. 2nd. New York: Academic Press, v.1, 1974. cap. 1, p.1-50. ISBN 978-0-12-677201-2.

OLSEN, S. et al. Creatine supplementation augments the increase in satellite cell and myonuclei number in human skeletal muscle induced by strength training. **Journal of Physiology-London,** v. 573, n. 2, p. 525-534, Jun 2006. ISSN 0022-3751.

OLVER, C. S. Erythropoiesis. In: WEISS, D. J. e WARDROP, K. J. (Ed.). **Schalm's Veterinary Hematology**. 6th. Ames, Iowa: Wiley-Blackwell, 2011. cap. 6, ISBN 047096183X.

PARIZA, M. W.; PARK, Y.; COOK, M. E. The biologically active isomers of conjugated linoleic acid. **Progress in Lipid Research,** v. 40, n. 4, p. 283-298, Jul 2001. ISSN 0163-7827.

PARK, Y.; PARIZA, M. Evidence that commercial calf and horse sera can contain substantial amounts of trans-10,cis-12 conjugated linoleic acid. **Lipids,** v. 33, n. 8, p. 817-819, 1998. ISSN 0024-4201.

PELLERIN, L. et al. Expression of monocarboxylate transporter mRNAs in mouse brain: Support for a distinct role of lactate as an energy substrate for the neonatal vs. adult brain. **Proceedings of the National Academy of Sciences of the United States of America,** v. 95, n. 7, p. 3990-3995, Mar 1998. ISSN 0027-8424.

PETERS, J. M. et al. Influence of conjugated linoleic acid on body composition and target gene expression in peroxisome proliferator-activated receptor alpha-null mice. **Biochimica Et Biophysica Acta-Molecular and Cell Biology of Lipids,** v. 1533, n. 3, p. 233-242, Oct 2001. ISSN 1388-1981.

PETERSEN, J. L. et al. Genome-Wide Analysis Reveals Selection for Important Traits in Domestic Horse Breeds. **Plos Genetics,** v. 9, n. 1, Jan 2013. ISSN 1553-7404.

PHILP, A.; MACDONALD, A. L.; WATT, P. W. Lactate - a signal coordinating cell and systemic function. **Journal of Experimental Biology,** v. 208, n. 24, p. 4561-4575, Dec 2005. ISSN 0022-0949.

POOLE, D. C.; ERICKSON, H. H. Cardiovascular function and oxygen transport: responses to exercise and training. In: KANEPS, K. W.;HINCHCLIFF, R. J*., et al* (Ed.). **Equine**

Exercise Physiology. Edinburgh: W.B. Saunders, 2008. cap. 4.1, p.212-245. ISBN 978-0-7020-2857-1.

POOLE, R. C.; HALESTRAP, A. P. Transport of lactate and other monocarboxylates across mammalian plasma-membranes. **American Journal of Physiology,** v. 264, n. 4, p. C761-C782, Apr 1993. ISSN 0002-9513.

PÖSÖ, A. R. Monocarboxylate transporters and lactate metabolism in equine athletes: A review. **Acta Veterinaria Scandinavica,** v. 43, n. 2, p. 63-74, 2002. ISSN 0044-605X.

PÖSÖ, A. R.; LAMPINEN, K. J.; RÄSÄNEN, L. A. Distribution of lactate between red blood cells and plasma after exercise. **Equine Veterinary Journal,** v. 27, n. S18, p. 231-234, 1995. ISSN 2042-3306.

PY, G. et al. Effects of streptozotocin-induced diabetes on markers of skeletal muscle metabolism and monocarboxylate transporter 1 to monocarboxylate transporter 4 transporters. **Metabolism-Clinical and Experimental,** v. 51, n. 7, p. 807-813, Jul 2002. ISSN 0026-0495.

RADIN, M.; EUBANK, M.; WEISER, M. Electronic measurement of erythrocyte volume and volume heterogeneity in horses during erythrocyte regeneration associated with experimental anemias. **Veterinary Pathology Online,** v. 23, n. 6, p. 656-660, 1986. ISSN 0300-9858.

RÄSÄNEN, L. A.; LAMPINEN, K. J.; PÖSÖ, A. R. Responses of blood and plasma lactate and plasma purine concentrations to maximal exercise and their relation to performance in standardbred trotters. **American Journal of Veterinary Research,** v. 56, n. 12, p. 1651-1656, Dec 1995. ISSN 0002-9645.

RAWSON, E. S.; PERSKY, A. M. Mechanisms of muscular adaptations to creatine supplementation. **International Sportmed Journal,** v. 8, n. 2, p. 43-53, 2007 2007. ISSN 1528-3356.

REEBEN, M. et al. MCT1 and CD147 gene polymorphisms in standardbred horses. **Equine Veterinary Journal. Supplement**, n. 36, p. 322-5, Aug 2006.

REED, G. H.; KENT, J. O.; WITTWER, C. T. High-resolution DNA melting analysis for simple and efficient molecular diagnostics. **Pharmacogenomics,** v. 8, n. 6, p. 597-608, Jun 2007. ISSN 1462-2416.

REED, G. H.; WITTWER, C. T. Sensitivity and specificity of single-nucleotide polymorphism scanning by high-resolution melting analysis. **Clinical Chemistry,** v. 50, n. 10, p. 1748-1754, Oct 2004. ISSN 0009-9147.

REVOLD, T. et al. Effects of training on equine muscle fibres and monocarboxylate transporters in young Coldblooded Trotters. **Equine Veterinary Journal,** v. 42, p. 289-295, Nov 2010. ISSN 0425-1644.

RIRIE, K. M.; RASMUSSEN, R. P.; WITTWER, C. T. Product Differentiation by Analysis of DNA Melting Curves during the Polymerase Chain Reaction. **Analytical Biochemistry,** v. 245, n. 2, p. 154-160, 1997. ISSN 0003-2697.

RODGERS, B. D.; GARIKIPATI, D. K. Clinical, agricultural, and evolutionary biology of myostatin: A comparative review. **Endocrine Reviews,** v. 29, n. 5, p. 513-534, Aug 2008. ISSN 0163-769X.

ROZEN, S.; SKALETSKY, H. Primer3 on the WWW for general users and for biologist programmers. In: (Ed.). **Bioinformatics methods and protocols**: Springer, 1999. p.365-386. ISBN 0896037320.

SALAMON, R. V. et al. Synthetic methods for obtaining conjugated linoleic acids (CLA) by catalysis. **Acta Univiversitatis Sapientiae, Alimentaria,** v. 5, p. 32-51, 2012. ISSN 2066-7744.

SANGUINETTI, C. J.; NETO, E. D.; SIMPSON, A. J. G. Rapid silver staining and recovery of PCR products separated on polyacrylamide gels **Biotechniques,** v. 17, n. 5, p. 914-922, Nov 1994. ISSN 0736-6205.

SAREMI, A. et al. Effects of oral creatine and resistance training on serum myostatin and GASP-1. **Molecular and Cellular Endocrinology,** v. 317, n. 1-2, p. 25-30, Apr 2010. ISSN 0303-7207.

SCHRÖDER, W.; KLOSTERMANN, A.; DISTL, O. Candidate genes for physical performance in the horse. **Veterinary Journal,** v. 190, n. 1, p. 39-48, Oct 2011. ISSN 1090-0233.

SCHUBACK, K.; ESSEN-GUSTAVSSON, B.; PERSSON, S. G. B. Effect of creatine supplementation on muscle metabolic response to a maximal treadmill exercise test in Standardbred horses. **Equine Veterinary Journal,** v. 32, n. 6, p. 533-540, Nov 2000. ISSN 0425-1644.

SCHUELKE, M. et al. Brief report - Myostatin mutation associated with gross muscle hypertrophy in a child. **New England Journal of Medicine,** v. 350, n. 26, p. 2682-2688, Jun 2004. ISSN 0028-4793.

SEWELL, D.; HARRIS, R. Effects of creatine supplementation in the Thoroughbred horse. **Equine Veterinary Journal,** v. 27, n. S18, p. 239-242, 1995. ISSN 2042-3306.

SKELTON, M. S. et al. Lactate influx into red blood cells of athletic and nonathletic species. **American Journal of Physiology-Regulatory Integrative and Comparative Physiology,** v. 268, n. 5, p. R1121-R1128, May 1995. ISSN 0363-6119.

SNOW, R. J.; MURPHY, R. M. Creatine and the creatine transporter: A review. **Molecular and Cellular Biochemistry,** v. 224, n. 1-2, p. 169-181, 2001. ISSN 0300-8177.

SZABÓ, G. et al. A deletion in the myostatin gene causes the compact (Cmpt) hypermuscular mutation in mice. **Mammalian Genome,** v. 9, n. 8, p. 671-672, Aug 1998. ISSN 0938-8990.

TACHIKUI, H. et al. Genomic organization and promoter activity of embigin, a member of the immunoglobulin superfamily. **Gene,** v. 240, n. 2, p. 325-332, Nov 1999. ISSN 0378-1119.

TAYLOR, C. F. Mutation scanning using high-resolution melting. **Biochemical Society Transactions,** v. 37, p. 433-437, Apr 2009. ISSN 0300-5127.

THOMAS, C. et al. Effects of acute and chronic exercise on sarcolemmal MCT1 and MCT4 contents in human skeletal muscles: current status. **American Journal of Physiology-Regulatory Integrative and Comparative Physiology,** v. 302, n. 1, p. R1-R14, Jan 2012. ISSN 0363-6119.

TOSCO, M. et al. A creatine transporter is operative at the brush border level of the rat jejunal enterocyte. **Journal of Membrane Biology,** v. 202, n. 2, p. 85-95, Nov 2004. ISSN 0022-2631.

TOZAKI, T. et al. A cohort study of racing performance in Japanese Thoroughbred racehorses using genome information on ECA18. **Animal Genetics,** v. 43, n. 1, p. 42-52, Feb 2012. ISSN 0268-9146.

TOZAKI, T. et al. A genome-wide association study for racing performances in Thoroughbreds clarifies a candidate region near the MSTN gene. **Animal Genetics,** v. 41, p. 28-35, Dec 2010. ISSN 0268-9146.

TOZAKI, T. et al. Sequence Variants at the myostatin Gene Locus Influence the Body Composition of Thoroughbred Horses. **Journal of Veterinary Medical Science,** v. 73, n. 12, p. 1617-1624, Dec 2011. ISSN 0916-7250.

VÄIHKÖNEN, L. K. et al. Lactate-transport activity in RBCs of trained and untrained individuals from four racing species. **American Journal of Physiology-Regulatory Integrative and Comparative Physiology,** v. 281, n. 1, p. R19-R24, Jul 2001. ISSN 0363-6119.

VÄIHKÖNEN, L. K.; HYYPPÄ, S.; PÖSÖ, A. R. Factors affecting accumulation of lactate in red blood cells. **Equine veterinary journal. Supplement**, n. 30, p. 443-7, Jul 1999.

VÄIHKÖNEN, L. K.; OJALA, M.; PÖSÖ, A. R. Age-related changes and inheritance of lactate transport activity in red blood cells. **Equine Veterinary Journal. Supplement**, n. 34, p. 568-72, Sep 2002.

VÄIHKÖNEN, L. K.; PÖSÖ, A. R. Interindividual variation in total and carrier-mediated lactate influx into red blood cells. **American Journal of Physiology-Regulatory Integrative and Comparative Physiology,** v. 274, n. 4, p. R1025-R1030, Apr 1998. ISSN 0363-6119.

VAN DER STOEP, N. et al. Diagnostic guidelines for high-resolution melting curve (HRM) analysis: An interlaboratory validation of BRCA1 mutation scanning using the 96-well LightScanner™. **Human mutation,** v. 30, n. 6, p. 899-909, 2009. ISSN 1098-1004.

VAN WIJLEN, R. P. J. Long-term conjugated linoleic acid supplementation in humans - effects on body composition and safety. **European Journal of Lipid Science and Technology,** v. 113, n. 9, p. 1077-1094, Sep 2011. ISSN 1438-7697.

VARGA, L. et al. Inheritance and mapping of Compact (Cmpt), a new mutation causing hypermuscularity in mice. **Genetics,** v. 147, n. 2, p. 755-764, 1997. ISSN 0016-6731.

VOSSEN, R. H. A. M. et al. High-Resolution Melting Analysis (HRMA)-More Than Just Sequence Variant Screening. **Human Mutation,** v. 30, n. 6, p. 860-866, Jun 2009. ISSN 1059-7794.

WANG, T.; LEE, H. G. Advances in Research on cis-9, trans-11 Conjugated Linoleic Acid: A Major Functional Conjugated Linoleic Acid Isomer. **Critical Reviews in Food Science and Nutrition,** v. 55, n. 5, p. 720-731, 2015. ISSN 1040-8398.

WILLIAMSON, S. A.; BEILHARZ, R. G. The inheritance of speed, stamina and other racing performance characters in the Australian Thoroughbred. **Journal of Animal Breeding and Genetics-Zeitschrift Fur Tierzuchtung Und Zuchtungsbiologie,** v. 115, n. 1, p. 1-16, Mar 1998. ISSN 0931-2668.

WITTWER, C. T. High-Resolution DNA Melting Analysis: Advancements and Limitations. **Human Mutation,** v. 30, n. 6, p. 857-859, Jun 2009. ISSN 1059-7794.

WITTWER, C. T. et al. High-resolution genotyping by amplicon melting analysis using LCGreen. **Clinical chemistry,** v. 49, n. 6, p. 853-860, 2003. ISSN 0009-9147.

YOUNG, L. E.; ROGERS, K.; WOOD, J. L. N. Left ventricular size and systolic function in Thoroughbred racehorses and their relationships to race performance. **Journal of Applied Physiology,** v. 99, n. 4, p. 1278-1285, 2005.

ZHOU, L. et al. Closed-tube genotyping with unlabeled oligonucleotide probes and a saturating DNA dye. **Clinical Chemistry,** v. 50, n. 8, p. 1328-1335, 2004. ISSN 0009-9147.

ZIMMERS, T. A. et al. Induction of cachexia in mice by systemically administered myostatin. **Science,** v. 296, n. 5572, p. 1486-1488, May 2002. ISSN 0036-8075.

ZIOUZENKOVA, O. et al. Peroxisome proliferator-activated receptors. **Current Atherosclerosis Reports,** v. 4, n. 1, p. 59-64, Jan 2002. ISSN 1523-3804.