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**CLINICOPATHOLOGIC FINDINGS IN THOROUGHBRED
HORSES DURING A HIGH SPEED – SHORT TERM
COMPETITION**

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Introduction

Blood plays an essential role during exercise. Its components provide transport of oxygen, electrolytes, hormones, water and nutrients to the exercising muscle, and at the same time provide removal of carbon dioxide and metabolism waste products, which result from exercise. Moreover, blood components are dynamically involved in the active reestablishment of acid-base imbalances created by exercise.

Throughout the years, various tests were created to evaluate changes of blood constituents related to exercise. The aim of these tests was to assess athlete's fitness condition, performance potential, and to investigate and correct poor performance. However, a wide range of changes in blood constituents are influenced by various factors like athlete's training condition, type of exercise, and sampling method, therefore throughout the years many studies have focused on different test combinations or on standardization of new tests.

The aim of this thesis was the evaluation of the occurrence of myopathy, hemolysis and hypercoagulation that occur in equine athletes after a high speed-short term competition. This is a three-year study, and for this purpose Thoroughbred horses that attend the Niballo horse race within Faenza City's medieval reenactment were included in the present study. Pre- and post-race blood samples were taken from each subject and assessed for clinicopathological data. The results have evidenced myopathy, oxidative stress, hemolysis and alteration of the coagulation-fibrinolysis axis, which occurred after the race in the cohort of the present study.

1. Exercise and muscular damage

Poor athletic performance is a common sign encountered in equine sport medicine. Evaluation and understanding of the causes that lead to this condition represented and still remains a current interest for veterinarians. Most of the studies in equine sport medicine regarding athletic performance were based on human sport medicine study designs. Two of the most studied topics in human sport medicine and equine sport medicine are represented by exercise related muscular damage and oxidative stress.

Many studies have been conducted on human and equine athletes to establish the cause and pathological mechanism of muscular damage related to exercise. These studies have considered various aspects, but the most relevant topics focused on anatomy and physiology of skeletal muscular during exercise, on the influence of various types of exercise on promoting skeletal muscle damage, and on metabolic changes during exercise that lead to myopathy.

1.1. Review of human sport medicine literature

Numerous studies in human literature have attempted to evaluate and understand the structural changes that occur with exercise in the skeletal muscle. The studies on specific characteristic of myosin, especially heavy chain myosin (HCM), led to the identification of several isoforms (Brooke M.H. & Kaiser K.K., 1970). With the aid of special methods, histochemical staining for oxidative enzymes, like adenosine

triphosphatases (ATPases), immunohistochemistry for HCM (Brooke M.H. & Kaiser K.K., 1974), and electrophoresis (Billeter R. et al., 1981), further studies on isolated single muscle fibers have evidenced that muscle fibers contain a single or multiple HCM isoforms, naming them pure type muscle fibers and hybrid type muscle fibers, respectively. Based on major MCH isoform, the pure type fibers are further divided in types: slow type I fiber, fast type fiber IIA, fast type fiber IID (formerly known as IIB) and fast type fiber IIX (Schiaffino S. & Reggiani C., 1994; Pette D. & Staron R.S., 2000).

The human skeletal muscle is composed of a mixture of slow and fast muscular fibers, and the proportion of these fibers can vary within different parts of the same muscle (Lexell J. et al., 1983). All types of exercise, but in particular ones that involve eccentric contraction, promote damage within fast type fibers because of their lower oxidative capacity and their shorter length (Lieber R.L. & Fridén J., 1999; van Wessel T. et al., 2010). In another study, Guerrero and colleagues, using diagnostic imaging techniques (Fig. 1-3), brought more data reinforcing the concept of higher susceptibility of fast type fiber to exercise-induced damage in comparison with slow type fibers (Guerrero M. et al., 2008).

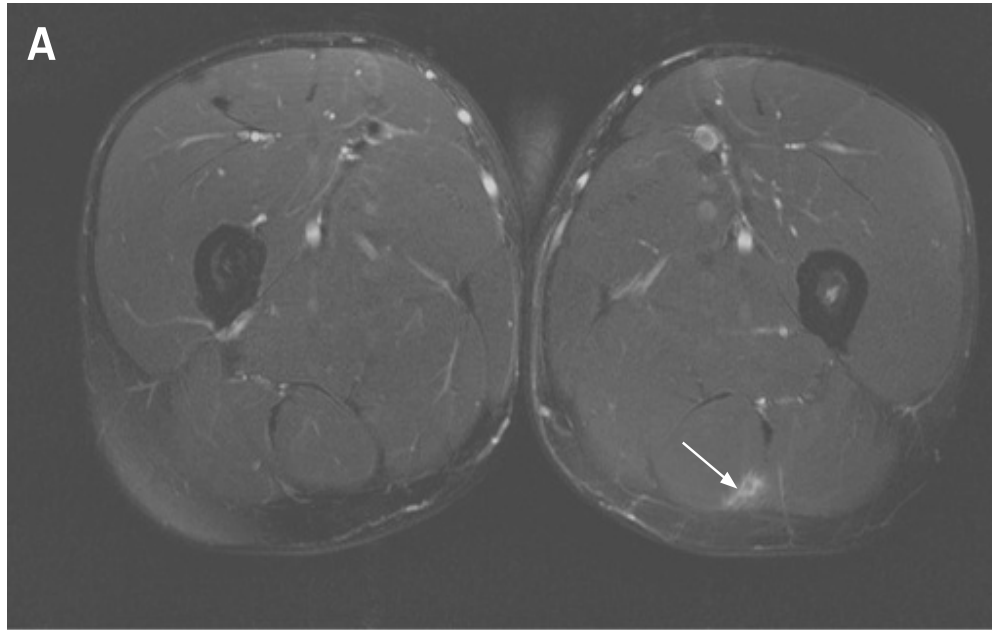


Figure 1 (Guerrero M. et al., 2008). Magnetic resonance image from an axial view showing the presence of a lesion on the biceps femoris, upper part of posterior side of right thigh. The white arrow is showing a muscular edema. This type of lesions are mainly present in the fast type fibers.

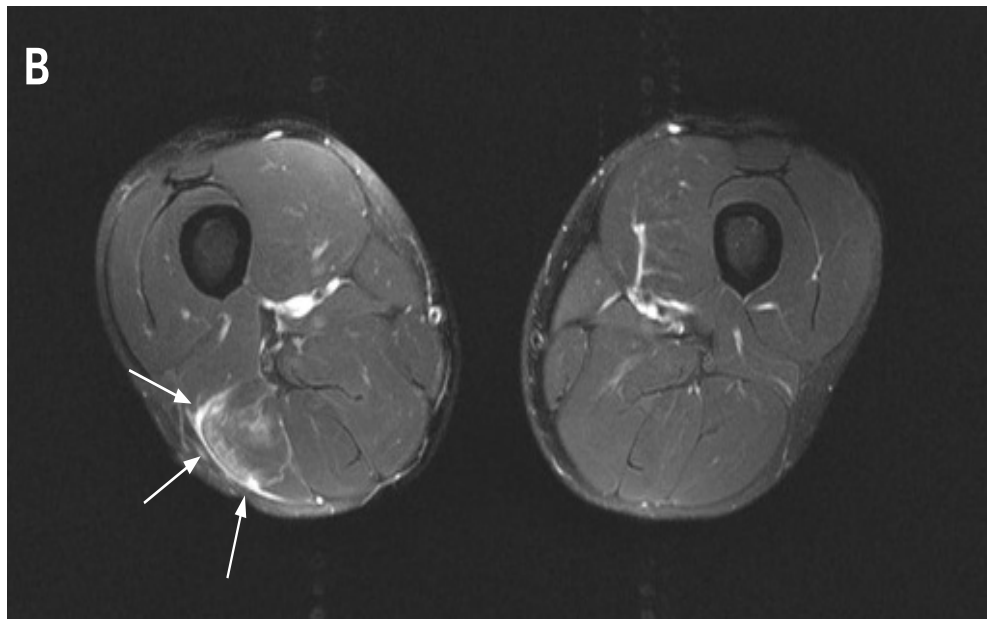


Figure 2 (Guerrero M. et al., 2008). Magnetic resonance image from an axial view showing a lesion present in the medial-distal part of the posterior side of the left thigh. The presence of edema is noted along with a fibrillar defect lesion in the long head of the biceps femoris. This is a more pronounced lesion, indicated by white arrows, and involves both, type I and type II fibers.

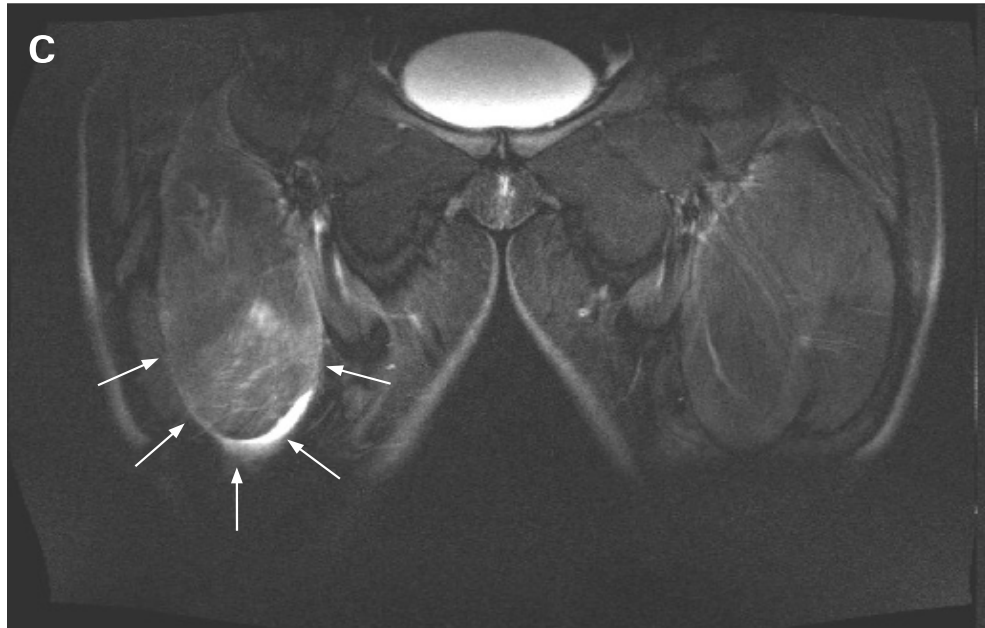


Figure 3 (Guerrero M. et al., 2008). Magnetic resonance image from a coronal view of the anterior side of the thigh. The arrows point out the presence of edema and a severe and extended fibrillar defect.

One of the first studies of sports-related muscular damage evidenced the occurrence of this phenomenon in athletes that are unaccustomed to exercise or in athletes that perform a sustained, intense workout (Hough T., 1900). Studies of human muscle mechanics have revealed that muscles can perform three types of contractions, which in the early 1900's were termed as myometric, isometric and plyometric (Hubbard A.W. & Stetson R.H., 1938). These terms were formed using prefixes from Greek language and added to the noun "metric", where "myo" means shorter, "iso" means the same, and "plyo" means larger. Later, in 2003, Faulkner explained how the scientific community and literature have changed the terminology, introducing new words for describing muscular contraction, "concentric" and "eccentric" (Faulkner J.A., 2003). Therefore, an eccentric muscular contraction presumes an action where the muscle lengthens under contraction, a concentric muscular contraction presumes an action where the muscle shortens, and an isometric muscular contraction presumes an action where the length of the muscle remains unchanged.

Numerous studies and reviews sustain that exercises that involve eccentric contractions have the most damaging effects on muscles. In a study from 2001, Proske and Morgan try to formulate and explain the events (Fig. 4) that lead to muscular damage following eccentric contractions, and promote the hypothesis of “popping sarcomere”. These authors sustain that a sudden change in the contracting force or a high intensity contraction lead to a non-uniform contraction of sarcomeres, and some of these sarcomeres are stretched at the point that they are damaged (Proske U. & Morgan D.L., 2001).

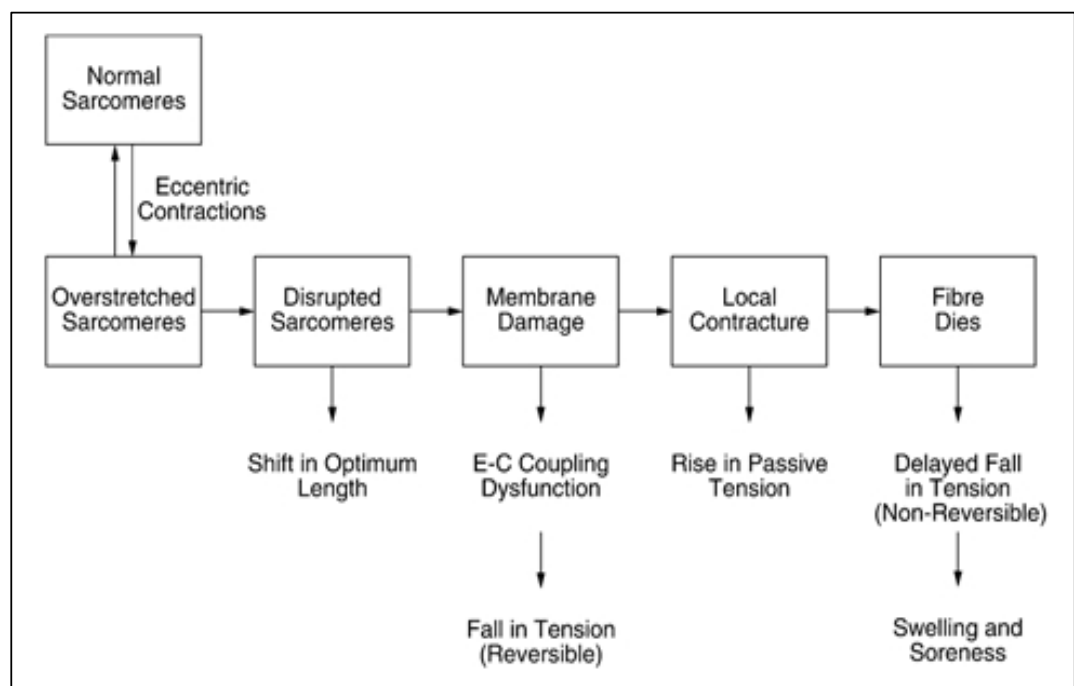


Figure 4 (Proske U. & Morgan D.L., 2001). Diagram of events that lead to muscular damage following eccentric contractions.

There are other studies that promote another hypothesis. After eccentric contraction that induces a structural change follows an impairment of calcium (Ca^{2+}) homeostasis (Proske U. & Morgan D.L., 2001; Tee J.C. et al., 2007). Taking inspiration from studies on mice (Duncan C.J. & Jackson M.J., 1987) and frogs (Duncan C.J., 1987), in human sport medicine it was hypothesized that the same mechanisms contribute and promote exercise-induced muscular damage. The damage induced by

exercise to the sarcoplasmic reticulum alters its function in Ca^{2+} homeostasis, which leads to accumulation of Ca^{2+} in the intracellular space and subsequent activation of Ca^{2+} overload phase. The defense mechanisms against Ca^{2+} are overwhelmed, and Ca^{2+} -dependent proteolytic and phospholipolytic enzymes are activated. These enzymes will damage myofibre structures, and contribute to muscular damage.

Numerous studies are focused on finding the ideal markers of muscular damage. One of the most used markers is serum creatine kinase (CK) because of its magnitude of increase compared to other proteins and the relative low cost of the assay. From the studies conducted until now it has been noted that there are many variables that influence the relationship between muscular damage and release of CK from muscles, and between CK and other markers of muscular damage (Clarkson P.M. & Hubal M.J., 2002). Other markers used to evaluate muscular damage are lactate dehydrogenase (LDH), aspartate transaminase (AST), carbonic anhydrase isoenzyme II, heart fatty acid binding protein, troponin (Sorichter S. et al., 1999), myosin heavy chains (Sorichter S. et al., 1999; Guerrero M. et al., 2008) and myosin light chains (Guerrero M. et al., 2008).

Other phenomenon that contributes in amplifying muscular damage is the inflammatory response secondary to the initial trauma to the muscular fibers (Fielding R.A. et al., 1993; Clarkson P.M. & Hubal M.J., 2002). The inflammatory cells promote the formation of free radicals, which lead to oxidative damage of the muscular cells, which will be further discussed in the oxidative stress chapter. From the numerous studies available in human literature it has been seen that the extent of inflammatory response secondary to exercise-induced muscular damage is influenced by various factors, like type of exercise (Kokot K. et al., 1988; Peeling P. et al., 2009) and group of muscles (Hirose L. et al., 2004).

Despite the abundant literature the exact mechanisms of damage, reparation and adaptation have not been fully understood yet (Howatson G. & van Someren K.A., 2008; Nikolaidis M.G. et al., 2008).

1.2 Review of equine sport medicine literature

In agreement with human sport medicine literature, in equine sport medicine there is an extensive specialized literature focused on exercise and its impact on skeletal muscle. A high percentage of these studies were concluded on Standardbred, Thoroughbred and endurance horses, and only a minor percentage on horses from other disciplines (Rivero J.-L. L., 2007).

As with human skeletal muscle, many studies were concluded to best identify the anatomy and physiology of the equine skeletal muscle. Immunohistochemistry, electrophoresis, staining for ATPase, were used to identify and classify MHC composition of the equine skeletal muscle fibers. Horses, like other mammals, have two main pure types of fibers, type I (slow oxidative) and type II (fast oxidative) (Fig. 5). Among the II type fibers, the horse has fast oxidative type IIA fibers and fast glycolytic type IIB fibers (Rivero J.-L. L.¹ et al., 1996). The type IIB fiber can be found named as type IIX in more recent studies. In another study, Rivero and colleagues, with the ATPase method, have evidenced the presence of a fourth type of fiber IIAB (IIAX) (Rivero J.-L. L.² et al., 1996). Other authors confirm the presence of the fourth type of hybrid fiber, type IIA/IIX. Moreover, these authors have demonstrated by succinic dehydrogenase (SDH) analysis of each type of fibers that type IIA/IIX

fibers have a more fast contraction ability and higher resistance to fatigue (Yamano S. et al., 2002).

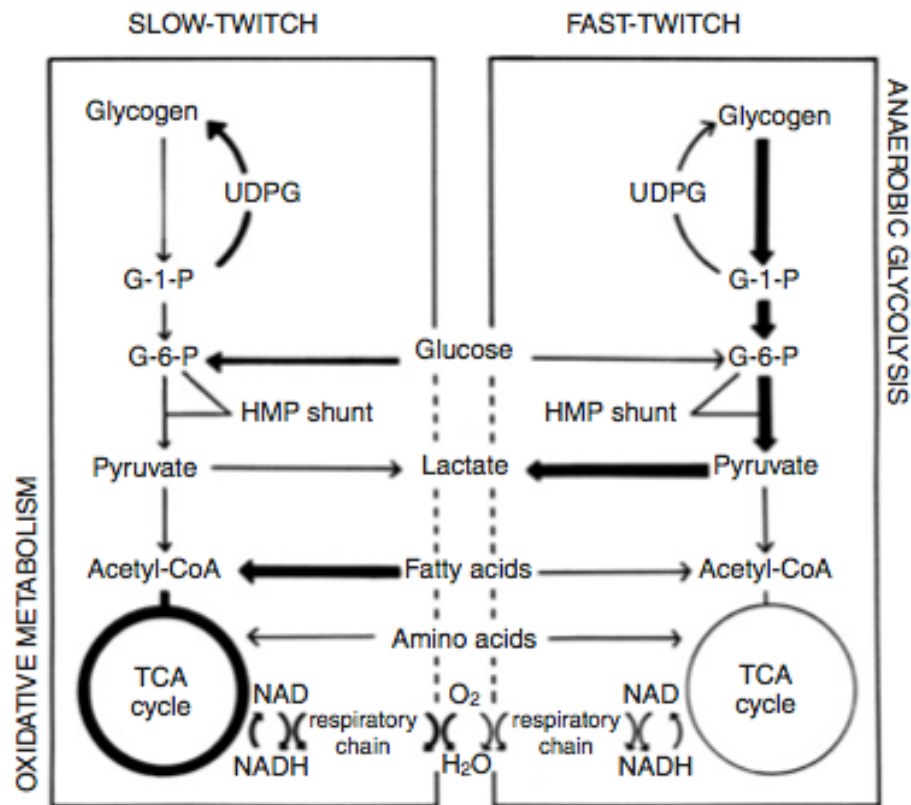


Figure 5. (Valberg S.J., 200) Metabolism in slow and fast twitch myofibers.

Numerous studies were performed to assess the influence of exercise and training protocols upon the transition and recruitment of muscular fibers. These studies have underlined that an increase in exercise duration and intensity will lead to the progressive transition from type I → type IIA → type IIB (IIX) (Hodgson D.R. et al., 1985; Valberg S., 1986; Essén-Gustavsson B. et al., 1995; Schuback K. & Essén-Gustavsson B., 1998). With the discovery of the fourth type of fiber, IIA/IIX, later studies have evidenced that higher intensity exercises leads to the recruitment of this type of fibers (Yamano S. et al., 2002; Rivero J.-L. L. et al., 2007).

Many articles in the equine sport medicine literature have studied different training schedules in order to prepare and improve the athletic condition of horses, and to avoid muscular damage (Rivero J.-L. L., 2007). The topic of exercise-induced muscular damage in horses is assessed with a wider focus on the markers of muscular injury. Terms like eccentric and concentric muscular contractions are not used in equine sport medicine. If in human literature exercises that presume eccentric contractions are considered high intensity exercises, in equine sport medicine the endurance race is considered as a high intensity exercise that requires superior athletic ability (Hinchcliff K.W. & Geor R.J., 2004).

In equine sport medicine literature it was hypothesized that imbalanced Ca^{2+} homeostasis might be involved in muscular damage related to exercise (Hodgson D.R., 1993). Ultrastructural alterations found in muscles from horses with rhabdomyolysis can lead to an imbalanced permeability of sarcoplasmic reticulum (Lindholm A. et al., 1974), which determines intracellular accumulation of Ca^{2+} (Byrd S.K. et al., 1989). This excessive accumulation of intracellular Ca^{2+} will determine the activation of enzymes that will contribute in amplifying muscular damage as seen in people (Proske U. & Morgan D.L., 2001). The Ca^{2+} hypothesis can be sustained by the studies on agents that interfere with the function of Ca^{2+} channels. Beech J. and collaborators have used phenytoin in horses and have managed to reduce the incidence of myopathy secondary to exercise, supposing that this agent could influence the permeability of sarcoplasmic reticulum (Beech J. et al., 1988).

The most frequently used makers used to detect muscular damage related to exercise are CK, LDH and AST. In one study, Anderson M.G. sustains that AST is released from cells due to a change in membrane permeability and not necessary due cellular breakdown. Moreover, the author sustains that CK is the most specific enzyme for muscular damage and it is parameter of choice in this type of studies (Anderson M.G., 1975). Later,

Volfinger L. and colleagues have studied the kinetics of CK in the horse and concluded that there are some differences between human and equine athletes, like the relationship between CK and athletic preparation encountered in human athletes, and bioavailability of CK in horses (Volfinger L. et al., 1994). Chiaradia L. and colleagues have studied exercise-induced muscular damage assessing CK and LDH, and in particular the isoenzymes specific of skeletal muscles. The authors have concluded that the intensity of exercise in their work led to lipid peroxidation, which was not intense enough to cause damage to muscular cells. In the same study the significant increase in LDH isoenzymes specific to myocardium lead to authors to hypothesize that beside skeletal muscles, myocardial muscles could be prone to exercise-induced damage (Chiaradia L. et al., 1998). In other study, Hargreaves B.J. and colleagues found AST and CK increased in association with decreased antioxidant status, suggesting leakage from damaged muscular cells following exercise (Hargreaves B.J. et al., 2002). Kim J. and colleagues in their study concluded that CK alone was not sensitive enough when compared to their findings on light and electron microscopy (Kim J. et al., 2005). Other authors studying carnosine have found it to be more selective than CK and AST plasma values in identifying muscular damage (Dunnett M. et al., 2002). Further studies are required to improve the evaluation of muscular damage in horses, perhaps by expanding the panel of markers for muscular damage.

Knowledge of exercise induced inflammatory reaction is rather limited in equine sport medicine. There are several studies that have demonstrated that exercise alter the immune response. In this study, besides demonstrating the negative effect of exercise on the immune response, the authors have revealed that post exercise samples showed an increase in leukocytes and neutrophils, as well as an increase in the production of reactive oxygen products (ROS) in non-stimulated leukocytes (Donovan

D.C. et al., 2007). There are studies that have documented the increase in myeloperoxidase (MPO) and elastase (ELT), enzymes contained mainly in the granules of neutrophils, that promote the formation of free radicals, which represents an efficient but non-selective mechanism of defense of white blood cells (Art T. et al., 2006; Lejeune J.P. et al., 2010; Serteyn D. et al., 2010). In the study of Serteyn D. and colleagues it has been also noted a correlation between MPO and ELT enzymes and CK, indicating that these enzymes can contribute in expanding the exercise-induced muscular damage (Serteyn D. et al., 2010).

2. Exercise and oxidative stress

The continuous research, in understanding the muscular damage related to exercise, led to the second most studied topic of sport medicine; exercise-induced oxidative stress. Exercise can induce an alteration to the oxidant/antioxidant balance causing oxidative stress, which has been suggested to be implicated in the pathogenesis of muscular damage, as seen previously, and in the pathogenesis of other exercise-related alterations, which will be further discussed.

2.1 Review of human sport medicine literature

The first study to investigate the oxidative stress induced by exercise in human athletes it was the study of Dillard C.J. and colleagues in 1978. They have measured in the expired air a marker of lipid peroxidation, pentane, and saw that administering vitamin E, as antioxidant, reduced the quantity of this biomarker in the expired air. It was evidenced that exercise induced lipid peroxidation, but the source of this peroxidation was not identified (Dillard C.J. et al., 1978). Since then, numerous studies and reviews were conducted on exercise-induced oxidative damage (Powers S.K. & Jackson M.J., 2007; Nikolaidis M.G. et al., 2008). Human literature on this topic assessed on human patients is rather limited, and most of these studies were conducted on rats because of the similarity between human and rat muscle, which may share the same pathways in oxidants production.

Free radicals that are generated by the cells are superoxide ($O_2^{\cdot -}$) and nitric oxid (NO). These free radicals are reactive can form reactive

oxygen species (ROS), which have been more extensively studied, and reactive nitrogen species (RNS). Continuous research has evidenced that these oxidants can be produced in many tissues, including muscles (Powers S.K. et al., 2011).

In the muscular cell ROS can be formed in various sites: mitochondrial site (Fernström M. et al., 2007; Sahlin K. et al., 2010), sarcoplasmic reticulum site (Xia R. et al., 2003), transverses tubules site (Espinosa A. et al., 2006), and membrane site (Pattwell D.M. et al., 2004).

There are several mechanisms that contribute in producing these oxidants and vary with the endogenous sites in the skeletal muscle. Within the mitochondria complex I (Lustgarten M.S. et al., 2012) and complex III (Muller F.L. et al., 2004) have been identified as sources of superoxide production within the electron transport chain. As previously seen, several muscular components can participate at the production of ROS through the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, which transfers electrons to molecular oxygen (Xia R. et al., 2003; Pattwell D.M. et al., 2004; Espinosa A. et al., 2006). Other pathway that generates ROS is represented by the phospholipase-A₂ (PLA₂). This enzyme cleaves mem

brane phospholipids, producing arachidonic acid, which is used as substrate by lipoxygenases to produce ROS (Zuo L. et al., 2004). Moreover, studies have evidenced that two types of PLA₂ exist, Ca²⁺-dependent and -independent, and it was hypothesized that during exercise the Ca²⁺-dependent is the one that is more actively involved in ROS production (Gong M.G. et al., 2006). Xanthine oxidase pathway represents another mechanism promotes ROS formation through oxidizing hypoxanthine to xanthine (Fig. 6) that will generate superoxide radicals (Sjödin B. et al., 1990; Hellsten-Westling Y., 1993; Hellstan Y. et al., 1997).

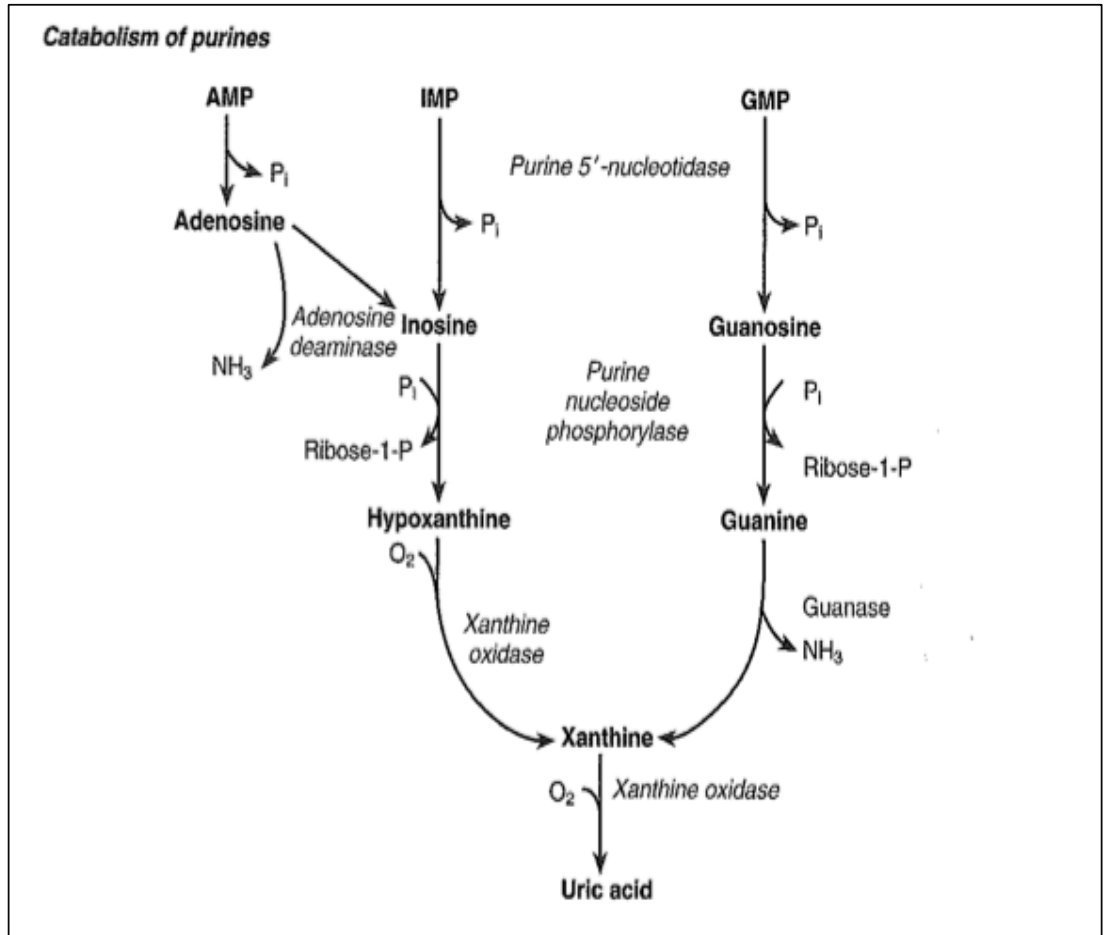


Figure 6. (Lamb E.J. & Price C.P., 2008) Catabolism of purines and formation of xanthine.

At muscular level the production of these oxidants can be further increased by several conditions encountered during exercise like, increased muscular temperature, decreased cellular pH and increased carbon dioxide (CO₂) tension (Reeder B.J. & Wilson M.T., 2001; Arbogast S. & Reid M.B., 2004).

Following eccentric exercise and muscular damage there can be an inflammatory response, as seen previously, which can promote further extension of the oxidative stress (Smolen J.E. et al., 1980; Kokot K. et al., 1988). The damage process-induced by exercise to muscles is extended to the capillaries of endothelium (Crenshaw A.G. et al., 1993), which were found to be rich in xanthine oxidase (Hellstan Y. et al., 1997). Similarly to a reperfusion injury process, the damaged endothelium will increase the

availability of xanthine oxidase, which will interact with present inflammatory cells, and increase ROS production (Hellstan Y. et al., 1997).

2.2 Review of equine sport medicine literature

In equine sport medicine literature in a 2008 review, Kirschvink and colleagues affirmed that there are three endogenous pathways related to exercise that lead to oxidative stress: electron transfer chain in the mitochondria, endogenous oxidant enzymes and oxidants that result from oxidative burst of inflammatory cells from the exercise-induced inflammatory process (Kirschvink N. et al., 2008).

The most numerous studies regarding oxidative stress in equine sport medicine were focused on endogenous oxidant enzymes, and in particularly xanthine oxidase. As seen previously during exercise, as the intensity increases, the transition between fiber types leads to the recruitment of fiber type IIB (IIX). These fibers have low content in adenosine triphosphate (ATP), and have fast glycolytic properties (Valberg S. & Essén-Gustavsson B., 1987). Intense exercise increases the demand for ATP exceeding the production ability of the organism. Subsequently there is a shift from the aerobic production to anaerobic production, which is more efficient. The anaerobic production of ATP can occur through anaerobic glycolysis (Fig. 7) and high-energy phosphorylation (Fig. 8). The result of these processes is accumulation of lactic acid and adenosine monophosphate (AMP). Excessive AMP accumulation and reduced re-phosphorylation ability of the organism, will initiate purine catabolism with production of ammonia and inosine

monophosphate (IMP). The anaerobic environment will lead to a conversion of xanthine dehydrogenase in xanthine oxidase. The later enzyme not only will breakdown hypoxanthine to uric acid, which represents a substrate for ROS production, but will continue this process even when metabolism is restored to aerobic pathway increasing the release of superoxide (Harris R.C. et al, 1991). In humans the breakdown end product of IMP is uric acid, but in horses the process ends further with the formation of allantoin (Räsänen L.A. et al., 1993).

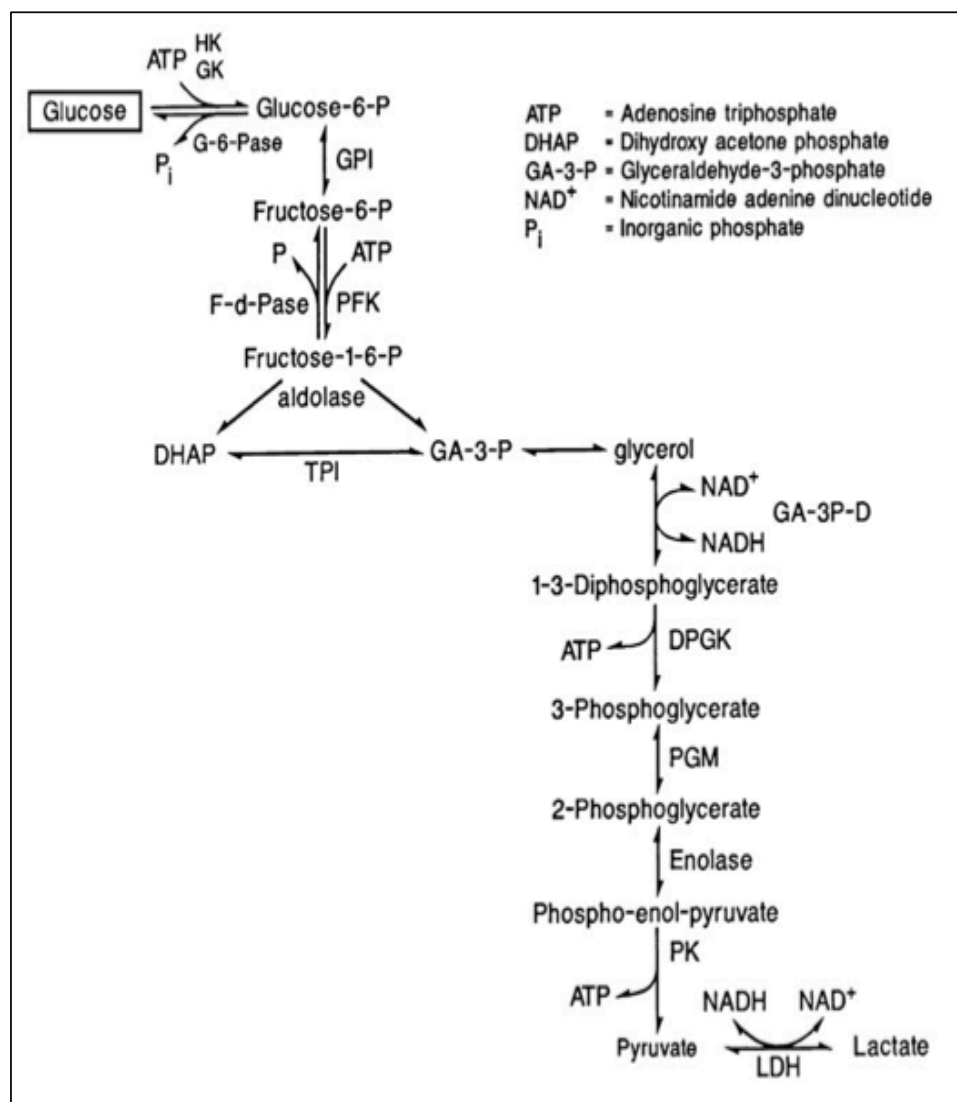


Figure 7. (Valberg S.J., 2008) Reaction scheme of anaerobic glycolysis

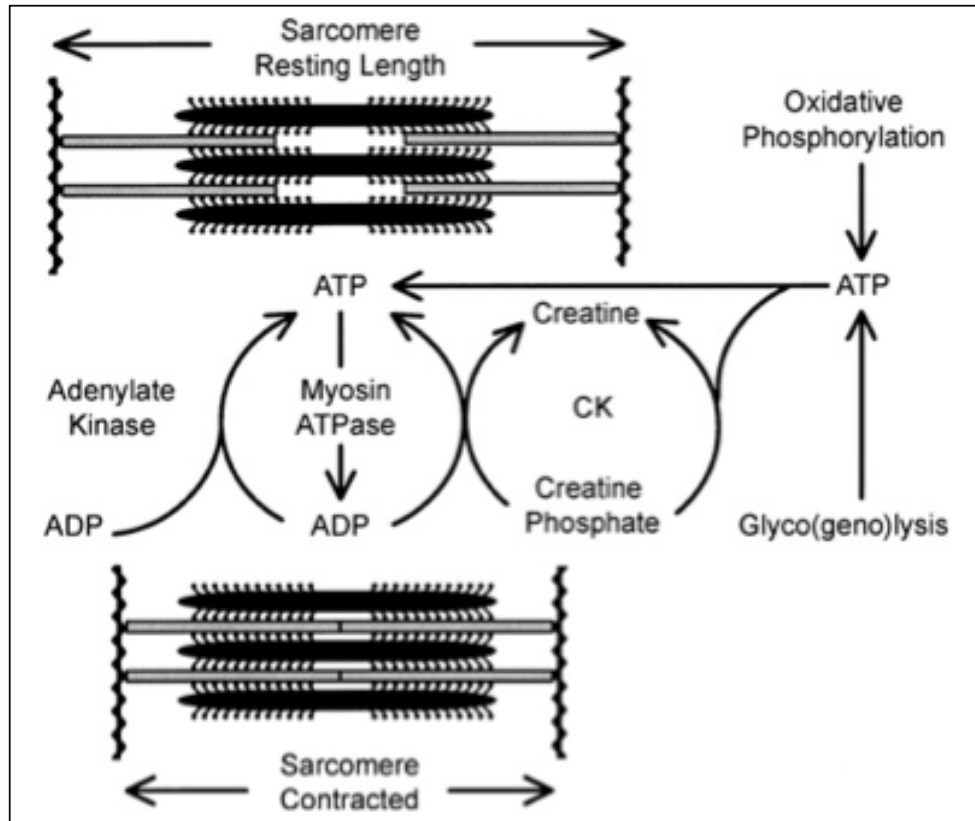


Figure 8. (Valberg S.J., 2008) Scheme of ATP production pathways.

Numerous studies were performed to evaluate the effect of variables, like training condition, type and intensity of exercise, on skeletal muscle metabolic response (Essén-Gustavsson B. & Valberg S., 1987; Valberg S. et al., 1989; Räsänen L.A. et al., 1995; Ronéus N. et al., 1995; Essén-Gustavsson B. et al., 1997; Ronéus N. et al., 1999; Evans D.L. et al., 2002; Viu J. et al., 2010). Schuback and Essén-Gustavsson have evidenced in their study that there is a positive correlation between increasing exercise intensity and increased depletion of ATP (Schuback K. & Essén-Gustavsson B., 1998). Harris R.C. and colleagues have suggested that ammonia (NH₃) could represent a better marker of anaerobic metabolism due to the fact that peaks more rapidly than uric acid (Harris R.C. et al., 1999). In a study from 2008 Kirschvink and

colleagues evidenced that the balance between oxidant and antioxidant status varies between breed, gender and age (Kirschvink N. et al., 2008).

Several studies focused on the damaging effect of exercise-induced oxidative stress. Chiaradia L. and colleagues have evaluated the exercise-induced oxidative stress and its damaging effects on muscles. Through the assessment of malondialdehyde, a product of lipid peroxidation, they have evidenced that exercise determined oxidative stress, but the extent of the oxidative stress was not great enough to damage skeletal muscle cells, as reflected by CK and LDH isoenzymes assessed in the study. They found a significant increase in LDH isoenzymes specific to myocardium hypothesizing the exercise-induced oxidative stress on myocardial muscle (Chiaradia L. et al., 1998). Other studies have demonstrated the exercise-induced oxidative damage by evidencing a positive correlation between decreased values of antioxidants and increased values of markers of muscle damage (White A. et al., 2001; Hargreaves B.J. et al., 2002).

3. Exercise-induced intravascular hemolysis

Intravascular hemolysis related to strenuous exercise represents another pathophysiological condition encountered and studied in human and equine athletes. In human sport medicine it has been postulated as a cause for sport anemia. In equine sport medicine this topic has been recently introduced thanks to the assessment of special markers, acute phase proteins (APPs).

3.1 Review of human sport medicine literature

Human sport medicine literature contains a large amount of studies and reviews, which are evidencing that intravascular hemolysis, can be related to exercise as well. Several causes have been incriminated for exercise-induced intravascular hemolysis, and among these the first cause that has been investigated, it is represented by mechanical damage to erythrocytes during exercise. This mechanism was firstly studied in runner athletes, and the crucial moment for erythrocyte destruction is represented by the moment when the athlete strikes his foot on the ground. In this phase of the exercise several variables are involved like, hardness of the running ground, distance, intensity of the stride, type of athletic foot equipment and foot temperature (Davidson R.J.L., 1964). For these reasons this mechanism was termed as “foot strike”. Later studies have identified mechanical destruction of erythrocytes in other sports that do not involve running, like swimming (Selby G.B. & Eichner E.R., 1986), and weight lifting (Schobersberger W. et al., 1990). Telford and colleagues sustain

that foot strike during running represents the major mechanical cause for exercise-induced hemolysis (Telford R.D. et al., 2003).

Beside mechanical damage, intravascular hemolysis can be caused by oxidative stress and hemorheological changes that occur with exercise. Erythrocytes are subject to oxidative damage due to their rich content in iron and increased contact with oxygen, especially during exercise. These two aspects represent essential elements in free radicals generation and subsequent oxidative damage. Moreover, free radicals generated by other mechanisms during exercise, as seen previously, can sum and amplify oxidative damage to erythrocytes. The oxidative stress is exerted especially on erythrocyte's membrane leading to a decreased deformability and consequently increased fragility (Huang Y. et al., 2000; Berzosa C. et al., 2011).

During exercise a series of hemorheological changes occur that lead to increased hematocrit (Hct) value. Plasma volume contraction, fluid shift, catecholamine-induced splenic contraction and subsequent release of stored erythrocytes and water loss with sweat, are conditions that increase blood viscosity and influence erythrocyte's resistance (Connes P. et al., 2013). Decreased deformability and hemorheological changes are mechanisms that affect mainly senescent erythrocytes (Robinson Y. et al., 2006).

Increased blood lactate from exercise may be responsible for increased erythrocyte fragility (Smith J.A. et al., 1997). More recently, Simonds and colleagues sustain that lactate does not interfere with erythrocytes deformability (Simonds M.J. et al., 2013). More studies are needed to fully elucidate the exact pathophysiological mechanisms of exercise-induced erythrocyte damage.

Other studies have evaluated the presence of exercise-induced intravascular hemolysis through the assessment of hemoglobin scavengers and acute phase reactants (Peeling P. et al., 2008; Peeling P. et al., 2009).

3.2 Review of equine sport medicine literature

Exercise-related intravascular hemolysis has been studied in equine sport medicine literature as well. In a recent study, Cywinska and colleagues have evidenced that in equine athletes, differently from what has been reported in human sport literature, male gender is more prone to intravascular hemolysis following exercise than female gender (Cywinska A. et al., 2011). In the middle of the 90's, Schott and colleagues describe for the first time the presence of hematuria and pigmenturia following exercise. The authors have noted that urinary changes increased in parallel with exercise intensity. The origin of pigmenturia was not specified, but it was hypothesized to be a consequence of muscular damage or hemolysis (Schott II H.C. et al., 1995).

In horses, the mechanical damage responsible for intravascular hemolysis was evaluated through studies conducted erythrocytes released by splenic contraction induced by exercise. Hanzawa and colleagues in series of studies on normal and splenectomised horses have evidenced that continuous accumulation and release of erythrocytes from the spleen makes them susceptible for mechanical and chemical stress. The mechanical stress is represented by the passage of erythrocytes through the splenic vessels during splenic contraction (Hanzawa K. et al., 1995; Hanzawa K. et al., 1999a).

Several factors contribute to the osmotic stress of erythrocytes. Boucher proposed that splenic storage of erythrocytes represents the major cause for increased osmotic fragility. Within the spleen erythrocytes are kept in a hypoxic environment, which determines erythrocyte's ATP depletion and subsequent increased osmotic fragility. These storage conditions are reflected by the release of "spiculated" erythrocytes (Boucher J.H. et al.,

1981). Later studies show contradictory results evidencing the lack of echinocytes in circulation following exercise, and that exercise-induced ATP depletion was similar for all erythrocytes, from the splenic pool and from outside the spleen (Smith J.E. et al., 1989; Snow D.H. & Martin V., 1990). Hanzawa and colleagues confirm the initial hypothesis that erythrocytes from splenic pool are more susceptible to lysis by evaluating the effect of different types of exercise on the erythrocytes from splenectomised and non-splenectomised horses, and by direct evaluation of hemolysis rate using a 0,56% hypotonic salt solution (Hanzawa K. et al 1999a; Hanzawa K. et al 1999b). Exercise-induced lactate accumulation, as well as changes in pH and temperature contribute and further extend the susceptibility of red blood cells to osmotic damage (Hanzawa K. et al., 1998; Hanzawa K. et al., 1999).

Exercise-induced oxidative stress, as seen in people, promotes intravascular hemolysis in horses by the same mechanisms. Matsuki and colleagues evidenced the effect of oxidative stress on erythrocytes related to exercise by assessing peroxidized phosphatidylethanolamine and MDA resulted from erythrocyte membrane lipid peroxidation (Matsuki N. et al., 1991). Avellini and colleagues demonstrated enhanced resistance of erythrocytes to oxidative stress in vitro by lower levels of MDA following antioxidant supplementation (Avellini L. et al, 1999).

Other studies have evaluated intravascular hemolysis-related to exercise by assessing acute phase proteins. Some of these proteins are positive acute phase reactants, but at the same time these proteins are hemoglobin scavengers as well. Due to this considerations, regardless of the inflammatory stimulus produced by intense exercise, a low value of these proteins reflect the occurrence of intravascular hemolysis following exercise. Among these reactants the most extensively studied acute phase protein is haptoglobin (Hp) (Willet K. & Blakmore D.J., 1979; Hanzawa

K. et al., 2002; Pellegrini Masini A. et al., 2003; Inoue Y. et al., 2005; Cywinska A. et al., 2011).

If in human medicine intravascular hemolysis has been postulated as a cause of sports anemia, in the horse, Inoue and colleagues have demonstrated by expanding the panel of acute phase proteins that exercise-induced intravascular hemolysis does not induce anemia (Inoue Y. et al., 2005).

4. Exercise and coagulation-fibrinolysis axis

It has been hypothesized that exercise has positive effect in maintaining health and prevents diseases such as coronary heart disease. Over the last two decades several studies has been conducted to understand the influence of exercise at various levels of coagulation and fibrinolysis processes, and how exercise can be of benefit from this point of view.

4.1 Review of human sport medicine literature

Hemostatic abnormalities related to exercise that have been extensively studied in human sport medicine are platelet activation, activation of the coagulation cascade and activation of fibrinolysis (Colwell J.A, 1986; Smith J.E., 2003).

Following exercise it was reported that platelet number increased due to mobilization from organs like spleen, bone marrow, and lungs (Dawson A. & Ogston D., 1969; Prisco D. et al., 1994). Tissue damage induced by exercise leads to exposure of collagen, which will determine platelet adherence and activation. The more intense the exercise is, the more extended the platelet activation is (Hanke A.A. et al., 2010). Thromboxane A₂, adenosine diphosphate (ADP), β -thromboglobulin and platelet factor 4, are mediators subsequently released to adherence and activation of platelets at the site of exposed collagen and will extend platelet activation process. Parts of these mediators are derived from other exercise-induced processes, like oxidative damage to phospholipids (Prisco D. et al., 1994; Smith J.E., 2003).

Coagulation cascade is activated by exercise probably by similar mechanisms. Several studies and reviews have evidenced that exercise induces a rise in coagulation factors, like factor VIII (FVIII) and von Willebrand factor (vWF) due to catecholamine stimulation and endothelial activation (Prisco D. et al., 1994; Rock G. et al., 1996; Smith J.E., 2003; Ribeiro J. et al., 2007). Fibrinogen represents another parameter that has been assessed in sport medicine. The conflicting results obtained by the studies so far are evidencing that exercise-induced activation of coagulation cascade has not been thoroughly understood yet (Mandalaki T. et al., 1977; Prisco D. et al., 1998; El-Sayed M. et al., 1999; Smith J.E., 2003).

Exercise influences fibrinolysis cascade as well. The activation of this process was documented in human sport literature with the assessment of fibrinolysis activators (tissue-type plasminogen activator), fibrinolytic inhibitors (plasminogen activator inhibitor), fibrinolytic agents (antithrombin III-ATIII), fibrin degradation products (d-dimers - D-D, fibrin degradation products) and fibrinogen degradation products (fibrinopeptide A) (Marsh N.A. & Gaffney P.J., 1982; Colwell J.A., 1986; Herren T. et al., 1992; Prisco D. et al., 1994; Prisco D. et al., 1998; Hilberg T. et al., 2003; Smith J.E., 2003). The fibrinolytic pathway is activated through multiple mechanisms like exercise-induced catecholamine stimulus, endothelial activation by oxidative damage, exercise-induced metabolic changes. It has been evidenced that the accumulation of lactate following exercise promotes the activation of fibrinolysis, and the intensity of fibrinolysis is correlated to lactate concentration and intensity of exercise (Womack C.J. et al., 2006).

Even though human sports literature is abundant regarding studies on exercise and its effect on hemostasis, the contradictory results shown by these studies suggest that further studies are needed to elucidate the effect of

exercise on the activation of coagulation and fibrinolysis cascades and the temporal relationship between these processes during exercise.

4.2 Review of equine sport medicine literature

Hemostasis topic in equine sport medicine has not been extensively studied as in human athletes. Nevertheless, several studies performed on horses in training and on horses performing different types of exercises, have evidenced that coagulation-fibrinolysis axis is influenced by exercise in equine athletes as well as in people (Ferguson E.W. et al., 1980; McKeever et al., 1990; Weiss D.J. et al., 1990; Monreal L. et al., 1995; Smith J.M. et al., 1997; Weiss D.J. et al., 1998; Kingston J.K. et al., 2002; Piccione G. et al., 2004a; Piccione G. et al., 2004b; Paltrinieri S. et al., 2008; Piccione G. et al., 2010; Assenza A. et al., 2013).

Some studies have evidenced that exercise can influence platelet function in horses. The mechanisms proposed for exercise-induced platelet aggregation are spontaneous platelet aggregation, shear stress-induced platelet activation, neutrophil-derived platelet activating factor, ADP released from erythrocytes (Weiss D.J. et al., 1997). The presence of neutrophil-platelet aggregates following exercise were identified in a few studies, but all of these studies failed to evidence of exercise-induced activated platelets (Weiss D.J. et al., 1998; Piccione G. et al., 2010). Kingston and colleagues have obtained the same results and failed to identify exercise-induced activated platelets using techniques described in human literature (Kingston J.K. et al., 2002). These neutrophil-platelet aggregates indicate exercise-induced platelet activation, but a failure to

identify activated platelets following exercise, demonstrates that further studies are required in the equine athlete to fully understand the influence of exercise on platelet function and its correlation with the coagulation-fibrinolysis axis during exercise.

Exercise-induced activation of coagulation and fibrinolysis cascades were studied and identified in equine athletes. Ferguson and colleagues identified increased fibrinolytic activity through the evaluation of euglobulin lysis time, plasma clot lysis time and the assessment of fibrin monomers (Ferguson E.W. et al., 1980). McKeever and colleagues have demonstrated an activation of the coagulation cascade induced by exercise by a reduced clotting time with no significant alterations in prothrombin time (PT) and activated partial thromboplastin time (aPTT), and activation of fibrinolysis through increased plasmin/plasminogen complex without a significant change in ATIII. These findings, previously seen in several studies in human literature, lead to the hypothesis that exercise might inhibit fibrinolytic factors (McKeever et al., 1990). In a later study Monreal and colleagues have evidenced concomitant activation of the coagulation and fibrinolysis cascades, expanding the panel of markers of hemostasis. Moreover, contrary to McKeever's study results, they have seen an increase in ATIII, which has been reported in human literature. Smith and colleagues have evidenced an exercise-induced activation of the coagulation cascade through the assessment of vWF. Despite the long half-life of this parameter, their results allowed them to hypothesize that exercise might induce microvascular trauma, which enhances coagulation and rapid consumption of vWF (Smith J.M. et al., 1997). Paltrinieri and colleagues in a study in which have validated a new method for evaluating hemostasis, have evidenced through thromboelastometry an increase in fibrinolysis following exercise (Paltrinieri S. et al., 2008).

Hemostatic alterations in horses following exercise are mainly attributed to endothelial activation of the factors involved coagulation-fibrinolysis

cascades. This activation process can be further extended by leukocytes, especially neutrophils by direct mechanisms, like neutrophil-platelet aggregates, and indirectly through neutrophil mediated ROS production. Continuous studies inspired by human literature allow to enlarge the knowledge of exercise-induced alteration of hemostasis in equine athletes, but the results obtained until now suggest that the equine athlete is different from the human athlete, and that more studies are needed in equine sport medicine.

Experimental part

5. Objectives

The purpose of the present study is the evaluation of the influence of an short term –high speed competition on equine athletes, and in particularly focusing on the assessment of metabolic alterations related to muscular damage, oxidative stress, intravascular hemolysis and hemostatic abnormalities, as seen in human sport medicine and more recently in equine sport medicine. These alterations were highlighted using more recent chemistry and coagulation markers (uric acid-UA, D-D, Fibrinogen, Hp, and iron profile parameters comprehensive of serum iron, Total iron binding capacity-TIBC, TIBC saturation and Ferritin).

6. Materials and Methods

6.1 Animals

Thirty Thoroughbred horses were included in the present study (15 geldings and 15 mares) of age between 4 and 12 years (mean 7 years). This study has been conducted during consecutive three-year manifestations. For each year 10 horses were included in the study, 5 of which have participated at the race of first manifestation and 5 at the race of the second manifestation. All the horses competed in a real high speed – short term competition, Palio di Faenza “Niballo” horse race. Prior to the race, all horses follow an athletic preparation. The horses start their training program three months before the competition. The training

schedule presumes long walks and canter, for the first two months, followed by twice a week competition simulations during the last month.

6.2 Niballo horse race

Niballo horse race is a manifestation within Faenza City's medieval historical reenactment. This manifestation is composed of two races that are held on the second Saturday of June and on the fourth Sunday of June. This competition presumes that two challengers start galloping at the same time and aim to touch with a spare for first a computerized dummy. The racetrack has an oval elliptical shape, and challengers gallop in opposite directions following an arch of a circle trajectory that closes the track at the point where the dummy is placed, at the same distance from the start point for both of the participants (Fig 9). Each horse has to run over across a distance of approximately 154 meters in 12 seconds for a total of eight times in about 1 hour. The 5 horses attending the competition challenge each other following a race schedule where each participant challenges the other four. Each challenge has the duration of roughly 2 minutes and it takes up to 15 minutes for one horse to challenge the other four participants. The horses attending this race start their athletic preparation on February with a canter training session for two months. After two months the training program presumes race simulations.

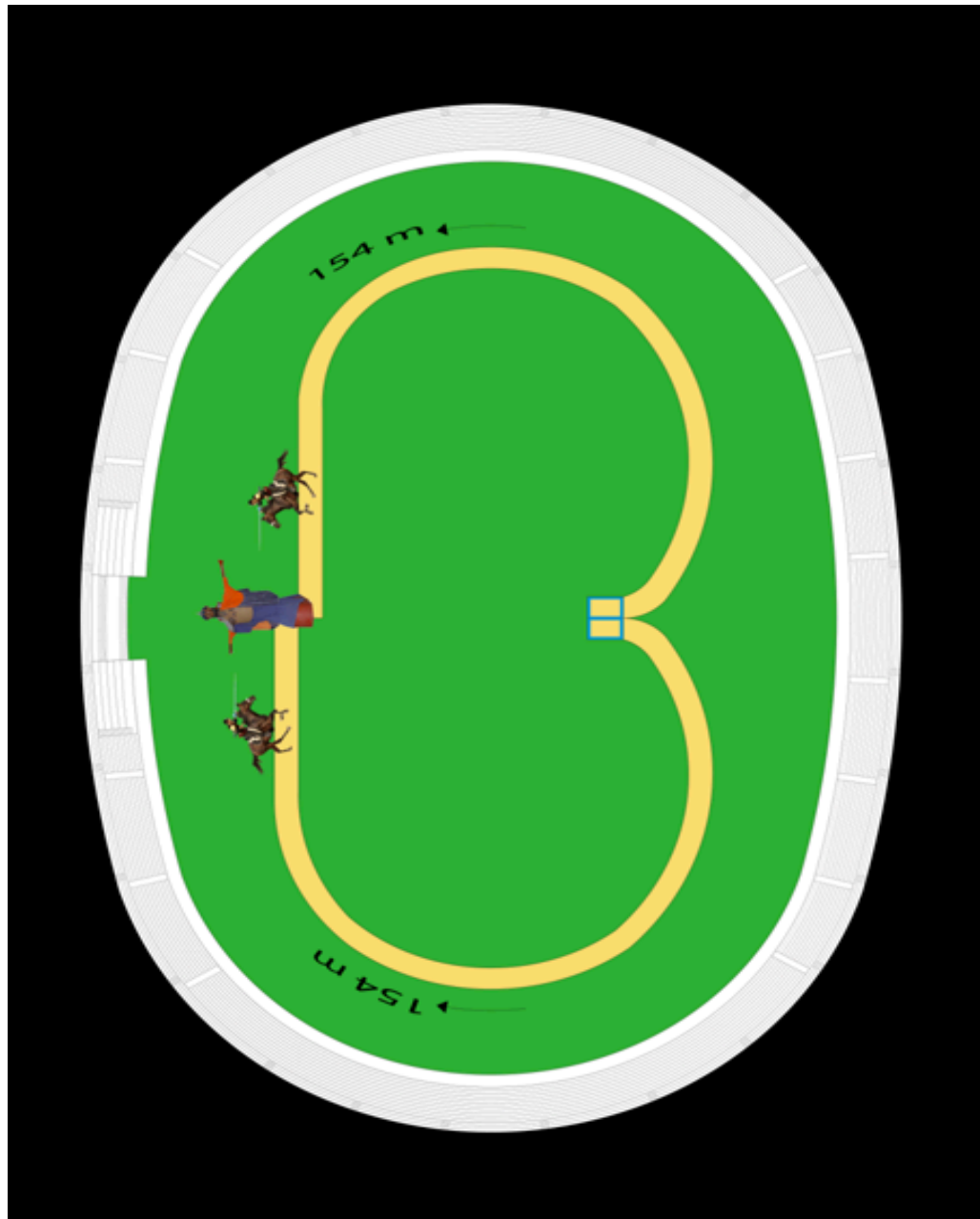


Figure 9.(<http://www.paliodifaenza.it>) The design of Faenza City's Niballo horse racetrack. The starting point is indicated by the blue square in the figure, and the end of the racetrack is at the point where the dummy is placed in the figure

6.2.1 Environmental conditions

Environmental conditions were recorded during each day of the manifestations in order to verify their influence on the obtained results.

These data were provided by ARPA Emilia-Romagna:

1. First year of study

First race: humidity 81%; temperature 19,4°C

Second race: humidity 47%; temperature 29,1°C

2. Second year of study:

First race: humidity 82%; temperature 21°C

Second race: humidity 38%; temperature 28,9°C

3. Third year of study:

First race: humidity 98%; temperature 17,6°C

Second race: humidity 47%; temperature 27,3°C

6.3 Blood samples

6.3.1 Blood sampling technique

Blood samples were obtained by venipuncture from the jugular vein with a “closed system” using vials with negative pressure S-Monovette® SARSTEDT. If the sampling method was considered to be unsatisfactory, a second sample was withdrawn using the same system from the opposite jugular vein, in order to prevent interferences with the analysis of parameters. For each individual a first sample was taken 24 hours before the race and the second sample within 30 minutes by the end of the race. These samples were taken at the same time with anti doping samples. For

each sample were used: **1 vial containing K₃EDTA anticoagulant**, **1 vial containing sodium citrate 1:9 anticoagulant**, and **1 plain vial**. All the vials were immediately refrigerated at +4°C and processed within 3 hours after withdraw at SEPAC VET Laboratory of Department of Veterinary Medical Science of Alma Mater Studiorum - University of Bologna.

6.3.2 Sample processing

Blood samples with K₃EDTA anticoagulant were collected using S-Monovette® Sarstedt EDTA KE/2.6 mL vials and delivered refrigerated at +4°C. At SEPAC VET Laboratory after vials were brought at room temperature a complete cell blood count (CBC) was performed using an automated hematology analyzer within 3 hours after withdraw.

Blood samples with sodium citrate anticoagulant were collected using S-Monovette® Sarstedt Coagulation 9 NC/5 mL vials (proportion blood and sodium citrate 1:9; sodium citrate solution 0.11 mol/L) and delivered refrigerated at +4°C. At SEPAC VET Laboratory these vials were centrifuged at 3000xG for 10 minutes. Plasma citrate supernatant was aspirated with single use pipette avoiding the layer above the buffy coat, in order to obtain platelet poor plasma. The obtained plasma citrate was stored frozen at -20°C until analysis. All these operations were concluded within 3 hours from withdraw.

Blood samples without anticoagulant were collected using S-Monovette® Sarstedt Serum Gel S/7.5 mL vials with cloth activator delivered refrigerated at +4°C. At SEPAC VET Laboratory serum was obtained from centrifugation of vials at 3000xG for 10 minutes. Serum was divided in 2 aliquots and stored frozen at -20°C until analysis. All these operations were concluded within 3 hours from withdraw.

6.4 Parameters

The following hematological, chemistry and coagulation variables were evaluated in the present study: on K₃EDTA samples a CBC comprehensive of hematocrit percentage (Hct; %), mean cell hemoglobin concentration (MCHC; gr%); on serum samples a chemistry profile comprehensive of Creatinine (Crea; mg/dL), Urea (mg/dL), Uric Acid (UA; mg/dL), Albumin (Alb; g/dL), Total Protein (TP; g/dL), Calcium (Ca; mg/dL), Phosphate (P; mg/dL), Sodium (Na; mEq/L), Potassium (K; mEq/L), Chloride (Cl; mEq/L), Gamma-glutamyl Transferase (GGT; U/L), Bile Acids, Bilirubin (Bil; mg/dL), plasma muscular enzymes (PMEs) - Creatine Kinase (CK; U/L), Aspartate Aminotransferase (AST; U/L), Lactate Dehydrogenase (LDH; IU/L), Iron profile parameters - Total Iron (Fe⁺⁺; µg/dL), Total Iron Binding Capacity (TIBC; µg/dL), TIBC Saturation (Sat; %), Acute Phase Proteins - Haptoglobin (HP; mg/dL), Ferritin (Ferr; mg/dL); on plasma citrate samples a coagulation profile comprehensive of Fibrinogen (Fib; g/L) and D-Dimers (D-D; µg/mL). All chemical and coagulation variables were assessed using previously validated methods and carried out on an automated analyzer Olympus AU 400.

6.4.1 Complete blood count

The CBC was performed using the Abbott Cell Dyn 3500 R automated hematology analyzer. Prior analysis, all samples were homogenized for 1 minute using Reamix 2789 Vortex instrument. For each sample a blood smear was made, which was used to evaluate platelet count microscopically.

6.4.2 Main methods used

6.4.2.1 Haptoglobin

Haptoglobin was assessed using an immunoturbidimetric method previously validated for horses in our laboratory (HAPTOGLOBIN OSR 6165, Olympus System Reagent).

Reagents, Calibration:

Reagent R1	Tris Buffer (PH 7,6) 78 mmol/l	Storage: 7 days at 2-8°C 2 days at 15-25°C
Reagent R2	Goat antibody-antihuman haptoglobin Preservative	Storage: 7 days at 2-8°C 2 days at 15-25°C
Multicalibrator	Serum Proteins 2 OLYMPUS Cat.No. ODR3023	Storage: 7 days at 2-8°C 2 days at 15-25°C
Control	Olympus control for Immunoturbidimetry ODC0014, ODC0015 e ODC0016	Storage: 7 days at 2-8°C 2 days at 15-25°C

Table 1. Reagents, Quality Control and Calibrators for the assessment of Haptoglobin.

Method principle: when the sample is mixed with the buffer solution R1 and antiserum solution R2, haptoglobin will react specifically with anti-haptoglobin antibodies to yield insoluble aggregates. The absorbance of these aggregates is proportional to the haptoglobin concentration in the sample.

Linearity: the linearity of the method is comprised between 30-400 mg/dL. The test has tolerance regarding prozone effect of 800 mg/dL.

On-board stability: reagents (R1 and R2) are ready to use and can be directly placed on the instrument. Reagents are stable, if unopened and stored between +2 - +8°C until expiration date. Reagents, once they are open and placed on the instrument, remain stable for 60 days.

Interferences:

Bilirubin: no significant interference until 40 mg/dL of added bilirubin

Hemolysis: added hemoglobin of 500 mg/dL lead to significant interference of 10%

Lipids: added lipid concentrations of 1000 mg/dL lead to significant interference of 20%

6.4.2.2 Uric Acid

Serum UA concentration was assessed using a colorimetric enzymatic test Uricase/PAP (Olympus System Reagent URIC ACID OSR 6136, OSR 6236).

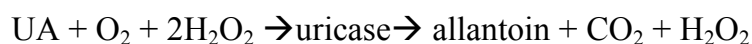
Reagents, Calibration:

Reagent R1	HEPES buffer (pH 7,5) 33 mmol/L	Storage: 2-8°C
Reagent R2	3,5 – DCHBS 3,25 mmol/L 4 – Aminofenazone 1,50 mmol/L Peroxidase ≥ 2 kU/L Uricase ≥ 400 U/L EDTA 0,72 mmol/L Ascorbate Oxidase ≥ 1,0 kU/L Preservative	Storage: 2-8°C
Multicalibrator	OLYMPUS System Calibrator	Storage: 2-8°C

Table 2. Reagents and Calibrations for AU assessment

Method principle: UA assessment is achieved through uricase reaction. Following, H_2O_2 formed will react with 3,5-dichloro-2-hydroxybenzenesulfonic-acid and 4-amminophenazone leading to the formation of a red-violet chinonimine as reaction indicator. These substances, reacting with a suitably buffered chromogen, develop a colored complex that is photometrical measurable and directly proportional to their concentration according to Lambert-Beer law. The color density allows an analytical evaluation of UA concentration.

Reaction scheme:



$2\text{H}_2\text{O}_2 + 3,5\text{-dichloride-2-hydroxybenzenesulfonic- acid} + 4\text{-amminophenazone}$

 $\text{N-(4antipiril)-3 chloro- nemonoimine+HCl} + 4\text{H}_2\text{O}_2$

Quality control: all control sera determined by this method can be used

Linearity: the method is linear for concentrations comprised between 0.5-30 mg/dL (89-1785 $\mu\text{mol/L}$) for serum and plasma.

6.4.2.3 Fibrinogen

Fibrinogen was assessed with a turbidimetric method (Turbidimetric Fibrinogen, MeDia diagnostics).

Reagents, Calibration:

Fibrinogen Reagent R1	Ammonium sulphate 0,42 mmol/L K₃EDTA 39,6 mmol/L Guanidine HCl 26,2 mmol/L pH 4,9	Storage: 2-6°C
Fibrinogen Reagent R2	Ammonium sulphate 1,57 mmol/L K₃EDTA 39,6 mmol/L Guanidine HCl 26,2 mmol/L pH 4,9	Storage: 2-6°C

Fibrinogen Standard	Lyophilized human Fibrinogen	Storage: 2-6°C Stability: 7 days
Fibrinogen Diluent	Citrate 15 $\mu\text{mol/L}$ Heparin 800 U/I Aminocaproic acid 0,1 mmol/L Bovine albumin 40 g/L Phosphate buffer pH 7,4	Storage: 2-6°C Stability: 7 days
Fibrinogen control, low level	Lyophilized human Fibrinogen 1,1 g/L	Storage: 2-6°C Stability: 7 days

Table 3. Reagents, Quality control and Calibrations for Fib assessment

Method principle: adding ammonium sulphate, EDTA and Guanidine hydrochloride, determine the precipitation of the Fibrinogen present in the plasma sample. The analyzer measures the resulting turbidimetric variation after previous assessment of “white sample” in order to avoid interferences from bilirubin, hemoglobin or chylomicrons.

Linearity: the method is linear from 0 to 9.0 g/L. Samples with superior Fibrinogen values must be diluted (1+2) with Fibrinogen Diluent and re-assessed.

Other data:

Temperature 25°C;

Wave length 340nm;

6.4.2.4 D-Dimers

D-Dimers were assessed with *Tina-quant® D-Dimer* method from Roche Diagnostics GmbH. This method uses a latex immunoturbidimetric principle. Immunologic-based methods developed in human medicine and applied for horses and other animal species are relying on the presence of cross-reactivity as shown in previous studies (Sandholm M. et al., 1995). Other studies have evidenced that these methods have similar or increased reliability compared to ELISA methods (Keeling D.M. et al., 1999).

It must be remembered that *in vivo*, complete degradation of fibrin to dimers D follows only after lysotherapy, for example tissue plasminogen mediated. For this reason anti-D-dimer antibodies bind to the so-called Oligomers X, where antigenic determinants are present and characterized by covalent links.

Reagents, Quality control:

Reagent R1 Buffer	TRIS/HCl Buffer 370 mmol/L NaCl 267 mmol/L pH 8,2	Storage: 2-8°C On-board stability: 28 days
Reagent R2 Anti-D-dimer- latex Suspension	Latex particles covered with human anti-D-dimer monoclonal antibodies (rat) 0,15% TRIS/HCl Buffer 10 mmol/L	Storage: 2-8°C On-board stability: 28 days
D-Dimer Control I/II (low control, high control)	Lyophilized human serum with listed D-dimer concentration (low 0,88 µg/dL, high 4,19 µg/dL)	Storage: 2-8°C Stability: 1 day at 15-25°C 14 days at 2-8°C

Table 4. Reagents and quality control for assessment of D-Dimers

Calibration (manual, 6 dilutions):

Zero Standard (f1)	Human serum matrix	Storage: 2-8°C Stability: 1 day at 15-25°C
D-Dimer Calibrator (f2)	Human fragments containing D-dimers in human serum matrix with listed concentration for each stock	Storage: 2-8°C Stability: 1 day at 15-25°C

Table 5. Calibrators for D-Dimers

Method principle:

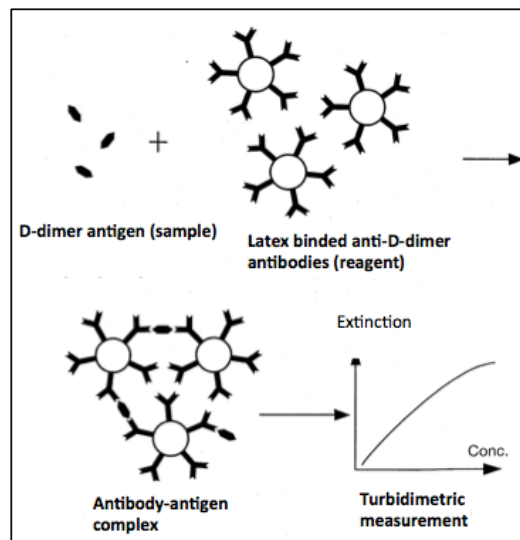


Figure 10. Scheme of method principle for D-dimers assessment by latex immunoturbidimetry

Linearity: the method is linear between 0,15 and 9,0 µg/mL. Concentrations above this limit need to be diluted with NaCl 0,9% (1:1).

Interferences:

Icterus (I): no significant interference until a I rate of 20 (conjugated and unconjugated bilirubin approximately 20 mg/dL);

Hemolysis (H): no significant interference until a H rate of 500 (hemoglobin approximately 500 mg/dL);

Lipemia (L) (Intralipid): no significant interference until a L rate of 750 (triglycerides approximately 1500 mg/dL);

No interference from rheumatoid factors < 100 UI/mL;

No interference from heparin < 1,5 UI/mL;

No documented interference from drugs (31 commonly used drugs);

High levels of IgM (e.g. myeloma) may lead to falsely increased results;

During lysotherapy (e.g. t-PA) increased D-Dimer fragments may lead to decreased results (prozone effect).

Other data:

Analytic sensibility: 0,04 µg/mL;

Temperature: 37°C;

Wave length: 0-700 nm.

6.4.2.5 Ferritin

For the assessment of ferritin it was used an immunoturbidimetric method of Olympus (Olympus System Reagent – FERRITIN, OSR 6150). This test is created for the measurement of human ferritin. Because of uncertain suitability of this test for horses, prior to the present study this test was validated for horses at the SEPAC VET Laboratory. The validation data are not reported in this study, but were similar to the human ones.

Method principle: latex agglutination reactions occur as a result of antibody-coated latex beads aggregating if antigen is present in sufficient quantity. Immune complexes formed in solution scatter light in proportion to their size, shape and concentration. Under conditions of antibody excess, increasing amounts of antigen results in higher scatter.

Turbidimeters measure the reduction of incident light due to reflection, absorption or scatter. The measurement of the decrease in light intensity transmitted (increase in absorbance) through particles suspended in solution as a result of complexes formed during the antigen-antibody reaction is the basis of this assay. The Olympus Ferritin reagent is a suspension of polystyrene latex particles, of uniform size, coated with polyclonal rabbit anti-ferritin antibody. When serum, containing ferritin, is mixed with the Olympus Ferritin reagent, an agglutination mixture occurs. This is measured spectrophotometrically on the analyzers.

Reagents, Quality control and Calibrator:

Ferritin Reagent R1-R2	TRIS Buffer (pH 8,2) 88 mmol/L Latex particles coated with rabbit anti-human ferritin Preservative	Storage and stability: 2-6°C On-board stability: 30 days at 2-8°C R2 mixed at weekly intervals
Calibrator	Serum protein Multi-calibrator OLYMPUS ODR 3021	Storage and stability: 7 days at 2-8°C 2 days at 15-25°C
Control	OLYMPUS ITA Control Sera ODC0014	Storage and stability: 7 days at 2-8°C 2 days at 15-25°C

Table 6. Calibrator, quality control and reagents for Ferritin

Sensitivity and linearity: the lowest detectable concentration of ferritin in serum was estimated at 6,4 µg/L. The test is linear within a concentration range of 8,0-450 µg/L. Samples with very high ferritin concentrations

(>20,000 IU/mL) can generate false low results without appropriate flags due to an excess of antigen in the sample (prozone effect).

Precision: the coefficients of variation within run and total are lower than 4,0.

Interferences:

Bilirubin: interference less than 5% for 40 mg/dL of bilirubin added;

Hemolysis: interference less than 10% for 5 g/L of hemoglobin added;

Lipemia: interference less than 10% for 1500 mg/dL of triglyceride added.

6.4.2.6 Total iron

For the assessment of the total iron it was used the direct colorimetric method of Olympus (Olympus System Reagent – IRON, OSR 6123s).

Method principle: the iron molecule in a buffer system with a pH of 4.8 is first liberated from transferrin, its carrier, and then it is reduced at the ferrous state. The ferrous iron ion will form with the specific compound FereneS 3-(2-piridil)-5,6-bis-[2-(5-furilsulphonic acid)]-1,2,4-triazine a stable dye complex, which color intensity is proportional to the iron quantity within the analyzed sample. The interference given by copper ions is eliminated with particular reacting conditions and using a specific chelate factor.

Reagents, quality control and calibration:

Total Iron Reagents	R1: acetate buffer 1,4 mol/l pH 4,8 Guanidine hydrochloride ≥ 4.5 mmol/l Chelate factor for copper ions R2: FereneS ≥ 20 mmol/l, Ascorbic acid ≥ 0.5 mol/l	Storage and stability 90 days at 2-8°C On board – 30 days
Calibrator	Multi-calibrator OLYMPUS SYSTEM CALIBRATOR Cat.N° .66300	Storage and stability 7 days at 2-8°C 2 days at 15-25°C
Control	Olympus Multi-control Cat.-N° ODC003, ODC004	Storage and stability : 7 days 2-8°C 2 days at 15-25°C

Table 7. Calibrator, quality control and reagents for Total Iron

Sensitivity and linearity: the lowest detectable concentration of iron is 0,5 mcg/dl. The test is linear within a concentration range of 1 - 100 mg/dl.

Precision:

The coefficients of variation (CVs) of the method were lower than 1% for intrassay and interassay repeatability.

Interfering substances:

Bilirubin: no significant interference for bilirubin concentration <15 mg/dl.

Hemoglobin: no significant interference for hemoglobin concentration <0,5 g/dl.

Lipemia: no significant interference for triglyceride concentration < 1000 mg/dl.

6.4.2.7 TIBC

The Total Iron Binding Capacity (TIBC) was obtained by the summation of Total Iron and UIBC concentrations. It was calculated automatically by the analyzer.

6.4.2.8 UIBC

For the determination of the Unsaturated Iron Binding Capacity (UIBC) it was used the photometric color test UIBC of Olympus (Olympus System Reagent, UIBC, OSR61205).

Method principle: the Fe^{2+} from reagent 1 reacts with Nitroso-PSAP from reagent 2 to form an intense green complex. If sample is added a part

or all of the iron ions bind specifically with transferrin at unsaturated iron binding sites at alkaline pH. The iron ions are thus not available for the color reaction with Nitroso-PSAP. The difference between the resulting changes in the measured absorbance with or without samples is equivalent to the iron quantity bound to transferrin (UIBC).

Reagents, quality control and calibration:

UIBC Reagent (R1 – R2)	Tris buffer (pH 8,1) 180 mmol/l Iron 6,9 µmol/l Nitroso-PSAP 176 µmol/l Hydroxylammonium chloride 36 mmol/l Preservative	Storage and stability: 2-6°C On board: 30 days
Calibrator	Multicalibrator OLYMPUS SYSTEM CALIBRATOR Cat.N°.66300	Storage and stability 7 days at 2-8°C 2 days at 15-25°C
Control	Olympus Multi-control Cat.-N° ODC003, ODC004	Storage and stability : 7 days 2-8°C 2 days at 15-25°C

Table 8. Calibrator, quality control and reagents for UIBC

Sensitivity and linearity: the lowest detectable concentration of UIBC is 13 mcg/dl. The test is linear within a concentration range of 55 – 550 µg/dl.

Precision: the within run and total variability of the method is represented by CVs lower than 4.

Interference:

Bilirubin: interference less than 6% with 40 mg/dl of bilirubin added.

Hemolysis: interference less than 10% with 2 g/l of hemoglobin added

Lipemia: interference less than 5% with 1000 mg/dl of lipids added.

6.4.2.9 Saturation

The percentage of Transferrin Saturation was calculated automatically by the analyzer using the following formula:

$$\text{Saturation} = \text{Total Iron} \times 100 / \text{TIBC}$$

6.4.3 Statistical analysis

All the data were managed with the following software:

MedCalc Statistical Software version 13.0.2 (MedCalc Software bvba, Ostend, Belgium; <http://www.medcalc.org>; 2014);

Microsoft Excel 2010;

Analyse-it vers. 2.21 (<http://www.analyse-it.com>).

Results were divided into Pre-race and Post-race groups and analyzed using descriptive statistics. The comparison between the two groups was made using Mann-Whitney U test; $p \leq 0.05$ was considered significant. Statistical analysis on post race samples was performed on corrected values, to exclude the influence of exercise-induced hemorheological changes, and on non-corrected values.

7. Results

As seen in human sport medicine literature (Ploutz-Snyder L.L. et al., 1995; Kargotich S. et al., 1998; El-Sayed M.S. et al., 2005) and equine sport medicine literature (McKeever K.H. et al., 1987; Naylor J. et al., 1993; McKeever et al., 1993; Nyman S. et al., 2002) following exercise hemorheological changes occur, and are characterized by fluid shift and plasma volume contraction. In agreement with these studies post-race values were corrected to possibly avoid the influence of hemoconcentration. For this purpose individual post-race albumin concentration was used to obtain a correction factor (CF) to recalculate post-race values according to the formula:

$$\text{Post-race corrected value} = \text{Post-race value} \times \frac{\text{Alb pre-race value}}{\text{Alb post-race value}}$$

The results of descriptive statistics applied to the variables assessed in this study are reported in table 9.

Other relevant findings induced by the competition were increased concentrations of PMEs [CK +962.8 % (Fig. 11); LDH + 64.1 % (Fig. 12)], Crt [+ 27.4 %; $p < 0.01$ (Fig. 13)] and UA [+ 1011.1 %; $p < 0.01$ (Fig. 14)]. Significant decreases in Hp concentration [- 26.6 %; $p < 0.01$ (Fig. 15)] associated to a significant increase of Ferr concentration [+ 342.5 %; $p < 0.01$ (Fig. 16)] were also detected with no other alteration of iron profile parameters (Fe, TIBC and Sat %). Hemostatic abnormalities were characterized by a significant decrease of Fib [- 11.3 %; $p < 0.05$ (Fig. 17)] accompanied by a non-significant increase of D-D concentrations [(+ 32.1 %; $p = 0.08$ (Fig. 18)].

Parameter	Mean		Deviation Standard		Median		Range		Normal distribution		Reference Intervalli
	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	
Hct %	40,41	47,37	2,14	3,56	40,6	48,25	37,2-43,7	40,7-53,4	0,4703	0,7165	32-52
Crea mg/dL	1,35	1,72	0,18	0,2	1,31	1,69	1,04-1,72	1,31-2,14	0,2905	0,9103	0,9-1,5
Urea mg/dL	42,3	34,97	23,9	5,73	33,47	33,5	24-100	24-51	<0,0001	0,2218	15-40
Alb g/dL	3,52	3,83	0,21	0,35	3,52	3,82	2,82-3,89	3-4,6	0,0065	0,7714	2,9-3,7
TP g/dL	6,09	5,97	0,46	0,46	6,14	6,02	5,18-7,08	5,1-6,87	0,9955	0,7074	5,9-7,3
Alb/Glob	1,48	1,50	0,28	0,26	1,46	1,471	0,97-2,08	1,04-1,94	0,9345	0,5949	0,7-1,3
Ca mg/dL	11,51	10,85	0,43	1,04	11,49	11,01	10,7-12,17	9,33-12,47	0,5492	0,2730	11,3-13,5
P mg/dL	3,81	2,65	0,56	0,46	3,8	2,65	2,9-5	1,8-3,8	0,8026	0,3418	1,75-4,9
Na mEq/L	135,25	125,55	2,67	9,21	136	126	130-140	107-143	0,4481	0,8781	135-143
K mEq/L	4	2,98	0,43	0,49	3,9	2,95	3,2-5,1	2-4	0,0745	0,9666	2,8-4,5
Cl mEq/L	98,9	86,75	2,25	7,36	99,5	87,5	94-102	69-97	0,0483	0,0809	98-104
GGT U/L	17,25	15,93	7,88	7,28	15,85	14,69	11-42,5	6,37-39,79	<0,0001	<0,0001	3,6-20,6
Bil mg/dL	2,4	1,83	0,84	0,73	2,31	1,68	1,18-4,88	0,88-3,94	0,01014	0,0069	1,2-4,5
CK U/L	135	1334,77	37,37	2322,79	135	687,5	69-210	206-11965	0,7843	<0,0001	90-270
AST U/L	312,2	363,93	81,79	139,35	301	334	108-633	207-949	<0,0001	<0,0001	60-280
LDH IU/L	523,13	817,2	104,09	341,01	517,5	735	335-765	529-2432	0,6653	<0,0001	240-970
UA mg/dL	0,20	2,08	0,1	1,57	0,18	1,6	0,08-0,5	0,24-6,91	0,0142	0,0005	0-0,5

Fe++ µg/dL	146,2	144,73	46	48,67	137	136,5	33-281	24-277	0,0356	0,2654	60-260
TIBC µg/dL	361,87	351,7	50,56	55,6	365	359,5	220-459	217-434	0,1507	0,1735	305-420
Sat %	41,13	39,87	14,34	10,63	40	40	9-85	7-60	0,0004	0,0374	12-70
Ferr	116,33	476,9	119,01	602,22	75,5	298	42-529	69-2694	<0,0001	<0,0001	30-100
Hp mg/dL	43,17	32,5	19,75	17,95	37,5	29,5	17-126	9-108	<0,0001	<0,0001	30-100
Fib g/L	3,98	3,53	0,8	0,73	3,83	3,4	3,06-6,64	2,66-5,9	0,0001	0,0002	1,6-4,1
D-D µg/mL	1,55	2,03	1,21	1,72	1,25	1,73	0,38-5,57	0,01-7,82	0,0001	<0,0001	0,2-0,48

Table 9. Useful parameters for descriptive analysis of clinicopathological data considered in the present study. **Mean pre/post**=mean values of pre-race and post-race data. **Standard deviation pre/post**=standard deviation of values for pre- and post-race data. **Median pre/post**=median of values for pre- and post-race data. **Range pre/post**=minimal and maximal values for pre- and post-race data. **Reference interval**=reference interval or variables used in the SEPACVET Laboratory.

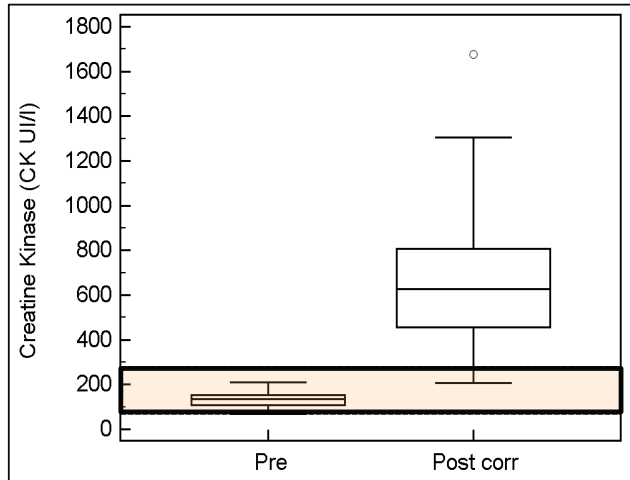


Figure 11. Statistical analysis of CK variable on pre- and post-race samples.

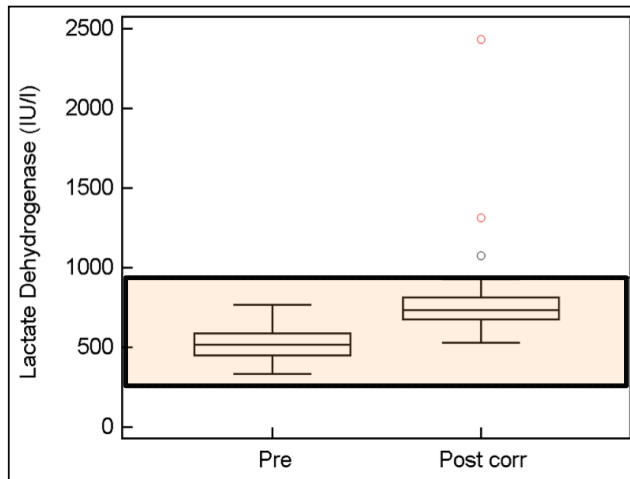


Figure 12. Statistical analysis of LDH variable on pre- and post-race samples.

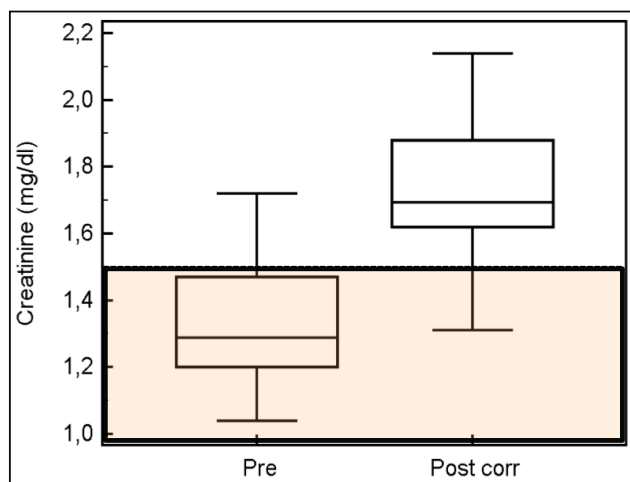


Figure 13. Statistical analysis of Crea variable on pre- and post-race samples.

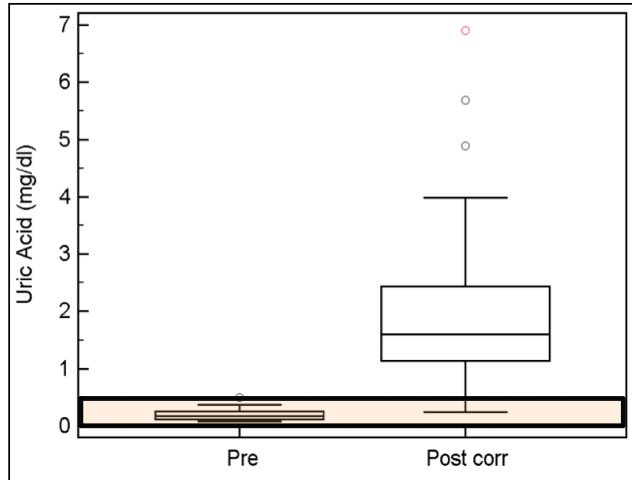


Figure 14. Statistical analysis of UA variable on pre- and post-race samples.

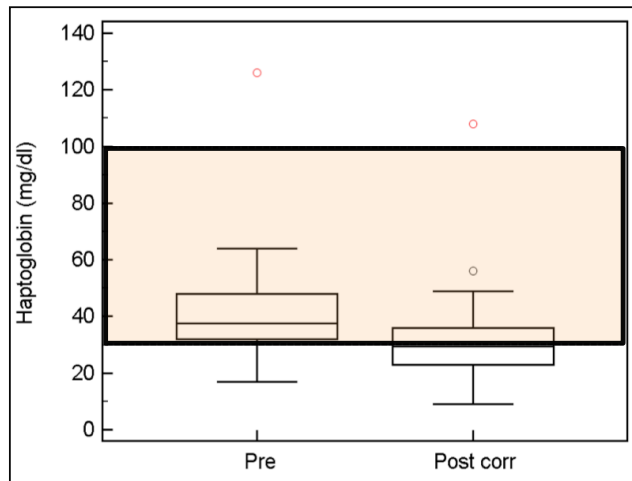


Figure 15. Statistical analysis of Hp variable on pre- and post-race samples

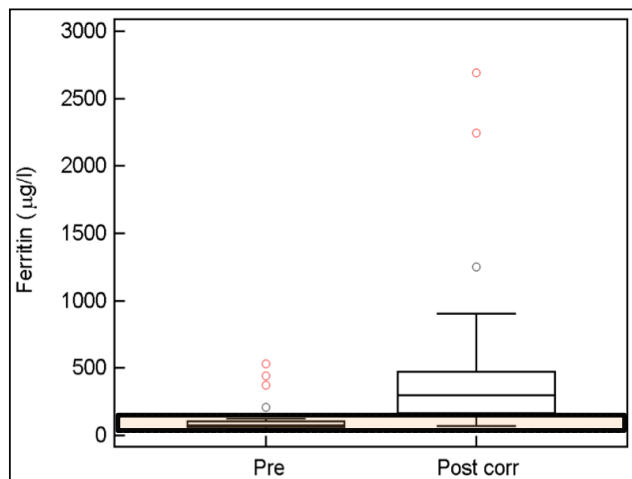


Figure 16. Statistical analysis of Ferr variable on pre- and post-race samples

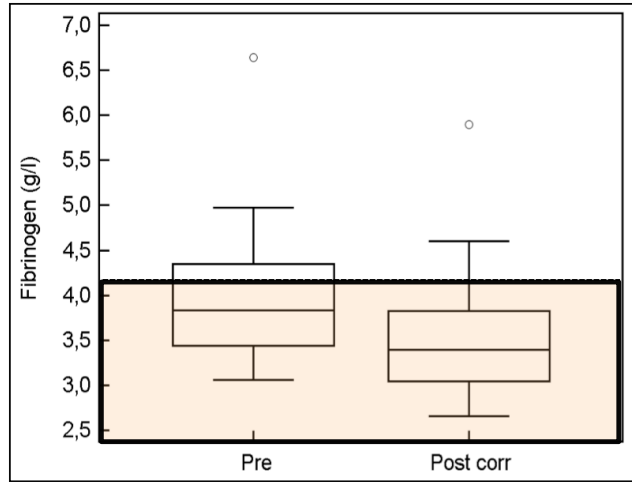


Figure 17. Statistical analysis of Fib variable on pre- and post-race samples

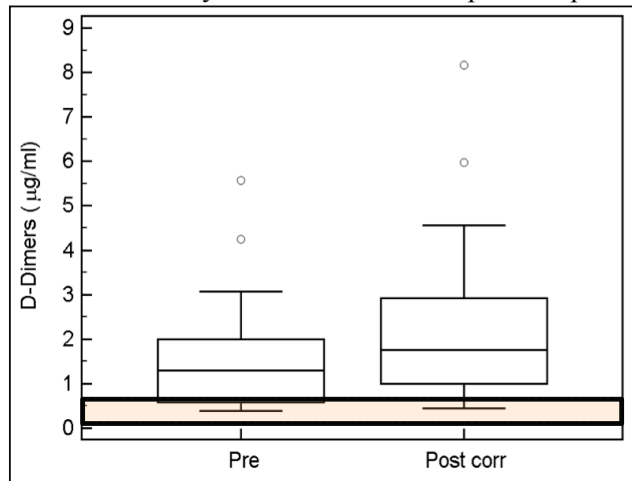


Figure 18. Statistical analysis of D-D variable on pre- and post-race samples

8. Discussion

The influence of exercise upon skeletal muscles and metabolism continues to represent a research interest point in human and equine sport medicine. From the review of human and equine literature it has been seen that there are many variables that could influence the response to exercise, and that the exact mechanism for most of the exercise-induced changes has not been fully understood.

This study proposed to highlight the occurrence of myopathy, oxidative stress and hemostasis activation, in athlete horses that attend a high intensity race.

Niballo horse race is a real high speed – short term competition that follows strict racing rules. The horses that attend this race undergo supra-maximal effort. This manifestation is held in June and high temperatures and increased humidity characterize the environmental conditions. In agreement with findings from human literature (Ploutz-Snyder LL et al, 1995; Kargotich S. et al., 1998; El-Sayed M.S. et al., 2005) and equine literature (McKeever K.H. et al., 1987; Naylor J. et al., 1993; McKeever et al., 1993; Nyman S. et al., 2002), hemodynamic changes have occurred in the horses that have attended this races, which have been evidenced by significant difference between pre-race and post-race concentration of the variables analyzed in the present study. Plasma volume contraction represents the first important finding in our study. Increased serum total protein and serum albumin concentrations in post-race samples can be explained by increased plasmatic concentrations due a loss of part of the water from plasma, which is commonly encountered in athletes. Fluid shift, the movement of fluid from intravascular space to extravascular space, represents another exercise-induced hemorheological change described in human and

equine sport medicine literature, manifested from a clinicopathological point of view by increasing total protein and albumin values. Human (Kargotich S. et al., 1998) and equine sport medicine studies (Cywinska A. et al., 2010) suggest that data should be analyzed only after correcting the values to avoid interference due to hemoconcentration. For this reason serum total protein or serum albumin can be used to obtain a correction factor (Cywinska A. et al., 2010). In the present study individual post race serum albumin concentration was used to obtain a correction factor, and subsequently all post-race values of the selected analytes were re-calculated.

Our results show that increments of serum albumin of (+ 8.9% mean variation) and serum total protein of (+ 8.6% mean variation) are accompanied by an increase of (+ 19.1% mean variation) of hematocrit value. This increase in hematocrit value, however, cannot be attributed only to plasma volume contraction and fluid shifts. Equine sport medicine studies report that a large number of erythrocytes are stored in the spleen. During exercise catecholamine-induced spleen contraction occurs and splenic pool erythrocytes are released into circulation leading to an increase in hematocrit value, which in our study resulted approximately two times increased compared with the one quantified by PT and Alb concentrations.

Other relevant finding evidenced by our results is represented by a significant increase of serum creatinine concentration ($p < 0,01$). During exercise β -adrenergic secretion will influence renal vascular smooth muscle determining the contraction of afferent arterioles. Renal perfusion is decreased and blood flow is directed to support other organs like brain, heart and muscles. In equine athletes it has been evidenced that renal blood flow can be decreased by exercise up to 70% (Schott H.C. et al., 1991). The renal function can be decreased to the limits of

physiologic, and when exercise decreases renal function beyond this limit acute azotemia occurs, which is a common complication recognized in athletes (Hodgson D.R. & Rose R.J., 1994). Even if scientifically it has not been proved, only hypothesized, another possible cause for increased serum creatinine level in post-race samples is represented by leakages from damaged muscular cells. Creatinine originates in the muscles from the breakdown of creatine and creatine phosphate, an energy store molecule (Valberg S.J., 2008). Therefore, other possibility, even if it is only a hypothesis, for the increased serum creatinine in post-race samples, could be attributed to creatinine leakage from damaged muscular cells

The leakage of muscular enzymes from damaged muscular cells represents other important finding of this study. The authors of the present study have assessed the CK, AST and LDH as markers of muscular damage. Post-race samples have evidenced significant increase in PME's (CK +962.8 % mean variation; LDH + 64.1 % mean variation) and indicated the presence of myopathy/muscular damage. This type of alteration remains generally sub-clinical, but a very small percentage of our cohort manifested clinically the exercise-induced myopathy. The review of studies regarding exercise-induced muscular damage in equine sport medicine has attributed this alteration to exercise-related oxidative stress (Hargreaves B.J. et al., 2002; Kirschvink N. et al., 2008; Viu J. et al., 2010). Chiaradia and colleagues have studied oxidative stress and myopathy following exercise (two gallop sessions with rider over for a 200 m distance). They have evidenced the presence of oxidative stress through the assessment of malondialdehyde but were not able to evidence of exercise-induced muscular damage with specific muscular isoenzyme markers (CK-MM and LDH-4). They have explained that the model of exercise used in their study caused oxidative stress, but was not

intense enough to cause muscular damage. In the same study the authors evidenced a moderate, but significant increase in LDH1/LDH2 ratio, suggesting that their type of exercise have induced damage to the myocardium muscle (Chiaradia E. et al., 1998). CK can have different tissue origins: CK-1 from brain, CK-2 and CK-3 from myocardium and skeletal muscle, and CK-Mt from the mitochondria of various tissues. LDH has five isoenzymes that can originate from myocardium or skeletal muscle (Stockham S.L. & Scott M.A., 2008). In our study we have not assessed specific isoenzymes markers of muscular damage, but the significant increase in our parameters without signs of hepatic damage support the muscular origin of our markers. Therefore, considering previous information, a contribution from myocardium damage following exercise for increased CK and LDH concentrations in our cohort cannot be ruled out in our study. Moreover, the significant increase in UA concentration in post race samples, allows the authors of the present study to affirm the presence of exercise-induced oxidative stress in the subjects of this study, and that the muscular damage evidenced by our results could be directly attributed to oxidative stress.

Other relevant finding of our study was the presence of laboratory hemostatic alterations due to exercise. An interesting finding regarded fibrinogen, which resulted decreased in the post-race data; however not significantly. After correction of all data, to avoid the influence of hemoconcentration, fibrinogen was significantly decreased (- 11.3 %; $p < 0.05$) in the post-race samples indicating that fibrinogenolysis occurred during the race. Post-race samples evidenced a non-significant increase in D-D concentration. Therefore, after correction of data, our results suggest the presence of a hypercoagulable state evidenced by a nearly significant increase of D-D ($p=0.08$). Due to the non-significant increase in D-D concentration we have considered Fib a more reliable parameter

suggestive of hypercoagulable state, which prevailed over the fibrinolysis state in these horses. However, previous study performed by some of the authors of the present study in other short term-high speed exercise settings (Dondi F. et al., 2010) evidenced that d-dimers increase significantly after race, as reported by other studies on endurance horses (Monreal L. et al., 1995). The equine sport medicine literature regarding the effect of exercise on hemostasis is rather limited. In a study of 1995, Monreal and colleagues have evidenced through their results a hypercoagulable state, with marked hypofibrinolysis and a slight hyperfibrinolysis, after 80 km endurance ride. The authors have indicated in their results that post-race samples have evidenced an increase in hematocrit values, but they don't specify correction of post-race values to exclude the influence of hemoconcentration (Monreal L. et al., 1995). Our results cannot be compared with the results from the above mentioned study due different type of exercise and increased number of markers that they have assessed, but we have excluded the influence of hemoconcentration, and our results are in agreement with human studies, which have evidenced a hypercoagulation state following exercise. Other studies, using different type of exercise and training sessions have evidenced increased blood coagulation following exercise (Piccione G. et al., 2004; Assenza A. et al., 2013). In one study hematocrit values were assessed before and after exercise (Piccione G. et al., 2004), and in the other only hemostatic parameters were assessed (Assenza A. et al., 2013). The exact mechanism by which exercise alters hemostasis has not been fully understood yet. In analogy with studies on sepsis syndrome in horses where decreased blood flow to the intestinal compartment initiates simultaneous coagulation and fibrinolysis (Monreal L. et al., 2000; Monreal L. & Cesarini C., 2009), catecholamine-mediated decreased blood flow to the spleen during

physical activity can be hypothesized as cause for exercise-induced hemostatic alteration. Similar to other studies results, our results showed that following exercise hemostatic alterations are reflected mainly by a hypercoagulable state. As discussed earlier, important hemorheological changes occur following exercise, for this reason further studies required to clarify pathophysiologic etiology of these alterations, should consider analysis of data after correcting results to exclude the influence of hemoconcentration post exercise.

The most important finding of our study was the presence of hemolysis related to exercise as stated by a significant decrease of Hp associated to a significant increase in Ferr concentration with no other alteration in iron profile parameters. In human sport medicine studies regarding hyperferritinemia related to exercise are present meanwhile data regarding hyperferritinemia in equine sport medicine are lacking. Both haptoglobin and ferritin are positive acute phase proteins. A possible explanation for increased ferritin concentration in post race samples could be attributed to the inflammatory response related to the exercise. Another possible explanation could be the presence of hemolysis since ferritin could be released by the damaged erythrocytes. The latter explanation could be further supported by the concomitant decrease in haptoglobin concentration noticed after the race. Decreased serum haptoglobin concentration during an acute phase response can be explained by the fact that beside a positive acute phase marker, haptoglobin is also a hemoglobin scavenger. According to studies from human (Peeling P. et al., 2009) and equine (Hanzawa K. et al., 2002) literature, decreased haptoglobin concentration in post race samples is suggestive of exercise-induced intravascular hemolysis. Haptoglobin-hemoglobin complexes that result from erythrocytes destruction are phagocytized by macrophages and transported to the liver. At hepatic

level these complexes are up-taken by endocytosis, which will lead to rapid increase in ferritin synthesis (Van Vlierberghe H. et al., 2004). Other explanation for decreased serum haptoglobin concentration in post race samples comes from human literature. Beside hemoglobin, haptoglobin is a scavenger of other molecules that contain heme, like myoglobin. Exercise-induced muscular damage leads to the leakage of myoglobin into the circulation, which will be bound to haptoglobin, as defense mechanism to prevent its toxic effect in the extracellular space (Nielsen M.J. et al., 2010). Therefore, decreased haptoglobin concentration, shown by our results, could be also attributed to the muscular damage evidenced by increased AST, CK and LDH concentrations in our results. Moreover, increased serum ferritin following exercise can be attributed to leakage from damaged muscular cells as evidenced by human literature.

Other possible cause for increased serum ferritin concentrations following exercise could be attributed to splenic contraction. Hyppä and colleagues have assessed the influence of various types of exercise on serum ferritin concentration. They have shown that moderate exercise without hemoconcentration increased ferritin concentration, and have hypothesized that exercise may alter membrane permeability, which could lead to ferritin leakage from spleen and liver due to their high content in ferritin (Hyppä S. et al., 2002). Leakage from hepatocytes is less probable in this case, because expect for a significant increase in LDH in post race samples, no other altered hepatic parameter was found.

In conclusion, both human and equine sport medicine literature have shown that the equation between skeletal muscle and metabolic responses to exercise still has many questions. New markers and new laboratory techniques can and are required to understand pathophysiological mechanisms, their clinical relevance and their

relationship with poor athletic performance.

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