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Blood biomarkers for the characterization of muscle damage induced by lower limb exercises

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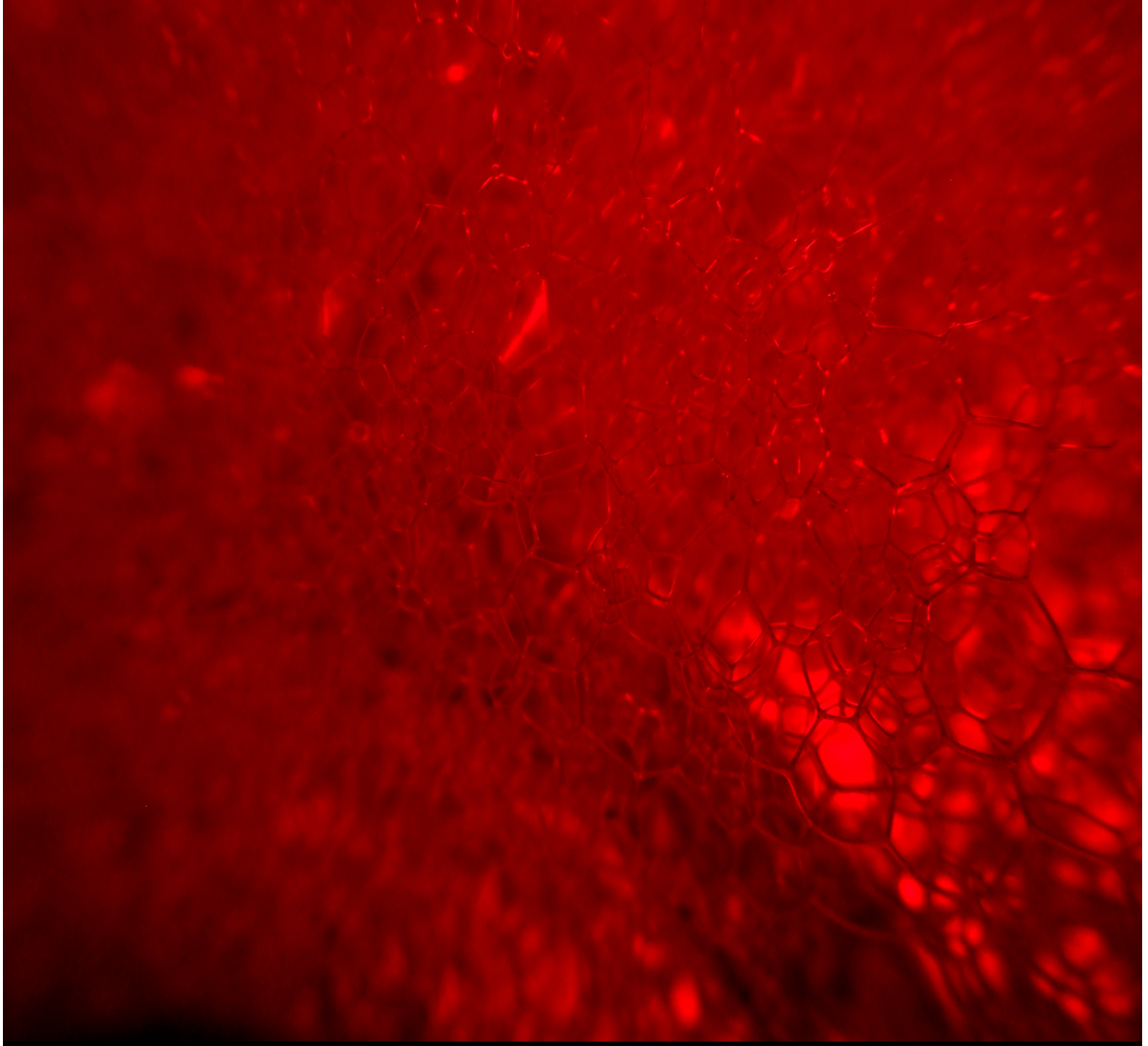

BIOMARCADORS SANGUINIS PER A LA CARACTERITZACIÓ DEL DANY MUSCULAR INDUÏT PER EXERCICIS DEL TREN INFERIOR

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Biomarcadors sanguinis per a la caracterització del dany muscular induït per exercicis del tren inferior

Blood biomarkers for the characterization of muscle damage induced by lower limb exercises

Tesi doctoral presentada per:

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A vosaltres, Sheila i Paula,
perquè sou el millor de la meva vida.

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> L'amor no és una paraula, no és una acció, no es defineix, no es limita. Com una certesa evident, s'imposa sense condicions.
>
> Alejandro Jodorowsky

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Prefaci

L'increment exponencial de la pràctica d'activitat física i esport de la població dels països occidentals s'ha associat a la necessitat d'augmentar la comprensió dels processos de resposta muscular a l'exercici, i d'optimitzar-ne els processos de recuperació. Una de les respostes a l'exercici, especialment quan aquest comporta contraccions excèntriques de certa intensitat, és el *dany muscular* o, més concretament, el *dany muscular induït per l'exercici* (EIMD). Així, el dany muscular i l'EIMD, amb 26.796 i 1351 entrades a PubMed respectivament (febrer 2016), són fenòmens sobre els quals la fisiologia ha focalitzat l'atenció en els últims anys.

Malgrat que certs processos inflamatoris puguin estar associats, el dany muscular no és el mateix que una lesió muscular. Les lesions musculars, generalment trencaments fibril·lars, s'observen, la majoria de vegades, a la unió miotendinosa i s'associen a una incapacitat muscular immediata i evident acompanyada d'una sensació de dolor aguda (80). Les lesions musculars produeixen la inhibició de la regeneració causada per la formació d'una cicatriu fibròtica, de manera que la funció mecànica del teixit es veu compromesa de manera notable. No obstant això, la majoria de vegades, l'execució d'exercicis que comporten la realització de contraccions excèntriques de certa intensitat desemboca en un dany a escala subcel·lular molt menys evident, amb la regeneració i adaptació funcional posteriors (36) a partir de la remodelació sarcomèrica (215).

L'EIMD produeix un cert nivell de pèrdua de la funció muscular que, tot i que normalment no es perllonga més de set dies, pot afectar decisivament la programació de l'entrenament esportiu. A més, un dels símptomes de l'EIMD és el dolor muscular d'aparició tardana (DOMS). La cinemàtica dels exercicis en els quals se sol·licita la musculatura que pateix DOMS es pot veure alterada i incrementar potencialment el risc de patir lesions (50). De la mateixa manera, aquesta sensació de dolor o desconfort muscular podria tenir un impacte negatiu en l'adherència a programes d'activitat física (101). Així, una comprensió més àmplia del fenomen de l'EIMD i un diagnòstic acurat poden ajudar a optimitzar les càrregues d'entrenament, reduir les lesions i augmentar l'adherència a programes d'activitat física.

Abstract

The aim of this thesis was to characterize lower limb exercise-induced muscle damage (EIMD) by analysing the time course and the magnitude of activity or concentration change of serum biochemical markers. For that purpose, lower limb EIMD was assessed by laboratory high intensity exercise (HIE) protocols (studies I, II and III) and field long duration exercise (LDE) protocols (studies IV and V).

High intensity exercise

In the first study (I), muscle damage induced by high intensity inertial half-squat exercise performed on a flywheel device was assessed through the serum evolution of muscle enzymes and fiber type-specific sarcomere proteins such as fast (type II) and slow (type I/ß) myosin (FM and SM respectively). Serum profiles were recorded at baseline and at different time points after exercise in ten healthy, physically active young men. The increase in serum muscle enzymes suggests increased membrane permeability of both fast (type II) and slow (type I) fibers, and the increase in FM reveals sarcomere disruption as well as increased membrane permeability of fast (type II) fibers. We concluded that an indirect molecular model of muscle damage could be constructed by measuring the time course of serum activity or concentration of muscle enzymes and proteins, according to their molecular weight, the fiber compartment in which they are located, and the fiber type (fast [type II] or slow [type I]) in which they are expressed.

During the second study (II) we applied the indirect molecular model of EIMD to assess the muscle response. Specifically, in a trained pole vaulter (PV) and a physical education student (PE), we investigated the effect of a leg press exercise leading to failure (LPF) on changes in serum activity of muscle enzymes and serum concentration of FM and SM, while simultaneously examining mechanical output components as indicators of the performance and fatigue developed throughout the exercise. The PV's exercise output revealed an explosive (power-oriented) profile leading to selective mild damage of fast fibers. In contrast, the PE exercise output showed a fatigue-resistant profile, which induced greater muscle enzyme activity and SM serum concentration, suggesting a higher extent of slow fiber damage.

In the last HIE study (III), we investigated the degree of damage inflicted on the hamstring muscles by an intensive voluntary eccentric exercise. We analysed

differences in the extent of damage between-subjects and within-subjects (limb-to-limb comparison). To do so, we assessed the relationship between serum biomarkers and other indirect markers of muscle damage. Thirteen males performed six sets of ten reps of eccentric unilateral leg curl with each leg. Results from this study challenge the notion that severe EIMD in humans is restricted to electrical stimulation protocols, since ten ('high responders') out of a total of thirteen subjects suffered severe muscle damage. Within-subject (limb-to-limb comparison) differences were also observed, revealing that experimental designs using contralateral limbs as a control should consider that different degrees of hamstring damage can be induced in the two legs. Sarcomeric mitochondrial creatine kinase (sMtCK) is a promising novel EIMD biomarker that allows identification of high responders. Finally, when muscle function is recovered, long-lasting increases in transverse relaxation time (T2) values suggest an adaptive process rather than damage.

Long duration exercise

The first LDE study (IV) applied the indirect molecular model of EIMD to assess the muscle response after mountain ultramarathon (MUM) in eight endurance-trained amateur athletes. From the results, we concluded that the increase in SM serum concentration after MUM may be indirect evidence of slow (type I) fiber-specific sarcomere disruptions.

Finally, the last study (V) based on LDE protocols compared serum changes of biomarkers of EIMD after a 35-km mountain trail race (MTR) and a 55-km MUM. The sample was composed of one group of ten amateur trained male athletes who ran a 35-km MTR and another group of six highly trained male athletes who ran a 55-km MUM. The results indicate that mountain running distance is related to deeper slow (type I) muscle fiber damage, even in highly trained individuals.

Resum

L'objectiu d'aquesta tesi ha estat caracteritzar el dany muscular induït per diversos exercicis del tren inferior mitjançant l'anàlisi de l'evolució temporal i la magnitud de canvi de l'activitat o la concentració de marcadors bioquímics sèrics. Amb aquesta finalitat, el dany muscular induït per diversos exercicis del tren inferior s'ha avaluat mitjançant protocols de laboratori basats en exercicis d'alta intensitat (HIE) (estudis I, II i III) i protocols de camp consistents en exercicis de llarga durada (LDE) (estudis IV i V).

Exercici d'alta intensitat

En el primer estudi (I), el dany muscular induït per un exercici de mig esquat inercial d'alta intensitat es va avaluar mitjançant l'evolució en sèrum de l'activitat d'enzims musculars i la concentració de proteïnes del sarcòmer específiques del tipus de fibra (ràpida [tipus II] o lenta [tipus I]), com ara les isoformes ràpida (tipus II) i lenta (tipus I o ß) de la miosina (FM i SM, respectivament). Es van registrar els perfils sèrics de deu homes joves, sans i físicament actius a l'inici de l'estudi i en diversos punts temporals després de l'exercici. Mentre que l'augment sèric d'enzims musculars va suggerir un increment de la permeabilitat de la membrana tant de les fibres lentes (tipus I) com de les ràpides (tipus II), l'increment d'FM va suggerir la disrupció del sarcòmer, i també un increment de la permeabilitat de la membrana de fibres ràpides (tipus II). Per tant, vam concloure que un model molecular indirecte de dany muscular induït es pot construir mitjançant l'avaluació de l'evolució temporal de l'activitat o la concentració sèrica d'enzims i proteïnes musculars, d'acord amb el seu pes molecular, el compartiment de la fibra en la qual es troben i el tipus de fibra (ràpida [tipus II] o lenta [tipus I]) en les quals s'expressen.

Durant el segon estudi (II) es va aplicar el model molecular indirecte d'EIMD per avaluar la resposta muscular. Concretament, es va avaluar, en un saltador de perxa entrenat (PV) i un estudiant d'educació física (PE), l'efecte d'un exercici de premsa de cames fins a l'extenuació (LPF). Els paràmetres mecànics de l'exercici es van utilitzar com a indicadors del rendiment i la fatiga induïda per LPF. El PV va revelar un perfil explosiu (orientat a la potència) que va induir a danys lleus i selectius de les fibres ràpides (tipus II). Per contra, el PE va mostrar un perfil resistent a la fatiga, i també una activitat enzimàtica superior i una elevada concentració sèrica d'SM, cosa que suggereix un grau més alt de dany de les fibres lentes (tipus I).

En l'últim estudi d'HIE (III), es va investigar el grau de dany induït per un exercici excèntric intensiu sobre la musculatura isquiotibial i es van analitzar les diferències en el grau de dany intersubjectes i intrasubjectes (comparació entre extremitats d'un mateix subjecte). Amb aquesta finalitat, es va avaluar la relació entre biomarcadors sèrics i altres marcadors indirectes de dany muscular. Tretze homes van realitzar sis sèries de deu repeticions de rull femoral unilateral i en van executar només la fase excèntrica amb cada cama. Atès que deu dels tretze participants en l'estudi van patir nivells elevats de dany muscular, els resultats desafien la noció segons la qual el dany muscular greu en humans es limita a protocols d'estimulació elèctrica. També es van observar diferències en la comparació entre extremitats, fet que va revelar que els dissenys experimentals que utilitzen l'extremitat contralateral com a control han de tenir en compte que es poden induir diversos graus de dany sobre la musculatura isquiotibial en ambdues cames d'un mateix subjecte. A més, la creatina quinasa mitocondrial del sarcòmer (sMtCK) sembla ser un nou i prometedor biomarcador d'EIMD que permet la identificació de *high responders*. Finalment, quan es recupera la funció muscular, increments persistents del temps de relaxació transversal (T2) suggereixen un procés d'adaptació/remodelació més que no pas dany muscular.

Exercici de llarga durada

El primer estudi d'LDE (IV) aplica el model molecular indirecte de dany muscular per avaluar la resposta del múscul després d'una ultramarató de muntanya (MUM) en vuit atletes (amateurs) entrenats en disciplines de resistència. Els resultats permeten concloure que l'augment de la concentració en sèrum d'SM després d'una MUM podria ser una evidència indirecta de disrupcions selectives del sarcòmer de fibres lentes (tipus I).

Finalment, a l'últim estudi (V) basat en protocols d'LDE es van comparar els canvis induïts per una cursa de muntanya de 35 km (MTR) i una MUM de 55 km en els nivells sèrics de biomarcadors de dany muscular. La mostra de l'estudi es va compondre d'un grup de deu homes (amateurs) entrenats en disciplines de resistència, que van competir a l'MTR, i d'un altre grup de sis homes amb nivells elevats d'entrenament, que van competir a la MUM. Els resultats indiquen que la major distància recorreguda a la MUM podria estar relacionada amb nivells superiors de dany muscular de les fibres lentes (tipus I), fins i tot en individus altament entrenats.

Abbreviations

ADP	Adenosine diphosphate
ALP	Alkaline phosphatase
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
ATP	Adenosine triphosphate
BFlh	Biceps femoris long head
BFsh	Biceps femoris short head
C-E	Concentric-eccentric
CK	Creatine kinase
CK-MB	Creatine kinase mb isoenzyme MB
CMJ	Counter movement jump
DOMS	Delayed onset muscle soreness
EIMD	Exercise-induced muscle damage
ELC	Eccentric leg curl
ELISA	Enzyme linked immunosorbent assay
FGC	Force-generating capacity
FM	Skeletal muscle fast (type II) myosin
fsTnI	Skeletal muscle fast troponin I
HIE	High intensity exercise
LC	Light chain
LDE	Long duration exercise
LDH	Lactate dehydrogenase
LPF	Leg press exercise leading to failure
MHC	Myosin heavy chain
MRI	Magnetic resonance imaging
MTR	Mountain trail race
MUM	Mountain ultra-marathon (> 42,195 km)
MVC	Maximal voluntary contraction
P_{max}	Maximum inertial concentric-eccentric power
RBE	Repeated bout effect
ROS	Reactive oxygen species
SEM	Standard error of the mean

SJ	Squat jump
SM	Muscle slow (type I/ß) myosin
sMtCK	Sarcomeric mitochondrial creatine kinase
ssTnI	Skeletal muscle slow (type I) troponin I
ST	Semitendinosus
TMB	Tetramethylbenzidine
VRS	Visual rating scale
γGT	Gamma glutamyltransferase

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1. INTRODUCTION

1.1 EXERCISE-INDUCED MUSCLE DAMAGE

Most people at some stage in their lives have probably experienced some kind of soreness or discomfort related to the performance of an unaccustomed activity, especially when involving high-force and/or high-velocity and/or high-strain eccentric contractions. The symptoms of muscle soreness and weakness experienced in the hours and days after novel eccentric exercise have been attributed to myofibrillar damage and are known in the scientific literature as 'exercise-induced muscle damage' (EIMD) (106). Although it has been one of the most important targets in sports science research, there is no established definition for EIMD. However, there is general agreement that it is a phenomenon characterized by a set of signs and symptoms (163) including 'delayed onset muscle soreness' (DOMS) (8), and an immediate and prolonged reduction in muscle function, most notably a reduction in force-generating capacity (FGC) (38).

1.1.2 Direct evidence of exercise-induced muscle damage

Direct evidence of EIMD, such as myofibrillar disruption and myofiber necrosis, requires histological analysis of muscle tissue (70). Fridén et al. (78, 79) provided the first evidence of morphological changes (i.e. Z-line disturbances) in the human contractile apparatus after an eccentric-biased exercise consisting in descending stairs repeatedly and eccentric cycling. Myofibrillar disruption is associated with structural changes to the t-tubule system and the sarcolemma (192). Immunohistochemical staining reveals degradation of cytoskeletal and myofibrillar proteins, which shows that part of the myofiber is necrotic (60, 123). Necrosis triggers an immune process characterized by an accumulation of inflammatory cells (monocytes/macrophages) observed 2-3 days post-EIMD within myofibers (118), which is a sign of degradation (163).

Histological evidence of separations of the extracellular matrix from myofibers and increased blood constituents in the extracellular space has also been reported (190). Increased efflux of muscle enzymes (especially creatine kinase [CK]) indicates that

extracellular matrix damage might increase sarcolemmal membrane permeability (190, 206).

The highest myofibrillar disruptions, observed histologically, have been found from one to four days following exercise. However they may also be observed immediately after exercise, as well as a week after exercise (79, 145, 174, 214). Myofibrillar disruptions that appear shortly after exercise signify damage, and the changes in myofibrillar structures observed some days into recovery may represent remodelling (214), especially if no significant changes in the FGC are found (81).

1.1.3 Exercise-induced muscle damage: mechanical and metabolic hypotheses

The events that occur as a consequence of eccentric exercise can follow two possible pathways: the mechanical stress and metabolic stress pathways (7, 10, 102). Although the exact mechanisms that underlie the phases in EIMD are not fully understood, while the initial muscle damage events have been reported to be mechanically-induced, the metabolic pathway may more adequately explain the persistent signs and symptoms of damage for several days following exercise.

Mechanical stress pathway

Muscle damage can occur as a consequence of mechanical loading on myofibers (102). Eccentric lengthening of sarcomeres is non-uniform (194) and results in overstretched myofilaments, which may no longer overlap within the sarcomere. Therefore, due to sarcomere inhomogeneity/disruption, passive structures (desmin and titin) absorb the additional tension, and Z-line streaming occurs (139, 170). This may cause the failure of the contractile machinery, manifesting a reduction in muscle FGC (171) (**Figure 1**).

Metabolic stress pathway

Metabolic stress induced by exercise is characterized by ischemia/hypoxia and an increase in heat, inorganic phosphates (and other metabolic waste accumulations), reactive oxygen species (ROS), and a decrease in pH. The disruption of calcium metabolism homeostasis due to an increase in the intracellular Ca^{2+} ions (135), stimulates proteolytic and phospholipolytic pathways leading to further myofibrillar

damage in the skeletal muscle (82). Eccentric exercise has been shown to lead to a loss of membrane integrity and an influx of Ca^{2+} into myofiber areas in rats (134). It has been recently proved in humans that there is a disruption in calcium homeostasis resulting from changes in the sarcoplasmic reticulum following a high intensity eccentric exercise consisting in 100 drop jumps (148). Elevated sarcoplasmic concentrations of Ca^{2+} activate several proteolytic and phospholipolytic pathways that degrade cytoskeleton, sarcolemma, mitochondria and contractile proteins (82) (**Figure 1**).

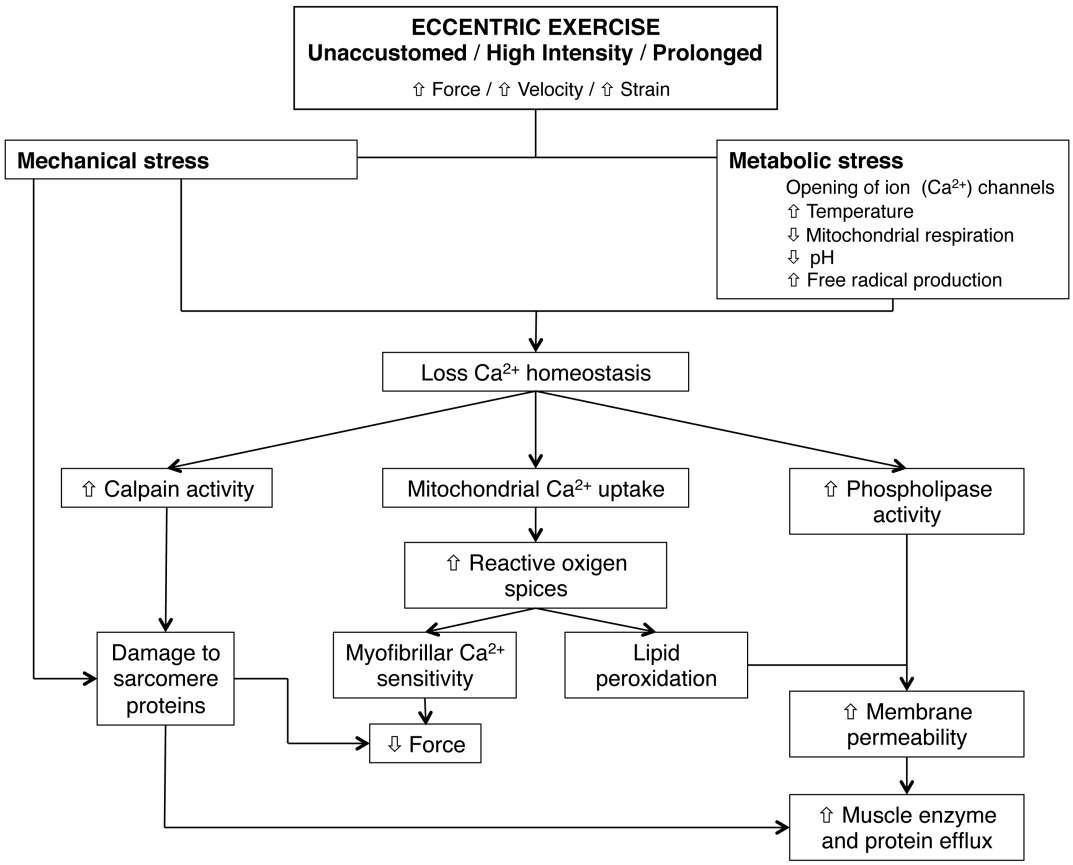


Figure 1. Theoretical model of exercise-induced muscle damage indicating hypothetical mechanisms which may be involved in increasing membrane permeability and the subsequent rise in muscle enzyme and protein efflux. Adapted from (4, 111).

1.1.4 Membrane permeability

By either mechanical or metabolic pathways, or by both, muscle damage is associated with increased membrane permeability, which results in leakage of muscle enzyme and proteins into the blood. Increased membrane permeability

provides a route into and out of the sarcoplasm that is distinct from the conventional route of endocytosis and exocytosis (134). Several hypotheses have been formulated about the mechanisms that drive increased membrane permeability;, however, they are mostly based on investigations performed with animal models.

McNeil and Khakee (134) first suggested that in rats performing eccentric exercise (running on a treadmill at an incline of 16º downhill), fiber membrane permeability was the result of membrane disruption due to mechanically induced stress. However, as it has been demonstrated that the resealing of artificially produced membrane disruptions occurs in less than a minute (14), alternative explanations for the membrane damage hypothesis have been proposed, which provide a more accurate explanation of the time course of muscle enzyme and proteins (peak at a minimum of 1 day following eccentric exercise). Increased membrane permeability may be caused by the activation of ion (Ca^{2+}) channels and a consequent increase in intracellular Ca^{2+}, phospholipase activity and ROS that peroxide membrane lipids (4) (**Figure 1**).

The membrane disruption mechanism could explain the increased membrane permeability and the subsequent muscle enzyme and protein leakage into the blood at early time points, and the activation of ion (Ca^{2+}) channels hypothesis may provide a better explanation of the persistent increased membrane permeability that leads to muscle enzyme and protein leakage several hours or days after eccentric exercise (106). Therefore, if Ca^{2+} homeostasis is not restored within a few days of exercise, irreversible damage to the myofibrillar structure and the cytoskeleton occurs, leading to partial necrosis of the myofiber (77, 82, 171).

1.1.5 Exercise-induced muscle damage assessment

Direct assessment of muscle damage requires histological examination of muscle via biopsy. However, given the invasiveness and difficulty of gathering human muscle tissue samples, indirect markers of muscle damage including perceived muscle soreness (DOMS), T2 signal intensity via magnetic resonance imaging (MRI) techniques, muscle function (i.e., decrements in FGC), and muscle-specific enzyme and sarcomere protein efflux to blood have been widely used as surrogate measures in EIMD investigations (55).

Indirect markers of exercise-induced muscle damage

The assessment of muscle damage requires reliable and valid indicators. This is definitely a major challenge because in human research there are no markers that are considered the 'gold standard' (163). It is necessary to assess the EIMD symptoms in a multifaceted way, and examine the relations within indirect markers with great caution.

Muscle function

Force-generating capacity

Although other markers of muscle function (for instance, jump capacity) have been used, changes in muscle function, measured as FGC, seem to be the most valid indirect marker of muscle damage (163, 204). FGC seems to reflect myofibrillar disruptions, inflammation and necrosis better than any other markers of muscle damage (163). Raastad et al. (174) reported a high correlation ($r = 0.89$) between the magnitude of decrease in maximal voluntary contraction (MVC) and the proportion of muscle fibers with ultrastructural disruptions.

FGC reductions that occur during or shortly after exercise may represent metabolic and/or central fatigue rather than damage, especially if concentric exercise protocols have been used (146). Because muscle fatigue is often recovered fairly quickly (within hours) (72) and muscle damage requires longer recovery periods before returning to baseline (depending on its degree > week) (163), persistent FGC decreases observed ≥ 24 h into recovery may be more representative of damage. Prolonged force loss in the days following eccentric exercise can usually last more than a week (146). Prolonged FGC recovery time results from the initial damage (mechanically-induced) during the exercise, and persistent damage several days following exercise results from the metabolic pathways involved in the regeneration process (55).

Magnetic resonance imaging

Changes in T2 signal intensity are thought to reflect increased extracellular water content. Thus, T2 increases after eccentric exercise are considered to indicate oedema in the muscle (55, 119). While short-lasting moderate increases in T2 found immediately after concentric or isometric exercise and returning to baseline values within 1 h post-exercise serve as an indicator of muscle recruitment (1, 73),

persistent increases in T2 from 24 h onwards following eccentric exercise are related to muscle damage (55, 182). Histological evidence of ultrastructural damage correlated significantly with increased MRI signal intensity 48 h following an eccentric biased exercise consisting in downhill running (157). Moreover, MRI is a safe non-invasive method to localize muscles with increased membrane permeability from which enzymes and proteins leak into the blood (119).

T2 values following eccentric exercise remain high when other indirect markers (i.e., FGC) of muscle damage have returned to baseline values (151). Specifically, increased T2 values have been found as long as 75 days (182) and 31 days (151) following (in both cases) an eccentric exercise of the elbow flexor muscles. Foley et al. (74) suggested that long-lasting T2 increases after swelling resolves and when other markers of EIMD have returned to baseline values cannot be attributed to extracellular water accumulation and may possibly reflect an adaptive process.

Perceived muscle soreness

DOMS, the most common symptom of EIMD, has been studied widely since the beginning of the 20th century (99). However, many unanswered questions relating to DOMS remain, and further research in this area is needed (50).

A hypothesis that has gained support from experimental findings in human undergoing DOMS is that mechanoreceptors, including muscle spindles, and altered processing of activity in large diameter afferents and C-tactile fibers contribute to the soreness following eccentric exercise (15, 141, 205). Specifically, changes in processing at the level of the spinal cord allow mechanoreceptors through large diameter afferents to access the pain pathway (170). It has been suggested that these mechanisms may underlie the mechanical allodynia[1] observed in DOMS.

[1]Central pain sensitization that leads to the triggering of a pain response from stimuli which do not normally provoke pain (141).

Perceived muscle soreness usually peaks 24-72 hours after exercise and subsides within a week (186). As an indirect marker, DOMS is a poor indicator of eccentric EIMD, and changes in other indirect markers of muscle damage are not necessarily accompanied by DOMS (154). Moreover, DOMS does not reflect histological evidences of myofibrillar disruptions (163) and presents considerable variability between subjects (in time course and peak magnitude) in response to the same exercise stimulus (106). However, measured as perceived muscle soreness, DOMS, has been widely used as an indirect marker of EIMD in the scientific literature.

Although in some of the studies in this thesis different indirect EIMD markers such as muscle function (measured as FGC), MRI and/or perceived muscle soreness (DOMS) have been used, the overall goal of the investigation was to assess the phenomenon of EIMD through the time course and magnitude of change of serum muscle biochemical markers (24). As a result, the next chapter in this introduction is dedicated to serum biomarkers of EIMD.

1.2 SERUM BIOMARKERS OF EXERCISE-INDUCED MUSCLE DAMAGE

Muscle enzymes and proteins leaked into blood following exercise may be used as EIMD biomarkers. A biomarker (biological/biochemical marker) is a measurable product or substance of an organism that is used as an indicator of a biological state to objectively measure physiological or pathogenic processes in the body that occur during health, disease or in response to pharmacological treatment (72). A surrogate endpoint has been defined as 'a biomarker intended to substitute for a clinical endpoint', the latter being 'a characteristic or variable that reflects how a patient feels, functions, or survives'. A surrogate endpoint is expected to predict clinical benefit or harm (or lack of benefit or harm) based on epidemiologic, therapeutic, pathophysiologic, or other scientific evidence (24). Biomarkers are often easier and cheaper to measure than 'true' endpoints (12): for example, it is easier to measure a subject's CK serum activity than use a muscle biopsy to measure the extent of muscle damage.

The criteria established by Hill (92) to analyse association in determining causation can be used for deciding the characteristics a biomarker needs to be a reliable surrogate endpoint (11) (**Table 1**).

Guidelines	Features of useful muscle damage biomarkers
Strength	A strong association between the effects of an exercise and the biomarker
Consistency	The association persists in different individuals, in different places, in different circumstances, and at different times
Specificity	The biomarker is associated with a specific muscle fiber type or compartment
Temporality	The time-course of changes in the biomarker and EIMD occur in parallel
Biological gradient (exercise 'dose' –responsiveness)	Increasing exercise volume and intensity (with the same contraction pattern) produce an increase in the biomarker levels
Plausibility	Credible mechanisms connect the biomarker, the muscle damage, and the type of exercise performed
Coherence	The association is consistent with the type of exercise performed and the biomarker
Experimental evidence	An intervention produces results consistent with the association
Analogy	There is a similar result to which we can adduce a relationship

Table 1. Austin Bradford Hill's guidelines that increase the likelihood that an association is causative. Adapted from (11).

Sorichter et al. (189) also described the features of an ideal biomarker of skeletal muscle fiber damage. One of these, which that group emphasized and is in accordance with Bradford Hill's criteria, was that the marker should be absolutely muscle fiber-specific. Nowadays, most of the biomarkers of EIMD used in the scientific literature are not fiber-type-specific and do not meet all the required features of a useful muscle damage biomarker. However, although most of the skeletal muscle enzymes and proteins do not meet all the required features of an ideal biomarker, they offer information about the functional status of muscle tissue, and vary widely in exercise conditions, thus offering a fast, accurate method for evaluating EIMD (21).

1.2.1 Exercise-induced muscle damage biomarkers

Enzymes

Serum skeletal muscle enzymes are indirect markers of the functional status of muscle tissue, and vary widely in exercise conditions. The use of blood muscle enzymes as biomarkers offers a fast, accurate method for evaluating the muscle damage caused by exercise (21). Although many enzymes leak into the blood from damaged muscles, we focus on CK and transaminases, aspartate aminotransferase and alanine aminotransferase (AST and ALT, respectively), which have been widely used as biomarkers (indirect markers) of EIMD (30-32).

Creatine kinase

Characteristics

CK is a dimeric globular enzyme of ~82 kDa which is found in both the sarcoplasm and mitochondria of the muscle fiber (13). CK buffers cellular adenosine triphosphate (ATP) and adenosine diphosphate (ADP) concentrations by catalysing the double-sided exchange of high-energy phosphate bonds between phosphocreatine and ADP produced during muscle contraction (32) (**Figure 2**). CK plays a key role in the energy homeostasis of cells with fluctuating energy requirements (201).

Isoenzymes

Structurally, CK has two polypeptide subunits: M, muscle type, and B, brain type. These subunits allow the formation of three tissue-specific isoenzymes in cytoplasm: CK-MB (cardiac and skeletal muscle), CK-MM (skeletal muscle) and CK-BB (brain). In the mitochondria there are two specific forms of mitochondrial CK (Mt-CK): ubiquitous Mt-CK (uMtCK) (expressed in various tissues such as brain and smooth muscle) and sarcomeric Mt-CK (sMtCK) (expressed in skeletal muscle) (180). While fast and slow fibers present almost equal CK activity (6), slow (type I) fibers have higher CK-MB and sMtCK (211).

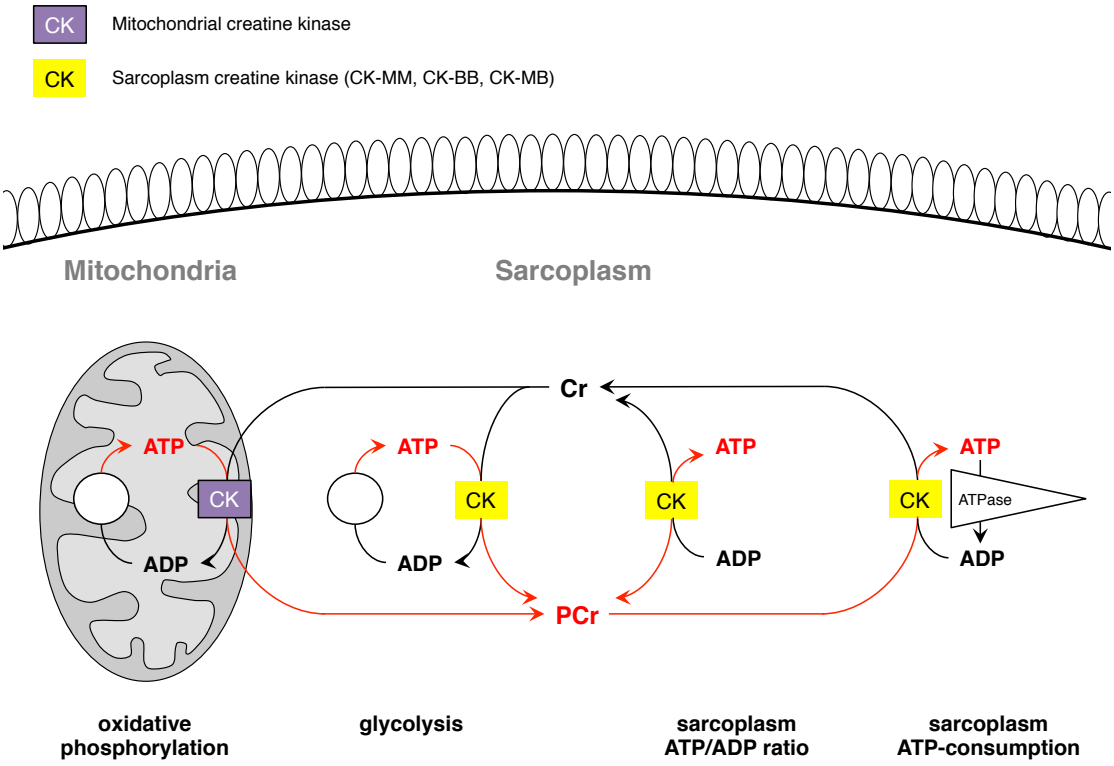


Figure 2. The creatine kinase/phosphocreatine system and the compartment-specific isoenzymes of creatine kinase (CK) found in mitochondria and sarcoplasm. Adapted from (180).

Location into the cell compartment

Three isoenzymes (CK-MM, CK-MB, CK-BB) are located in the cytoplasm and two isoenzymes (uMtCK, sMtCK) in the mitochondria, providing specific information on injured tissue because of their tissue distribution (30) (**Figure 2**). Mitochondrial CK isoenzyme (Mt-CK) is an octameric enzyme localized in the intermembrane and cristae space of the mitochondria (66). Moreover, sMtCK is present only in striated muscle (164).

Clinical reference values

Baseline levels of serum CK in general population are in the range of 35 to 175 $IU \cdot L^{-1}$ (13), and are higher in trained athletes (98). Specifically, reference intervals for CK in trained athletes from different sports were 82-1083 $IU \cdot L^{-1}$ in males and 47-513 $IU \cdot L^{-1}$ in females (140).

Mechanisms of appearance and clearance from blood

When exercise leads to muscle damage, CK appears in the blood via the pathway mentioned above (increased membrane permeability). The CK flux into serum is a physiological reaction to exercise but there is a breakpoint at 300-500 $IU \cdot L^{-1}$ (32). Leaked CK into the interstitial space is taken by the lymphatic system and released into blood circulation (22). Although not all the mechanisms implicated in CK clearance from the blood are known (13) it is commonly accepted that the reticuloendothelial system is responsible for this function and that the clearance time depends on subject's training status and the intensity and duration of exercise (59).

Peak magnitude and temporal response following eccentric exercise

Time course to CK serum peak following eccentric exercise shows a wide variability between studies. Peak magnitude is determined by many factors related to exercise (such as intensity and duration), as well as by genetics, the subject's training status and/or previous exposure to exercise and the muscles involved (13, 30, 32, 106). Focusing on lower limb exercise protocols, regular athletic resistance exercises performed bilaterally and downhill running, evoke the smallest magnitude and fastest peak CK response (~ 1000 $IU \cdot L^{-1}$, 1 day after exercise), followed by isolated (unilateral) high intensity eccentric contractions of the knee extensors (range: 200 to 25.000 $IU \cdot L^{-1}$, at 1-4 days after exercise), and, finally, ultra-marathon running over extreme distances (> 40.000 $IU \cdot L^{-1}$, immediately after the exercise) and neuromuscular electrostimulation of the knee extensors (range: 1350-85.000 $IU \cdot L^{-1}$, at 4 days after exercise) (**Table 2**). Thus, the contrasting findings on CK time course and peak magnitude challenge the appropriateness of comparing fiber responses from studies using quite different eccentric exercise protocols.

Authors	Exercise (mode)	Sets and repetitions	Intensity	Peak magnitude (time to peak)
Davies et al. (62, 63)	Barbell half-squat (bilateral)	10 x 10 (-)	70% of subject's body mass	~ 800 IU·L^{-1} (1 day)
Malm et al. (130)	Treadmill downhill running at 4º or 8º	1 x 45 min (each condition of 4º and 8º)	4º: 50% of $VO2_{max}$ 8º: maximum tolerated speed	~ 1000 IU·L $^{-1}$ (1 day)
Nurenberg et al. (157)	Treadmill downhill running at 8º	1 x 30 min	8 km·h^{-1}	200-1200 IU·L $^{-1}$ (range: 12-36 hours)
Trappe et al. (196)	Isolated knee extension (unilateral)	10-14 x 10 (1 min)	120% of concentric 1-RM	~ 5000 IU·L^{-1} (5 days)
Stupka et al. (191)	Leg press and isolated eccentric leg extension (unilateral). ROM: 15-90º	Leg press: 3 x 12 Leg extension: 10 x 10 (3 min)	Leg extension: 120% of concentric 1-RM	♀: 200-1400 IU·L^{-1} ♂: 300-2100 IU·L^{-1} (range: 4 days)
Bourgeois et al. (29)	Leg press and isolated eccentric leg extension (unilateral)	6 x 10 (-)	Leg extension: 80-85% of concentric 1-RM	~ 750 IU·L^{-1} (1 day)
Beaton et al. (17)	Eccentric isokinetic leg extension (unilateral). ROM: 60-120º	30 x 10 (1 min)	30º·s^{-1}	~ 300 IU·L $^{-1}$ (2 days)
Paulsen et al. (162)	Eccentric isokinetic leg extension (unilateral). ROM: 35-105º	30 x 10 (30 sec)	30º·s^{-1}	~ 200-25.000 IU·L^{-1} (range: 4 days)
Child et al. (51)	Eccentric isokinetic leg extension (unilateral). ROM: from prone position, almost full extension and flexion	1 x 70 (10 sec between each rep)	1.75 rad·s^{-1}	~ 16.000 IU·L^{-1} (4 days)
Fouré et al. (76)	Neuromuscular electrostimulation. Hip and knee angles joints secured at 90º and 100º, respectively	1 x 40 contractions (each leg)	Maximal level of evoked force according to the pain threshold	1350-85.000 IU·L^{-1} (range: 4 days)
Skenderi et al. (185)	246-km continuous running race			~ 43.000 IU·L^{-1} (after the race)

Table 2. Characteristics from the studies in which creatine kinase (CK) were used for the assessment of muscle response to lower limb eccentric exercise. ROM, range of movement. 1-RM, one repetition maximum.

Transaminases

Characteristics

AST and ALT are both enzymes of 90 kDa (175). AST catalyses the interconversion of aspartate and α-ketoglutarate to oxaloacetate and glutamate, and ALT catalyses the reversible transfer of α-amino group of alanine to the α-keto group of ketoregularic acid to generate glutamate and pyruvate. These reactions occur between the mitochondria and the cytosol, and provide energy to cells (30). It has been reported that slow (type I) fibers have higher AST activity (177).

Location in the cell compartment

ALT is found in the cytoplasm and AST is found both in the cytoplasm and the mitochondria. These enzymes are mainly in the skeletal and myocardial muscle, liver and erythrocytes (30, 210).

Clinical relevance and reference values

Although AST and ALT increases may be due to muscle and/or liver damage, when more specific hepatic injury markers such as alkaline phosphatase (ALP) and gamma glutamyltransferase (γGT) remain unaltered, EIMD can be distinguished from liver damage (20). The transaminases AST and ALT are released at a constant rate, and their usual healthy levels represent the equilibrium between the normal turnover of hepatocytes and clearance of enzymes from blood (28). According to Nathwani et al. (142), normal serum values for AST range between 10-40 $IU \cdot L^{-1}$, and ALT between 20-65 $IU \cdot L^{-1}$.

Mechanisms of appearance and clearance from blood

When exercise leads to muscle damage, transaminases, especially AST, appear in blood via the pathway mentioned previously (increased membrane permeability). Although in animal models it has been suggested that muscle-released transaminases, specifically AST, could reach the circulating blood directly from the intracellular compartment, leaked muscle enzymes are mainly transported via the lymph into the blood stream because of the extremely low capillary permeability of muscle tissue (127).

Peak magnitude and temporal response following eccentric exercise

Although the etiologic bases for transaminase increases are not well understood, and the reliability of AST (and especially ALT) as EIMD biomarkers still remains unclear, numerous studies following cycling or running ultra-endurance events have reported serum increases in transaminases, using AST and ALT as muscle damage biomarkers (20, 68). Studies using serum transaminases as EIMD biomarkers are displayed in **Table 3**, where it can be observed that most studies analysing long distance events found AST and ALT serum peaks immediately after exercise. Petersson et al. (168) reported serum increases of AST and ALT between 2 and 5 days after a whole body weightlifting program in men who were not experienced with strength exercises and who had no evidence of liver disease. Large increases of transaminases are also described in patients hospitalized under 'extreme exercise' conditions (i.e., vigorous squatting session or long distance run) (142) **Table 3**. Time course to serum peak seems to differ between ultra-endurance exercise (immediately after exercise) and resistance exercise (range 2-4 days after exercise) **Table 3**.

Authors	Exercise (mode)	Sets and repetitions	Intensity	Peak magnitude (time to peak)
Skenderi et al. (185)	246-km continuous running race			AST: ~ 1200 IU·L^{-1} (post) ALT: ~ 250 IU·L^{-1} (post)
Klapcińska et al. (113)	48-hours flat Ultra-Marathon			AST: ~ 800 IU·L^{-1} (post) ALT: ~ 220 IU·L^{-1} (post)
Nathwani et al. (142)	'Extreme exercise' (vigorous squatting or long distance run)	NR	NR	AST: 2466 (range: 421-3967) IU·L^{-1} ALT: 497 (range: 115-712) IU·L^{-1} (at hospitalization)
Nosaka et al. (152)	Eccentric biceps curl (unilateral). ROM: 50-170º	1 x 24 (15 sec between each rep)	Maximal	AST: 105 IU·L^{-1} (4 days)
Clarkson et al. (56)	Eccentric biceps 'preacher' curl (bilateral)	2 x 25 (5 min, 12 sec between each rep)	Maximal	AST: 113 IU·L^{-1} (4 days) ALT: 45 IU·L^{-1} (4 days)
Oakley et al. (158)	Eccentric isokinetic knee flexion (unilateral)	5 x 10 (1 min)	NR	AST: 144 IU·L^{-1} (3 days) ALT: 45 IU·L^{-1} (3 days)
Fonda et al. (75)	Drop jumps (bilateral), Leg curl (bilateral)	5 x 10 (1 min) each exercise	Drop jumps: 0.6 m height, Leg curl: 70% and 130% of concentric 1-RM	AST: 47 IU·L^{-1} (4 days)

Table 3. Characteristics from the studies in which aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) were used for the assessment of muscle response to exercise. NR, none reported. ROM, range of movement. 1-RM, one repetition maximum.

Sarcomere proteins

Although other skeletal muscle sarcomere proteins such as α-actin (131), troponin I (187), and troponin isoforms, including fast troponin (fsTnI) and slow troponin I (ssTnI) (46), have been used as biomarkers of EIMD, the next section focuses on myosin and the studies which have used β/slow myosin heavy chain (MHC) fragments as an EIMD biomarker.

Myosin

Characteristics

With a molecular mass of ~493 kDa (95), sarcomeric myosin is a contractile protein that slides along actin, hydrolyses adenosine triphosphate (ATP) and produces movement (167). It is composed by three functional subdomains: (1) the motor domain which interacts with actin and binds ATP, (2) the neck domain which binds light chains and calmodulin, and the tail domain which serves to anchor and position the motor domain so that it can interact with actin (181). Myosin molecules can be dissociated into six polypeptide chains: two heavy chains (MHC), coiled around each other forming a double helical structure with a molecular weight of ~200 kDa each, and two pairs of light chains (LC) or calmodulin with a molecular weight of ~15-26kDa (85), called essential or alkali LC and regulatory or phosphorylatable LC respectively (178).

Isoforms and fiber type

Human skeletal muscle fibers can be differentiated histochemically into slow (type I) and fast (type II), which can be further subdivided into types IIa and IIx (23, 207) depending on its myosin heavy chain (MHC) isoforms (167). The existence of numerous MHC isoforms (MHC1 or ß/slow, MHC2A and MHC2X or fast) differentially distributed in different fibers makes MHC the best available marker for fiber typing (178). MHC2 isoforms are characteristic of fast (type II) fibers from skeletal muscle only, and the MHC1 isoform is found in slow (type I) fibers from both cardiac and skeletal muscle. MHC isoforms revealed the existence of ‘pure’ (only one MHC isoform) and ‘hybrid’ (two or more MHC isoforms) muscle fibers (166) (for a review see Schiaffino and Reggiani (178)).

Location in the fiber compartment

Myosin is located in the sarcomere, conforming the thick filament in a bipolar disposition throughout the A-band, and cross-linked at the center by the M-band (2).

Appearance in blood following eccentric exercise

Mair et al. (129) first described β/slow MHC fragments as EIMD biomarkers of slow (type I) fibers and established the upper reference limit of β/slow MHC fragments in plasma at 300 $\mu U \cdot L^{-1}$. According to its time course, there seems to be a delay in the release of β/slow MHC fragments in serum once the exercise is completed (129, 172, 187, 188). The first significant increases in serum levels of β/slow MHC fragments appear 24 h after exercise (172, 188) but the serum peak shows a wide variability ranging from 48 h (172) to 6 or 9 days after exercise (129) (**Table 4**). However, while eccentric exercise laboratory studies involved recreationally active subjects (physical education students), Prou et al. (172) analysed muscle response in highly trained triathletes (14.8 hours per week of training). The magnitude of increase in β/slow MHC fragments has been reported to indicate degradation of myofibrils as well as increased membrane permeability (129).

Authors	Exercise (mode)	Sets and repetitions	Intensity	Peak magnitude and/or time course
Mair et al. (129)	Eccentric leg extension (unilateral) in an exercise rack	7 x 10 (3 min)	110% of MVC	Started to rise from the 2^{nd} day and peaked the 6^{th}-9^{th} day after exercise (no values available)
Prou et al. (172)	Nice Triathlon	Swimming: 4 km Cycling: 120 km Running: 32 km		Baseline: 142 ± 69 $\mu U \cdot L^{-1}$ Post: 156 ± 116 $\mu U \cdot L^{-1}$ 48 h: 2603 ± 1405 $\mu U \cdot L^{-1}$ 4 days: 1002 ± 523 $\mu U \cdot L^{-1}$
Sorichter et al. (187)	Eccentric leg extension (unilateral) in an exercise rack	7 x 10 (3 min)	150% of MVC	Peak values between 1 and 4 days (median: 330, range: 168-454 $\mu U \cdot L^{-1}$)
Sorichter et al. (187)	Treadmill downhill running at 16% incline	1 x 20 min	70% $VO2_{max}$	Peak values at 48 h (median: 1021, range: 457-1540 $\mu U \cdot L^{-1}$)
Sorichter et al. (188)	Eccentric leg extension (unilateral) in an exercise rack.	7 x 10 (3 min)	150% of MVC	Before: 93 $\mu U \cdot L^{-1}$ 1 day after: 416 $\mu U \cdot L^{-1}$ 4 days after: 458 $\mu U \cdot L^{-1}$

Table 4. Characteristics from the studies in which β/slow myosin heavy chain (MHC) fragments were used for the assessment of muscle response to lower limb eccentric exercise. MVC, maximal voluntary contraction.

New tendencies in exercise-induced muscle damage serum biomarkers

In view of the features that an ideal serum biomarker should have (189), a number of studies have recently focused on biomarkers of skeletal muscle fast (type II) fibers. Chapman et al. (46) and Dahlqvist et al. (61) proposed serum increases of fsTnI as indirect markers of fast (type II) fibers. In relation to degrees of muscle injury established by magnetic resonance images, Guerrero et al. (2008) suggested increases in the blood stream of muscle myosin isoforms, fast (type II) and slow (type I / ß) (FM and SM respectively) as valid and reliable biomarkers of muscle injury because of their fiber specificity.

1.3. THE EXTENT OF EXERCISE-INDUCED MUSCLE DAMAGE

Based on an extensive review, Paulsen et al. (163) were the first authors to propose categorizing the degree of EIMD as mild, moderate and severe, according to FGC reduction and CK serum activity:

- Mild EIMD is characterized by small reductions in FGC (< 20%), CK activity lower than 1000 $IU \cdot L^{-1}$, and full recovery is normally complete within 48 hours.
- Moderate EIMD corresponds with sensible FGC declines (20-50%), CK activity higher than 1000 $IU \cdot L^{-1}$, and/or recovery is complete between 2 and 7 days following exercise.
- Severe EIMD is characterized by a large reduction in FGC (> 50%), CK activity higher than 10.000 $IU \cdot L^{-1}$, and long-lasting recovery (> week).

1.3.1 Variables influencing the extent of exercise-induced muscle damage

Exercise intensity

As stated above, eccentric exercise, especially when involving high intensity contractions (i.e., high-force and/or high-velocity and/or high-strain) can cause muscle damage. The relationship between the different intensity factors with the extent of EIMD is examined next.

Eccentric contraction force

In electrically stimulated extensor digitorum longus muscles in mice, it was observed that the extent of damage was related to the peak force developed during eccentric contractions (132). In humans, the role of eccentric exercise force magnitude on the muscle damage extent has been widely studied on the upper extremities (i.e., elbow flexors; biceps brachii). Specifically, Nosaka and Newton (153), and Peake et al. (165) indicated that exercise intensity played an important role in the extent of muscle damage by comparing a single bout of maximal and submaximal (50% and 10% of maximum isometric strength respectively) eccentric contractions of the elbow flexors. However, the influence of eccentric peak torque on the extent of muscle

damage is controversial. Chapman et al. (45) found no relationship between the eccentric peak torque, measured during an exercise consisting in eccentric contractions of the elbow flexors, and the level of muscle damage. In contrast, high specific torque eccentric contractions (via electrical stimulation) of the knee extensors were found to induce greater changes in markers of muscle damage (26).

Eccentric contraction velocity

The commonly held belief amongst athletes and coaches that high velocity exercise results in superior levels of muscle damage is supported by most human experiments comparing fast versus slow eccentric contractions (42-44, 183). Only one study has found a greater magnitude of muscle damage after a slow velocity protocol (161), and one other reported no differences between velocities analysed for muscle damage markers (16). However, it should be taken into account that all the experiments comparing the effect of fast versus slow eccentric contractions over the extent of muscle damage involved the elbow flexors, so extrapolating those observations to other muscle groups may be misleading. Moreover, although different studies using animal models have also found a relationship between the eccentric contraction velocity and the extent of muscle damage (132, 203), Brooks and Faulkner (35) stated that eccentric velocity was only relevant for the degree of muscle damage during lengthening contractions with a large strain.

Eccentric contraction strain

One of the most critical factors for EIMD is high strain (i.e., muscle lengthening beyond the optimum length for force-generation) (139, 171). In animals, it has been proved that the extent of muscle damage is highly dependent on the eccentric strain magnitude (123, 124, 193, 208) and that muscle length before stretch may influence muscle damage by significantly increasing the fiber strain magnitude (37). Those observations were confirmed in human experiments at different muscle lengths (range of motion) involving the elbow flexors (109, 144, 155) and the knee extensors (52); these studies reported similar results in so far as more force was lost after subjects exercised with muscle initially set at a longer length. For example, in an exercise consisting of eccentric contractions of the knee extensors at short muscle length (160º to 80º) and long muscle lengths (120º to 40º), Child et al. (52) reported a greater FGC decline after exercise at the longer muscle length.

The large variances observed in studies analysing the influence of exercise intensity over the extent of muscle damage may be due to the disparity in methods (exercise protocol, muscle function variables measured, subjects' training background, if any, and muscle group/s involved). The large variation in muscle damage indices within and between studies makes it difficult to reach a definitive conclusion on the contribution of eccentric exercise intensity factors. Therefore, although the extent of muscle damage is related to exercise intensity factors such as high-force and/or high-velocity and/or high-strain, many other variables such as the mode of muscle activation, genetic variability, age, gender, the muscle group involved and/or the novelty of eccentric exercise may also substantially influence the extent of EIMD (106, 163).

Mode of muscle activation

The notion that muscle damage necessarily occurs following voluntary eccentric exercise in humans has been strongly challenged. Recently it was shown that the extent of muscle damage in humans was greater following electrically stimulated contractions (severe disturbances of Z-lines, myofibers and extracellular matrix followed by a large loss of FGC and myofiber necrosis) (60) than following voluntary eccentric contractions (minor myofibrillar disruptions and minor reductions in FGC) (81, 136). Moreover, extremely large CK serum increases (range: 1350 to 85.000 $IU \cdot L^{-1}$) have been found following isometric electrical stimulation-induced muscle damage of the knee extensors (76) (**Table 2**). According to Paulsen et al. (163), exercise involving electrically stimulated contractions can induce severe EIMD and, although large variations in the responses of healthy individuals are expected, regular athletic training involving voluntary eccentric contractions (i.e., resistance exercise) typically causes mild EIMD. Intensity factors related to the eccentric component of the resistance exercise such as high-force and/or high-velocity and/or high-strain, may increase the degree of muscle damage from mild to moderate.

Muscle group involved

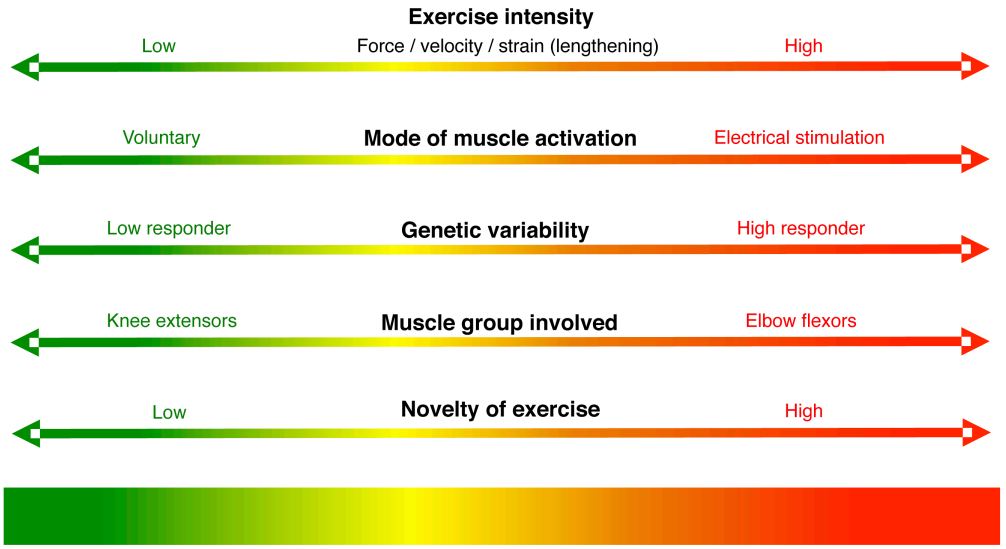
It has been stated that knee extensors are less predisposed to muscle damage following high intensity eccentric contractions, since their exposure to eccentric contractions in daily activity is one of the main factors determining susceptibility (48). Moreover, because of the differences in the architecture of the arm and leg muscles, it is possible that mechanical stress per muscle unit differs between these two muscle groups when doing eccentric exercises of the same intensity (121). Changes

in muscle length during eccentric contractions appear to be another contributing factor (48). Experiments involving maximal isokinetic voluntary contractions of the elbow flexors have reported high levels of EIMD, reflected by large, long-lasting decreases in FGC (≥ 50%, 1 day after exercise) (46, 120) and sharp CK serum increases (9698 ± 4270 $IU \cdot L^{-1}$, 4 days after exercise) (120). Moreover, Chapman et al. (46) found significant increases in sfTnI from baseline (5.5 ± 5.2 $ng \cdot mL^{-1}$) to 14.6 ± 12.8 $ng \cdot mL^{-1}$, and 89.5 ± 71.4 $ng \cdot mL^{-1}$, at 1 to 4 days following eccentric exercise, indicating selective fast (type II) fiber damage.

Fiber susceptibility

In animal models, it has been proved that fast (type II) fibers are more susceptible to EIMD (125, 199) due to their morphological and biomechanical characteristics such as their less elastic titin isoform (97), lower desmin content (53), narrower Z-discs (128), shorter fiber length (122) and lower optimum length for tension (34) than slow fibers. In human studies, histological (79, 108) and serum biochemical (46) evidence of selective fast (type II) fibers damage following intense eccentric exercise has also been found.

Hydhal and Hubal (106) propose that muscle response to eccentric contractions functions on a continuum along which exercise intensity (i.e., high-force and high-velocity and/or high-strain) has a direct influence over the extent of muscle damage, but that other modifying factors (genetic variations, muscle group involved and/or novelty of exercise) are also crucial and must be carefully analysed (**Figure 3**). Further human studies are necessary to clarify how eccentric exercise intensity affects the extent of muscle damage.



	Mild muscle damage	Moderate muscle damage	Severe muscle damage
Force-generating capacity reduction	< 20%	20-50%	> 50%
Creatine kinase activity	< 1000 IU·L^{-1}	> 1000 IU·L^{-1}	> 10.000 IU·L^{-1}
Time to recovery	48 h	2-7 days	> week

Figure 3. Individual response to a bout of eccentric exercise is dependent on several factors, including exercise intensity, the mode of muscle activation, genetic variability, the muscle group involved and the novelty of the exercise. Lines with bidirectional arrows indicate that response under each condition can occur anywhere along the continuum, e.g., high exercise intensity results in a greater extent of muscle damage than low exercise intensity but may also result in a less damaging stimulus under circumstances of voluntary contractions, low genetic responding, low novelty, etc. Adapted from (106, 163).

Genetic variability: high versus low responders

Large inter-individual variation in response to eccentric exercise is commonly reported (45, 47, 58, 105, 152, 162). Based on the extent of muscle damage individuals, can be characterized as ‘low’, ‘moderate’ or ‘high’ responders (163). The ‘repeated bout effect’ (RBE), defined as the adaptation whereby a single bout of eccentric exercise protects against muscle damage from subsequent eccentric bouts (133), and may last some months following the first bout (156), have been proposed as one of the mechanisms responsible for the variations between individuals. Aside from age or gender, other contributing factors that could explain those large variations include genetic variability across individuals (54, 104, 212, 213) and training status (32, 162, 200).

1.4 MUSCLE DAMAGE INDUCED BY LONG DURATION EXERCISE

Exercise volume (i.e., duration) may also substantially influence the extent of muscle damage. It is well known that prolonged exercise can lead to muscle damage, especially if there is a component of eccentric work, as in running (9). For this reason, long distance running under laboratory or field conditions has been widely used as an EIMD model.

1.4.1 Level running distance and muscle damage

Functionally, in order to support the weight of the body against gravity, most lower limb muscles work eccentrically for some part of the normal gait cycle during flat running. When this eccentric work of the 'anti-gravity' muscles (knee extensors, muscles of the anterior and posterior tibial compartments and hip extensors) is performed over a long distance, muscle damage is likely to occur. Histological evidence of myofibrillar disruption (202) and necrosis (91) has been found in endurance runners after the completion of a long-distance running competitive event such as the athletic marathon (42,195 km).

Regarding running distance, Noakes (149) indicates that increases in serum muscle enzymes following a running event are related more to duration than to intensity. During level running it has been well established that the total running distance covered or the time spent running is related to the amount of Ca^{2+} accumulation and the extent of muscle damage (159). Furthermore, the degree of muscle enzyme leakage was found to be dependent on running distance (159). During a 48-h flat running ultra-marathon, increased serum levels of CK, LDH, AST and ALT indicate that active muscle suffers a significant degree of sarcolemmal damage with increased running distance (113). Moreover, in ultra-endurance events (100-km) a significant degree of sarcolemmal damage is caused during the run (160) and can lead to extraordinarily high serum enzyme levels, as demonstrated during a 246-km ultra-endurance event in which participants' mean values of CK increased from 178 ± 18 at the baseline to 43.763 ± 6764 $IU \cdot L^{-1}$ immediately after the race (185). Therefore, the longer the distance run, the greater the stress and damage on muscle tissue.

1.4.2 Mountain trail running

There is no consensus about the definition of mountain running competitions, which is a semantic debate beyond the scope of the present thesis. The term 'mountain trail race' (MTR) has been generically used to refer to competitive long-distance off-road runs from 15 to > 90 km, which are performed in a mountain context and involve a great cumulative elevation gain (uphill and downhill) (64). The term 'mountain ultra-marathon' (MUM) has been specifically associated to MTR over a longer distance than the marathon (> 42,195 km) (137). While distance and the total elevation gain (climb distance^{-1} ratio) are the main extrinsic indicators of effort in MTR, the pace running is the most important performance parameter.

These kinds of competition present an exclusive physiological stress profile induced through combined fatigue and muscle damage: They have experienced a great rise in popularity during the last decade (94) and offer a unique opportunity for field-specific assessments of EIMD in humans.

1.4.3 Mountain trail running as an exercise-induced muscle damage model in exercise physiology

In spite of the popularity of these strenuous events, very little is known about the structural damage inflicted on the muscle fibers of the athletes who participate. It is generally agreed that mountain running competition causes muscle damage due to neuromuscular fatigue (138) and the numerous eccentric contractions completed during the downhill phases (67, 130). When running downhill, the eccentric component increases because the peak flexion angles become significantly greater, and this is a much stronger stimulus for damage than level or uphill running (67).

As previously indicated, the change in muscle function, measured as reduced FGC, has been proposed to be a reliable and valid indirect marker of muscle damage, reflecting myofibrillar disruption, inflammation and necrosis (174). Protocols analysing downhill running, as encountered in trail races, normally induce mild to moderate muscle damage (33, 71, 130). Field studies analysing the neuromuscular fatigue and damage induced by long distance mountain running have reported significant decreases in FGC both immediately and several days after a MUM. Specifically, (138) reported a 35% reduction in baseline MVC torque shortly after a

166-km MUM and this reduction was still significant (9% below baseline levels) five days after the competition, which is consistent with a moderate EIMD process.

1.4.4 Serum biomarkers of muscle damage and mountain trail running

Biochemical assessment of muscle damage has also been used in mountain trail competitions, but the relationship between distance and muscle enzyme efflux is unclear when comparing CK values obtained by different studies analysing the physiological consequences of MUM (**Table 5**).

Authors	Sample	Mountain ultra-marathon distance and negative elevation gain	CK ($IU \cdot L^{-1}$)
Hoffman et al. (93)	40 women and 176 men	161-km 7000 m	After: range: 1500 to 264.300, median: 20.850, mean: 32.956
Millet et al. (138)	22 experienced ultra-marathon runners	166-km 9500 m	Baseline: 144 ± 94 After: 13.633 ± 12.626
Saugy et al. (176)	15 experienced ultra-marathon runners	330-km 24.000 m	Baseline: 112 ± 33 After: 3719 ± 3045

Table 5. Creatine kinase (CK) values obtained in different studies analysing Mountain ultra-marathon physiological consequences.

The inconsistency between CK activity and the MUM distance displayed in **Table 5** may be due to the high complexity of mountain trail competitions (total elevation gain), environmental factors (weather and surface conditions), and the pacing strategy used by runners, especially during downhill running when the eccentric component is exacerbated (67). Moreover, when competing over very long distances, athletes tend to experience high levels of fatigue and use a more conservative pacing strategy in order to finish the race. For example, Saugy et al. (176) reported that most of the participants in a 330-km MUM (*Tor des Géants*) walked the last part of the competition due to nociceptive feedback and sleep deprivation-induced fatigue.

Sarcomere proteins have also been used as biomarkers of muscle damage induced by mountain running. Koller et al. (116) used β/slow (type I) MHC fragments as a muscle fiber type-specific damage biomarker. That group found β/slow MHC fragment increases in plasma in trained individuals following a MUM (67-km distance; 30-km downhill running) and although slow (type I) MHC fragments are common to skeletal and cardiac muscle, the damage was mainly related to slow (type I) fibers of skeletal muscle.

2. RESEARCH AIMS and HYPOTHESES

2.1 AIMS

2.1.1 General aims

The aim of this thesis was (I) to characterize the muscle damage induced by different lower limb exercises by analysing the time course and the magnitude of activity or concentration change of serum leaking enzymes and investigate into new biomarkers, as well as (II) to examine its relationship with other indirect markers of muscle damage.

2.1.2 Specific aims

The specific aims of this thesis were:

Preliminary aim

1. To construct an indirect molecular model of EIMD by measuring the time course of serum activity or concentration of muscle enzymes and proteins, according to their molecular weight, the fiber compartment in which they are located, and the fiber type (fast [type II] or slow [type I]) in which they are expressed (study I).

Functional aims

2. To analyse the extent of lower limb muscle damage induced by high intensity exercise under laboratory conditions by measuring serum levels of biomarkers (studies I, II and III).
3. To analyse the extent of lower limb muscle damage induced by long duration exercise under competitive conditions (i.e., mountain trail running) by measuring serum levels of biomarkers (studies IV and V).
4. To examine the influence of mountain trail distance and runners' training status on the serum levels of muscle damage biomarkers (study V)

2.2 HYPOTHESES

Study I

1. Myosin isoforms are a valid and highly sensitive biomarker of exercise-induced sarcomere damage.
2. A model of mild EIMD can be constructed by measuring the time course of the serum activity or concentration of muscle enzymes and proteins following high intensity concentric-eccentric (C-E) inertial exercise, according to their molecular weight and the fiber compartment in which the enzyme or protein is located.
3. By measuring fiber type-specific sarcomere proteins such as FM and SM, the type of fibers damaged by exercise can be assessed.
4. Fast (type II) and slow (type I) fibers are damaged in a similar manner during high intensity C-E inertial exercise.

Study II

1. A C-E leg press exercise leading to failure (LPF), a high intensity and volume exercise, induces both fast (type II) and slow (type I) fibers damage.
2. A national level pole vault athlete, as a power athlete, is expected to have higher proportions of fast (type II) fibers in the knee extensors; therefore, high power outputs during C-E leg press exercise leading to failure (LPF) are expected.
3. In contrast, a physical education student is likely to have similar proportions of fast (type II) and slow (type I) fibers in the knee extensors; therefore, high fatigue-resistant outputs during LPF can be expected.
4. The LPF output is related to the type of fibers damaged.

Study III

1. Severe hamstring muscle damage can be induced in humans following an intensive unilateral (performed separately with both legs) eccentric leg curl exercise (ELC).
2. Different degrees of muscle damage can be expected between-subjects following an intensive unilateral ELC (performed separately with both legs).

3. Similar degrees of muscle damage can be expected within-subjects (limb-to-limb comparison) following the same intensive unilateral ELC (performed separately with both legs).
4. sMtCK is a valid and highly sensitive biomarker of exercise-induced skeletal muscle damage.

Studies IV and V

1. Since there is evidence of muscle damage after prolonged mountain running, and since endurance-trained athletes have higher proportions of slow (type I) fibers, competing in a mountain trail race (MTR) or a mountain ultra-marathon (MUM) (> 42.195 km) induces slow (type I) fiber-specific sarcomere disruptions.
2. Mountain trail distance (exercise duration) is related to the extent of slow (type I) fiber muscle damage.
3. Training status is related to a muscle protective effect reflected by a lower release of muscle enzymes and type-specific sarcomere proteins to blood stream and/or an enhanced clearance.

3. METHODS

This thesis presents data from five consecutive studies. Lower limb EIMD was assessed by laboratory high intensity exercise (HIE) protocols (studies I, II and III) and by field long duration exercise (LDE) protocols (studies IV and V). Here we summarize the methods used in laboratory HIE and field LDE studies. For further details, the reader is referred to the corresponding article.

3.1 Subjects

Forty-nine healthy male (48) and female (1) subjects gave written, informed consent to participate in the experiments. The main characteristics of the subjects are displayed in **Table 6**.

Study		Subjects	n	Age (years)	Mass (kg)	Height (cm)
HIE	I	Physical education students	**10**	28.6 ± 6.1	73.3 ± 6.9	175 ± 6.5
	II	Pole vault athlete	1	22.8	73.8	1.72
		Physical education student	1	22.5	69.9	1.71
			2			
	III	Physically active young men	**13**	22.9 ± 2.1	74.0 ± 5.8	177 ± 6.2
LDE	IV	Trained mountain runners				
		Men	7	39.8 ± 3.3	76.9 ± 7.9	179 ± 5.2
		Woman	1	39.1	67	173
			8			
	V	Mountain runners				
		Moderately trained	10	37.7 ± 7.4	73.9 ± 9.4	178 ± 3.4
		Highly trained	6	34.0 ± 5.2	71.3 ± 8.8	176 ± 7.3
			16			

Table 6. Main characteristics of the subjects from the five studies composing the thesis (mean ± standard deviation). HIE, high intensity exercise (laboratory protocols). LDE, long duration exercise (field protocols).

3.1.1 High intensity exercise

Subjects from laboratory HIE studies (I, II and III) were asked not to perform any exercise either during the week before or during the experimental period. No subjects had suffered myotendinous injuries in the past year and, with the sole exception of the pole vault athlete, the subjects were recreationally active but had not been involved in any regular resistance-training program for at least six months prior to the experiments.

3.1.2 Long duration exercise

Subjects from field LDE studies (IV and V) were healthy, trained mountain running competitors who had not suffered muscle injuries in the six months before participating in the studies. In order to avoid bias, no instructions were given regarding the type of training performed the week before the competitions in which the field LDE studies were performed, but competitors were asked about this matter in order to better interpret baseline data. Physical activity until 48 hours after the race was limited and massages were prohibited.

3.1.3 Ethical approval of research

The studies complied with the standards of the World Medical Association (Declaration of Helsinki) and approval was given by the Ethics Committee of the Catalan Sports Council (Government of Catalonia) (0099S/690/2013).

3.2 Study design

Both laboratory HIE and field LDE studies were carried out with baseline tests, the exercise protocol or competition, and repeated post-tests; i.e., observational pre-post design from a single group or two groups of subjects (**Table 7**, **Table 8**, **Table 9**, and **Table 10**).

3.2.1 High intensity exercise

	Baseline		Post-exercise					
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
Hours	-0.5	0	24	48	72	96	120	144
Tests		**EXERCISE**						
Blood sample	X		X	X				X
Perceived muscle soreness	X		X	X	X	X	X	X
Jump (SJ and CMJ)	X		X	X				X
Max. Power (concentric and eccentric)	X							X

Table 7. Schematic overview of experimental procedures of laboratory high intensity exercise study I.

	Baseline		Post-exercise	
		Day 0	Day 1	Day 2
Hours	-0.5	0	24	48
Tests		**EXERCISE**		
Blood sample	X		X	X
Power (concentric and eccentric)		X		

Table 8. Schematic overview of experimental procedures of laboratory high intensity exercise study II.

	Baseline		Post-exercise						
		Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Hours	-0.5	0	24	48	72	96	120	144	168
Tests		**EXERCISE**							
Blood sample	X		X	X	X				X
Perceived muscle soreness	X		X	X	X	X	X	X	X
Magnetic resonance imaging	X		X						X
Maximal voluntary contraction	X		X	X	X				X

Table 9. Schematic overview of experimental procedures of laboratory high intensity study III.

3.2.2 Long duration exercise

	Baseline		Post-exercise		
	Day -1	Day 0		Day 1	Day 2
Hours	-24	0	1	24	48
Tests		**EXERCISE**			
Blood sample	X		X	X	X

Table 10. Schematic overview of experimental procedures of field long duration exercise studies (IV and V).

3.3 Choice of muscle groups

In laboratory studies, lower limb muscles (specifically knee extensors (quadriceps femoris) and/or flexors (hamstring muscles)), were chosen because they are crucial for human locomotion and sport performance, and also because these muscle groups are widely used in exercise physiology research. In field studies involving mountain running competitive events, these muscle groups also have a fundamental role.

3.4 Exercise protocols

In order to replicate what happens in the sports context, during the laboratory HIE studies EIMD protocols consisting in regular resistance training exercises were applied. The leg curl and (especially) the squat are frequently used exercises in the field of strength and conditioning. The main characteristics of the laboratory HIE studies are displayed in **Table 11**. In field LDE studies, strenuous mountain running competitive events were used as EIMD models.

	Exercise characteristics		
Study	I	II	III
Exercise	Vertical half-squat	Horizontal half-squat	Prone leg curl
Machine (device)	Inertial	Pneumatic	Weight stack
Main muscle group	Knee extensors	Knee extensors	Hamstring (isolation)
Secondary muscle groups	Hip and ankle extensors	Hip and ankle extensors	None
Contraction type	Con-Ecc	Con-Ecc	Ecc
Mode	Bilateral	Bilateral	Unilateral
Rep velocity	Maximum (power oriented)	Maximum (power-endurance oriented)	3 seconds
Sets and reps	7 x 10	9 x failure	6 x 10 (each leg)
Rest (min)	3	3	3
Load	Highest resistance level (minimum cone radius)	75% 1-RM	120% 1-RM
Intensity (eccentric)	↑↑	↑↑	↑↑↑

Table 11. Lower limb exercises characteristics from laboratory high intensity exercise studies. Con, concentric. Ecc, eccentric. 1-RM, one repetition maximum.

3.5 Blood sampling and processing

A 5- or 8-mL blood sample was drawn from an antecubital vein and allowed to clot for 30 minutes in a tube (SST II Advance, Becton Dickinson Vacutainer Systems, UK). Blood was centrifuged at 3000 *g* for 10 minutes at 4ºC. Three 200 µl aliquots of serum were immediately pipetted into Eppendorf tubes and stored at -80ºC until analysed.

Biochemical analyses of CK, AST and ALT were performed in an Advia 2400 automatic device (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), following the method of the International Federation of Clinical Chemistry Committee's primary reference procedures for the measurement of catalytic activity concentrations of enzymes (184).

CK-MB analysis was performed using a Dimension Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) with a sensitivity limit of 0.5 $ng \cdot mL^{-1}$, following the method described by Vaidya and Beatty (197). An Enzyme Amplified Sensitivity Immunoassay (EASIA) from Diasource (Biosource, Louvain-la-Neuve, Belgium) was used to measure IL-6 serum concentrations with a sensitivity limit of < 2 pg/mL. Serum cTnI determinations were done in a Dimension Clinical Chemistry System automatic device (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) with an analytical measurement range of 0.017 to 40 $ng \cdot mL^{-1}$. Serum concentration of sMtCK was measured by using a commercial enzyme linked immunosorbent assay (ELISA) kit SEC386Hu (Cloud Clone Corp., Houston, TX, USA) according to the manufacturer's protocol.

To measure FM and SM serum concentrations an ELISA-sandwich using monoclonal antibodies (all Sigma Aldrich, Poole, UK) developed by the Department of Physiological Science I from the University of Barcelona was used. Briefly, two plates (Corning 96-well EIA/RIA, Sigma Aldrich, Poole, UK) were coated overnight at 4ºC, one plate with monoclonal anti-myosin (skeletal, fast) clone My-32 and another plate with monoclonal anti-myosin (skeletal, slow) clone NOQ7.5.4D. Monoclonal anti-myosin (skeletal, fast) clone My-32 localizes an epitope on the MHC 2 and monoclonal anti-myosin (skeletal, slow) clone NOQ7.5.4D recognizes an epitope located on the heavy meromyosin portion of ß/slow MHC. The plates were blocked with block buffer (Super Blocking Buffer, Thermo Fisher Scientific Inc., Rockford, IL, USA). A calibration curve of a 6-point serial dilution from 0 to 250 ng was obtained with commercial pure myosin of porcine muscle M0273. Each sample (10 µL) was then loaded by triplicate into the wells of the plate. Washes were done with phosphate buffered saline (PBS), pH 7.4, 10mM. The ELISA for both fast (type II) and slow (type I/ß) myosin (FM and SM respectively) plates was then completed by adding anti-myosin polyclonal antibody M7523, and afterwards, by adding mouse anti-IGG linked to peroxidase A6154. Incubation steps (60 min at 37ºC) were performed immediately after adding the antibodies and/or samples. Finally, tetramethylbenzidine (TMB) (Sigma Aldrich, Poole, UK) liquid substrate system for

ELISA was added and allowed to react at room temperature. The reaction was then stopped with H_2SO_4, and the quantity of FM and SM was measured spectrophotometrically (Synergy 2 Multi-Mode Microplate reader, Biotek Instruments, Winooski, VT, USA) at 450nm. Sample myosin concentrations were determined by interpolation from the calibration curve ($r^2 > 0.9$). Samples were analysed in triplicate with an intra-assay coefficient of variation (CV) below or equal to 10% for FM and SM respectively.

All these analyses were conducted at the Hospital Clínic (Barcelona, Spain) and at the Department of Physiological Science I at the University of Barcelona (Barcelona, Spain).

3.6 Magnetic resonance imaging

MRIs (3 T scanner; Siemens, Erlangen, Germany) were performed ~30 min prior to exercise and 24 hours and 7 days following exercise. Subjects were supine on the MR-gurney with head outside the MR-bore and thighs covered with one 32- and two flexible 4-channel coils respectively in the proximal and distal segments. A custom-made foot-restrain device was used to standardize and fix limb position, and to avoid any compression of thigh muscles. Twelve cross-sectional images of the thigh of both legs were obtained, starting at the very distal margin of the ischial tuberosity, and using the following scan sequences: (a) axial fat-suppressed proton density, TR 3000 ms, TE 30-33, eco train 4, slice thickness 3.5 mm, gap 28 mm, FOV 400x290 mm, matrix 320x180 and ipat 2; (b) axial T2 mapping, TR 1000 ms, TE (18, 36, 54, 72, 90, 108), eco train 6, FOV 400x400 mm, matrix 256x256, slice thickness 3.5 mm and gap 28 mm. A parametric image was generated from the T2 mapping sequence using the Leonardo workstation (Siemens, Erlangen, Germany). Scout images and anatomical landmarks were obtained to ensure identical positioning in pre- and post-scans.

T2 of hamstring muscles (semitendinosus [ST]), and biceps femoris long head [BFlh]) and short head [BFsh]) from both legs were measured using eFilm Lite v.3.1 software (Merge Healthcare, Chicago, IL). Using the fat-suppressed images to detect any confounding artefact (i.e., vessels, fat), a circular region of interest (ROI) was selected for individual hamstring muscles in each of the T2 mapping images where muscles were visible. Following pre-exercise scan analysis, the same-size circular

ROIs were placed in the T2 images of the post-exercise scan, to ensure the same positioning as in the pre-exercise analysis. In the evaluations, the images containing areas at 30% (proximal), 50% (middle) and 70% (distal) of thigh length from upper border of ischial tuberosity (0%) to the lower border of the tibial plateau (100%) were used (117). The same researcher performed the MR imaging scan and the T2 calculation. High interrater reliability has been previously reported with intraclass correlation coefficients ranging from 0.87 to 0.94 (39).

3.7 Muscle soreness

A 10-point visual rating scale (VRS) was used to quantify muscle soreness in lower limb muscles. Each number on the scale was accompanied by descriptive words for soreness, from 0 to 10 (intolerably intense). Participants were asked to fill in the VRS before and after the exercise, at the same time of day. In experiments involving the knee extensors, subjects were asked to fill in the VRS during a body weight squat to a knee joint angle of 90º, and in experiments involving the knee flexors, subjects stretched and contracted (isolated unloaded knee flexion from a stand up position) to assess general soreness throughout the hamstring muscles.

3.8 Muscle function

Muscle function of knee extensors and knee flexors (hamstring muscles) was assessed by different FGC tests in studies I and III.

3.8.1 Force-generating capacity

Maximum power test

A maximum inertial concentric-eccentric (C-E) power test (P_{max}) was developed as an indicator of the specific dynamic FGC of the knee extensors. During the P_{max} test, dynamic muscle work was characterized using the evolution of force, displacement, and velocity data, sampled at a frequency of 100 Hz by MuscleLab 4020e from the force sensor and linear encoder (Ergotest Technology, Langesund, Norway). The test consisted in a half-squat exercise that participants started from a 90º knee angle static position using an adjustable pulley on the flywheel inertial resistance device. Three submaximal repetitions were performed to initiate rotation of the flywheel,

which were immediately followed by five maximal voluntary C-E repetitions, with no pause. The three highest power repetitions were selected, and their average concentric and eccentric power was calculated and established as maximum C-E power.

Jump test

Jump tests were used to evaluate lower body dynamic explosive strength. A MuscleLab 4020e electronic contact mat (Ergotest Technology) was used to determine the squat jump (SJ) and countermovement jump (CMJ) flight time. For the SJ, the participants were positioned on the contact mat with a knee angle of 90° and were not allowed to jump until 4 s passed; for the CMJ, they performed a fast flexion movement of the knee joint, followed by a maximum-effort vertical jump. For both tests, the participants retained the hands-on-hips position until the final phase of the jumps. The best of three trials was recorded for further analysis.

Maximal voluntary contraction test

In study III, FGC was measured as MVC; i.e., average force in a 1-s window when a force plateau had been established (195). MVCs of the knee flexor muscles were measured for each leg with a force gauge connected to an A/D converter system, MuscleLab 4020e (Ergotest AS, Langesund, Norway). Subjects were prone with the hip joint at 40º of flexion and with the knee joint at 30º of flexion and received verbal encouragement during the test to ensure maximal effort. Subjects performed two isometric MVCs of 3 to 5 seconds with a 1-min rest between contractions, and if any countermovement was evident, an additional MVC was measured.

3.9 Statistics

Data were tested for approximation to a normal distribution using the Shapiro-Wilk test. Asymmetrically distributed data were log- or sqrt-transformed before analysis.

One-way (studies I, II, and IV) and two-way (study III) repeated measure analysis of variance (ANOVA) followed by a paired t-test with a Bonferroni correction was performed to identify statistically significant changes from baseline. Repeated measure analysis of covariance (ANCOVA) (group x time [covariate: training hours

per week]) (study V) followed by a paired t-test with a Bonferroni correction was performed to identify statistically significant changes from baseline.

Transformed data that still did not show a normal distribution were analysed using Friedman's test followed by the Wilcoxon signed rank test with a Bonferroni correction to identify significant changes from baseline.

Associations between variables of interest were assessed using Pearson's correlation coefficient test or Spearman's rank order correlation coefficient (choice dependent on Shapiro-Wilk test for Gaussian distribution).

The unpaired t-test and the Mann-Whitney test (choice dependent on Shapiro-Wilk test for Gaussian distribution) were used to analyse differences between groups.

Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. The level of significance was set at $P < 0.05$ (studies I, II, III and V) or $P < 0.01$ (study IV).

The statistical analysis was conducted using SPSS version 20.0 (SPSS Statistics, IBM corp., Armonk, NY, USA) statistical analysis software.

4. STUDIES

The studies were based on the aims and hypothesis proposed and will be presented as five studies, as follows.

HIE, laboratory studies: I, II, III.

LDE, field studies: IV, V.

4.1 Study I

Muscle enzyme and fibre type-specific sarcomere protein increases in serum after inertial concentric-eccentric exercise

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Muscle enzyme and fiber type-specific sarcomere protein increases in serum after inertial concentric-eccentric exercise

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Muscle damage induced by inertial exercise performed on a flywheel device was assessed through the serum evolution of muscle enzymes, interleukin 6, and fiber type-specific sarcomere proteins such as fast myosin (FM) and slow myosin (SM). We hypothesized that a model of muscle damage could be constructed by measuring the evolution of serum concentration of muscle proteins following inertial exercise, according to their molecular weight and the fiber compartment in which they are located. Moreover, by measuring FM and SM, the type of fibers that are affected could be assessed. Serum profiles were registered before and 24, 48, and 144 h after exercise in 10 healthy and recreationally active young men. Creatine kinase (CK) and CK-myocardial band isoenzyme increased in serum early (24 h) and returned to baseline values after 48 h. FM increased in serum late (48 h) and remained elevated 144 h post-exercise. The increase in serum muscle enzymes suggests increased membrane permeability of both fast and slow fibers, and the increase in FM reveals sarcomere disruption as well as increased membrane permeability of fast fibers. Consequently, FM could be adopted as a fiber type-specific biomarker of muscle damage.

Although there is reasonable evidence that unaccustomed or vigorous muscle activity, especially that involving eccentric contractions, can cause exercise-induced muscle damage (EIMD), the notion that muscle damage necessarily occurs following voluntary eccentric exercise in humans has been much challenged (Paulsen et al., 2012). Recently, it was proven that the extent of muscle damage in humans was greater following electrically stimulated contractions (severe disturbances of Z-lines, myofibers, and extracellular matrix followed by a loss of force-generating capacity and myofiber necrosis; Crameri et al., 2007) than following voluntary eccentric contractions (minor myofibrillar disruptions and minor changes in force-generating capacity; Gibala et al., 2000; Crameri et al., 2007; Mikkelsen et al., 2009). Although large variations in the responses of healthy individuals are expected, exercise involving regular athletic training with voluntary eccentric contractions (e.g., resistance exercise) typically causes mild EIMD [i.e., a small reduction in force-generating capacity (< 20%) and creatine kinase (CK) activity lower than 1000 U/L] and full recovery is normally complete within a few days (Paulsen et al., 2012).

Direct evidence of EIMD requires histological analysis of muscle tissue (Faulkner et al., 1993). However, in the context of sports activity, such an invasive technique is rarely used. Although they do not always accurately reflect the extent of EIMD, indirect markers are widely used, including delayed onset muscle soreness, decrements in force-generating capacity, and muscle-specific enzyme and protein efflux to blood (Clarkson & Hubal, 2002). The mechanisms involved in the appearance of different muscle enzymes and proteins in blood following eccentric exercise still remain unclear but depend on features such as their structure, molecular weight, and charge or membrane charge; the cellular compartment in which the enzyme or protein is located; the pathway the enzyme or protein follows into the blood; and the clearance rate of the enzyme or protein (Lindena & Trautschold, 1983; Mair et al., 1992; Sorichter et al., 1999; Dahlqvist et al., 2013).

Increased membrane permeability provides a route into and out of the sarcoplasm that is distinct from the conventional route of endocytosis and exocytosis. McNeil and Khakee (1992) suggested that in rats, it could be caused by the loss of sarcolemmal integrity as a result of eccentric exercise (running on a treadmill at an incline of 16° downhill). Also in rats, following electrical stimulation, increased membrane permeability can be related to the excitation-induced Ca^{+2} uptake (Mikkelsen et al., 2004) and several days after eccentric exercise, as shown in mouse models of Duchenne muscular dystrophy, membrane permeability can remain high because of the activation of ion channels (Allen

et al., 2005). Enzymes leaked from muscle are then mainly transported via the lymph into the blood stream because of the extremely low capillary permeability of muscle tissue (Lindena & Trautschold, 1983). One of the enzymes that are released from the sarcoplasm through permeabilized membranes is CK, which has been widely used as a serum muscle damage biomarker. However, there is no evidence that CK levels reliably reflect the degree of muscle damage (Fridén & Lieber, 2001) and it is not fiber type specific (Chapman et al., 2013). So, a muscle fiber-specific biomarker is required to evaluate the damage induced by exercise according to muscle fiber type. Recently, Chapman et al. (2013) proposed skeletal muscle fast and slow troponin I as fiber type-specific biomarkers and indicated that type II (fast twitch) fibers are more susceptible to eccentric EIMD, in agreement with other findings (Lieber & Friden, 1988). However, skeletal muscle troponin I has a soluble precursor pool in the sarcoplasm (3–4% of total skeletal muscle troponin I), which seems to be responsible for its rapid release from the muscle to the blood stream (Sorichter et al., 1997), and could lead to misleading results because its early appearance in serum cannot be exclusively related to sarcomere disruptions.

In relation to degrees of muscle injury established by magnetic resonance images, Guerrero et al. (2008) proposed increases in the blood stream of muscle myosin isoforms, fast (FM) and slow (SM), as ideal biomarkers of muscle injury because of their fiber specificity. Myosin is a sarcomere protein, with a molecular mass of 493 kDa (Holtzer & Lowey, 1959) that slides along actin, hydrolyses ATP and produces movement. Myosin has different heavy and light isoforms depending on the fiber type (Pette & Staron, 2000). While FM is characteristic of skeletal muscle only, SM is found in both cardiac and skeletal muscle. A moderate serum rise in FM or SM, below the injury levels described in Guerrero et al. (2008), could indicate sarcomere disruption of a specific fiber type because of to its molecular mass, its structural localization, and the absence of a soluble cellular myosin pool (Sorichter et al., 1997).

It has been reported that most human limb skeletal muscles are composed of a mixture of slow (type I) and fast (type II) fibers (Lexell et al., 1983). Autopsy studies of knee extensor muscles show that the percentage of type II fibers account for a 54.5% of vastus lateralis, 45.7% of vastus intermedius, 49.4% of vastus medialis, and 57.7% of rectus femoris (Garrett et al., 1984). In vastus lateralis biopsies performed on young men ($n = 95$; 21.2 ± 2.2 years), 59.8% of muscle fibers were type II (Staron et al., 2000). Because of this mixed fast and slow muscle fiber composition, we must expect different degrees of serum increase of FM and SM, depending on the intensity, volume, and type of muscle contraction during exercise.

Sports and exercise movements are a combination of concentric and eccentric (C-E) muscle contractions. Moreover, the magnitude of the eccentric muscle contraction in most sports actions (e.g., sprinting or jumping) is related to the previous concentric phase. Since the presentation of the flywheel principle (Berg & Tesch, 1998), many sports trainers and physicians use devices based on the inertia of rotating flywheels. These allow highly specific sports exercises to be performed in which the eccentric contraction is directly dependent on the preceding concentric contraction or, if desired, brief episodes of eccentric overload can be induced (Tesch et al., 2004). Flywheel inertial resistance devices have proved effective in promoting early skeletal muscle hypertrophy and strength gain because of the high torques required to stop the rotation (Tesch et al., 2004).

In the present study, we used an exercise design that achieves high-intensity biphasic C-E intervention of the knee extensor muscles, to replicate what occurs in several sports actions. For the first time, the physiological response to this exercise performed on a flywheel inertial resistance device was assessed through the time course evaluation of serum muscle enzymes, muscle myosin isoforms, serum muscle inflammation biomarker (interleukin 6; IL-6), perceived muscle soreness, and dynamic force-generating capacity. We hypothesized that a model of mild EIMD can be constructed by measuring the time course of the serum concentration of muscle proteins following inertial exercise, according to their molecular weight and the fiber compartment in which the enzyme or protein is located. Moreover, by measuring fiber type-specific sarcomere proteins such as FM and SM, the type of fibers affected could be assessed.

Methods

Participants

Ten healthy men [mean ± standard deviation (SD): age 28.6 ± 6.1 years; height 175 ± 6.5 cm; bodyweight 73.3 ± 6.9 kg], who had not suffered any myotendinous injuries for 1 year before the experiments, volunteered to participate in the study. All were recreationally active but had not been involved in any training program for at least 6 months prior to the experiments. They were asked not to perform any exercise during the week before and throughout the experimental period.

The experiment was conducted in accordance with the code of ethics of the World Medical Association (Declaration of Helsinki) and approval was given by the Ethics Committee of the Catalan Sports Council (*Generalitat de Catalunya*) (0099S/690/2013).

All the participants were informed about the purpose of the study, known risks, and possible hazards associated with the experimental protocol before recruitment and each gave written consent.

Inertial exercise design

In order to evaluate muscle response to high-intensity voluntary C-E exercise, the participants were required to perform seven sets of 10 maximum-intensity repetitions of the half-squat exercise in a flywheel inertial resistance device (Portable VersaPulley™, Heart Rate Inc., Costa Mesa, California, USA). The device is comprised of a cone attached above a flywheel, and as the flywheel and cone

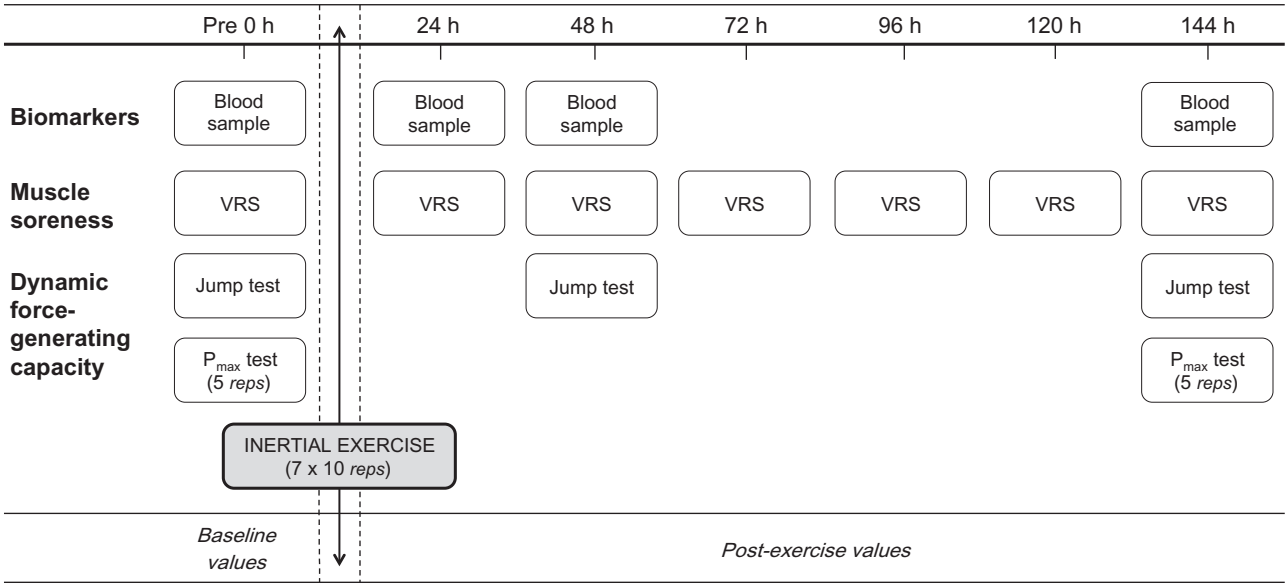

Fig. 1. Schematic overview of experimental procedures. VRS, visual rating scale (perceived muscle soreness). P_{max} test, inertial half-squat maximum power test (five repetitions). Exercise (7×10), inertial half-squat exercise (7 sets of 10 repetitions).

spin, a tether winds and unwinds around the cone. The concentric action unwinds the tether and the eccentric action occurs during rewinding. The flywheel inertial resistance device was configured with the tether threaded through a pulley attached to a platform. The end of the tether was attached to a shoulder and waist harness, so the subjects could pull vertically on the tether. For the flywheel inertial resistance, the arm pulley was set at the highest resistance level, which corresponds to the minimum cone radius.

The participants started from a 90° knee angle static position and made three submaximal repetitions immediately before 10 high-intensity voluntary C-E repetitions, with no pause. Three minutes rest was given between sets. In order to achieve high-intensity voluntary C-E repetitions, the individual maximum execution rhythm (MER) was set as the proper lifting cadence and the participants were verbally encouraged to follow it during each repetition and set. Moreover, and despite volitional aspects, during inertial exercise, resistance is at the momentary maximum ensuring a maximal C-E effort at any given point in the range of motion (Tesch et al., 2004).

Inertial exercise kinetics assessment

During inertial C-E exercise, dynamic muscle work was characterized using the evolution of force, displacement, and velocity data, sampled at a frequency of 100 Hz by MuscleLab 4020e from the force sensor and linear encoder (Ergotest Technology, Langesund, Norway; Tesch et al., 2004). Moreover, an electronic metronome (Qwik Time QT-3 Quartz, 1 X S006p, Qwik TUNE®; Moras et al., 2009), which emits a regular beep at a pace calibrated in beats per minute (bpm), was used to control the exact repetition rate, set as MER.

Dynamic force-generating capacity and perceived muscle soreness

Before the inertial exercise, the participants completed a jump test and an inertial half-squat maximum power (P_{max}) test as indicators of the specific dynamic force-generating capacity of the knee extensors. The jump test was repeated 48 h and 144 h post-exercise, while the P_{max} test was performed only before and 144 h after completion of the exercise, as it could affect the results of blood muscle damage biomarkers. Moreover, the subjects were instructed to assess their muscle soreness on a visual rating scale (VRS) both before exercise and 24, 48, 72, 96, 120, and 144 h post-exercise (Fig. 1).

Jump test

Jump tests have been used as lower body dynamic explosive strength evaluation tools. A MuscleLab 4020e electronic contact matt (Ergotest Technology) was used to determine the squat jump (SJ) and countermovement jump (CMJ) flight time. For the SJ, the participants were positioned on the contact mat with a 90° knee angle and were not allowed to jump until 4 s had passed, while for the CMJ, they performed a fast flexion movement of the knee joint, followed by a maximum-effort vertical jump. For both tests, the participants retained the hands-on-hips position until the final phase of the jumps. The best of three trials was recorded for further analysis.

P_{max} test

To assess inertial P_{max}, the participants started the half-squat test from a 90° knee angle static position using an adjustable pulley on the flywheel inertial resistance device. Three submaximal repetitions were performed to initiate rotation of the flywheel, which were immediately followed by five maximal voluntary C-E repetitions, with no pause. The three highest power repetitions were selected, and their average time duration was calculated and converted to a MER in bpm (Moras et al., 2009) for each participant.

Muscle soreness

A 10-point VRS was used to quantify muscle soreness in the knee extensor muscles during a body weight squat to a 90° knee joint angle. Each number on the scale was accompanied by descriptive words for soreness, from 0 (none) to 10 (intolerably intense). Participants were asked to fill in the VRS 24, 48, 72, 96, 120, and 144 h after the exercise, at the same time of day.

Blood sampling and processing

To study how inertial exercise affects fiber structure and which types of muscle fibers are involved the most in maintaining performance during exercise, FM and SM were measured in serum. EIMD has previously been related to an increased serum concentration of muscle enzymes, so we measured CK, creatine kinase-myocardial band isoenzyme (CK-MB), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Furthermore, the inflammatory response was assessed as IL-6 serum levels.

Five milliliters of blood was obtained from the volunteers before the inertial exercise, and 24, 48, and 144 h after the exercise (Fig. 1). The samples were centrifuged at 2000 *g* for 10 min at 4 °C. Serum aliquots were stored at −80 °C until analysis. Serum was used to measure enzymatic activities and FM and SM levels. Biochemical analyses of CK, AST, and ALT were performed in an Advia 2400 automatic device (Siemens Medical Solutions Diagnostics, Tarrytown, New York, USA), following the method of the International Federation of Clinical Chemistry's Committee primary reference procedures for the measurement of catalytic activity concentrations of enzymes (Siekmann et al., 2002).

CK-MB analysis was performed using a Dimension Clinical Chemistry System (Siemens Healthcare Diagnostics), following the method described by Vaidya and Beatty (1992). An enzyme amplified sensitivity immunoassay from Diasource (Biosource, Louvain-la-Neuve, Belgium) was used to measure IL-6 serum concentrations with a sensitivity limit of < 2 pg/mL.

To measure FM and SM serum concentrations, we developed an enzyme-linked immunosorbent assay (ELISA sandwich) using monoclonal antibodies (all Sigma Aldrich, Poole, UK).

Two plates (Corning 96-well EIA/RIA, Sigma Aldrich, Poole, UK) were coated overnight at 4 °C, one plate with monoclonal anti-myosin (skeletal, fast) clone My-32 and another plate with monoclonal anti-myosin (skeletal, slow) clone NOQ7.5.4D. The plates where blocked with block buffer (Super Blocking Buffer, Thermo Fisher Scientific Inc., Rockford, Illinois, USA). A calibration curve of serial dilution from 0 to 250 ng was obtained with commercial pure myosin of porcine muscle M0273. Washes were done with phosphate buffered saline, pH 7.4, 10 mM. The ELISA for both FM and SM plates was then completed by adding anti-myosin polyclonal antibody M7523, and afterwards, by adding mouse anti-IGG linked to peroxidase A6154. Incubation steps (60 min at 37 °C) were done immediately after adding the antibodies and/or samples. Finally, tetramethylbenzidine (Sigma Aldrich) liquid substrate system for ELISA was added and allowed to react at room temperature. The reaction was then stopped with H_2SO_4, and the quantity of FM and SM was measured spectrophotometrically (Synergy 2 Multi-Mode Microplate reader, Biotek Instruments, Winooski, Vermont, USA) at 450 nm. Sample myosin concentrations were determined by interpolation from the calibration curve ($r^2 > 0.9$). Samples (10 µL) were analyzed in triplicates with an intra-assay coefficient of variation (CV) below 9% for FM and below 6% for SM. Linearity of FM assay was 80% and 90% for the SM assay.

Statistical analysis

Distributions were considered for each of the variables, with the normality of continuous variables assessed using the Shapiro–Wilk test. As the CK and CK-MB activity data were asymmetrically distributed, these values were log-transformed before analysis. One-way repeated-measures analysis of variance was used to identify the effect of time on muscle enzyme activities, FM and SM levels, perceived muscle soreness, and muscle dynamic force-generating capacity. If significant effects were found, a post-hoc Bonferroni-corrected Student's *t*-test was applied. The Wilcoxon signed rank test was used on log-transformed data that still did not show a normal distribution (CK-MB). Correlations between variables of interest were calculated using Pearson's correlation coefficient. Intra-participant variability was expressed as the coefficient of variation: CV (%) = 100 × SD/mean value. Data are presented as mean ± standard error of the mean ($n = 10$), unless otherwise stated. The level of significance was set at $P < 0.05$. All the statistical analysis was conducted using the SPSS version 20.0 (SPSS Statistics, IBM Corp., Armonk, New York, USA) statistical analysis software.

Results

Inertial exercise kinetics

Exercise kinetics was assessed to evaluate the magnitude of the eccentric phase of the inertial exercise and how concentric contractions influence eccentric ones. The kinetics (displacement, velocity, and force) of the inertial exercise carried out on the flywheel inertial resistance device provided us the average and peak power (Fig. 2). The results showed that both average and peak power were strongly correlated between concentric and eccentric muscle contractions ($r = 0.940$; $P > 0.001$ and $r = 0.912$; $P = 0.001$). Concentric average power (530 ± 130 watts) was 40% greater than eccentric average power (320 ± 80 watts; $P > 0.001$), and concentric peak power (1100 ± 260 watts) was 39% greater than eccentric peak power (670 ± 160 watts; $P = 0.001$; Fig. 3). The average intra-participant variability in displacement (produced by knee flexion–extension) during the exercise was 4% in both the concentric and the eccentric phase.

Dynamic force-generating capacity and perceived muscle soreness

P_{max} test

With regard to dynamic force-generating capacity, the average displacement during the half-squat inertial P_{max} test was similar before inertial exercise (concentric: 39.17 ± 4.24 cm, and eccentric: 36.99 ± 4.99 cm) and at 144 h after inertial exercise (concentric: 38.94 ± 3.33 cm, and eccentric: 37.92 ± 3.47 cm). MER was also similar between the pre-inertial exercise P_{max} test (43 ± 4 bpm) and the 144 h post-inertial exercise P_{max} test (44 ± 2 bpm). In contrast, while significant differences were observed between average and peak eccentric power exerted in the pre-inertial exercise (0 h), P_{max} test ($P = 0.03$) and in the 144 h post-inertial exercise P_{max} test ($P = 0.004$), no significant differences were observed between the average and peak concentric power in the pre-inertial exercise (0 h) P_{max} test and 144 h post-inertial exercise P_{max} test (Fig. 4).

Jump test

The results showed that lower body dynamic explosive strength evaluated by the jump tests was not significantly modified by inertial exercise. At 48 and 144 h, the SJ and CMJ values were slightly lower than the pre-exercise

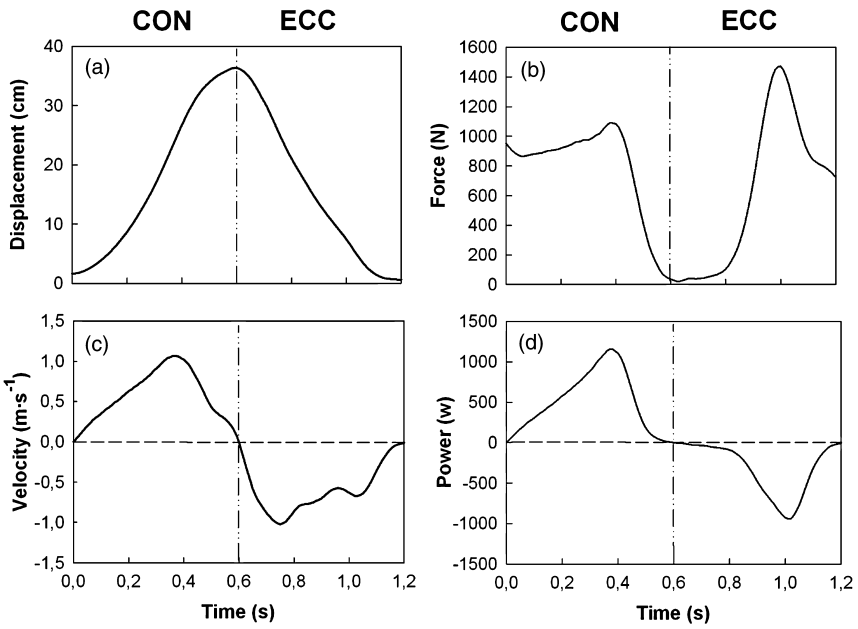

Fig. 2. Inertial exercise kinetics and kinematics in one participant's C-E repetition. Displacement applied to the flywheel during the knee flexion–extension (a). Velocity applied to the flywheel during displacement (b). Force applied to the flywheel during displacement (c). Power applied to the flywheel as a product of force and velocity (d). Vertical dashed–dotted line denotes the C-E deflection.

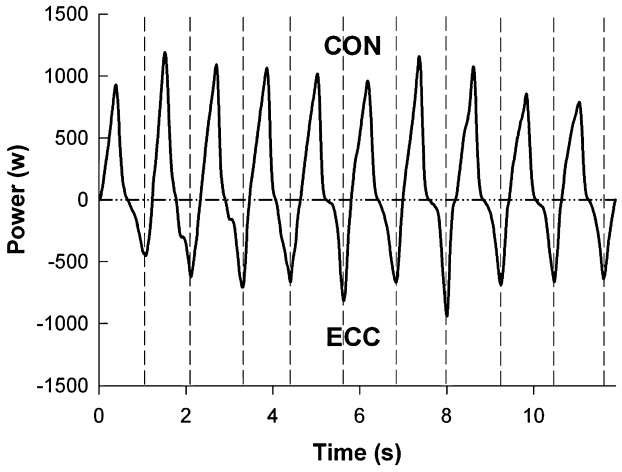

Fig. 3. Power (watts) applied by a participant during one set of inertial exercise. Note that concentric power is greater than eccentric power. Horizontal dashed–dotted line denotes the C–E deflection. Vertical dashed line delimits repetitions. CON, concentric; ECC, eccentric.

jump test values. SJ values were 31.84 ± 4.71 cm pre-exercise, 31.17 ± 3.99 cm at 48 h post-exercise, and 30.69 ± 4.32 cm at 144 h post-exercise (a decrease of 2.1 and 3.6%, respectively). CMJ were 36.73 ± 5.12 cm pre-exercise, 35.19 ± 5.04 cm at 48 h post-exercise, and 35.25 ± 5.91 cm at 144 h post-exercise (a decrease of 4.2% and 4.0%, respectively).

Muscle soreness

The average values for perceived muscle soreness during a body weight squat to a 90° knee joint angle were higher 24 h after the inertial exercise, remained at the higher values at 48 h, and declined to control values at 96 h (Fig. 5).

Serum muscle enzymes, FM and SM, and IL-6

To analyze the muscle response and which types of fibers were most involved in inertial C-E exercise, we evaluated changes in FM and SM and enzymes in serum over time. After the exercise, there was a progressive increase in average FM serum levels, which reached its highest value at 48 h, and remained significantly elevated at 144 h. In contrast, SM showed similar blood concentrations at all the times analyzed after the exercise compared to the values obtained before the inertial exercise (Table 1). Individual analysis of relative FM and SM serum concentrations was also performed. After the exercise, there were nonuniform increases in FM serum levels in nine of the participants (range 35% to 259% from baseline values). Only one participant showed no FM changes (< 6% from baseline values). The pattern of increase to the relative FM peak was also different between the participants. While five of them reached the serum peak 144 h post-exercise, three reached it at 48 h and only one at 24 h. With regard to SM, only three participants showed slight increases until the relative peak at 24 and 48 h (range 23% to 60% from baseline values) following inertial exercise (Fig. 6). The FM/SM ratio was significantly increased by 51% ± 21% ($P = 0.02$) at 48 h and by 96% ± 36% ($P = 0.007$) 144 h post-exercise (Table 1).

All enzyme activities prior to inertial exercise were within the normal range (Table 1). After exercise, the

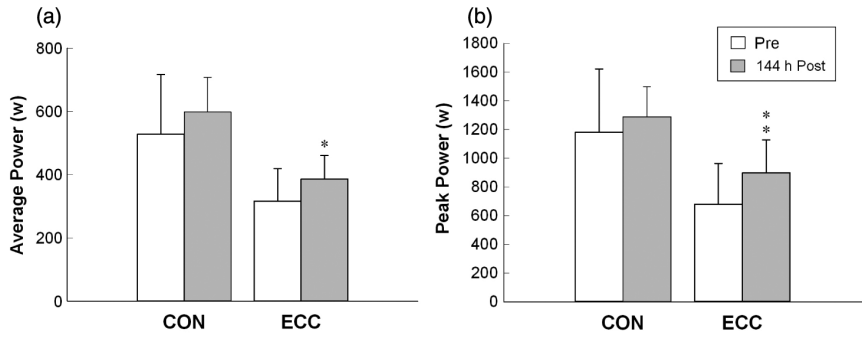

Fig. 4. Concentric and eccentric average (a) and peak (b) power increases between pre (0 h) and 144 h post-exercise in P_{max} test ($n = 9$). * and **, Significantly different from the pre-exercise value at $P < 0.05$ and $P < 0.01$, respectively.

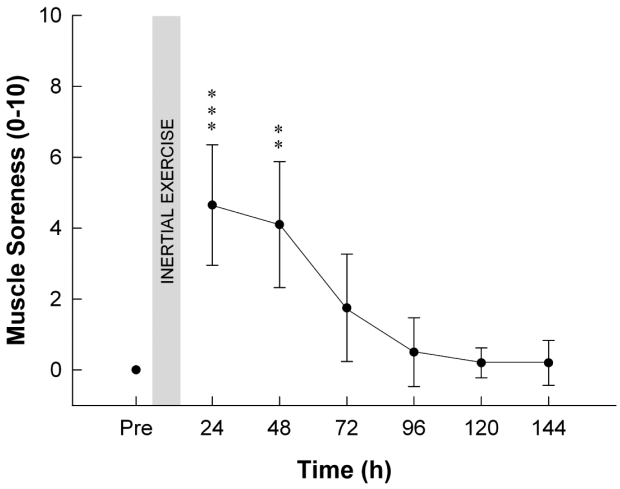

Fig. 5. Changes in muscle soreness. ** and ***, Significantly different from the pre-exercise value at $P < 0.01$ and $P < 0.001$, respectively.

time course of serum activity of CK, CK-MB, and AST were similar, while ALT showed no variations. Serum CK levels were 2.9-fold higher at 24 h ($P = 0.01$) and returned to baseline values 144 h after exercise. CK-MB showed a significant increase at 24 h ($P = 0.005$) and returned to baseline values 48 h post-exercise. The behavior of both AST and ALT was different from that of CK, CK-MB: despite a slight nonsignificant increase at 24 h in serum AST concentration, activity remained constant throughout the rest period after the exercise (Table 1). However, total pooled data from CK and AST activities were highly correlated ($r = 0.769$; $P > 0.001$; Fig. 7). The IL-6 serum levels of two participants prior to the inertial exercise were higher than the normal range (≤ 5 pg/mL). No changes were seen in the average values of IL-6 following the exercise. However, it is interesting to note that five participants showed nonuniform increases in IL-6 serum levels (range 31% to 1150% from baseline values), while the other five participants showed no changes from baseline values (Fig. 8).

Discussion

We examined the influence of inertial high-intensity voluntary C-E exercise on knee extensor muscles and how it affects the different types of muscle fibers. To this end, we chose a half-squat exercise on a flywheel inertial resistance device, and FM and SM as fiber type-specific sarcomere proteins that are potential biomarkers of muscle damage.

Our results show for the first time in a well-characterized inertial exercise that fast and slow fibers respond differently following a high-intensity voluntary C-E exercise based on a movement similar to many sports actions (e.g. jumping, sprinting). We found an increase in the FM isoform in blood, indicating a sarcomere disruption of fast fibers; while the absence of change in the serum SM isoform and the serum increase in both AST (albeit nonsignificantly) and CK-MB suggested that slow fibers were mainly affected at the sarcolemmal level. Moreover, because CK is not fiber type specific, the changes in total CK activity cannot be related exclusively to increased membrane permeability of fast fibers.

The inertial C-E exercise that we studied induces significant perceived muscle soreness, and increases in serum CK concentrations. Neither effect is large, suggesting that mild EIMD was inflicted (Paulsen et al., 2012). The CK increases are very similar to those reported in prior studies, which induced mild EIMD using voluntary eccentric exercise protocols involving the knee extensors (Bourgeois et al., 1999; Davies et al., 2011). Interestingly, although only mild EIMD was produced, sarcomere disruptions of fast fibers were suggested by FM serum increases. Other studies using greater muscle work volumes (200 eccentric contractions) found higher CK activity following exercise without histological evidence of myofiber damage (Mikkelsen et al., 2009). This is probably because CK presents large inter- and intra-individual variability and only provides evidence of muscle damage in a dichotomous fashion: damaged or not damaged (Fridén & Lieber, 2001) and minor sarcomere disruptions can indeed occur without significant changes in the

Table 1. Changes in serum concentration of the muscle enzyme and fibre-type-specific proteins before (0 h) and 24, 48 and 144 h after inertial exercise

	Pre (0 h)	24 h	48 h	144 h
FM (μg/L)	1082.8 ± 203.9	1391.0 ± 254.8	1562.4 ± 241.9*	1485.6 ± 157.7**
SM (μg/L)	1887.9 ± 247.7	1893.0 ± 245.9	2067.9 ± 241.4	1738.9 ± 259.8
FM/SM ratio	0.58 ± 0.09	0.73 ± 0.11	0.77 ± 0.10*	0.98 ± 0.12**
CK (U/L)	221.4 ± 42.7	652.7 ± 198.3**	416.2 ± 126.9	196.1 ± 50.2
CK-MB (ng/mL)	1.32 ± 0.27	1.83 ± 0.26**	1.18 ± 0.25	1.31 ± 0.41
AST (U/L)	25.10 ± 2.77	32.90 ± 3.57	29.50 ± 3.06	23.00 ± 2.62
ALT (U/L)	21.30 ± 2.81	22.50 ± 2.79	21.40 ± 2.41	22.80 ± 2.99

Data are presented as mean ± SEM. FM, fast myosin. SM, slow myosin. CK, creatine kinase. CK-MB, creatine kinase-myocardial band isoenzyme. AST, aspartate aminotransferase. ALT, alanine aminotransferase. * and **, Significantly different from the pre-exercise value at $P < 0.05$ and $P \leq 0.01$, respectively.

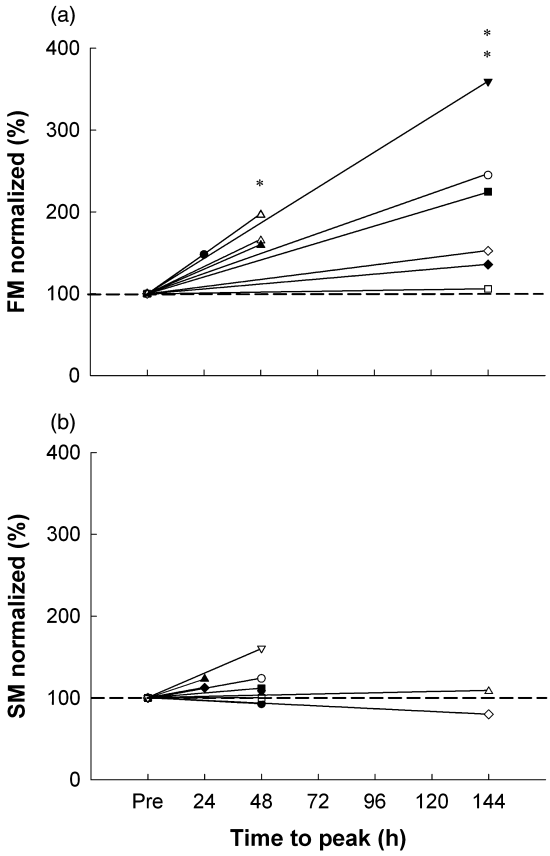

Fig. 6. Individual changes in serum concentration of FM (fast myosin) (a) and SM (slow myosin) (b), until serum peak at 24, 48, or 144 h following inertial exercise. Data are normalized to baseline values (100%). * and **, Significantly different from the pre-exercise value at $P < 0.05$ and $P < 0.01$, respectively.

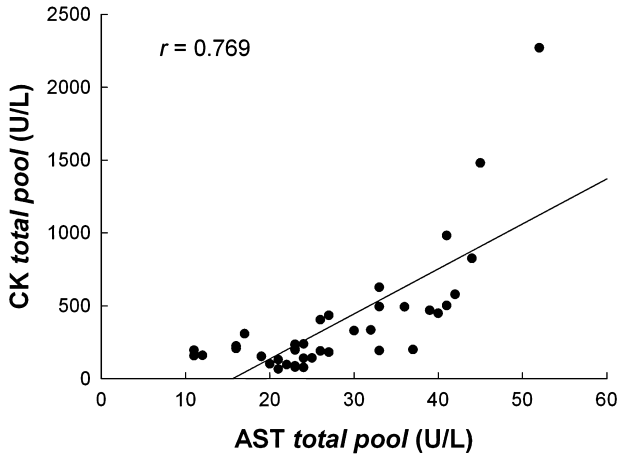

Fig. 7. Association between serum creatine kinase (CK) and aspartate aminotransferase (AST) in the total pool of data (pre, 24, 48, and 144 h post-exercise; $P > 0.001$).

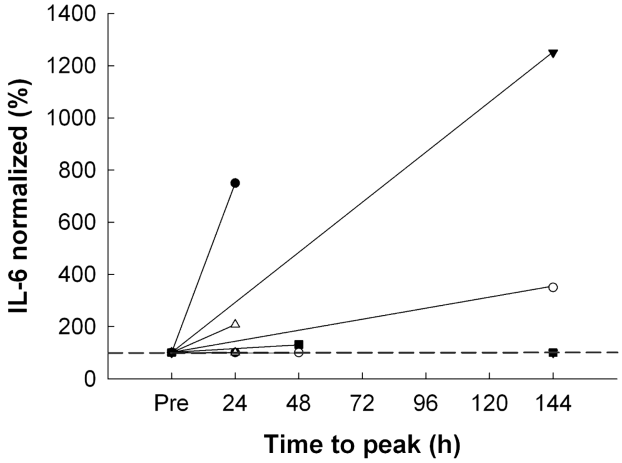

Fig. 8. Individual data of changes in serum concentration of IL 6 (interleukin-6), until serum peak at 24, 48, or 144 h following inertial exercise. Data are normalized to baseline values (100%).

force-generating capacity (Gibala et al., 2000). Moreover, eccentric contractions of the leg muscles can lead to serum increases in IL-6 for several days following exercise (Paulsen et al., 2012). However, in the present study, no clear inflammatory response was seen following the inertial exercise, as the serum concentration of IL-6 showed large inter- and intra-individual variability in the magnitude of and the time to the serum peak in five of the participants. Furthermore, no correlation was found between IL-6 and enzyme or protein serum concentrations.

With regard to the inertial exercise kinetics, our results showed that values for concentric average and peak

power during maximal inertial exercise are greater than eccentric values. However, the eccentric average and peak power is highly dependent on the preceding concentric phase because of the kinetic energy of the rotating flywheel (Tesch et al., 2004). In addition, as previous studies have established causality between eccentric exercise and changes in indirect markers of muscle damage (Clarkson & Hubal, 2002), it seems reasonable to relate the increases in perceived muscle soreness and CK mostly to the eccentric phase of the inertial exercise. Most evidence indicates that fast fibers cannot be selectively recruited for eccentric contractions over a wide range of effort and speed (Chalmers, 2008), and during inertial exercise resistance is at its momentary maximum, thereby ensuring a maximal C-E effort at every point in the range of motion (Tesch et al., 2004). Therefore, fast and slow fibers should be recruited and damaged in a similar manner during maximal C-E inertial exercise.

Consequently, we can expect that serum concentrations of FM and SM should increase in a similar manner. Nevertheless, our results showed that only FM serum concentration was significantly raised after the inertial exercise: no differences were observed in SM serum levels. The increases in the FM serum concentration indicate sarcomere disruption of fast fibers. This is in agreement with Chapman et al. (2013) and Dahlqvist et al. (2013), who proposed troponin I as a fiber type-specific biomarker of sarcomere damage, despite the fact that only Chapman et al. (2013) found increases in fast skeletal troponin I in healthy participants. Moreover, increases in serum FM support the claim that fast fibers are more susceptible to eccentric EIMD (Lieber & Friden, 1988) because of their morphological and biomechanical characteristics; less elastic titin isoform, less desmin content, narrower Z-discs, and shorter optimum length for tension than slow fibers (for a review see Schiaffino & Reggiani, 2011).

FM and SM levels in serum have been used before as muscle injury biomarkers (Guerrero et al., 2008). An increase in FM serum levels and the FM/SM ratio were related to grade I muscle injury, diagnosed by magnetic resonance. Although our results show higher baseline serum levels of both FM and SM than we have described previously as reference values, the FM/SM ratio before exercise (0.58), which is considered a control value, was similar to that found before (Guerrero et al., 2008). The increase in the FM/SM ratio confirmed that mainly fast fiber sarcomeres were disrupted after the inertial exercise. Furthermore, although FM was significantly increased in serum 48 and 144 h after the inertial exercise, the FM/SM ratio was below the levels described by Guerrero et al. (2008) (2.20), which indicated an absence of grade I muscle injury. It must be pointed out that in Guerrero et al. (2008), serum myosin concentration was determined by Western blot technique and, in the present study, was determined by ELISA, which allowed for faster but also reliable assessments of myosin isoforms serum levels.

Although the SM serum concentration remained almost unaltered in all the individuals, indicating that the sarcomere integrity of slow fibers was not compromised, sarcolemmal damage to the skeletal muscle slow fibers cannot be ruled out because of the increased serum activity of CK-MB.

CK is a poor indicator of sarcomere damage (Fridén & Lieber, 2001) and is not fiber type specific (Chapman et al., 2013), but the significant increase in serum total CK and CK-MB activity at 24 h could be evidence of increased membrane permeability because of their sarcoplasmic (cytosolic) location (Dahlqvist et al., 2013).

The present study also reported no changes in ALT serum concentration and only nonsignificant serum increases in AST 24 h after the inertial exercise; but a strong association between CK and AST. AST could play a role similar to that of CK and CK-MB as a biomarker of increased membrane permeability, as it is also found in the sarcoplasm (cytosol). Moreover, while fast and slow fibers present almost equal CK activity (Apple & Tesch, 1989), slow fibers have higher CK-MB (Yamashita & Yoshioka, 1991) and AST (Schantz & Henriksson, 1987) activity. As a consequence, a 24 h rise in CK-MB and AST could be more precisely associated with increased membrane permeability of slow fibers, which suggests that this kind of fiber is also recruited and damaged at the sarcolemmal level by inertial exercise.

The fact that the serum peak in AST and, especially CK and CK-MB coincided at 24 h could be associated with their similar molecular weight (82 kDa, 87 kDa, and 92 kDa, respectively). Their mainly sarcoplasmic location could also explain their similar serum profiles, as they reach the interstitium because of an increase in the membrane permeability of both fast and slow fibers. It is known that membrane permeability increases during eccentric exercise by mechanical stress-induced membrane disruptions (McNeil & Khakee, 1992). However, as it has been demonstrated that the resealing of artificially produced membrane disruptions occurs in less than a minute (Bansal et al., 2003), increased membrane permeability caused by the activation of ion (Ca^{+2}) channels and a consequent increase in reactive oxygen species that affect membrane lipids (Allen et al., 2005) may more adequately explain muscle enzyme and protein leakage several hours or days after eccentric exercise.

The time course of FM was different. The average serum FM concentrations were significantly elevated at 48 h, as previously found (Guerrero et al., 2008), and levels were still high at 144 h after inertial exercise. Moreover, the analysis of individual cases showed non-uniform changes in the magnitude and the time of the

serum peak, as reported by Mair et al. (1992) and Sorichter et al. (1997) with beta (slow) myosin heavy-chain fragments. It has been suggested that the first event after exercise is a degradation process in which protease calpain initiates the turnover of fiber proteins by releasing them from their filamentous sarcomere structure (Goll et al., 2003). Degradation is then completed by the ubiquitin–proteasome system (Eble et al., 1999). One reason for the late increase in serum FM is that sarcomere protein turnover is slower than that of sarcoplasmic proteins (Neti et al., 2009) and total maximum calpain activity is augmented 48 h after eccentric exercise, reaching a level three times higher after 144 h (Kanzaki et al., 2010). Another reason for the late increase in serum FM and its delayed peak values could be the absence of a soluble myosin heavy-chain precursor pool (Sorichter et al., 1997). The cascade of reactions that results in activated skeletal muscle protein metabolism is a complex process that could explain the differences between the time course of FM and muscle enzymes.

Finally, eccentric power output (average and peak power) was enhanced 144 h post-exercise, when FM serum concentration was still significantly above baseline values. Following the proposal of Paulsen et al. (2012), in the present study, only mild EIMD was inflicted, as force-generating capacity, expressed as jumping ability, only suffered a slight decrease and eccentric power output was significantly improved 144 h post-exercise. Furthermore, enzyme serum activity and perception of muscle soreness rose only moderately. Minor sarcomere disruptions can indeed occur without significant changes in the force-generating capacity (Gibala et al., 2000), probably because force can be transmitted near a few disrupted sarcomeres, as it can be transmitted laterally through neighboring myofibrils, across the sarcolemma, and through the extracellular matrix (Grounds et al., 2005). So, if the structural components involved in lateral force transmission remain undamaged, only slight changes in muscle function are expected (Raastad et al., 2010). Yu et al. (2004) propose that the sarcomere alterations observed 7 days after eccentric exercise could indicate muscle adaptation and regeneration, rather than damage. Accordingly, 144 h post-exercise, FM serum levels could be related to a sarcomere remodelling process rather than damage. Finally, in inexperienced participants, as in the present study, there is an improvement in the ability to apply force after a single strength training session that is related to motor learning factors (Selvanayagam et al., 2011). The magnitude of the mild EIMD, the lateral force transmission, the relationship between time-persistent high FM serum levels and both sarcomere turnover and adaptation, and the early strength gains caused by neural factors explain the 144 h post-exercise eccentric power output enhancement.

In short, the present study shows for the first time that high-intensity C-E inertial exercise of the knee extensors, involving a highly specific movement similar to several sports actions, induces a different level of damage in fast and slow fibers. Interestingly, while an increase in muscle damage biomarkers such as CK-MB could indicate increased membrane permeability of slow fibers, FM serum increases revealed sarcomere disruption as well as increased membrane permeability of fast fibers. Moreover, FM is sensitive even when the EIMD is mild, which demonstrates that skeletal muscle myosin isoforms could be adopted as fiber type-specific biomarkers of muscle damage. Our results support a model of muscle damage based on the serum evolution of muscle enzymes and muscle FM and SM according to their molecular weight and the fiber compartment in which they are located.

Perspectives

Although direct evidence of muscle damage is histological, in a sports context, the analysis of EIMD is essentially based on indirect evidence such as the measurement of serum biomarkers, especially CK, which is not an ideal biomarker. Recently, myosin isoforms were shown to be reliable biomarkers of muscle injury in patients who were previously diagnosed by magnetic resonance and ultrasonography (Guerrero et al., 2008). For the first time, the present study reports a close relationship between inertial exercise and serum increases in FM, which indicate sarcomere disruptions as well as increased membrane permeability of the fast fibers in physically active and healthy individuals. This original finding could be useful for researchers who study EIMD and need a fiber type-specific biochemical marker of skeletal muscle obtained through a minimally invasive technique. Additionally, myosin isoforms have favorable release times and stability in blood. Thus, they provide a highly sensitive biomarker of sarcomere damage, which is easy to use in diagnoses that are not performed immediately after exercise.

Key words: Inertial exercise, exercise-induced muscle damage, knee extensor, fast myosin, creatine kinase, dynamic force-generating capacity.

Acknowledgements

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4.2 Study II

Fast fibre damage after leg press exercise leading to failure: the case of a pole vaulter

Fast fibre damage after leg press exercise leading to failure: the case of a pole vaulter

Brief report: case study *(single case-control study)*

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Pole vaulter fast fibre damage

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Abstract

Objective. The aim of this study was to investigate, in a trained pole vaulter (PV) and a physical education student (PE), the effect of a leg press exercise leading to failure (LPF) on changes in serum activity of muscle enzymes and serum concentration of fast (FM) and slow (SM) myosin isoforms, while simultaneously examining mechanical output components as indicators of performance and fatigue developed throughout exercise.

Methods. Observational comparison of response in a single control (PE) - case (PV) study. Differences between the participants' exercise outputs were examined by unpaired *t*-test or Mann-Whitney test and serum levels of muscle enzymes and myosin isoforms were analyzed at baseline and 24 and 48 hours after LPF.

Results. Exercise output analyses showed that the PV's average fatigue index was significantly higher ($P = 0.004$). Moreover, during the first six sets, the concentric average power exerted by the PV was significantly ($P < 0.01$) higher (range: 14% to 35%) than that of the PE. The PV only showed acute mild increases of serum creatine kinase (CK) and FM 24 hours after exercise. In contrast, the PE presented persistent serum rises of several muscle enzymes and SM until 48 h after exercise.

Conclusions. The PV's exercise output revealed an explosive (power-oriented) profile leading to selective mild damage of fast fibres. In contrast, the PE exercise output showed a fatigue-resistant profile, which induced greater muscle enzyme activity and SM serum concentration, suggesting a higher extent of slow fibre damage.

Keywords: leg press, muscle failure, creatine kinase, myosin isoforms, power output.

Introduction

Although the physiological responses to exhausting leg press exercise leading to failure (LPF) have been well-documented recently in recreational endurance-trained athletes with a high mean percentage of slow (type I) fibres (65±12%),[1] care should be taken when generalizing the results to power athletes, who are expected to have higher proportions of fast (type II) fibres. Fast fibres generate high peak power and contract with high shortening speed, mainly determined by the myosin isoform,[2] and their energy failure implies a great decrease in muscle power output, especially at fast movement rates.[3] In this sense, LPF is primarily used for muscle strength and hypertrophy, inducing selective fatigue of fast fibres and then progressive recruitment of slow (type I) fibres, which decreases mechanical efficiency in the final part of the exercise.[1] Since resistance training, when it is exhaustive and has a demanding eccentric component, typically causes mild muscle damage,[4] indirect evidence of few myofibrillar disruptions from both fast and slow fibres can be expected following LPF.

The purpose of this study was therefore to investigate, in a trained pole vaulter (PV), the effect of LPF on changes in serum activity of muscle enzymes and serum concentration of myosin isoforms, as indirect biomarkers of sarcomere disruptions of fast and slow fibres,[5] while simultaneously examining the mechanical output components as indicators of performance and fatigue developed throughout exercise. Besides, the PV case study was compared against a control subject who had similar characteristics to those described in previous studies using exhausting leg press exercise models.[1]

Methods

Participants

Two participants were recruited for the study: a national level, under-23, pole PV (age 22.8 years, weight 73.8 kg, height 1.72 m) who trained 12 hours per week, had six years of athletic training experience, and a personal best of 4.85 m; and a physical education student (PE) (age 22.5 years, weight 69.9 kg, height 1.71 m) who performed 6-7 hours per week of physical activity consisting of endurance running and recreational football training. The study conformed to the World Medical Association's code of ethics (declaration of Helsinki) and approval was given by the Ethics Committee of the Catalan Sports Council (*Generalitat de Catalunya*) (0099S/690/2013).

Design

Single control-case study

Blood analyses

Blood samples of 5 mL were collected before exercise (baseline) and 24 and 48 h after exercise. Serum was obtained and analyzed for fast and slow myosin (FM and SM respectively), creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT), and creatine kinase MB isoform (CK-MB).[5]

One repetition maximum assessment

After three warm-up sets, a resistance was chosen that was thought to be slightly below the concentric one repetition maximum (1-RM), and participants were instructed to perform one repetition in a pneumatic leg press (Air300, Keiser Corporation, Fresno, CA, USA). Participants started the test from a knee 90°-angle static position using the adjustable seat of the pneumatic machine, and performed a concentric extension to reach the full extension of 180º against the resistance. Following increases in resistance between trails were adjusted to minimize the total number of attempts (3-4) required before the 1-RM was obtained.

Exercise

Participants performed 9 sets of concentric-eccentric repetitions until failure at a workload equivalent to 75% of concentric 1-RM in the pneumatic leg press, which allows for constant resistance throughout the whole range of motion independently of the velocity of exercise. A 3-min rest between sets was given. The participants were encouraged to complete the whole range of motion of every repetition as rapidly as possible. The concentric and eccentric work, velocity and power of each repetition were recorded using a linear encoder integrated to MuscleLab 4020e (Ergotest Technology AS, Langesund, Norway) system. Fatigue index (%) (FI) was calculated as follows:

([Max Power – Min Power] / Max Power) x 100

The participants were provided with visual feedback (MuscleLab software) and verbal encouragement in order to maximize power output and achieve muscle failure.

Statistical analysis

The normality of each variable was tested using the Shapiro-Wilk test. The unpaired *t*-test or Mann-Whitney test (the choice was dependent on a normality test for Gaussian distribution) were used to test differences between subjects' mechanical output variables. Friedman's test with post-hoc Wilcoxon signed-rank tests with a Bonferroni correction was used to test differences between the PV's average power output in the first set and the rest of the sets.

Results

The 1-RM was 320 kg for the PV and 250 kg for the PE. The PV performed higher total work during exercise, but no average work differences were found between participants (Fig. 1[a]). The fatigue index was greater in the PV in every set, and significant differences were found between the participants' average fatigue index ($P = 0.004$) (Fig. 1[b]). The PV applied higher concentric average velocity during the first sets of the LPF (Fig. 1[c]). Compared to the concentric average power exerted by the PV during the first set of the LPF, significant ($P < 0.01$) reductions (range: -12% to -19%) were found during the exercise, with the exception of sets 2 and 6, in which no significant differences were observed. Moreover, during the first six sets the concentric average power exerted by the PV was significantly ($P < 0.01$) higher (range: 14% to 35%) than that of the PE (Fig. 1[d]).

A clearly different response in biochemical markers was observed between participants. While, the PV showed slight serum increases in CK (from 183 to 405 $IU \cdot L^{-1}$) and CK-MB (from 0.5 to 1.6 $ng \cdot mL^{-1}$) at 24 h, and a clearly decreasing trend to enzyme baseline activity values at 48 h after exercise, the PE presented sharp serum rises, over the clinical normality range, of CK-MB (from 0.5 to 4.4 $ng \cdot mL^{-1}$) at 24 h, and of CK (from 142 to 1000 $IU \cdot L^{-1}$) and AST (from 24 to 41 $IU \cdot L^{-1}$) at 48 h after exercise (Fig. 2). The PV only showed mild increases in serum FM (from 1557 to 1998 $\mu g \cdot L^{-1}$) at 24 h and remained high (1928 $\mu g \cdot L^{-1}$) 48 h after exercise. In contrast, the PE presented moderate serum rises of SM until a peak (from 1303 to 1892 $\mu g \cdot L^{-1}$) 48 h after exercise (Fig. 3).

Discussion and conclusions

This case study presents unique data from a highly trained PV. The results indicate that selective, mild, fast-fibre damage was induced following LPF.

Pole vault competitors have similar characteristics to sprint athletes, since a high approach speed is necessary in this track and field event,[6] so a high percentage of fast fibres is expected in these athletes. Unfortunately, there are no histochemical studies involving trained pole vault athletes, but Korhonen et al.[7] stated that young sprint trained competitors (18-33 years) present a high relative fast-fibre percentage in the vastus lateralis area (59±6%).

During the first two sets of LPF, the PV developed a high average power and velocity, which are related to the fast-fibre capacity to generate great power output and contract with elevated shortening speed.[3] LPF required a maximal effort from the PV, as reflected by a clear decrease in power output, observed from the third set onwards, which indicates energy failure and selective fatigue of fast fibres.[1] Evident reductions in total work output per set also suggested progressive recruitment of slow (type I) fibres, with a decrease in mechanical efficiency.[1] In contrast, the PE showed a significantly lower power output, but an extraordinary capacity for maintaining its average power throughout exercise, which could be related to slow fibres' specialization for fatigue-resistant response during continuous activity.[2] Although fatigue was not as evident as in the PV, a marked reduction in mechanical work probably reflected greater recruitment and progressive decrease in slow fibres' efficiency.

Interestingly, the exercise output profile was in accordance with the serum biochemical response of both participants. In the PV, selective recruitment and fatigue of fast fibres led to damage of those fibres, which was suggested by FM increases observed 24 h after LPF. Slight FM increases in serum have been previously related to mild exercise-induced muscle damage (few myofibrillar disruptions).[5] In contrast, the PE moderate serum SM increases after exercise, which suggested slow-fibre damage,[8] probably related to higher recruitment and fatigue of these types of fibres during exercise. Both FM and SM serum levels were high 48 h after LPF because of myosin complex degradation metabolism.[9] The PV's slight FM increases in serum were accompanied by marginal CK activity rises at 24 h, returning to almost baseline values at 48 h after LPF, which is indirect evidence of a metabolic recovery status,[10] probably related to strength training adaptations. In contrast, the PE showed clinically relevant increases in CK, CK-MB and AST until 48 h after exercise, which suggests a greater extent of muscle damage. Moreover, sharp serum increases in CK-MB and AST activities reinforced the notion that slow fibres were damaged, since these enzyme activities are higher in slow fibres.[11 12]

In conclusion, biochemical response seems to be closely related to exercise output. We can say that the PV's exercise output revealed an explosive (power-oriented) profile, leading to selective, mild damage of fast fibres. In contrast, the PE exercise output showed a fatigue-resistant profile, which induced greater muscle enzyme activity and SM serum concentration, and suggests a greater extent of slow fibre damage.

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Figure captions

Fig. 1. Leg press leading to failure output variables comparison between a pole vaulter (PV) and a physical education student (PE) at baseline and 24 and 48 h after exercise. Total work output (sum per set) and average work ± standard deviation per set (a), fatigue index per set and average fatigue index ± standard deviation of the whole exercise (b), average concentric (positive values) and eccentric (negative values) velocities ± standard deviation per set (c), and average concentric power ± standard deviation per set (d). #Significant difference between participants at $P < 0.01$. *Significantly lower than the first set of values at $P < 0.01$.

Fig. 2. Muscle enzyme serum activity comparison between a pole vaulter (PV) and a physical education student (PE) at baseline and 24 and 48 h after exercise. Creatine kinase (CK) (a), creatine kinase MB isoform (CK-MB), (b), aspartate aminotransferase (c), and alanine aminotransferase (d). A dashed line indicates the upper limit of clinical normality values.

Fig. 3. Fibre-type-specific sarcomere proteins' serum concentration comparison between the pole vaulter (PV) and physical education student (PE) at baseline and 24 and 48 h after exercise. Fast myosin (FM), and slow myosin (SM). Data are normalized to baseline values.

Figure 1

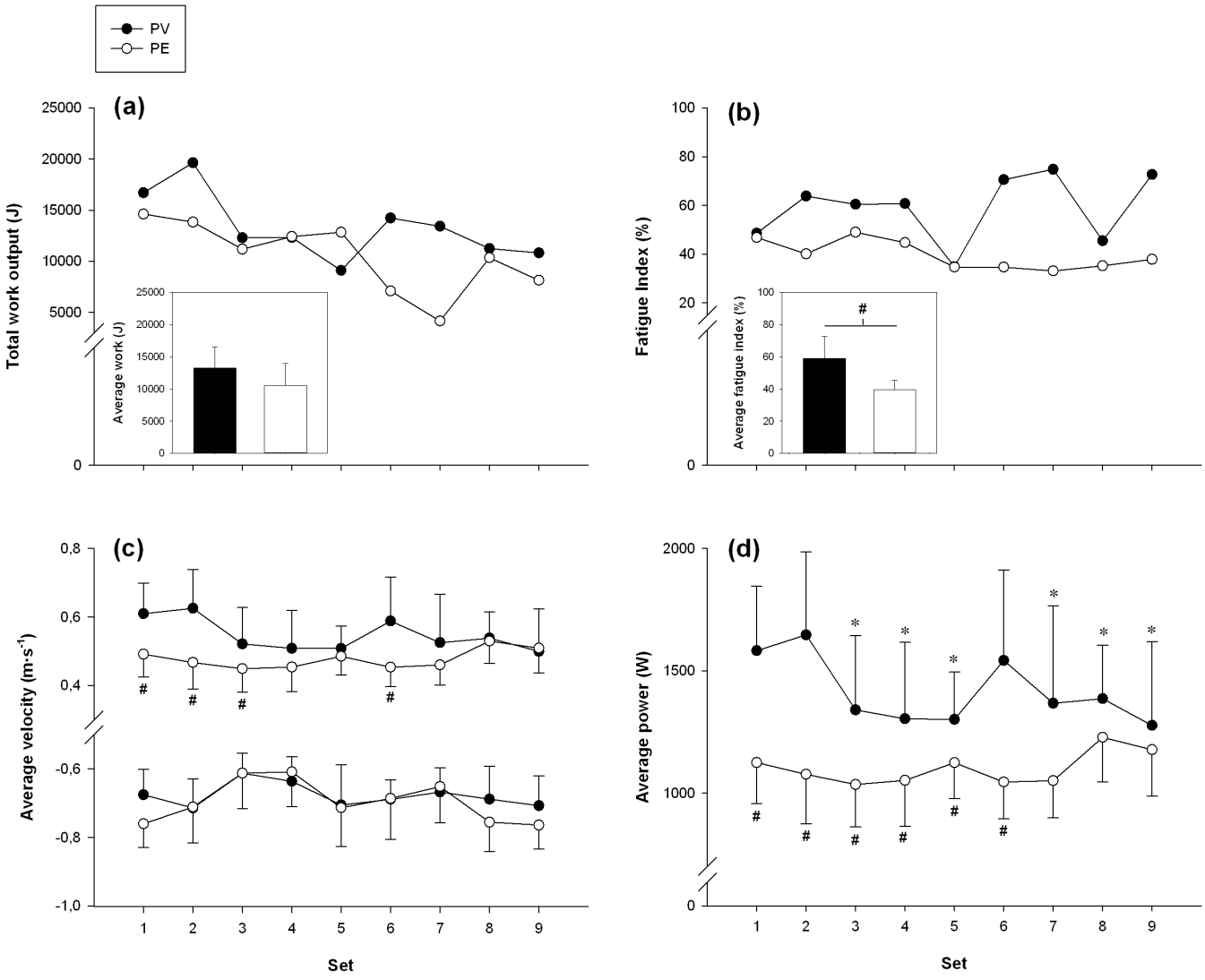

PV
PE
(a)
Total work output (J)
25000
20000
15000
10000
5000
0
Average work (J)
(b)
Fatigue Index (%)
100
80
60
40
20
Average fatigue index (%)
#
(c)
Average velocity (m·s-1)
0,8
0,6
0,4
-0,6
-0,8
-1,0
(d)
Average power (W)
2000
1500
1000
*
1 2 3 4 5 6 7 8 9
Set

Figure 2

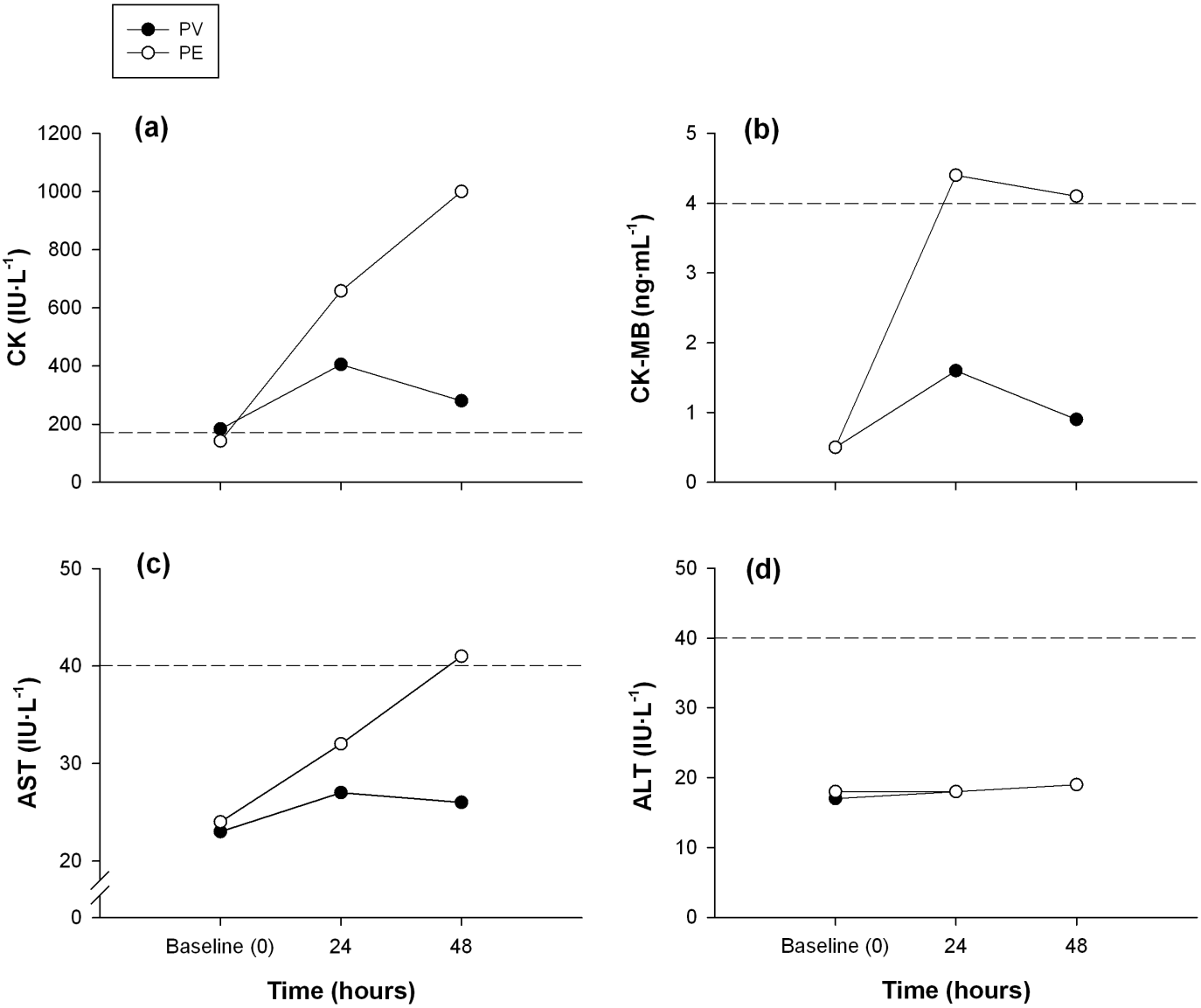
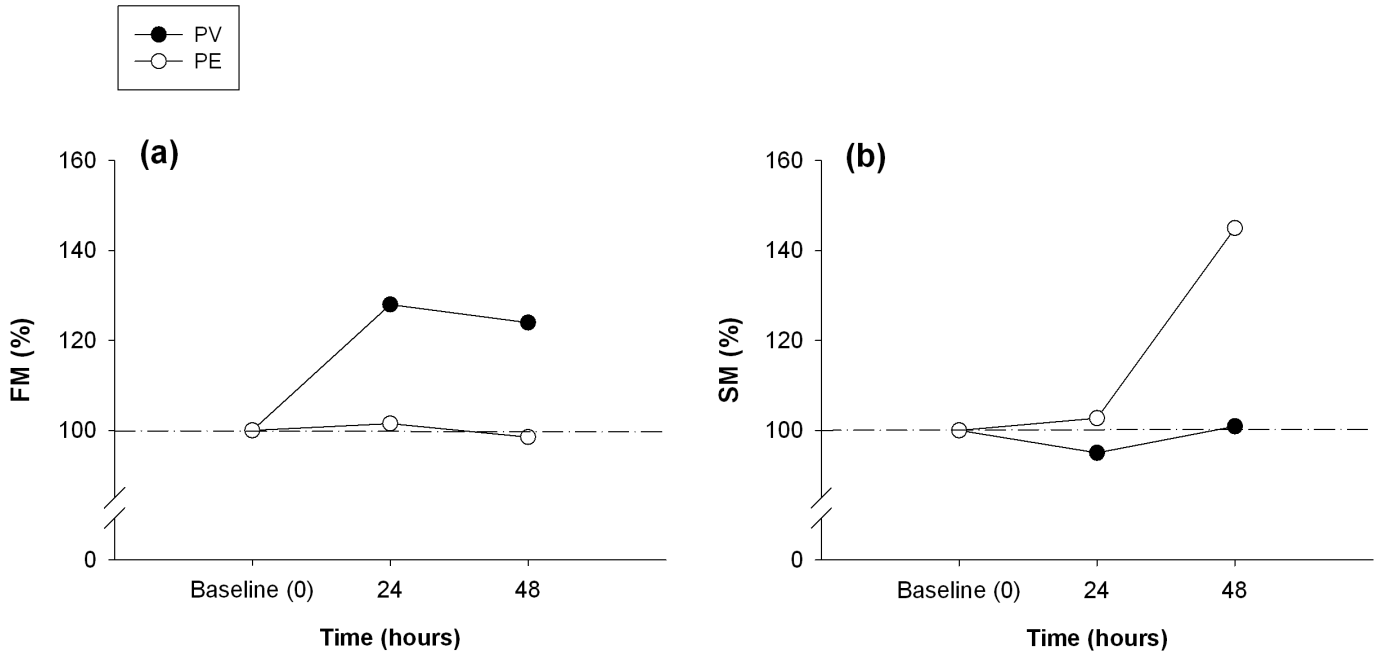

PV
PE
(a)
CK (IU·L-1)
1200
1000
800
600
400
200
0
(b)
CK-MB (ng·mL-1)
5
4
3
2
1
0
(c)
AST (IU·L-1)
50
40
30
20
0
Baseline (0)
24
48
Time (hours)
(d)
ALT (IU·L-1)
50
40
30
20
10
0
Baseline (0)
24
48
Time (hours)

Figure 3


PV
PE
(a)
160
140
120
100
0
FM (%)
Baseline (0)
24
48
Time (hours)
(b)
160
140
120
100
0
SM (%)
Baseline (0)
24
48
Time (hours)

4.3 Study III

Functional assessment of differences between and within subjects in the extent of hamstring muscles damage following intensive eccentric exercise

Functional assessment of severe hamstring muscle damage following intensive eccentric exercise: differences between and within subjects

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Running head

Severe hamstring muscle damage

Correspondence

Joan Aureli Cadefau

Keywords: severe muscle damage, sarcomeric mitochondrial creatine kinase, high responders, force-generating capacity, magnetic resonance imaging

ABSTRACT

Purpose: The aims of this study were to investigate the degree of damage inflicted on the hamstring muscles by intensive voluntary eccentric exercise and to analyse differences in the extent of between-subject and within-subject damage (limb-to-limb comparison).

Methods: Thirteen males performed six sets of ten reps of eccentric unilateral leg curl with each leg. Before and at regular intervals for 7 d after exercise, force-generating capacity (FGC) was measured with maximal isometric voluntary contraction (MVC). Serum enzyme levels, magnetic resonance imaging (MRI) transverse relaxation time (T2) and perceived muscle soreness were also assessed and compared against FGC.

Results: Two groups of subjects were identified according to the extent of exercise-induced muscle damage (EIMD) reflected by sharp, long-lasting FGC declines: high responders (n = 10) and moderate responders (n = 3). Significant differences were also found in FGC within-subjects (limb-to-limb comparison) from the high responder group. Changes in serum enzyme levels after exercise were correlated with MVC losses. Sarcomeric mitochondrial creatine kinase (sMtCK) increases were only observed in high responders. The MRI T2 analysis revealed that the semitendinosus (ST) was the hamstring muscle most damaged by exercise. ST T2 values obtained 7 d after exercise from the leg whose hamstring muscles showed larger FGC decline were correlated to MVC reductions, but no correlations were found between T2 values and MVC reductions in the leg whose hamstring muscles showed the smaller FGC decline.

Conclusion: The results challenge the notion that severe EIMD in humans is restricted to electrical stimulation protocols. Within-subject (limb-to-limb comparison)

differences reveal that experimental designs using contralateral limbs as a control should take into account that different degrees of hamstring damage can be induced in each leg. sMtCK is a promising novel EIMD biomarker that allows identification of high responders. When muscle function is recovered (i.e., when MVC returns to baseline values) the long-lasting increases in T2 values suggest an adaptive process rather than damage.

INTRODUCTION

Repeated eccentric contractions (i.e., lengthening muscle actions) can lead to muscle damage, and it may take days or weeks for the muscles to recover (8) (13, 29, 48). This type of damage has been named ‘exercise-induced muscle damage’ (EIMD) (47). Muscle damage induced by extreme regimes of eccentric exercise present signs such as myofibrillar disruptions (22, 23) and myofiber necrosis (25, 33). Those signs are commonly assessed by histological examination of muscle tissue via biopsy; however, this invasive technique is rarely used when hamstring muscles are involved. In those cases, the combined evaluation of EIMD symptoms such as prolonged loss of force-generating capacity (FGC) (8, 49), delayed onset muscle soreness (14, 39), enzyme leakage from damaged fibers (42, 45), and increase in muscle proton transverse relaxation time (T2) (21, 32) can be a valid alternative. Specifically, FGC seems to reflect myofibrillar disruptions, inflammation, and necrosis better than any other markers of muscle damage (47). In this regard, a high correlation ($r = 0.89$) was reported between the magnitude of decrease in maximal isometric voluntary contraction (MVC) and the proportion of muscle fibers with ultrastructural disruptions (49). Based on the association found in several studies between loss of FGC and myofibrillar disruptions, Paulsen et al. (47) suggested using the term ‘severe’ EIMD when a large loss of FGC (≥ 50% reduction) and/or long-lasting recovery (> 1-week) were found following muscle damaging protocols. However, although FGC provides reliable and valid information about the extent of muscle damage, it does not offer any evidence of the location of the damage process, either in the muscle or in the fiber structure. Therefore, when hamstring muscle response to intensive eccentric exercise is evaluated, and when ‘severe-EIMD’ is likely, proxy markers of muscle damage such as magnetic resonance imaging (MRI) and serum biochemical markers are needed to obtain a more accurate picture of the phenomenon.

The hamstring muscles comprise the biceps femoris long head (BFlh), the biceps femoris short head (BFsh), the semimembranosus (SM), and the semitendinosus (ST). These muscles present anatomical, architectural, and functional differences from each other (53). MRI can identify changes in each muscle and, in particular, T2 provides a quantitative index of muscle activation (30) and damage (20, 32). Because the degree of response to intensive eccentric exercise in different muscles is likely to vary (35), MRI can be a powerful tool for identifying differences among hamstring muscle damage. Unilateral intensive eccentric leg curl has been shown to be effective in isolating the ST muscle (30, 37), and it seems reasonable to assume that when most of the eccentric work relies on a single isolated muscle, the extent of muscle damage is exacerbated.

Serum muscle enzyme activities following eccentric exercise have been widely used as indirect biochemical markers of muscle damage (7). Muscle enzyme leakage to the bloodstream has been associated with increased membrane permeability (29, 45). The combination of different proxy markers such as serum biomarkers and T2 can be applied to identify muscles from which enzymes are being released into the circulation (32).

Mitochondria have recently been recognized as key players in cellular regulatory systems such as Ca^{2+} management and apoptosis (40), and magnetic resonance spectroscopy has indicated that impaired mitochondrial function may be a consequence of EIMD. Because mitochondrial creatine kinase (MtCK) activity is believed to be active as an energy sensor by coupling cellular energy state to cell apoptosis (50), we measured serum levels of sarcomeric mitochondrial creatine kinase (sMtCK) as a potential biomarker of mitochondrial damage from the sarcomere.

The present study is part of a multidisciplinary research project dedicated to analysing hamstring muscle response to eccentric exercise. The purpose of this study was to investigate the degree of damage inflicted on the hamstring muscles by an intensive voluntary eccentric exercise and to analyse differences in the extent of hamstring muscle damage between subjects and within subjects (limb-to-limb comparison). In this study, analyses of the FGC of the hamstring muscles have been combined with evaluations of muscle enzyme leakage and MRI.

MATERIALS AND METHODS

Subjects. Thirteen healthy male students (mean ± SD, age = 22.9 ± 2 yr, height = 1.77 ± 0.6 m, weight = 74 ± 6 kg) with no history of hamstring injuries gave written informed consent to participate in the study. Subjects' dominant leg was determined by asking them their preferred leg when kicking a ball. The fitness level of the subjects varied according to their daily physical activity; two subjects were sedentary and the other eleven were "moderately active" to "physically active" (exercising 2 – 5 exercising $d \cdot wk^{-1}$) (Table 1). Subjects were involved in systematic heavy resistance strength training for at least six months prior to the experiments. Moreover, they were asked not to perform any exercise during the week before or at any time during the experimental period. The study complied with the code of ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Ethics Committee of the Catalan Sports Council (*Generalitat de Catalunya*) (099S/690/2013).

Experimental design. Changes in muscle function were examined for 1 wk following a bout of unilateral eccentric hamstring curls performed with each leg. The recovery of the muscle FGC was assessed by repeated tests of isometric MVC during a knee flexion from a prone position. The first MVC test was performed before the eccentric exercise to establish the baseline values, and the rest of the tests were repeated 24, 48 and 72 h and 7 d after exercise.

In order to obtain a detailed biochemical monitoring of the muscle enzyme leakage, blood samples were scheduled immediately before assessment of FGC. To conduct a non-invasive analysis of the physiological changes that occur in the muscles recruited during exercise, MRI were also obtained just before assessment of FGC at baseline and at 24 h and 7 d after exercise. Finally, perceived muscle soreness in

the hamstring muscles was also assessed by using a visual rating scale (VRS) before and 24, 48, 72 h and 4, 5, 6 and 7 d after exercise (Fig. 1).

Eccentric exercise. Subjects performed six sets of 10 eccentric unilateral hamstring leg curls (Prone Leg Curl Technogym^{TM}, Italy) at 120% of their 1-repetition maximum (1-RM) with each leg, with a 3-min rest between sets. The 1-RM assessment and the exercise protocol are described elsewhere (30, 37).

Force-generating capacity. FGC was measured as MVC; i.e., average force in a 1-s window when a force plateau had been established (Tesch, 2004 #165). MVC of the hamstring muscles were measured for each leg with a force gauge connected to an A/D converter system, MuscleLab 4020e (Ergotest AS, Langesund, Norway). Subjects were prone with the hip joint at 40º of flexion and with the knee joint at 30º of flexion, and were verbally encouraged during the test to ensure maximal effort. Subjects performed two isometric MVCs of 3 to 5 seconds with a 1-min rest between contractions, and if any countermovement was evident, an additional MVC was measured. According to the percentage of reduction of FGC and its time course, the muscle damage experienced by the subjects was classified into severe EIMD (i.e., large reduction in MVC of > 50% of baseline values, and/or recovery was not completed in 1 week) or moderate EIMD (i.e., notable MVC declines of 20-50% of baseline values, and recovery was completed between 48 h and 7 d) (Paulsen, 2012 #489) during the repeated MVC tests following eccentric exercise. The leg that showed the larger loss of MVC was considered to have the greater hamstring muscle damage.

Muscle soreness. A 10-point VRS was used to quantify muscle soreness in the hamstring muscles. Each number on the scale was accompanied by descriptive words for soreness, from 0 (none) to 10 (intolerably intense). Subjects stretched and

contracted (isolated unloaded knee flexion from a stand up position) to assess general soreness in the whole hamstring muscles.

Blood analysis. An 8-mL blood sample was drawn from an antecubital vein. The blood was allowed to clot for 30 min at room temperature (21ºC) and was then centrifuged at 3000*g* for 10 min at 4ºC. After separation, serum aliquots were stored at -80ºC until analysis. Measurements of creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were performed on an Advia 2400 automatic device (Siemens™ Medical Solutions Diagnostics, Tarrytown, NY, USA). Creatine kinase MB isoform (CK-MB) analysis was performed following the method described by (Vaidya, 1992 #647) using a Dimension Clinical Chemistry System (Siemens™ Healthcare Diagnostics, Tarrytown, NY, USA) with a sensitivity limit of 0.5 ng/mL. Serum concentration of sMtCK was measured by using a commercial ELISA kit SEC386Hu (Cloud Clone Corp., Houston, TX, USA) according to the manufacturer's protocol.

Magnetic resonance imaging. MRIs (3 T scanner; Siemens, Erlangen, Germany) were performed ~30 min prior to exercise and 24 hours and 7 days following exercise. Subjects were supine on the MR-gurney with the head outside the MR-bore and thighs covered with one 32- and two flexible 4-channel coils respectively in the proximal and distal segments. A custom-made foot-restraint device was used to standardize and fix limb position, and to avoid any compression of thigh muscles. Twelve cross-sectional images of the thigh of both legs were obtained, starting at the very distal margin of the ischial tuberosity, and using the following scan sequences: (a) axial fat-suppressed proton density, TR 3000 ms, TE 30-33, eco train 4, slice thickness 3.5 mm, gap 28 mm, FOV 400x290 mm, matrix 320x180 and ipat 2; (b) axial T2 mapping, TR 1000 ms, TE (18, 36, 54, 72, 90, 108), eco train 6, FOV 400x400 mm, matrix 256x256, slice thickness 3.5 mm and gap 28 mm. A parametric

image was generated from the T2 mapping sequence using the Leonardo workstation (Siemens, Erlangen, Germany). Scout images and anatomical landmarks were obtained to ensure identical positioning in baseline and post-scans.

T2 of hamstring muscles (semitendinosus [ST]), and biceps femoris long head [BFlh]) and short head [BFsh]) from both legs were measured using eFilm Lite v.3.1 software (Merge Healthcare, Chicago, IL). Using the fat-suppressed images to detect any confounding artefact (i.e., vessels, fat), a circular region of interest (ROI) was selected for individual hamstring muscles in each of the T2 mapping images where muscles were visible. Following pre-exercise scan analysis, the same-size circular ROIs were placed in the T2 images of the post-exercise scan, to ensure the same positioning as in the pre-exercise analysis. In the evaluations, the images containing areas at 30% (proximal), 50% (middle) and 70% (distal) of thigh length from upper border of ischial tuberosity (0%) to the lower border of the tibial plateau (100%) were used (30). The same researcher performed the MR imaging scan and the T2 calculation. High intertester reliability, with intraclass correlation coefficients ranging from 0.87 to 0.94, has previously been reported (9).

Statistics. For variables that were normally distributed (Shapiro-Wilk test), a one-way repeated-measures ANOVA followed by a paired *t*-test with a Bonferroni correction was performed to identify statistically significant changes from baseline. The exception was CK-MB, for which Friedman's test, followed by Wilcoxon signed rank test with a Bonferroni correction, was used. A two way repeated-measures ANOVA (leg dominance x time) was performed to identify the main effects of leg dominance over MVC. A two-way repeated-measures ANOVA (muscle region x time) followed by a paired *t*-test with a Bonferroni correction was performed to identify statistically significant differences between T2 values of hamstring muscles after exercise. Differences between FGC reductions and T2 values from each leg were

assessed using the unpaired T-Test. Differences between subgroups of subjects were assessed using the Mann-Whitney test. Associations between variables of interest were assessed using Pearson's correlation coefficient test or Spearman's rank order correlation coefficient (the choice depending on the Shapiro-Wilk test for Gaussian distribution). Data are presented as means ± standard error of the mean (SEM) unless otherwise stated. The level of significance was set at $P < 0.05$. The statistics were performed with SPSS version 20.0 (SPSS Statistics, IBM Corp., Armonk, NY, USA).

RESULTS

Force-generating capacity. According to the MVC data, the muscle damage experienced by the subjects was classified into severe or moderate, following the criteria presented above (see methods). Subjects were then grouped into 'high' (n = 10) or 'moderate' (n = 3) responders according to the degree of muscle damage experienced (severe or moderate respectively). With the sole exception of subjects 5, 6 and 8, the leg whose hamstring muscles showed the larger decrease in FGC was the non-dominant one, but no significant effect of leg dominance (dominant or non-dominant) was found. Time-related changes in MVC from high and moderate responders are shown in Fig. 2. High responders' legs whose hamstring muscles showed the greater FGC reduction suffered significant MVC decreases of 34 ± 7%, 52 ± 7%, 39 ± 8%, and 38 ± 7% at 24, 48, 72 h and 7 d after exercise respectively. Unexpectedly, in high responders, the leg whose hamstring muscles showed the smaller reduction in MVC only presented statistically significant decreases of 18 ± 6% and 34 ± 9% at 24 and 48 h respectively, and the percentage of MVC reduction differed significantly between legs at 48 h and 7 d after exercise (Fig. 2). The three subjects (subjects 1, 7, and 9) classified as moderate responders showed a surprisingly low loss of FGC in both legs, and significant differences were found in MVC declines between groups at 48 h and 7 d after exercise (Fig. 2) (Fig. 6).

Muscle soreness. High responders' perceived muscle soreness during stretching and contracting (isolated unloaded knee flexion from a stand up position) was significantly elevated over baseline values at every time-point analysed. Muscle soreness increased after exercise and peaked at 72 h, with a value of 7.8 ± 0.3 arbitrary units (a.u.). Significant differences were found between high and moderate responders and between the hamstring muscles of the legs that showed the larger and the smaller loss of FGC at 72, 96 and 120 h after exercise (Fig. 3). Significant

correlations were found between high responders' legs whose hamstring muscles showed the larger FGC reduction, MVC declines, and muscle soreness at 48 h ($r = -0.726$; $P = 0.008$) and 72 h ($r = -0.637$; $P = 0.026$) after exercise.

Creatine kinase. Serum CK levels of high responders followed a sharp increasing pattern until 72 h after exercise, when peak activity was observed (45,455 ± 9922 $U \cdot L^{-1}$ [range: 530 – 95,920 $U \cdot L^{-1}$]), and then a decreasing trend was shown 7 d after exercise (13,990 ± 3622 $U \cdot L^{-1}$ [range: 4198 – 42,972 $U \cdot L^{-1}$]). Serum CK values of the moderate responders followed a biphasic pattern, increasing at 24 h after exercise, declining slightly at 48 h, and then increasing again until its peak at 7 d after exercise (701 ± 163 $U \cdot L^{-1}$ [range: 447 – 1005 $U \cdot L^{-1}$]). Significant differences were seen in serum CK activity between the two groups at 48, 72 h and 7 d after exercise (Fig. 4) (Fig. 6). Significant correlations were found between reductions of MVC and CK at 48 h ($r = -0.720$; $P = 0.006$) and 7 d ($r = -0.566$; $P = 0.044$) after exercise.

Creatine kinase-MB isoenzyme. Serum CK-MB levels of high responders showed only a significant increase at 72 h after exercise (5.81 ± 3.3 $ng \cdot mL^{-1}$ [range: 0.6 – 35.1 $ng \cdot mL^{-1}$]) and no differences were found between groups (Fig. 4).

Sarcomeric mitochondrial creatine kinase. Serum sMtCK concentrations of high responders increased until a significant peak at 72 h (547 ± 115 $ng \cdot mL^{-1}$ [range: 0 – 918 $ng \cdot mL^{-1}$]) and remained significantly elevated 7 d after exercise (237 ± 53 $ng \cdot mL^{-1}$ [range: 76 – 616 $ng \cdot mL^{-1}$]). Significant differences were found between groups at 72 h and 7 d after exercise (Fig. 4). Significant correlations were found between peak reductions of MVC and sMtCK at 48 h ($r = -0.691$; $P = 0.009$) and 7 d ($r = -0.572$; $P = 0.041$) after exercise.

Aspartate aminotransferase. High responders' AST activity showed a sustained increase above the clinically normal range (5 – 40 $IU \cdot L^{-1}$) until a peak at 72 h after

exercise (691 ± 191 IU·L^{-1} [range: 21 – 1813 IU·L^{-1}]), and 7 d after the exercise it was still significantly higher than baseline values (422 ± 96 IU·L^{-1} [range: 132 – 1097 IU·L^{-1}]). Significant differences were found between groups at 48, 72 h and 7 d after exercise (Fig. 4). A significant correlation was found between peak reductions of MVC and AST at 48 h (r = -0.753; *P* = 0.003).

Alanine aminotransferase. Serum ALT activity in high responders (n=10) showed a sharp increase above the clinically normal range (5 – 40 IU·L^{-1}) at 72 h, but was only statistically significantly elevated 7 d after exercise (183 ± 191 IU·L^{-1} [range: 23 – 365 IU·L^{-1}]). Significant differences were found between groups at 48, 72 h and 7 d after exercise (Fig. 4). Significant correlations were found between peak reductions of MVC and ALT at 48 h (r = -0.698; *P* = 0.008) and 7 d (r = -0.578; *P* = 0.039) after exercise.

Magnetic resonance imaging. Significant T2 increases of 146%, 172% and 186% were found in all sections of ST (proximal [30%], middle [50%], and distal [70%] respectively) from high responders 7 d after exercise (Fig. 6). Moderate T2 increases of 67% revealed that BFsh from high responders was also damaged 7 d after exercise. No T2 differences in BFlh with regard to baseline were found. Significant differences between groups were found in T2 values hamstring ST from both legs 7 d after exercise. Surprisingly, no T2 differences were found between the leg's hamstring muscles that showed the larger and the lower loss of FGC following exercise (Table 1). Analyses of variance revealed no T2 differences between ST muscle regions. Finally, the T2 values obtained 7 d after exercise from all ST sections (proximal [30%], middle [50%], and distal [70%], respectively) for the leg whose hamstring muscles showed the greater FGC decline were correlated to MVC reductions but, in contrast, no correlations were found between T2 values and MVC

reductions in the leg whose hamstring muscles showed the smaller FGC decrease (Fig. 5).

DISCUSSION

Sharp, long-lasting decreases of FGC (observed until 7 d after exercise) accompanied by long-lasting severe increases of circulating enzymes (i.e., > 10,000 $IU \cdot L^{-1}$), T2 values and perceived muscle soreness in ten of the subjects from the sample (n = 13) were consistent with a severe EIMD process. These results challenge the notion that severe muscle damage in humans is restricted to electrical stimulation protocols (15, 21).

Force-generating capacity

Between-subjects. We first classified the 13 subjects into groups on the basis of the leg that suffered the larger loss in MVC following intensive hamstring eccentric exercise. This is a reliable measure that is considered the best indirect marker of the extent of EIMD (28, 47, 55). Two groups of subjects were identified according to the extent of EIMD reflected by peak FGC declines: high responders (n = 10, average peak MVC loss = 52%, severe EIMD) and moderate responders (n = 3, average peak MVC loss = 21%, moderate EIMD), assuming that the number of fibers with ultrastructural and myofibrillar disruptions is reflected by the reduction in FGC (33, 34, 47, 49).

In a sample of young sedentary subjects, the intensive eccentric leg curl exercise protocol was shown to induce elevated levels of EIMD (FGC was not fully recovered 1 w after exercise) (30). However, in the present study, in which most of the subjects were active, the same exercise protocol (plus one more set) did not induce severe EIMD in all subjects: in three, EIMD was ‘only’ moderate (47). Even when subjects are exposed to a standardized exercise protocols, a large inter-subject variability exists in the severity of FGC loss (1, 24, 46). In this study, groups did not differ from each other according to any demographic or baseline measure, although there

seemed to be differences in the levels of sport activity and training. Although the contributing factors to the large between-subject variations in individual responses remain unclear, one of the most widely supported theories is that low and/or moderate responders show a smaller decrease of muscle function because they have executed high-force eccentric work using the same muscle group over a period of some months (43). This phenomenon is commonly referred to as the 'repeated-bout effect' (RBE) (36). In the present study, the RBE was probable since the sport activities carried out by moderate responders were athletics (participants 1 and 9) and football (participant 7). Both athletics practitioners were used to performing long-distance sprints, and it is well known that hamstring muscle activity rises with increasing running speed (2, 31), and that posterior tight muscles act eccentrically during both the late swing and the terminal stance phases of the running cycle (53, 57). Participant 7 was involved in a regular football-training program in which long-distance sprints also occur and his participation in an on-field hamstring-injury prevention program cannot be ruled out. Moreover, moderate responders were active (participant 7) or moderately active (participants 1 and 9), and since activity (training) status seems to be a contributing factor to variations in individual responses to eccentric exercise (17, 23, 46), it seems reasonable to assume that their activity levels have a protective effect against EIMD. Although other contributing factors such as certain genetic factors (27) cannot be ruled out, the RBE conferred by the type of sport and the activity level (training status) seem to be the factors that explain the smaller decline of hamstring muscle function in moderate responders.

Within-subjects (high responders limb-to-limb comparison). Experimental designs based on a single group observational comparison of response by using contralateral limbs have been used (11). However, this kind of model is based on the assumption that changes in markers of muscle damage between limbs are similar, but our study also found significant differences in muscle function within subjects (limb-to-limb

comparison). To the best of our knowledge, this is the first study comparing hamstring muscle damage within subjects (limb-to-limb comparison) after the same unilateral leg curl exercise performed with each leg. In this regard, high responders showed significant differences in MVC reductions between legs after exercise. As found in previous studies, these differences in the magnitude of EIMD were not influenced by lower limb dominance (26). It might be speculated that hamstring muscles are not equally resilient to EIMD following eccentric exercise; however further investigation is needed to confirm or dismiss this suggestion.

Serum enzymes

Between-subjects. Differences between subjects (high and moderate responders) suggested by FGC losses were confirmed by the differences in the increases in CK and other serum enzyme activities or concentrations after exercise. It has been stated that CK, to some extent, reflects the amount of myofibrillar damage, especially when damage becomes severe (33). Moreover, large increases in CK activity observed 4-5 days after exercise may reflect a segmental myofiber necrosis (33). The correlation between CK serum levels and MVC (FGC losses) found 7 d after exercise supports the notion that long-lasting CK increases are related to fibermyofiber necrosis. Moreover, the large CK activity increases found in high responders can be explained by certain genetic factors. For example, it has been stated that the subjects with homozygous myosin light chain kinase (MLCK) 49T and heterozygotes for the MLCK C37885A rare allele had large amounts of CK after eccentric exercise (12). Hubal et al. (27) reported that variations in single nucleotide polymorphisms in chemokine ligand 2 (CCL2) and its chemokine receptor 2 (CCR2) were related to large CK increases.

Of particular interest were the serum increases in sMtCK, because, in contrast to the other biochemical markers, the increases were only significant in high responders. The mitochondria have recently been recognized as key players in cellular regulatory systems like Ca^{2+} management and apoptosis (40). Specifically, MtCK have been reported to play the role of an energy sensor, coupling cellular energy state to cell apoptosis (50). The significant serum increases in sMtCK found in high responders are likely to be indicative of mitochondrial swelling and of disruption and decline in muscle respiratory capacity (10) and, given the correlations observed between sMtCK and MVC (FGC reductions) at 48 h and especially 7 d after exercise, it seems reasonable to assume that FGC reductions reflect a number of myofibrils that have died due to an apoptotic process triggered by large sMtCK increases. Moreover, it has been demonstrated in rats that prolonged exercise induces mitochondrial damage but that training status has a protective effect on the mitochondria against exercise (10) via a specific reduction of the mitochondrial Ca^{2+} uptake (5) and an increase in antioxidant capacity (54). Although it has only been proved in animal models (*Sprague-Dawley* rats) (10), it seems reasonable to assume that moderate responders' training status might confer a protective effect on the mitochondria against eccentric EIMD. Consequently sMtCK could be adopted as a novel and promising EIMD biomarker, sensitive to training status and suitable for the recognition of high responders.

Long-lasting increases in AST and ALT were only found in high responders. Although AST and (especially) ALT are often considered specific markers of liver injury, they are ubiquitously present in most tissues, including skeletal muscle (6, 16). Moreover, hypertransaminasemia is commonly present in patients with high CK levels resulting from extreme exercise (38). However, hepatic damage cannot be ruled out because more specific hepatic injury markers such as alkaline phosphatase (ALP) and gamma glutamyltransferase (γGT) (3) have not been measured.

Magnetic resonance imaging

Within-hamstring (muscle comparison). Muscle damage was localized in three hamstring muscles (BFlh, BFsh, and ST) by using MRI T2 relaxation time. In both groups of subjects (high and moderate responders), and in accordance with Kubota et al. (30) and Mendiguchia et al. (37), the T2 results suggested that the individual responses of the hamstring muscles following the eccentric leg curl exercise differed between hamstring muscles; the ST was the most damaged, probably because of its architectural characteristics. Specifically, ST is a fusiform muscle with long fascicle lengths and a small physiological cross-sectional area (56), which explains its major contribution to eccentric knee-flexion exercise (37) and its higher sensitiveness to eccentric exercise (30).

Within-Semitendinosus (muscle region comparison). Uniform changes were found across all the ST regions measured (proximal [30%], middle [50%], and distal [70%]). Although Kubota et al. (30) found non-uniform changes among ST regions following intensive eccentric exercise, these differences were only found at 48 and 72 h following exercise. As in our study, those authors found uniform changes across the measured ST regions at 24 h and 7 d after eccentric leg curl exercise.

Between-subjects. In contrast to moderate responders, ST and to a lesser extent BFs in high responders showed long-lasting T2 increases 7 d after exercise in the leg whose hamstring muscles showed the greater FGC decline. T2 increases have been correlated to ultrastructural abnormalities observed by histology in humans after downhill running (44) and illustrate an oedematous process (13, 18, 35, 52) which is consistent with the higher extent of muscle damage reflected by persistent FGC reductions 7 d after exercise.

Within-subjects (high responders limb-to-limb comparison). Interestingly, all the ST regions measured from the leg whose hamstring muscles showed the greater FGC decline were correlated with MVC reductions but, in icontrast, no correlations were found between T2 values and MVC reductions in the leg whose hamstring muscles showed the smaller FGC decline. It has been shown that following eccentric exercise T2 values remain elevated, even when other markers of muscle damage have returned to baseline values (13). Specifically, elevated T2 values have been found as long as 75 days (51) and 31 days (41) following (in both cases) eccentric exercise of the elbow flexor muscles. Foley et al. (19) suggested that long-lasting T2 increases after other markers of EIMD have returned to baseline values might reflect an adaptive process rather than damage. Accordingly, since the muscle function in the leg whose hamstring muscles showed the smaller FGC decline was recovered 7 d after exercise (i.e., MVC returned to baseline values), it seems reasonable to relate its long-lasting increases in T2 values to an adaptive process. Although T2 has been identified as a relevant biomarker of muscle damage (4, 20, 32), the results of the present study reinforce the notion that MRI must be accompanied by muscle function assessments when muscle damage is analysed.

In summary, the results of indirect markers of EIMD obtained in ten of the subjects from the sample (n = 13) challenge the notion that severe muscle damage in humans is restricted to electrical stimulation protocols (15, 21). However, the results showed a wide range of FGC loss (from 7% to 84%), reflecting a different degree of EIMD between subjects (high and moderate responders) which may be related to the subjects' level of sport activity and training status. The results also challenge the assumption that changes in markers of muscle damage are similar between limbs, since within-subject (limb-to-limb comparison) differences were also found in the high responder group. Experimental designs using contralateral limbs as a control should take into account that different degree of hamstring damage can be induced between

legs, at least in high responders. Changes in the serum enzyme activities or concentrations after exercise were in accordance with the between-subject differences (high and moderate responders) in the extent of EIMD reflected by FGC reductions. Furthermore, and although further investigation is needed, it seems that sMtCK is a promising novel EIMD biomarker that allows identification of high responders. The MRI analysis revealed that ST was the hamstring muscle most damaged by the eccentric leg curl exercise, and uniform T2 changes were observed across sections of this muscle. Finally, since the muscle function in the leg whose hamstring muscles showed the smaller FGC decline was recovered 7 d after exercise (i.e., the MVC returned to baseline values), it seems reasonable to relate its long-lasting increases in T2 values to an adaptive/remodeling process rather than to damage.

FIGURE CAPTIONS

Figure 1. Schematic overview of experimental design.

Figure 2. Mean (± SEM) values of percentage of maximal voluntary contraction (MVC) from the hamstring muscles of the leg that showed the larger (a) and the smaller (b) loss of force-generating capacity following exercise. *, ** and *** : significantly different from baseline value at $P < 0.05$, $P < 0.01$ and $P < 0.001$, respectively. $^{\#}$ Significant difference between groups at $P < 0.05$. $^{\phi}$ and $^{\phi\phi\phi}$. Significant difference between high responders' legs at $P < 0.05$ and $P < 0.001$ respectively.

Figure 3. Mean (± SEM) values (a.u., arbitrary units) of perceived muscle soreness from the hamstring muscles of the leg that showed the larger (a) and the smaller (b) loss of force-generating capacity following exercise. * Significantly different from baseline value at $P < 0.05$. $^{\#}$ Significant difference between groups at $P < 0.05$. $^{\phi}$ Significant difference between high responders' leg at $P < 0.05$.

Figure 4. Mean (± SEM) values of (a) CK, creatine kinase (note the logarithmic scale on the y-axis); (b) CK-MB, creatine kinase MB_{mass} isoenzyme; (c) AST, aspartate aminotransferase; (d) ALT, alanine aminotransferase; (e) sMtCK, sarcomeric mitochondrial creatine kinase; (f) CRP, C-Reactive protein. *, ** and ***: Significantly different from baseline value at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. $^{\#}$, $^{\#\#}$ Significant difference between groups at $P < 0.05$ and $P < 0.01$ respectively.

Figure 5. Correlation between percentage of change of maximal voluntary contraction (MVC) and semitendinosus (ST) T2 muscle section (30%, proximal; 50%, middle; 70%, distal) values from the hamstring muscles of the leg that showed the larger (a) and the smaller (b) loss of force-generating capacity 7 days after exercise (n = 13). r_s : Spearman correlation coefficient.

Figure 6. Representative T2-weighted magnetic resonance images of the middle section (50% of thigh length) from a moderate responder (subject 7) (left) and a high responder (subject 3) (right) before and 24 h and 7 d after eccentric exercise (Biceps femoris long head, BFlh, Biceps femoris short head, BFsh, and Semitendinosus, ST). Values of percentage of maximal voluntary contraction (MVC) from the hamstring muscles of the leg that showed the larger and the smaller loss of force-generating capacity (LL FGC and SL FGC, respectively) and values of CK (creatine kinase) at baseline and at regular intervals for 7 d after exercise. Note that both subjects play the same sport (football) but present different activity levels (high versus medium) (Table 1).

TABLES

Table 1. Characteristics of the subjects' physical activity levels.

Subject	Activity level	Sport activity
1	Medium	Athletics, recreational
2	Low	Indoor football, recreational
3	Medium	Football, recreational
4	Medium	Indoor football, recreational
5	Low	No exercise
6	Low	Basketball, recreational
7	High	Football, active
8	Low	No exercise
9	Medium	Cycling and athletics, recreational
10	Medium	Water polo, active
11	Medium	Running, recreational
12	High	Roller hockey, active
13	Medium	Football, recreational

Activity levels: low, two exercise bouts per week; medium, two to four exercise bouts per week, high, more than four exercise bouts per week.

Table 2. Mean values (± SEM) of T2 (ms) from hamstring muscles before and after unilateral eccentric curls.

			T2 (ms)	
			Leg	
Muscle (Section)	Time	Group	LL FGC	SL FGC
BFsh	Baseline	High responders (n=10)	43.4 ± 2.2	45.7 ± 1.5
(70%, distal)	24 h		49.8 ± 2.6	60.2 ± 4.2***
	7 d		72.6 ± 9.6*	73.7 ± 11.6*
	Baseline	Moderate responders (n=3)	47.2 ± 9.3	47.9 ± 7.7
	24 h		50.8 ± 9.7	49.9 ± 8.1
	7 d		48.0 ± 8.5	46.7 ± 7.4
ST	Baseline	High responders (n=10)	48.0 ± 1.7	51.8 ± 1.9
(30%, proximal)	24 h		110.7 ± 42.3	117.3 ± 25.6
	7 d		117.9 ± 14.6***	154.0 ± 19.8***
	Baseline	Moderate responders (n=3)	44.8 ± 9.1	52.6 ± 8.1
	24 h		54.8 ± 12.8	68.4 ± 10.6
	7 d		49.6 ± 18.9$^{\#}$	59.7 ± 9.1$^{\#}$
ST	Baseline	High responders (n=10)	44.4 ± 1.9	43.2 ± 2.3
(50%, middle)	24 h		113.4 ± 48.3	89.4 ± 17.9
	7 d		119.8 ± 20.0***	122.9 ± 15.0***
	Baseline	Moderate responders (n=3)	43.2 ± 8.2	46.9 ± 7.5
	24 h		49.5 ± 9.3	53.5 ± 8.5
	7 d		43.7 ± 14.9$^{\#\#}$	45.3 ± 6.8$^{\#}$
ST	Baseline	High responders (n=10)	43.5 ± 0.8	46.3 ± 1.9
(70%, distal)	24 h		62.4 ± 8.7	72.8 ± 8.7
	7 d		124.6 ± 21.2***	143.2 ± 17.7***
	Baseline	Moderate responders (n=3)	43.7 ± 8.6	48.2 ± 7.5
	24 h		47.1 ± 9.1	49.1 ± 7.5
	7 d		44.0 ± 17.9$^{\#\#}$	45.3 ± 6.0$^{\#}$

Only T2 (ms) values from hamstring muscle sections that showed significant time-related changes from baseline are shown (Biceps femoris short head, BFsh, and Semitendinosus, ST). T2 values from the hamstring muscles of the leg that showed the larger and the smaller loss of force-generating capacity following exercise, LL FGC and SL FGC respectively. *, ** and *** Significantly different from baseline value at $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively. $^{\#}$, $^{\#\#}$ Significant difference between groups at $P < 0.05$ and $P < 0.01$. Note that no differences between legs were found.

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Figure 1

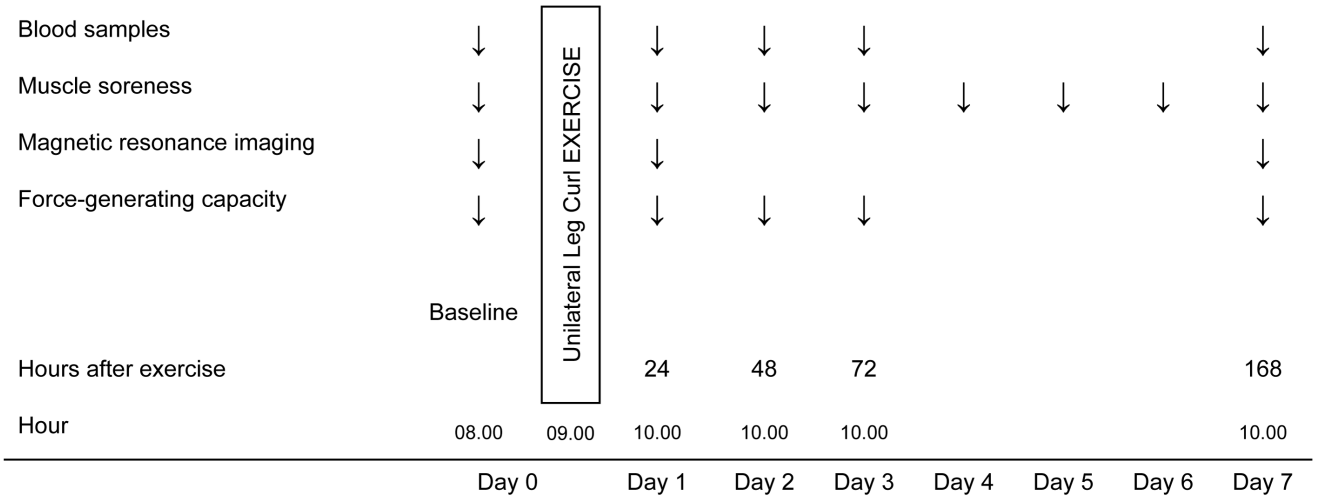

Blood samples
Muscle soreness
Magnetic resonance imaging
Force-generating capacity
Baseline
Unilateral Leg Curl EXERCISE
Hours after exercise
24
48
72
168
Hour
08.00
09.00
10.00
10.00
10.00
10.00
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7

Figure 2

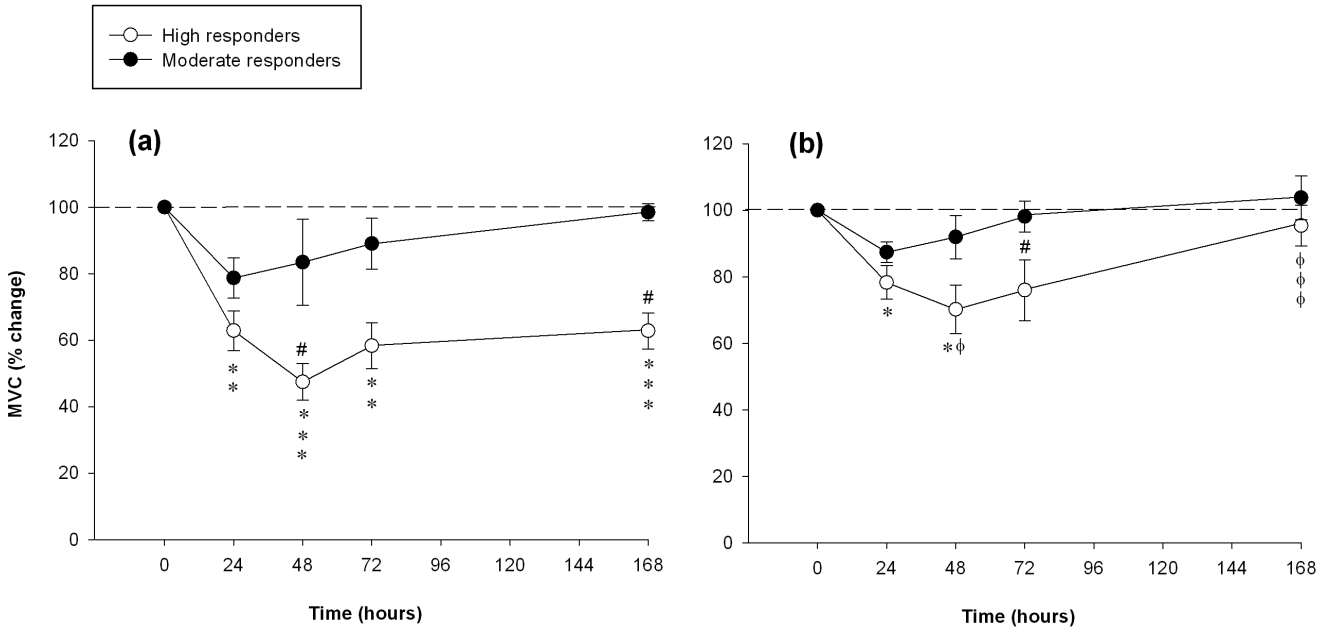

High responders
Moderate responders
(a)
(b)
MVC (% change)
Time (hours)
Time (hours)
0
20
40
60
80
100
120
0
24
48
72
96
120
144
168

Figure 3

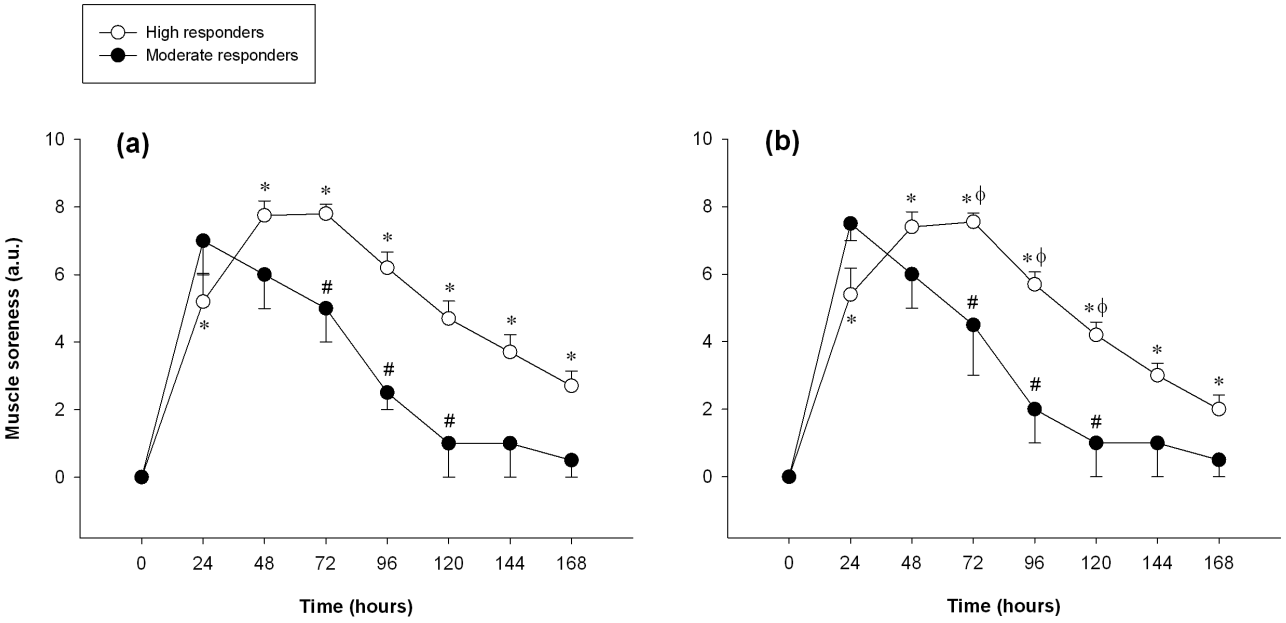

High responders
Moderate responders
(a)
(b)
Muscle soreness (a.u.)
Time (hours)
0
24
48
72
96
120
144
168
2
4
6
8
10
*
#
*ϕ

Figure 4

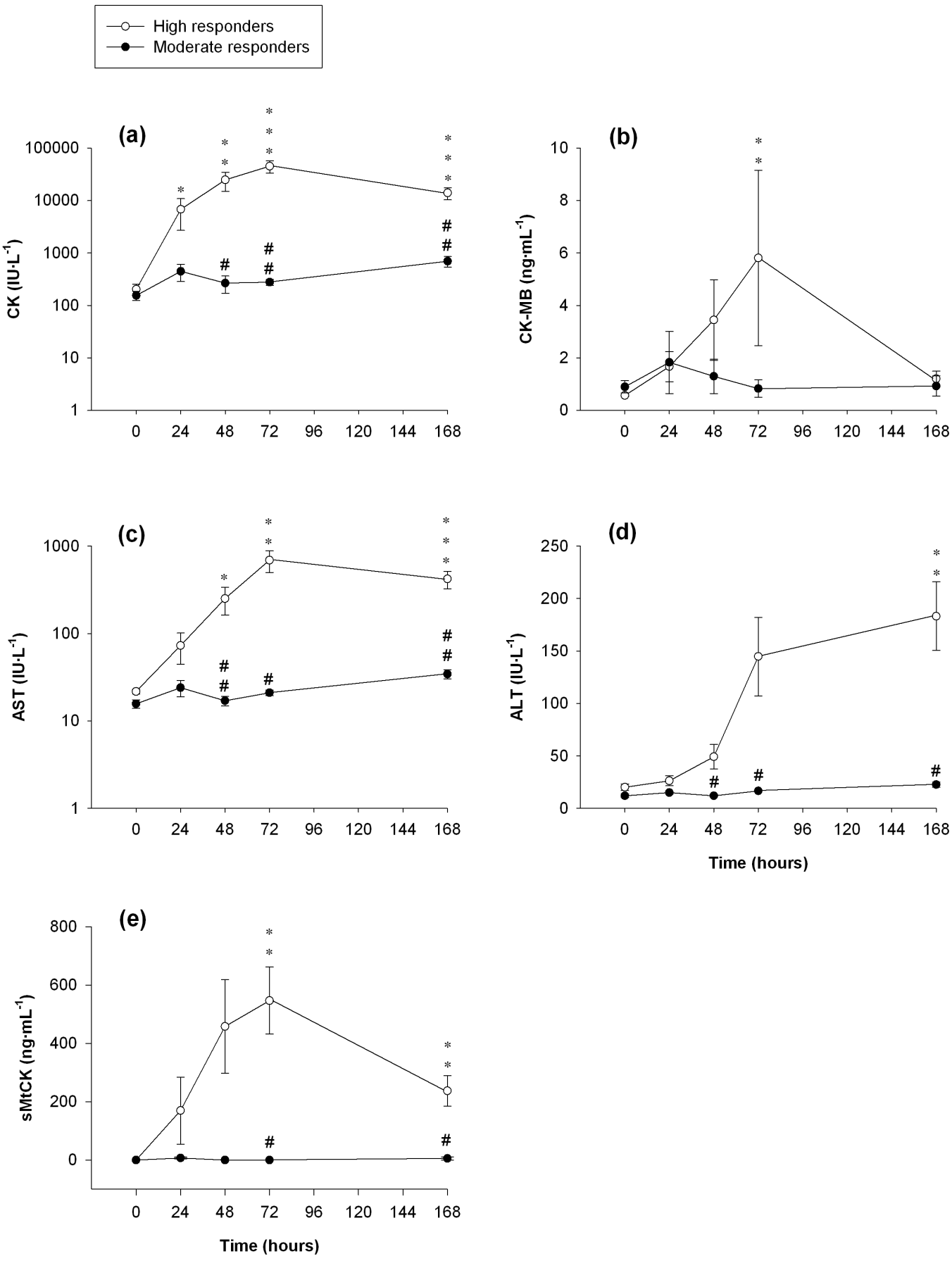

High responders
Moderate responders
(a)
CK (IU·L-1)
100000
10000
1000
100
10
1
0 24 48 72 96 120 144 168
(b)
CK-MB (ng·mL-1)
10
8
6
4
2
0
0 24 48 72 96 120 144 168
(c)
AST (IU·L-1)
1000
100
10
1
0 24 48 72 96 120 144 168
(d)
ALT (IU·L-1)
250
200
150
100
50
0
0 24 48 72 96 120 144 168
Time (hours)
(e)
sMtCK (ng·mL-1)
800
600
400
200
0
0 24 48 72 96 120 144 168
Time (hours)

Figure 5

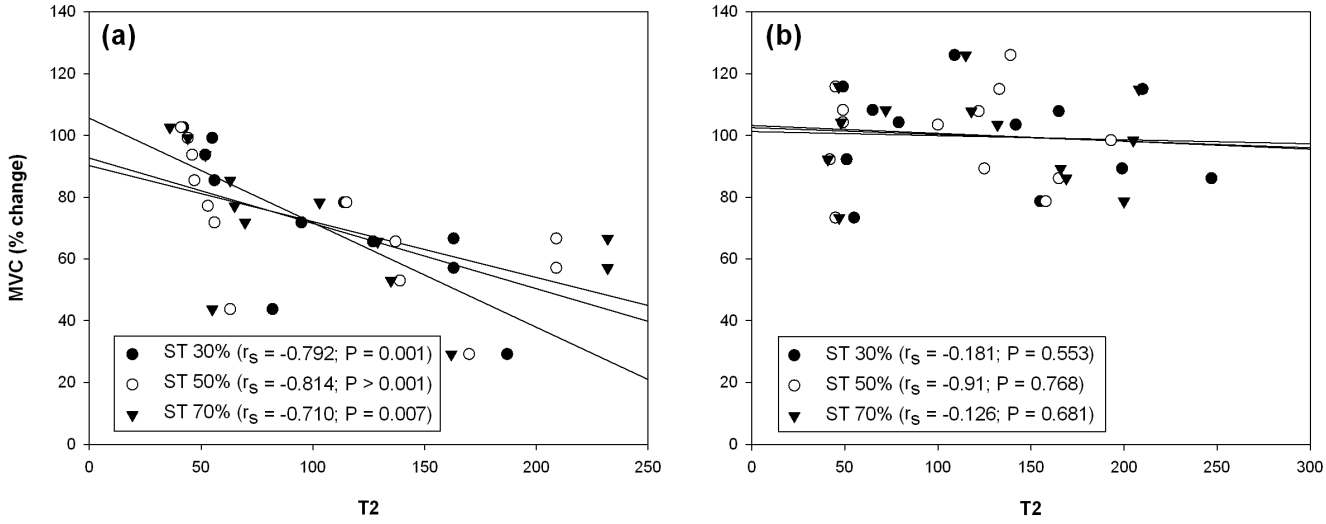
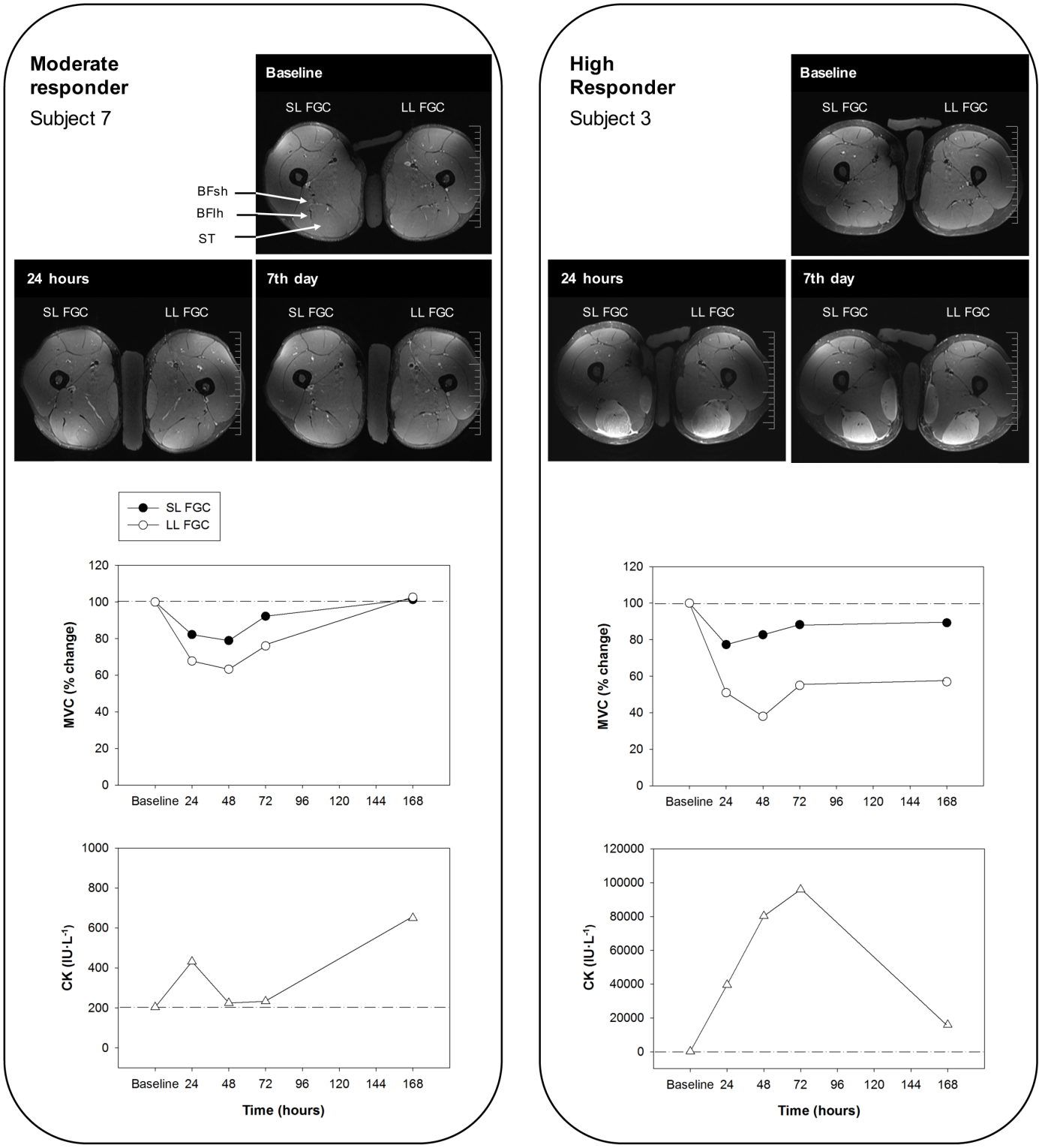

(a)
MVC (% change)
T2
ST 30% (r_s = -0.792; P = 0.001)
ST 50% (r_s = -0.814; P > 0.001)
ST 70% (r_s = -0.710; P = 0.007)
(b)
T2
ST 30% (r_s = -0.181; P = 0.553)
ST 50% (r_s = -0.91; P = 0.768)
ST 70% (r_s = -0.126; P = 0.681)

Figure 6


Moderate responder
Subject 7
Baseline
SL FGC
LL FGC
BFsh
BFlh
ST
24 hours
7th day
High Responder
Subject 3
MVC (% change)
CK (IU·L-1)
Time (hours)
Baseline 24 48 72 96 120 144 168

4.4 Study IV

Sarcomere disruptions of slow fibre resulting from Mountain Ultramarathon

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http://dx.doi.org/10.1123/ijspp.2014-0267

Human Kinetics
ORIGINAL INVESTIGATION

Sarcomere Disruptions of Slow Fiber Resulting From Mountain Ultramarathon

Gerard Carmona, Emma Roca, Mario Guerrero, Roser Cussó, Alfredo Irurtia, Lexa Nescolarde, Daniel Brotons, Josep L. Bedini, and Joan A. Cadefau

Objective: To investigate changes after a mountain ultramarathon (MUM) in the serum concentration of fast (FM) and slow (SM) myosin isoforms, which are fiber-type-specific sarcomere proteins. The changes were compared against creatine kinase (CK), a widely used fiber-sarcolemma-damage biomarker, and cardiac troponin I (cTnI), a widely used cardiac biomarker. ***Methods:*** Observational comparison of response in a single group of 8 endurance-trained amateur athletes. Time-related changes in serum levels of CK, cTnI, SM, and FM from competitors were analyzed before, 1 h after the MUM, and 24 and 48 h after the start of the MUM by 1-way ANOVA for repeated measures or Friedman and Wilcoxon tests. Pearson correlation coefficient was employed to examine associations between variables. ***Results:*** While SM was significantly (P = .009) increased in serum 24 h after the beginning of the MUM, FM and cTnI did not change significantly. Serum CK activity peak was observed 1 h after the MUM (P = .002). Moreover, serum peaks of CK and SM were highly correlated (r = .884, P = .004). ***Conclusions:*** Since there is evidence of muscle damage after prolonged mountain running, the increase in SM serum concentration after a MUM could be indirect evidence of slow- (type I) fiber-specific sarcomere disruptions.

Keywords: eccentric contraction, creatine kinase, muscle myosin isoforms

Mountain ultramarathons (MUM) are competitive events consisting of walking and running on mountain trails over a great cumulative elevation gain and over a longer distance than the athletic marathon (>42.195 km). Distance and cumulative elevation gain are the main determinants of MUM difficulty. Long-distance trail competitions have risen in popularity over the last few years.[1] However, the acute physiological responses to extreme endurance events still remain unclear. It is known that MUM competitions are strenuous and generally include negative slopes, so long distances are run downhill. It has been stated that strenuous exercise can result in muscle damage,[2] which is particularly exacerbated if eccentric contractions are performed (for a review see Proske and Allen[3]). Downhill running increases the eccentric component because the peak flexion angles are significantly greater, and it is a much stronger stimulus for damage than level or uphill running.[4] Therefore, it seems reasonable to relate most of the muscle damage to the negative-slope phases of the trail. MUM is a great opportunity for field-specific assessments of a physiologically stressful competitive event that induces muscle damage.[5,6]

Direct evaluation of muscle damage involves histological examination of muscle tissue by biopsy. However, in a sports context, the analysis of exercise-induced muscle damage is essentially based on proxy markers such as measurements of enzyme activity in blood, especially the activity of creatine kinase (CK). Previous studies evaluated the muscle damage induced by MUMs[5,6] and revealed large increases in total CK concentrations. However, CK is not a specific biomarker of skeletal muscle.[7] Koller et al[8] used slow (type I) myosin heavy-chain (MHC) fragments, and Melin et al[9] used beta MHC as muscle-fiber-specific damage biomarkers. Those groups found increases in this protein in plasma after mountain-running events. Although slow (type I) MHC fragments and beta MHC are common to skeletal and cardiac muscle, the damage was mainly related to slow (type I) fibers of skeletal muscle. However, the results found by Koller et al[8] and Melin et al[9] were highly unspecific, since plasma levels of MHC fragments were not compared with any cardiac-specific biomarker. Since it has been stated that strenuous exercise could induce a significant release of cardiac proteins such as troponin into the bloodstream,[10] it seems reasonable to assume that proteins found in cardiac and skeletal muscle, such as MHC fragments and beta MHC, could also be released from myocardium to blood.

Recently, myosin isoforms have been proposed as fiber-type-specific biomarkers of muscle damage that would represent indirect evidence of sarcomere disruptions.[11] However, Carmona et al[11] observed selective release of fast myosin isoforms (FM) after high-intensity knee-extensor exercise, but no changes in slow-myosin-isoform (SM) serum concentration were reported. SM is found in both cardiac and skeletal muscle, and FM is characteristic of fast skeletal muscle. Limb skeletal muscles are composed of slow (type I) and fast (type II) fibers,[12] but adult skeletal muscles shows plasticity and can undergo conversion between different fiber types in response to exercise.[13] Endurance athletes tend to have a predominance of slow (type I) fibers.[14,15] For these reasons, we hypothesized that serum increases in myosin isoforms, especially in SM, in endurance-trained participants after a MUM could indicate not only the extent but also the type of fiber affected. Furthermore, in the current study, the lack of specificity of SM was minimized by analyzing the changes in serum concentration of cardiac troponin

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I (cTnI), a widely used myocardial-specific biomarker. It has been shown that cTnI is released after prolonged exercise. However, in contrast to myocardial infarction, in which cTnI is usually over 0.6 ng/mL and remains stable in blood for at least 5 days, cTnI release after prolonged exercise does not achieve such high serum levels and returns to baseline within 24 to 48 hours.[16] To the best of our knowledge, this is the first field study to use a combination of myosin isoforms and cTnI to assess indirectly the muscle damage induced by a MUM.

The aim of this study was to investigate changes in serum concentration of myosin isoforms after a MUM. SM and FM were compared with CK, a widely used biomarker of exercise-induced muscle damage. The lack of specificity of SM was countered with the measurement of serum cTnI concentration. Since there is evidence of muscle damage after prolonged mountain running,[5,6] we hypothesized that a specific increase in SM serum concentration after a MUM would be indirect evidence of slow (type I) -fiber sarcomere damage in endurance-trained mountain runners.

Methods

Participants

We initially recruited 17 endurance runners, 14 men and 3 women. However, due to bad weather conditions during the competition, only 8 subjects decided to complete the study: 7 men and 1 woman (mean ± SD; men, n = 7, 39.8 ± 3.3 y, 178.7 ± 5.2 cm, 76.9 ± 7.9 kg; women, n = 1, 39.1 y, 173.0 cm, 67.0 kg). All of the participants were experienced white nonprofessional athletes (mean training regimen, 450.0 ± 210.31 min/wk of endurance training) who were specifically trained for MUM. All were healthy and had incurred no muscle injuries in the 6 months before the study. To avoid bias, no instructions were given about the type of training performed the week before the competition, but athletes were asked about it to better interpret the baseline serum levels of enzymes and contractile proteins. Physical activity after the race was limited and massages were prohibited. The study conformed to the Declaration of Helsinki for medical research, participants provided written informed consent, and the research was approved by the ethics committee of the Catalan Sports Council (Government of Catalonia).

Design

The study design used observational comparison of response in a single group of endurance-trained amateur athletes.

Methodology

The participants ran in the "Cavalls del Vent" MUM in 2012, an official competition organized by Salomon Nature Trails. It was a circular route with an official length of 84.84 km (~85 km) and a total cumulative elevation gain of 12,180 m. The start of the race was at 755 m above sea level and the maximum summit achieved during the trail was 2520 m (Figure 1). Each runner's average speed was calculated according to the total distance run divided by his or her official time.

Four blood samples were obtained: 1 day before the competition (pre), less than 1 hour after finishing the competition (post), and, because a significant degree of damage can occur during the race, 24 and 48 hours after the beginning of the MUM. A 5-mL blood sample was drawn from an antecubital vein. Blood was allowed to clot for 30 minutes in a tube (SST II Advance, Becton Dickinson Vacutainer Systems, UK) before being centrifuged at 3000 *g* for 10 minutes at 4°C. Three 200-µL aliquots of serum were stored at –80°C until analysis.

CK determinations were performed in an Advia 2400 automatic device (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA), and cTnI determinations were made in a Dimension Clinical Chemistry System automatic device (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) with an analytical measurement range of 0.017 to 40 ng/mL. To obtain muscle myosin-isoform concentrations in serum, we developed an enzyme-linked immunosorbent assay (ELISA-sandwich), which is described elsewhere.[11] Briefly, a calibration curve was obtained by a serial dilution from 0 to 250 ng of pure myosin from porcine muscle M0273, and the ELISA was completed by using monoclonal antimyosin (skeletal, fast) clone My-32, monoclonal antimyosin (skeletal, slow) clone NOQ7.5.4D, antimyosin polyclonal antibody M7523, and mouse anti-IGG linked to peroxidase A6154 (all Sigma Aldrich, Poole, UK). Intra-assay coefficients of variation were below 8% for FM and below 7.5% for SM. The linearity of the FM assay results was 80%, and it was 90% for the SM assay.

Statistical Analyses

The normality of each variable was tested using the Shapiro-Wilk test. As SM and CK were asymmetrically distributed, these variables were log-transformed before analysis. One-way repeated-measures ANOVA was used to identify the effect of time on CK activity and SM and FM serum levels. When any significant main effects were found, pairwise *t*-test comparisons with a Bonferroni correction

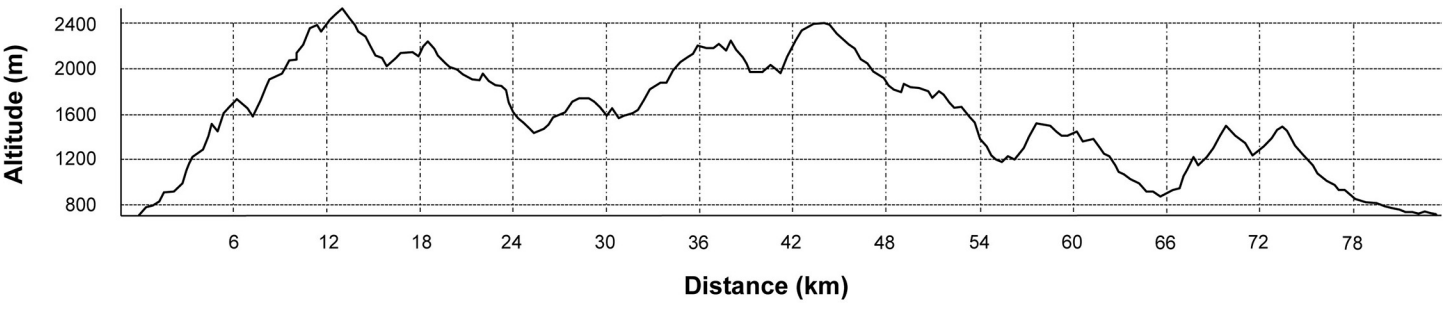

Figure 1 — Altitude profile of the entire mountain ultramarathon and the distance scale.

were used. The time course of changes in cTnI serum concentration was evaluated with the use of Friedman and Wilcoxon nonparametric tests. Effect sizes (ES) (Cohen *d*) were calculated to determine the practical difference between baseline values and serum peaks of enzymes and proteins. ES values of above 0.8, 0.8 to 0.5, 0.5 to 0.2, and lower than 0.2 were considered large, moderate, small, and trivial, respectively. Pearson correlation coefficient was employed to evaluate the association between the variables of interest. Data are presented as mean ± standard error of the mean unless otherwise stated. The level of significance was set at $P < .01$. All statistical analyses were conducted using SPSS version 20.0 statistical-analysis software (SPSS Statistics, IBM Corp, Armonk, NY).

Results

The week before the competition, participants reported the use of similar training strategies, based on a decrease in training volume (km/wk) and an increase in intensity (running average velocity). At the competition, only 1 participant finished the MUM (~85 km). The rest of the participants left the competition at different points along the trail due to bad weather conditions: temperature, range 0.9°C to 13.1°C; rain, range 0 to 6.1 mm/h; humidity, range 90% to 97%; and wind speed (east-southeast), range 1.1 to 5 m/s. The total distance (km) covered and official time (h:min:s) of each participant who decided to carry on with the study protocol were determined by the last official control point passed just before leaving the MUM. Individual average speed (km/h) was calculated according to these results (Table 1). With respect to biochemical markers, the average serum CK activity at baseline was in the clinically normal range (35–175 U/L) and rose significantly from 132 ± 22 U/L (pre) to a peak of 2052 ± 860 U/L (ES = 3.02) less than 1 hour after finishing the MUM. Average CK serum activity remained significantly elevated 24 hours (1345 ± 651 U/L) (ES = 2.59) after the beginning of the competition, but a clear decreasing trend was observed (Figure 2). The only woman who participated in the study until the end and also completed the whole long-distance trail (participant number 7) had the highest values of CK in serum in all samples (Table 1). Almost all participants were in the clinically normal range of cTnI (<0.017–0.050 ng/mL), or slightly above, in all analyzed samples (Table 1). No significant increase was seen in average cTnI serum concentrations 1 hour after the MUM (from 0.018 ± 0.001 ng/mL to 0.067 ± 0.028 ng/mL), and values returned to baseline 1 day after the trail (Figure 2). However, the average values of cTnI were highly biased, because participant number 7 showed an almost 10-fold increase in cTnI serum concentration 1 hour after the competition, which remained around 4-fold elevated 24 and 48 hours after the start of the MUM (Table 1). A nonsignificant slight increase in average FM serum concentration was observed 1 hour after the competition (from 1508 ± 222 µg/L to 1731 ± 204 µg/L), which remained stable until 24 hours after the beginning of the competition (1744 ± 250 µg/L) and returned to baseline values at 48 hours (1520 ± 318 µg/L) (Figure 1). Average FM serum values of all time points analyzed were in the previously established normal range (> 1000 µg/L).[11] Finally, no changes in SM were found until 24 hours after the start of the MUM, when SM serum concentration rose significantly from 1443 ± 390 µg/L to 3743 ± 1110 µg/L (ES = 1.34). SM serum activity remained nonsignificantly elevated 48 hours after the initiation of the competition (2828 ± 762 µg/L). Average baseline SM serum levels were in the normal range (> 2000 µg/L).[11] (Figure 2). The SM serum peak at 24 hours after the start of the MUM was also highly correlated with the CK serum peak found 1 hour after the competition ($r = .884$; $P = .004$) (Figure 3). Finally, SM was not correlated with cTnI.

Discussion

To our knowledge, this is the first study to assess the utility of the myosin isoforms SM and FM as serum biochemical markers of fiber-specific muscle damage induced by a MUM in experienced endurance runners. SM and FM were compared against CK, a widely used biomarker of exercise-induced muscle damage, and cTnI, a specific biomarker of cardiac damage. The novel finding of the current study was that only SM serum levels were significantly raised, while FM serum levels remained almost unaltered after the MUM competition. Another remarkable finding was that CK and SM serum peaks were highly correlated.

In the absence of myocardial infarction, muscle injury, or disease, large and time-sustained increases in blood CK activity have been widely accepted as a biomarker of muscle damage.[17] As expected, the MUM induced large increases in CK serum levels, up to an almost 16-fold rise 1 hour after the competition. Significant CK serum increases in healthy and well-trained participants have been previously documented after MUM competitions.[5] Large variability in serum CK concentrations among participants is common and has been previously described in experienced ultramarathoners after 24 hours of treadmill running[18] and after a 166-km MUM.[6] The reason for this variability still remains unclear, but it has been suggested that susceptibility is mainly related to genotype characteristics.[19] The CK serum peak 1 hour after the MUM and its recovery over the following 48 hours were also consistent with previous studies.[6] However, CK levels provide a gross indication of muscle-fiber damage, because they cannot identify the magnitude of damage[20] or the type of fibers affected.[7]

Although direct evidence of muscle damage is histological, force-generating capacity is considered a reliable and valid marker of muscle damage.[21] It has moreover been demonstrated that running a MUM induces large decreases in the knee-extensor force-generating capacity (a 35% decrease after a 166-km MUM), which are related to fatigue and muscle damage.[6] Furthermore, different studies have recently proposed sarcomere proteins such as troponin as proxy fiber-type-specific biomarkers of muscle damage.[7,22] According to the observed time course, molecular mass, and fiber compartment in which these proteins are located, their elevation in serum suggests more severe damage, signifying sarcomere damage.[22] As fiber-type-specific sarcomere proteins, SM and FM could allow for indirect diagnosis of sarcomere damage and the type of fiber affected.[11] Because MUM running has been demonstrated to induce muscle damage,[6] the significant increases in SM serum concentration seen 24 hours after the start of the MUM suggested selective slow (type I) fiber damage, and due to its elevated molecular weight (493 kDa) and its fiber intrasarcomeric compartmentalization, increased serum SM could indicate sarcomere disruptions of those fibers. Moreover, since cTnI showed a nonsignificant serum increase 1 hour after the competition and returned to almost baseline values 24 hours after the beginning of the MUM, SM serum increases in healthy individuals can be mainly related to skeletal-muscle slow-fiber damage, rather than myocardial damage. Furthermore, there was no statistical relationship between SM and cTnI. However, further research is needed on cTnI and the distance of ultraendurance events, because the only participant who completed the whole MUM (participant number 7) showed the highest increases in cTnI in every sample analyzed after the competition.

Table 1 Concentrations of Serum Creatine Kinase, Slow Myosin, and Cardiac Troponin I 1 Day Before the Competition (Pre), Less Than 1 Hour After Finishing the Competition (Post), and 24 and 48 Hours After the Beginning of the Mountain Ultramarathon for Each Participant

					Creatine Kinase (U/L)				Slow Myosin (μg/L)				Cardiac Troponin I (ng/mL)			
Participant	Gender	km	Time (h:min:s)	Av speed (km/h)	Pre	Post	24 h	48 h	Pre	Post	24 h	48 h	Pre	Post	24 h	48 h
1	M	41	09:39:18	4.25	70	793	933	611	856	703	1674	1333	0.017	0.029	0.017	0.017
2	M	26	06:47:33	3.83	108	363	356	214	811	920	1741	1832	0.017	0.022	0.017	0.017
3	M	26	06:47:16	3.83	156	350	196	126	628	505	1233	963	0.017	0.040	0.017	0,017
4	M	41	09:10:12	4.47	74	2691	1118	468	3097	3159	9812	5543	0.018	0.034	0.017	0.018
5	M	53	10:26:24	5.08	173	856	553	259	842	1225	1928	1162	0.017	0.029	0.017	0.017
6	M	53	12:09:28	4.36	179	2542	1156	531	3338	3205	4553	3979	0.017	0.079	0.017	0.017
7	F	85	11:48:48	7.26	233	7643	5819	2722	988	1404	6999	6361	0.027	0.260	0.115	0.101
8	M	53	12:09:32	4.36	59	1175	626	288	984	1400	2007	1454	0.017	0.043	0.017	0.017

Abbreviations: M, male; F, female; km, kilometers of mountain ultramarathon completed; Av, average.

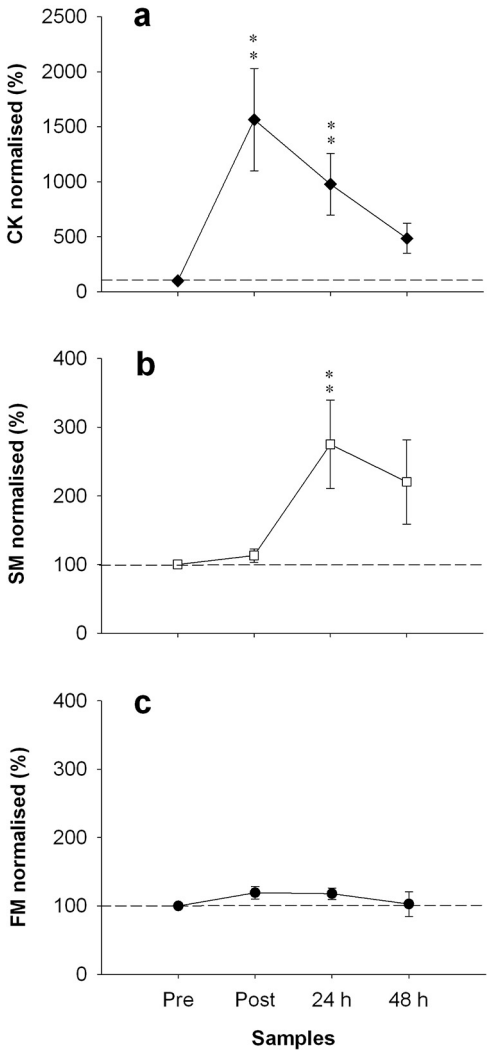

Figure 2 — Changes in (a) serum concentration of creatine kinase (CK) (n = 8), (b) slow myosin (SM) (n = 8), and (c) skeletal-muscle fast myosin (FM) (n = 7) 1 day before the competition (pre), less than 1 hour after finishing the competition (post), and 1 and 2 days after the mountain ultramarathon. Data are normalized (mean ± standard error of the mean) to pre-mountain-ultramarathon values (100%). **Significantly different from preexercise value at $P < .01$.

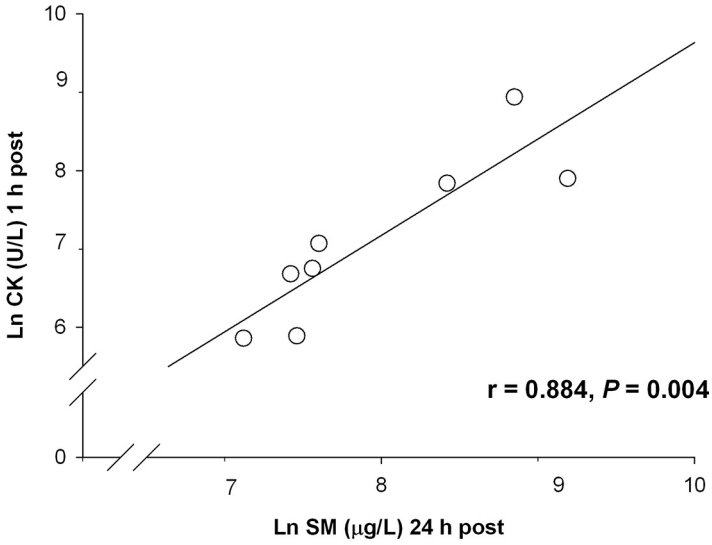

Figure 3 — Association between serum creatine kinase (CK) (natural log) peak activity 1 hour after finishing the long-distance trail competition (post) and slow myosin (SM) (natural log) peak concentration 24 hours after the start of the mountain ultramarathon (n = 8). *r,* Pearson correlation coefficient.

No changes in FM serum concentration were seen after the MUM, which indicates that the fast fibers suffered no damage or, at least, less damage than the slow fibers. Certainly, it has been proved by histology that fast fibers are most susceptible to eccentric contractions,[23] and downhill running, which is a mainly eccentric activity, induces muscle damage.[4] However, since endurance runners have greater levels of slow (type I) fibers,[14,15] it is reasonable to assume that slow (type I) fibers are predominantly recruited and damaged during a MUM. However, the hypothesis that a MUM also induces fast (type II) -fiber damage cannot be completely ruled out because CK is non–fiber-type-specific. Moreover, baseline FM serum levels were over the previously described normal range,[11] a phenomenon that can be attributed to the type of training (higher intensity and less volume) performed by the runners the week before the race, but this is only speculation. Future studies in this area should carefully analyze the training strategies used the week before the competition.

Due to its mainly sarcoplasmic location, CK is thought to indicate increased membrane permeability after membrane disruptions at early time points[24] and the peroxidation of membrane lipids caused by an increase in reactive oxygen species and the activation of ion (Na^+ and Ca^{2+}) channels for several days after exercise.[25] The peak value for CK serum was found 1 hour after the MUM. This could be explained by the long duration of the competition, causing a significant degree of membrane damage during the run.[26] It has been demonstrated that the resealing of artificially produced membrane disruptions occurs in less than a minute,[27] but CK efflux may occur during the run due to a continuous process of membrane disruption followed by rapid resealing. The CK serum peak post-MUM and its recovery kinetics over the following 2 days were consistent with previous studies.[6] Nevertheless, the average 1-hour post-MUM CK serum peak was lower than that found after longer distance races.[28] In this respect, and according to Waskiewicz et al,[28] since the volume of exercise increases the metabolic demands for intracellular Ca^{2+},[26] and it is associated with muscle-damage indices, it seems reasonable to assume that augmented membrane permeability is related to the distance covered, as can be seen in Table 1. While participants 2 and 3, who completed 26 km, showed mild increases in serum CK activity post-MUM, participant 7, the only one who completed the whole MUM (~85 km), presented the greatest increases in CK serum activity after the competition. In contrast, exercise intensity, expressed as average speed (km/h) at which the participants ran the MUM, showed no trend, since participants left the competition at different points along the route (see Table 1). Further research with a larger sample is therefore needed in this area to clarify the relationship between biochemical markers of muscle damage and both distance (km) and average speed (km/h).

Myosin isoforms have a different serum time course than CK.[11] Sarcomere-protein turnover is longer than that of sarcoplasm proteins,[29] so the SM serum peak 1 day after the MUM can be explained by the increased activity of calpain 2 days after exercise.[30] Calpain is a Ca^{+2}-dependent protease. The long duration of the

MUM may have led to large increases in intracellular Ca^{+2} during the run, which would accelerate calpain degradation and lead to the significant increases in SM serum 1 day after the MUM. Calpain removes myosin from the filamentous structure of the sarcomere.[31] At this time, increased membrane permeability due to the activation of stretch-activated ion channels[25] could lead to a release of large proteins into the interstitium. Once in the interstitial space, proteins are mainly transported via the lymphatic system into the bloodstream, because the capillary membranes in skeletal muscle are almost impermeable to proteins.[7,32] The muscle-fiber compartment in which SM is located and its complex degradation process could explain the delayed increases in serum of SM.

Finally, CK and SM serum peaks occurred with 1-day difference but were strongly correlated. Membrane damage could accompany sarcomere disruptions, due to the tight connection between myofibrils, cytoskeleton, and membranes.[33] Therefore, it seems that membrane damage could be related to subsequent fiber sarcomere disruption of slow fibers.

Practical Implications

The current study shows that SM could provide indirect information about fiber-type-specific sarcomere damage 1 day after a MUM, and since the serum peak of myosin isoforms is not reached until 1 day after a MUM, they could be used in diagnoses that are not made immediately after the competition. Although fiber specificity cannot be determined by CK, its serum activity 1 hour after a MUM seems to be related to the subsequent SM serum response. MUM trainers and runners should be aware that the total distance covered could be related to muscle damage, and sharp SM serum increases suggest that a longer recovery may be needed. Further research regarding MUM distance covered, training and performance variables, and damage degree inflicted to skeletal-muscle slow (type I) fibers is needed.

Conclusions

In summary, since there is evidence of muscle damage after prolonged mountain running, an increase in SM serum concentration after a MUM could be indirect evidence of selective slow (type I) -fiber-specific sarcomere disruptions.

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4.5 Study V

Skeletal muscle sarcomere damage threshold is related to distance in Mountain Trail competitions

1 Cover page

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Title page

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Abstract

The aim of this study was to compare changes after a 35-km mountain trail race (MTR) and a 55-km mountain ultra-marathon (MUM) in serum activity or concentration of muscle damage biomarkers including creatine kinase (CK), creatine kinase MB isoform (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), cardiac troponin I (cTnI) and fast and slow myosin isoforms (SM and FM respectively).

One group (n=10) of amateur trained men who ran a 35-km MTR and another group (n=6) of highly trained men who ran a 55-km MUM volunteered for the study. Changes in the serum levels of muscle damage biomarkers were assessed before, 1 hour after competitions, and 24 and 48 hours after the beginning of the races by repeated measures analysis of covariance (ANCOVA), including training hours per week as a covariate.

The results showed that distance determines SM release, which signifies sarcomere damage of slow fibres following a 55-km MUM, even when the race was undertaken by highly trained athletes. The first changes in SM serum obtained at 24 hours were correlated with CK, CK-MB and AST activities or concentrations registered 1 hour after the competitions. These results indicate that mountain running distance is related to deeper slow (type I) muscle fibre damage, even in highly trained individuals.

Introduction

Although physiological responses to mountain trail running competitions has become an important topic in sports science research in recent years, very little is known about the structural damage to the muscle fibres of athletes who participate in these strenuous events. While there is no consensus about the definition of these competitions (Millet, 2011), the term ‘mountain trail race’ (MTR) has been generically used to refer to competitive long-distance runs from 15 to > 90 km, which are performed in a mountain context and involve a great cumulative elevation gain (uphill and downhill) (Easthope et al., 2010), and the term ‘mountain ultra-marathon’ (MUM) has been specifically associated with MTR over a longer distance than the standard marathon (> 42.195 km) (Carmona et al., 2015; Saugy et al., 2013).

There is general agreement that mountain trail running leads to muscle damage because of strenuous competitive conditions, which cause fatigue, and the numerous eccentric contractions completed during the downhill phases (Millet et al., 2011). However, concerning muscle damage, an important question remains unanswered: is there a muscle damage threshold related to distance? During flat running, it has been well-established that the total running distance covered or the time spent running is related to the extent of muscle damage (Overgaard et al., 2004; Waskiewicz et al., 2012). Specifically, during ultra-endurance running, it has been stated that increased serum levels of muscle enzymes indicate that active muscle suffers a significant degree of sarcolemmal damage with increased running distance (Overgaard, Lindstrom, Ingemann-Hansen, & Clausen, 2002).

Biochemical assessment of muscle damage has also been used in mountain trail competitions, but the relationship between distance and muscle enzyme efflux is less clear (Saugy et al., 2013). This could be because of the high complexity of mountain trail competition characteristics (total elevation gain), environmental factors (weather and surface conditions), and the pacing strategy used by runners, especially during downhill running when the eccentric component is exacerbated (Eston, Mickleborough, & Baltzopoulos, 1995). Besides, muscle enzymes are not fibre-type-specific (Friden & Lieber, 2001) and, due to their mainly sarcoplasmic location, their appearance in blood can only suggest a loss of sarcolemma integrity (Noakes, 1987). To overcome these limitations, the use of serum levels of fast and slow (FM and SM respectively) myosin isoforms as indirect biomarkers of fibre-type-specific sarcomere damage, have been used previously (Guerrero et al., 2008; Carmona et al., 2014). Concretely, significant increases in SM, suggesting sarcomere disruptions of slow (type I) fibres, were found in MUM participants who completed different total distances of the whole trail (Carmona et al., 2015).

The aim of the present study was to investigate the influence of mountain running distance on structural muscle damage by comparing the time course response of muscle enzymes and sarcomere fibre-type-specific proteins in two groups of mountain runners performing a 35-km MTR or a 55-km MUM.

Methods

Participants

Ten men who ran a 35-km MTR [mean ± standard deviation] (age 37.7 ± 7.4 years, weight 73.9 ± 9.4 kg, height 177.6 ± 3.4 cm), and six men who ran a 55-km MUM (age 34.0 ± 5.2 years, weight 71.3 ± 8.8 kg, height 176.2 ± 7.3 cm) volunteered for the study. The group that ran the 35-km MTR was composed of amateur, trained runners (7.0 ± 1.1 hours per week) with at least 3 years of mountain training experience. The other group, which ran the 55-km MUM, was composed of amateur and/or sponsored highly trained runners (12.6 ± 3.0 hours per week), with at least 5 years of mountain training experience. All participants were healthy and had not suffered any muscle and/or tendon injury 6 months before the study. Physical activity after the competitions was limited, and massages were prohibited. All participants were asked about the type of training performed the week before the race to better understand the baseline values of serum enzymes and fibre-type-specific proteins. The study conformed to the Declaration of Helsinki for medical research and participants provided written informed consent. The study was previously approved by the Ethics Committee of the Catalan Sports Council (0099S/690/2013).

Mountain trail race and mountain ultra-marathon

Both competitions, 35-km MTR and 55-km MUM, were included in the *Volta a la Cerdanya Ultrafons*® mountain endurance competitions in 2013, and had similar total elevation gains of 2089 and 2259 m respectively. Average

negative slopes were 8.9% for the 35-km MTR and 8.0% for the 55-km MUM (see the mountain trail profiles in Figure 1). Environmental conditions for the 35-km MTR were 8.9ºC and 92% relative humidity (RH) at the start (9:00) of the race and 13.9ºC and 59% RH at the end of the race (between 13:00 and 15:00). Environmental conditions for the 55-km MUM were 10.4ºC and 79% RH at the start of the race, and 10.8ºC and 83% RH at the end of the race (between 15:00 and 17:00).

****Figure 1 near here****

Blood sampling

Blood samples of 5 mL were drawn from an antecubital vein by standard venipuncture before the competition (pre), 1 hour after (post), and 24 and 48 hours after the beginning of the race(s). Samples were allowed to clot for 30 min and then centrifuged at 3000 x *g* for 10 min. Three aliquots of serum were obtained and stored at -80ºC until they were analysed for enzymatic activity or concentration, cardiac troponin I concentration, and myosin isoform concentration.

Biochemical assays

Automatized analyses of creatine kinase (CK), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were performed in an Advia 2400 (Siemens Medical Solutions Diagnostics, Tarrytown, NY, USA). Creatine kinase MB isoform (CK-MB) and cardiac troponin I (cTnI) analyses were performed using a Dimension Clinical Chemistry System (Siemens Healthcare Diagnostics, Tarrytown, NY, USA), with an analytical measurement range of

0.5–300 ng·mL^{-1}, and 0.017–40 ng·mL^{-1}, respectively. The concentration of myosin isoforms, FM and SM, was measured using the enzyme-linked immunosorbent assay (ELISA sandwich). Briefly, two plates (Corning 96-well EIA/RIA, Sigma Aldrich, Poole, UK) were coated overnight at 4ºC with capture monoclonal antibodies (all Sigma Aldrich, Poole, UK), anti-myosin (skeletal, fast) clone My-32 and anti-myosin (skeletal, slow) clone NOQ7.5.4D, for FM and SM assessment respectively. The plates were then washed 3 times (phosphate buffered saline, pH 7.4, 10 mM) and blocked with block buffer (Super Blocking Buffer, Thermo Fisher Scientific Inc., Rockford, Illinois, USA) before being incubated (60 min at 37ºC). After a wash step, samples (10 µL) were added by triplicate, and a calibration curve of 6-point serial dilution, from 0 to 250 ng, of commercial pure myosin of porcine muscle M0273 was obtained. To complete the ELISA, anti-myosin polyclonal antibody M7523 was used as the primary antibody, and mouse ant-IGG linked to peroxidase A6154 as the secondary antibody. Finally, myosin concentrations (µg·L^{-1}) were obtained by the interpolation of the calibration curve (r^2 > 0.95). Intra-assay coefficients of variation were 10.0% and 5.5% for FM and SM respectively. The linearity of the FM assay was 80% and 90% for SM.

Statistical analysis

Data were tested for approximation to a normal distribution using the Shapiro-Wilk test. Not normally distributed data, such as CK, CK-MB, AST and cTnI, were log- or sqrt-transformed before analysis. Repeated measures analysis of covariance (ANCOVA), with distance groups (35-km MTR and 55-km MUM) as an independent variable, was used to identify the effect of time (pre, post,

24 and 48 hours following the competition) on serum biomarkers of muscle damage (CK, CK-MB, AST, ALT, cTnI, FM and SM). Training hours per week was included as a covariate, because the training status of the runners could have a pronounced effect on time-related serum changes of muscle biochemical markers. When significant effects were found, a post-hoc test was performed by applying a paired t test with a Bonferroni correction for the time per distance effect. Spearman's rank order correlation analysis was used to assess relationships between variables of interest. The differences between groups were analysed by using the unpaired t test or Mann-Whitney test (depending on the variable distribution). Data are presented as mean ± standard error of the mean (SEM), unless otherwise stated. The level of significance was set at $P < 0.05$. The statistical analysis was conducted using SPSS version 20.0 (SPSS Statistics, IBM corp., Armonk, NY, USA) statistical analysis software.

Results

Training and performance

The training status (training hours per week) of the 55-km MUM group was significantly 80% (P = 0.04) higher than in the 35-km MTR group. No differences were seen in the years of mountain running training experience. As expected, the official running time was significantly (P = 0.04) higher for the group who ran the 55-km MUM, but no differences were found in the average running velocity ($km \cdot h^{-1}$) (Table 1).

****Table 1 near here****

Serum muscle enzymes activity

Serum CK activity of the 35-km MTR group showed significant increases at every time point analysed after the trail, until a peak of 832 ± 267 $U \cdot L^{-1}$ (P = 0.003) 48 hours after the beginning of the MTR. However, although eight of the 35-km MTR participants' peak CK activity was obtained at 24 hours, participants 1 and 6 showed high peak values at 48 hours (Table 2). Serum CK activity of the 55-km MUM group increased significantly and peaked (1238 ± 201 $U \cdot L^{-1}$; P < 0.001) at 24 hours, returning to non-significantly different values from the baseline 48 hours after the beginning of the MUM. The comparison between groups showed that CK activity was significantly 164% (P < 0.001) higher 1 hour after finishing the competition for the group who ran 55-km MUM. No other differences were found between the groups (Figure 2). There were no differences between groups in pooled CK peak activity, independently of the time to peak (Table 2).

Serum concentration of CK-MB was significantly elevated in both groups following the race until its peak at 24 hours (6.15 ± 1.2 $ng \cdot mL^{-1}$; $P < 0.001$, and 15.2 ± 2.1 $ng \cdot mL^{-1}$; $P < 0.001$, for the 35-km MTR and 55-km MUM groups respectively) and returning to non-significantly different values from the baseline 48 hours after the beginning of the competitions. The comparison between groups showed that CK-MB concentration was significantly 251% ($P < 0.001$) and 147% ($P = 0.003$) higher 1 hour after and 24 hours after the beginning of the competition for the group who ran 55-km ($P = 0.003$) (Figure 2). Moreover, the magnitude of the CK-MB peak concentration, independently of its time course, was significantly different between groups ($P < 0.001$) (Table 2).

Serum AST levels were significantly increased in both groups at every time point analysed following the competition. Serum peaks were at 24 hours (49 ± 4 $U \cdot L^{-1}$, $P < 0.001$ and 63 ± 7 $U \cdot L^{-1}$, $P < 0.001$ for the 35-km MTR and 55-km MUM groups respectively) and no differences between groups were detected. No significant time-related changes and no differences between groups were seen in ALT serum activity (Figure 2; Table 2).

****Figure 2 near here****

Serum muscle myosin isoforms concentration

While the serum SM concentration of the 35-km MTR group showed no changes from baseline values, the SM serum concentration of the 55-km MUM group was significantly increased at every time point analysed following the competition, peaking at 48 hours (4766 ± 916 $\mu g \cdot L^{-1}$; $P < 0.001$).

Accordingly, the comparison between groups showed significant higher time-related and time-independent peak values of serum SM for the group that ran the 55-km MUM (Figure 3; Table 2). Furthermore, in both groups, the first significant increases in enzyme activity (CK and AST), registered 1 hour after the competition, were significantly correlated with the first SM serum significant increase obtained at 24 hours after the beginning of the trail (Figure 5). Time-independent serum peaks of SM and CK-MB were also correlated ($r = 0.76$; $P < 0.001$). No significant changes from baseline values were seen in average FM serum concentration over time in any of the groups. However, participants 2 and 3 from the 35-km MTR, and 14 and 16 from the 55-km MUM, showed high relative serum peaks at different time points (Table 2). Finally, the 55-km MUM group had significantly higher FM serum concentrations throughout the experimental period, even at the baseline (Figure 3).

****Figure 3 near here****

Serum cardiac troponin I

While no significant changes were seen in the cTnI serum concentration for the 35-km MTR group at any time point analysed, individual peak values of cTnI showed increases over the clinical normality range (0.050 $ng \cdot mL^{-1}$) for seven of the 35-km MTR group (Table 2). A significant cTnI peak of 0.143 ± 0.07 $ng \cdot mL^{-1}$ ($P = 0.03$) was found in the group that ran the 55-km MUM 1 hour after finishing the race, returning to baseline values at 24 hours after the beginning of the MUM. A comparison between the groups showed no

significant time-related or time-independent peak differences between the cTnI serum concentrations (Figure 4; Table 2).

****Figure 4 near here****

****Figure 5 near here****

****Table 2 near here****

Discussion

To the best of our knowledge, this is the first study that analyses the effect of mountain running distance on structural muscle damage by comparing the muscle biochemical response to two mountain running events of different lengths (35-km MTR versus 55-km MUM). Furthermore, because different training levels were found between the groups of competitors, the influence of training status on biochemical indices of muscle damage was also analysed. The main finding of the present study was that (I) distance was the main determiner of SM release, signifying sarcomere damage of slow fibres following a MUM, even when the race was undertaken by highly trained athletes. Other relevant findings were that (II) excepting CK-MB, only minor differences in enzymatic activities after the competitions were found between groups, (III) the first changes in SM serum obtained at 24 hours were correlated with CK, CK-MB and AST activities or concentrations registered 1 hour after the competitions, and (IV) cTnI was only significantly elevated in serum following the 55-km MUM.

The release of sarcomere proteins, such as myosin, from muscle fibre to the bloodstream has been reported previously as indirect evidence of sarcomere damage (Guerrero et al., 2008; Carmona et al., 2014). In the present study, the 55-km MUM induced elevations of SM in serum that suggest the presence of slow (type I) fibre sarcomere damage. In contrast, the fibre damage inflicted by the 35-km MTR was mainly located at membrane level, because only muscle enzyme efflux to the bloodstream was observed, which indicates increased sarcolemmal permeability (for a review see (Hyldahl & Hubal, 2013).

No significant increases in sarcomere fibre-type-specific proteins were found in the 35-km MTR group. Notably, although significant differences were found in the training status between groups (significantly, training was 80% higher in the group that ran the 55-km MTR), in this case, no muscle fibre sarcomere protective effect seems to be provided by training status. It has been proved in humans during flat running that endurance training status only has a minor influence on muscle damage indices and, especially, on Ca^{2+} accumulation in the human vastus lateralis after running 20 km (Overgaard et al., 2004). Progressive intercellular Ca^{2+} accumulation is important in mediating two forms of muscle damage: increased sarcolemmal permeability (Mikkelsen, Fredsted, Gissel, & Clausen, 2004), and degradation of sarcomere proteins due to the activation of calcium-dependent proteases (Goll, Thompson, Li, Wei, & Cong, 2003). Therefore, as accumulation of Ca^{2+} is dependent on the time of exposure to exercise (Overgaard et al., 2004; Overgaard et al., 2002), it seems reasonable to assume that the greater the distance, and consequently duration, of a mountain trail competition, the greater the muscle damage. Moreover, both competitions, 35-km MTR and 55-km MUM, had similar average negative slopes and no significant differences were seen between the years of training experience, and, especially, between the average velocities in which both groups ran the mountain races. Therefore, it can be concluded that although other factors that were not measured during the races, such as pacing strategy, may substantially influence the degree of muscle damage, in the present study the distance, and consequently the duration of the exercise (86% higher for the 55-km MUM group) were the main factors inducing slow (type I) fibre sarcomere damage.

Regarding enzymatic serum activities obtained after the competitions, significant increases were found and similar time courses were observed in both groups. With the exception of CK-MB, which will be discussed later, only minor significant time-related differences in CK and AST serum activities were found between groups 1 hour after finishing the races. Moreover, no differences between groups were found in serum CK and AST activities obtained from 24 to 48 hours after the beginning of the races, because the activities of enzymes from the 55-km MUM group tended to stabilize in blood at 48 hours. It has been stated that enzyme clearance time depends, among other factors, on athlete's training status (Brancaccio, Lippi, & Maffulli, 2010) and since the 55-km MUM participants were highly trained, it cannot be ruled out that they presented enhanced clearance rates.

Another remarkable finding is that CK, CK-MB and AST serum activities from all participants 1 hour after both the 35-km MTR and the 55-km MUM were correlated to the serum concentration of SM registered 24 hours after the beginning of the competitions. Although fast (type II) and slow (type I) fibres present similar CK activity (Apple & Tesch, 1989), competitive distance runners tend to have greater levels of slow fibres (Harber & Trappe, 2008; Secher, Mizuno, & Saltin, 1984), and it is reasonable to assume that slow fibres are predominantly recruited and damaged during a mountain race. Therefore, CK serum activity could be related to slow fibres contractile activity. The correlation between SM and both CK-MB and AST could be explained by the fact that slow (type I) fibres have higher activity of those enzymes (Schantz & Henriksson, 1987; Yamashita & Yoshioka, 1991). Time-

independent serum peaks of CK-MB and SM were also correlated, which indicates a close relationship between the magnitudes of appearance of these biochemical markers. This could be related to the fact that muscle-type creatine kinase subunit (M-CK) interacts with sarcomere structure (Hornemann et al., 2003). Moreover, CK-MB was the only enzyme whose concentration was significantly higher for the group that ran the 55-km MUM until 24 hours after the beginning of the race. When it is not possible to analyse the presence of fibre-type-specific sarcomere proteins in serum, and myocardial damage is ruled out, sharp increases in CK-MB serum concentration could be more precisely related to slow-fibre damage.

Prolonged endurance exercise has been related to cTnI release in healthy individuals (Mingels et al., 2009). In the present study, only the group that ran the 55-km MUM showed significant increases in cTnI, which suggests that run length, and consequently exercise duration, could be closely related to cTnI release. However, average values of the 55-km MUM group are largely biased, since participant 14 presented 0.505 $ng \cdot mL^{-1}$ following the race (see Table 2) and there were no differences in cTnI peaks between groups. The mechanisms of prolonged exercise-induced cardiac troponin release are not yet fully understood, but some hypotheses have been developed (Clarke, Caldwell, Chiao, Miyake, & McNeil, 1995; Koller & Schobersberger, 2009). However, it seems likely that prolonged exercise-induced cardiac troponin release is a benign process (Shave et al., 2010), especially when its serum levels return to baseline within 24–48 h (Mehta et al., 2012), as in the case of the present study.

Finally, although fast (type II) fibre damage cannot be ruled out, especially because some individuals (participants 2 and 3 from the 35-km MTR, and 14 and 16 from the 55-km MUM) showed large FM increases following the competitions, no significant time-related changes were seen in average serum FM concentration. It is also remarkable that the group that ran the 55-km MUM showed higher FM values than the 35-km MTR group at every time-point analysed, even at baseline. The training strategies used several days before the competition by the 55-km MUM participants, based on a drastic reduction of running km per week and allowing for an increase in running intensity (i.e. fast running series), could induce damage to fast fibres. As previously found, when muscle force-generating capacity is fully recovered, persistent high serum levels of FM could indicate sarcomere remodelling rather than damage (Carmona et al., 2014). This is in accordance with previous findings indicating that myofibrillar disruptions that appear shortly after exercise signify damage, and the changes in myofibrillar structures observed some days into recovery may represent remodelling (Yu, Carlsson, & Thornell, 2004). Therefore, the high FM baseline values of the 55-km participants may represent a remodelling process induced by a previous increase in training intensity several days before the competition.

Conclusions

In summary, although the reproducibility of this kind of field studies is low because of the complex characteristics of mountain trail competitions (i.e. total elevation gain), and important performance variables (e.g. pacing strategy) were not analysed, to the best of our knowledge, this is the first

study to indicate that mountain running distance is related to biochemical indices of muscle damage. Physicians, trainers and runners should be conscious that competing in mountain running events longer than the standard marathon distance (> 42.195 km) is related to deeper slow (type I) muscle fibre damage, even in highly trained individuals.

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S0735-1097(10)01705-5 [pii]

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TABLES

Table 1

Variables	Distance group	
	35-km MTR	55-km MUM
Training		
Years of mountain running training	6.1 ± 1.3	8.1 ± 1.2
Training hours ($h \cdot wk^{-1}$)	7.0 ± 1.1	12.6 ± 3.0*
Performance		
Official running time (h:min:sec)	3:50:50 ± 0:08:38	6:52:38 ± 0:34:22*
Average running velocity ($km \cdot h^{-1}$)	9.22 ± 0.33	8.29 ± 0.69

Training and performance characteristics of participants by distance group. Values are means ± SEM. * Significantly different from the 35-km MTR group value at $P < 0.05$.

Table 2

	Participant	Peak CK Activity ($IU \cdot L^{-1}$) [time of peak][a]	Peak CK-MB Concentration ($ng \cdot mL^{-1}$) [time of peak][a]	Peak AST Activity ($IU \cdot L^{-1}$) [time of peak][a]	Peak cTnI Concentration ($ng \cdot mL^{-1}$) [time of peak][b]	Peak SM Concentration (% Change from Baseline) [time of peak][c]	Peak FM Concentration (% Change from Baseline) [time of peak][c]
35-km MTR	1	2755[48 h]	5.3[24 h]	90[48 h]	0.037[Post]	0	7[Post]
	2	411[24 h]	3.9[Post]	35[24 h]	0.192[Post]	15[Post]	47[24 h]
	3	588[24 h]	2.8[24 h]	40[24 h]	0.070[Post]	17[Post]	73[Post]
	4	588[24 h]	6.4[24 h]	48[24 h]	0.067[Post]	6[48 h]	16[24 h]
	5	900[24 h]	11.4[24 h]	66[24 h]	0.107[Post]	4[48 h]	19[Post]
	6	1855[48 h]	9.1[24 h]	64[48 h]	0.096[Post]	20[24 h]	0
	7	702[24 h]	9.3[24 h]	50[24 h]	0.089[Post]	30[48 h]	9[24 h]
	8	1599[24 h]	5.9[24 h]	64[24 h]	0.028[Post]	31[48 h]	0
	9	322[24 h]	5.3[24 h]	43[Post]	0.037[Post]	1[48 h]	11[Post]
	10	388[24 h]	3.3[Post]	40[Post]	0.117[Post]	0	0
55-km MUM	11	1425[24 h]	13.2[24 h]	86[24 h]	0.113[Post]	28[24 h]	36[48 h]
	12	687[24 h]	21.3[24 h]	54[24 h]	0.038[Post]	80[48 h]	8[Post]
	13	1612[24 h]	17.2[24 h]	83[24 h]	0.098[Post]	109[24 h]	36[Post]
	14	1119[24 h]	18.9[24 h]	51[24 h]	0.505[Post]	156[24 h]	69[48 h]
	15	1889[24 h]	19.4[Post]	62[24 h]	0.089[Post]	130[48 h]	34[Post]
	16	940[48 h]	10.9[Post]	41[Post]	0.017	75[24 h]	100[48 h]
35-km MTR (mean value)		1011	6.3	54	0.084	12	18
55-km MUM (mean value)		1279	16.8*	63	0.143	96*	47

Individual peak values [time of peak] for main variables. Time-independent mean values of each group are shown. Creatine kinase (CK), creatine kinase-myocardial band (CK-MB), aspartate aminotransferase (AST), cardiac troponin I (cTnI), slow myosin (SM), fast myosin (FM).

[a] See Figure 2

[b] See Figure 4

[c] See Figure 3

*, Significant difference between groups at $P < 0.001$

Figure captions

Figure 1. Altitude profile and the distance scale in km of the 35-km mountain trail race (35-km MTR) (a) and the 55-km mountain ultra-marathon (55-km MUM) (b).

Figure 2. Changes in enzyme activities of creatine kinase (CK) (a), creatine kinase MB isoform (CK-MB) (b), aspartate aminotransferase (AST) (c), and alanine aminotransferase (ALT) (d). Values are means ± SEM. ** and *** Significantly different from baseline at $P < 0.01$, and $P < 0.001$, respectively. $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$, Significant differences between groups at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Figure 3. Changes in serum concentration of slow myosin (SM) (a) and fast myosin (FM) (b). Values are means ± SEM. *** Significantly different from baseline at $P < 0.001$. $^{\#}$, $^{\#\#}$ and $^{\#\#\#}$, Significant differences between groups at $P < 0.05$, $P < 0.01$, and $P < 0.001$, respectively.

Figure 4. Changes in serum cardiac troponin I (cTnI) concentration. Clinical normality range (< 0.050 ng·mL^{-1}). Values are means ± SEM. *, Significantly different from baseline at $P < 0.05$.

Figure 5. Spearman rank correlation coefficients (r_s) between serum mean values of SM, slow myosin; CK, creatine kinase (a); CK-MB, creatine kinase MB isoform (b); and AST, aspartate aminotransferase (c). Note that serum values of CK, CK-MB and AST are from 1 hour after the trail and SM serum values are from 24 hours after the beginning of the competitions.

Figure 1

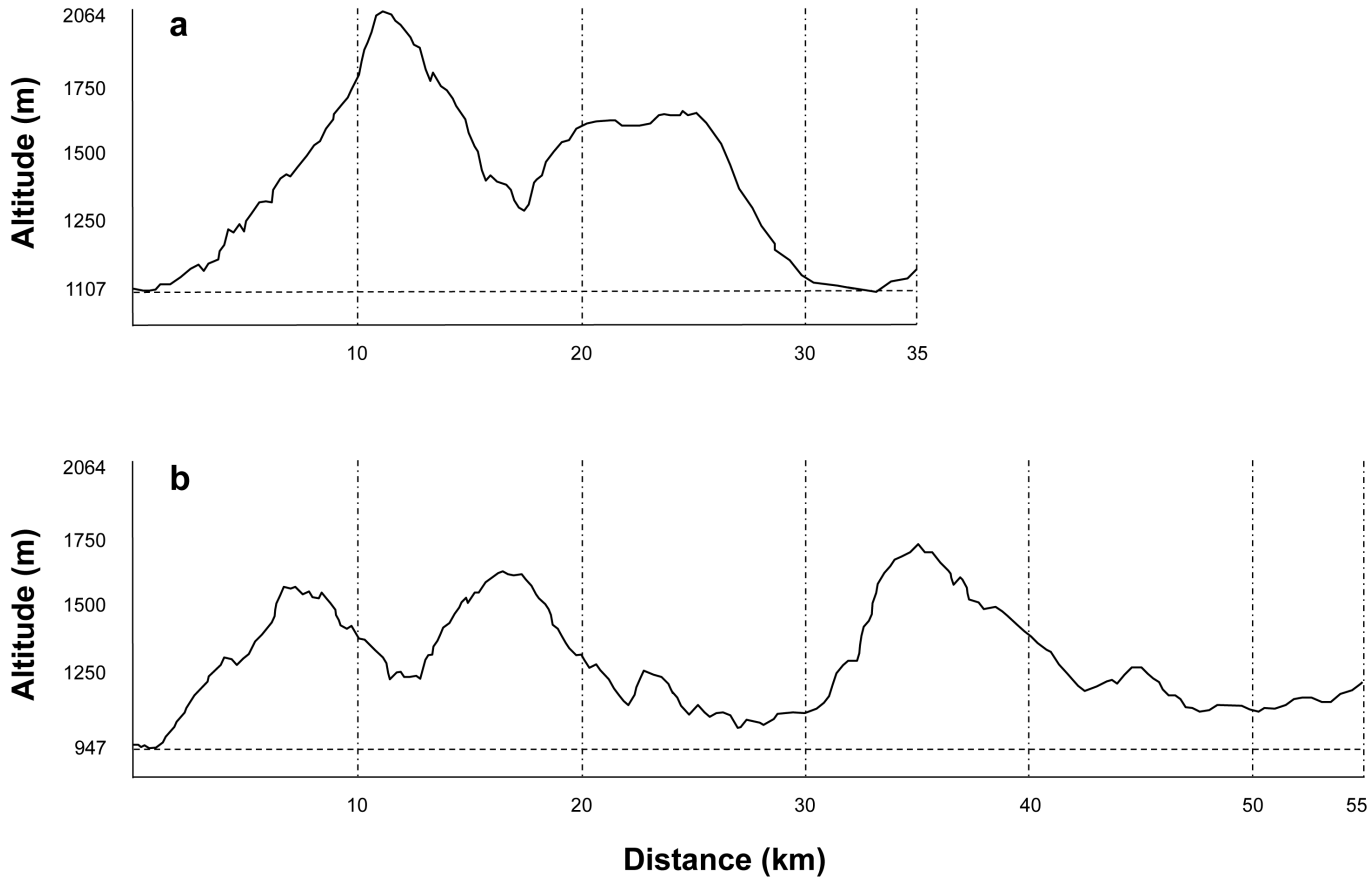

a
2064
1750
1500
1250
1107
Altitude (m)
10
20
30
35
b
2064
1750
1500
1250
947
Altitude (m)
10
20
30
40
50
55
Distance (km)

Figure 2

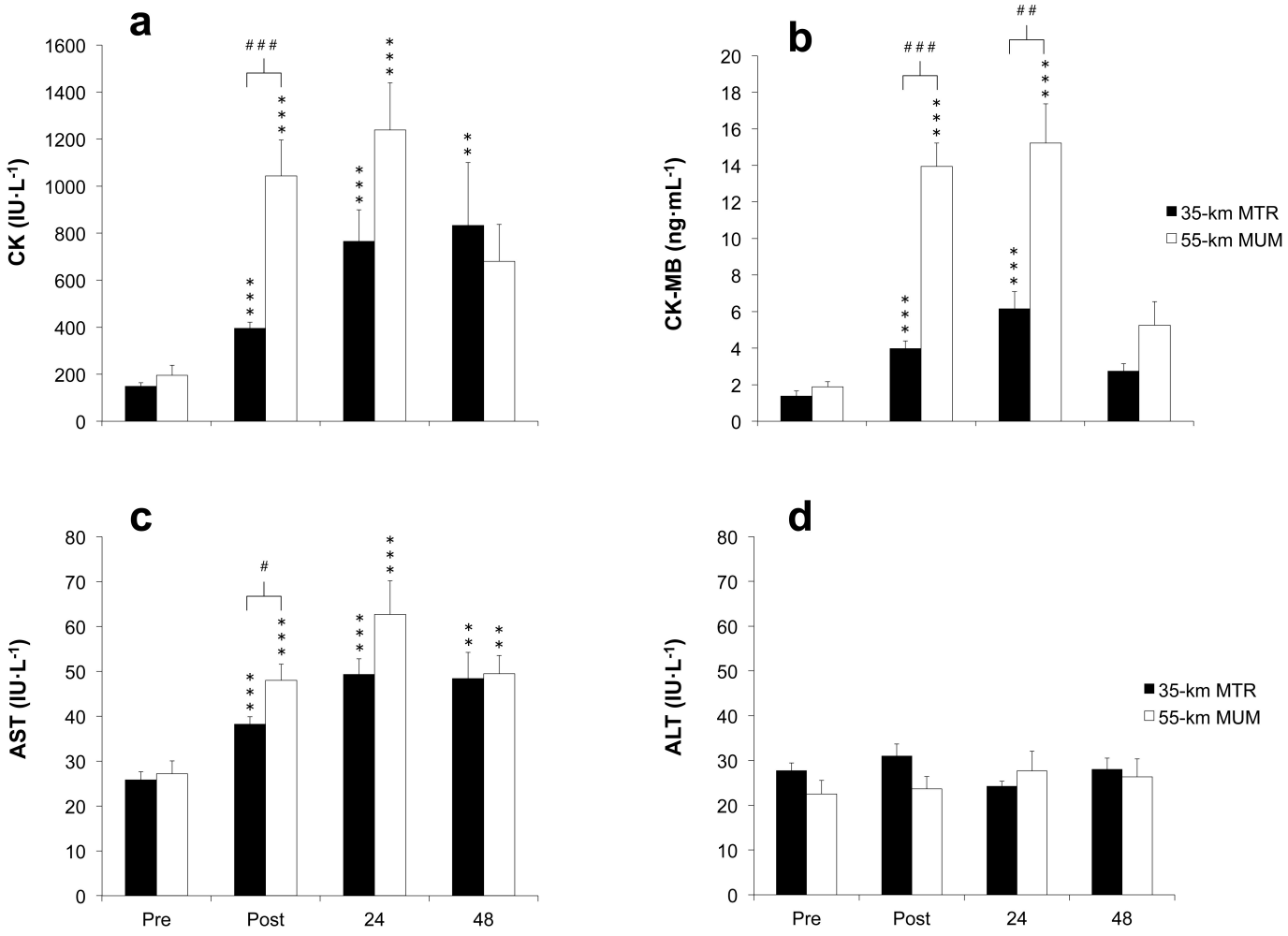

a
CK (IU·L-1)
1600
1400
1200
1000
800
600
400
200
0
###
***
***
***
***
**
b
CK-MB (ng·mL-1)
20
18
16
14
12
10
8
6
4
2
0
###
##
***
***
***
***
35-km MTR
55-km MUM
c
AST (IU·L-1)
80
70
60
50
40
30
20
10
0
#
***
***
***
***
**
**
Pre
Post
24
48
d
ALT (IU·L-1)
80
70
60
50
40
30
20
10
0
35-km MTR
55-km MUM
Pre
Post
24
48

Figure 3

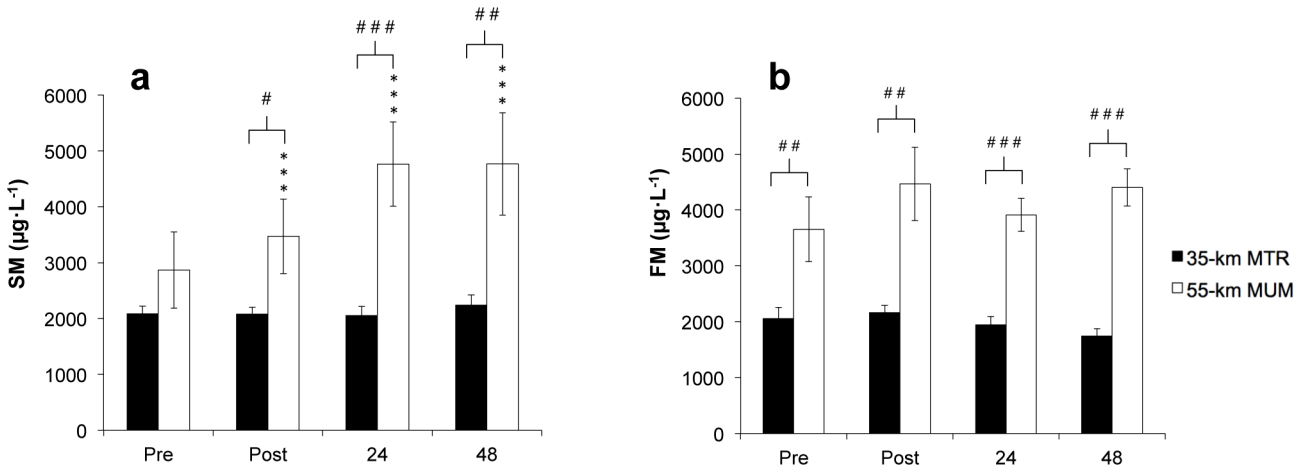

a
SM (µg·L-1)
6000
5000
4000
3000
2000
1000
0
Pre
Post
24
48
#
###
##
****
b
FM (µg·L-1)
35-km MTR
55-km MUM

Figure 4

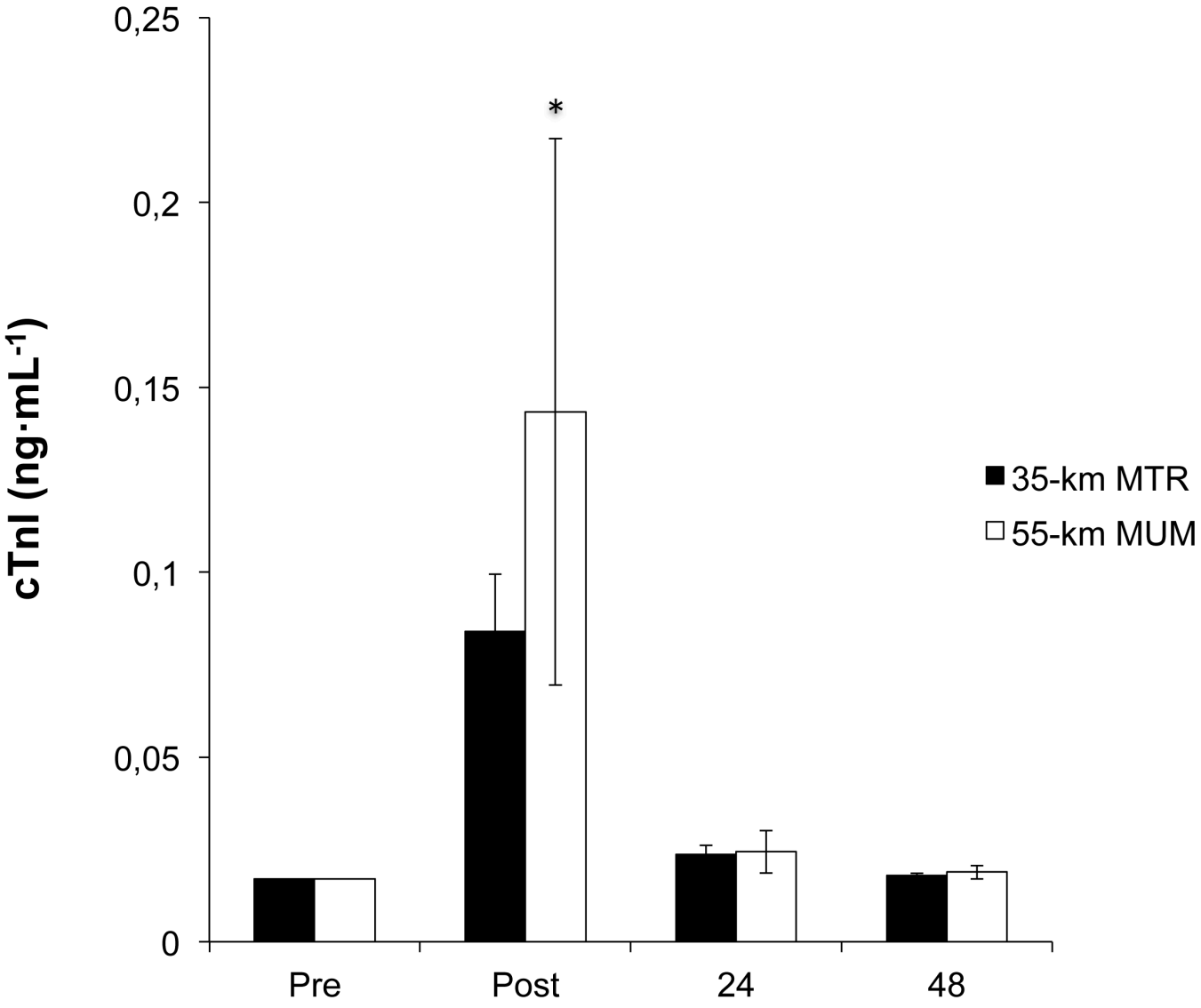
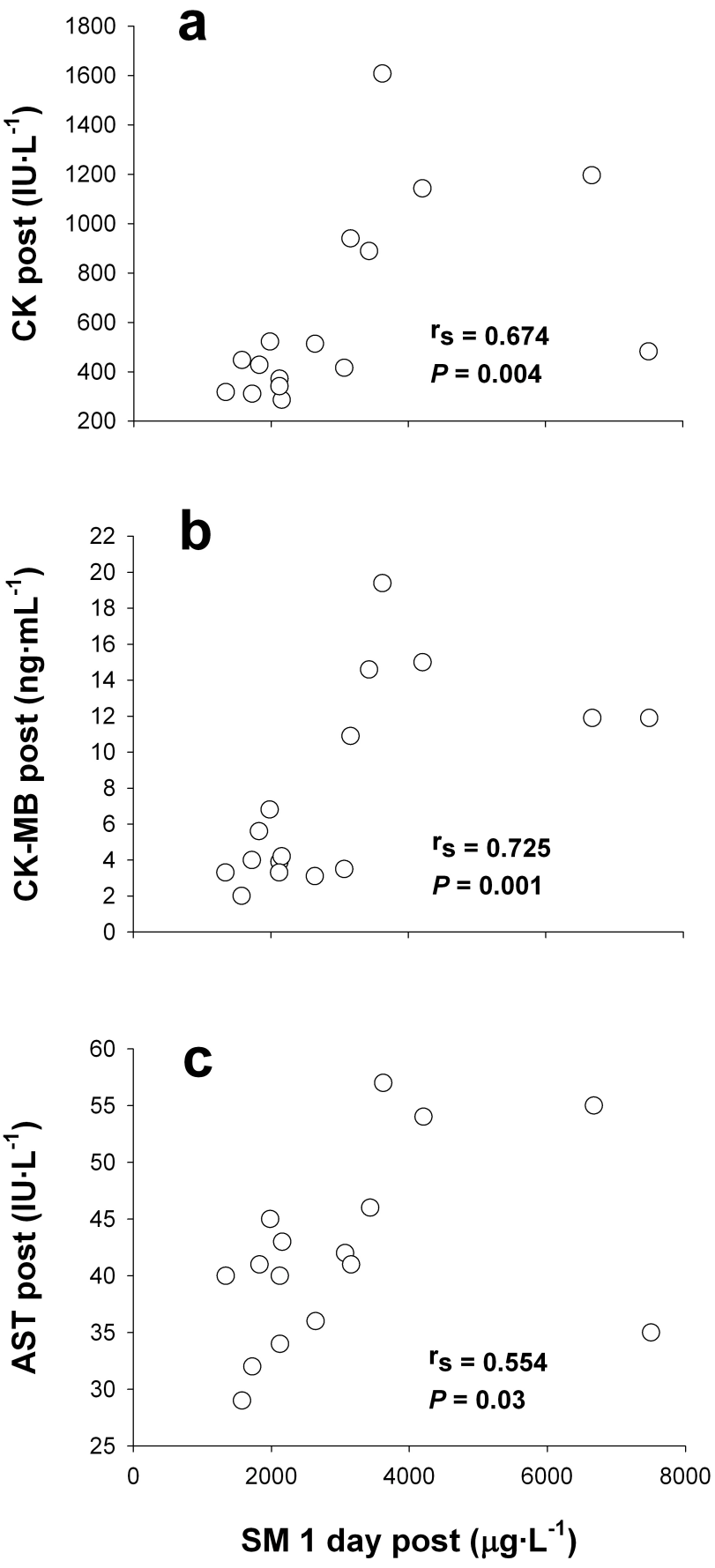


Figure 5


a
CK post (IU·L-1)
1800
1600
1400
1200
1000
800
600
400
200
rs = 0.674
P = 0.004
b
CK-MB post (ng·mL-1)
22
20
18
16
14
12
10
8
6
4
2
0
rs = 0.725
P = 0.001
c
AST post (IU·L-1)
60
55
50
45
40
35
30
25
rs = 0.554
P = 0.03
0
2000
4000
6000
8000
SM 1 day post (µg·L-1)

5. RESULTATS i DISCUSSIÓ

5.1 Cap a un model molecular indirecte de dany muscular induït per l'exercici (EIMD)

A partir dels resultats obtinguts als diversos estudis exposats en aquesta tesi (I, II, III, IV i V), es proposa establir un model molecular indirecte d'EIMD. El perfil temporal i la magnitud d'aparició en sèrum dels diversos enzims i proteïnes del múscul esquelètic després de l'execució d'un exercici que indueixi dany muscular sembla dependre no només de la tipologia (volum i intensitat) de l'exercici, sinó també del seu pes molecular i de la seva compartimentació estructural a la fibra muscular. A més, l'ús de les isoformes de la miosina permet no només ubicar el dany estructural (afectació del sarcòmer), sinó també discernir quina tipologia de fibra (ràpida [tipus II] o lenta [tipus I]) muscular s'ha vist afectada principalment per l'exercici (**Figura 14**).

5.1.1 Enzims

Tal com s'ha indicat al llarg d'aquesta tesi, està àmpliament acceptat per la comunitat científica que l'augment de l'activitat o de la concentració enzimàtica en sèrum després de l'exercici excèntric és un indicador de l'increment de la permeabilitat de la membrana de la fibra muscular (106, 160), ja sigui per disrupció (134) o per l'activació dels canals iònics (Ca^{2+}) amb increment dels nivells de les espècies reactives de l'oxigen (ROS) i el desencadenament consegüent dels processos de peroxidació lipídica del sarcolemma (4). No obstant això, fenòmens com ara grans pics d'activitat sèrica de creatina quinasa (CK) (> 10.000 $IU \cdot L^{-1}$) més enllà de les 48 hores posteriors a l'exercici (estudi III), increments per damunt del llindar de la normalitat clínica de la concentració en sèrum de l'isoenzim MB de la CK (CK-MB) (estudis IV i V) o augments significatius de la CK mitocondrial sarcomèrica (sMtCK) (estudis III, IV i V) mereixen una atenció especial, ja que són fets que poden ser indicatius d'un procés de dany muscular que vagi més enllà de l'increment de la permeabilitat de la membrana. Per tant, el perfil temporal i la magnitud d'aparició en sèrum dels diversos enzims analitzats permeten determinar el grau d'afectació muscular del tren inferior en funció de la tipologia d'exercici realitzat.

Creatina quinasa: de l'increment de la permeabilitat de la membrana a la necrosi de la fibra muscular

En dos dels estudis de laboratori basats en protocols d'HIE (estudis I i II), es van trobar increments —significatius en el cas de l'estudi I— de l'activitat sèrica de la CK al cap de 24 hores, la qual va tornar a valors de normalitat 48 hores després de l'exercici. L'única excepció va ser el subjecte control de l'estudi II, en el qual es va registrar el pic sèric d'activitat de la CK 48 hores després de l'exercici (**Figura 4**). D'altra banda, en els estudis de camp basats en protocols d'LDE (estudis IV i V), es van descriure increments significatius a partir d'una hora després de la finalització de l'exercici, els quals es van mantenir al cap de 24 hores. 48 hores després de l'inici de l'exercici, es va observar una clara tendència a la baixa en l'activitat en sèrum de la CK, amb l'excepció del grup de corredors moderadament entrenats que va córrer l'MTR de 35 km (estudi V) (**Figura 4**).

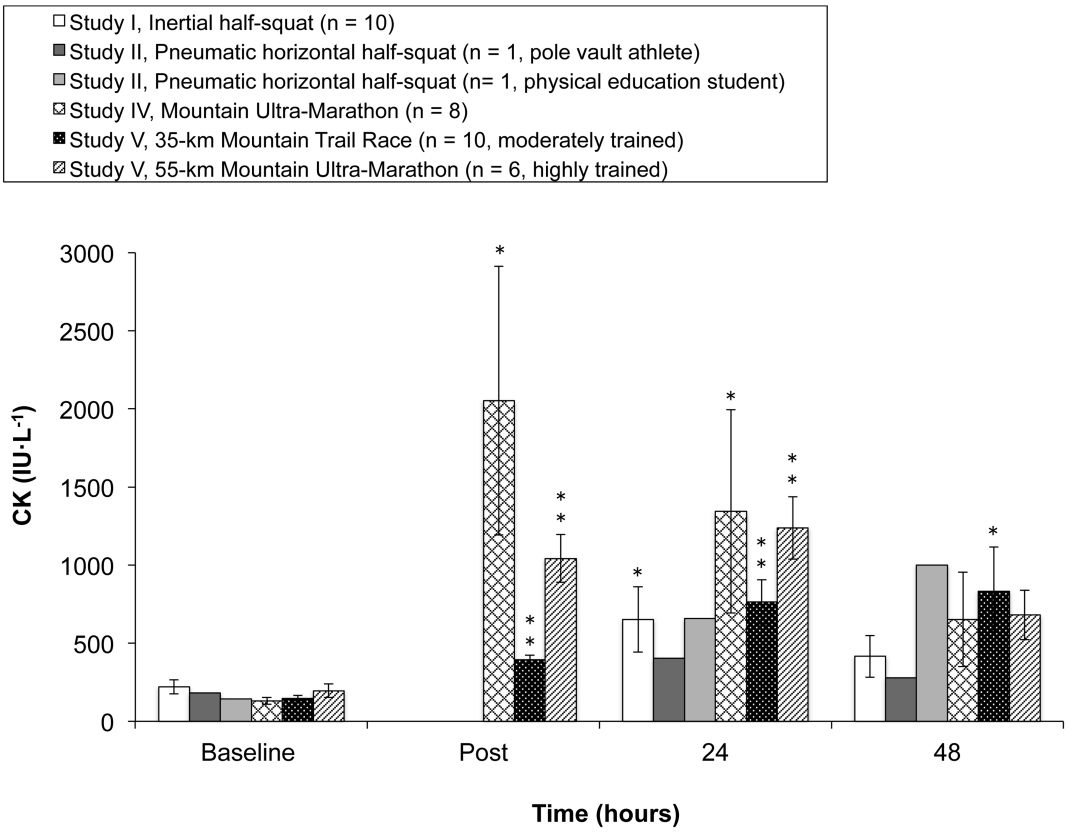


Figura 4. Mitjana ± error estàndard de la mitjana (SEM) de l'activitat de la creatina quinasa (CK) obtinguda en dos dels estudis de laboratori basats en protocols d'exercici d'alta intensitat (HIE) (estudis I i II) i dels estudis de camp basats en exercicis de llarga durada (LDE) (estudis III i IV). *, ** Significativament diferent respecte als valors basals a escala $P < 0,01$, i $P < 0,001$, respectivament.

Contràriament al que s'ha exposat fins ara, —increments moderats de l'activitat sèrica de la CK amb una marcada tendència a la baixa a partir de les 48 hores posteriors a l'exercici—, en l'estudi III, els subjectes classificats com a *high responders* van mostrar pics d'increment de l'activitat sèrica de la CK molt superiors als que es poden observar a la **Figura 4**, tant pel que fa a la seva magnitud (45.455 ± 11.721 $IU \cdot L^{-1}$) com al curs temporal fins al pic (72 hores després de l'exercici) (**Figura 5**).

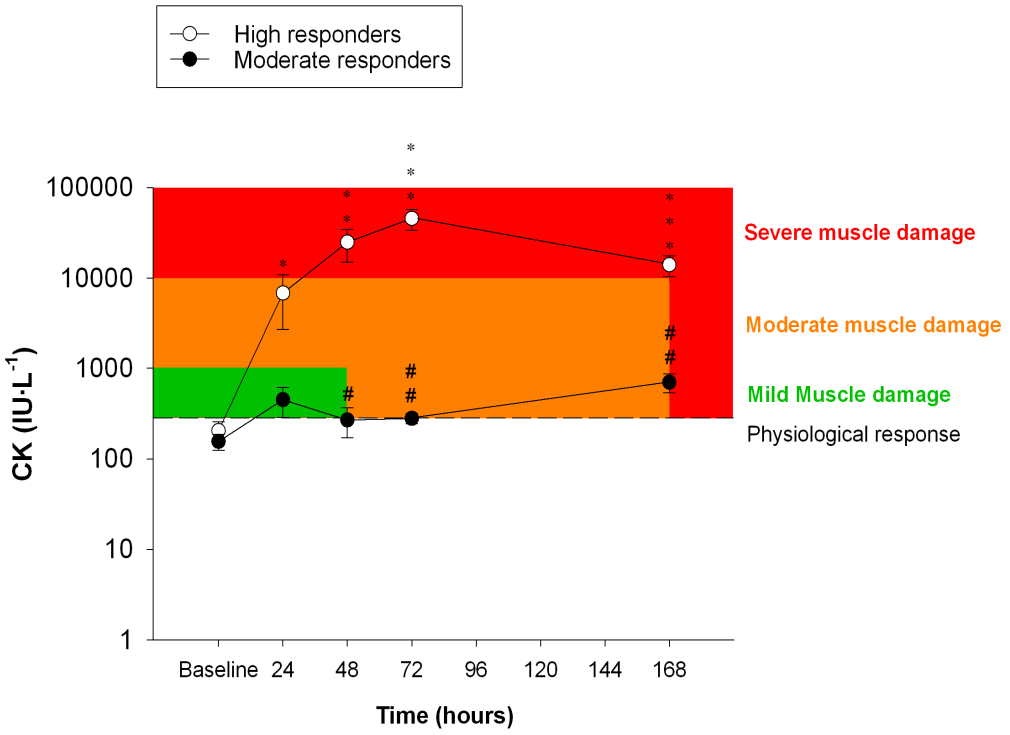


Figura 5. Mitjana ± error estàndard de la mitjana (SEM) de l'activitat de la creatina quinasa (CK) obtinguda en dos grups de subjectes (*high and moderate responders*) (estudi III). L'eix y es mostra en escala logarítmica. La línia discontínua marca la frontera entre la resposta fisiològica i el dany muscular lleu (*mild muscle damage*). *, ** Significativament diferent respecte als valors basals a escala P < 0,05, P < 0,01, i P < 0,001, respectivament. #, ## Diferències significatives entre grups a escala P < 0,05 i P < 0,01, respectivament. Adaptat de (32, 47, 57, 163).

Mentre que els pics sèrics de CK observats durant les primeres 24 hores després de l'exercici indiquen un increment de la permeabilitat de la membrana, és més probable que els pics produïts més enllà dels 4-5 dies posteriors a l'exercici es trobin relacionats amb un procés de necrosi en alguns segments de les fibres musculars (7, 162). Així, doncs, els nivells de CK observats a partir de les 48 hores i fins als set dies posteriors a l'exercici en la població de subjectes categoritzada com a *high*

responders a l'estudi III indiquen molt possiblement la necrosi d'alguns segments de les fibres musculars afectades per l'exercici excèntric. La CK, en certa mesura, reflecteix la quantitat de dany, especialment quan l'EIMD esdevé greu (120).

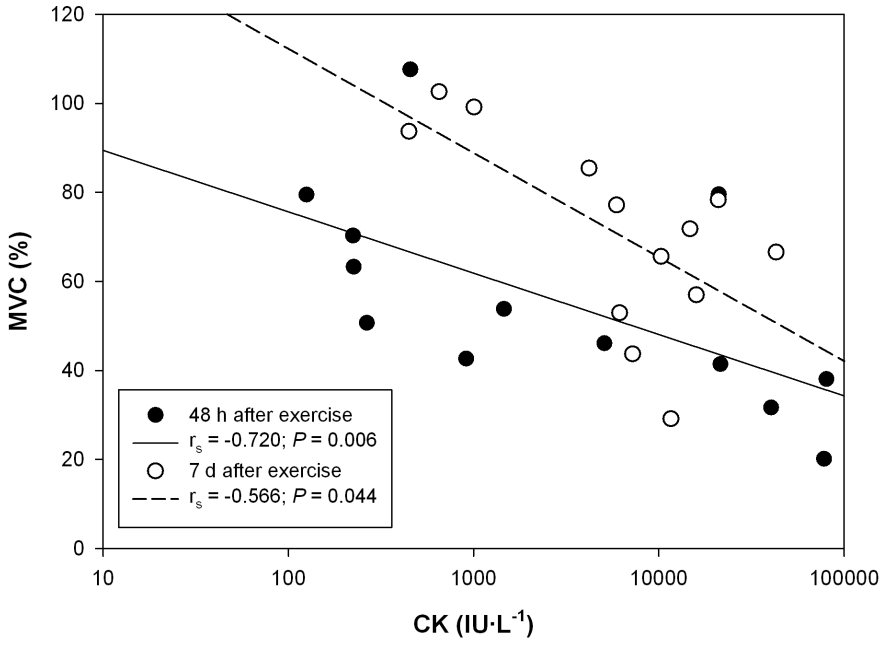


Figura 6. Coeficients de correlació de Spearman (r_s) entre els valors de força isomètrica voluntària màxima (MVC) de la cama que va patir el nivell de pèrdua de capacitat de generació de força (FGC) més accentuat i la creatina quinasa (CK) obtinguts 48 hores i set dies després de l'exercici de rull de cames excèntric (estudi III, n = 13). L'eix x es mostra en escala logarítmica.

Finalment, les correlacions obtingudes entre els increments de CK i les davallades marcades de la capacitat de generació de força (FGC) observades també en l'estudi III reforcen la noció segons la qual els nivells sèrics de CK reflecteixen la quantitat de dany, especialment quan l'EIMD esdevé greu i és probable la necrosi d'alguns segments de les fibres musculars afectades per l'exercici (**Figura 6**).

Isoenzim MB de la creatina quinasa i disrupció del sarcòmer de les fibres lentes (tipus I)?

Les diverses correlacions trobades entre l'activitat o concentració de diversos enzims amb la concentració en sèrum de la isoforma lenta (tipus I o ß) de la miosina (SM) suggereixen que elevacions significatives de CK-MB podrien estar relacionades amb el dany estructural del sarcòmer de les fibres lentes (tipus I). Concretament, a l'estudi V es reporta una correlació elevada entre el pic sèric de CK-MB registrat una hora després de l'exercici i la concentració sèrica pic d'SM observada 24 hores després de l'inici de l'LDE ($r = 0.725$, $P = 0.001$). A més, el *pool* total de valors de CK-MB obtinguts una hora després de l'exercici dels estudis de camp basats en protocols d'LDE (estudis IV i V) també presenta una correlació elevada amb els nivells d'SM registrats 24 hores després de l'exercici (**Figura 7**).

El CK-MB està format per les subunitats M i B (30). La subunitat M de la CK es troba a la regió M (també anomenada banda M) del sarcòmer (103). La regió M és el centre de múltiples processos metabòlics, mecanosensitius i de proteòlisi intracel·lular (proteasoma) (2, 96). Tots aquests processos sostenen l'homeòstasi de la fibra i l'activitat contràctil mitjançant el manteniment de la integritat sarcomèrica per donar resposta a les demandes energètiques durant la contracció i adaptar-se a diversos estímuls bioquímics i biomecànics (103). D'aquesta manera, increments en sèrum de CK-MB per sobre del llindar de normalitat clínica (4 $ng \cdot mL^{-1}$) podrien estar relacionats amb l'afectació de la integritat d'aquesta estructura sarcomèrica. D'altra banda, atès que s'ha provat que les fibres lentes (tipus I) presenten nivells de CK-MB més elevats que no pas les fibres ràpides (tipus II) (211), un increment significatiu de la concentració en sèrum de CK-MB es podria associar al dany de les fibres lentes (tipus I).

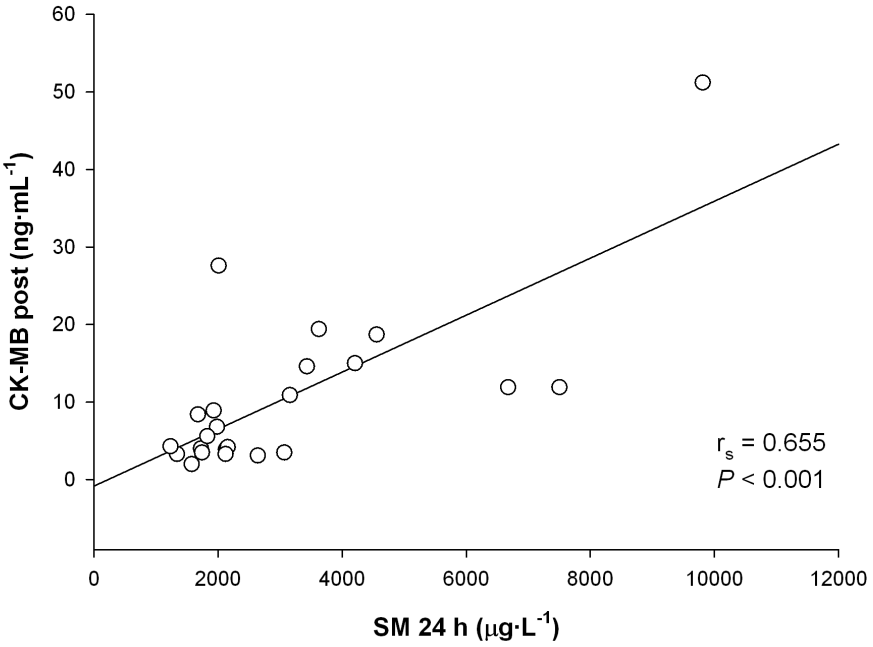


Figura 7. Coeficient de correlació de Spearman (r_s) entre els *pools* totals (n = 24) (estudis IV i V) de l'isoenzim MB de la creatina quinasa (CK-MB) registrats una hora després de l'exercici i els nivells de miosina lenta (tipus I o ß) (SM) obtinguts 24 hores després de l'inici de l'exercici. Els valors del subjecte 7 de l'estudi IV (CK-MB: 245 ng·mL^{-1}; SM: 6999 µg·L^{-1}) s'han exclòs de la representació gràfica.

Les diverses característiques del CK-MB, com ara la ubicació de la subunitat M al sarcòmer (96, 103) o la seva presència superior a les fibres lentes (tipus I) (211) unides amb les correlacions elevades trobades amb l'SM, suggereixen que increments significatius d'aquest isoenzim, per sobre del llindar de normalitat clínica (4 ng·mL^{-1}), podrien indicar no només un increment de la permeabilitat de la membrana, sinó també un dany més profund relacionat amb l'aparell contràctil (regió M del sarcòmer) de les fibres lentes (tipus I).

Creatina quinasa mitocondrial sarcomèrica: de l'efecte protector de l'entrenament a l'apoptosi de la fibra muscular

En els estudis de camp basats en protocols d'LDE (estudis IV i V) s'observen increments significatius d'sMtCK a partir d'una hora després de la finalització de l'exercici, els quals es van mantenir 24 hores després de l'exercici. L'única excepció va ser el grup de corredors que presentaven nivells d'entrenament més elevats (*highly trained*) en l'estudi V (**Figura 8**). Tal com s'ha demostrat amb rates (Sprague-Dawley), l'exercici de llarga durada indueix a dany mitocondrial, però el nivell d'entrenament exerceix un efecte protector sobre els mitocondris (49) a partir d'una reducció específica de la captació de Ca^{2+} (27) i un increment de la capacitat antioxidant (198). Aquest fet, encara que només s'hagi provat en un model animal, podria explicar les concentracions menors d'sMtCK observades en l'estudi V en els corredors que presentaven nivells d'entrenament més elevats (*highly trained*).

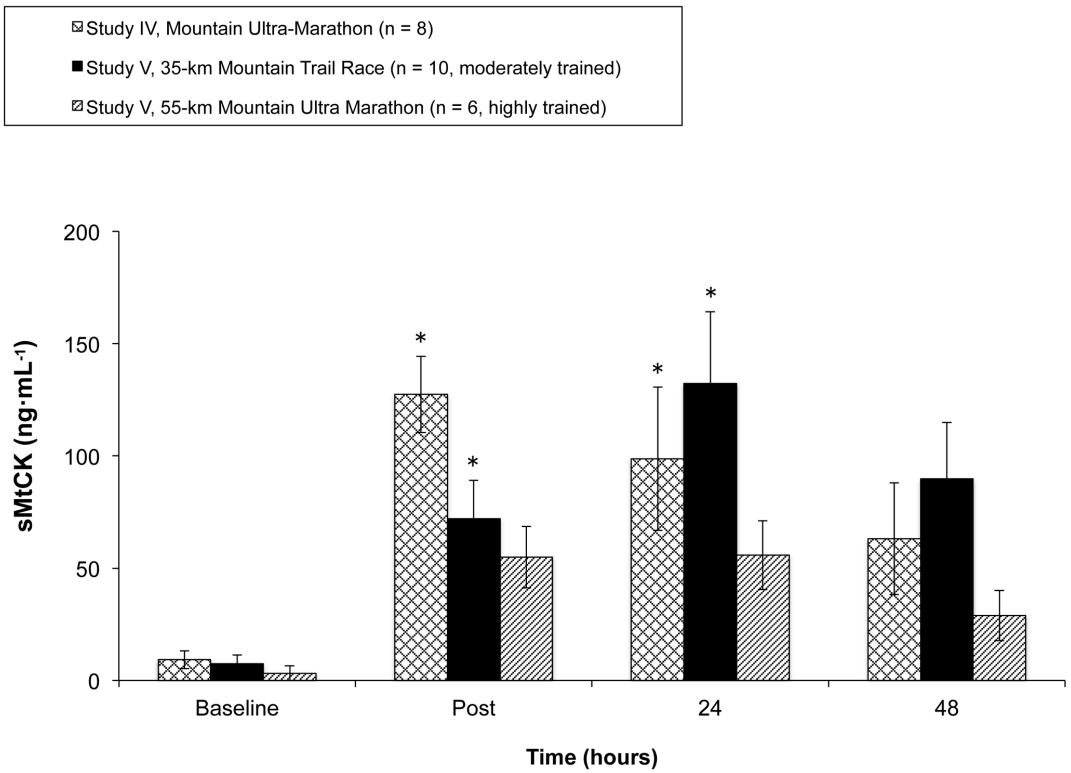


Figura 8. Mitjana ± error estàndard de la mitjana (SEM) de la concentració de creatina quinasa mitocondrial del sarcòmer (sMtCK) obtinguda en els estudis de camp basats en exercicis de llarga durada (estudis IV i V). * Significativament diferent respecte als valors basals a escala $P < 0,05$.

D'altra banda, en l'estudi III es van trobar valors de concentració sèrica d'sMtCK molt superiors als obtinguts en els estudis de camp basats en protocols d'LDE (estudis IV i V). A més, també es van trobar notables diferències significatives entre els grups de subjectes (*high versus moderate responders*) (**Figura 9**).

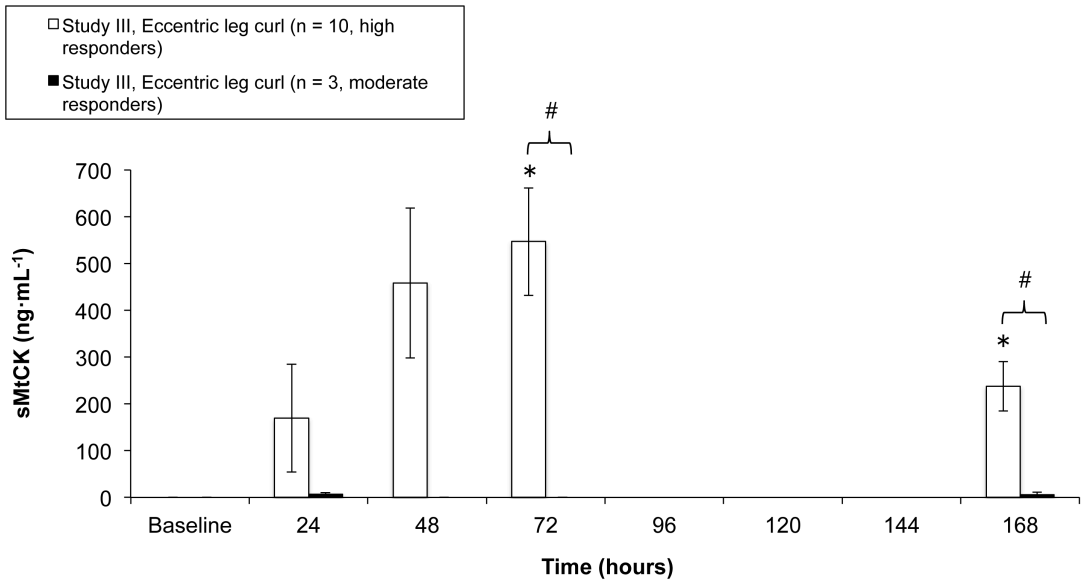


Figura 9. Mitjana ± error estàndard de la mitjana (SEM) de la concentració de creatina quinasa mitocondrial del sarcòmer (sMtCK) obtinguda en l'estudi III. * Significativament diferent respecte als valors basals a escala $P < 0,05$. # Diferència significativa entre grups a escala $P < 0,05$.

Recentment, els mitocondris han estat reconeguts com a agents clau en els sistemes de regulació cel·lular, com la gestió del Ca^{2+} i els processos d'apoptosi (147). Concretament, sembla que l'sMtCK desenvolupa el paper de sensor de l'estat energètic de la fibra muscular i que, en funció d'aquest, desencadena el procés d'apoptosi (180). Així, és probable que grans increments d'sMtCK, com en el cas de l'estudi III, siguin indicatius d'inflamació i disrupció mitocondrial, amb la reducció consegüent de la capacitat respiratòria de la fibra muscular i, en última instància, l'apoptosi de segments de la fibra muscular (49). A més, s'ha descrit una correlació elevada entre els decrements d'FGC i la disrupció miofibril·lar (163, 174). En conseqüència, s'ha assumit que la magnitud de pèrdua d'FGC reflecteix el nombre de fibres afectades (120). Ateses les diverses correlacions mostrades en l'estudi III entre els valors d'sMtCK i la força isomètrica voluntària màxima (MVC) (decrements d'FGC) al cap de 48 hores i, especialment, set dies després de l'exercici, sembla

raonable pensar que les pèrdues sensibles d'FGC són un reflex d'un cert nombre de miofibril·les que han mort per apoptosi (112, 169) (**Figura 10**).

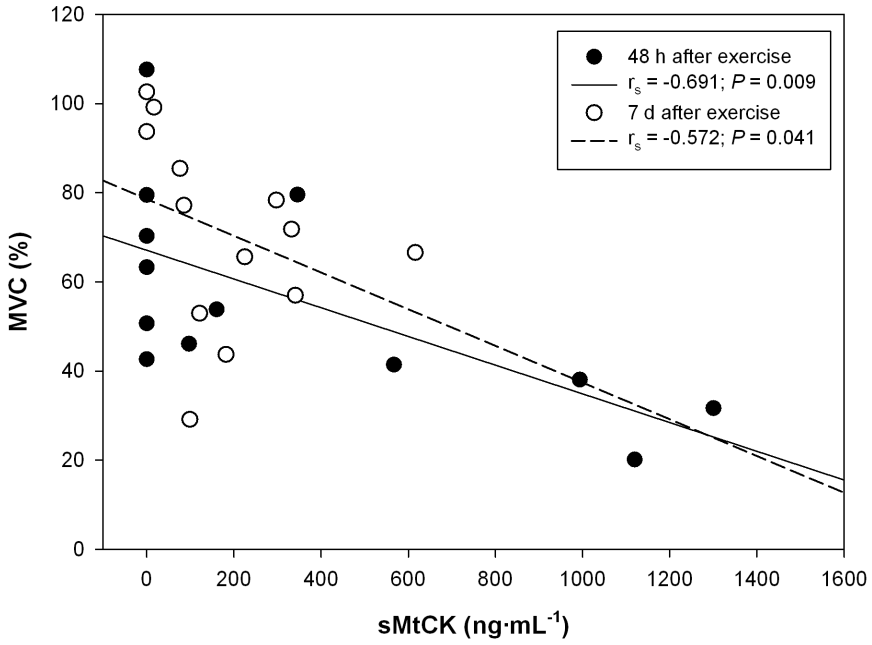


Figura 10. Coeficients de correlació de Spearman (r_s) entre els valors de força isomètrica voluntària màxima (MVC) de la cama que va patir el nivell de pèrdua de capacitat de generació de força (FGC) més accentuat i la creatina quinasa (CK) obtinguts 48 hores i set dies després de l'exercici de rull de cames excèntric (estudi III, n = 13).

5.1.2 Isoformes de la miosina

En dos dels estudis de laboratori basats en protocols d'HIE (estudis I i II), es van trobar increments (significatius en el cas de l'estudi I) de la concentració sèrica de la miosina ràpida (tipus II) (FM) al cap de 48 hores. L'única excepció va ser el subjecte control de l'estudi II, en el qual no es van observar canvis en els nivells d'FM en cap dels punts temporals analitzats. D'altra banda, en els estudis de camp basats en LDE (estudis IV i V), es van descriure increments significatius de la miosina lenta (tipus I o ß) (SM) a partir de les 24 hores posteriors a l'inici de l'exercici. Així, doncs, en termes generals, es pot concloure que, mentre que els LDE indueixen majoritàriament a increments d'SM, els HIE provoquen augments d'FM (**Figura 11**).

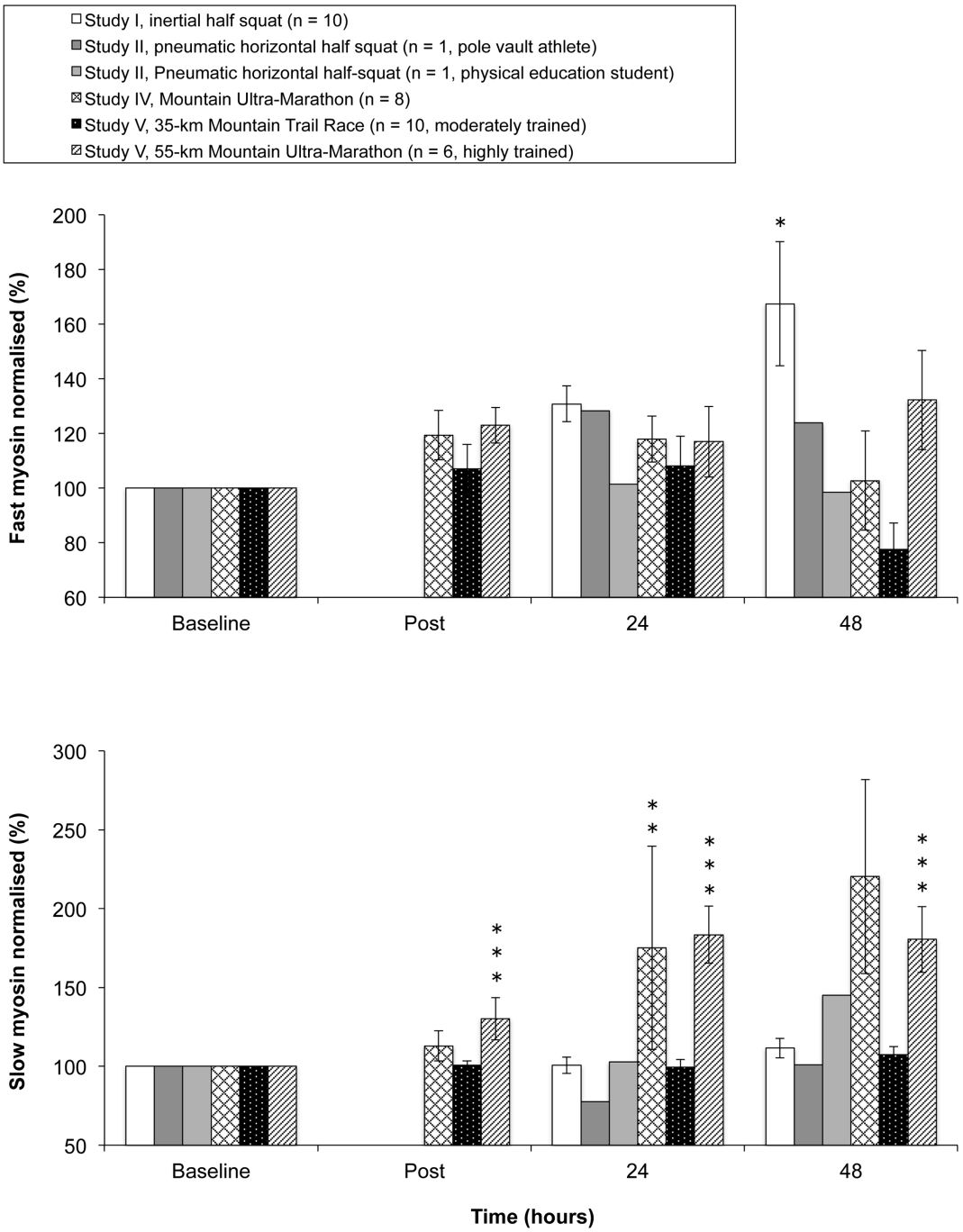


Figura 11. Mitjana ± error estàndard de la mitjana (SEM) de la concentració de miosina ràpida (tipus II) i lenta (tipus I o ß) obtinguda en dos dels estudis de laboratori basats en protocols d'exercici d'alta intensitat (HIE) (estudis I i II) i dels estudis de camp basats en exercicis de llarga durada (LDE) (estudis III i IV). *, **, *** Significativament diferent respecte als valors basals a escala $P < 0,05$, $P < 0,01$, i $P < 0,001$, respectivament.

Així, doncs, en els estudis I, II, IV i V, mentre que augments sèrics d'enzims com la CK indiquen un increment de la permeabilitat de la membrana (sarcolemma) (106), increments significatius de miosina suggereixen un dany més greu relacionat no només amb el sarcolemma, sinó també amb la disrupció del sarcòmer (40, 41). Aquesta afirmació té el suport de diversos estudis anteriors sobre el fenomen de l'EIMD en els quals també es va utilitzar com a biomarcador la concentració sèrica de diverses proteïnes del sarcòmer, com ara la α-actina (131) o la troponina I (187), i també les seves respectives isoformes ràpida (fsTnI) i lenta (ssTnI) (46, 61), o els fragments de la cadena pesada de la miosina (MHC) lenta (tipus I o ß) (129, 187, 188). A més, les isoformes de la miosina ja s'havien mostrat com a biomarcadors vàlids de lesions musculars diagnosticades per ressonància magnètica (MRI) i ecografia. De la mateixa manera, les isoformes de la miosina no només es presentaven com una eina de diagnòstic del grau de lesió muscular (graus I, II i III), sinó que també permetien diferenciar quina tipologia de fibra s'havia vist afectada principalment (86). En aquest sentit, els resultats obtinguts als diversos estudis d'aquesta tesi suggereixen que la miosina també pot ser un biomarcador vàlid i fiable de dany muscular (fins i tot quan aquest és de caràcter lleu) induït per diverses tipologies d'exercici. A més, les isoformes de la miosina són biomarcadors específics de fibra que permeten identificar quina tipologia de fibra (ràpida [tipus II] o lenta [tipus I]) s'ha vist afectada principalment per l'exercici (40, 41).

Curs temporal d'aparició en sèrum

En els diversos estudis realitzats, el pic sèric d'isoformes de la miosina es va trobar entre les 24 i les 48 hores posteriors a l'exercici (**Figura 11**). Aquests resultats són coincidents amb els exposats prèviament per Guerrero et al. (86). A més, en l'estudi I, en el qual es van arribar a mesurar les concentracions sèriques de miosina fins a 144 hores després de l'exercici, es va observar que l'FM es mantenia elevada de manera significativa fins gairebé una setmana després de l'exercici (40). L'FM i l'SM, per tant, presenten un curs temporal en sèrum diferent del dels enzims. S'ha suggerit que després de l'exercici excèntric s'inicia un procés de proteòlisi en què la proteasa calpaïna allibera les proteïnes contràctils de l'estructura filamentosa del sarcòmer (84). Llavors, el procés de degradació de les proteïnes de l'aparell contràctil com la miosina es completa amb el sistema ubiquitina-proteasoma (65). Una raó que podria explicar els increments sèrics de la concentració de miosina més enllà de les 24 hores posteriors a l'exercici és que el procés de recanvi de les proteïnes sarcomèriques requereix més temps que no pas el de les proteïnes no

contràctils del sarcoplasma (143). A més, l'activitat màxima de la calpaïna augmenta unes 48 hores després de l'exercici excèntric i arriba a nivells fins a tres vegades més elevats al cap de 144 hores (110), fet que podria explicar l'obtenció de valors significativament elevats de miosina fins gairebé una setmana després de l'exercici (vegeu l'estudi I). La cascada de reaccions que tenen com a resultat la degradació de proteïnes de l'aparell contràctil del múscul esquelètic és un procés complex que sembla que determina el curs temporal d'aparició de les isoformes de la miosina en sèrum.

Atès que la miosina presenta un pes molecular de 493 kDa (95) i el teixit muscular presenta una permeabilitat capil·lar baixa, sembla raonable pensar que la seva entrada al corrent sanguini es produeix per via limfàtica (22, 126, 127). Aquest fet també contribuiria a la seva aparició tardana en el sèrum després de l'exercici excèntric.

Finalment, atesa l'estabilitat elevada en sang de la miosina (86), fins a 144 hores després de l'exercici en el cas de l'FM (40), en estudis futurs caldrà clarificar el curs temporal dels mecanismes de depuració responsables del retorn dels valors sèrics de les isoformes de la miosina als nivells registrats de manera prèvia a l'exercici.

Disrupció i remodelació

Mentre que els increments significatius d'isoformes de la miosina observats fins a 48 hores després de l'exercici excèntric suggereixen una disrupció del sarcòmer (40) (41), els valors elevats de manera persistent fins gairebé una setmana després de l'exercici indicarien un procés de remodelació, especialment quan els valors d'FGC han recuperat els nivells basals (**Figura 12**). Això està en línia amb estudis anteriors en què, mitjançant l'observació a través de microscopi immunoelectrònic de mostres de teixit muscular, s'afirma que les disrupcions miofibril·lars observades poc després de l'exercici indiquen dany de l'aparell contràctil, mentre que els canvis en les estructures miofibril·lars observats després de diversos dies de recuperació podrien representar un procés de remodelació (214).

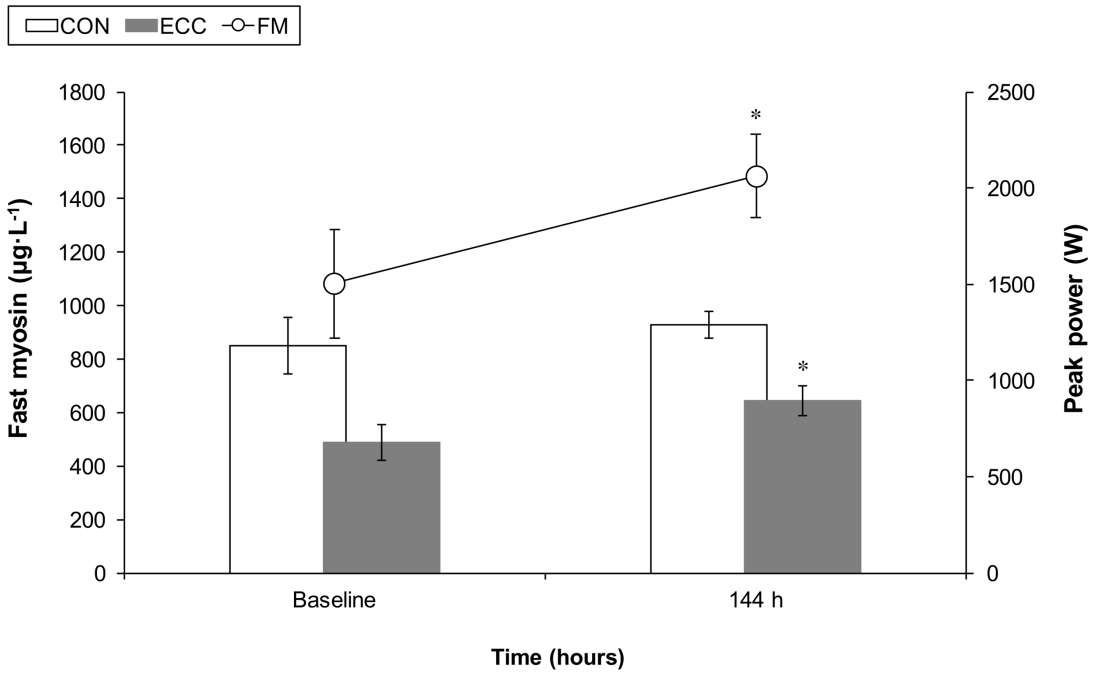


Figura 12. Mitjana ± error estàndard de la mitjana (SEM) de la concentració de miosina i potències inercials màximes (resultats estudi I). CON, concèntric. ECC, excèntric. FM, miosina ràpida (tipus II). * Significativament diferent respecte als valors basals a escala P < 0,01.

5.1.3 Limitacions del model. La problemàtica de les fibres híbrides

Mitjançant models animals (mamífers), Hämäläinen i Pette (88) van descriure les fibres «híbrides» en músculs esquelètics «normals» (no sotmesos a canvis en els patrons d'estimulació neural mitjançant electrostimulació). Aquest tipus de fibres del múscul esquelètic expressen més d'una isoforma de l'MHC, mentre que les fibres «pures» n'expressen només un tipus (179). S'ha demostrat en humans que les fibres híbrides coexpressen diversos tipus d'MHC: MHC ß/slow i 2A, i MHC 2A i 2X (25) (115). Aquestes expressen un fenotip de fibres en transformació (5) com les descrites en condicions d'immobilització induïda per lesió (87) o com a resposta a l'entrenament (100). D'aquesta manera, l'aparició en sèrum d'FM o SM podria indicar no només la disrupció sarcomèrica de fibres pures (ràpides o lentes), sinó també de fibres híbrides que expressen diferents tipus d'MHC. Per a aquest model molecular d'EIMD, mentre que les fibres híbrides MHC 2A i 2X no representen cap tipus de problema, ja que l'anticòs monoclonal utilitzat les reconeix de manera

genèrica com a fibres ràpides (tipus II), el dany potencial provocat sobre les fibres híbrides MHC 1 ß/slow i 2A podria induir a interpretacions errònies. Cal destacar, però, que en el vast lateral de subjectes sedentaris joves (23-31 anys), només s'ha trobat un 8 % de fibres híbrides que coexpressen MHC 1 ß/slow i 2A (115), i en subjectes moderadament actius no s'han trobat indicis d'aquesta tipologia de fibres híbrides (114). D'aquesta manera, el biaix al qual podria induir un dany potencial de les fibres híbrides MHC 1 ß/slow i 2A es veuria pal·liat de manera notable en els estudis basats en exercicis del tren inferior a intensitats elevades, ja que durant la seva realització, la mostra emprada incloïa, principalment, participants joves i moderadament actius. Per contra, hi ha discordança entre estudis amb humans sobre el percentatge de fibres híbrides MHC 1 ß/slow i 2A en subjectes entrenats en resistència. Mentre que Klitgaard et al. (114) van reportar que un 36 % de les fibres analitzades del vast lateral de subjectes entrenats en resistència coexpressaven MHC ß/slow i MHC 2A, Putman et al. (173) no van trobar indicis d'aquesta tipologia de fibres (o percentatges extremament baixos [<1 %]) també al vast lateral de subjectes entrenats en resistència. Pel que fa al gastrocnemi lateral, s'ha descrit un ~6-7 % de fibres híbrides MHC 1 ß/slow i 2A en atletes de mig fons i fons (rang: 800-10.000 m) (90) i de cros atlètic (89). Per tant, i malgrat la manca de consens entre estudis, atès que en subjectes entrenats en disciplines de resistència la presència de fibres híbrides MHC 1 ß/slow i 2A podria arribar a ser relativament elevada, els increments sèrics d'SM indicarien no només dany de fibres lentes (tipus I), sinó també de fibres híbrides MHC 1 ß/slow i 2A.

5.1.4 Resum

En els estudis de laboratori I i II, basats en protocols d'HIE en què s'han emprat exercicis globals del tren inferior amb gran participació dels extensors del genoll (mig esquat), s'han obtingut increments lleus de l'activitat o concentració enzimàtica en sèrum (p. ex., CK ≤ 1000 $IU \cdot L^{-1}$) 24 hores després de l'exercici. En els estudis de camp IV i V, basats en protocols d'LDE en què s'han utilitzat curses competitives de muntanya, s'han obtingut increments moderats de l'activitat o concentració enzimàtica en sèrum (p. ex., CK 1000 - 2000 $IU \cdot L^{-1}$) entre una i 24 hores després de l'exercici. En ambdós casos (HIE i LDE), el curs temporal i la magnitud dels augments són coherents amb un procés d'increment de la permeabilitat de la membrana (106, 160). Els resultats indiquen que els extensors del genoll presenten una resistència elevada al dany muscular induït per HIE (106), especialment quan

els exercicis són de caràcter global i multiarticular. L'exposició a contraccions excèntriques de la cadena extensora del tren inferior durant les activitats diàries (48, 107) i la seva arquitectura (18, 209) sembla que són els factors determinants del seu nivell baix de susceptibilitat.

Els increments posteriors a l'exercici de la concentració sèrica del CK-MB, per sobre del llindar de normalitat clínica, observats en els estudis de camp IV i V i basats en protocols LDE podrien suggerir un dany de l'aparell contràctil (afectació de la regió M del sarcòmer) de les fibres lentes (tipus I).

D'altra banda, en l'estudi de laboratori III, basat en un protocol d'HIE en què s'ha emprat un exercici analític (monoarticular, amb aïllament elevat del múscul semitendinós) d'alta intensitat (120 % de la repetició màxima [1-RM] concèntrica) de la musculatura flexora del genoll (isquiotibials), és probable que grans pics sèrics d'activitat/concentració enzimàtica (p. ex., CK > 10.000 $IU \cdot L^{-1}$; > sMtCK 500 $ng \cdot mL^{-1}$) registrats més enllà de les 72 hores posteriors a l'exercici i encara significativament elevats als set dies es trobin relacionats amb processos de necrosi (7, 162) i apoptosi (112, 169) d'alguns segments de les fibres musculars. Els isquiotibials (flexors del genoll) són més susceptibles a l'EIMD excèntric que no pas els extensors del genoll (48).

Pel que fa a les isoformes de la miosina, en els estudis de laboratori I i II, basats en protocols d'HIE en els quals s'han emprat exercicis globals del tren inferior amb gran participació dels extensors del genoll (mig esquat), s'han obtingut increments (significatius en el cas de l'estudi I) de la concentració d'FM en sèrum 48 hores després de l'exercici. En els estudis de camp IV i V, basats en protocols d'LDE en els quals s'han utilitzat curses competitives de muntanya, s'han obtingut increments significatius de la concentració sèrica d'SM entre les 24 i 48 hores posteriors a l'exercici. En cap dels estudis no s'han trobat increments (significatius) concurrents d'FM i SM. En ambdós casos (HIE i LDE), els augments d'FM o SM són coherents amb un procés de disrupció del sarcòmer de les fibres ràpides (tipus II) (40) o lentes (tipus I) (41), respectivament. És interessant destacar que l'anàlisi estadística del *pool* total de concentracions en sèrum d'isoformes de la miosina dels estudis basats en protocols d'HIE (estudis I i II) i els estudis basats en LDE (estudis IV i V) confirma aquesta tendència de dany selectiu (**Figura 13**).

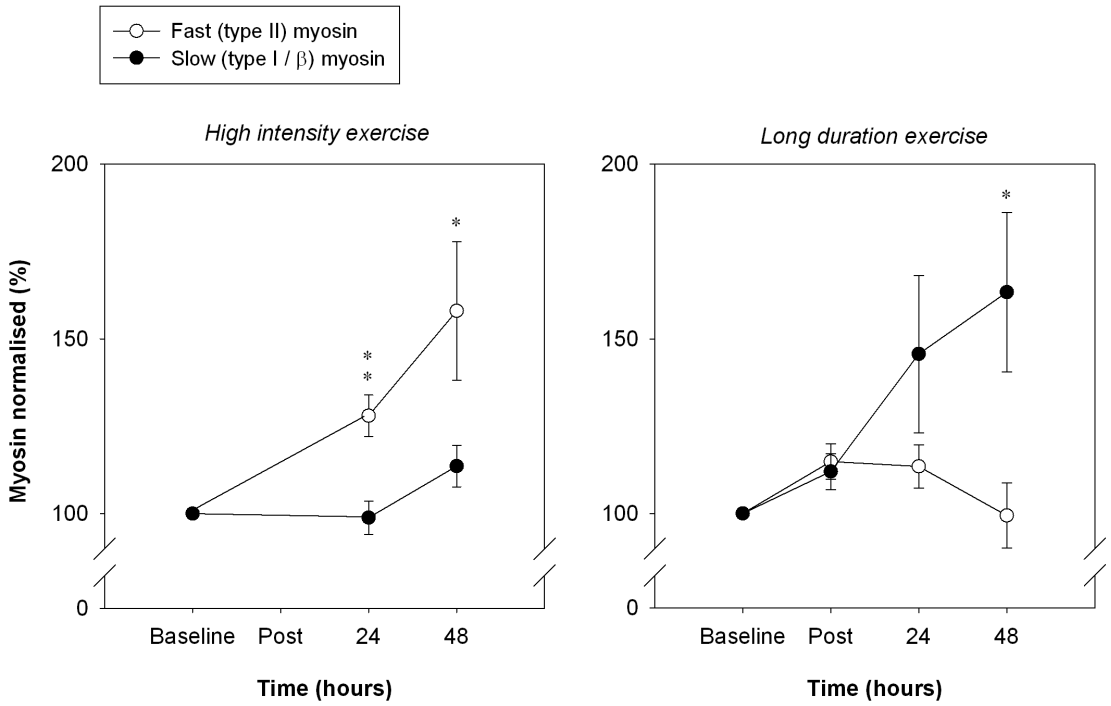


Figura 13. Mitjana ± error estàndard de la mitjana (SEM) del *pool* total de concentracions de miosina ràpida (tipus II) i lenta (tipus I o ß) obtingut dels estudis basats en exercicis d'alta intensitat (HIE) (n = 12) (estudis I i II) i de llarga durada (LDE) (n = 24) (estudis III i IV). Les dades estan normalitzades respecte als valors basals (100 %). *, ** Significativament diferent respecte als valors basals a escala P < 0,01, i P < 0,001, respectivament .

Valors d'isoformes de la miosina elevats de manera persistent, fins gairebé una setmana després de la realització de l'exercici excèntric, poden ser indicatius d'un procés de remodelació sarcomèrica més que no pas de disrupció (214), especialment quan els valors d'FGC ja s'han recuperat (40).

Finalment, a la **Figura 14** es resumeix el model molecular d'EIMD. Fonamentat inicialment en les propostes de diversos autors (3, 4, 13, 19, 30, 61, 65, 83, 84, 106, 127, 129, 134, 160, 180, 189), s'ha desenvolupat en base als resultats de l'estudi I (40) i, a partir d'aleshores, s'ha enriquit mitjançant els resultats de la resta d'estudis (II, III, IV i V) que composen la present tesi. El model es basa en el pes molecular i la compartimentació estructural en la fibra muscular dels diversos enzims i isoformes de la miosina, i també en el seu perfil temporal i la seva magnitud d'aparició en sèrum. En la **Figura 14** es mostren els mecanismes hipotètics que podrien estar

involucrats en l'aparició d'aquests enzims i proteïnes en el sèrum a partir d'un estímul primari com són les contraccions excèntriques desenvolupades en els exercicis d'alta intensitat (estudis I, II i III) o llarga durada (estudis IV i V).

A la **Figura 14** es mostra com mentre els increments sèrics moderats de CK indiquen un augment de la permeabilitat de la membrana (106, 160), per disrupció (134) o per l'activació dels canal iònics (Ca^{2+}) (4), augments de FM o SM són coherents amb un procés de dany més profund, relacionat amb la disrupció del sarcòmer de les fibres ràpides (tipus II) (40) o lentes (tipus I) (41), respectivament. Finalment, a la **Figura 14** també es reflecteix com increments sèrics de sMtCK poden ésser indicatius d'un dany miofibril·lar profund basat en la inflamació i disrupció mitocondrial i, en última instància, l'apoptosi de segments de la fibra muscular (49).

Així doncs, a partir d'aquest model es pot caracteritzar el dany muscular en funció del tipus d'exercici excèntric (alta intensitat o llarga durada). No obstant, cal tenir en compte que el grau de dany muscular que pot induir un exercici està altament determinat per factors individuals de tipus genètic (54, 104, 150, 213), pel nivell d'activitat (entrenament) (69, 81, 162) o per l'efecte protector del *repeated bout effect* (*RBE*) (133), de forma que no es pot realitzar una universalització d'aquests resultats en poblacions amb característiques diferents a les dels subjectes que han composat les mostres dels diversos estudis.

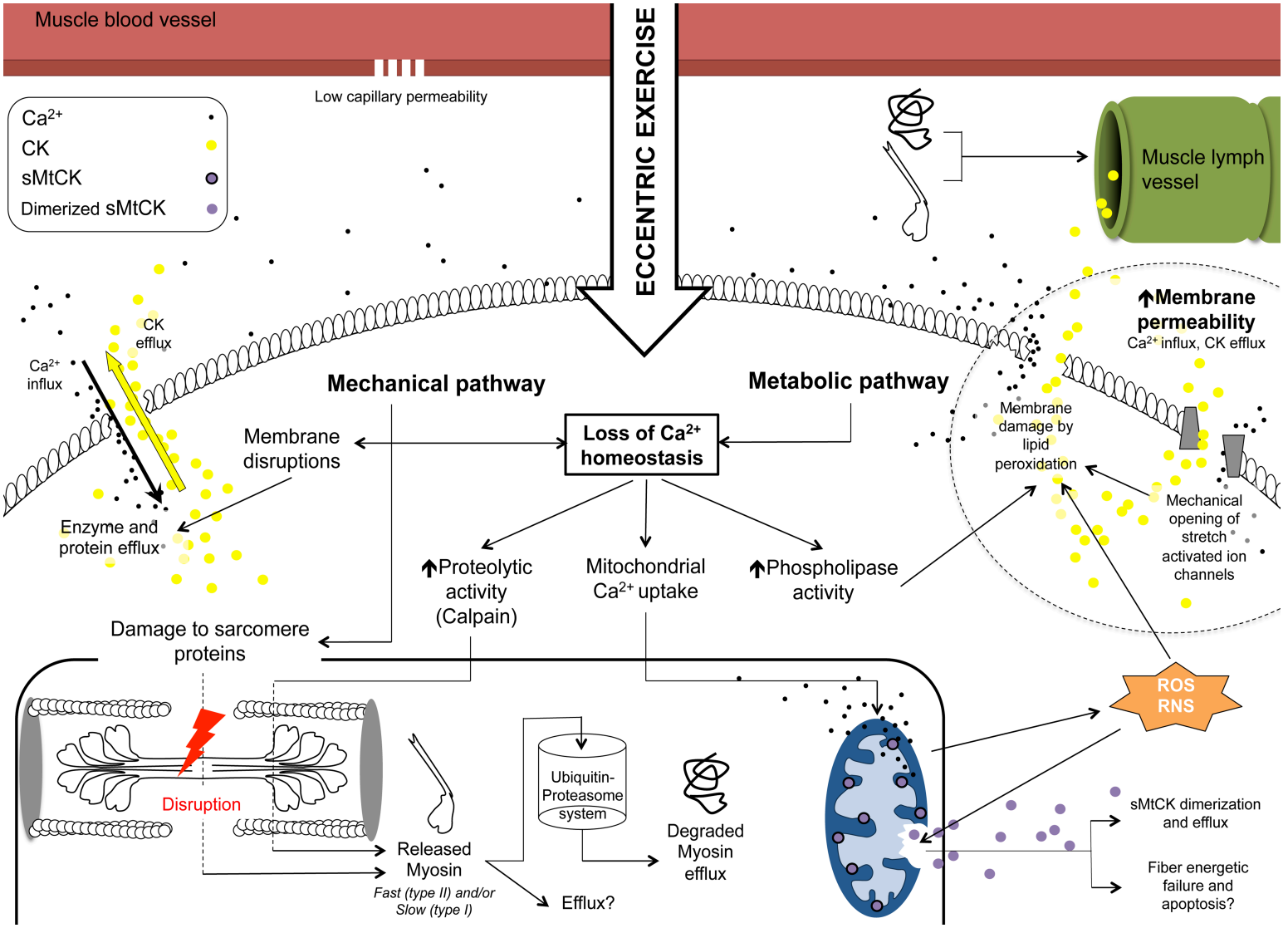


Figura 14. Model molecular indirecte de dany muscular induït per l'exercici (EIMD) que indica els mecanismes hipotètics que hi podrien estar involucrats. Ca^{2+}, calci (ions). CK, creatina quinasa. sMtCK, creatina quinasa mitocondrial sarcomèrica. ROS, espècies reactives de l'oxigen, RNS, espècies reactives del nitrogen. Basat en (3, 4, 13, 19, 30, 40, 61, 65, 83, 84, 106, 127, 129, 134, 160, 180, 189)

6. CONCLUSIONS

6.1 Indirect molecular model of exercise-induced muscle damage

- FM and SM serum increases revealed sarcomere disruption as well as increased membrane permeability of fast (type II) and slow (type I) fibers respectively. Consequently, myosin isoforms can be adopted as fiber type-specific biomarkers of muscle damage.
- An indirect model of EIMD can be constructed based on the serum evolution of muscle enzymes and muscle FM and SM according to their molecular weight, the fiber compartment in which they are located and, in the case of myosin isoforms, the fiber type in which they are expressed.

6.2 Application of the indirect molecular model of exercise-induced muscle damage for exercise characterization

6.2.1 High intensity exercise

- An increase in FM serum concentration following inertial exercise suggests selective fast (type II) fiber damage.
- The exercise output of the pole vault athlete revealed an explosive (power-oriented) profile leading to selective mild damage of fast (type II) fibers. In contrast, the exercise output of the physical education student showed a fatigue-resistant profile, which led to a higher extent of slow (type I) fiber damage.
- Although large differences between subjects (high and moderate responders) can be expected according to the sporting activity and the activity level (training status), intensive eccentric unilateral leg curl exercise induces severe EIMD.

- Changes in the serum enzyme activities or concentrations after exercise were in accordance with the differences between-subjects (high and moderate responders) in terms of the extent of EIMD reflected by FGC reductions.
- sMtCK is a promising novel EIMD biomarker of myofibrillar apoptosis which is able to identify high responders.

6.2.2 Long duration exercise

- An increase in SM serum concentration following MUM (42.195 km) indicates selective slow (type I) fiber damage.
- Although the reproducibility of field studies is low because of the complex characteristics of mountain running competitions, distance may be related to biochemical indices of muscle damage.
- When competing in a MUM, training status does not seem to have any effect on serum changes in EIMD biomarkers.

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ANNEX

19th annual Congress of the European College of Sports Science, 2014, Amsterdam (Netherlands). Abstract published in book of abstracts, MO-PM22 Exercise & Muscle Metabolism, p 178.

Skeletal muscle fast myosin increases in serum after maximal concentric-eccentric inertial exercise

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Introduction

We hypothesized that a model of muscle damage could be made by measuring the time course of the serum concentration of muscle enzymes and proteins following inertial exercise, according to their molecular weight and their fibre compartmentalization. Moreover, by measuring fibre-type specific sarcomere proteins, fast myosin (FM) and slow myosin (SM), the type of fibres that are affected could be assessed (1).

Methods

Ten recreationally active men were required to perform 7 sets of 10 maximum intensity concentric-eccentric (C-E) repetitions of a half-squat exercise in a flywheel inertial resistance device (Portable VersaPulley™, Heart Rate Inc., Costa Mesa, CA). The exercise dynamic muscle work was characterized using the time course of force, displacement and velocity data, sampled at a frequency of 100 Hz from the force sensor and linear encoder of MuscleLab 4020e (MuscleLab, Ergotest Technology, Langesund, Norway) (2). The muscle damage effect of this exercise was assessed through the evolution of serum muscle enzymes and fibre-type specific myosin isoforms. Serum profiles of creatine kinase (CK), creatine kinase MB isoenzyme (CK-MB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), and myosin isoforms (FM and SM), were measured before and 24, 48 and 144 h post-exercise.

Results

Muscle enzymes, CK, CK-MB and, to a lesser extent, AST, were early increased in serum (24 h), and returned to baseline values at 48 h post-exercise. FM was late increased in serum (48 h) and remained elevated until 144 h post-exercise. SM serum concentration showed no significant changes.

Discussion

The maximal C-E inertial exercise of the knee extensors, involving a highly specific movement similar to several sports actions, induces a different level of damage in fast and slow fibres. Interestingly, while an increase in muscle damage biomarkers like CK, CK-MB, and to a lesser extent AST, indicated increased membrane permeability, FM serum increases revealed sarcomere disruption as well as increased membrane permeability of fast fibres. Consequently, FM could be adapted as a fast-fibre biomarker of muscle damage. The results support a model of muscle damage based on the serum time course of muscle proteins according to their molecular weight and their fibre compartmentalization, depending on exercise and fibre-type.

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Monday, 01 December 2014

Confirmation of presentation and acceptance for the Young Investigators Award 2014

To whom it may concern,

Gerard Carmona presented the Mini-oral titled "SKELETAL MUSCLE FAST MYOSIN INCREASES IN SERUM AFTER MAXIMAL CONCENTRIC-ECCENTRIC INERTIAL EXERCISE" at the 19th Annual Conference of European College of Sport Science, Amsterdam, The Netherlands 2014.

The abstract "SKELETAL MUSCLE FAST MYOSIN INCREASES IN SERUM AFTER MAXIMAL CONCENTRIC-ECCENTRIC INERTIAL EXERCISE"" by **Gerard Carmona** was also accepted for the Young Investigators Award 2014 at the 19th Annual Conference of European College of Sport Science, Amsterdam, The Netherlands 2014.

Yours sincerely,

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