Release of $\alpha$-actin into serum after skeletal muscle damage

A Martínez-Amat, H Boulaiz, J Prados, J A Marchal, P Padial Puche, O Caba, F Rodríguez-Serrano, A Aránega

**Objective:** The skeletal muscle protein $\alpha$-actin was investigated in the serum of subjects with severe skeletal muscle damage to assess its utility as a reliable and predictive marker of muscle damage.

**Methods:** Serum samples were obtained from 33 healthy controls and 33 patients with severe skeletal muscle damage, defined by a total creatine kinase value of $>500$ IU/l (Rosalki method). Troponin I, troponin T, and myoglobin concentrations were determined by immunoassay and $\alpha$-actin concentrations by Western blot and densitometry.

**Results:** The mean serum concentration of $\alpha$-actin in controls and patients with skeletal muscle damage was $600.9$ and $1968.51$ ng/ml, respectively, a statistically significant difference. Sera of patients with muscle damage showed higher levels of $\alpha$-actin than of troponin or myoglobin. No significant difference in troponin I levels was observed between the groups.

**Conclusions:** According to these results, $\alpha$-actin was the most significant skeletal muscle damage marker analysed and may be an ideal candidate for the identification of all types of myofibre injury, including sports injuries. Our findings support the use of $\alpha$-actin as a marker alongside other currently used biological proteins.
was the presence of a total CK value of >500 IU/l by the Rosalki method (Beckman Instruments, Madrid, Spain).

Control assays were performed in 33 serum samples collected at the Granada Regional Blood Bank from 24 healthy males (72.7%) and nine healthy females (27.3%) with a mean age of 54.5 and 76.3 years, respectively. These donors ranged in age from 22 to 79 years.

Serum samples were obtained by venopuncture and collected in tubes with separator gel (Venoject II, Terumo Europe, Leuven, Belgium). After clotting, they were centrifuged for 10 min at 5000 rpm (Beckman Instruments Centrifuge). The sera were then aliquoted into 1.5 ml Eppendorf tubes for the determinations.

**Determination of total CK activity**

The enzymatic kinetic method was used to determine total CK activity. In the reaction, the CK catalyses the transfer of a phosphate group from creatine phosphokinase to adenosine diphosphate. The subsequent formation of adenosine triphosphate is measured by using two associated reactions, catalysed by hexokinase and glucose-6-phosphate dehydrogenase, which produce nicotinamide adenine dinucleotide. This CK assay contains the activator monothioglycerol.

**Immunooassay determination of troponin I (cTnI), troponin T (cTnT), and myoglobin**

Troponin I, troponin T, and myoglobin were determined by chemiluminescent immunooassay. For troponin I and myoglobin, an Access sandwich-type immunoassay (Beckman Instruments) was used. A sample was added to a glass reaction vessel with alkaline phosphatase-conjugated anti-troponin I and anti-myoglobin monoclonal antibodies along with paramagnetic particles coated with anti-troponin I and anti-myoglobin monoclonal antibodies. Cardiac troponin and human myoglobin bind to the antibody in the solid phase, whereas the antibody-alkaline-phosphatase conjugate reacts with different antigenic sites on cardiac troponin and myoglobin molecules. After incubation, separation in a magnetic field and washing removes materials not bound to the solid phase. A chemiluminescent substrate, Lumi-Phos 530, was added to the reaction vessel and a luminometer was used to measure the light generated by the reaction. The production of photons is inversely proportional to the amount of enzymatic conjugate present at the end of the reaction and, consequently, to the concentration of cardiac troponin I and myoglobin in the sample. The amount of analyte in the sample was determined using a multipoint calibration curve.

For the troponin T determination, the Elecsys 2010 troponin T test (Roche Diagnostic, Indianapolis, IN) was used, which is performed in 18 min at 37°C. In a first incubation step, a sandwich-type complex was formed by the sample, a specific biotinylated monoclonal antibody against troponin T, and a ruthenium chelate labelled (chelate tris(2,2′-bipyridul)ruthenium(II), (Ru(bpy)2+3) specific monoclonal antibody against troponin T.

**Detection of α-actin by Western blot**

Sarcomeric α-actin was determined by Western blot. A sample of serum (5 µl) from each study subject was dissolved in Laemmli’s sample buffer (62.76 mm Tris-HCl pH 6.8, 1% 2-mercaptoethanol, 1% SDS, 10% glycerol, and 0.01% bromophenol blue) at 1:5 ratio, boiled for 5 min, microfuged for 1 min, and analysed by SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) in a Mini Protein II cell (Bio-Rad, Hercules, CA) at 60 mA for 1 h at room temperature. The gels with samples of serum were run in duplicate in all cases. The gels for immunoblot analyses were separated electrophoretically and transferred to a nitrocellulose membrane by applying a current of 20 V at room temperature for 30 min. The blots were treated with blocking solution (20 mM Tris, 0.9 NaCl, 10% non-fat milk) for 3 h at room temperature and then reacted with a 1:2000 dilution of anti-sarcomeric α-actin monoclonal antibody (Alpha-Sr-1 Clones, Dako, Glostrup, Denmark). Primary antibodies were incubated overnight at 4°C. Membranes were washed (15 min in 5% TBST) and incubated with horseradish peroxidase conjugated rabbit anti-mouse IgG (1:2000, Sigma-Aldrich, St Louis, MO) for 1 h at room temperature, followed by additional washes (15 min in 5% TBST). Proteins were visualised by enhanced chemiluminescence (ECL, Bonus, Amersham, Little Chalfont, UK).

**Densitometric analysis of α-actin**

Densitometric analysis was carried out by scanning x-ray images of the membranes (Fluorine-S Multilmager, Bio-Rad). The resolution of the images was 100 pixels per inch. A densitometry image analysis software package (Quantity 1, Bio-Rad) was used. The procedure was repeated for every sample processed in this study. A digital image was obtained formed by 66 wells, 33 with control group and 33 with skeletal muscle damage group samples.

A standard curve was obtained by immunoblotting with an amount of known proteins (nanograms of pure α-actin) and densitometrically quantifying the bands obtained.

### Table 1 Determination of levels of CK, troponin (cTnT, cTnI), myoglobin, and α-actin in serum

<table>
<thead>
<tr>
<th></th>
<th>Muscle damage group</th>
<th>Control group</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK (IU/l)</td>
<td>2041 ± 2194.5 (1015)</td>
<td>105.5 ± 103.9* (79)</td>
</tr>
<tr>
<td>TnI (ng/ml)</td>
<td>0.035 ± 0.031 (0.030)</td>
<td>0.0213 ± 0.019 (0.016)</td>
</tr>
<tr>
<td>TnT (ng/ml)</td>
<td>0.047 ± 0.075 (0.010)</td>
<td>0.0072 ± 0.0253* (0.001)</td>
</tr>
<tr>
<td>Myoglobin (ng/ml)</td>
<td>891 ± 887.449 (633.1)</td>
<td>28.867 ± 24 (18 9062)</td>
</tr>
<tr>
<td>α-Actin (ng/ml)</td>
<td>1968.5 ± 515.25 (2119.9)</td>
<td>600.90 ± 532.97* (514)</td>
</tr>
</tbody>
</table>

All data are expressed as mean ± SEM of four measurements. The significance of the differences (*) between the control and muscle damage groups was determined by the Wilcoxon test. A significant difference was considered when p<0.001. The median value is indicated in parentheses.

*Figure 1 Representation of mean TnI and TnT levels in control group and muscle damage group sera. *Indicates significant differences between the groups.*
A mean myoglobin value of 891.815 ng/ml (range: 3.633–58.6 ng/ml) was detected in the muscle damage group sera, significantly (p<0.001) higher than the value of 28.869 ng/ml (range: 0.00–1.359 ng/ml) in the control sera (table 1).

Circulating α-actin was detected by immunoblotting as a band located at 43 kDa (fig 2). The serum protein concentration was determined by densitometric study of the α-actin bands. The mean serum α-actin level in the muscle damage group was 1968.51 ng/ml (range: 854–2.394 ng/ml), significantly (p<0.001) higher than the concentration of 600.90 ng/ml (range: 0.00–1.359 ng/ml) in the control group (table 1, fig 3).

**RESULTS**

Sera of the muscle damage group showed a mean CK activity in serum of 2041.9 IU/l (range: 517–8943 IU/l), significantly (p<0.001) higher than the level of 105.5 IU/l (range: 15–545 IU/l) found in the control sera (table 1).

The muscle damage group showed a mean troponin T (cTnT) value of 0.035 ng/ml (range: 0.001–0.160 ng/ml) compared with 0.0213 ng/ml (range: 0.001–0.085 ng/ml) for the control group, a non-significant difference (fig 1). There was a significance (p<0.001) difference between the mean value of troponin T (cTnTI) in the muscle damage group (0.047 ng/ml; range: 0.001–0.220 ng/ml) and that in the control group (0.0072 ng/ml; range: 0.001–0.139 ng/ml) (table 1).

A mean myoglobin value of 891.815 ng/ml (range: 3.633–58.6 ng/ml) was detected in the muscle damage group sera, significantly (p<0.001) higher than the value of 28.869 ng/ml (range: 79.8–5.7 ng/ml) found in the control sera (table 1, fig 2).

**DISCUSSION**

Major questions have been raised in sports medicine about the diagnosis of skeletal muscular damage. Are there any faithful, reliable, sensitive, and reproducible markers of muscular injury? Do variations in these biological markers correlate with the severity of histologic injury? Are increases in these markers associated with overtraining? There appears only to be a consensus that intense physical exercise induces injury of the muscle fibres, and that the severity of these injuries depends on the duration of the exercise, its characteristics, and the training level of the individual.

Clinical assessment of exercise induced muscle injuries is very difficult. The severity and evolution of these structural injuries are highly variable, and they are characterised by simple residual pains in muscle masses and sometimes by a persistent sensation of heaviness in the lower limbs. Because the clinical expression of this pathology is non-specific, its impact in sports remains difficult to estimate.

The ideal marker should be muscle specific with a high intracellular concentration, and it should be rapidly released in the event of injury and be stable, maintaining its elevated concentration in blood for an adequate time period. It should have a high diagnostic sensitivity, especially in the first few hours after the injury, and have a diagnostic specificity as close to 100% as possible.

Variation in CK activity is currently used as an indicator of muscle damage. The present study demonstrated differences in total CK activity between healthy individuals and those with muscle damage. However, Kuipers and Keizer concluded that it can never be considered a reliable marker of muscle damage; because the presence of total CK in plasma does not faithfully reflect the extent and nature of muscle injury due to interferences produced by isozymes (CKMM, CKMB, and CKBB) of different origin. Bigard reported that the extent of exercise induced muscle damage cannot be assessed solely on the basis of variations in CK activity. The lack of sensitivity and reproducibility of CK activity has led to
Biological diagnosis of muscle damage is based on immunoassay determination of the serum activity of sarcoplasmic enzymes. The detection of even small amounts of proteins bound to intracellular structures always indicates necrosis. Thus, troponins and α-actin have been used as biochemical markers to detect heart damage. Troponin isoforms (cTnI and cTnT) are confirmed as heart damage markers but are not useful as skeletal muscle damage markers.

a prudent attitude towards the utility of this biological parameter.

Troponins are used as markers of heart muscle damage.20 21 We observed significant differences in serum cTnT levels between healthy and muscle damaged individuals but no significant differences were observed in cTnI values. Various authors have confirmed the specificity of the cTnI17 18 and cTnT18 isoforms as heart damage markers but their detection after skeletal muscle damage has not been considered useful. On the other hand, due to its cardiac specificity, cTnI can be used to differentiate between heart and skeletal muscle injuries,19 avoiding false positives and increasing the reliability of results.

Muscle damage markers used to date are not sufficiently sensitive for early detection, and the muscle protein α-actin may serve this purpose. It is abundant in the cytosolic compartment of the cell,20 unlike other proteins such as troponin (there are seven monomers of α-actin for each molecule of troponin),21 and it can be detected up to 72 h after its release.22 Moreover, α-actin is detected 1 h after the onset of muscle damage symptoms, whereas troponin is released late into the bloodstream.20 21

Our study has demonstrated the utility of this protein to detect skeletal muscle injury, with statistically significant (p<0.001) differences in serum α-actin levels between individuals with muscle damage and controls. These results can be applied in sports medicine because, according to Féasson,11 exercise does not induce variations in α-actin levels. Therefore, the detection by Western blot of circulating α-actin in patients with skeletal muscle damage may reflect cell damage. Although based on a heterogeneous study sample, the results obtained represent preliminary evidence of the value of α-actin as an early marker of muscular damage.

In conclusion, our results indicate that α-actin may be a good marker to be applied alongside other currently used biological markers for the early identification and diagnosis of skeletal muscle damage. The key characteristics of α-actin for this purpose are its abundant presence and stability over time.

Authors’ affiliations
A Martinez-Amat, J A Marchal, F Rodriguez-Serrano, Department of Health Sciences, University of Jaén, E-23071 Jaén, Spain
H Boulaiz, J Prados, O Cabo, A Aranega, Basic Cardiovascular Research Section, Department of Morphological Sciences, School of Medicine, University of Granada, E-18012 Granada, Spain
P Paladí Puche, Faculty of Sciences of Physical Activity and Sports, Department of Physical and Sports Education, University of Granada, E-18220 Granada, Spain
This study was supported by the Andalusian Sports Medicine Center (C.A.M.D.) through project no. 2003/319563
Competing interests: none declared

What is already known on this topic
What this study adds
α-Actin is an abundant protein in the muscle, where it represents >20% of all cell proteins. In this study, we demonstrate α-actin efficiency as a marker of muscle damage suggesting its potential use as a new marker of skeletal muscle damage. As our results show, α-actin was the most significant skeletal muscle damage marker analysed. We therefore propose it as candidate for the identification of all types of myocardial injury.

REFERENCES