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## CORRECTION

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There was an error in the article by Guerrero *et al* published in the July issue of the journal (Guerrero M, Guiu-Comadevall M, Cadefau JA, *et al.* Fast and slow myosins as markers of muscle injury. *Br J Sports Med* 2008;**42**:581–4). Table 1 was omitted from the article. The table is reproduced online at <http://bjism.bmj.com/supplemental/>.

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**FAST AND SLOW MYOSINS AS MARKERS OF MUSCLE INJURY**

**Key words:** muscle injury, serum muscle markers, fast myosin, slow myosin.

**Word count:** 3000

## **ABSTRACT**

**Objective:** The diagnostic of muscular lesions suffered by athletes is done through a clinical diagnosis accompanied by confirmation tests using imaging techniques on the lesion (ultrasonography and/or magnetic resonance) as well as laboratory techniques for blood analysis that detect usually the presence of non-specific muscle markers .

The aim of the study is the evaluation of fast and slow-twitch fibres injury using specific muscle markers for those fibres.

**Methods:** Blood samples were obtained from 51 non-sport people and 36 sportsmen with skeletal muscle injury. Western blood was performed to determine fast and slow myosin and creatin kinase. Skeletal muscle damage was diagnosed according to physical examination, ultrasonography and magnetic resonance and biochemical markers criterion.

**Results:** The imaging tests have been shown to be excellent for the detection and confirmation of Grade II and III lesions. However, Grade I lesions often remain unconfirmed by these techniques. Grade I lesions show higher fast myosin than slow myosin with very low CK increase. Grade II and III show high values of both myosin.

**Conclusions:** The evaluation of fast and slow myosin in the blood 48 hours after the lesion occurs has been seen to be a good parameter for the detection of Type I lesions specially, based on the fact that fast myosin is an exclusive skeletal muscle marker. The correct diagnosis of Grade I lesions can promote prevention of the injury's progression in athletes undergoing continual training sessions and competitions, thus aiding sports physicians in their decisions.

**Key terms for indexing purpose:** muscle injury, serum muscle markers, fast myosin,

slow myosin.

## **INTRODUCTION**

Muscle is sensitive to the protocols of contraction and work to which it is submitted, since its structure is prepared to support these protocols and to adapt to new situations of force. However, if integrity is affected, to a greater or lesser extent, by overload, with tears occurring which we call muscle lesions. These lesions can, again to a greater or lesser degree, produce incapacity to continue exertion of the force.

Extenuating unaccustomed exercise and high-force eccentric action leads to skeletal muscle damage, with changes in muscle structure and function. It induces damage to muscle fibre membranes [1, 2] myofibrillar disruption [3] and sarcoplasmic reticulum vacuolization [4].

Such exercise-induced muscle damage activates a cascade of reactions that result in an activated skeletal muscle protein metabolism. The protease calpain is activate immediately after exercise. Calpain initiates the metabolic turnover of myofibrillar proteins by releasing them from their filamentous structure [5]. Although calpain does not degrade actine and myosin it contributes to their release [6]. This allows the detection of such proteins in peripheral blood after cleavage, using specific assays such as troponin I (TnI) and myosin heavy chains (MHC) [7,8]. Soricher et al. [9] exposed the features of an ideal marker of skeletal muscle fibre injury. One of these was that the marker should be absolutely muscle-fibre specific to allow reliable diagnosis of skeletal fibre type injury. None of the markers analyzed by these authors is muscle-type specific. The markers habitually used, such as CK, H-FABB, Mb, TnI or  $\alpha$ -actin [10], in addition to not being totally specific for skeletal muscle, reach a maximum value before 10 hours have elapsed after the origin of the lesion and decrease considerably before 24 hours

have elapsed after the stress situation. The greater part of lesions is produced on holidays, so that it is very easy for these 10-12 critical hours for carrying out analysis of the patient to have elapsed. Often, the recently produced lesions are not accompanied by pain, and a day later can be enough for the markers of low molecular weight to have degraded and left no trace in the serum. The troponins are proteins that are very specific in terms of fibre type, being of low molecular weight but susceptible of being rapidly proteolyzed, which may be the reason why they have a very short half-life in blood [11].

Skeletal muscle is a tissue with a heterogeneity of fibre types, type I and II, the proportion of which varies with the type of muscle and even within the different regions of the type of muscle [12]. Some of the contractile proteins present different isoforms according to the type of fibre. One of these is myosin, which can present different heavy and light chains isoforms, according to whether the fibre type is fast or slow [13].

Myosin presents an ideal profile as a parameter to study and is directly assignable to the grade of the lesion since, due to its high molecular weight, its appearance in blood can only be explained by a fibre lesion. Fast myosin is only characteristic of fast skeletal muscle, while slow myosin is only common to skeletal and cardiac muscle. The maximum value of slow myosin in blood has been measured by Schiaffino and Reggiani [14] and presents its maximum 48 and 72 hours after the lesion.

The aim of the present paper is to evaluate muscle lesions, using as markers fast and slow myosins present in the serum of athletes 48 hours after having suffered a lesion. The efficiency of this maker will be compared with the detection of the lesion by ultrasonography (US), magnetic resonance (MR) and other traditional serum markers.

## **METHODS**

**Materials:** Monoclonal anti-myosin (skeletal, fast) clone My-32 (Sigma), Monoclonal anti-myosin (skeletal, slow) clone NOQ7.5.4D (Sigma). Agarose (Sigma). Protein A (Sigma). Loading buffer Nupage<sup>R</sup> LDS sample buffer (Novex).

**Subjects:** 36 young male athletes, aged 18-25, practising the sports of athleticism, horse jockeying, tennis, football, basketball and the pentathlon were chosen on the basis of having suffered some pain and/or injury. Controls were sedentary males of 18-55 years old. This study was approved by the ethics committee of the University of Barcelona and the ethics committee of the Hospital Clinic i Provincial of Barcelona.

Muscle injuries were classified into three categories according to clinical findings: grade I (Doms and elongation, very little muscle tear); grade II (fibrillar disruption, moderate muscle tear); grade III (fibre disruption, evident muscle tear).

2 ml of blood were obtained from the 51 controls and from the athletes 48 hours after having suffered a muscle problem. The serum was used for myosin determination.

**Treatment of the sample:** After obtaining 2 ml of blood in a Vacutainer<sup>R</sup> tube, it was centrifuged at 2000xg at 4°C for 10 minutes. The serum could be kept at -80°C without any loss of myosin for 15 days. Serum protein was determined by the Bradford method [15]. Myosin as a blood marker is present at very low concentration compared with other serum proteins. For that reason it was concentrated from the serum sample by

immunoprecipitation with A protein linked to agarose-antibody. The pellet was resuspended with 15  $\mu$ l of loading buffer (60 mM HCl-Tris, 10% glycerol, 2% SDS, 5%  $\beta$ -mercaptoethanol, 0.025% blue of bromophenol, pH 6.8). It was centrifuged and warmed for 10 min to 70°C. The sample (about 25  $\mu$ l) was prepared to be carried out on a Nupage<sup>R</sup> Novex<sup>R</sup> 3-8% Tris-acetate gels and immunodetection.

One or two fast-myosin and slow-myosin standards were loaded in every gel. Electrophoresis was run in X Cell SureLock<sup>R</sup> Electrophoresis Cell (Invitrogen) at 150 V for 1h at room temperature. The gel was cut in strips of 7 cm x1.2 cm. The gel for immunoblot analyses were transferred to a PVDF sequencing membrane (Immobilon<sup>TM</sup> P<sup>SQ</sup> Millipore). The transference was done at 33 V for 70 min at room temperature.

The blots were treated with SuperBlocking Buffer in PBS (Pierce) at 0.01% to Tween20 for 1 hour and a half at room temperature and they reacted one gel with 1:90.000 anti-myosin fast monoclonal antibody and the other gel with 1:150.000 anti-myosin slow monoclonal antibody. Membranes were washed with 0,01% Tween 20 at PBS 6 times for 5 minutes and incubated with horseradish peroxidase conjugated rabbit anti-mouse IgG (1:90.000) for 1 hour at room temperature, followed by additional washes (5 minutes 6 times in 0,01% Tween 20 at PBS). Each strip was mixed with 80  $\mu$ l of a mix (1:1) of Ultra Supersignal for 5 min. Proteins were visualized by enhanced chemiluminescence (SuperSignal<sup>R</sup> West Dura Trial Kit. Pierce). After drying the strips they were printed on the Hyperfilm TM ECL for 10 min. This process was prepared with two samples. One was for detection of fast-myosin and the other for slow-myosin. The films were scanned with a Hewlett Packard Scanjet 5200C and quantification was done Quantity One 1-D ( BioRad).

**Fast and slow-myosin standard:** The protein standards were prepared from fast and slow rabbit muscle (TA for fast-fibres and Soleus for slow fibres). Purified myofibrils were obtained according to the method described by Hasten et al., [16]. After carrying out this process, we got myofibrils suspension of about 1 mg/ml of protein. The suspension was denatured with the loading buffer 1:1 v/v for 2 min at 100°C. 70 µg of protein was applied to an acrylamide gel (7.5% T, 2.5% C) to separate the proteins. The electrophoresis was run for 1h 15 min at 150 V. The presence of myosin was localized by staining a gel with Coomassie Blue G-250 R-250 BioRad. The bands from an undyed gel were cut and electroluted with a commercial electroluter (BioRad mod. 422) during 60 mA, 5h with the electrolution buffer (25 mM HCl-tris, 192 mM glicine, 0.1% SDS, pH 6.8). We obtained 0.93 µg/µl of fast myosin and 1.55 µg/ µl of slow myosin. The myosins were diluted with 10 mM HCl-Tris, 300 mM NaCl, 2 mM EDTA, pH 6.8. Standards were kept in fractions of 50 µl at -30°C.

**Measurement of enzymatic activities:**

Creatine Kinase (CK) was determined by a Technicon DAX System autoanalyzer, Clinical Method, Bayer Publication n0. SM4-1140L95 (1995) according to the method of Szasz et al. [17].

***Imaging evaluation***

The echographies were carried out at the CEARE and in the Ultrasonographic Department of the FIATC Clinic. Toshiba Medical System ultrasonography equipment with multi-frequency probe (Just-Vision in CEARE, PowerVision in FIATC). The MRs

were done at the Department of Magnetic Resonance of the Corachán Clinic, using a Siemens Symphony device (1.5 TESS).

Both types of soundings obtain results whose evidence increases in direct proportion with the extent of the lesion grade. Thus, in Grade I lesions US usually images the lesion (haematic suffusion and defect of some fibres) two or three days after the accident, while MR shows muscular oedema from the first moment. Grade II lesions show oedema and fibrillar defect both in US and in MR, and Grade III lesions image a greater defect associated with haematoma and muscle. Both US and MR serve as evolutive control of the lesion, observing how the oedema goes away and fibrillar repair appears.

### ***Statistical analysis***

The values of the each marker at controls and different grades of injury were compared by analysis of variance (Anova). They were considered significant at  $P < 0.05$ .

## RESULTS

### 1. MR Images:

Figure 1 shows three MR images of different grades of muscle lesion. 1A): Grade I lesion in the upper part of the posterior side of the right thigh. We observe, in the axial image, a zone of muscle oedema (signal increase) transduced by the recent biceps femoris tear (arrows). 1B): Grade II lesion in the medial-distal part of the posterior side of the left thigh. We observe, in the axial image, an area of oedema (signal increase) and fibrillar defect transduced by the recent biceps femoris lesion (long head) (arrows). 1C): Grade III lesion in the distal part of the anterior side of the thigh. We observe, in the coronal image, a large area of oedema (signal increase) and extensive fibrillar defect of the rectus femoris, in the distal part (arrows).

### 2. US images

Figure 2 shows the ultrasonographic correspondence of the lesions shown in Figure 1. 2A): Grade I lesion. The US shows, in transverse section, the area of fibrillar defect located between the biceps femoris and the semitendinous. 2B): Grade II lesion. The US shows, in transverse section, the most extensive area of the fibrillar defect and haematic suffusion in the long head of the biceps femoris. 2C): Grade III lesion. The US shows, in longitudinal section, complete muscle defect of the rectus femoris (arrows).

### 3. Clinical diagnosis, marker enzymes, and myosin levels in normal and injured

Diagnostic	Number of samples	US	MR	CK ( U/l)	MYOSIN (µg/l)		Fast/Slow ratio
					Fast	Slow	
<b>Normal</b>	51	---	----	102±8	625±62	1535±166	0.3
<b>Grade I</b>	12	(-) o (+)	----	202±22	2880±159	1281±197	2.2
<b>Grade II</b>	16	++	++	482±47	3432±402	3722±700	0.9
<b>Grade III</b>	10	+++	+++	739±245	8055±2200	6518±124	1.2

**muscles**

**TABLE I. Comparison of the results obtained by different technologies of muscle injuries**

Data are expressed as means ± SEM. Statistical Anova showed to be extremely significant for every parameter (columns).  $P < 0.001$

Table I shows the normal values of the sera of the control group of athletes and of the athletes with different grades of muscle lesions. Our results indicate that in the normal state the concentration of fast and slow myosin in blood did not surpass 1000 µg/ml of fast myosins and 2000 of slow myosins, showing a fast/slow ratio of 0.3. The patients diagnosed with Grade I lesions, which are not imaged by US or MR, showed high levels of fast myosin (greater than the slow myosins), showing a fast/slow ratio greater than 2. The CKs were found practically within the limits of normality. In the Grade II and III lesions, which were diagnosed by US and MR, an increase in both fast and slow myosins was observed, although a fast/slow ratio near 1 was exhibited. The

concentration of slow myosins in comparison with fast myosins increased in direct proportion with the seriousness of the lesion. The CKs also showed an increase in the same direction as the muscle lesion, increasing in the same way as the slow myosins, showing itself to be a very good marker for type II and type III lesions, especially.

## **DISCUSSION**

The different human muscles are made up of a mixture of slow and fast fibres, approaching 50%, unlike what occurs in some animals who have muscles with 90% of only fast fibres and others with 90% of only slow fibres. Concretely, the vastus lateralis of young athletes between the ages of 15 and 18 years and of caucasian race have 36.5% of slow type fibres and 63.5% of fast type fibres, and of \*these, 52.3% are type IIa, 8.1% type IIb and 3.1% of type IIc. [18] The existence of mixed muscles in humans makes lesions the cause of the entrance of slow and fast myosins into blood. However, due to the fact that resistance to lesions and to fatigue in the two types of fibres is not the same, slow or fast myosin heavy chain (MHC) could be found in blood according to the type of lesioned fibres. In general, the rapid fibres are more easily fatigued and more sensitive to the lesion. Therefore, we can expect that the fast fibres that tire more rapidly release fast MCH before the slow fibres in the face of less intense exercises. The slow MCHs will flow under more fatiguing conditions and probably their presence in blood will be the expression of a more important lesion.

On the other hand, the presence of fast MCH in blood signals the fact that only skeletal muscle is affected and thus constitutes an absolutely specific marker. The presence of slow MHC could indicate the presence of muscle lesion in skeletal and/or heart muscle. However, given the fact that the patient types undergoing the test are athletes in which a cardiac lesion is ruled out, the detection of MHC in blood would respond as a slow fibre lesion marker, with the consequences that this information would be able to contribute.

We have developed a method to detect myosins in blood based on specific recognition by fast and slow myosin anti-bodies. We studied a group of athletes who practice different sports and who presented for consultation with muscle pains. A medical examination was given to these patients, as well as an US, an MR, and certain blood analyses which included evaluation of the presence of CK used as a usual muscle marker, as well as slow and fast myosins. We also studied 51 people who did not practice any sport either sporadically or for pleasure and who were catalogued as normal.

Thus, as shown, 48 hours after the onset of the muscle problem, the CK markers show a small increase in the Grade I lesions. Only fast myosin is a marker with very high values suggesting that Grade I lesions are mainly produced in Type II fibres. The results of the US and MR in the Grade I lesions are confused/ambiguous in some cases, yielding negative or positive results that fail to assure total recognition of the lesion. The US and MR techniques in Grade II and Grade III lesions are highly effective, since they detect the lesions via images.

In the Grade II lesions, the serum markers show an increase in CK, with the greatest increases being those of both types of myosin, which can reach as much as 10 times the normal value (in some case). The Grade III lesions are well detected by the CK markers, and also by both types of myosin.

Our conclusions are that the use of fast myosin provides a highly sensitive marker for Grade I lesions, at least equal to the MR sounding and greater than US and clinical

diagnosis. On the other hand, fast myosin is a totally specific marker for skeletal muscle, a property not shared by any of the markers in use today, such as CK and myoglobins. Myosin also presents the advantage of being more sensitive and more stable in blood, since it presents a maximum 48 hours after the lesion and lasts longer, so that it is easier to use it in diagnoses not carried out at that moment, and it can be used as a parameter to follow the evolution of the muscle lesion. Therefore, we conclude that the determination of fast and slow myosins is a good system to aid the diagnostic of muscle lesions, especially for those that are difficult to detect by other procedures.

#### **ACKNOWLEDGEMENTS**

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**FIGURES**

**Figure 1**

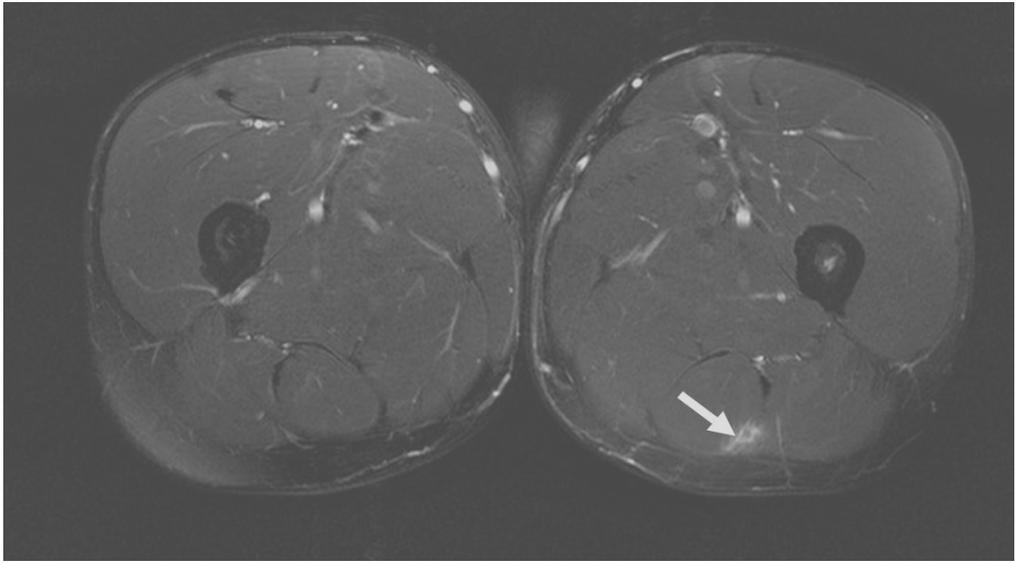
**Magnetic resonances of different grades of muscle lesion:**

A) Grade I lesion. B) Grade II lesion. C) Grade III lesion.

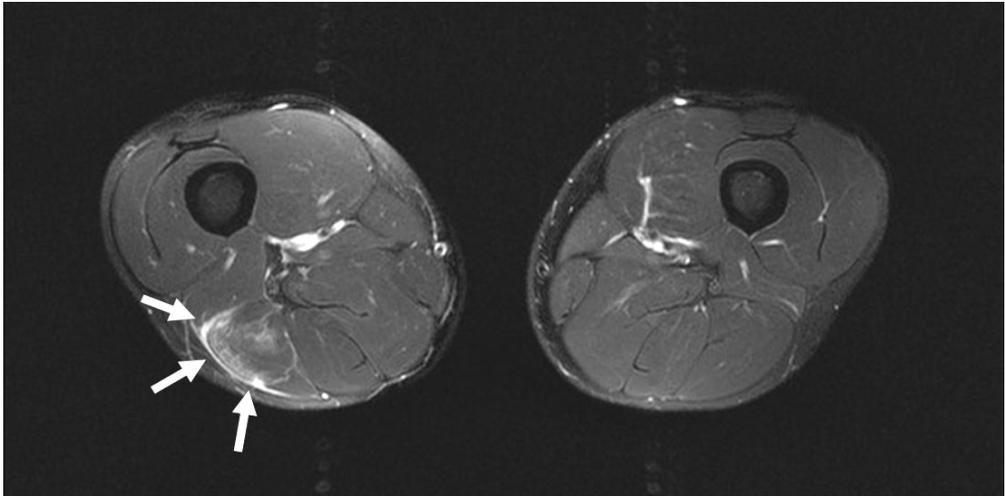
**Figure 2**

**Ultrasonography of different grades of muscle lesion:**

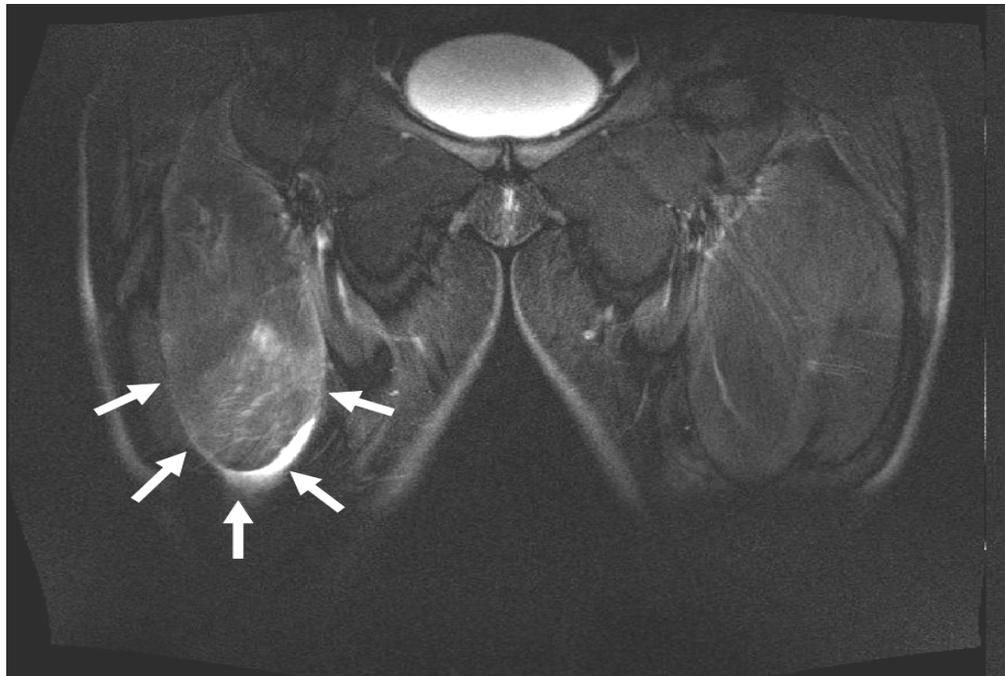
A) Grade I lesion. B) Grade II lesion. C) Grade III lesion.



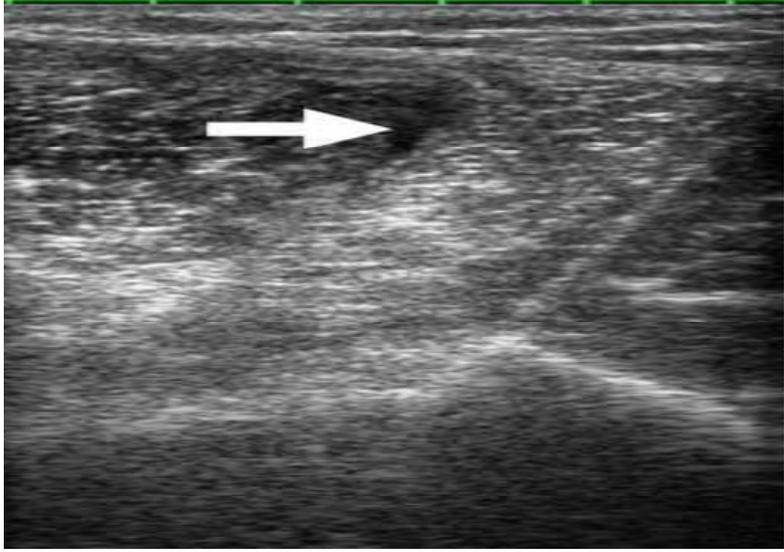
**A**



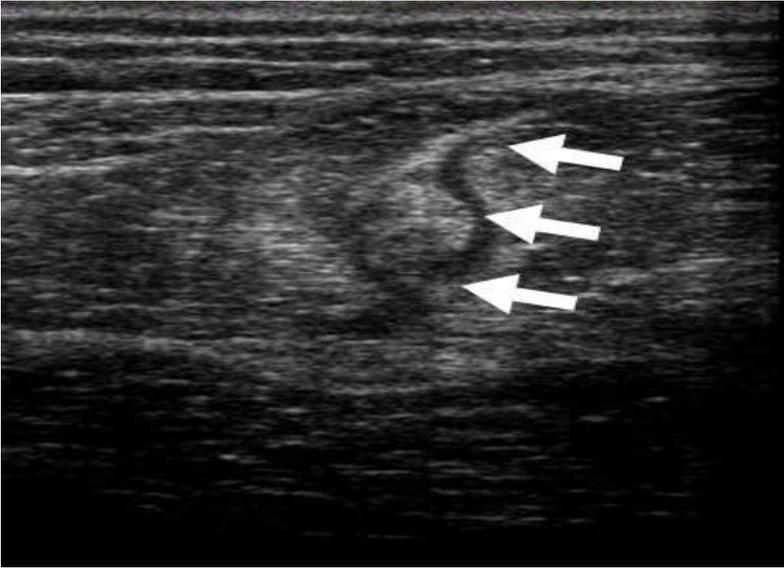
**B**



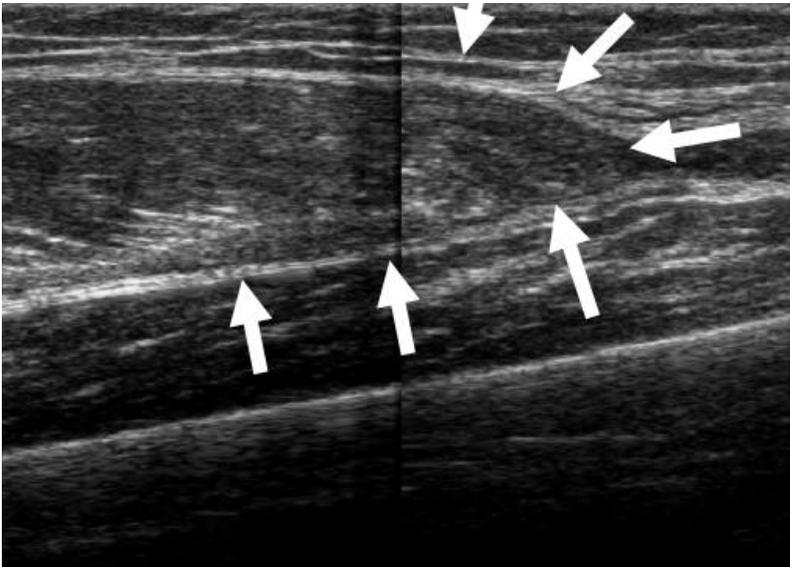
**C**



**A**



**B**



**C**