

**MECHANISMS OF HUMAN SKELETAL  
MUSCLE REMODELING IN RESPONSE TO  
CONCENTRIC AND ECCENTRIC LOADING  
PARADIGMS**

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A thesis submitted to the Manchester  
Metropolitan University in accordance with the  
requirements of the degree of Doctor of  
Philosophy

2014

## **Abstract**

It is common knowledge that resistance exercise promotes muscle growth (hypertrophy) and increased strength and function: thus, regular exercise can help minimize the loss of muscle mass and function in healthy ageing. Skeletal muscle can contract by either shortening or lengthening (*concentrically or eccentrically, respectively*). A substantial number of studies focused on the effect of concentric versus eccentric training protocols on muscle morphological and functional changes: eccentric contractions are generally thought to result in more increased muscle hypertrophy and strength, because of the higher force produced by the muscle and the more severe exercise induced muscle damage, which may lead to a stronger adaptations in muscle remodeling and repair processes. Study 1 shows that ECC and CON exercise protocols lead instead to similar gains in muscle size, but through different architectural remodeling mechanisms: moreover, acute contraction-specific molecular responses have been characterised. Study 2 and Study 3 were then performed in order to gain novel insights into the relationship between these morphological adaptations and the metabolic responses (MPS, muscle protein synthesis) of human skeletal muscle in response to chronic ECC vs. CON loading paradigms. Study 2 was first carried out in order to validate the use of deuterium oxide isotope tracing technique for measuring changes in MPS in free-living subjects over longer-term periods (compared to normal AA infusion studies) of resistance exercise. After assessing the feasibility of deuterium oxide tracing technique in measuring MPS response during resistance-training protocols, study 3 investigated the chronic responses in MPS to ECC vs. CON loading in two

different sites of the human vastus lateralis, presenting novel insights into MPS and skeletal muscle homogeneity, attempting to link MPS changes to the different mechanisms of muscle morphological remodelling occurring after ECC vs. CON training.

## *Symbols and Acronyms*

**Å** = Angstroms

**AA** = Amino Acid(s)

**ACSA** = Anatomical Cross-Sectional Area

**ADP** = Adenosine Di-phosphate

**AKT** = Protein Kinase B

**APE** = Atoms Percent Excess

**ATP** = Adenosine Tri-phosphate

**BSA** = Bovine Serum Albumin

**BMI** = Body Mass Index

**Ca<sup>++</sup>** = Calcium

**CO<sub>2</sub>** = Carbon Dioxide

**CON** = Concentric (*exercise / contraction*)

**COPD** = Chronic Obstructive Pulmonary Disease

**CPS** = Collagen Protein Synthesis

**CSA** = Cross-Sectional Area

**CT** = Computer Tomography

**DEXA** = Dual-Energy X-ray Absorptiometry

**D<sub>2</sub>O** = Deuterium Oxide

**ECC** = Eccentric (*exercise / contraction*)

**ECG** = Electrocardiography

**EDTA** = Ethylenediaminetetraacetic acid

**EGTA** = ethyleneglycol-bistetraacetic acid

**eIF4E** = Eukaryotic translation initiation factor 4E

**EMG** = Electromyography

**ERK<sup>1/2</sup>** = Extracellular Signal-Regulated Kinase

**FAK** = Focal Adhesion Kinase

**FOXO** = Forkhead Box O protein

**FSR** = Fractional Synthetic Rate

**F-V** = Force-Velocity Relationship

**GC-MS** = Gas-Chromatography Mass Spectrometry

**GSK3 $\beta$**  = Glycogen Synthase Kinase-3 Beta

**$^2\text{H}$**  = Deuterium

**$^2\text{H}_2\text{O}$**  = Deuterium Oxide

**HCl** = Hydrogen Chloride

**IGF-1** = Insulin-like Growth Factor 1

**I $\kappa$ B** = I $\kappa$ B Kinase

**IRMS** = Isotope-Ratio Mass Spectrometry

**Lf** = Fascicle Length

**L-T** = Length-Tension Relationship

**MAFbx** = Atrogin-1

**MAPK** = Mitogen Activated Protein Kinase

**MCME** = n-Methoxycarbonyl Ethyl Ester

**MGF** = Mechano-Growth Factor

**MHC** = Myosin Heavy Chain

**MLC** = Myosin Light Chain

**MT** = Muscle Thickness

**mTOR** = Mammalian Target of Rapamycin

**MTJ** = Myo-tendineous Junction

**mm** = Millimetre

**mM** = Millimola

**MRI** = Magnetic Resonance Imaging

**MuRF-1** = Muscle Ring Finger Protein-1

**MU** = Motor Unit

**MVC** = Maximal Voluntary Contraction

**MyoPS** = Myofibrillar Protein Synthesis

**N $_2$**  = Nitrogen

**NaF** = Sodium Fluoride

**NaOH** = Sodium Hydroxide

**NH $_4$ OH** = Ammonium Hydroxide

**p70S6K** = p-70S6 Kinase

**p38** = p38 MAP kinase

**p90RSK** = Ribosomal S6 Kinase p90 (part of MAPK family)

**PA =  $\theta$**  = Pennation Angle

**PCA** = Perchloric Acid

**PCSA** = Physiological Cross-Sectional Area

**PHAS-1** = 4EBP1 = Eukaryotic Translation Initiation Factor 4E-binding protein

**PI3K** = Phosphoinositide 3-Kinase

**PVDF** = Polyvinylidene fluoride

**RET** = Resistance Exercise Training

**RF** = Rectus Femoris Muscle

**RMS** = Root Mean Square

**ROM** = Range Of Movement

**RT** = Resistance Training

**SEM** = Standard Mean Error

**SD** = Standard Deviation

**TBST** = Tris Buffered Saline with Tween 20

**TC/EA-IRMS** = High Temperature Conversion Elemental Analyser IRMS

**TSC1-TSC2** = Tuberos Sclerosis Complex 1-2

**VI** = Vastus Intermedius Muscle

**VM** = Vastus Medialis Muscle

**VL** = Vastus Lateralis Muscle

**VOL** = Muscle Volume

*Dedicato a Mamma e Papa'.*

*“...fatti non foste a viver come bruti, ma per seguir virtute e  
canoscenza”*

*[Dante Alighieri, Inferno, Canto XXVI]*

# MECHANISMS OF HUMAN SKELETAL MUSCLE REMODELING IN RESPONSE TO CONCENTRIC AND ECCENTRIC LOADING PARADIGMS

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"The power of locomotion is that which contracts and relaxes the muscles whereby the members and joints are moved, extended or flexed. [...] Each muscle has its own peculiar purpose and it obeys the decree of the composite sense" (*Avicenna, early 11<sup>th</sup> century*).

## **Introduction**

### *1. Skeletal muscle structure*

Skeletal muscle is a very unique tissue: its capacity to generate force and to enable purposeful movement plays a key role in locomotion. Not only skeletal muscle is the human's body most abundant tissue (40-45 % of its total composition), but it also has the peculiar ability to adapt to a large variety of exogenous or endogenous stimuli, such as physical training, unloading, feeding and substrate availability, clinical and pathological conditions. Skeletal muscle fibres are highly specialised multinucleated cells that have the capacity to contract; these cells are arranged hierarchically within the



muscle, from the largest scale (*microns*,  $10^{-6}$  m,  $\mu\text{m}$ ) to the smallest one (*angstroms*,  $10^{-10}$  m,  $\text{\AA}$ ) (Fig 1).

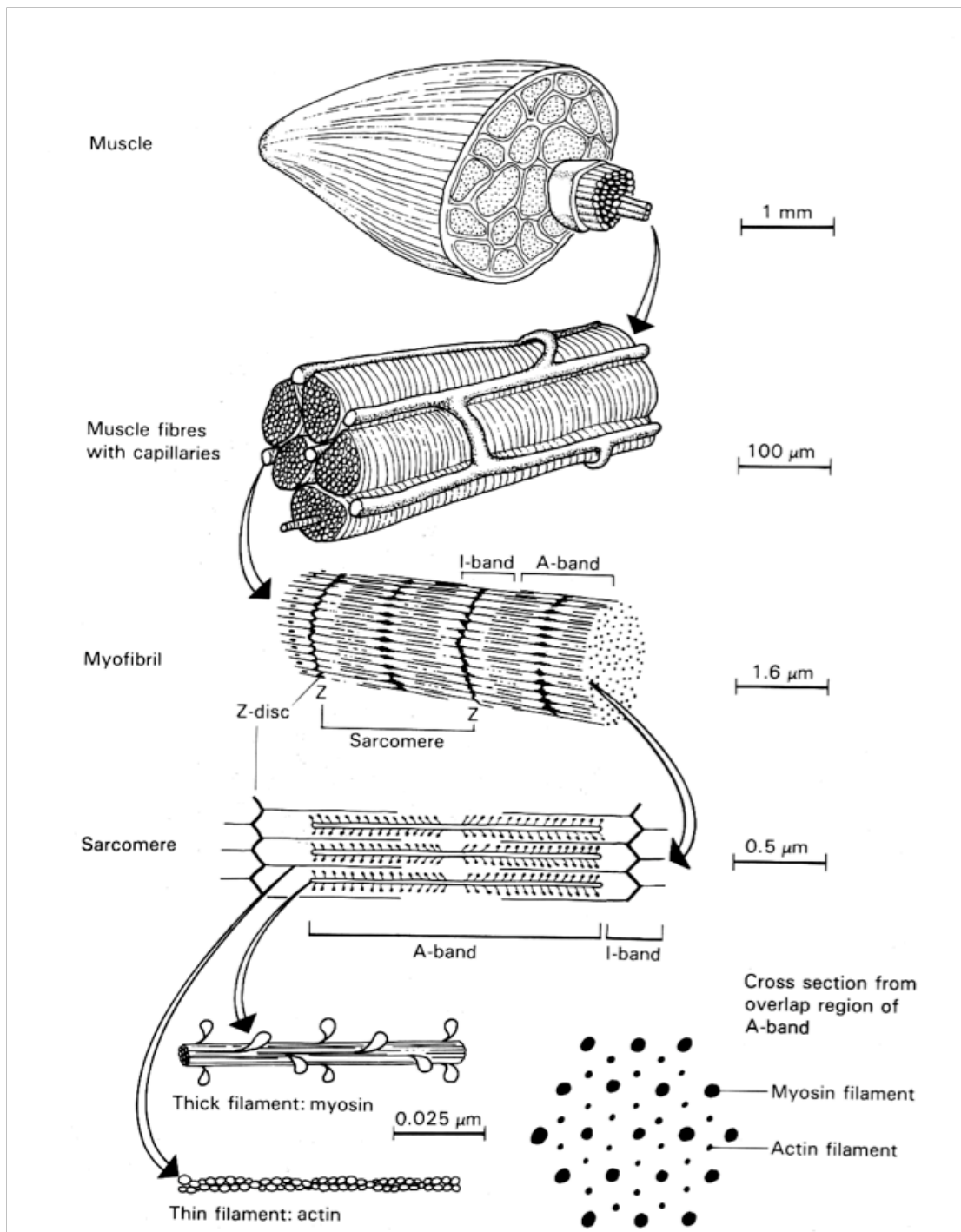


Fig 1. (from Billeter & Hoppeler, in Koomi et al., *Strength and Power in Sports*, 2003)

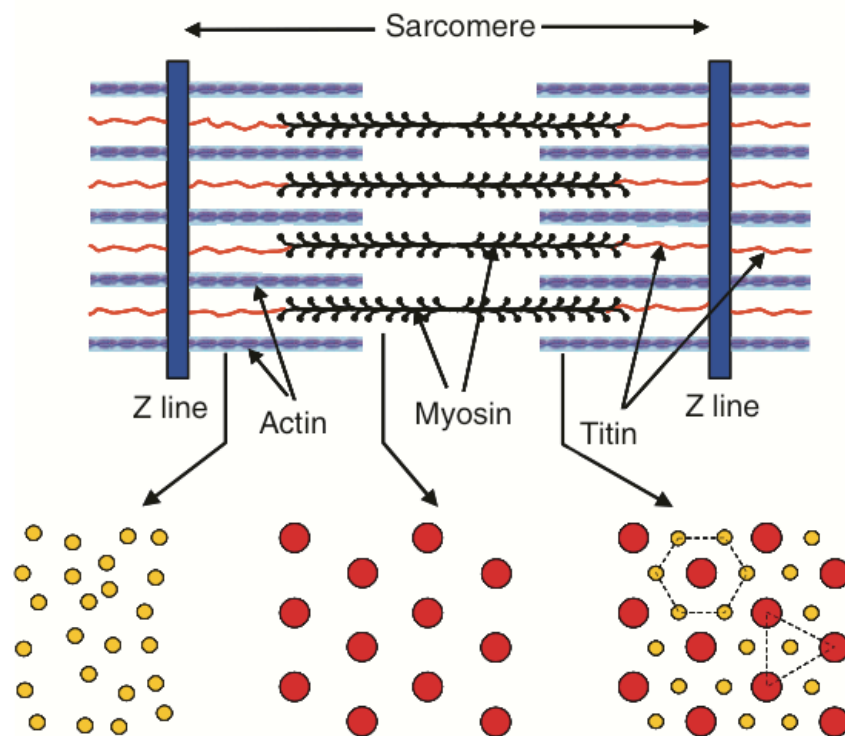
Skeletal muscle presents different layers of collagenous connective tissue

that divide and link muscle fibres together at the same time. The muscle *fascia* surrounds the muscle itself: right below the fascia, a thinner layer of connective tissue, the *epimysium*, covers the whole muscle. Underneath there are bundles of 10 to 100 of muscle fibres, the muscle fascicles, surrounded by the *perimysium*. Another thinner matrix of connective tissue called *endomysium* divides each single fibre from another one. The cell membrane surrounding each muscle fibre is known as *sarcolemma*; the fluid enclosed within the fibre and the sarcolemma is called *sarcoplasm*. A muscle fibre is composed of *myofibrils* arranged in parallel, which are simply 1µm diameter strings of *sarcomeres*, subunits of the myofibrils arranged in series. Thus, muscle's contractile unit is the *sarcomere* (*from 2 to 2.5 µm long*), composed by a specific arrangement of two main protein filaments, *actin* and *myosin*. Bundles of 100 to 400 *myofilaments* form myofibrils and about 2000 myofibrils form an adult muscle fibre.

### *1.1 The Sarcomere: contractile and structural proteins*

Sarcomeres are the functional units of muscle contraction, and can be seen as very little bricks placed one next to the other forming a muscle segment (the myofibril). As mentioned, each sarcomere is composed of myofilaments, one relatively thick called myosin and one thinner called actin. Myosin presents an anti-parallel tail-to-tail arrangement and is composed of two heavy chains (MHCs) and two light ones (MLCs) which combine together to form the thick myofilament structure. Two main myosin fragments can be distinguished: the globular head represents the actin-binding site while the tail conglomerates with other myosin tails. Actin is a globular protein

composed by two strands of polymerized actin monomers forming a double helical arrangement. This thin filament is attached to each Z line (the “borders” that delimit a sarcomere) and runs towards the centre of the sarcomere (Fig 2).



*Fig 2. Geometrical organisation of a sarcomere and actin and myosin spatial arrangement (from Jones DA et al. 2004)*

Two different proteins are in close interaction with actin: troponin and tropomyosin, which regulate the relationship between actin and myosin. Tropomyosin lies on seven actin subunits and blocks the sites where the myosin head can bind. Troponin, instead, is positioned every seven monomers of actin and consists of three different components, each of them with a specific role: troponin T binds tropomyosin, troponin C is receptive for

calcium ions ( $\text{Ca}^{++}$ ) and Troponin I represents the inhibitory subunit. The characteristic interaction between all this whole complex of proteins will be discussed in the cross-bridge cycle session.

Actin and myosin “interdigitate” between each other in a distinctive hexagonal arrangement, in order to produce muscle shortening and generate muscle force: each myosin filament is surrounded by six actin filaments and so each thick filament may bind to any of the six thin filaments. Conversely each actin filament can interact with three different myosin filaments (Fig 1 and 2). Various regions of the sarcomere can be described (Fig 3): the myosin filaments are situated mostly in the middle of the sarcomere, area known as A-band (H is the zone where there is no overlap between the different protein filaments); the region containing the actin filaments, called I-band surrounds the A-band on its sides and is situated next to the Z line, that represents the end of the single sarcomere unit, where the thin filaments are held in a square array. Therefore, one sarcomere dimension can be calculated from one Z- band to the other.

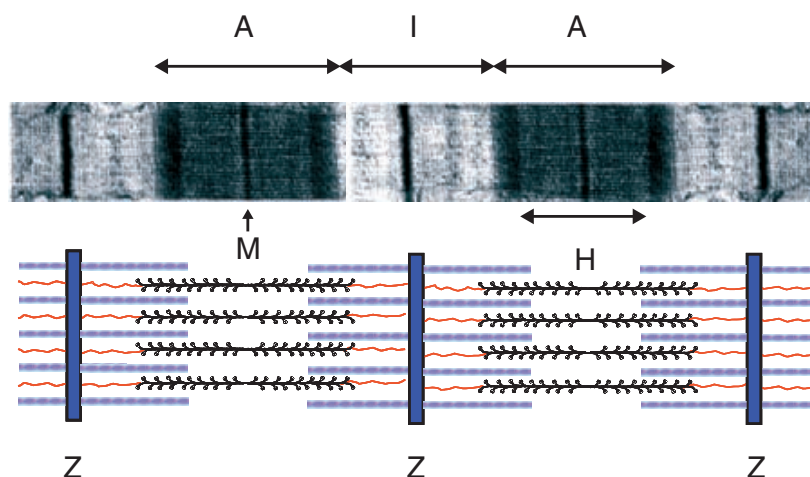


Fig 3. Sarcomere's structure (from Jones et al. 2004)

### 1.2 The cytoskeletal complex: structural proteins

There are a variety of additional structural proteins necessary for muscle contraction, as their task is to maintain the architecture of the sarcomere: these proteins and structures form the *Cytoskeleton*. The ultimate role of the whole cytoskeletal complex is to maintain the orientation of actin and myosin within the sarcomere (*endosarcomeric cytoskeleton*) and the alignment of myofibrils between each other (*exosarcomeric cytoskeleton*). Two of the main structural proteins of the sarcomere are titin and nebulin. The first is responsible to connect myosin to the Z band, keeping myofilaments aligned and giving longitudinal steadiness to the sarcomere; nebulin, instead, regulates the length of actin filaments. Another sarcomeric structural protein,  $\alpha$ -actinin, binds the actin filaments together at the Z line.

The cytoskeletal structures developed outside the sarcomere include proteins called *intermediate fibres* and *focal adhesions*. Intermediate fibres proteins, such as desmin, link adjacent myofibrils and are responsible for the alignment of Z lines. Focal adhesions connect intracellular proteins to extracellular space, connecting myofibrils to sarcolemma and muscle fibres to the myotendineous junction. Dystrophin belongs to the focal adhesions family and its task is to link the contractile apparatus to the sarcolemma.

### 1.3 The cross-bridge cycle: the sliding filament theory

As previously mentioned, myosin interacts with actin to generate force: when

the globular heads of myosin interdigitate with the actin corresponding site; the phenomenon takes the name of *cross-bridge cycle*. The cycle is consisted of three main phases: detachment, activation and attachment of myosin globular head (Enoka, 2008). Cycle begins with ATP (adenosine triphosphate) binding to the myosin head causing the detachment of the latter from actin; myosin head is weakly attached to actin at this state and ATP has been hydrolysed to adenosine diphosphate (ADP) + an inorganic phosphate. Successively,  $\text{Ca}^{++}$  binds to troponin, freeing the myosin-binding sites of the actin filament, so that the globular head can attach to actin: the inorganic phosphate is released whilst the power stroke of the cross-bridge cycle occurs, resulting in exertion of force at the contractile site. Myosin filaments are arranged so that actin filaments can slide between them and thus myosin can bind to actin at the same time; this is possible because while thick filaments remain fixed, thin filaments can slide in and out (Jones et al. 2004). These mechanisms constitute the sliding filament theory of muscle contraction (Huxley, 1974).

## *2. Functional and mechanical properties of skeletal muscle*

The observations regarding the interactions between contractile proteins, and consequently their influence on the behaviour of skeletal muscle contraction have been of great interest since the past century. In order to better understand the mechanisms of muscle force generation, we need to acknowledge that the force developed by a muscle is strictly related to the variation of muscle length and the velocity of movement.

### 2.1 Length-tension (L-T) relationship

Muscle force generation is dependent on the interaction between the contractile protein filaments and, consequently, the production of force is proportional to the extent of myosin and actin overlap. The force developed by a muscle during an isometric contraction varies with the change in length of the muscle itself. The classical studies by Gordon and colleagues (Gordon et al. 1966) and Sir Andrew F Huxley (Huxley & Simmons, 1971; Huxley, 1974) describe the relationship between muscle tension development at different muscle length on frog skeletal muscle fibres. The sarcomere *Length-Tension* curve (Fig 4) presents an ascending part (where force increases as sarcomere length does), a small plateau (where force is constant for specific sarcomere length) followed immediately by the descending limb (where force decreases as sarcomere length increase more).

As described by Gordon, Huxley and Julian (Gordon et al. 1966b), as the muscle fibre is stretched, no active muscle force is developed at a length of 3.65  $\mu\text{m}$  (micrometres): this occurs because no interdigitation between myosin and actin filaments is possible at this length, as thick filaments are 1.65  $\mu\text{m}$  long and thin filaments are 2  $\mu\text{m}$  long. Thus, there is no overlap of any contractile protein unless sarcomere length decreases and contractile filaments are able to interact and to produce force. In frog skeletal muscle fibre the tension generated begins to grow as sarcomere length goes from 3.65  $\mu\text{m}$  to 2.2  $\mu\text{m}$ : at that point, tension reaches a plateau (*maximum tetanic*

tension) between 2.2 and 2  $\mu\text{m}$  of sarcomere length. The plateau region results in greater filament overlap and tension is constant as no more cross-bridge interactions are able to occur. If the sarcomeres are further shortened, the actin filaments from one side of the sarcomere overlap with the ones of the opposite side, resulting a “double-overlap” situation, which affects the cross-bridges formation and, as a consequence, the force generated by the contractile material. As a result, the tension registered between 2 and 1.7  $\mu\text{m}$  declines as the sarcomere shortens: after further shortening (sarcomere length  $< 1.7 \mu\text{m}$ ) the myosin filaments come into contact with sarcomeres Z line and fibres and tension production dramatically drops. Human skeletal muscle L-T relationship behaves very similarly to the frog’s one, but optimal sarcomere length (where maximum tetanic tension can be developed) has been found between 2.6 and 2.8  $\mu\text{m}$  in humans (Walker & Schrodt, 1974), because of the longer actin filaments of human sarcomeres compared to frog (myosin length does not change).

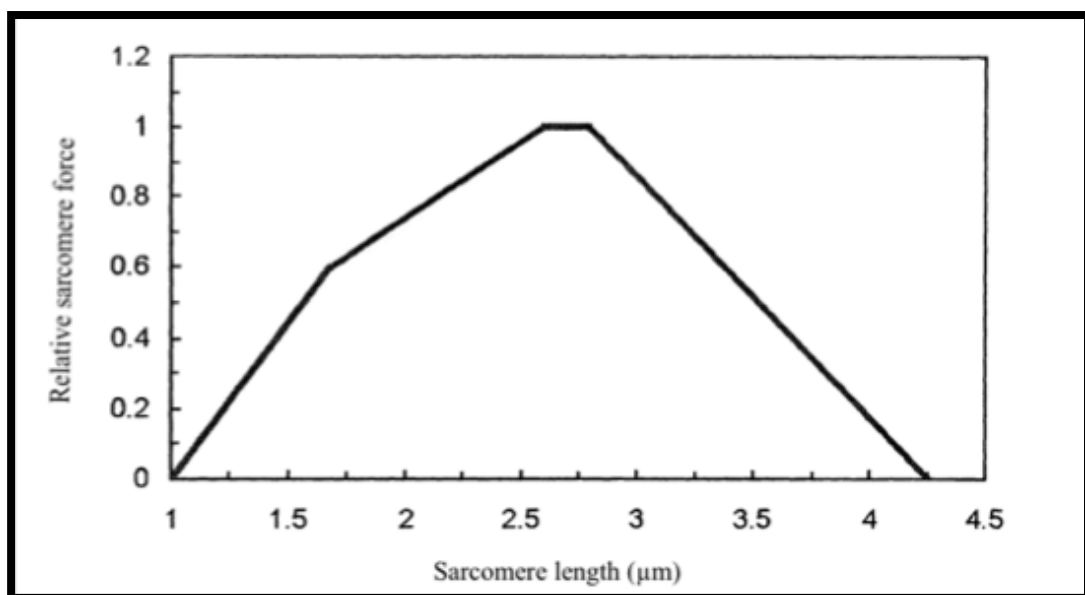
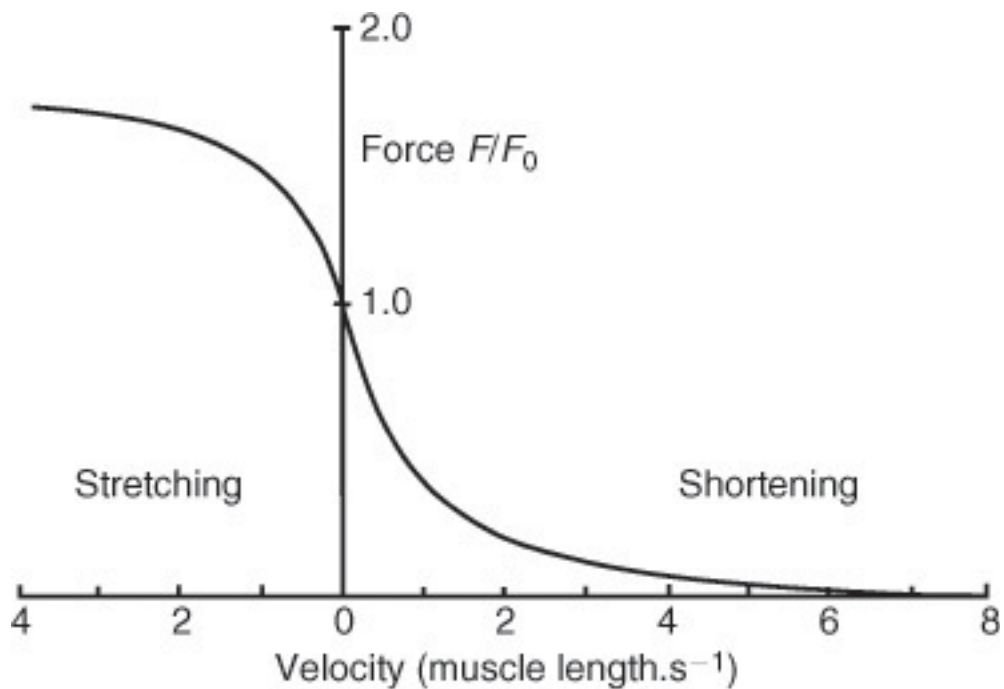


Fig 4. Length tension relationship in human skeletal muscle fibres (from Carter,



## 2.2 Force-Velocity (F-V) relationship

The force developed by a muscle is not just dependent on sarcomere length and cross-bridges formation, but it can also vary with the velocity of contraction. Hill first (Hill, 1938) and Katz just one year later (Katz, 1939) clearly showed how force values change with the increase or decrease of velocity of shortening and lengthening of skeletal muscle. Their findings have been used since then to describe the force-velocity relationship of a muscle (Fig 5).



*Fig 5. Force-velocity relationship of skeletal muscle during lengthening and shortening phases (from Jones et al. 2004).*

The voluntary muscle activation necessitated to lift a certain load usually results in a contraction, in which the muscle begins to shorten. The movement occurring when muscle exerts force while shortening is known as

*concentric contraction*. Hill's work (1938) described the behaviour of skeletal muscle force when the velocity of shortening changes, thus during concentric actions. The shortening part of the F-V relationship is a rectangular hyperbola (Fig 5) and shows that muscles generate less force with increasing the velocity of shortening. Therefore, as the load required to be lifted becomes lighter, the velocity of movement becomes greater until muscle reaches its maximum velocity of shortening, at which F will be equal to 0. Conversely, the more the velocity of contraction diminishes the greater is the force developed. Thus, the heavier is the load to lift, the slower will be the velocity of shortening, until the latter will get close to 0 value. The force generated when the velocity of movement is 0 takes the name of *isometric force* (isometric = having equal dimension, thus the muscle exerts tension but with no real joint movement involved); this also represents the maximum value of force that can be generated while the muscle shortens.

As the load applied on a muscle increases, it will reach a point where "the external force on the muscle is greater than the force the muscle can generate" (Lieber, 1992). Consequently, the muscle is forcibly lengthened even if it may be fully activated. The movement occurring when a muscle exerts tension while lengthening is known as *eccentric contraction*. Katz's work on relationship between force and speed of contraction in muscle (1939) has been complementary to Hill's findings: while Hill showed how force behaves related to the velocity of shortening of a muscle, Katz well presented what occurs to tension generation capacity when a muscle is lengthened. By examining the left part of the F-V curve (Fig 5 - stretching part, i.e. eccentric contraction), it can be easily observed that: - 1) not only

the maximum tension that can be generated while the muscle actively lengthens is greater than the one developed in isometric and shortening contractions, but also that - 2) the absolute tension does not depend of lengthening velocity.

Regarding the first point, previous studies (Katz, 1939, Lombardi & Piazzesi, 1990) showed *in vitro* that with the increase of lengthening velocity, muscle force developed rises until it reaches a plateau at a value close to 1.8 times the maximum isometric one. Even though *in vivo* the force values differs from the *in vitro* situation (for reasons that will be explained in the next sections), eccentric muscle force still registers greater values than the ones generated by either isometric or concentric actions (1.2 times circa) (Aagaard et al., 2000 Westing et al., 1988).

### *2.3 Theories on the mechanics of shortening vs. lengthening contractions*

The F-V relationship explains how muscular force behaves when a muscle shortens or lengthens at different velocities. What mechanisms lay behind the varying of force generation between velocities and types of contraction?

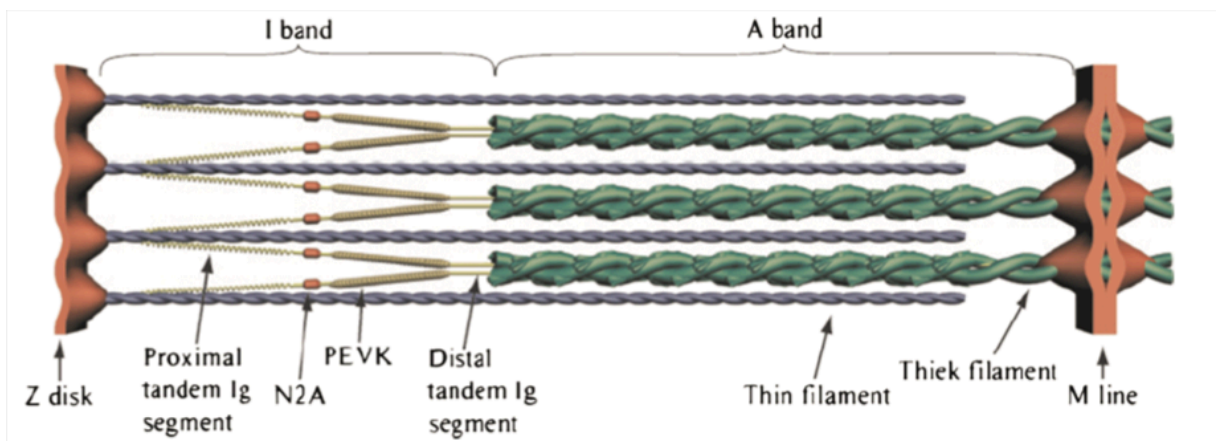
Observing the F-V curve, during shortening contractions, the force developed is always less than the isometric one (maximum value): this occurs because, the quicker the movement, the less is the number of cross-bridges that will form. The greater the velocity of contraction, the shorter is the time in which myosin can bind to actin. Moreover, during a fast movement, the S2 complex of myosin (i.e the flexible fragment of the myosin tail close to the globular

head) will not be fully extended, resulting in a S2 portion compression and in consequent less pulling force applied by the thick filament on actin.

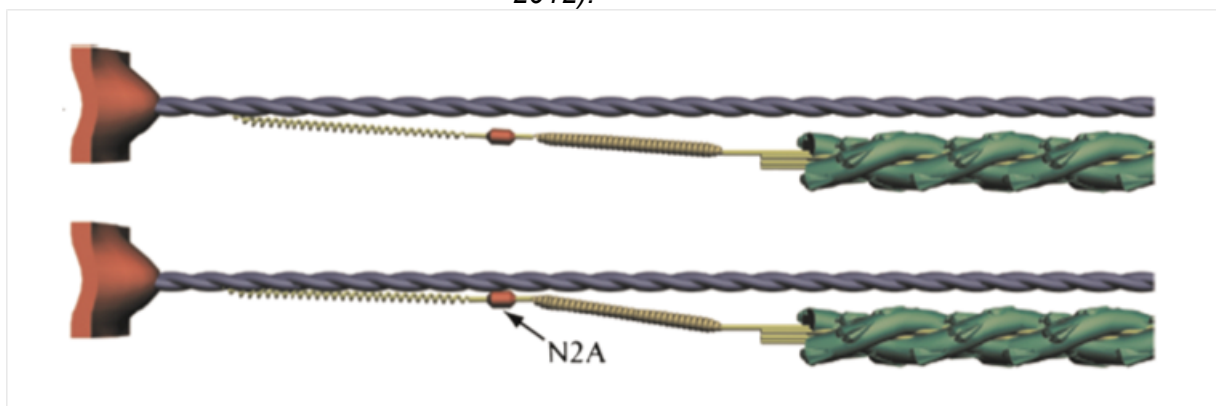
When velocity of movement becomes a value closer to 0, then not only a larger number of cross-bridges will be attached, but furthermore myosin S2 complexes are fully stretched and able to pull onto the actin and to produce bigger values of force. If the muscle is performing an eccentric contraction, the values of force developed exceed the ones during concentric and isometric actions. According to Huxley's model (1974), this increase in force could be due to a further greater stretch of S2 portions of myosin, occurring at first at slow velocity of stretching (Jones et al. 2004). As the lengthening becomes faster, fewer myosin heads will be able to bind to actin, but a good number of them will just remain into the attached position; if these cross bridges are stretched even more, they will inevitably be forced to detach. The proposed mechanism is that these thick filament heads are able to re-bind to the thin filament very quickly, and maybe representing the reason why muscles are able to develop high values of force during long lengthening contractions and at lower energetic cost.

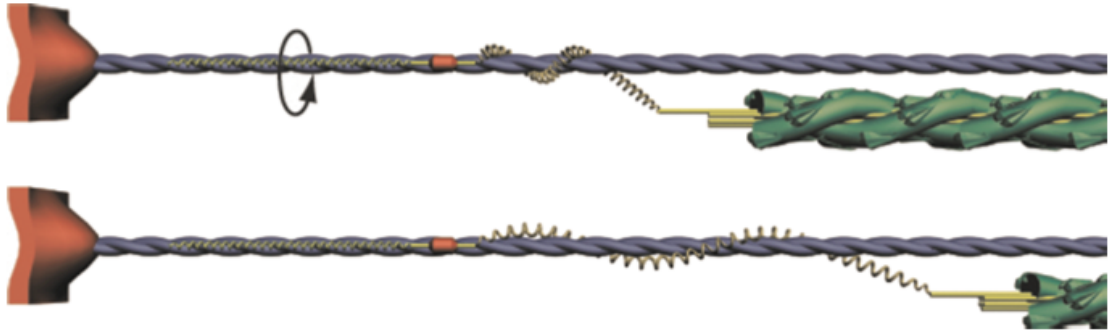
The latter explanation may provide a key of interpretation of why there is an enhancement of force during lengthening contractions; nevertheless, few other issues of Huxley's model are still poorly understood to the present day (Herzog et al., 2008), such as the depression of force with shortening and the low cost of force generation when muscle actively stretches. The work by Nishikawa (Nishikawa et al., 2012) suggested that the giant protein titin (Fig 6) could be involved in muscular contraction mechanics, acting as an internal spring able to store and release elastic potential energy. It has been

demonstrated that, during the cross-bridges cycle, actin rotates as myosin translates, (R. S. Morgan, 1977) (Fig 9). The theory proposed is the one where titin is seen as a “winding filament”, which is activated by  $Ca^{++}$  release and winds upon the actin filament when the latter is rotated by myosin translation in cross-bridges (Monroy et al., 2012) (Fig 7 & 8). The winding filament theory is explained below.



*Fig 6. Half-sarcomere illustration with layout of titin represented. Titin is formed by two segments (Ig and PEVK) connected by the N2A portion. (from Nishikawa et al. 2012).*





*Fig 7-8. Role of titin in active muscle. In the first picture titin is firstly represented in its resting position at low  $Ca^{++}$  concentration, then when calcium ions concentration becomes substantial, N2A portion binds to the actin filament, shortening and stiffening the titin spring. When cross-bridges cycle starts, the PEVK segment is winded on the actin filament by the thin filament rotation occurring while myosin translates (Morgan, 1977). In active sarcomeres, the PEVK segment is therefore stretched, and this enhances the force generated by a sarcomere, as titin acts as a spring, releasing stored elastic energy.  
(from Nishikawa et al. 2012)*

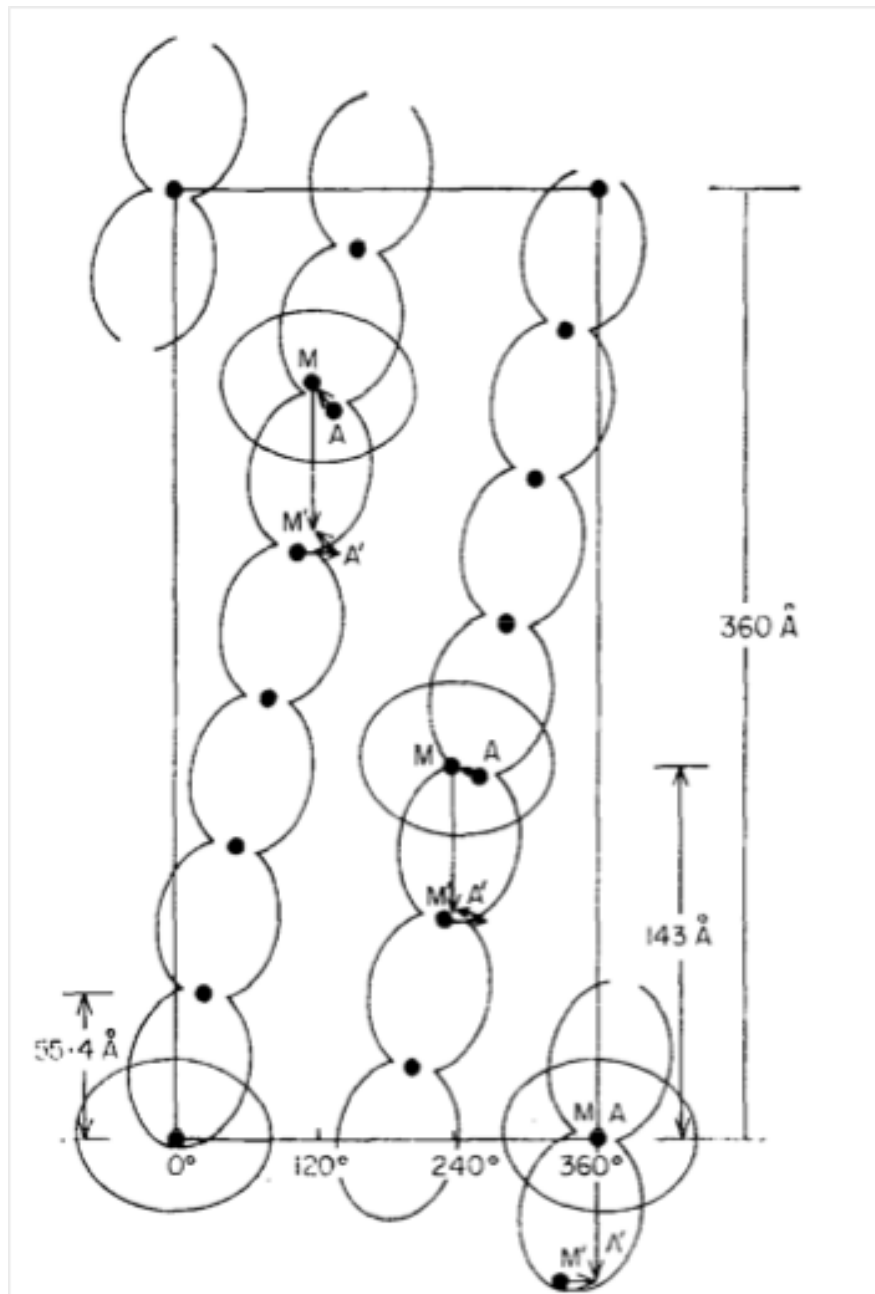


Fig 9. Representation of the superimposed turning of actin helix by three myosin filaments during cross-bridge phenomenon (from Morgan, 1977).

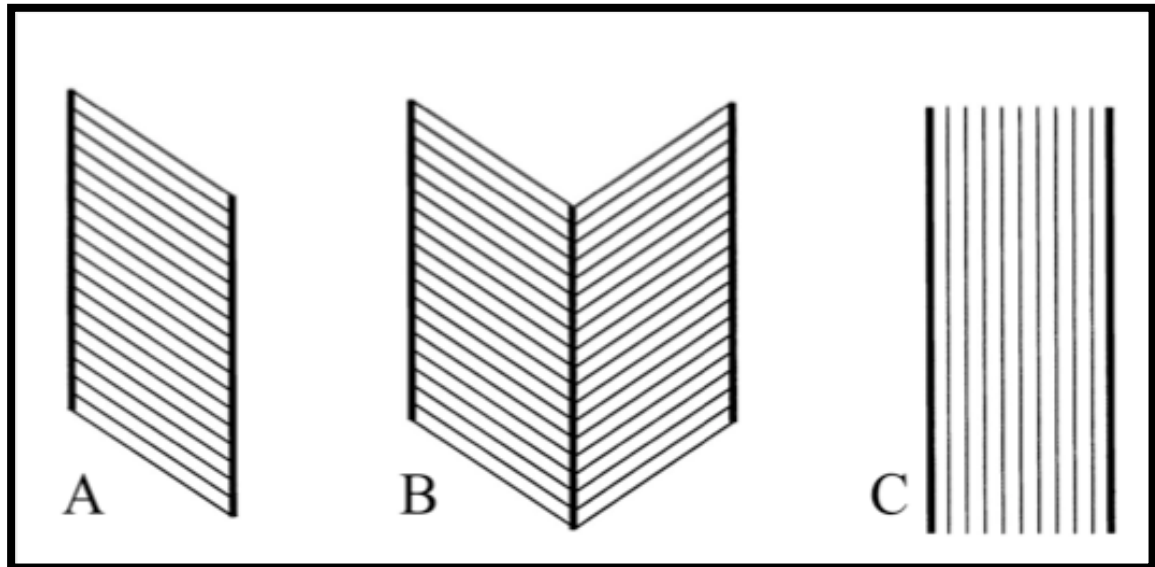
### 3. Muscle architecture

The functional features of skeletal muscle presented above explain how a muscle works in order to produce force and how the latter is dependent on velocity of contraction and muscle length. The internal macroscopic design of how muscle fascicles are arranged within the muscle is described as *muscle architecture* and it can influence the functional properties of muscles (Narici & Maganaris, 2006).

#### 3.1 Fascicle architectural arrangements and their significance

Skeletal muscle presents two main typologies of architectural models: generally, *parallel fibred* muscles are distinguished from *pennate fibred* ones (Fig 10). In parallel fibred muscles, fascicles run longitudinally and in the same direction of muscle-tendon unit action line whereas, pennate muscles fascicles are placed to form an angle (*pennation angle*) with the muscle-tendon line. If all the fibres in a muscle insert onto the tendon aponeurosis with the same pennation angle (PA), then the muscle is classified as *unipennate*; when different PA could be found within the same muscle, then the term *multipennate* is used to describe this specific structural arrangement (Fig 10). Most of the muscles of the human body appear to show pennate architecture (Friederich & Brand, 1990).





*Fig 10. Different architectural arrangement of skeletal muscle: muscle A is pennate, muscle B is regarded as multipennate while muscle C presents a parallel fibred arrangement (from Campbell & Wood, 2002)*

Pennation angle does influence fibre length (Narici & Maganaris 2006) as it constrains the muscle fibres to extend to only part of the whole muscle length. As fibre length is dependent on the number of sarcomeres in series that form the fibre itself, shorter fibres (with less serial contractile units) will present a slower velocity of shortening and this phenomenon influences the speed of contraction of the whole muscle. On the other hand, pennation angle plays an important role in the spatial arrangement of muscle fascicles, allowing more fibres to be placed within the muscle (Gans & Bock, 1965; Gans, 1982). This reflects the existence of more sarcomeres arranged in parallel (Kawakami et al., 1993; Reeves et al., 2009) and also means that pennate muscles are able to develop higher contractile force. However, this gain of contractile units in-parallel could also represent a problem for muscle force exertion, as the fascicle are thus placed in a non-longitudinal

arrangement, if related to the muscle-tendon line of action. Thus, because muscle force must act through the tendon and then onto the bone to ultimately create joint movement, the force developed in the direction of the fibres will have to be transferred longitudinally to the tendon aponeurosis. The total amount of “force transmitted” (or also force “loss”) could be calculated with planar geometrical models, by using trigonometric formulas (Fig 11). The first theoretical model was suggested by philosopher Nicolas Steno; in one of his “Lemma” of his “Specimen of elements of myology” (1667) it is stated that “*when a muscle contracts, its acute angles widen*”, therefore affirming the fact that muscle do show pennation angle and that it rises upon contraction influencing force production. Following a good amount of previous studies/reviews on muscle architecture and contraction (Gans & Bock 1965, Alexander & Vernon 1975, Gans 1982, (Wickiewicz et al., 1983, (Fukunaga et al., 1997), Narici summarised (Narici, 1999) the functional significance of muscle architecture (which can be easily evaluated in vivo by using ultrasound imaging technique). In Fig 11, a two-dimensional model of pennate muscle is presented.

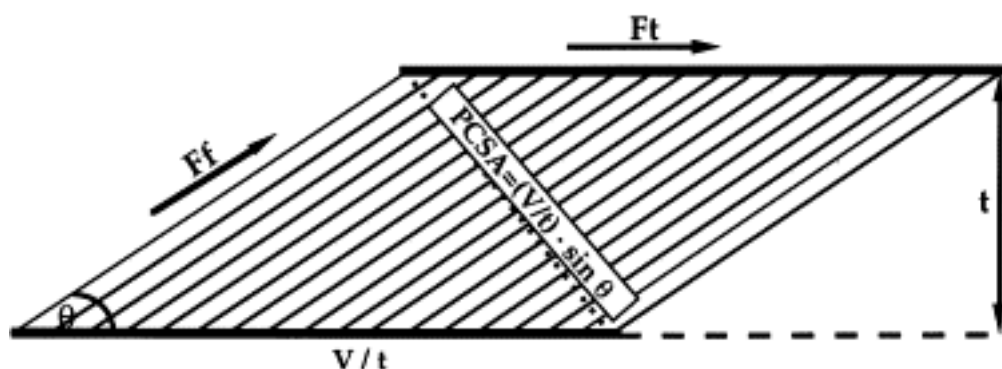


Fig 11. Pennate muscle scheme taken from Narici (1999).

“The quantity that best describes the capacity of muscle to generate maximum contractile force and transmit it through its distal tendon is the physiological cross-sectional area (PCSA) of a muscle” (Narici & Maganaris, 2006). PCSA is defined as the “cross-section that cuts all the fibre at the right angle” (Haxton, 1944; Alexander & Vernon 1975; Narici, 1999) and it represents a good measure of the amount of sarcomeres arranged in parallel within the muscle (Haxton, 1944). By using trigonometry, PCSA can be calculated as:

$$PCSA = (V/t) \cdot \sin \theta \quad (\text{Narici 1999; Alexander \& Vernon 1975})$$

where  $V$  is muscle volume,  $t$  the distance between the two tendon aponeuroses and  $\theta$  is the angle of pennation. Therefore we can also calculate the force acting on the muscle-tendon line ( $F_t$ ):

$$F_t = F_f \cdot \cos \theta$$

where  $F_f$  represents the force acting along the fascicles.

According to what explained above, despite the trade-off between force gain and loss caused by pennation angle, it is suggested that the force transmitted to the tendon will remain positive as long as the PA will not exceed values of 45 degrees (Alexander & Vernon 1975).

### *3.2 The influence of muscle architecture on muscle functional properties*

Consequently to what explained in the section above (i.e. a pennate muscle will have shorter fibres but more contractile material packed in-parallel,

compared to a parallel-fibred muscle) it can be stated that a small change in  $L_f$  in pennate muscles will affect the L-T relationship of the muscle. The length- tension curve of pennate muscle will have a higher peak in force but a reduced width than a parallel-fibred one; a pennate muscle of the same mass, because of the greater number of sarcomeres in parallel, has a greater PCSA than a parallel-fibred skeletal muscle. Hence the force development is greater because of the greater presence of contractile units per gram of tissue. F-V relation is also affected by changes in muscle architecture: as previously said velocity of shortening is proportional to fascicle length ( $L_f$ ).

If two muscles with same  $L_f$  and PA but with different PCSA are compared, the L-T curve will show differences between muscles in terms of in force developed with a consequent increase in maximum tetanic tension in the muscle with a greater PCSA. The muscle with a smaller PCSA will show also lower values of force at the same velocities of shortening in the F-V relationship (Lieber, 1992).

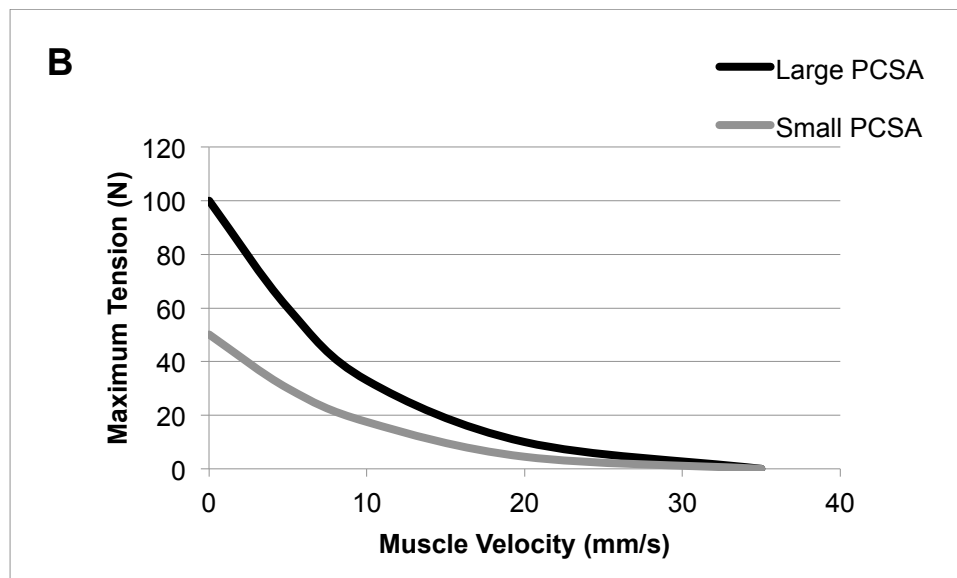
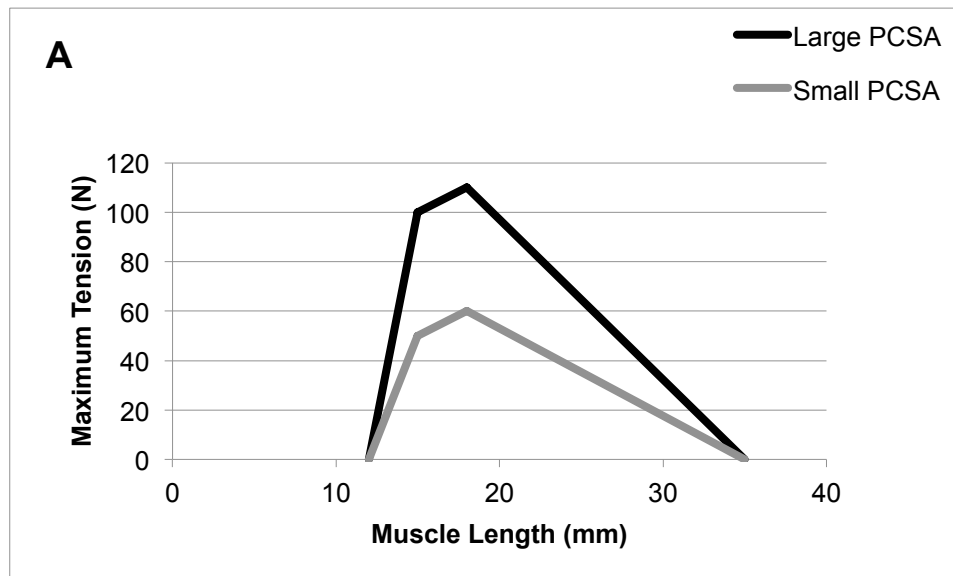
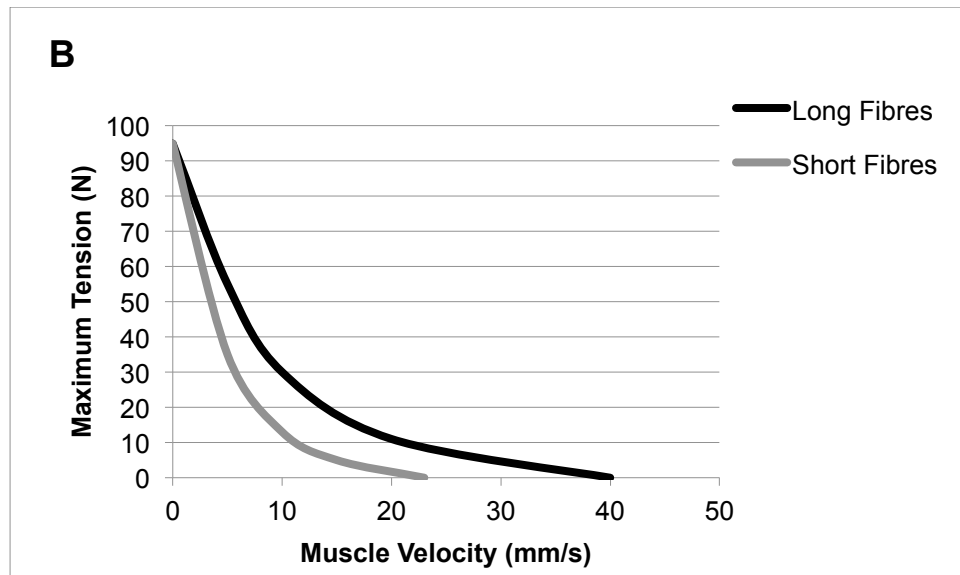
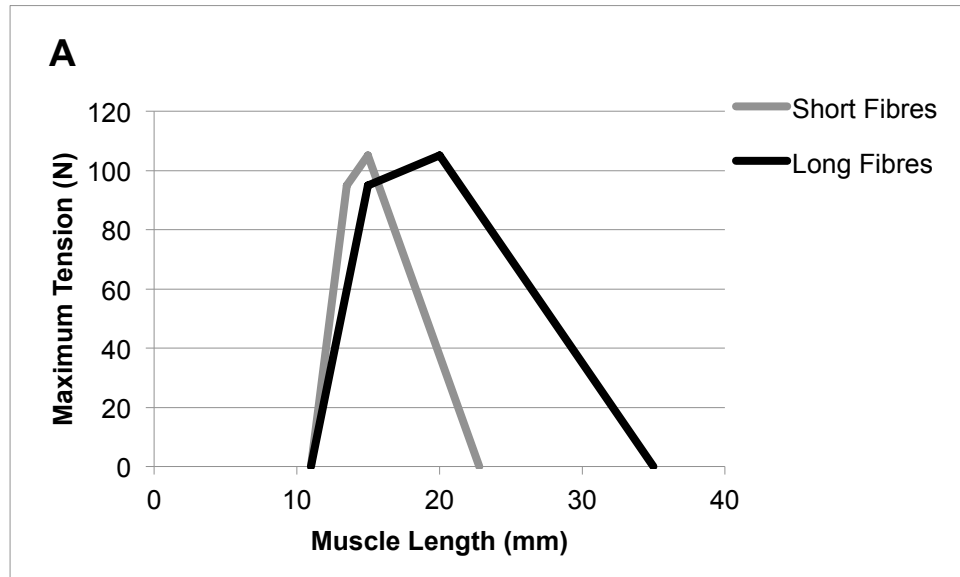


Fig 12. L-T curve (A) & F-V relation (B) for two muscles with same  $L_f$  and pennation angle but different PCSA (from Franchi MV 2010; readapted by Lieber & Fridén, 2000)

If instead two muscles have the same PCSA and pennation angle but different  $L_f$ , dissimilarities in absolute muscle range of movement will be found, but the peak of absolute force will be equal. L-T curves will show the

same maximum tension but the longer-fibred muscle curve will be much wider. Furthermore, the value of maximum shortening velocity of this muscle will be higher (F-V relationship).



*Fig 13. L-T curve (B1) and F-V relation (B2) in two muscles with same PCSA and pennation angle but with different Lf (from Franchi MV 2010; readapted by Lieber & Frieden ,2000).*

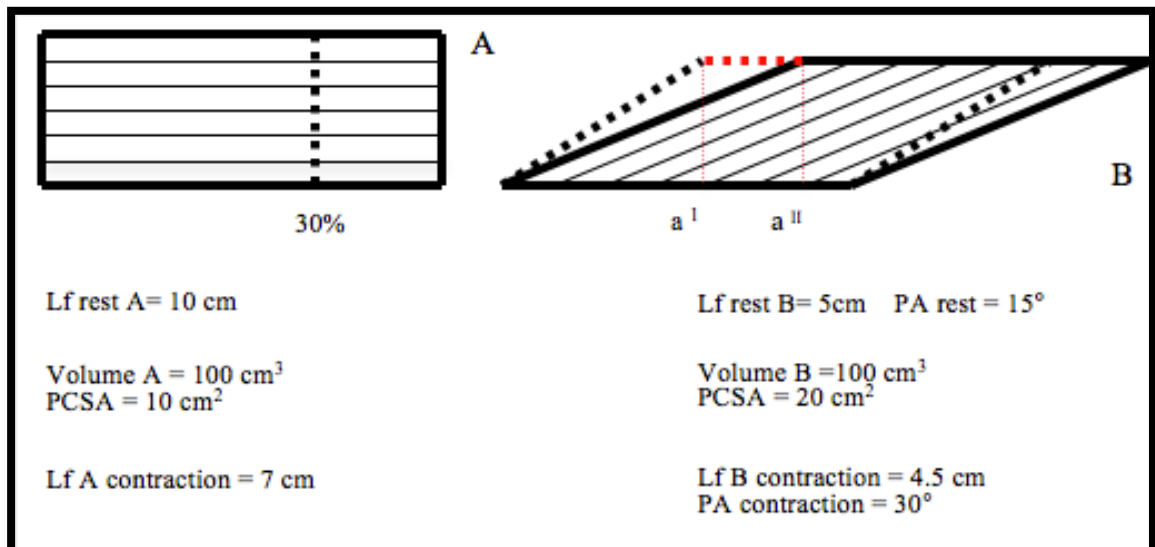


Fig 14. (from Franchi, 2010). Hypothetical comparison between a parallel fibred (A) and a pennate muscle (B) of the same Volume = 100 cm<sup>3</sup>. Values of Lf, PA and PCSA at rest are shown above.

Assuming that during contraction the fibres of both muscles shorten by 30% of their resting length (43), the fibres muscle A will shorten from 10 to 7 cm while those of muscle B will shorten from 5 to 4.5 cm. Assuming that this shortening occurs in 1 s, for muscle A shortening will be  $Lf_{rest} - Lf_{contraction} = 3 \text{ cm/s}$ . For muscle B, PA will increase from  $\theta = 15^\circ$  to almost  $\theta' = 30^\circ$  during contraction (as shown by Narici et al (1999)).

Thus the resulting shortening of muscle B expressed per second will be equal to  $[a' - a''] / s$ , as shown below:

$$\begin{aligned}
 a' &= Lf_{rest} * \cosine \theta \\
 a' &= 5 * \cosine 15 = 4.85 \text{ cm} \\
 a'' &= Lf_{contraction} * \cosine \theta' \\
 a'' &= 4.5 * \cosine 30 = 3.045 \text{ cm}
 \end{aligned}$$

$[a' - a''] / s = 1.8 \text{ cm/s}$  which is Muscle B maximum velocity of shortening.

Hence, it can be concluded that in comparing a parallel fibred to a pennate muscle of the same Volume, the former will have a greater velocity of shortening of the latter.

Therefore, muscles with relatively long fibres are muscles with high contraction velocities and large excursions; conversely muscles with large PCSA are able to develop greater tensions (Lieber, 1992) (Fig 15).

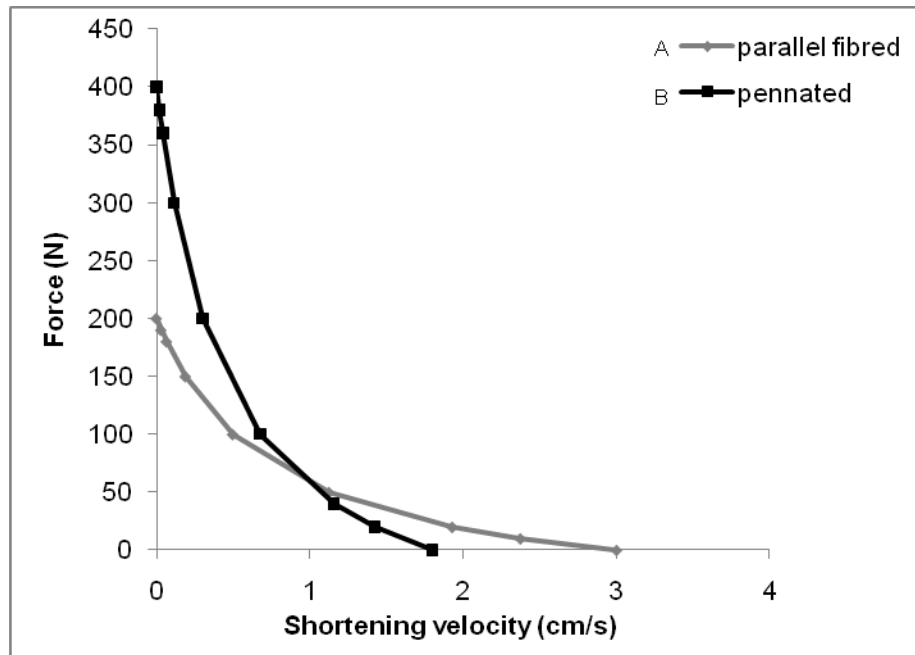


Fig 15. F-V relationships for parallel fibred vs. pennated muscle with same volume (from Franchi MV 2010).

#### 4. Muscular adaptations to resistance training

Resistance exercise is used to increase muscle size and strength and also has clinical importance in rehabilitation and treatment of muscle mass loss (Wackerhage & Atherton, 2006). Resistance training is defined as a specialized method of conditioning performed in order to obtain specific muscular adaptations.

There are many types of resistance training methods, but two main typologies are classically recognised: these are usually described as *isotonic*<sup>1</sup> or *isokinetic* loading modalities. Isotonic loading is the most

<sup>1</sup> The common use of the term isotonic to describe this type of training is not strictly correct as a true isotonic contraction requires constant loading imposed on the muscle and not constant external weight since the latter, because of the change in moment arm with joint movement, does not result in constant load experienced by the muscle. The recent use of cams in training machines is a rough attempt to achieve constant muscle loading but this is far from optimal, as expected from a true isotonic contraction as described by Wilkie (Wilkie, 1956).



common way of resistive exercise (can be also referred as “conventional training”) and it consists in lifting or lowering the same external load throughout the muscle range of movement. It is generally performed using free weights or standard weight lifting machines. Isokinetic mode is instead carried out on specific isokinetic machines, on which the angular velocity of the movement is pre-set and does not change through the whole range of movement (ROM) of the exercise. This theoretically permits to apply maximal force (at that specific angular velocity) for the whole muscle ROM.

A large amount of studies have demonstrated that both isotonic and isokinetic exercise lead to increase in muscle strength, power and size in response to overloading. Those adaptations can summarily be distinguished as of *morphological* and *neuromuscular* nature.

#### *4.1 Morphological adaptation to strength training*

Changes in whole-muscle size (muscle volume) and cross-sectional area are the primary morphological adaptations to increased loading. The increase in cross-sectional area (CSA) (hypertrophy) is largely due to an increase in the CSA of the muscle fibres (Macdougall et al., 1980). Many studies reported changes in muscle CSA and volume (Folland & Williams, 2007) following a resistance-training regimen both in young (Narici et al., 1989; Young et al., 1983) and older adults (Harridge et al., 1999; Narici et al., 2005; Reeves et al., 2004). Accordingly, fibre hypertrophy reflects an addition of contractile material as a result of enhanced protein synthesis after exercise (David J Glass, 2003), and consequently this is expected to lead to an increase in

muscle strength. Muscle hypertrophy has been observed preferentially in type 2 fibres after strength training (Hather et al., 1991); their hypertrophy is comparatively faster and their atrophy comparatively quicker than that of type 1 fibres during training and detraining.

#### *4.1.2 Satellite cells*

What is the mechanism for which the muscle fibres can actually grow in size? Plasticity of skeletal muscle is connected to the role of a group of myogenic precursor cells called “satellite cells”; their peculiarity is to be located between the basal lamina and the sarcolemma of muscle fibres, anatomically distinct from the myonuclei (Harridge, 2007). As Zammit (Zammit et al., 2006) defined them, satellite cells are “muscle stem cells that can give rise to a differentiated cell type and maintain themselves by self-renewal”. When activated (usually during the growth-repair cycles), they proliferate and fuse with the muscle fibres, dividing their nuclei in two parts: one part will be incorporated into the muscle cells, the other withdraws from the cell cycle in order to replace satellite cells previously used (Zammit et al., 2006). It has been proposed that these events occur in order to maintain the myonuclear domain of the muscle cell (Kadi et al., 1999; Petrella et al., 2006) which is the ratio of fibre size to number of nuclei. However, it has been demonstrated that, during hypertrophy, muscle fibres can grow in size no more than 17% (Kadi et al., 2004) without adding more nuclei through the satellite cells-cycle. This suggests that muscle fibres can still hypertrophy without increasing myonuclear number, as shown by McCarthy & Esser (2007), but

beyond a certain limit, as satellite cells activation becomes vital for muscle growth regulation.

Also, counting that is known that satellite cells number can be increased by strength training (Kadi et al., 2004), a recent study by Bruusgaard et al. (Bruusgaard et al., 2010) showed that the myonuclei added to the muscle fibres through satellite cells activation seem to be protected from apoptosis occurring during muscle atrophy processes, contributing to maintain a good myonuclear domain even after a period of muscle disuse. This “muscle memory” is thought to be important in old people, as the ability of adding new myonuclei is impaired: thus, resistance training at an early age, and successively increasing in satellite cells activation may represent a benefit.

#### *4.2 Neuromuscular adaptation to strength training*

It is common knowledge that voluntary contractions require control of the muscle by the nervous system, which stimulates muscle fibres through motor units recruitment and rate coding. A motor unit (MU) is defined as a set of muscle fibres controlled by the same motoneuron: when the impulse coming from the nervous system runs through the motoneuron axons and reaches the fibres, provided it is above the threshold of activation of that particular MU, it produces a muscle twitch. The size and the speed of the twitch is mainly dependent on the type and number of muscle fibres belonging to a single unit: motor units, therefore, differentiate between themselves according to the contractile and metabolic properties as well as the number of fibres they contain. If an increase in muscle size is the main result of the adaptations in muscle morphology, an increase in muscle strength is instead

influenced in by neural factors as well as by hypertrophy. This explains why in the early stages of a strength training program there is dissociation between the increase in muscle strength (which may be observed within the first few days of training) and the gains in muscle mass (Häkkinen et al., 2001, Jones & Rutherford, 1987), detectable only after few weeks of training by using imaging techniques like CT, DEXA, MRI. However, recent studies investigating the molecular (Bickel et al. 2005) and architectural (Seynnes et al., 2007) responses to resistance exercise training (RET) exercise have shown that the molecular adaptations to muscle loading appear within 12 hours of a training bout and signs of hypertrophy can be detected within 10 days of RET.

The main neural adaptations to strength training are generally referred to an increase in neural drive, which is the result of an increase in MU recruitment (Aagaard et al., 2000), and an increase in firing frequency (Sale, 1988). Several studies have also shown a decrease in antagonist muscle activation following RET (Häkkinen et al. 1998) and this can be a potentially additional mechanism contributing to the increase in agonist muscle strength with RET (Narici et al., 1996).

##### *5. Muscle growth and skeletal muscle hypertrophy: cellular signalling pathways involved and anabolic response*

The regulation of mammalian skeletal muscle size is a very complicated process: the peculiar characteristic of this specific tissue it is the ability to adapt to unloading/overloading states through atrophy/hypertrophy

mechanisms, respectively. Atrophy occurs when a muscle presents a decrease in cell size, mainly caused by the loss of sarcomeric proteins, organelles and cytoplasm (Sandri, 2008); hypertrophy, instead, “is defined as an increase in muscle mass, which comes as a result of an increase in size, as opposed to the number, of pre-existing skeletal muscle fibres” (Glass, 2005). Resistance exercise provides a unique stimulus for muscle growth. Thus, a muscle adapts to overloading by increasing in size and strength as a result of improved rate of protein synthesis and through the incorporation of satellite cells into the muscle fibres (satellite-cell-mediated mechanism) (Harridge, 2007; Glass, 2003; Sandri, 2008).

**What are the factors regulating the size of muscle mass? What are the processes, the cascade of events involved in muscle hypertrophy?**

### *5.1 Key cellular signalling pathways in muscle growth and hypertrophy*

This section will review the molecular mechanisms that lie behind the control of muscle growth and the events occurring during muscle hypertrophy, mostly in response to resistance training.

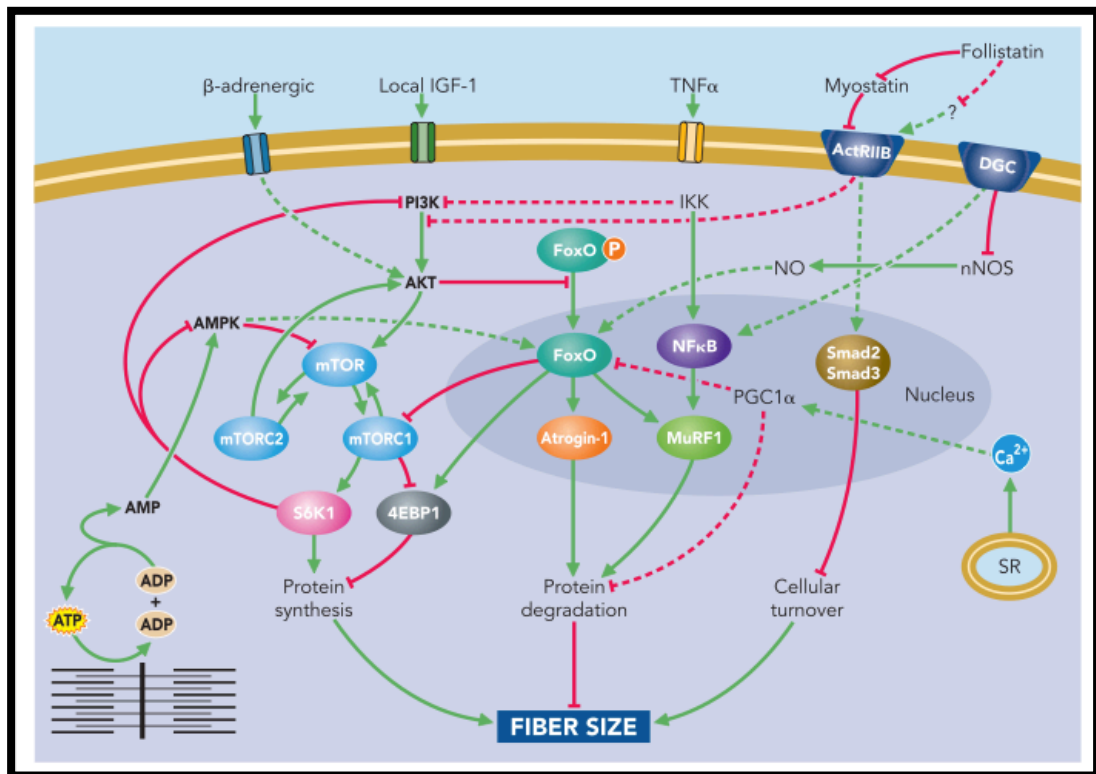


Fig 15. Major cellular signalling pathways regulating muscle fibre size (from Sandri, 2008)

### 5.1.2 Hypertrophy via IGF-1/AKT pathway

Insulin-like growth factor 1 (IGF-1) is among the most important growth-promoting factors of skeletal muscle: secreted primarily from the liver, under GH control, is also locally synthesized by the skeletal muscle itself, in distinct IGF-1 splicing products (Harridge, 2007; Sandri, 2008). In vitro, an increase of the gene encoding IGF-1 was found in work-induced hypertrophy (DeVol et al., 1990) and also IGF-1 has been demonstrated to induce protein synthesis in cultured muscle cells (Rommel et al., 2001). Mice with over-expression of IGF-1 present muscle hypertrophy (Musarò et al., 2001)

whereas IGF-1 knockout mice show smaller muscles compared to normal ones, suggesting the importance of the anabolic effect of this protein on muscle cells (Wackherage & Atherton, 2006). The local production of an IGF-1 isoform has been observed in animal muscle by Yang (Yang et al., 1996), suggesting that this splice variant (successively named MGF, mechano-growth factor) was important in local regulation of muscle growth in response to stretch. However, human muscle data show a less clear correlation between IGF-1, MGF expression and resistance training; also, even if these data suggest that IGF-1 could be one of the major factors regulating muscle mass, this does not prove it is the only important element involved in hypertrophy in response to strength training.

IGF-1 also induces an increase in muscle size by stimulating the PI3K/Akt signalling pathway (Glass, 2005) that results in activation of targets promoting protein synthesis. PI3K is a lipid kinase, which produces phosphatidylinositol-3, 4, 5-triphosphates that binds Akt; activation of the serine-threonine kinase Akt has been demonstrated to induce hypertrophy, protein synthesis, gene transcription (Glass, 2003; Glass, 2005; Sandri, 2008). Akt activity has been associated to exercise in vivo (Turinsky & Damrau-Abney, 1999, Nader & Esser, 2001) and to passive stretch (Sakamoto et al., 2003) in rat contracting muscle: this suggests that mechanical tension might be a crucial stimulus in Akt activation in contracting muscle. However, even it remains to be established how mechanical stress is converted into Akt activation, it is needed now to clarify the downstream targets of Akt likely leading to protein synthesis.

### *5.1.3 The Akt/mTOR pathway*

Akt activation upregulates different signalling pathways relevant to muscle hypertrophy: one of these is represented by the stimulation of the mammalian target of rapamycin (mTOR), a kinase that has been observed to integrate and regulate different cell signals and to play a key role in muscle growth (Sandri, 2008). mTOR influence on muscle growth has been studied in vivo by Bodine (Bodine et al., 2001) showing that rapamycin (which inhibits mTOR) blocked muscle hypertrophy and regeneration in response to overload. As said, Akt activates mTOR: this is an indirect process though. Akt has been demonstrated to disrupt tuberous sclerosis complex-1 and-2 proteins (Tsc1- Tsc2), which can inhibit mTOR, by phosphorylating Tsc2; therefore, Akt inhibits the Tsc1-Tsc2 complex, which will be thus unable to inhibit mTOR (Inoki et al., 2002).

### *5.1.4 The mTOR/S6K pathway*

mTOR is part of two multiprotein complexes (Sandri, 2008): mTORC1, required for S6K and PHAS-1 (also known as 4EBP1) signalling, and mTORC2, required for Akt-FoxO signalling. S6K (p70S6 kinase) is therefore the first mTOR target: Ohanna (Ohanna et al., 2005) showed that S6K is necessary for achievement of normal size of muscle fibres, as in a knockout model muscle cells were smaller. Baar & Esser (Baar & Esser, 1999) observed an increase of S6K activity and translation after high intensity contractions in rats. However, S6K true role on protein synthesis is still



unclear. MacKenzie and colleagues (Mackenzie et al., 2007; MacKenzie et al., 2009), stressing the importance of the role of amino acids intake after resistance exercise in the increase of protein synthesis rate, observed an increase of mVps34 protein activity 3 hours after resistance training. They suggested that mVps34, which controls amino acids signalling to mTOR and S6K, could take part in this hypertrophy/protein synthesis pathway acting as an amino acids sensor to mTOR after exercise.

mTORC1 second target is represented by PHAS-1: mTOR seems to inhibit this protein, which is a negative regulator of a protein initiation factor called eIF-4E (Glass, 2005), promoting therefore likely new protein synthesis.

mTORC2 seems instead to enhance Akt activity influencing Akt-FoxO signalling pathway: Akt inhibits FoxO activity, which promotes several muscle atrophy pathways (Sandri, 2008). In conclusion, although most of the studies have been carried out in animals, mTOR pathways are recognised as crucial during the regulation of muscle growth and hypertrophy.

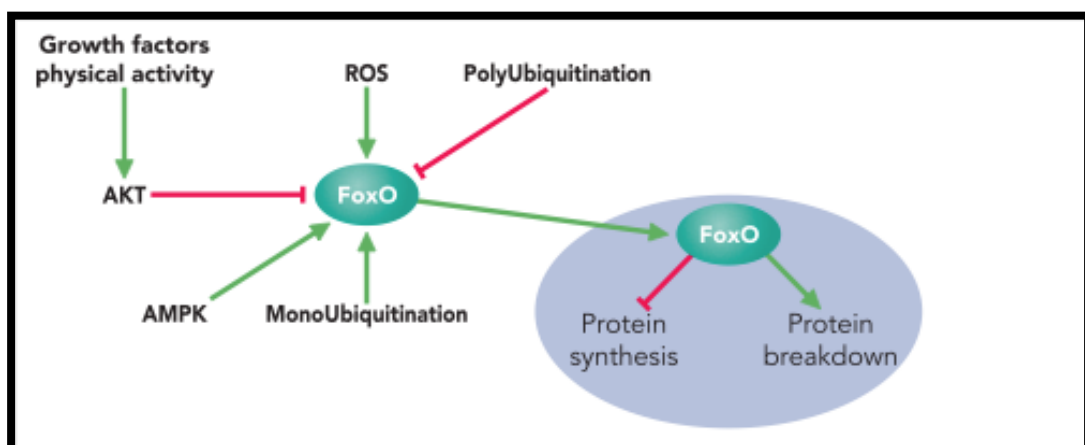


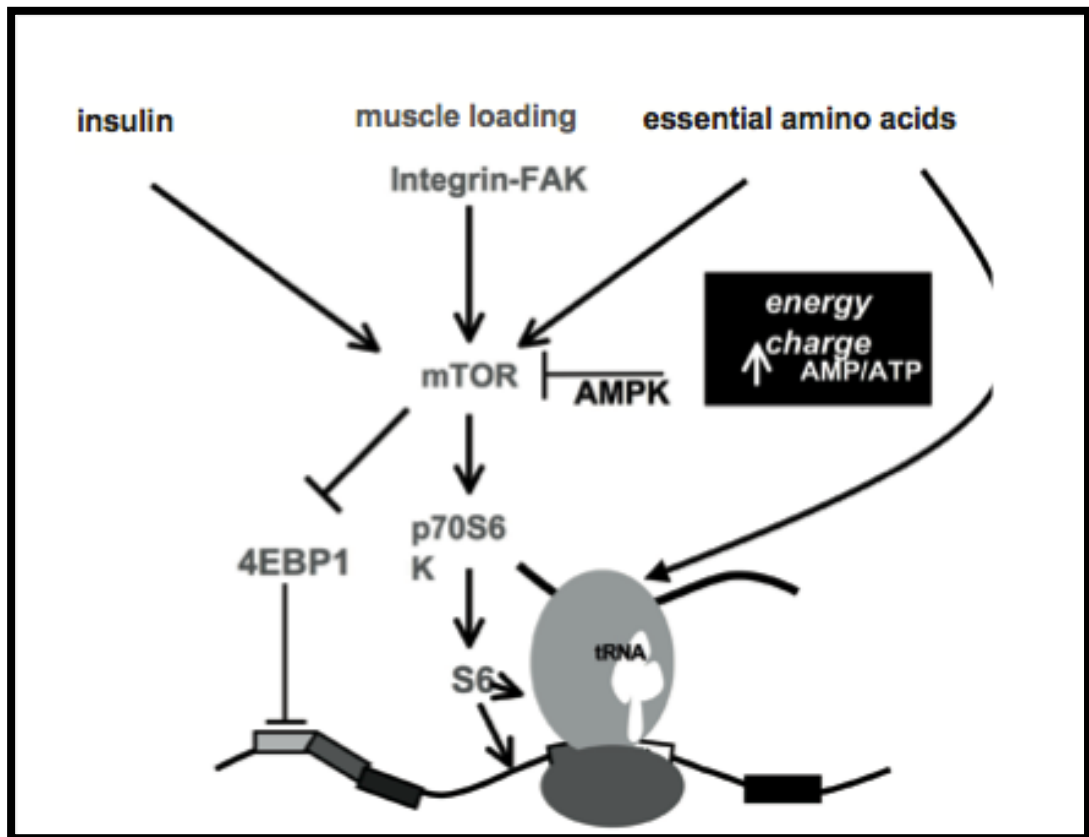
Fig 16. Role of FOXO group on muscle protein breakdown regulation (from Sandri, 2008).

### *5.1.5 The Akt/GSK3 $\beta$ pathway*

Glycogen-synthase kinase 3 $\beta$  is a substrate of Akt and it has been shown to modulate hypertrophy by being inhibited by Akt itself (Glass, 2003). GSK3 $\beta$  has been observed to block protein translation initiated by eIF-2B protein in cardiac hypertrophy (Hardt & Sadoshima, 2002), and thus that may suggest that PI3K/Akt/GSK3 $\beta$  pathway could induce protein synthesis in muscle independently of the mTOR pathway.

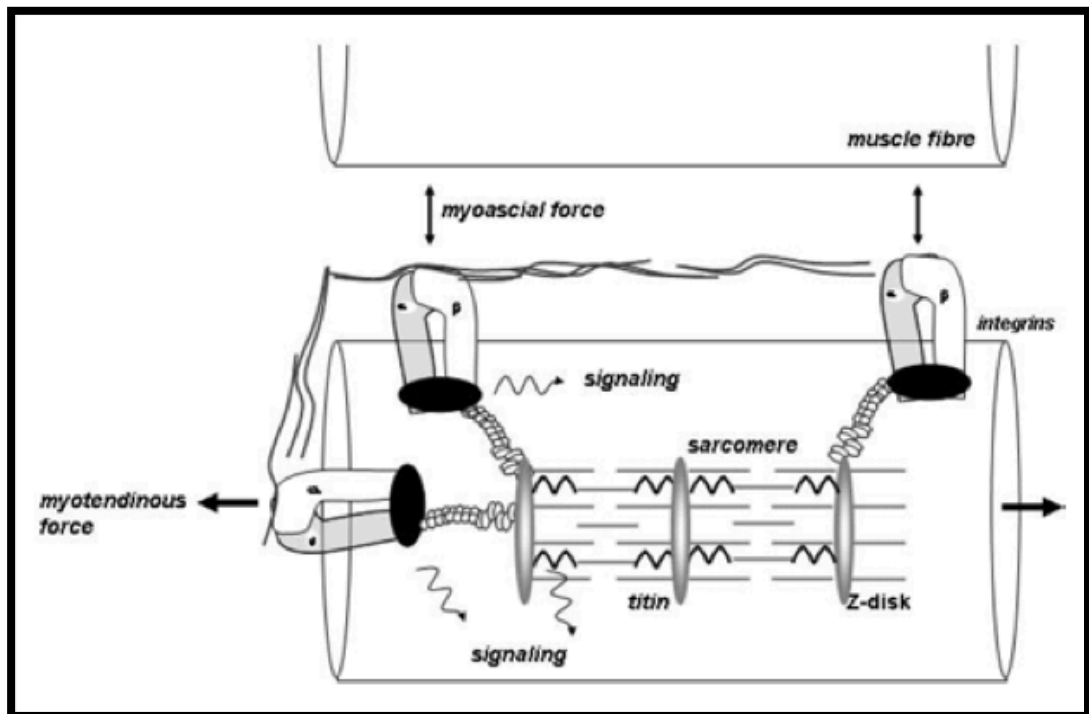
### *5.1.6 Mechanical transduction: FAK and MAPK*

As suggested, different sensors of muscle mechanical activity seem to be able to transform tension into signals that induce activation of muscle actin promoter (Rennie et al., 2004).



*Fig 17. Main pathways integrating physiological responses to external stimuli ultimately controlling ribosomal activity and the initiation of translation (from Fluck, 2012).*

Focal adhesion kinase (FAK), a protein situated in the sarcolemma, could be one of the sensor of mechanical strain: after muscle loading there is a significant increase of FAK and its tyrosine phosphorylation status (Gordon et al., 2001). Its transcription factor, the serum response factor (SRF) appears to be linked to the expression of the muscle protein itself as Fluck and colleagues (Flück et al., 1999) showed in stretch-induced hypertrophy of muscle in roosters.



*Fig 18. Mechanotransduction in skeletal muscle: the deformation of integrins caused by mechanical forces applied onto the muscle cells activates the cascade of mechanotransduction signalling (from Durieux et al., 2007).*

Mitogen activated protein kinases (MAPKs) seem to be intensity dependent and related to muscle tension as well as FAK. These proteins are involved in cell differentiation by integrating extracellular cues such as mechanical and metabolic signals into downstream regulation of gene expression (Mauro et al., 2002). In isolated animal muscle, Wretman (Wretman et al., 2001) showed differences in MAPK phosphorylation in response to either concentric or eccentric contraction modes. This contraction-mode specificity in MAPK activation is also illustrated by the work of Martineau & Gardiner (Martineau & Gardiner, 2001), which showed that phosphorylation of MAPK (ERK  $\frac{1}{2}$ , and JNK) but not p38 kinase, is specifically phosphorylated in relation to peak tension of rat muscle. On the basis of these findings on animal skeletal muscle, the hypothesis is put forward that the rate of peak tension, rather than the absolute mechanical load, affects the intracellular

signalling responses. Although these intracellular signalling processes control the activity of transcription factors and the expression of genes, the downstream activation of transcription factors, which are held responsible for the remodelling of skeletal muscle with hypertrophy, remains to be addressed.

### *5.2 Muscle protein synthesis in response to exercise*

Skeletal muscle is an extremely adaptive tissue, which remodels in response to “metabolic and locomotory demands of exercise” (P J Atherton & Smith, 2012).

Muscle performance is connected to the turnover of contractile proteins: the growth of skeletal muscle mass is tightly dependent on the balance between new proteins synthesis and existing proteins degradation (Sandri, 2008). In adult muscle hypertrophy is so reached through an increase in new protein accumulation and a decrease in protein breakdown (“net protein accretion” – Wackerhage & Atherton, 2006), and later through the role played by satellite cells. In healthy recreationally active individuals, muscle protein breakdown (MPB) is higher than protein synthesis (MPS) in the fasted state; conversely, the opposite scenario is found in the fed state and, transitorily, after exercise (Atherton & Smith, 2012). Resistance training is a crucial stimulus for muscle hypertrophy: initially, straight after exercise, there is an increase in protein breakdown, which occurs until feeding (Phillips et al., 1997). Thus, food intake during recovery has been shown to cause net protein gain and increase in muscle mass (Tipton et al., 1999). Also, an increased intake of

amino acids leads to a consequent increase in protein synthesis, which, following resistance exercise, may remain elevated for a longer period compared to just feeding (72h after training versus 2-3h after ingestion). However, MPS response to both feeding and exercise is also limited in duration by the “muscle-full effect” (Atherton et al., 2010): in response to feeding, after a latency of circa 30 minutes, MPS is increased, reaching an anabolic peak 1.5 hours post-feeding, returning then to baseline values after 2 hours. At this point, muscle anabolic activity is “shut down” despite the elevated amount of circulating amino acids. When exercise is the main stimulus for anabolic response instead of feeding, MPS might be sustained for up to 4 hours after exercise in the fasted state (Kumar et al., 2009) and up to 24 hours in presence of amino acids (Cuthbertson et al., 2006).

It is suggested that the size of the MPS acute response to exercise is dependent on the intensity and the workload (Atherton & Smith, 2012). It has been shown by Kumar and colleagues (2009) that MPS rises in responses to intensities of exercise greater than 60% of the one repetition maximum (1RM) while the same exercise performed at 40% 1RM or less intensity did not result in any significant anabolic response. On the other hand, Burd and colleagues (Burd et al., 2010) demonstrated that even low intensity exercise resulted in protein synthesis accretion, if performed to failure, compared to heavy load resistive exercise. (30% vs. 90% work matched). The proposed mechanism of why such adaptation has been observed is to be found in the nature of muscle fatigue: if the exercise is indeed performed to failure, type II fibre are thought to be recruited more as the muscle works towards exhaustion (Burd et al., 2012).

### *5.2.1 Stable isotope tracing techniques and D2O method development*

Measurement of muscle protein turnover can be assessed from muscle biopsic tissue by using stable isotope tracing techniques (Rennie et al., 1982). Stable isotopes do not present any radioactive decay and are made of “heavy atoms”: “they are identical to their endogenous counterparts but can be distinguished by their mass difference using mass spectrometry techniques” (Atherton & Smith, 2012). By collecting biological samples it is therefore possible to measure the incorporation of stable isotope labelled amino acids into new proteins, thus assessing MPS in skeletal muscle (Rennie et al., 1982). If a known amount of labelled (tracer) and unlabelled (tracee) amino acids is supplied (via primed-costant infusion), labelled and unlabelled amino acids will mix into new synthesized proteins: MPS can therefore be assessed by measuring the tracer/tracee ratio (protein enrichment) and compared to the precursor enrichment (Gasier et al., 2010). Generally, the choice of the labelled amino acid to be used is related to the method of measurement. Stable isotopes of Carbon ( $^{13}\text{C}$ ) or Nitrogen ( $^{15}\text{N}$ ) are commonly used with isotope ratio mass spectrometry (IRMS) of specific gases ( $\text{CO}_2$  or  $\text{N}_2$ , respectively): the advance in stability and sensitivity of these machines in the last decades has resulted in the refining of the technique, allowing laboratories to obtain greater resolution of acute responses to MPS (Atherton et al., 2010). The disadvantages of this technique are represented by the restricted measurement periods (maximum of several hours) and the fact that this could lead to an overestimation of

protein fractional synthetic rate (FSR) in short time period analyses (Gasier et al., 2009). Moreover, the method is invasive and is commonly performed in a controlled environment, which does not help to investigate what could happen in free-living conditions. The latter concern is the reason why a new methodology is required to understand if acute FSR values could be also translated to a free-living environment. Recently, the use of deuterium oxide ( $^2\text{H}_2\text{O}$  – “heavy water” or D2O) has been developed to measure MPS in cell culture and in vivo (Dufner et al., 2005; Dufner & Previs, 2003) and also in humans in response to endurance training and feeding (Robinson et al., 2011). Initially introduced in the early forties, the use of D2O it is now of great interest, although it had been put aside for many years, likely due to the scarce availability of deuterium in the past decades. This methodology now offers the possibility of measuring MPS over weeks and months, in free-living conditions, just by ingesting heavy water (Gasier et al., 2010, Atherton & Smith, 2012) (the mechanism are presented in Chapter 2 and 3).

#### *6. Concentric (CON) and Eccentric (ECC) contractions: fundamental differences and adaptations to CON vs. ECC RET*

As presented above, it is known that skeletal muscle can contract by either shortening or lengthening (*concentrically* or *eccentrically*, respectively). During concentric contractions the muscle shortens and exerts a force at the muscular-tendinous junction, causing a change in the joint angle as well. Eccentric contractions occur very often in everyday common activities (walking downstairs where the quadriceps is working eccentrically to



maintain an erect posture and prevent from falling) and they usually act as a force in opposition to a concentric contraction by a muscle working across a given joint to protect joints from damage.

The two types of contractions present many different features: the presence of specific mechanisms of force generation at contractile proteins level constitutes one of the reasons of why greater production of force is observed during active lengthening of the muscle compared to shortening (please see section 2.3). Moreover, different neuromuscular activation strategies have been observed when comparing concentric to eccentric contractions (Enoka, 1996, Aagaard et al., 2000, Nardone et al., 1989). Recent investigations have shown greater cortical activity when eccentric voluntary movements are performed compared to concentric actions (Fang et al., 2001, Kwon & Park, 2011).

Different morphological and functional adaptations of skeletal muscle to CON vs. ECC exercise have been previously reported (Jones & Rutherford 1987) and it has been claimed that eccentric training results in a greater increase in muscle strength and mass due to the higher loading that ECC actions can bear (Cook & McDonagh, 1995; Westing et al., 1988).

Regarding neuromuscular adaptations to training, eccentric actions show faster neural adaptations in response to training (Hortobágyi et al., 1996) and lower muscle electrical activity than concentric movements with analogous value of force (Tesch et al., 1990).

It has been also suggested that shortening and lengthening contractions result in differential sarcomere addition, likely due to contraction-specific ultra

structural changes and different degree of exercise-induced muscle damage (Newham et al., 1983; Proske & Morgan, 2001; Schoenfeld, 2012) where ECC exercise leads to an increase in the number of serial sarcomeres in animals (Butterfield et al., 2005; Williams & Goldspink, 1971). Thus, specific adaptations to ECC or CON training could also influence the remodeling process of muscle architecture (greater serial sarcomeres addition reflects an increase in muscle fascicle length).

### *7. Main studies: rationales, hypotheses and open issues*

According to what presented above, it is clear that skeletal muscle seems to specifically adapt to multi-variety of stimuli: however, several postulates could be made regarding the mechanisms that appear to regulate the remodelling of skeletal muscle:

- a) skeletal muscle presents a fine hierarchical organisation which allow the contractile material to properly act as a force generator
- b) indeed, the interdigitation between myosin and actin is the major determinant of muscle force production: this interaction, together with both the length of muscular fascicles and the velocity of shortening/lengthening, influences the functional properties of skeletal muscle
- c) resistance exercise is a unique stimulus that triggers skeletal muscle capacity to adapt, resulting in various training responses: morphological (gains in muscle mass through increase of anabolism causing fibre hypertrophy), architectural (changes in PA and Lf), neuromuscular (increase in recruitment of motor units), functional (increase in strength and power).

Different typologies of exercise lead to loading-specific adaptations (i.e. ECC vs. CON are an example)

d) the adaptations mentioned above can be seen as the result of molecular mechanisms of skeletal muscle cells remodelling, which appear to be vary in response to specific muscle actions. However, even though many studies have focused on skeletal muscle intracellular behaviour in response to loading in vitro and mainly on animal muscle, few have investigated these mechanisms in human skeletal muscle.

Therefore, while acknowledging these four points, the need of further examination of the mechanisms of skeletal muscle remodelling in humans, in response to specific loading protocols, seems justified.

The first study presented in chapter 1 investigates both the acute responses to pure eccentric versus a pure concentric exercise single bouts and the result of 10 weeks of the same loading in terms of morphological, architectural, functional and molecular adaptations of the vastus lateralis muscle in young volunteers.

Since the classical works that described the behaviour of skeletal muscle during shortening and lengthening actions (Hill, 1938; Katz, 1939) and the mechanics of force generation (Huxley, 1971), a substantial number of studies aimed to clarify the many different aspects of these two types of contraction both in animal models (Williams et al., 1988; Wretman et al., 2001; Martineau & Gardiner,, 2001; Adams et al., 2004; Butterfield et al., 2005; Heinemeier et al., 2007) and human muscle (Hakkinen et al., 1981, Newham et al., 1983; Jones & Rutherford, 1987; Higbie et al., 1996;

Hortobagyi et al., 1996; Seger et al., 1998; Aagaard et al., 2000; Moore et al., 2005; Cuthbertson et al., 2006; Blazevich et al., 2007; Reeves et al., 2009; Mueller et al., 2009; Mueller et al., 2011; Moore et al., 2012). However, among all these references, most of them have not clearly shown or even suggested a link between changes in muscle morphology and the molecular signalling which may be responsible for some particular adaptations in response to the two contraction modes. Previous studies have investigated the influence of either ECC or CON (or both) exercise on muscle architecture remodeling (Blazevich et al., 2007; Guilhem et al., 2011; Baroni et al., 2013), but none of these works focused on the actual mechanisms that could lay behind such peculiar adaptations. Furthermore, most of the previously cited articles have matched contractions for work and because ECC contractions can develop more force than CON ones, then it follows that CON training should consist of more repetitions compared to ECC one when matched for work. To the best of the author's knowledge, no study has instead tried to match eccentric and concentric actions for relative equivalent loading stimulus (different loading but same exercise intensity): such matching would then be useful to clarify the real contribution of these loading modes to muscular hypertrophy. So far, only Reeves and colleagues (Reeves et al., 2009), comparing the functional and architectural responses to conventional (concentric + eccentric) training vs. pure eccentric training, successfully attempted to match the two different training modes for relative maximal load (80% of either CON or ECC phase, respectively) (further explanation will be found in chapter 1). Last but not least, although effects of ECC and CON contractions on muscle signalling have been object of research in animal

models, a very low number of studies focused on these signalling / anabolic / genomic responses in humans (Boppart et al., 1999; Gibala et al., 2000; Chen et al., 2003; Moore et al., 2005; Kostek et al., 2007); furthermore, all these previously cited works examined the acute changes to ECC vs. CON single bouts or very short RET period (i.e. only 5 days for the work by Gibala and colleagues) but without attempting to clarify the possible links between acute and chronic adaptations (which are of crucial interest, as also stressed in a recent review by Murton & Greenhaff (Murton & Greenhaff, 2013)).

Therefore, the aim of study 1 was to compare the outcomes of pure CON vs. ECC exercise training, on human muscle, in terms of architecture, morphology and functional aftereffects, and associate those to muscle cell signalling responses. The hypothesis put forward was that shortening vs. lengthening, chronically applied and matched to balance the relative loading inducement, would result in distinct outcomes in muscle morphology, function and architecture with possible mechanical and biochemical mechanisms as a cause of definite adaptations.

Even though study 1 attempts to highlight some of the possible biological mechanisms underlying the contraction-specific remodelling of muscle size and architecture, cellular signalling pathways only show how transcriptional and translational processes may be regulated in the anabolic response after exercise, but without providing an actual quantification of new protein synthesis occurred. In contrast, as mentioned in section 5.2.1, dynamic “snapshots” of MPS can be assessed by using stable isotopes (Rennie et al., 1982). Moreover, recent new developed tracer methods (as deuterium oxide) allow measures of cumulative tracer incorporation into proteins over a period

of several weeks or months (Robinson et al., 2011; Atherton & Smith, 2012), providing the actual quantification of muscular anabolic response to definite interventions, rather than just portraying a likely activation/regulation of transcription and translation (i.e. as cell signalling data describe). Traditionally, calculation of a fractional synthetic rate (FSR) of MPS has been gauged by using venous/arterial labeled AA infusion with addition of multiple biopsy collection: nevertheless, not only this technique must be performed in controlled environment but it also implicates short-study duration, only permitting acute evaluations of muscular anabolism. Therefore, it is appreciable how a less limiting and longer-term methodology, as the one that deuterium oxide tracer could offer, can be used to determine MPS in 'free-living' subjects.

Thus, the aim of study 2 was to investigate the efficacy of D<sub>2</sub>O for quantifying progressive and cumulative MPS under regular and exercised states (with the intention of evaluate its likely sensitivity to enlighten muscle anabolism during short training periods). Our hypothesis is that D<sub>2</sub>O combined with IRMS technique (pyrolysis - isotope ratio mass spectrometry, which permits high-precision assessment of hydrogen or oxygen stable isotopes) could be used to differentiate the fractional synthesis profiles (myofibrillar, collagen, sarcoplasmic) of muscle proteins over a short-term resistance exercise protocol (4 bouts over 8 days). Hence, by doing this, while being able to avoid AA infusion via cannulation (and thus to avoid limitations caused by time and controlled setting), we would attempt to clarify and calculate MPS in free-living conditions and over a period in which hypertrophic responses cannot be detected by commonly used imaging techniques (MRI, DXA, CT).

As intents and objectives of the first two projects have been considerably clarified, study 3 design falls directly into places as a main consequence. After testing the efficacy of D<sub>2</sub>O method with resistance training in study 2, one of the main purposes for study 3 was to evaluate what study 1 could not provide: the quantification of muscle protein synthesis in response to an ECC vs. CON loading protocol, measured via IRMS and deuterium oxide stable isotope tracer young healthy males.

Hence, this final study was conceived particularly to unravel a few unsolved issues regarding shortening and lengthening contractions in humans. Firstly, literature still furnishes controversial findings about the real contribution to increase in muscle size brought by the two loading modes (Wernbom et al., 2007; Roig et al., 2009): our study 1 suggests contraction-specific hypertrophic patterns, which need further investigation. Secondly, the mechanisms of differential architectural remodeling appear to indicate loading-specific sarcomere addition (Kawakami et al., 1993; Reeves et al., 2009): even though works in animal muscle showed that stretching or even lengthening contractions seem to facilitate the increase in fibre length by sarcomerogenesis in series (Williams et al., 1988; Williams, 1990; Morgan & Talbot, 2002; Butterfield et al., 2005; Zöllner et al., 2012) and preferably at the end of muscle fascicles (Williams & Goldspink, 1971) it remains still unknown where and how this new contractile material is placed throughout human muscle length. Furthermore, so far no study in humans has tried to ascribe potential heterogeneous responses to a training intervention in the same muscle (i.e. evaluating adaptations to exercise in two different part of the same muscle).

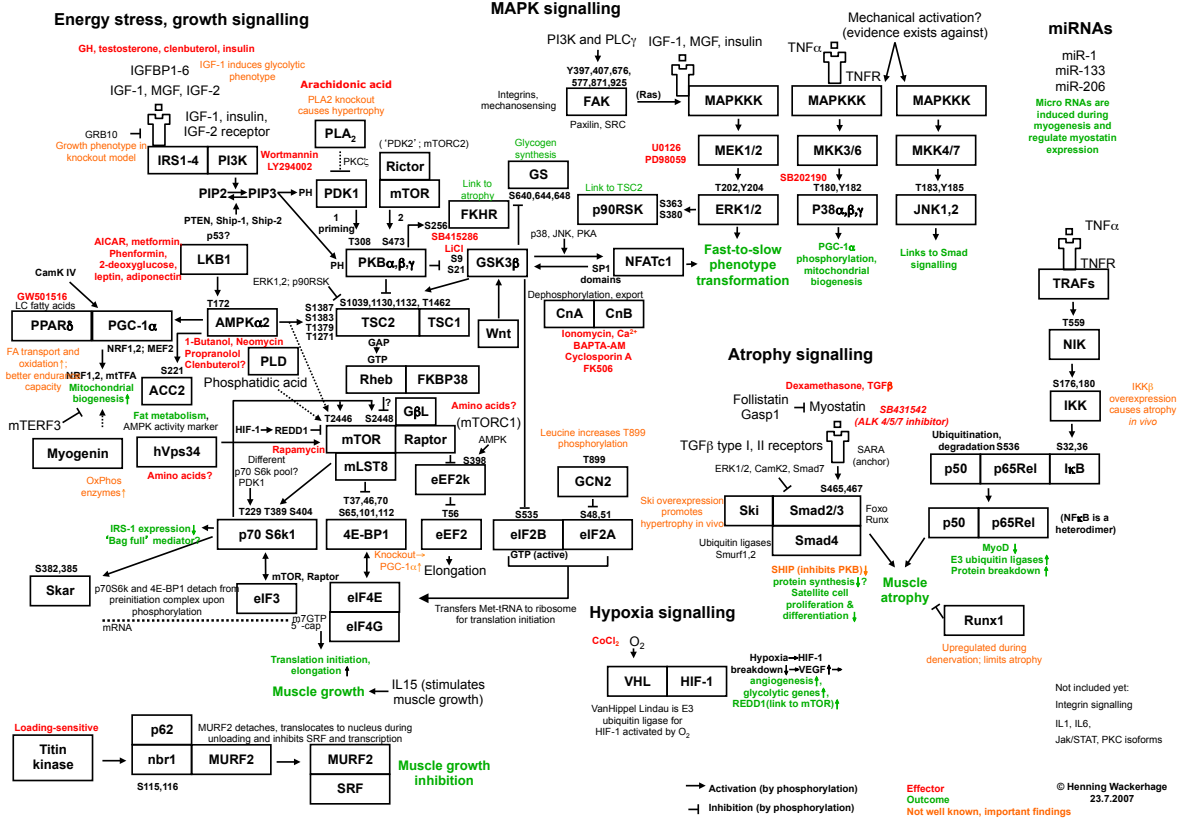
Hence, we recruited health young males that performed different unilateral training (one leg training eccentrically, while the other exercised only concentrically) for 8 weeks: we aimed to collect muscle samples at different VL muscle sites (VL mid-muscle belly and closer to -4cm away from-myotendineous junction (MTJ)) and at different time-points (baseline- 4 weeks- post-training) in order to gauge progressive architectural, functional and anabolic adaptations to the training protocols.

The aim of study 3 was therefore to investigate distinct changes in MPS related to shortening vs. lengthening loading over a period of 8 weeks while attempting to fill the gap in our understanding of the relationship between acute and chronic muscular changes to ECC vs. CON RET. Examining muscle homogeneity throughout its length (mid-length vs. MTJ biopsies), regarding its biochemical responses to different mechanical stimuli, it was surely of great interest; the hypothesis put forward was that eccentric and concentric contractions will lead to different preferential increase in MPS through the muscle length, likely showing differences between muscle sites as result of contraction-dependent regional increase in muscle mass (i.e. please see chapter 1 – results section). Eccentric and Concentric training should likely lead to similar morphological, functional and architectural changes previously seen in study 1 and thus new insight of the mechanisms describing chronic exercise features could be investigated in study 3.



# Appendix

## Signal Transduction Pathways involved in the Regulation of Skeletal Muscle Phenotype and Size



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## Chapter 1

# Architectural, functional, and molecular responses to concentric and eccentric loading in human skeletal muscle

The work presented in this chapter has been published as:

**Franchi et al.** *Acta Physiol*, Volume 210, Issue 3, 642-654, 2014.

## **ABSTRACT**

**Aim:** Architectural, functional, and molecular responses of human skeletal muscle were investigated in response to concentric (CON) or eccentric (ECC) resistance training programs (RT).

**Methods:** Twelve young males performed 10 weeks of concentric (CON) or eccentric (ECC) resistance training (RT) (n = 6 CON, 6 ECC). An additional 14 males were recruited to evaluate acute muscle fascicle behavior (while performing leg press exercise) and molecular signalling in biopsies collected from vastus lateralis (VL) after 30 min of single bouts of CON or ECC exercise. VL volume was measured by magnetic resonance imaging. Muscle architecture (fascicle length, Lf; pennation angle, PA) was evaluated by ultrasonography. Muscle remodelling signals to CON or ECC loading (MAPK/AKT-mammalian target of rapamycin (mTOR) signalling) and inflammatory pathway (TNF $\alpha$ /Murf-1-MAFbx) were evaluated by immunoblotting.

**Results:** Despite the ~1.2 fold greater load of the ECC group, similar increases in muscle volume (+8% CON and +6% ECC) and in maximal voluntary isometric contraction (+9 % CON and +11 % ECC) were found after RT. However, increases in Lf were greater after ECC than CON (+12 vs. +5%) while increases in PA were greater in CON than ECC (+30 vs. +5%). Distinct architectural adaptations were associated with preferential growth in the distal regions of VL for ECC (+ECC +8% vs. +CON +2) and mid-belly for CON (ECC +7 vs. CON +11%). While MAPK activation (p38MAPK, ERK1/2, p90RSK) was specific to ECC, neither mode affected AKT-mTOR or inflammatory signalling 30 min after exercise. **Conclusion:**

Muscle growth with CON and ECC RT occurs with different morphological adaptations reflecting distinct fibre fascicle behaviour and molecular responses.



## Introduction

Skeletal muscles can contract by shortening (concentric) or lengthening (eccentric) (Joyce et al. 1969; Joyce & Rack 1969). “Conventional” resistance exercise training, using commercial exercise machines is the most common form of resistance-exercise, consisting of lifting and lowering a constant external load. Thus, conventional resistance exercise training combines CON (lifting-phase) and ECC (lowering-phase) actions. According to the force-velocity (F-V) relationship, each value of force and velocity on a given curve should belong to the same level of neural activation (Bigland & Lippold, 1954; Camilleri & Hull, 2005; Chow & Darling, 1999). Yet, this requirement is not met by conventional RT as the same external load is displaced during both lifting and lowering phases. Thus, motor units must be de-recruited in the ECC part to enable the load to be lowered (Reeves et al. 2009); as such the load used for conventional training is limited by the CON muscle action. Therefore, to ensure that the ECC component of resistance training is not under-loaded, it would be necessary that both shortening and lengthening phases follow the physiological force-velocity curve i.e., the absolute load should be greater for the ECC than the CON contraction (Katz 1939), theoretically involving the same level of neural activation between contraction modes. Nonetheless, to our knowledge comparisons of pure CON to ECC exercise with such matching to equalize the relative loading stimulus, meeting a fundamental premise of the F-V relation, have not yet been made.

A recent investigation (Reeves et al. 2009) provided evidence that distinct loading patterns also lead to distinct architectural adaptations to exercise training, as suggested by Hortobágyi *et al.* (1996). In this previous study (Reeves et al., 2009), the architectural responses to muscle loading in older-aged individuals undergoing conventional vs. ECC only training regimes were compared. After 14-wk of training the authors noted a greater increase in muscle fibre (fascicle; Lf) length in the ECC only group compared to the conventional RT group. Conversely increases in pennation angle (PA) were only evident following conventional RT, but not after ECC only exercise. Furthermore, since conventional RT involves mixtures of both CON and ECC contractions, the architectural responses to “pure” ECC or CON contractions performed on standard isotonic machines with such matching for relative loading stimulus are unknown.

Distinct architectural adaptations to ECC vs. CON contractions also raise the question as to what could be the molecular basis of this phenomenon. Since both human and pre-clinical work has provided evidence of distinct molecular responses to e.g. CON vs. ECC contractions, it is likely that similar mechanisms underlie the different architectural adaptations. This hypothesis seems supported by the recent observation that ECC vs. CON growth of cardiomyocytes is regulated via ERK1/2 MAPK signalling (Kehat et al. 2011), demonstrating that acute signalling differences in response ECC vs. CON exercise could underlie the ensuing distinct architectural adaptations. In another investigation, using isolated rat muscle Wretman et al. (Wretman et al. 2001) reported greater increases in phosphorylation of ERK 1/2 and p38

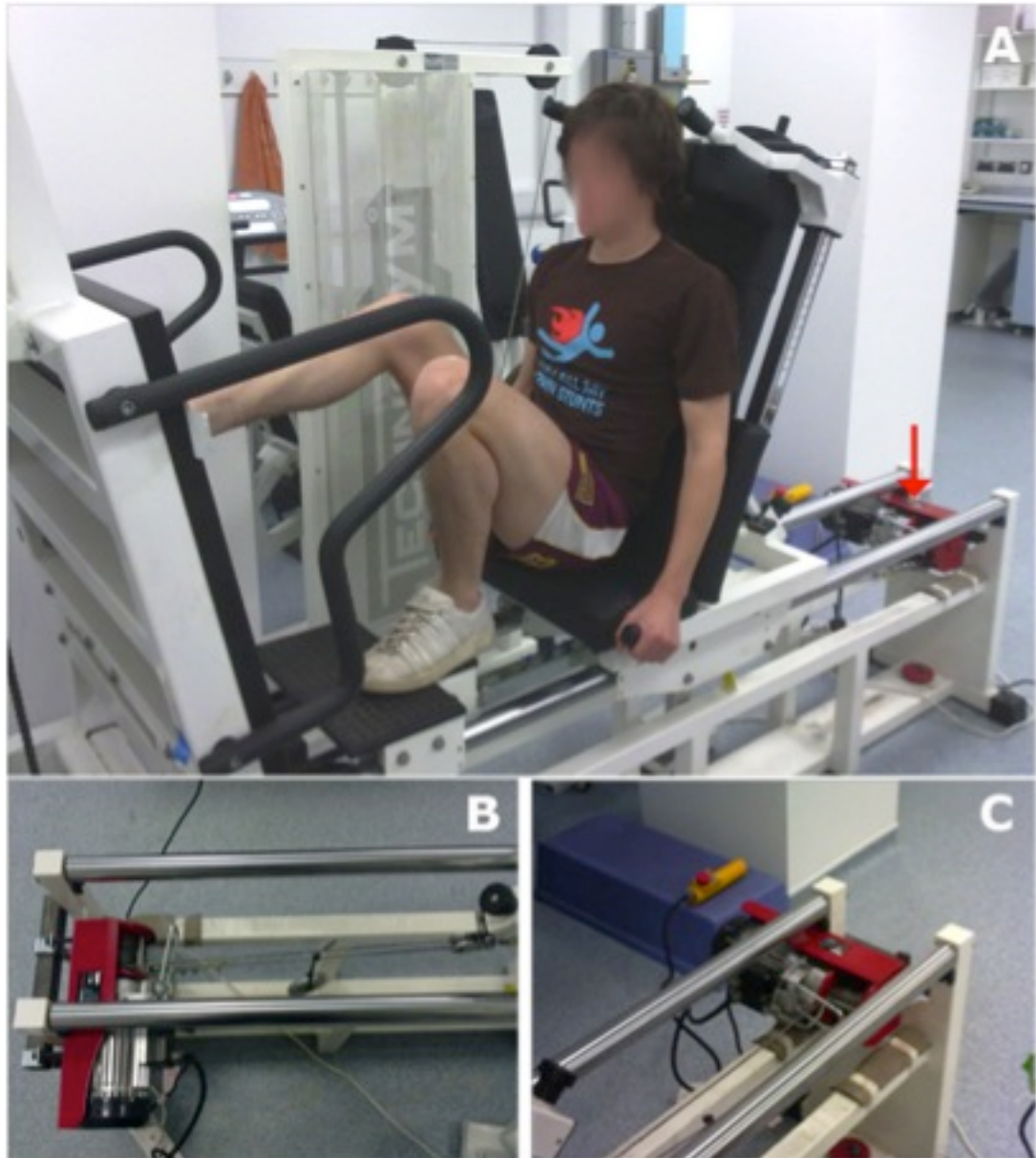
MAPKs induced by ECC vs. CON contractions. In addition Martineau et al. (Martineau & Gardiner 2001), observed that activation of MAPKs activation was quantitatively related to muscular tension with ECC contraction providing the greater stimulus. Finally, microarray analyses in young men (Kostek et al. 2007) demonstrated distinct responses to *acute* CON vs. ECC contractions, suggesting that contraction-specific muscle remodelling results both from distinct signalling and genomic responses to CON vs. ECC exercise. Nonetheless, the relationships between MAPK (or other) signals and that of the distinct architectural basis of skeletal muscle hypertrophy in response to CON vs. ECC exercise, remains unknown.

Therefore, the aim of the present study was to compare the effects of pure CON vs. ECC exercise training in terms of architecture, morphology and functional outcomes, and relate this to muscle cell signalling responses potentially ascribing the distinct structural and functional adaptations to CON vs. ECC training. The hypothesis put forward was that different mechanical stimulus (shortening vs. lengthening), chronically applied and matched to balance the relative loading inducement, would result in distinct adaptations in muscle morphology, function and architecture: possible underlying mechanical and biochemical mechanisms may be involved in these distinct remodelling processes.

## **METHODS**

We recruited 12 young men ( $25\pm 3$  y, height =  $182\pm 8.5$  cm, mass =  $71.9\pm 8.5$  Kg; means  $\pm$  SD) not partaking in resistance exercise training to undergo a 10-week resistance exercise training program. Based on their maximum

isometric knee extension torque, they were divided (matched for baseline strength) into two training groups: EG (ECC, n=6, 25±3 y) or CG (CON, n=6, 25±3 y). Resistance exercise training was carried out with a leg-press machine (Technogym, Gambettola Italy) modified to enable performance of either an ECC only (EG) or CON only (CG) contractions. This was achieved using an electric engine attached to the back of the leg-press (Fig 1): in the EG, the chair was pulled back with a cable that connected the electric winch to the weight stack via a steel cable, ensuring that subjects did not exert any force with their quadriceps to perform what would otherwise have been the concentric component of the exercise. When the chair was released, it enabled the subject to lower the training load under control through an ECC contraction of the quadriceps. Conversely the CG performed a CON-only movement consisting of lifting the load. In this case the engine operated only during lowering of the load, ensuring that subjects did not exert any force with their quadriceps to perform what would have otherwise been the eccentric component of the exercise.



**Fig 1.** Subject on the Technogym leg press modified ad hoc with the special electric engine visible on the right corner indicated by the arrow (Fig 1 A). The electric winch attached to the chair via steel cable; Fig 1B shows how the winch was connected to the chair (the red arrow indicates a counterweight that prevented the cable from becoming too slack and getting damaged); Fig 1C presents the site where the engine was placed

The timing of the contraction was slightly different for the two groups: the CG were asked to complete the contraction in  $\sim 2$  s, whereas this time period was  $\sim 3$  s for the EG. This time difference ( $\sim 2$  s CG vs  $\sim 3$  s EG) was necessary to ensure that the load was indeed lowered under control in the EG. The

training period for the first study was performed, after a familiarisation session, three times per week for 10-wk and people trained both legs but unilaterally. Both training and acute exercise bouts on the leg press machine involved the main extensor muscles of the lower limbs. The training load used was of 80% of the concentric (CG) or 80% of the eccentric (EG) 1RM, with 4 series of a minimum of 8 to a maximum of 10 repetitions with one-minute rest in between the sets. The 1RM was assessed after a warm-up program performed on the leg press machine using a very light weight that allowed the subject to easily perform 8 to 10 repetitions (concentrically or eccentrically). Then, the protocol followed for both contraction phases was the one suggested by Baechle & Earle (1994). This study was approved by the ethical committee of the health care science faculty of the Manchester Metropolitan University and conformed to the requirements of the Declaration of Helsinki. Volunteers were informed of the purpose of the study, the experimental design and procedures involved and all the potential risks involved before giving their written consent

#### *Measurement of electromyographic (EMG) activity*

VL integrated EMG was measured as representative of the knee extensors to provide an indication of neural drive to this muscle group during the tests performed on the Cybex dynamometer. Two surface electrodes (10 mm diameter) were placed next to each other on the lower third of the VL muscle with a 20 mm centre-to-centre electrode distance. These two electrodes were arranged in a “bi-polar” configuration with a third electrode, the “ground”,

placed on a bone area (the patella bone in this case). The skin was shaved and conditioned using a special skin preparation gel (Nuprep™) to reduce skin impedance (using an electrode impedance tester - Oxford medical ltd, Medilog, UK) below 5,000 Ohms. In order to reproduce the same electrode positioning in the successive recording sessions, measurements were taken and anatomical spots (bone processes, tendon and muscle insertions) were used to know exactly the right portion of VL for the surface electrodes to be placed. Acquisition of the surface EMG signal was obtained through the Biopac A/D acquisition system at a sampling frequency of 2000 Hz –and filtered through a bandwidth of 10-500 Hz. The root mean square (RMS) was calculated from the raw EMG over a 200 ms time frame where the peak of torque was expressed during the isometric MVC trials. During the 1RM assessment, EMG was monitored in order to support our assumption that CON and ECC 1RMs would have resulted in similar neural drive: the RMS was calculated over a 200 ms time frame during the mid-portion of the contraction phase.

#### *Magnetic Resonance images (MRI)*

Axial plane scans of the thigh were taken before (1 week) and post-training (4-5 days) using a 0.25 Tesla magnetic resonance imaging (MRI) scanner (Esaote G-scan, Italy). A T1-weighted Spin Echo protocol was used (repetition time 900 ms, echo time, 26 ms, number of excitation 2, Field of View 200x200 mm, slice thickness 10 mm, gap between slices, 1.0 mm). Participants were asked to lie supine on the MRI bed and to insert their leg into a circular coil. Due to the scanning area of the coil, the thigh was imaged

in 3-4 separate sections. Markers were placed on the thigh from the patella to the hip to denote different sections and avoid overlap. Axial plane scans along the entire length of the VL were collected; on average, the number of axial scans obtained in each subject was the same for the baseline and post-training periods (~34). From these scans the contours of the VL muscle of each MRI scan were digitized using the Osirix image analysis software and, subsequently, VL Muscle Volume was calculated as follows:

$$\text{Volume}_{VL} (\text{cm}^3) = \sum \text{ACSA} \cdot (\text{slice thickness} + \text{gap between slices}).$$

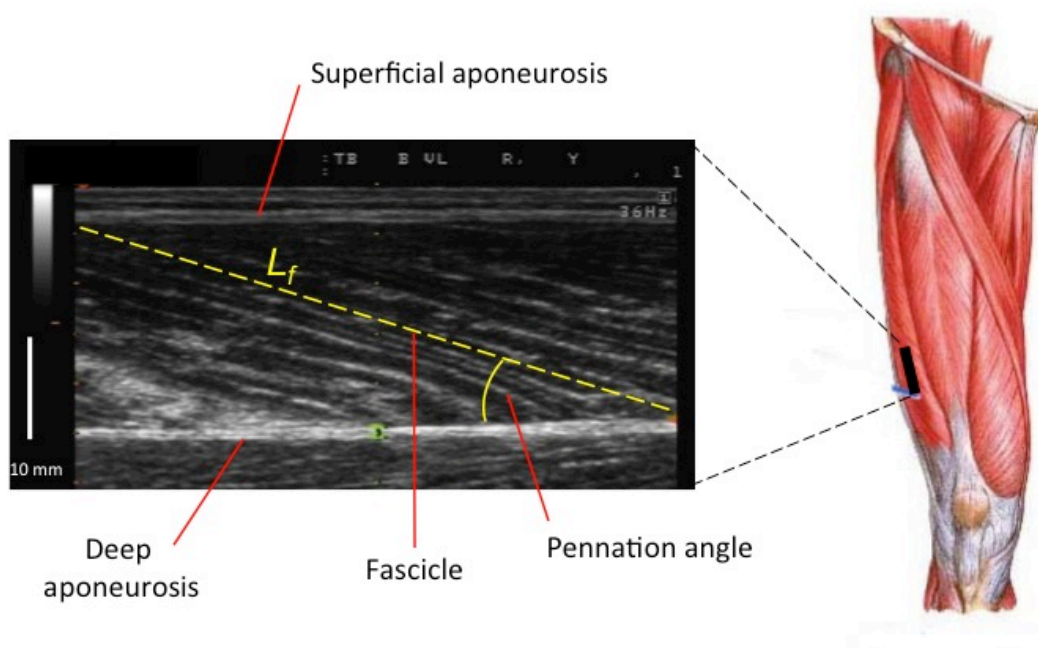
Regional VL hypertrophy was calculated after training by obtaining the baseline and post-exercise average values of the first 5 axial scans where the VL muscle was visible starting from the hip/knee joint (proximal and distal portions respectively) and the 5 scans around the peak of ACSA (muscle mid portion): from these mean values, the percentage increase in ACSA was calculated for the 3 different regions of the VL muscle.

### *Muscle (VL) Architecture*

Before (1 week) and after training (4-5 days), VL muscle architecture i.e., Lf and PA were measured (by the same investigator) from images obtained *in vivo* at rest using B-mode ultrasonography (Mylab 70, Esaote Biomedica, Italy), with a 100 mm, 10-15 MHz, linear-array probe. Resting ultrasound images were taken at a specific joint angle (150°), corresponding almost to full knee extension (180°), while the participant was seated on the Cybex



Norm dynamometer chair; the transducer was aligned in the fascicle plane in order to be able to visualize an optimal portion of fascicles on the ultrasound screen. The muscle architectural parameters were quantified from the ultrasound scans using the image analysis software, ImageJ 1.42q (National Institutes of Health, USA). The visible portion of the fascicle length was directly measured using this software. In some instances, a small portion of the fascicle extended off the ultrasound window and it was necessary to estimate this non-visible portion using a linear extrapolation of fibres and aponeuroses (Erskine et al. 2009). Pennation angle was measured as the intersection between fascicles and the deep tendon aponeurosis (Fig 2). The reliability of these ultrasound techniques has been published (Intra Class Correlation value = 0.99)(Reeves et al. 2004) ; images were collected and digitally analysed by the same operator.



**Fig 2.** VL ultrasound image captured at rest: pennation angle and the visible part of a muscle fascicle is shown.

### *Muscle function*

Participants were familiarized with all the devices and procedures involved in the study before the actual test sessions: the exercise-training participants were asked to perform contractions in a seated position on the reclining chair of the Cybex Norm dynamometer (hip angle = 85°, hip angle at supine position = 0°). The lower leg was strapped to a pad situated at the end of the Cybex lever arm and the knee joint center of rotation was aligned with the dynamometer fulcrum. The torque produced on the Cybex dynamometer was sampled into an analogue to digital acquisition system (Biopac System, Inc. California) at a frequency of 200 Hz and displayed on the screen of an Apple computer (Mac. G4). Maximum isometric torque of the knee extensor muscle group was evaluated by participants performing an isometric maximum voluntary contraction (MVC) at every 10° (0.175 rad) from 90° to 150° (from 1.57 to 2.62 rad) of knee joint angle (180° = full extension). Two MVCs were recorded at each joint angle with 2 min separating each contraction and the highest torque produced was used to assess MVC changes from pre to post training.

### *Acute behavioural and molecular responses to CON and ECC contraction*

An additional untrained 14 men (25±4 y, height = 184±7 cm, mass = 74±4 Kg) were recruited and divided into two groups (CG acute, n=7, 26±4 y and

EG acute, n=7, 25±4 y) to perform a single bout of ECC or CON exercise, adopting the same design of the training study (same load-repetitions-sets combination). Vastus lateralis (VL) muscle biopsies were collected in these additional volunteers before and 30 min after exercise for signalling purpose: this time was specifically chosen as MAPK activation appears to be transient (Nader & Esser 2001). Ultrasound scans were also acquired during a single CON or ECC contraction performed on the leg-press device. Measures of fascicle length and pennation angle were recorded from screen captures during contractions and analysed in an identical fashion to in the training study.

#### *Immunoblotting*

Post exercise biopsies were processed in a similar fashion to as previously described (Atherton et al, 2010). Briefly, ~20 mg of muscle was snipped in ice-cold buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaF, 10 mM  $\beta$ -Glycerophosphate disodium salt, 1 mM EDTA, 1 mM EGTA, 1 mM activated  $\text{Na}_3\text{VO}_4$  (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at  $10 \mu\text{l} \cdot \mu\text{g}^{-1}$  of tissue. Homogenates were rotated for 10 min and the supernatant collected by centrifugation at  $13,000 \times g$  for 5 min at 4°C. The supernatant (sarcoplasmic fraction) was used for immunoblot analysis: protein concentrations were determined using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE-US) and adjusted to  $1 \mu\text{g} \cdot \mu\text{l}^{-1}$  in 3× laemmli. Each sample was loaded onto pre-cast 12% Bis-Tris Criterion XT gels (BioRad, Hemel Hempstead, UK) at 15  $\mu\text{g}$  per lane and separated

electrophoretically at 200 V for 1 h. Proteins were then wet-transferred at 100 V for 1 h onto polyvinylidene difluoride (PVDF) membranes (0.22  $\mu$ m), blocked for 1 hour in 2.5% skimmed milk in 1 $\times$  Tris-buffered saline/ Tween-20 (TBS-T), and then incubated in 1 $^\circ$  antibodies (1:2000 dilution in 2.5% BSA in TBS-T) rocking overnight at 4 $^\circ$ C. For phosphorylation of MAPK p38 (Ser189/207), p90RSK (Thr359/Ser363), ERK1/2 (Thr202/Tyr204), p70S6K (Thr389), Akt (Ser473), p65 (Ser536) and pan-actin antibodies were obtained from Cell Signaling Technology, Inc. (MA, US), 4E-BP1 (Ser65/Thr70) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA-US) and MAFbx, Murf-1 (C-terminal region) from ECM Bioscience (KY, US). For total amount of TNF $\alpha$ , p65 and I $\kappa$ B $\alpha$  antibodies were obtained from Cell Signaling Technology, Inc. (MA, US) The next day, membranes were washed 3 $\times$ 5 min in TBS-T, incubated in HRP-conjugated 2 $^\circ$  antibody (New England Biolabs, Hertfordshire, UK; 1:2000 in 2.5% BSA in TBS-T) at room temperature for 1 h, before 3 $\times$ 5 min washes in TBS-T. Membranes were exposed to chemiluminescent HRP Substrate (Millipore Corporation, Billerica, MA-US) for 5 min and bands quantified by Chemidoc XRS (BioRad, Hertfordshire, UK). Software measures were taken to prevent pixel saturation; loading anomalies were corrected to Pan-Actin.

### *Statistical analysis*

Differences for group (CG vs. EG, the training groups and CG1 vs. EG1, the acute study groups) and time (baseline vs. post-training / baseline vs. post-exercise) were analyzed using a two-way factorial analysis of variance test

using GraphPad PRISM software (version 5.0d; GraphPad software Inc. San Diego, CA). Significant interactions between groups and time were located by Bonferroni post-hoc test. The delta ( $\Delta$ ) training values (percentage increases) were statistically tested between groups using an independent t-test, that was also used to compare baseline differences between CG and EG for physiological parameters. A power calculation was performed: our current sample size has a beta level of 0.8 (i.e., power of 80%) for the training study (12 participants) using the parameter of pennation angle and a beta level of 0.9 (i.e., power of 90%) for the acute study (14 participants) using the parameter of p38 MAPK.

## **RESULTS**

### *EMG of CON-ECC 1-RM, maximum lifting or lowering ability (1-RM) and training load*

The means of baseline EMG values for CG and EG 1-RM are presented in Table 1. As mentioned, to test our assumption that both CON and ECC contractions belonged to the same Force-Velocity curve, EMG activity was measured during performance of a single concentric and eccentric 1-RM in each subject to evaluate if the two phases correspond to a similar level of neural activation. In support of our assumption, no significant difference existed in neural activation during the performance of the CON or ECC only exercise. As expected, regarding the maximum lifting or lowering ability data, the baseline and post-training 1-RM was higher in the EG than the CG (Table

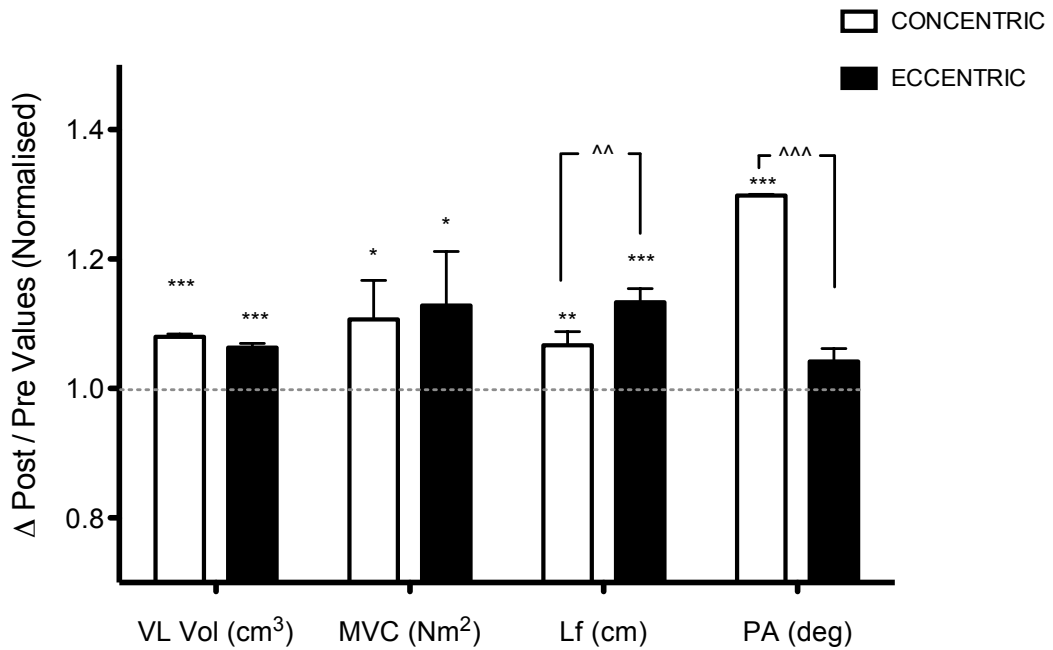
1). The pre-to-post training increase in 1-RM was statistically significant in both groups, but with no significant difference in the percentage increase between the EG and CG.

CG 1RM (Kg)			EG 1RM (Kg)			Load ratio	
Pre	Post	Δ%	Pre	Post	Δ%	Pre	Post
192 ± 16	262 ± 30	36*	233 ± 13	337 ± 9	44*	1.21	1.29
EMG (mV)			EMG (mV)				
0.33 ± 0.1			0.31 ± 0.1				

**Table 1.** Maximum lifting or lowering ability changes for the CON Group (CG) and the ECC one (EG) and EMG values recorded at baseline 1RM leg-press for concentric and eccentric phases. Load ratio is also showed and calculated as the ratio of pre and post ECC/CON training loads. (Pre = baseline, Post = Post-training) values are means ± SEM (\* P<0.05, pre-to-post difference).

#### *Muscle morphology and architecture and maximum voluntary contraction*

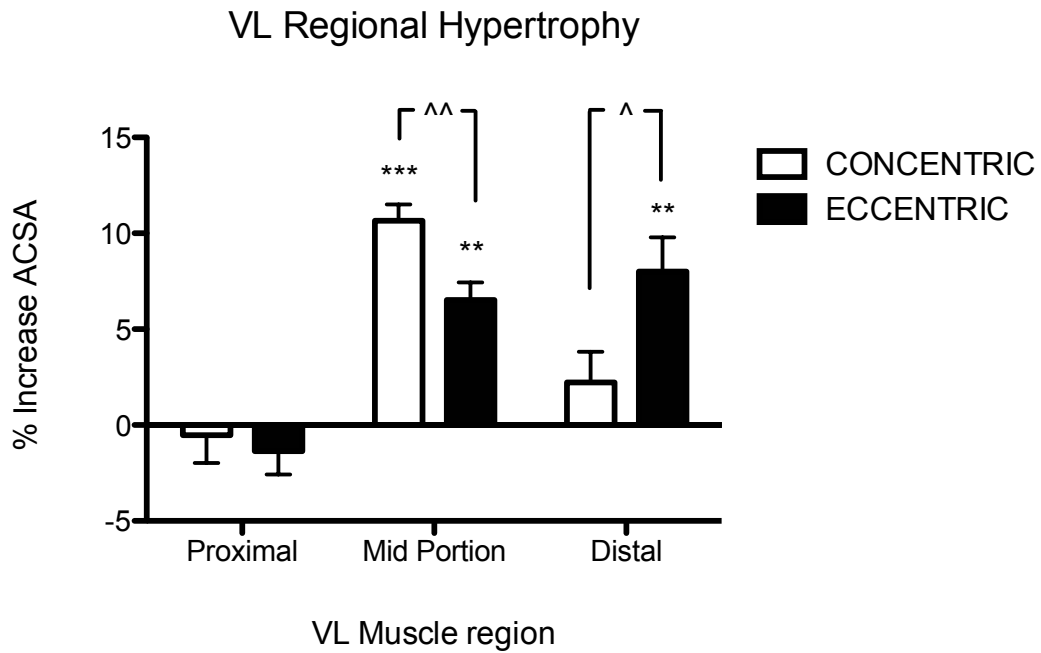
After training, both groups showed an increase in VL muscle volume but the change was similar between the EG (6 ± 0.4%, mean ± SEM, P < 0.0001) and CG (8 ± 0.5%, P < 0.0001). However, Lf increased significantly more (P<0.01) in the EG (12 ± 2%, P < 0.0001) compared to the CG (5 ± 1%, P < 0.01); conversely PA increased significantly less (P<0.01) in the EG (5 ± 1%, P > 0.05) than the CG (30 ± 0.5%, P < 0.0001) group. Maximum voluntary contraction (MVC) peak amplitude changed in both groups similarly (significant pre-to-post difference, P<0.05) (EG = 11 ± 8%, P < 0.05, CG = 9 ± 6% increase, P < 0.05; Figure 3).



**Fig 3.** Post/Pre training ratios of muscle volume, isometric MVC and muscle architecture in the concentric and eccentric exercise groups. Y = 1 represent the baseline value. Data normalized to pre values; means  $\pm$  SEM (\*  $P < 0.05$  \*\*  $P < 0.001$  \*\*\*  $P < 0.0001$  - ^^, ^^ = significantly different between groups:  $P < 0.01$  and  $P < 0.001$ , respectively).

### Regional hypertrophy of VL muscle in response CON or ECC training

Differences in localized hypertrophy were observed in response to 10-wk of either CON or ECC resistance exercise (Figure 4). While both loading modalities induced similar effects on ACSA % increase/decrease in the proximal area (EG =  $-1 \pm 1\%$ , mean  $\pm$  SEM, and CG =  $-0.5 \pm 1\%$ ) a significant difference was found in both mid portion (EG =  $7 \pm 1\%$ , and CG =  $11 \pm 1\%$ ,  $P < 0.01$ ) and distal part of vastus lateralis (EG =  $+8 \pm 2\%$  versus CG =  $+2 \pm 1.5\%$ ,  $P < 0.05$ ) between the two types of training.



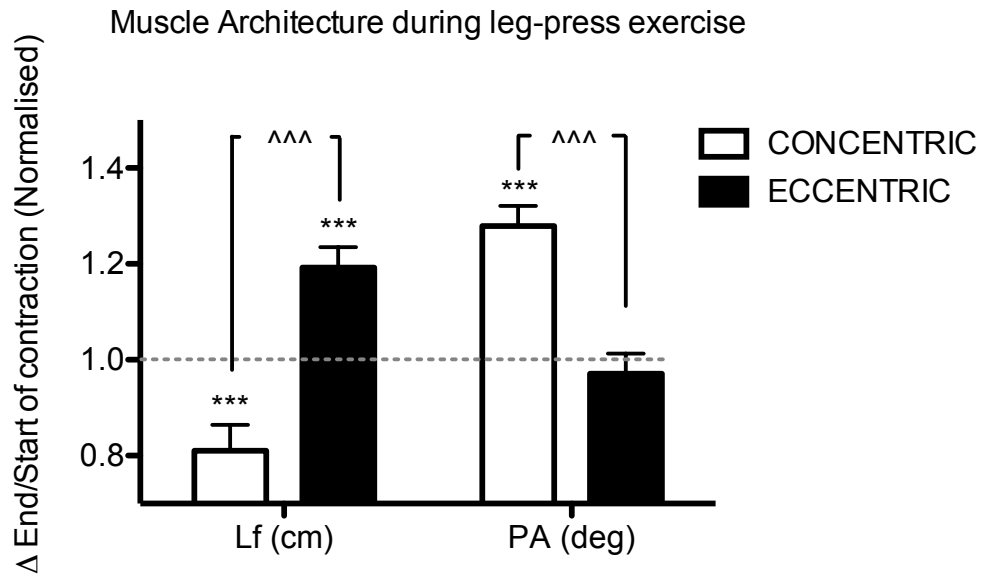
**Fig 4.** Regional Hypertrophy of VL muscle (ACSA = Anatomical Cross Sectional Area) after concentric and eccentric training. Data are means  $\pm$  SEM (\*\* $P < 0.01$  \*\*\* $P < 0.001$  - ^, ^^ = significantly different between groups:  $P < 0.05$ ,  $P < 0.01$ ).

#### *Architectural behaviour of VL muscle during performance of CON vs. ECC contractions*

Following discovery of such distinct architectural adaptations, we recruited a second cohort to interrogate possible mechanical reasons for these findings, with the aim of determining fascicle behaviour during CON and ECC contractions. Differential behaviour was observed in Lf and PA during CON and ECC resistance exercise performed with leg-press (Figure 5). During ECC, fibres lengthened during performance of ECC exercise (Lf =  $+19 \pm 2\%$ , mean  $\pm$  SEM, from start to end of contraction,  $P < 0.0001$ ) whereas during CON there was a substantial fascicle shortening in Lf (Lf =  $-19 \pm 2\%$ ,  $P < 0.0001$ ). Similarly, while PA remained similar from the start to the end of ECC



(PA =  $-3 \pm 1\%$ ,  $P > 0.05$ ), it showed a substantial increase during CON (PA =  $+28 \pm 1\%$ ,  $P < 0.0001$ ).

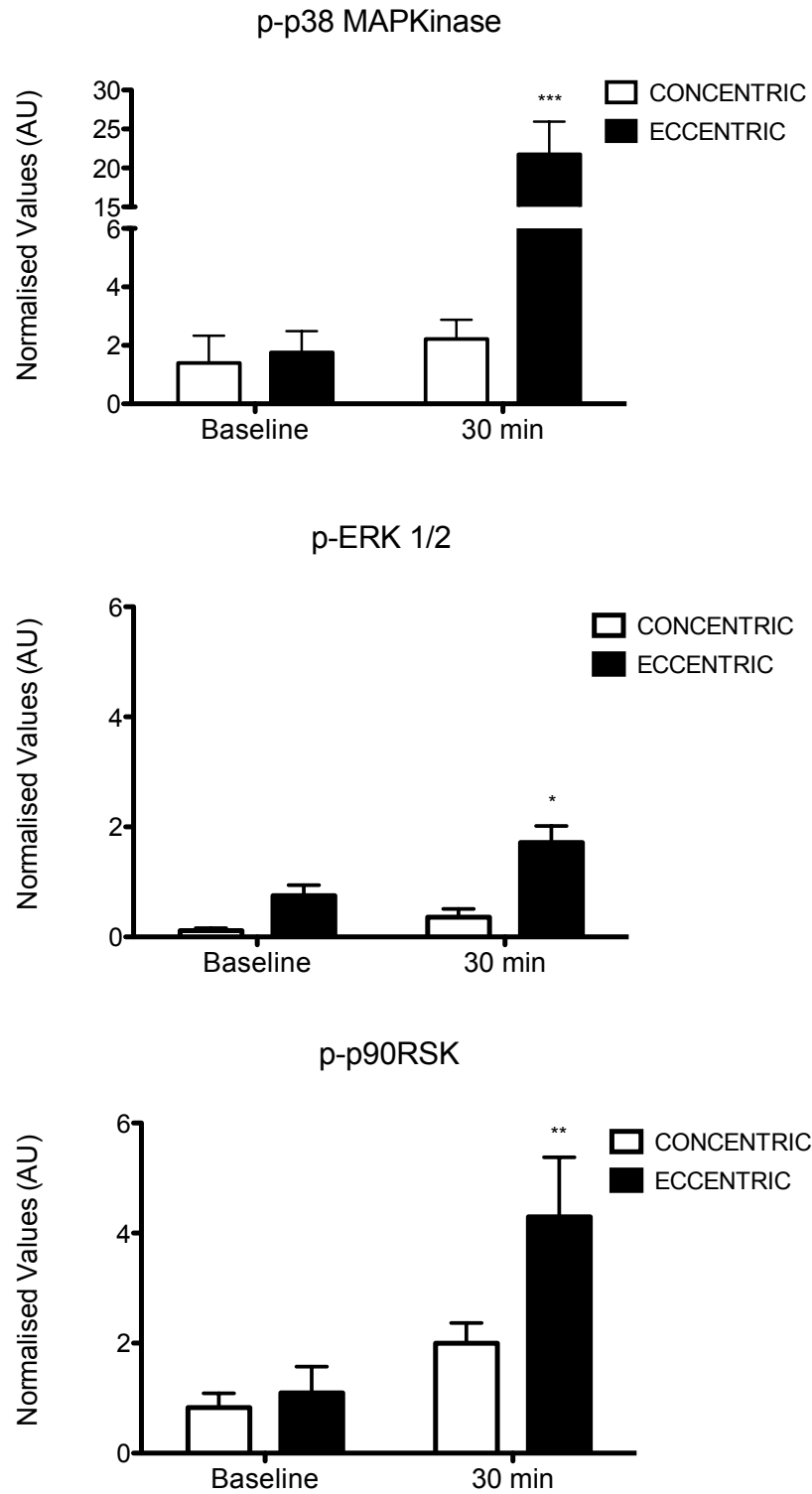


**Fig 5.** Muscle architectural behavior during a concentric and eccentric contraction performed on the leg-press device. Y = 1 represent the baseline value. Data normalized to pre values; means  $\pm$  SEM (\*\*\*)  $P < 0.0001$  - ^^^ = significantly different between groups  $P < 0.0001$ ).

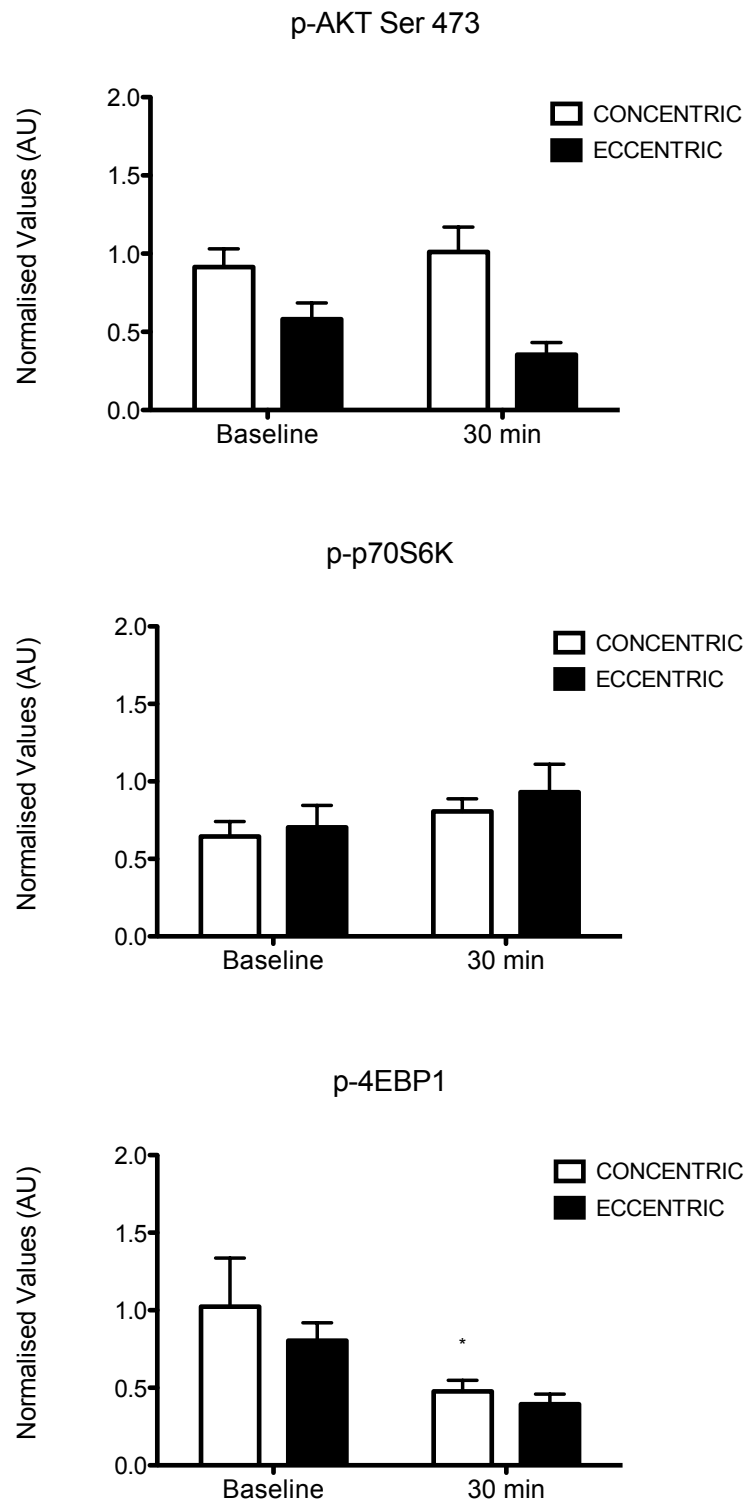
#### *Acute MAPK, AKT-mTOR and Inflammatory/breakdown signaling responses to a single acute bout of CON vs. ECC exercise*

By taking biopsies 30 min following this single bout of CON or ECC we were also able to interrogate intramuscular signalling purported to be involved in exercise adaptations. Significant increases in phosphorylation of mitogen activated protein kinases (MAPKs) i.e., p-38MAPK, ERK1/2 and p90RSK (Figure 6) were found 30 min after ECC resistance exercise (p38MAPK =  $20 \pm 4$ -fold, ERK1/2 =  $2 \pm 0.3$ -fold, p90RSK =  $3 \pm 1$ -fold) but not after CON

resistance exercise. In contrast, there was no modulation in the phosphorylation of Akt (Ser473) and mammalian target of rapamycin (mTOR) substrate p70S6K 30 min after CON or ECC exercise, although a significant suppression ( $P < 0.05$ ) in activation of 4-EBP1 was found only after CON exercise (Figure 7). Non-significant changes in the activation of the TNF $\alpha$ /Murf-1-MAFbx pathway (TNF $\alpha$ , p-p65, p65, I $\kappa$ B $\alpha$ , p-MurF-1, p-MAFbx) were found 30 min after CON or ECC exercise.



**Fig 6.** MAPK Molecular responses (phosphorylation) at 30 minutes after either a single concentric or eccentric training session. Data are means  $\pm$  SEM. (\*  $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ )



**Fig 7.** Akt, p70S6K and 4EBP1 molecular responses (phosphorylation) at 30 minutes after either a single concentric or eccentric training session. Data are means  $\pm$  SEM (\*  $P < 0.05$ ).

## DISCUSSION

In the present study we compared, for the first time, the structural remodelling of human skeletal muscle in response to pure CON and ECC loading while attempting to link these with the molecular signalling pathways implicated in muscle remodelling (MAPKs, mTOR etc). Furthermore, whilst previous investigations focused on morphological and architectural responses to CON and ECC resistance training matched for work-load (Blazevich et al. 2007; Higbie et al. 1996; Moore et al. 2012), no study has yet, to the best of our knowledge, matched the CON and ECC phases for the same relative load while monitoring neural drive in order to meet one of the fundamental requirements of the F-V relationship (Bigland & Lippold 1954; Camilleri & Hull, 2005; Chow & Darling, 1999). Hence, our training loads were matched to the same percentage of the CON and ECC repetition maximum (i.e., CON 1RM and ECC 1RM) and EMG values revealed similar levels of neural activation for both CON and ECC 1-RM (Table 1). The ECC group/CON group training load ratio remained between the 1.21 to 1.29 range (Table 1), this confirms previous observations of the greater forces associated with ECC than CON *in vivo* (Aagaard et al. 2000; Westing et al. 1988). These findings support our contention that both shortening and lengthening phases of our resistance exercise paradigms belonged to the same F-V curve. Although 1RM assessment is a sort of an “unrefined” method, the fact remains that 1RM is still recognised as ‘golden standard’ in training studies. Furthermore, the best available technique to assess neural drive *in vivo* is still EMG. In this investigation, integrated EMG (i.e. result of recruitment and recoding) was similar in the two different conditions. The

authors would like to emphasise that in the present study the load was not matched for neural activation but rather, EMG was recorded in relatively equal external loads and similar EMG values were found.

Muscle hypertrophy *per se*, was an expected consequence of our resistance training protocols. However, muscle volume showed similar changes after both training modes (ECC = +6 and CON = + 8%, non significant difference between groups). Although both concentric and eccentric exercise programs have shown to induce gains in muscle mass there seems to be insufficient evidence of the superiority of either these two types of contraction (Wernbom et al. 2007). Nevertheless, the similar changes in muscle volume in the present study were considered unexpected, as not only ECC training load was higher, but also because ECC training has been suggested to produce greater hypertrophy and strength than CON training (increase in muscle fibre size, Hortobágyi et al. 1996) and associated trends towards whole muscle greater CSA (Roig et al. 2009). If the predominant promoter of muscle hypertrophy were the mechanical stimulus, one would expect to find a greater hypertrophy in the ECC group due to the higher training load (i.e. higher mechanical stimulus). However, this was not the case, indicating that the intensity of mechanical stimulus may not be the sole determinant for muscle hypertrophy; rather, this might be governed by the type of contraction performed (ECC/CON) and also that other factors blunting muscle hypertrophy may be at play in ECC contractions.

Interesting differences in muscle architecture using ultrasound were also found as result of the different training regimens. Although Lf increased in both groups, the ECC group showed a significantly greater gain in Lf compared to the CON one, while training produced an increase in PA after both types of training but the increase in pennation angle in the ECC group was much lower than in the CON one (Fig. 3). These findings suggest that addition of serial sarcomeres occurs in response to muscle lengthening scenarios (e.g. Holly et al. 1980; Reeves et al. 2009; Seynnes et al. 2007), and herein mainly, as a result of the ECC component. Instead, increases in PA occur to bundle more contractile units along the tendon aponeurosis (Gans & Bock 1965; Kawakami et al. 1993) primarily reflecting muscle shortening, principally as a result of the CON component. Finally, while these findings of distinct architectural responses are allied with those reported by Reeves et al. (Reeves et al. 2009) in older humans after ECC vs. conventional training, our current evidence for distinct architectural adaptations to *pure* CON vs. ECC in younger individuals is the first report of its kind and reveals that adaptations following conventional RT are dominated by the concentric component (at least in older men), perhaps reflecting the greater loading stimulus of the CON phase compared to the ECC one, when applied using standard gym equipment.

Intriguingly, while both groups showed a similar overall increase in VL muscle volume, the regional morphological patterns of muscle hypertrophy induced by the two loading modes differed substantially. For instance, while ECC exercise promoted greater muscle hypertrophy (as measured by

changes in ACSA by MRI) in the distal portion compared to CON, increases in the mid VL muscle were greater for CON than ECC (Fig. 4). We contend that evidence of these differences in the regional distribution of hypertrophy along the muscle belly, reflect a differential addition of sarcomeres in series and in parallel. Pennation of muscle fibres allows greater packing of sarcomeres in parallel along the tendon aponeurosis (Gans & Bock 1965). Hence, the finding that CON training promoted a large increase in pennation (30%), with little increase in fascicle length (5%), strongly suggests that CON training leads to hypertrophy mainly through addition of sarcomeres in-parallel. As indicated by the increase in ACSA, this phenomenon seems to mainly occur in the central region of the VL, which, because of the bell-shaped distribution of muscle ACSA, comprises a large portion (~ 60%) of the whole VL volume. Instead, when training involved muscle stretch, i.e. with ECC training, hypertrophy occurred mainly through an elongation of fascicles (12%) and with little increase in pennation angle (5%), suggesting preferential addition of sarcomeres in-series. The increase in ACSA over a larger portion (about 2/3) of the muscle belly (central and distal regions) associated with preferential increase in fascicle length suggests that the addition of new sarcomeres in series occurred over a large portion of the muscle belly. It remains to be established where along muscle fibres sarcomere were added but it is probable that this occurred at the periphery since early (Williams & Goldspink 1971) as well as recent (Allouh et al. 2008) observations showed (directly or indirectly), preferential addition of sarcomeres in series at the periphery of muscle fibres in response to stretch overload, and in response to developmental growth, as satellite cell



frequency and concentration seems particularly high at the ends of muscle fibres (Allouh et al. 2008). Although this accordance between architectural and morphological adaptations to training seems reasonable, a limitation of the present study is that ultrasound scans were taken just from the middle of the muscle belly with the assumption that changes in architecture observed in this region would be representative of changes along the whole muscle. This may not be the case, as pennation angle might have increased more closer to the myotendineous junction after ECC exercise (i.e. causing the greater VL distal hypertrophy). However, although in principle it could be argued that limiting the ultrasound scans to a single muscle site may not also be representative of other changes occurring in other muscles, it must be acknowledge that Vastus Lateralis presents a more uniform architecture throughout its length compared to other heads of the quadriceps (i.e. Vastus intermedius (VI) presents inhomogeneous architecture, Blazeovich et al. 2006). Moreover, a very recent publication investigating the changes in muscle architecture between different sites of the four heads of the quadriceps, observed how the adaptations in muscle CSA, thickness and PA were quite consistent between the Vasti and only significantly different if compared to Rectus Femoris (RF) changes size and architecture (Ema et al. 2013). These results could be explained by the fact that RF is a bi-articular muscle, differing from the vasti anatomically and biomechanically. The region of VL investigated in the present study (VL mid length) coincides with the site in which the largest CSA value was observed. Furthermore, our aim was to show different responses brought by the two different loading paradigms: the choice of the muscle site is supported by the study by Ema et al. (2013) in

which VL is the muscle that showed less inhomogeneous changes between CSA and architecture throughout the muscle hence we have reason to believe that this site could still be the best representative of the whole quadriceps.

Blazevich and colleagues (2007) similarly reported an increase of Lf in response to ECC exercise in the first 5 weeks of training but it could be argued whether the architectural adaptations of the present study do continue overtime, as it appears that in Blazevich's study these adaptations did not occur beyond the 5 weeks period. Thus, whilst Blazevich and colleagues confirmed the early architectural adaptations phenomenon previously reported by Seynnes et al. (2007), our present work suggests that these changes are still detectable after 10 weeks of RET. Further investigation is needed in order to assess if these architectural responses will be observed of different magnitude (i.e. similar or milder) after 5 weeks of RET.

Despite the distinct global (i.e., whole-muscle volume) hypertrophy responses between CON and ECC training groups, functional (strength) adaptations revealed similar increases in isometric MVC for both groups (CON 9%; ECC 11%). Although this similarity in the strength increase seems paralleled by the changes in muscle volume, it does raise the question of why, despite the greater training load (1.2 fold) of the ECC group, VL hypertrophy was similar. Possible causes of this finding and of the different architectural adaptations to ECC and CON training may be linked to muscle damage caused by ECC contractions and to distinct signalling pathways

involved in ECC and CON.

Following such intriguing findings in the adaptive features of VL muscle after 10-wk of CON vs. ECC, we chose to investigate the acute changes of muscle architecture in response to single CON or ECC exercise bouts by recruiting a second subject group. As expected, based upon the behaviour of the muscle-tendon unit, during CON contractions fascicles shortened (by -19%) and lengthened (+19%) during ECC contractions. We speculate that the contraction-specific VL fascicle length change (i.e., marked fascicle shortening during CON and marked fascicle lengthening during ECC) is a primary cause of the differential architectural adaptations and that such adaptations start from the first training session after CON and ECC bouts, as suggested by Seynnes *et al.* (Seynnes *et al.* 2007) who showed that such differences in muscle architecture can be detected at very early stages of training.

Although distinct cell signalling responses to CON and ECC in humans have yet to be established, in the present study we observed increases in phosphorylated MAPK e.g. p-38 MAPK, ERK 1/2 and p90RSK in the ECC but not CON. Similarly, reputed differences in the signalling response of muscle cells have been observed in animal models (Martineau & Gardiner 2001; Wretman *et al.* 2001). Furthermore, ERK1/2 expression was previously shown to regulate CON vs. ECC growth pathways in cardiomyocytes, suggesting MAPKs are involved in regulating architectural remodelling processes in muscle tissue (Kehat *et al.* 2011). In this latter study, cardiomyocytes isolated from mice lacking ERK showed an increase in

length of the cardiomyocytes (ECC growth) whereas cardiac cells isolated from mice over-expressing MEK1 (a MAPK-Kinase, ERK1/2 up-regulator) showed a preferential growth in myocyte thickness (CON growth). Recruitment of this second study group allowed us to interrogate the effects of an acute bout of CON or ECC upon intramuscular signalling proteins associated with adaptation to exercise, with particular focus on MAPK's. In doing this, we observed a specific MAPK activation only in response to ECC (which lead to a preferential increase in Lf), although, a similar and simple relationship between ERK1/2 and determination of architectural adaptation in human skeletal muscle could not be confirmed, unless it is the reverse of the mechanisms occurring in cardiac muscle. Further work is needed to define this.

Another clue as to why these architectural differences may exist is that ECC leads to a greater degree of damage than CON (Byrne et al. 2004), with greater myofibrillar disruption occurring with ECC (Schoenfeld 2012). However, we found no significant increases in TNF $\alpha$ /Murf-1-MAFbx pathway (p-TNF $\alpha$ , p-p65, p65, I $\kappa$ B, p-MurF-1, p-MAFbx) 30 min after exercise. This supports the notion that activation of MAPK in response to ECC occurred independently of muscle damage/inflammation, acting through MAPK (Kramer & Goodyear 2007; Murton et al. 2008) and more likely through mechano-transduction mechanisms (although we cannot exclude the likely onset of muscle damage/inflammation phenomena after our single biopsy time point). Furthermore, despite the fact that we observed no differences in whole-muscle hypertrophy between ECC and CON (despite the marked architectural adaptive differences), we measured activation of growth

signalling (mTOR substrates). Apart from suppression of 4EBP1 30 min post CON only (Atherton et al. 2005), no other signals were modulated by 30 min post CON or ECC. Clearly, the energy stress after exercise, which governs the latency in muscle protein synthesis responses (Cuthbertson et al. 2006), may have prevented us from evaluating MAPK and AKT-mTOR cross-talk. However, no further biopsies were taken, which represents a study limitation. It must be acknowledge that performing acute and chronic studies in different groups precluded interrogation of correlative links e.g. between MAPK phosphorylation and architectural adaptations. Nonetheless, the acute exercise data revealing substantial contraction-dependent divergence in mechanical and molecular responses and the chronic data revealing divergent architectural/ morphological adaptations are highly robust. It could also be argued that one limitation of this study is the different time under tension curve found in the two types of contractions: as stated, the greater time under tension in the eccentric mode was specifically chosen to enable to perform the lengthening/lowering of the load phase in safety. Nevertheless, as previous studies have shown (Burd et al. 2012) the greater time under tension curve is associated to increased anabolic response. If this was the case in this study, we should have observed differences in hypertrophic response (i.e. ECC Vol > CON Vol), which did not occur. Burd and colleagues investigated the anabolic responses to different time under tension comparing 1 second contractions vs. 6 seconds ones (6-fold greater) whereas the present investigation used 2 sec vs. 3 sec (0.5-fold difference): it is likely that this relatively smaller difference in time under tension was not sufficient to trigger different hypertrophic adaptations, as the morphological

data are much more reflecting the findings presented by Adams et al. (2004) that showed same increase in muscle size to ECC, CON and Isometric training of the same duration. Moreover, although the greater time under tension could reflect/suggest a likely increased physiological blood flow restriction occurring during the ECC phase, which should therefore result in a higher stimulation of hypertrophic signalling and/or greater muscle volume (Meyer et al. 2006), in this study no differences have been found in terms of anabolic signalling between the two training regimes. Hence we may conclude that this difference in time under tension was not sufficient to modulate a differential anabolic response.

## **Conclusions**

This study has shown that CON and ECC training paradigms lead to divergent structural adaptations, supported by different myogenic responses. ECC training leads to a marked increase in fascicle length (~1.5 fold) with no significant change in pennation angle while CON training induces a 3-fold increase in pennation angle, with little (<1-fold) change in fascicle length. These results suggest that ECC training seems to promote the addition of sarcomere in series whereas CON training favours the addition of sarcomere in parallel. This differential pattern of sarcomere addition induced by the two types of training, as inferred by the increase in fascicle length and pennation angle, seems also reflected by the distribution of muscle hypertrophy along the VL muscle belly, predominant in the mid to distal regions for ECC training and predominant in the mid-belly region for CON training. The different muscle remodelling induced by CON and ECC training may be associated

with distinct MAPK responses to the two contraction modes. The similar hypertrophy with ECC and CON RT may be explained by the greater myofibrillar disruption caused by ECC loading, followed by possible activation of inflammatory pathways likely antagonizing muscle hypertrophy.

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## Chapter 2

### **A validation of the application of D<sub>2</sub>O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein sub-fraction synthesis in free-living humans**

The work presented in this chapter has been published as:

**Wilkinson D.J.\***, **Franchi M.V.\***, Brook M., Narici M.V., Williams J.P., Mitchell W.K., Szewczyk N.J., Greenhaff P.L., Atherton P.J., Smith K. (2014) A validation of the application of D<sub>2</sub>O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein sub-fraction synthesis in humans. *American Journal of Physiology Endocrinology and Metabolism*  
**\*Equal First Authors**

*(Contribution to the study: leading all the resistance training programme, acquisition and analysis of all the functional, morphological and architectural data, collection assistance and storage and treatment of all the muscle samples collected during the study, help on myofibrillar and collagen FSR analysis, composition of the manuscript).*

## **Abstract**

**Aim:** Traditional  $^{13}\text{C}$ -amino acid tracing methods necessitate sustained bed-rest and intravenous cannulation (thus restricting studies to approximately 8h) and cannot inform on cumulative aspects i.e. diurnal muscle protein synthesis (MPS). Therefore, we tested the efficacy of the  $\text{D}_2\text{O}$  tracer for quantifying MPS over a period not feasible with  $^{13}\text{C}$  tracers and too short to quantify morphological changes in muscle mass in response to resistance exercise training (RET).

**Methods:** Eight men ( $22\pm 3.5\text{y}$ ) undertook one-legged resistance-exercise over 8-d ( $4\times 8\text{-}10$  repetitions: 80%-1RM every second-day, to yield internal 'non-exercised' vs. 'exercise' leg-comparisons) with Vastus Lateralis biopsies taken bi-laterally: 0, 2, 4 and 8-d. After day 0 biopsies, participants consumed a  $\text{D}_2\text{O}$  bolus (150ml; 70-Atoms%); saliva was collected daily in order to evaluate  $\text{D}_2\text{O}$  body-water enrichment. Fractional synthetic rates (FSR) of myofibrillar (MyoPS), sarcoplasmic (SPS) and collagen (CPS) protein-fractions were measured by GC-Pyrolysis-IRMS and TC/EA-IRMS.

**Results:** Body-water was initially enriched at 0.16-0.24 APE and decayed at  $\sim 0.009\%.\text{d}^{-1}$ . In the non-exercised-leg, MyoPS was:  $1.45\pm 0.10\%.\text{d}^{-1}$ ,  $1.47\pm 0.06\%.\text{d}^{-1}$ ,  $1.35\pm 0.07\%.\text{d}^{-1}$  at 0-2, 0-4 and 0-8d respectively ( $\sim 0.05\text{-}0.06\%.\text{h}^{-1}$ ). MyoPS was greater in the exercised-leg compared to the control (Ex leg: 0-2d  $1.97\pm 0.13\%.\text{d}^{-1}$ , 0-4d  $1.96\pm 0.15\%.\text{d}^{-1}$ ;  $P<0.01$ , 0-8d  $1.79\pm 0.12\%.\text{d}^{-1}$ ;  $P<0.05$ ). CPS was slower than MyoPS, but followed a similar pattern, with the exercised-leg tending to yield greater FSR's (0-2d;  $1.14\pm 0.13\%.\text{d}^{-1}$  vs.  $1.45\pm 0.15\%.\text{d}^{-1}$ , 0-4d;  $1.13\pm 0.07\%.\text{d}^{-1}$  vs.  $1.47\pm 0.18\%.\text{d}^{-1}$ ,

0-8d;  $1.03 \pm 0.09\% \cdot d^{-1}$  vs.  $1.40 \pm 0.11\% \cdot d^{-1}$ , rest vs. exercise leg). SPS was unchanged.

**Conclusion:** D<sub>2</sub>O is applicable to quantify diurnal MPS in humans and detect short-term anabolism/[catabolism].

## **Introduction**

Skeletal muscle is the body's largest tissue and its well functions are crucial for maintenance of metabolic health. Muscle is not only responsible for locomotory tasks, but also acts as a major glucose disposal sink and a fuel reservoir for other organs in times of fasting and stress (Wolfe, 2006). Thus, unsurprisingly, the decline of muscle mass with ageing (sarcopenia) or during acute or chronic illness (i.e. sepsis, COPD, cancer cachexia, organ failure (Lang, Frost, & Vary, 2007; Schols, Broekhuizen, Weling-Scheepers, & Wouters, 2005; Toth, LeWinter, Ades, & Matthews, 2010; Williams et al., 2012)) leads to frailty, loss of mobility and independence, in addition to increased risk of cardiovascular and metabolic disease (e.g. type II diabetes; (Srikanthan, Hevener, & Karlamangla, 2010) or in severe cases, even death (Arango-Lopera, Arroyo, Gutiérrez-Robledo, Pérez-Zepeda, & Cesari, 2013)).

Over recent years, considerable effort has been focused on research into techniques and interventions for understanding the regulation of skeletal muscle mass and minimizing loss of muscle mass with ageing and disease (Jones et al., 2009; B. E. Phillips, Hill, & Atherton, 2012). Despite this, effective treatments to mitigate muscle loss remain limited. Indeed, despite extensive investment in pharmaceutical interventions (Barillaro, Liperoti, Martone, Onder, & Landi, 2013; Onder, Della Vedova, & Landi, 2009) the current most effective and safe means by which to maintain or increase muscle mass remains resistance exercise training (RET) with accompanying protein nutrition (Churchward-Venne, Burd, Phillips, & Research Group, 2012; Häkkinen, Kraemer, Newton, & Alen, 2001; Ivey et al., 2000; V. Kumar

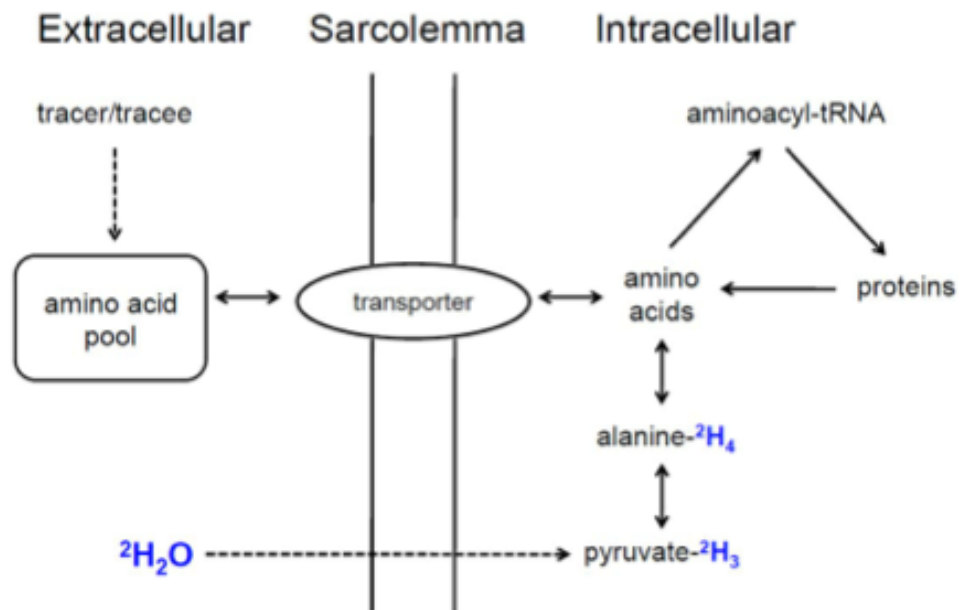
et al., 2012; Parise & Yarasheski, 2000). The hypertrophic response to RET is regulated via cumulative post-exercise increases in muscle protein synthesis (MPS) (Holm et al., 2010; Vinod Kumar et al., 2009; S. M. Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997), which after several weeks/months leads to muscle hypertrophy that is quantifiable using imaging techniques such as DXA, ultrasound, MRI or CT.

Traditionally, MPS has been measured *in vivo* using stable isotope tracers of amino acids (AA) with either heavy carbon ( $^{13}\text{C}$ ), deuterium ( $^2\text{H}$ ) or nitrogen ( $^{15}\text{N}$ ) motifs. The incorporation of these labeled AA's into muscle protein, allows for the calculation of a fractional synthetic rate (FSR) of MPS (Rennie et al., 1982; Yarasheski, Smith, Rennie, & Bier, 1992). While providing great utility, these methods are not without their inherent limitations. For instance,  $^{13}\text{C}$  AA tracers only permit measurements to be performed over short durations (typically <8-12h) and require preparation of sterile infusions (often expensive), venous/arterial cannulation and multiple biopsy collection, all within a controlled laboratory environment. Hence, the need to develop less restrictive and longer-term approaches to determine MPS in 'free-living' humans seems well justified. New methodologies should therefore result sensitive enough to detect changes in MPS and anabolic and/ or catabolic scenarios over periods in which it is not plausible to measure changes in muscle mass via imaging techniques.

Recent advances in mass spectrometry, in particular the development of Pyrolysis - isotope ratio mass spectrometry (IRMS) systems for high precision measurement of hydrogen and oxygen stable isotopes (Hilkert, Douthitt, Schlüter, & Brand, 1999; Ripoche, Ferchaud-Roucher, Krempf, &



Ritz, 2006), have led to the recent re-introduction of the first stable isotope tracer used in metabolic research; deuterium oxide ( $D_2O$  or 'heavy water'; (Rittenberg & Schoenheimer, 1937; Schoenheimer & Rittenberg, 1935). Upon oral ingestion,  $D_2O$  equilibrates within the body water pool (~20mins; (Belloto et al., 2007)), and through exchange with hydrogen, the deuterium can be incorporated into multiple metabolic pools and tissues (Fig 1); the use of intra-venous administration is thus avoided and this and allows subjects to administer tracer whilst performing normal activities.  $D_2O$  has been successfully integrated into the measurement of protein synthesis in both animal models (Belloto et al., 2007; Busch et al., 2006; De Riva, Deery, McDonald, Lund, & Busch, 2010; Dufner et al., 2005; H G Gasier et al., 2011; Kasumov et al., 2011; Miller, Robinson, Bruss, Hellerstein, & Hamilton, 2012; Yuan et al., 2008) and humans (Busch et al., 2006; Heath G Gasier, Fluckey, Previs, Wiggs, & Riechman, 2012; MacDonald et al., 2013; Previs et al., 2004; Robinson, Turner, Hellerstein, Hamilton, & Miller, 2011). Nonetheless, to date few studies have concentrated on its application and validity in the arena of human muscle.



*Fig 1. D<sub>2</sub>O incorporation into protein pool through hydrogen exchange (from Gasier et al. 2010)*

Robinson *et al.*, (2011) reported that it is possible to measure MPS over extended periods (i.e. 6 weeks) in groups of free-living adults, highlighting its unique suitability for determining longer-term MPS. Furthermore, Gasier *et al.*, (2012) showed stimulation of myofibrillar protein synthesis (MyoPS) but not mixed muscle protein synthesis 24 h following a single exercise bout highlighting the importance of delineating fraction specific differences. Nonetheless, the authors of this study did not measure responses over longer periods in an attempt to resolve the temporal and cumulative relationship between successive bouts of RE and MPS, the latter being the key underlying feature regulating muscle hypertrophy. Finally, both the Gasier and Robinson studies used relatively large doses of D<sub>2</sub>O to be able to chart MPS due to sensitivity issues involved with GC-MS as an analytical

tool. In comparison, IRMS adds at least 100-fold increases in sensitivity for measuring tissue isotopic enrichment compared to GC-MS, as demonstrated by MacDonald et al., (2013) who showed that MyoPS could be measured over a period of 4-14 days following a small single bolus of 150mls D<sub>2</sub>O in healthy adults under rested conditions.

Hence, our objective was to assess the utility of D<sub>2</sub>O for quantifying temporal and cumulative MPS under habitual and stimulated conditions (in order to assess its potential sensitivity to inform on anabolic/ anti-catabolic interventions). Volunteers performed unilateral exercise (Kim, Staron, & Phillips, 2005) where one leg was exercised and the contralateral served as internal control: consequently, we tested the efficacy of D<sub>2</sub>O for determining cumulative MPS at rest and in response to exercise.

We hypothesized D<sub>2</sub>O alongside IRMS techniques could be used to distinguish the synthesis profiles of muscle protein fractions (myofibrillar, collagen, sarcoplasmic) over a period of short-term RET (4 bouts over 8 days). Our rationale for this was: (i) it is unfeasible to use AA tracers over this time, (ii) it would inform about diurnal MPS in real-life settings, and (iii) it is a period too short to quantify increases in muscle mass using conventional morphometric techniques as a consequence of RET in healthy, young volunteers.

## **Material and Methods**

### *Subject characteristics and ethics*

Eight young healthy males (22±3.5y; body mass index (BMI) 23.5±0.8 kg·m<sup>-2</sup>) were recruited. All volunteers were screened by means of a medical

questionnaire, physical examination and resting ECG, with exclusions for metabolic, respiratory and cardiovascular disorders or other symptoms of ill health. Subjects had normal blood chemistry, were normotensive (BP<140/90) and were not prescribed any medications; all subjects performed activities of daily living and recreation but did not routinely participate in any formal exercise. All subjects gave their written, informed consent to participate after all procedures and risks were explained. This study was approved by The University of Nottingham Ethics Committee and complied with the Declaration of Helsinki.

### *Study Procedures*

Volunteers were asked to refrain from heavy exercise for the 72h before the start of the study and performed no exercise over the course of the study other than that prescribed as part of the protocol. A week before the first visit, strength was assessed for the training leg using an isokinetic dynamometer (Isocom, Isokinetic Technologies, Eurokinetics, UK), with 1-repetition maximum (1RM) also assessed (Dominant leg; Technogym, Gambettola, Italy), in addition muscle architecture was assessed by ultrasound (MyLab 70, Esaote Biomedica, Italy). The same measurements were performed 8 days later following the final training and biopsy session. Participants then completed a unilateral resistance exercise training programme over a period of 8 days, training consisted of single leg knee extension exercise (4 x 8 reps at 80% 1RM) performed on day 0, 2, 4, 6 and 8. 20g of whey protein isolate (Pro-Isolate Tech, Muscletech, Ontario, Canada) were provided post exercise to ensure sufficient substrate to

support the increased demands of exercise on MPS processes. Bilateral biopsies (non-exercised and exercised) of *m. vastus lateralis* were taken under sterile conditions using the conchotome biopsy technique (Dietrichson et al., 1987) with 1% lidocaine (B. Braun Melsungen, Germany) as local anaesthetic, on days 0 (Basal), 2, 4 and 8. A rested biopsy was taken prior to the exercise bout to avoid acute stimulation from the exercise and whey protein feed. Muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold saline and then frozen in liquid N<sub>2</sub> and stored at -80°C until further analysis. Single venous blood samples were collected on day 0 and day 8 into lithium-heparin coated tubes, these were immediately cold centrifuged at 3,200 rpm, the plasma fraction was then aliquoted and frozen at -80°C until analysis. Immediately post biopsy on day 0, participants provided a saliva sample (collected in sterile plastic tubes) and were asked to consume a single 150ml oral bolus of D<sub>2</sub>O (70 Atoms%, Sigma Aldrich, Poole, UK), this was performed with the aim to label the body water pool to ~0.2%. To monitor the body water enrichment throughout the study, each participant was asked to provide a single daily saliva sample collected at midday at least 30mins after their last meal or drink. These were collected in sterile plastic tubes and kept refrigerated, participants were asked to bring these to each training session. Upon receipt of saliva samples, they were immediately cold centrifuged at 16,000g to remove any debris that may be present; they were then aliquoted into 2 ml glass vials and frozen at -20°C until analysis. A detailed schematic of the study protocol is provided in figure 2.

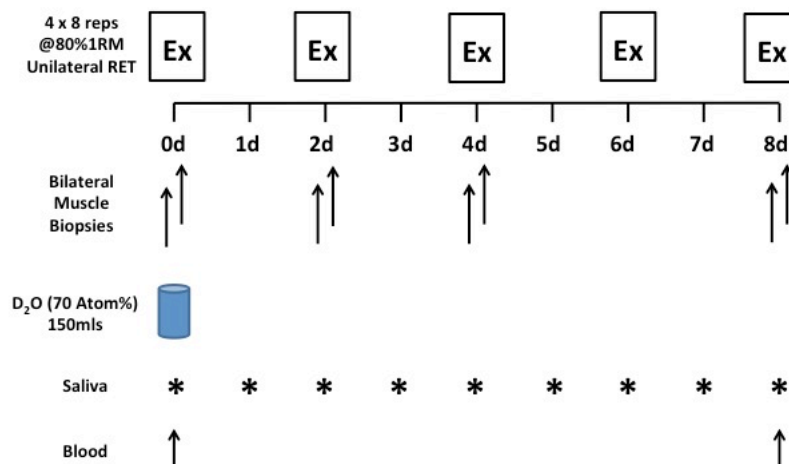


Fig 2. Study design scheme

### Body Water Enrichment

Body water enrichment was determined through direct liquid injection of saliva samples (0.1  $\mu$ l volume) into a High Temperature Conversion Elemental Analyser (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an Isotope Ratio Mass Spectrometer (IRMS, Delta V Advantage, Thermo, UK). To minimize the effect of carryover between samples, each was injected a minimum of 4 times. To validate the accuracy of the TC/EA for measuring body water enrichment from saliva the analysis was repeated with two participant's saliva sets using GC-Pyrolysis-IRMS (Trace GC isolink Delta V Advantage, Thermo Scientific, Hemel Hempstead, UK) and a modification of the protocol by (Mahsut et al., 2011). Briefly 100 $\mu$ l of each saliva sample was incubated with 2  $\mu$ l of 10N NaOH and 1  $\mu$ l of acetone for 24 h at room temperature; this high pH incubation leads to the exchange of deuterium from water with the hydrogen positions

on the acetone. Following incubation the acetone was extracted into 200µl of n-heptane and 0.5 µl of the heptane phase was injected into the GC for analysis. A standard curve of known D<sub>2</sub>O enrichment was run alongside the saliva samples for calculation of enrichment. Further validation was also provided via analysis of water extracted from day 0 and 8 plasma. In addition, 50-100 µl aliquots of plasma were placed in the cap of inverted auto-sampler vials; these were then placed on a heating block set at 90 °C for 2h. Water distillate was then collected by rapidly cooling the vials on ice for 10 min, and was transferred to fresh vials for direct liquid injection into the TC/EA, as for the saliva.

*Isolation and derivatization of myofibrillar, sarcoplasmic and collagen protein fractions*

For isolation of myofibrillar, sarcoplasmic and collagen fraction ~30-50 mg of muscle was used. The muscle was homogenised in ice-cold homogenisation buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaF, 10 mM β-Glycerophosphate disodium salt, 1 mM EDTA, 1 mM EGTA, 1 mM activated Na<sub>3</sub>VO<sub>4</sub> (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10 µl·µg<sup>-1</sup> of tissue. Homogenates were rotated for 10 min and the supernatant collected by centrifugation at 13,000 × g for 5 min at 4°C. The myofibrillar pellet was solubilised in 0.3M NaOH, separated from the insoluble collagen by centrifugation and the myofibrillar protein was precipitated with 1M perchloric acid (PCA). The sarcoplasmic proteins were precipitated from the initial homogenate supernatant fraction with 1M PCA, and separated by centrifugation. The insoluble collagen fraction was washed

sequentially with 0.3M NaOH, 70% ethanol, 0.5 M acetic acid, 0.5 M acetic acid, and isolated by centrifugation. Protein-bound AA from myofibrillar, sarcoplasmic and collagen were released using acid hydrolysis by incubating in 0.1M HCl in Dowex H<sup>+</sup> resin slurry overnight before being eluted from the resin with 2M NH<sub>4</sub>OH and evaporated to dryness. The AA were then derivatized as their n-methoxycarbonyl methyl esters (MCME) according to the protocol of Husek (Husek & Liebich, 1994), with slight modification. The dried samples were resuspended in 60µl distilled water and 32µl of methanol, following a brief vortex, 10µl of pyridine and 8µl of methylchloroformate were added. Samples were vortexed for 30s and left to react at room temperature for 5 min. The newly formed MCME AA's were then extracted into 100µl of chloroform; any remaining water was removed from the sample with addition of a molecular sieve. Incorporation of deuterium into protein bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage, Thermo Scientific, Hemel Hempstead, UK).

#### *Plasma alanine enrichment*

Plasma (200µl) proteins were precipitated with 100% ethanol and the supernatant evaporated to dryness, reconstituted in 0.5M HCl and the lipid fraction removed using ethyl acetate extraction; alanine was then converted to its MCME derivative as described above. Enrichment (MPE) of alanine was then determined using gas chromatography-mass spectrometry (GC-MS; MD800, Fison, UK) and single ion monitoring of m/z 102, 103, 104, 105, 106.



### *GC-pyrolysis IRMS deuterium analyses*

For GC-Pyrolysis-IRMS analysis of acetone and MCME alanine, samples were separated on a DB-wax column (30m x 0.32mm x 0.25µm, Agilent J&W, USA), following splitless injection. The oven temperature programme for acetone was: start at 50°C hold for 2mins; ramp at 30°C/min to 240°C and hold for 2mins, and for alanine was: start at 70°C and hold for 3 mins; ramp at 10°C/min to 240°C hold for 15mins. The separated samples were then passed through a high temperature (1420°C) conversion reactor where the analytes were converted to H<sub>2</sub> gas before being directed to the IRMS where the <sup>2</sup>H/<sup>1</sup>H ratio was determined. For TC/EA analysis of body water enrichment, following direct liquid injection into the TC/EA where samples were immediately converted to H<sub>2</sub> gas, sample gases were directed to the IRMS where the <sup>2</sup>H/<sup>1</sup>H ratio was determined. The deuterium isotopic enrichment provided as δ<sup>2</sup>H was converted to atom % using the following equation:

$$Atom\% = \frac{100 \times AR \times (\delta^{2H} \times 0.001 + 1)}{1 + AR (\delta^{2H} \times 0.001 + 1)}$$

Where, AR represents the absolute ratio constant for deuterium based on the VSMOW standard and equates to 0.00015595. This was then converted to atom % excess (APE) by correcting for baseline sample i.e. background enrichment.

### *Calculation of fractional synthetic rate (FSR)*

The fractional synthetic rate (FSR) of myofibrillar (MyoPS), sarcoplasmic (SPS), collagen (CPS) protein synthesis was determined from the

incorporation of deuterium labeled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7) as the surrogate precursor labelling between subsequent biopsies. In brief, the standard equation:

$$\text{FSR } (\% \cdot \text{d}^{-1}) = [(\text{APE}_{\text{Ala}})] / [(\text{APE}_{\text{P}}) \times t] \times 100$$

Where,  $\text{APE}_{\text{Ala}}$  = deuterium enrichment of protein bound alanine,  $\text{APE}_{\text{P}}$  = precursor enrichment and  $t$  is the time between biopsies, was used.

### *Muscle architecture and strength assessment*

We also analyzed changes in muscle architecture as these represent a technique with sufficient resolution to detect subtle changes in muscle architecture (albeit not muscle mass) after only short-periods of exercise training (Alegre, Jiménez, Gonzalo-Orden, Martín-Acero, & Aguado, 2006). Exercised leg Vastus Lateralis ultrasonographic images were obtained at rest using B-mode ultrasonography (Mylab 70, Esaote Biomedica, Italy), with a 100 mm, 10-15 MHz, linear-array probe. Ultrasound images were taken at a specific point at full knee extension, while the participant was lying supine. The transducer was aligned in the fascicle plane to capture an optimal portion of fascicles. The muscle architecture parameters fascicle length (Lf) and pennation angle (PA) were quantified by the same unblinded investigator (MVF) from the ultrasound scans using ImageJ 1.42q (National Institutes of Health, USA). The visible portion of the fascicle length was directly assessed using this software. Pennation angle was measured as the intersection between fascicles and the deep tendon aponeurosis. Isometric muscle

strength was measured in a sitting position using an isokinetic dynamometer (Isocom, Isokinetic Technologies, Eurokinetics, UK) throughout a range of 6 knee joint angles from 90° to 40°, with full extension corresponding to 0°. Subjects were seated in the dynamometer chair and secured into position using straps across the chest. Contractions lasted for 4 s, with a rest period of 45 s between contractions, and a rest period of 90 s between knee joint angle changes. The maximum isometric torque value (MVC) was chosen for data analysis.

### *Statistical Analyses*

Descriptive statistics were produced for all data sets to check for normal distribution (accepted if  $P > 0.05$ ) using a Kolmogorov-Smirnov test. All data are presented as mean  $\pm$  SEM. Differences within groups (ex type x time) were detected by repeated measures one way (time) and two-way (ex type x time) ANOVA with a Bonferroni correction using GraphPad Prism software (Version 5, La Jolla, San Diego CA). Differences for muscle strength and architectural measures were analysed by paired t-test. Correlations were assessed using Pearson Product Moment Correlation Coefficient. The alpha level of significance was set at  $P < 0.05$ .

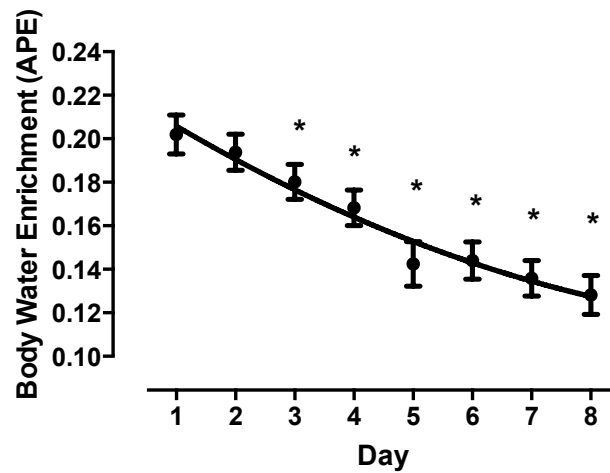
## **Results**

### *Body water and plasma alanine enrichment*

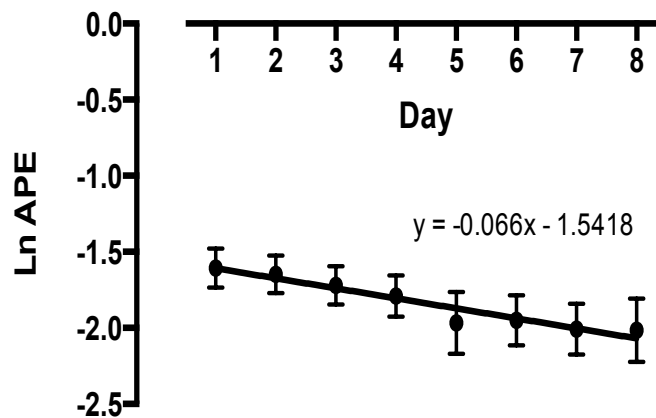
Mean body water enrichment over the 8 day period is presented in figure 3A. A single bolus of 150ml of 70% D<sub>2</sub>O led to a body water enrichment of 0.202

$\pm 0.009\%$  24h post ingestion (range: 0.162 – 0.237%). Body water enrichment followed an exponential decay pattern (figure 3A), decaying slowly and significantly over the 8 day period at a rate of approximately  $0.009\% \cdot d^{-1}$ . Raw atoms per enrichment (APE) values were natural logarithm converted to determine the mean decay constant (see figure 3B), from this a mean half-life for body water elimination of  $11 \pm 0.9$  days was calculated.

**A**



**B**



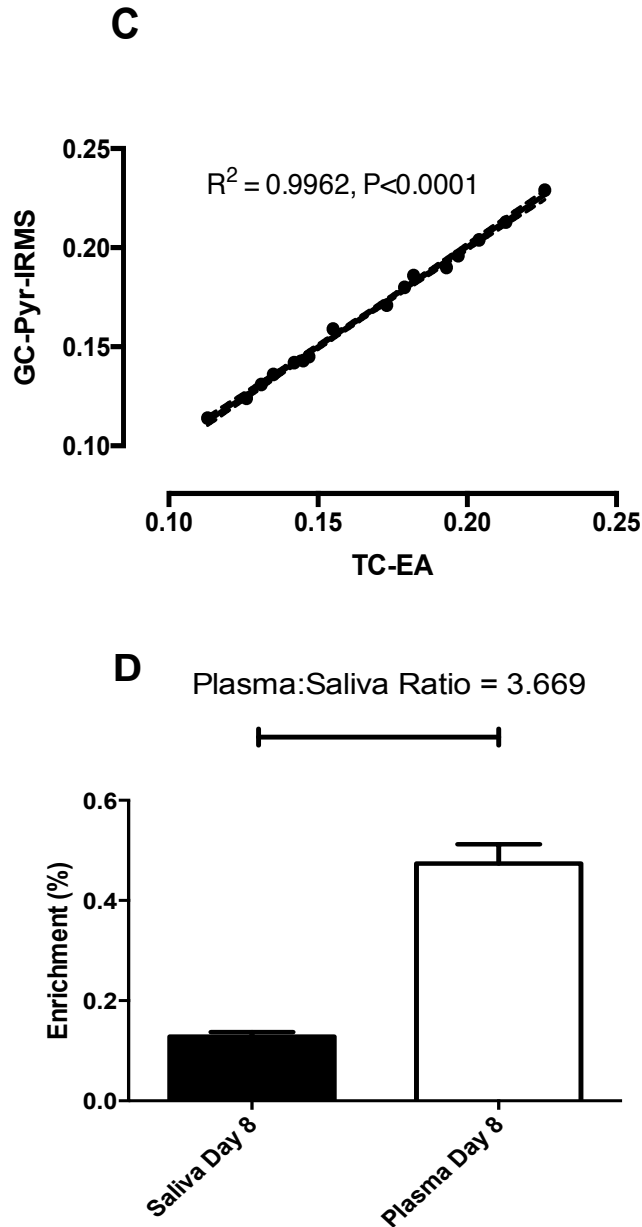


Figure 3: A) Exponential time-course of body water enrichment over 8 days following oral bolus of 150mls  $D_2O$  (70 atoms %). B) Natural logarithm transformed body water enrichment for calculation of decay constant and elimination half-life. C) Correlation of two different body water analytical techniques using TC-EA direct liquid injection, and GC-Pyr-IRMS. D) Comparison of plasma alanine enrichment and saliva enrichment at day 8. \* indicates significantly different from initial day 1 timepoint,  $P < 0.01$

Participant 1 BW (APE)		Participant 2 BW (APE)	
TC/EA	GC- Pyr- IRMS	TC/EA	GC- Pyr- IRMS
0.229	0.226	0.204	0.204
0.213	0.213	0.197	0.196
0.190	0.193	0.182	0.186
0.180	0.179	0.173	0.171
0.159	0.155	0.113	0.114
0.145	0.147	0.135	0.136
0.131	0.131	0.142	0.142
0.124	0.126	0.145	0.143

	Saliva Water Day 8 (APE)	Plasma Water Day 8 (APE)
<b>Participant 1</b>	0.124	0.124
<b>Participant 2</b>	0.145	0.144
<b>Participant 3</b>	0.128	0.126
<b>Participant 4</b>	0.090	0.090
<b>Participant 5</b>	0.154	0.157
<b>Participant 6</b>	0.106	0.106
<b>Participant 7</b>	0.165	0.166
<b>Participant 8</b>	0.114	0.112

*Table 1: Comparison between MS-techniques for measuring body water enrichment following D<sub>2</sub>O ingestion and the use of saliva as a surrogate for plasma.*

Saliva samples from 2 subjects were prepared via a modification of a method described by Mahsut et al., (2011), where deuterium from water is transferred onto the hydrogen positions of acetone under high pH incubation. These samples were then analysed via GC-Pyr-IRMS. This was performed to validate that there was no dilution in enrichment with saliva due to

contaminants, which may be present. Table 1 and figure 3C provides a comparison of the APE for the two participants as measured using both the direct liquid injection method for TC-EA and the GC acetone exchange methods, there was no difference between the two methods, showing highly significant correlation between the two techniques ( $P < 0.0001$ ,  $R^2 = 0.9962$ , Figure 3C) underlining the validity of TC-EA method. Furthermore, water extracted from day 8 plasma samples provided further validity for the method (Table 1).

It has been demonstrated in the literature, based on GC-MS mass isotopomer distribution analysis (MIDA) calculations, that an average of 3.7 deuterium's are incorporated onto alanine from  $D_2O$ , out of a possible 4 carbon-hydrogen bonds, following a bolus of  $D_2O$  in mammals (Dufner et al., 2005; Previs et al., 2004). Hence this correction factor is used in the precursor-product equation to calculate FSR when body water enrichment is being used as the surrogate precursor. To validate this for our own calculations the alanine enrichment in 8-day plasma samples was determined by GC-MS, the mean plasma alanine to saliva body water ratio was calculated as 3.67 (figure 3D), therefore confirming the utility of 3.7 as a correction factor for use in calculation of FSR in this study.

#### *Fractional Synthetic Rates of MyoPS, SarcPS and CollagenPS*

Figures 4A and B show the mean  $\delta^2H$  in protein bound alanine over the course of the 8-day training period: an exponential non-linear pattern of incorporation was observed. Breaking this down into sections of time we identify linear kinetics are maintained up to 4 days (Figure 4A) after which it

switches to non-linear from 4-8 days (Figure 3B). Therefore the standard precursor-product calculation (as detailed in the methods) was only valid for FSR up to 4 days, after which a non-linear model was applied to the data whereby;

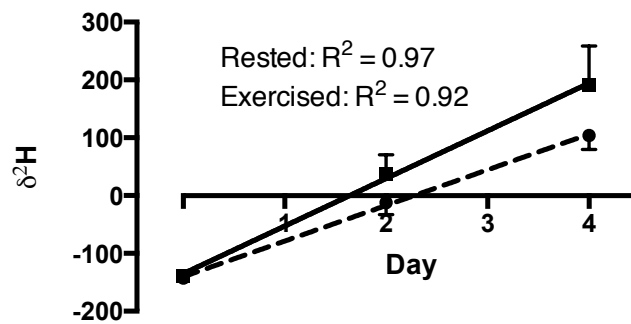
$$\text{FSR} = -\text{Ln} \left( \frac{1 - \left[ \frac{\text{APE}_{\text{Ala}}}{\text{APE}_{\text{P}}} \right]}{t} \right)$$

Using this equation, in the rested leg, MyoPS was  $1.45 \pm 0.10\% \cdot \text{d}^{-1}$ ,  $1.47 \pm 0.06\% \cdot \text{d}^{-1}$ ,  $1.35 \pm 0.07\% \cdot \text{d}^{-1}$  at 0-2, 0-4 and 0-8 d respectively. Which when calculated as  $\% \cdot \text{h}^{-1}$  represented between  $0.052 - 0.061\% \cdot \text{h}^{-1}$ , this rate is identical to the rates that are observed using traditional AA stable isotope tracers (Smith, Patterson, & Mittendorfer, 2011). MyoPS was significantly increased in the exercised leg at 0-2 d;  $1.97 \pm 0.13\% \cdot \text{d}^{-1}$ , 0-4 d;  $1.96 \pm 0.15\% \cdot \text{d}^{-1}$  ( $P < 0.01$ ; Figure 3A) and 0-8 d;  $1.79 \pm 0.12\% \cdot \text{d}^{-1}$  ( $P < 0.05$ ). Figure 4D illustrates the temporal pattern for stimulation of MyoPS over the 8-day training period. FSR in the exercised leg is significantly increased above that of the rested leg at 2-4 days ( $P < 0.001$ ) after which it rapidly falls by  $\sim 40\%$  from 4-8 days ( $P < 0.01$ ). CollagenPS was, as expected, overall slower than MyoPS, but followed a similar pattern over the 8 days, with the exercised leg showing a trend for greater FSR (2 d;  $1.14 \pm 0.13\% \cdot \text{d}^{-1}$  vs.  $1.45 \pm 0.15\% \cdot \text{d}^{-1}$ , 4 d;  $1.13 \pm 0.07\% \cdot \text{d}^{-1}$  vs.  $1.47 \pm 0.18\% \cdot \text{d}^{-1}$ , 8 d;  $1.03 \pm 0.09\% \cdot \text{d}^{-1}$  vs.  $1.40 \pm 0.11\% \cdot \text{d}^{-1}$ ; Figure 5A). However, due to a problem with sample recovery during collagen sample extraction, there were a number of missing data points therefore it was not possible to run statistical analyses on this data set.

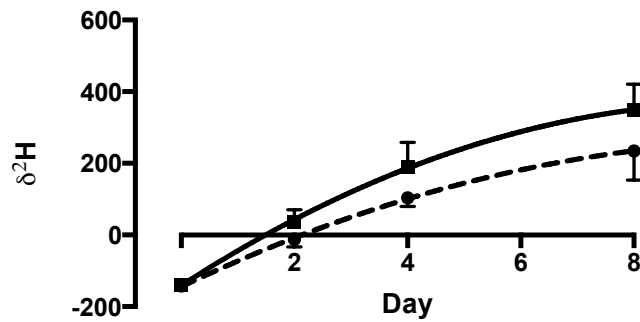


Sarcoplasmic PS, as with Collagen PS was found to be increased in the exercised leg at both 0-2, 0-4 and 0-8 d;  $1.93 \pm 0.15\% \cdot d^{-1}$  vs.  $1.48 \pm 0.17\% \cdot d^{-1}$ ,  $1.67 \pm 0.11\% \cdot d^{-1}$  vs.  $1.22 \pm 0.07\% \cdot d^{-1}$ , and  $1.88 \pm 0.23\% \cdot d^{-1}$  vs.  $1.39 \pm 0.23\% \cdot d^{-1}$  respectively (Figure 5B), however this was not statistically significant.

**A**



**B**



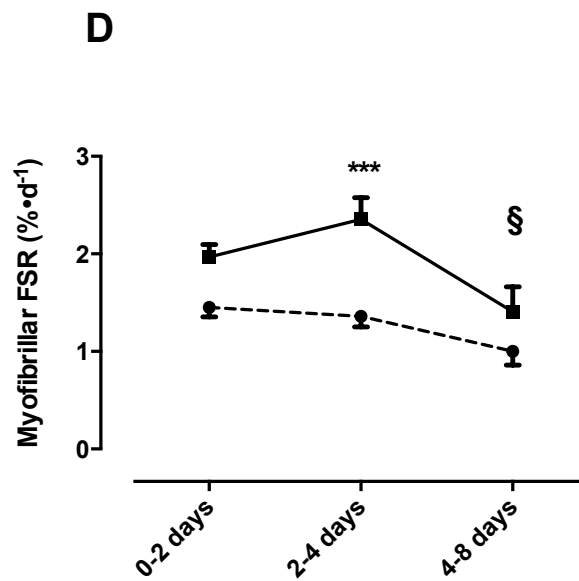
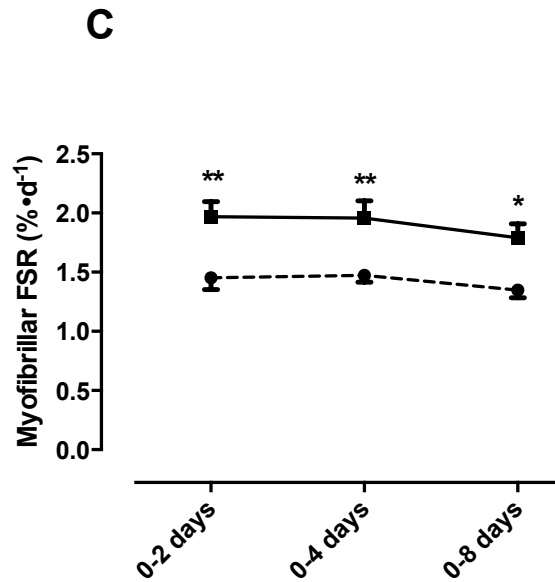


Figure 4: A) Linear incorporation of deuterium ( $\delta^2H$ ) into protein bound alanine over the first 4 days. B) Illustration of non-linear incorporation of deuterium into protein bound alanine beyond 4 days. C) Myofibrillar protein synthesis rates in exercised (solid line) and non-exercised (dotted line) legs over 8 day training period. D) Temporal pattern of myofibrillar protein synthesis in exercised and non-exercised legs. \*\*\* indicates significantly different from rested leg at same time point  $P < 0.001$ , \*\*  $P < 0.01$ , \*  $P < 0.05$ . § indicates significantly different from previous time point in same leg  $P < 0.05$

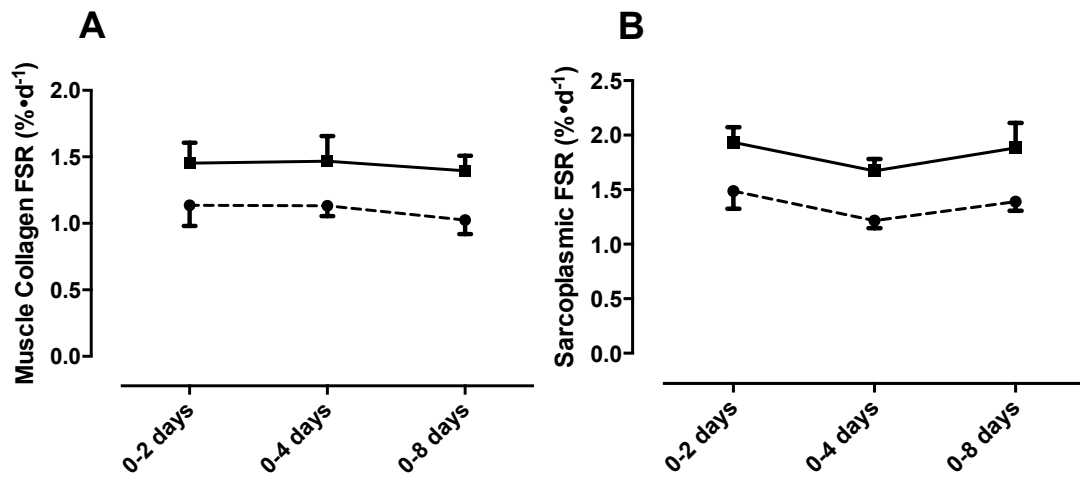


Figure 5: A) Muscle collagen and B) Sarcoplasmic protein synthesis in exercised (solid line) and non-exercised (dotted line) legs over 8 day training period.

### Strength and muscle architecture

At 8 days we observed significant increases in fascicle length ( $1.08 \pm 0.16\%$ ;  $P < 0.001$ ) and pennation angle ( $1.83 \pm 0.16\%$ ;  $P < 0.001$ ) as measured using ultrasonography. There was a small increase in MVC of  $3.4 \pm 3.5\%$ , but this was not statistically significant.

### Discussion

The purpose of the present study was to assess the efficacy of using the D<sub>2</sub>O tracer to determine the temporal synthesis of muscle protein sub-fractions at 'rest' (ie. In normal conditions, not trained) and in response to short term RET, in the form of successive exercise bouts. Additionally, we aimed to demonstrate the utility of this method for providing short-term resolution of the regulation of muscle mass under conditions where traditional AA tracer approaches are untenable and where changes in mass due to RET are not

yet quantifiable with standard image measurement techniques (ultrasound, MRI, DXA). Indeed, although we observed increases in fascicle length and pennation angle at the end of 8 days, suggestive of increased hypertrophic response, this change was very small, increasing by 1.08% and 1.8% respectively. Furthermore, we aimed to validate the use of IRMS for quantifying these responses rapidly and robustly. Here we show for the first time that using a single bolus of D<sub>2</sub>O we are able to detect synthesis rates of multiple protein fractions (MyoPS, CollagenPS and SarcPS) at rest and also increases in synthesis in response to an anabolic stimulus (exercise) vs. an internal non-exercised contralateral leg. This difference was detectable within 48 h of the first exercise bout, gradually declining from 2-8 days. This highlights the applicability of D<sub>2</sub>O for quantifying cumulative MPS over periods when detection of an increase in muscle mass (via imaging techniques) is not feasible.

RET remains the most feasible tool for increasing or maintaining muscle mass in both healthy young and old individuals (Häkkinen et al., 1998; Raue et al., 2012), in addition to frail elderly and clinical populations (Cadore et al., 2013; Lønbro et al., 2013; Stene et al., 2013). Hypertrophy associated with resistance exercise is caused primarily by cumulative increases in MPS leading to net protein accretion (P J Atherton & Smith, 2012). Using traditional AA tracer techniques, resistance exercise has been shown to induce increases in MPS following a single bout (Holm et al., 2010; Vinod Kumar et al., 2009; S. M. Phillips et al., 1997) which can be augmented by the addition of a protein feed post exercise (West et al., 2011), and with

adequate nutrition can be sustained for up to and beyond 24h (Cuthbertson et al., 2006; Miller et al., 2005; S. M. Phillips et al., 1997). It was our aim to measure participants in a 'free-living' normal environment, such that these studies were not controlled for feeding, with the exception of the protein taken post exercise, to provide a "maximal stimulus" to ensure the study was not confounded by individuals consuming insufficient protein with which to sustain MPS. Indeed, following the first bout of RET we observed that MyoPS was increased by ~36% compared to the non-exercised leg after 48h (Figure 4C). These data support the findings of the acute AA tracer work (acute infusions on different study days), which substantiates that with adequate nutrition, MPS can be maintained for greater than 24 h post exercise (Cuthbertson et al., 2006; Miller et al., 2005).

In the present data we further validate the use of D<sub>2</sub>O in measuring turnover in skeletal muscle (H G Gasier et al., 2011; Heath G Gasier et al., 2012; MacDonald et al., 2013; Robinson et al., 2011) by highlighting for the first time, the efficacy of the D<sub>2</sub>O tracer for monitoring the cumulative and temporal responses of protein synthesis to an anabolic stimulus (RET). Over 8 days of RET, MyoPS peaked between 2 and 4 days. Following this initial stimulation, there was a drop in FSR of ~40% from 4-8 days, which could be suggestive of a 'muscle full' effect (P J Atherton & Smith, 2012; Philip J Atherton et al., 2010; Bohe, Low, Wolfe, & Rennie, 2001; Millward, Bowtell, Pacy, & Rennie, 1994) where the muscle has achieved a new set-point of accommodation, requiring greater stimulation to sustain growth. Finally, although not significant there was a slight drop in MyoPS in the non-exercised leg over the 8 days (Figure 3D), this may have been influenced by

the 'free-living' nature of the design, for example no strict controls on diet and exercise were imposed on the participants. We conclude that future studies of this kind would thus benefit from diet and activity monitoring.

The importance of studying distinct muscle protein sub-fractions can be seen via the preferential acute increase in myofibrillar MPS in response to resistance vs. mitochondrial MPS after endurance exercise- both prophetic of ensuing adaptations to each exercise mode (Wilkinson et al., 2008). As with MyoPS, both CPS and SPS has been shown to be stimulated in a similar fashion immediately post resistance exercise, highlighting the coordinated response of muscle proteins to stimulation by exercise (Miller et al., 2005). In the present study, we have seen similar coordinated effects. SPS was greater, albeit not quite significantly ( $P=0.14$ ,  $0.07$ ,  $0.16$  for 0-2, 0-4, 0-8d respectively), over the 8-day training period in the exercised compared to the non-exercised leg. Our present data on CPS supports other published data (Babraj et al., 2005; Miller et al., 2005) demonstrating that muscle collagen turns over at a slower rate than the myofibrillar and sarcoplasmic fractions (~25% lower than myofibrillar in the non-exercised state). As expected, CPS rates showed a similar pattern of change over the 8 day period to the myofibrillar fraction, however due to sample processing problems, we were unable to produce complete data sets for all subjects such that statistical representation of the data is limited. Despite this, we show this approach can be used for the quantification of multiple muscle fractions in a single study, further refinements to the protocol, sample processing and the use of tandem

MS will permit measurement of the turnover of additional protein pools, e.g. mitochondrial protein fractions.

It is also necessary to compare our *absolute* FSR's to those captured using traditional  $^{13}\text{C}$  methodologies, in order to qualify their quantitative utility. We obtained 'rested' FSR for MyoPS of  $\sim 1.25\text{-}1.47\ \%\cdot\text{d}^{-1}$ , which equates to  $\sim 0.05\text{-}0.06\ \%\cdot\text{h}^{-1}$ , representative of that usually achieved with the use of  $^{13}\text{C}$  AA tracers (Smith et al., 2011). This was also observed with 'rested' SPS rates which were  $\sim 0.051\text{-}0.062\ \%\cdot\text{h}^{-1}$  in the present study, similar to rates quoted within the literature using  $^{13}\text{C}$  tracers (Philip J Atherton et al., 2010; Miller et al., 2005). Whilst CPS rates were slightly higher than some values quoted in previous literature ( $\sim 0.042\text{-}0.047\ \%\cdot\text{h}^{-1}$  here compared with  $\sim 0.018\text{-}0.025\ \%\cdot\text{h}^{-1}$ ; (Miller et al., 2005, 2006)), CPS rates of up to  $0.06\ \%\cdot\text{h}^{-1}$  have been reported (Holm et al., 2010). Therefore, using the present approaches we arrive at values approximating those expected.

Herein, we have validated a highly sensitive stable isotope tracer approach for measuring human MPS using  $\text{D}_2\text{O}$ . In one of the only other humans studies of its kind, Gasier *et al.* (2012) showed that by providing a 300ml bolus of 70%  $\text{D}_2\text{O}$ , they were able to measure increased rates of MyoPS following a single bout of resistance exercise 24h post exercise using the same unilateral model as the present study. To do this, the authors utilized GC-MS for measuring both bound protein and body water deuterium enrichment, reporting that due to the low levels of enrichment in the body water ( $<0.5\%$ ) accurate data quantification was difficult, due to limitations of

the GC-MS approach they used. Here we show that by giving a single 150ml bolus of D<sub>2</sub>O and using a combination of TC/EA-IRMS and GC-Pyr-IRMS we are able to sensitively and accurately measure body water and bound protein alanine enrichment to an accuracy of 1-3 ‰. The substantial increase in measurement sensitivity provided by IRMS allows us to uncover subtle temporal differences in response to exercise not possible with standard GC-MS. Indeed, ‰ changes over 2 days in the present study ranged between 116-176 ‰; such large changes suggest that measurements of MyoPS may be possible over even shorter periods (e.g. several hours, given the appropriate D<sub>2</sub>O dosing protocol) with similar levels of accuracy using IRMS. Per hour, current ‰ changes would be equivalent to 2-3 ‰, however by increasing the D<sub>2</sub>O dosing 3-fold, it is likely that rates of MyoPS could be measured over periods of hours, enhancing the utility of this approach and potentially replacing the need for i.v AA tracers for measurement of protein turnover in certain situations.

To ease the burden on the participants within the present study, we chose to provide only a single small bolus of D<sub>2</sub>O (150mls) at the beginning of the study, based on the experimental design provided by MacDonald et al., (2013), where a single 100ml D<sub>2</sub>O bolus was applied to measure MyoPS over a period of 4-14 days. Many published uses of D<sub>2</sub>O for monitoring metabolic turnover in both animals and humans usually provide regular daily doses of D<sub>2</sub>O following an initial large bolus, this is implemented in an attempt to mimic that of AA tracers whereby a large prime and continuous infusion is provided to maintain an isotopic steady state. However, it is



possible to measure protein synthesis in the non-steady state conditions (Garlick, McNurlan, Essén, & Wernerman, 1994; Rennie, Smith, & Watt, 1994). Due to the slow turnover of water in the body, half-life is reported as ~7-10 d (Heath G Gasier, Fluckey, & Previs, 2010; MacDonald et al., 2013) and was calculated as a mean of  $11 \pm 0.9$  d in the present study, MacDonald et al., (2013) has shown that D<sub>2</sub>O elimination follows a slow exponential decay after bolus ingestion, as was observed here (figure 2A). This slow decay maintains body water enrichment within adequate levels for continuous incorporation into muscle protein and measurement of MPS over periods of a week and potentially longer. The use of IRMS, with its high measurement sensitivity for isotopic abundance, ensures that body water enrichment only needs to be raised by a small amount ~0.2% compared to the 1-2% quoted by other researchers (Robinson et al., 2011). Furthermore, this also minimizes the potential for the onset of the reported side effects from D<sub>2</sub>O, such as dizziness and nausea. This function of slow D<sub>2</sub>O elimination combined with high measurement accuracy makes this technique ideally suited for use within populations, where regular D<sub>2</sub>O administration or high doses of D<sub>2</sub>O may be problematic (e.g. frail elderly) or contraindicated (e.g. children or critical care patient populations), highlighting the wide-ranging applicability of this tracer technique.

## **Conclusion**

The present study demonstrates for the first time that it is possible, by using only a single 150ml bolus of D<sub>2</sub>O, to measure the temporal response of FSR within multiple muscle protein sub-fractions over a short-period; furthermore,

this investigation validates the approach in response to an anabolic stimuli (RET), from which we could determine the temporal and cumulative anabolic responses to exercise (in comparison to the contralateral non-exercise control leg). The increased sensitivity provided by GC-Pyr-IRMS allows accurate measurement of MyoPS, Sarcoplasmic PS and Collagen PS over as little as 2 days, whilst also being able to robustly detect a stimulation of protein synthesis. Therefore, we reaffirm that D<sub>2</sub>O is a valid tracer approach for measuring MPS and muscle anabolism in extended 'free-living' situations, and will have wide application to assess the efficacy of clinical/ nutritional/ exercise interventions for maximizing mass/attenuating atrophy. Finally, despite holding much promise, the extent to which D<sub>2</sub>O can be utilized as a tool to "predict" chronic anabolic outcomes, remains to be determined.

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## Chapter 3

### **Chronic changes in muscle protein synthesis in response to eccentric and concentric loading measured in two different regions of the human *vastus lateralis* muscle**

The work presented in this chapter will soon be submitted to a peer-reviewed journal for publication (*Franchi MV et al. 2014, manuscript in preparation*).

## Introduction

There is a wealth of literature on concentric and eccentric exercise and it is fascinating how these two types of actions differ in terms of the mechanisms of muscle contraction and of the responses to acute and chronic exercise. Nevertheless, especially on humans, the mechanisms that regulate the changes in skeletal muscle morphology/architecture in response to concentric and eccentric loading have not yet been fully elucidated.

It is common knowledge that lengthening (eccentric) contractions can generate greater muscle force than isometric and shortening (concentric) contractions (Katz, 1939; Westing et al., 1991; Cook & McDonagh, 1995); for this reason (i.e. possibility to train with greater loads), there is a general hypothesis that eccentric exercise may have the potential to promote larger increases in muscle size and strength compared to concentric and isometric training (Roig et al., 2009). However, Wernbom and colleagues (Wernbom et al., 2007) suggested that if the two types of loading are performed at sufficient intensity and work volume, then it is difficult to establish which is the best training mode, as significant hypertrophy is reached in either case. The most important studies that compared ECC vs. CON training and the correspondent increase in muscle size underwent the author's scrutiny and have been organised in table 1: it is clear that up to now, the results are considerably controversial.

Nonetheless, recent studies suggest that the changes in muscle morphology in response to concentric and eccentric training are governed by different remodelling mechanisms (Murton et al., 2008; Hyl Dahl & Hubal, 2013;

Franchi et al., 2014). Indeed, distinct differences in the geometrical remodelling of muscle architecture have been found in response to these two modes of training (Reeves et al., 2009; Duclay et al., 2009; Potier et al., 2009; Baroni et al., 2013). As very recently demonstrated by Franchi and colleagues (2014) human skeletal muscle displays a similar hypertrophy in response to ECC and CON training (matched for relative maximum load), but through two divergent mechanisms of architectural remodelling. Training with lengthening contractions promoted a greater increase in fascicle length (Lf) compared to concentric training, whereas shortening contractions preferably induce an increase in pennation angle. These mechanisms suggest a differential addition of new contractile material along the muscle: longitudinal muscle growth (i.e. Lf increase) due to the addition of new sarcomeres in-series (Williams & Goldspink, 1971; Williams, 1990; Lynn & Morgan, 1994), while an increase in PA likely reflects an addition of new sarcomeres in-parallel (Gans, 1982; Kawakami et al., 1993; Narici, 1999; Narici & Maganaris, 2007, Reeves et al., 2009).

Hence the recent observations of Franchi et al. (2014) provided additional evidence that different patterns of muscle hypertrophy are obtained by CON and ECC training and these are achieved through distinct architectural adaptations.

Furthermore, disparities in the regional distribution of changes in anatomical cross sectional area (ACSA) were found in response to ECC vs. CON training (Franchi et al., 2014). While ECC resulted in a preferential hypertrophy in the distal regions of the vastus lateralis muscle (close to myotendinous junction, MTJ), CON training tends to favour hypertrophy

around mid muscle belly. Not only this could reflect the aforementioned changes in muscle architecture, but it also suggests heterogeneities in muscle growth along the muscle belly in response to the two loading modes. . Although early studies by Williams and Goldspink (Williams & Goldspink, 1971; Goldspink, 1985; Williams et al., 1988) on rodent muscles suggested that serial sarcomeres are added at the end of the muscle fibres, it remains to be shown whether such adaptations also occur in human skeletal muscle and if so, where new contractile material is added, and in particular, in response to ECC or CON loading.

In terms of changes in muscle protein synthesis in response to ECC and CON work, there is no strong evidence of superiority of either mode on the increase of anabolic signalling and fractional synthetic rates (FSR) of human skeletal muscle (Phillips et al., 1997; Cuthbertson et al., 2006). Phillips and colleagues showed a substantial similarity in mixed protein synthesis increase between shortening and lengthening exercise (unilateral training of 8 sets of either 8 ECC or CON repetitions, at 80% of 1RM CON), measured at 3, 24 and 48 hours after the RET session (112%, 65% and 34% of MPS increase, respectively, compared to baseline). Cuthberston and colleagues measured both the anabolic signalling activation (Akt and p70S6K) and the myofibrillar (MPS) and sarcoplasmic (SPS) FSRs in response to dynamic shortening or lengthening exercise (12 minutes of continuous stepping up to knee height with one leg –i.e. CON action- and down with the contralateral limb – i.e. ECC- while carrying 25% of the subject's body weight): phosphorylation of the anabolic targets together with MPS and SPS increased at the same time points (measured at 6 and 24 hours after the

exercise bout) regardless the contraction mode. However, Moore and colleagues (Moore et al., 2005) revealed that, when ECC vs. CON exercise were matched for work (performed maximally on an isokinetic dynamometer), the increase in myofibrillar FSR was more robust (4.5 hours after exercise) in the ECC compared to the CON trained leg. The latter findings may suggest a temporal difference in the changes in MPS, as ECC might result in more protein synthesis accretion in the acute state after exercise. However, no differences were observed between the two types of contractions in myofibrillar FSR at 8.5 hours time point.

Regarding different signalling pathways, the differences between shortening and lengthening contractions seem to be more relevant. The work of Franchi et al. (2014) showed an activation of the MAPK family immediately after ECC but not CON contractions (30 minutes after exercise bout). Previous animal findings support the idea of contraction-specific MAPK activation to ECC vs. CON contractions (Wretman et al., 2001; Martineau & Gardiner, 2001): Wretman and colleagues demonstrated that MAP Kinases ERK<sup>1/2</sup> and p38 are phosphorylated differently respective to the exercise mode (i.e. p38 was found highly increased after ECC compared to CON, supporting the human findings described by Franchi's study). The work by Martineau and Gardiner (2001) suggested that MAP Kinases (ERK<sup>1/2</sup>, p38 and JNK) might be activated in a muscle tension-dependent manner (eccentric > isometric > concentric > passive stretch), thus proposing that MAPK phosphorylation could be a reflection of the magnitude of mechanical stress applied to the muscle. Hence, ECC may trigger specific cascades of cellular responses, which CON exercise does not seem to.

Even though the works presented so far provide valuable information, the fact remains that current research has centred mostly on acute changes in cellular signalling and metabolism of skeletal muscle related to resistance exercise, attempting to link these to chronic muscular adaptations. Whilst the investigation of these acute cascade of events is needed to better clarify what mechanisms could drive muscle cells adaptive response to RET, a major open question is how these changes occur during a chronic time period, as suggested by Atherton and Smith (Atherton & Smith, 2012) and Murton and Greenhaff (Murton & Greenhaff, 2013).

Regarding this last consideration, the use of deuterium oxide labelled water ( $D_2O$ ) has been recently validated to measure protein fractional synthesis rates after chronic period of RET in humans in free-living conditions (Wilkinson & Franchi et al. 2014). By using  $D_2O$  therefore, chronic changes in muscle protein synthesis as a result of eccentric vs. concentric training protocols could be assessed, attempting to link previously reported acute adaptations (hours after exercises) to the ones occurring during weeks of RET.

To the author's knowledge, so far no study attempted to assess chronic changes in protein synthesis after exercise in two different sites of the same muscle and whether the type of exercise influenced the location and pattern of addition of sarcomeres.

Therefore, the main aim of the present study was to investigate whether different training modalities lead to differential accretion in MPS along the length of the muscle, due to different muscle remodelling mechanisms in response to ECC and CON RET. In order to investigate this, myofibrillar

protein synthesis (by using D<sub>2</sub>O stable isotope technique) of the VL muscle in response to 8-weeks ECC vs. CON loading protocols was evaluated at two different muscle sites: one at mid-belly and the other at the distal part of the VL (4 cm above the myotendinous junction).

*Table 1. Summary of studies comparing ECC vs. CON and the effect on increase in muscle size.*

<b>Contracti on type</b>	<b>Muscle group</b>	<b>Volunte ers</b>	<b>Intensit y</b>	<b>Duration - Frequen cy Reps x Sets</b>	<b>% ACSA - Vol - Girth</b>	<b>Referen ce</b>
Isotonic ECC vs. CON. vs. Isometric	Quadriceps	Young Men	80% 1 CON RM	12 wks - 3 x wk 8 x 4 unilateral RET	similar % ACSA for ECC CON and ISO (+5%) (data acquired from CT scans)	<i>Jones &amp; Rutherford (1987)</i>
Isokinetic Ecc vs. Con	Quadriceps	Young Men and Women	90% maxima l CON power	4 wks - 3 x wk 50 reps per session	%t type II fibres ACSA CON > ECC	<i>Mayhew et al. (1995)</i>
Isokinetic Ecc vs. Con	Quadriceps	Young Women	MVC	10 wks - 3 x wk 10 x 3 unilateral RET	% ACSA ECC > CON (6.6 vs. 5 %) (sum of 7 CSAs from MRI scans)	<i>Higbie et al. (1996)</i>
Isokinetic ECC vs. CON	Quadriceps	Young Men	MVC	12 wks 3 x wk 8-12 reps 4-6 sets unilateral RET	% ACSA type II fibres ECC > CON	<i>Hortoba gyi et al. (1996)</i>



Isokinetic ECC vs. CON	Quadriceps	Young Men	MVC	10 wks - 3 x wk 10 x 4 unilateral RET	similar % ACSA mid belly for ECC vs. CON Distal %ACSA ECC > CON (from MRI)	<i>Seeger et al. (1998)</i>
Isokinetic ECC vs. CON	Quadriceps	Young Men and Women	MVC	12 wks 3 x wk 8-12 reps 4-6 sets unilateral RET	% ACSA type II fibres ECC > CON	<i>Hortoba gyi et al. (2000)</i>
Isokinetic ECC vs. CON	Quadriceps	Young Men	MVC	10 wks 3 x wk 30 reps per session	no differences between ECC and CON for % ACSA (measured by MRI)	<i>Blazevic h et al. (2007)</i>
Isokinetic ECC vs. CON	Quadriceps/Bic eps femoris Elbow flexors/extenso rs	Young Women	MVC	5 months 3 x wk 30 reps per session unilateral RET	no differences in fat free mass for ECC and CON (acquired by DEXA)	<i>Nickols- Richards on et al. (2007)</i>
Isokinetic ECC vs. CON matched for work	Elbow Flexors	Young Men	MVC	9 wks ECC 20 reps as start and up to 60 reps by the end CON was matched for work	similar increase in muscle % CSA (pQCT)	<i>Moore et al. (2012)</i>
Isotonic ECC vs. CON matched for relative maximum load	Quadriceps	Young Men	%80 1RM ECC and CON respecti ve to the training group	10 wks 3 x wk 8-10 reps 4 sets unilateral RET	similar increase % vast us lateralis VOL for ECC and CON	<i>Franchi et al. (2014)</i>

Isokinetic ECC vs. CON	Elbow flexors	Young Men	MVC	12 wks 2.5 x wk 24 reps per session	ECC only increased% A CSA and type II fibres ACSA	<i>Vikne et al. (2006)</i>
Isokinetic ECC vs. CON	Elbow flexors	Young Men	MVC	7 wks 4 x wk	ECC only increased in muscle girth	<i>Komi &amp; Buskirk (1972)</i>
Isokinetic ECC vs. CON	Quadriceps	Young Men	MVC	6 wks - 3 x wk 10 reps per session unilateral RET	no significant pre-to-post changes in muscle girth for ECC and CON	<i>Duncan et al. (1989)</i>
Isotonic Conventio nal vs. CON vs. ECC	Quadriceps	Young Women	65 % 1RM CON	10 wks - 3 x wk 10 x 3 Bilateral RET	similar ECC and CON % Muscle girth	<i>Ben-Sira et al. (1995)</i>

## Methods

We recruited eight healthy young males ( $y$   $23 \pm 5.4$ , height =  $186 \pm 6.5$ cm, mass =  $84.5 \pm 13.8$  Kg; BMI =  $24 \pm 4$   $\text{kg} \cdot \text{m}^{-2}$  means  $\pm$  SD) not involved in resistance exercise protocol to undergo an 8-week resistance exercise-training program.

All participants were screened by medical questionnaire with exclusion criteria included joint diseases and metabolic, respiratory, cardiovascular impairments. All subjects gave written, informed consent. This study was approved by The University of Nottingham Ethics Committee and complied with the Declaration of Helsinki.

### Study Design Summary

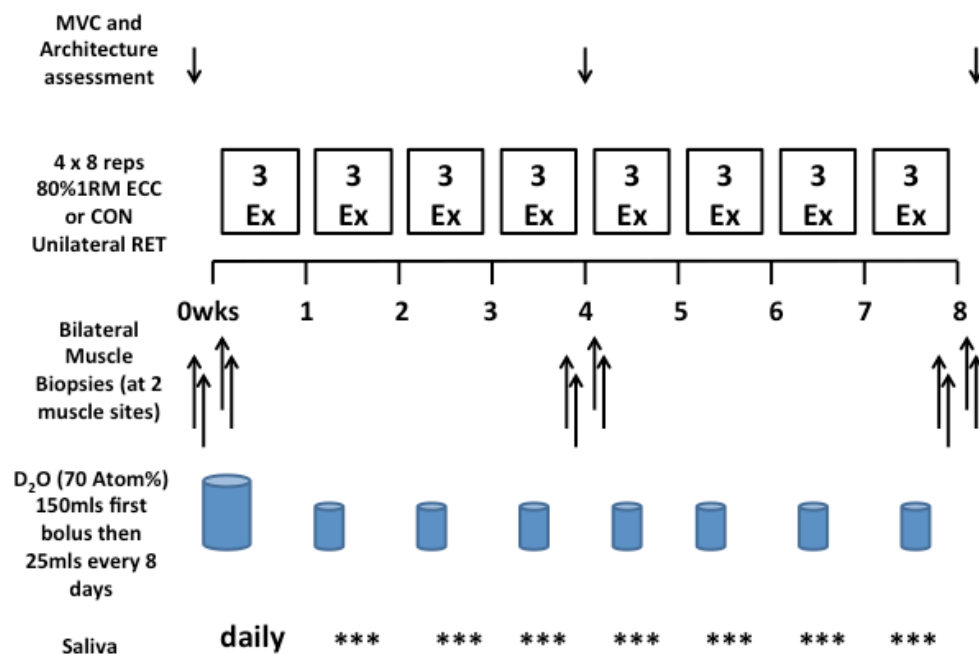


Fig1. Study design scheme

Vastus lateralis architecture was assessed on both legs by ultrasound technique within one week of commencement of training and on the final day of the study. VL muscle architecture i.e., Lf and PA were measured from images obtained *in vivo* at rest using B-mode ultrasonography (MyLab 70, Esaote Biomedica, Italy), with a 100 mm, 10-15 MHz, linear-array probe. Bilateral knee extensor strength was assessed isometrically by isokinetic dynamometer (Isocom, Isokinetic Technologies, Eurokinetics, UK). One-repetition maximum (1RM) was assessed for both ECC (1RM ECC) and CON legs (1RM CON) using the same protocol as described in our previous study (Franchi et al. 2014); 1RM was then re-checked on the first training day before the start of exercise. Volunteers were asked to refrain from heavy exercise for the 72h before the start of the study and performed no RET for the lower limbs over the course of the study other than that prescribed as part of the study protocol.

Using the conchotome technique (Dietrichson et al. 1987) (i.e. collection of muscle samples via surgical forceps), biopsies were taken from both *musculi vastus laterales* (VLs), from two different positions within each muscle ('DISTAL' close to the myotendinous junction (MTJ) and 'MID', close to the mid-belly) on day 0 (basal, pre-training) and after weeks 4 and 8, i.e. in total 12 biopsies were taken per participant. The mid-belly of VL was identified as that region with uniform and maximal cross sectional area (CSA) close to the midpoint of a line joining greater trochanter of the femur to joint-space of the knee (i.e. 50% of VL length). Basal biopsies were taken on the mid-sagittal line of the muscle in the mid-belly (MID) and 15mm anterior to the mid-sagittal line at 40 mm from the MTJ (DISTAL). Subsequent biopsies were

performed at week 4 and, at week 8, 30 mm posterior to the basal DISTAL biopsy and 30 mm proximal to the basal MID. This represents, to the best of our knowledge, the first human in-vivo study to collect tissue from close to the MTJ. All biopsies were performed under local anaesthesia (1% Lignocaine, B. Braun Melsungen, Germany) and with aseptic technique. Ultrasound scanning with Phillips iU22 and 40 mm Phillips L9-6 linear array transducer (Phillips Healthcare, Reigate, United Kingdom) permitted determination of MTJ and muscle boundaries. Reduced muscle CSA in the DISTAL region necessitated ultrasound guidance throughout infiltration of local anaesthesia and execution of conchotomy.

The basal (rested) biopsies were collected prior to any exercise bout to avoid acute interferences on muscle signalling provided by exercise. Muscle was rapidly dissected free of fat and connective tissue, washed in ice-cold saline solution and frozen in liquid N<sub>2</sub> and stored at -80°C until further analysis. A detailed schematic of the study protocol is provided in figure 2.

Immediately post biopsy on day 0, participants gave a saliva sample (collected in sterile plastic tubes) and ingested a single 150ml oral bolus of D<sub>2</sub>O (70 Atoms%, Sigma Aldrich, Poole, UK), labelling the body water pool to ~0.2%. To monitor the body water enrichment throughout the study, each participant was asked to provide a single daily saliva sample (for the first 8 days, then 2-3 times a week from day 9 until the end of the study i.e. week 8) collected at midday at least 30mins after their last meal or drink. These were collected in sterile plastic tubes and kept refrigerated, participants were asked to bring these to the following training session. Upon receipt of saliva samples, they were immediately cold centrifuged at 16,000g to remove any

debris that may be present; they were then aliquoted into 2 ml glass vials and frozen at  $-20^{\circ}\text{C}$  until analysis (as described by Wilkinson & Franchi et al. 2014)

### *Training Protocol*

Resistance exercise training was carried out with a customised leg-press machine (Technogym, Gambettola Italy) specifically adapted to perform either ECC only or CON only contractions (please see Franchi et al. 2014 for more information about the training device and protocols). Participants completed an 8-weeks exercise-training programme consisting of unilateral RET of the main extensor muscles of the lower limbs. Training load was evaluated as 80% of the concentric or 80% of the eccentric 1RM and people trained both legs but unilaterally: thus, each leg was randomly assigned to a specific exercise type so one leg performed concentric only exercise whilst the opposite trained only eccentrically. The protocol followed for RET itself (i.e. 4 series of a minimum of 8 to a maximum of 10 repetitions with one-minute rest in between the sets, time of contraction  $\sim 2$  s CON vs.  $\sim 3$  s ECC) was exactly the same as reported by a previous investigation of our research group (Franchi et al. 2014).

### *Muscle architecture and maximum voluntary contraction assessment*

Changes in muscle architecture were evaluated by analysis of ultrasonographic images (US) of vastus lateralis muscle obtained at rest using B-mode ultrasonography (MyLab 70, Esaote Biomedica, Italy), with a 100 mm, 10-15 MHz, linear-array probe. Resting US images were taken while the participant was lying on a bed for examination (corresponding to full knee extension). The US images were taken at the middle of VL length, with

the mid-point of the probe placed longitudinally exactly on the 50% of VL length and on the mid-sagittal line of the muscle while the participant was lying supine. The transducer was then aligned in the fascicle plane to capture an optimal portion of fascicles (Reeves et al., 2009). Images were collected and digitally analysed by the same unblinded operator (MVF). Quantification of muscle architectural adaptations, as assessment of fascicle length (Lf), pennation angle (PA), measured as the intersection between fascicles and the deep tendon aponeurosis) values, and muscle thickness (MT), measured as the perpendicular distance between the superficial and the deep tendon aponeurosis, was performed by using ImageJ 1.42q software (National Institutes of Health, USA). The visible portion of the fascicle length was directly assessed, then, when a little portion of the fascicle extended off the ultrasound window, an estimation of the non-visible part was performed using a linear extrapolation of fibres and aponeuroses (Franchi et al. 2014). Muscle thickness (MT) was measured as it has been previously established as a reliable indicator of changes in muscle mass/volume (Miyatani et al., 2002; Takai et al., 2013) and thus it is thus regarded as a valid index of muscle hypertrophy induced by resistance training.

Isometric maximum voluntary contraction (MVC) was determined by isokinetic dynamometer (Isocom, Isokinetic Technologies, Eurokinetics, UK): volunteers were asked to perform isometric contractions at different knee joint angles from 90° to 50°, with full extension corresponding to 0°. Subjects were seated (hip angle = 85°, hip angle at supine position = 0°) secured into position using straps across the chest. The lower leg was strapped to the pad of the Isocom lever arm and the knee joint center of rotation was aligned with

the dynamometer fulcrum. Contractions lasted for 4 s, with a rest period of 30s between contractions, and a rest period of 90 s between knee joint angle changes. The maximum isometric torque value produced was chosen to assess MVC changes between 3 different time points (baseline, 4weeks, 8weeks –post training).

### *Body Water Enrichment*

Body water enrichment was assessed by direct liquid injection of saliva samples (0.1 µl volume) into a High Temperature Conversion Elemental Analyser (TC/EA; Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an Isotope Ratio Mass Spectrometer (IRMS, Delta V Advantage, Thermo, UK). To reduce the carryover effect between different samples, each saliva sample was injected for at least 3 times.

A validation of the accuracy of the TC/EA for measuring body water enrichment from saliva has been reported in a previous publication from our lab (Wilkinson & Franchi et al. 2014).

### *Isolation and derivatization of myofibrillar protein fractions*

This procedure has been described extensively by Wilkinson and colleagues (2014). Summarising briefly, in order to isolate the myofibrillar fraction from the collected tissue ~30-40 mg of muscle was used. The tissue was homogenised in ice-cold homogenisation buffer (50 mM Tris-HCl (pH 7.4), 50 mM NaF, 10 mM β-Glycerophosphate disodium salt, 1 mM EDTA, 1 mM EGTA, 1 mM activated  $\text{Na}_3\text{VO}_4$  (all Sigma-Aldrich, Poole, UK)) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK) at 10



$\mu\text{l}\cdot\mu\text{g}^{-1}$  of tissue. Homogenates were rotated for 10 min and the supernatant collected by centrifugation at  $13,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ . The myofibrillar pellet was solubilised in 0.3M NaOH, separated from the insoluble collagen by centrifugation and the myofibrillar protein was precipitated with 1M perchloric acid (PCA). Protein-bound AA from myofibrillar, were released using acid hydrolysis by incubating in 0.1M HCl in Dowex  $\text{H}^{+}$  resin slurry overnight before being eluted from the resin with 2M  $\text{NH}_4\text{OH}$  and evaporated to dryness. The AA were then derivatized as their n-methoxycarbonyl methyl esters (MCME) according to the protocol of Husek (Husek & Liebich, 1994), with slight modification. The dried samples were resuspended in 60 $\mu\text{l}$  distilled water and 32 $\mu\text{l}$  of methanol, following a brief vortex, 10 $\mu\text{l}$  of pyridine and 8 $\mu\text{l}$  of methylchloroformate were added. Samples were vortexed for 30s and left to react at room temperature for 5 min. The newly formed MCME AA's were then extracted into 100 $\mu\text{l}$  of chloroform; any remaining water was removed from the sample with addition of a molecular sieve. Incorporation of deuterium into protein bound alanine was determined by gas chromatography-pyrolysis-isotope ratio mass spectrometry (Delta V Advantage, Thermo Scientific, Hemel Hempstead, UK).

#### *GC-pyrolysis IRMS deuterium analyses*

For GC-Pyrolysis-IRMS analysis of MCME alanine, samples were separated on a DB-wax column (30m x 0.32mm x 0.25 $\mu\text{m}$ , Agilent J&W, USA), following splitless injection. The oven temperature programme for alanine was: start at  $70^{\circ}\text{C}$  and hold for 3 mins; ramp at  $10^{\circ}\text{C}/\text{min}$  to  $240^{\circ}\text{C}$  hold for 15mins. The separated samples were then passed through a high

temperature (1420°C) conversion reactor where the analytes were converted to H<sub>2</sub> gas before being directed to the IRMS where the <sup>2</sup>H/<sup>1</sup>H ratio was determined. For TC/EA analysis of body water enrichment, following direct liquid injection into the TC/EA where samples were immediately converted to H<sub>2</sub> gas, sample gases were directed to the IRMS where the <sup>2</sup>H/<sup>1</sup>H ratio was determined. The deuterium isotopic enrichment provided as δ<sup>2</sup>H was converted to atom % using the following equation:

$$Atom\% = \frac{100 \times AR \times (\delta^{2H} \times 0.001 + 1)}{1 + AR (\delta^{2H} \times 0.001 + 1)}$$

Where, AR represents the absolute ratio constant for deuterium based on the VSMOW standard and equates to 0.00015595. This was then converted to atom % excess (APE) by correcting for baseline sample i.e. background enrichment.

*Calculation of fractional synthetic rate (FSR)*

The fractional synthetic rate (FSR) of myofibrillar (MyoPS) protein synthesis was determined from the incorporation of deuterium labeled alanine into protein, using the enrichment of body water (corrected for the mean number of deuterium moieties incorporated per alanine, 3.7) as the surrogate precursor labelling between subsequent biopsies. In brief, the standard equation:

$$FSR (\% \cdot d^{-1}) = [(APE_{Ala})] / [(APE_P) \times t] \times 100$$

Where, APE<sub>Ala</sub> = deuterium enrichment of protein bound alanine, APE<sub>P</sub> = precursor enrichment and *t* is the time between biopsies, was used.

### *Statistical Analyses*

All data are presented as mean  $\pm$  SEM. Differences within groups (ex type x time) were detected by repeated measures with a two-way (ex type x time) factorial analysis of variance with a Bonferroni correction using GraphPad Prism software (Version 6, La Jolla, San Diego CA). The delta ( $\Delta$ ) training values (percentage increases) were statistically tested between groups using an independent t-test. The alpha level of significance was set at  $P < 0.05$ .

## **Results**

### *Muscle morphology and architecture*

Changes in muscle architecture (i.e. fascicle length, *Lf*, and pennation angle, *PA*) and thickness (MT) were investigated at baseline and after 4 weeks and 8 weeks of unilateral ECC or CON training regime. After 4 weeks, *Lf* increased considerably ( $P < 0.05$ ) in the ECC trained leg ( $4.5\% \pm 0.6\%$ ; *means  $\pm$  S.E.M.*) and significantly more ( $P < 0.01$ ) compared to the leg performing CON training ( $2.0\% \pm 0.5\%$ ) (Fig 3). Conversely, CON presented a greater change in *PA* ( $P < 0.05$ , time significance) compared to ECC ( $9\% \pm 0.9$  vs.  $3.5\% \pm 1$ , respectively,  $P < 0.01$ ) (Fig 4). Similar trends of muscle architectural adaptations were observed at the end of the training period (8 weeks): ECC showed an increase in *Lf* of  $9\% \pm 0.8\%$  ( $P < 0.0001$ ), significantly greater ( $P < 0.01$ ) than that produced by CON exercise ( $4\% \pm 0.4$ ). *PA* significantly increased in both ECC and CON trained legs ( $5.7\% \pm 0.7\%$ ,  $P < 0.05$ , vs.  $17.6 \pm 1.3$ ) although the change was significantly smaller for ECC compared to CON ( $P < 0.01$ ).

Muscle thickness (Fig 5) showed a very pronounced increase after ECC and CON, both at 4 ( $8.2\% \pm 1.1\%$  vs.  $10.5\% \pm 0.7\%$ , respectively,  $P < 0.001$ ) and 8 weeks ( $12.5\% \pm 1.1\%$  vs.  $15.7\% \pm 0.7\%$ , respectively,  $P < 0.001$ ). The increase in thickness was significantly different between 4 and 8 weeks ( $P < 0.05$  vs.  $P < 0.01$ , ECC and CON respectively); however, statistically, no differences were observed for muscle thickness between the two exercise interventions at any time point.

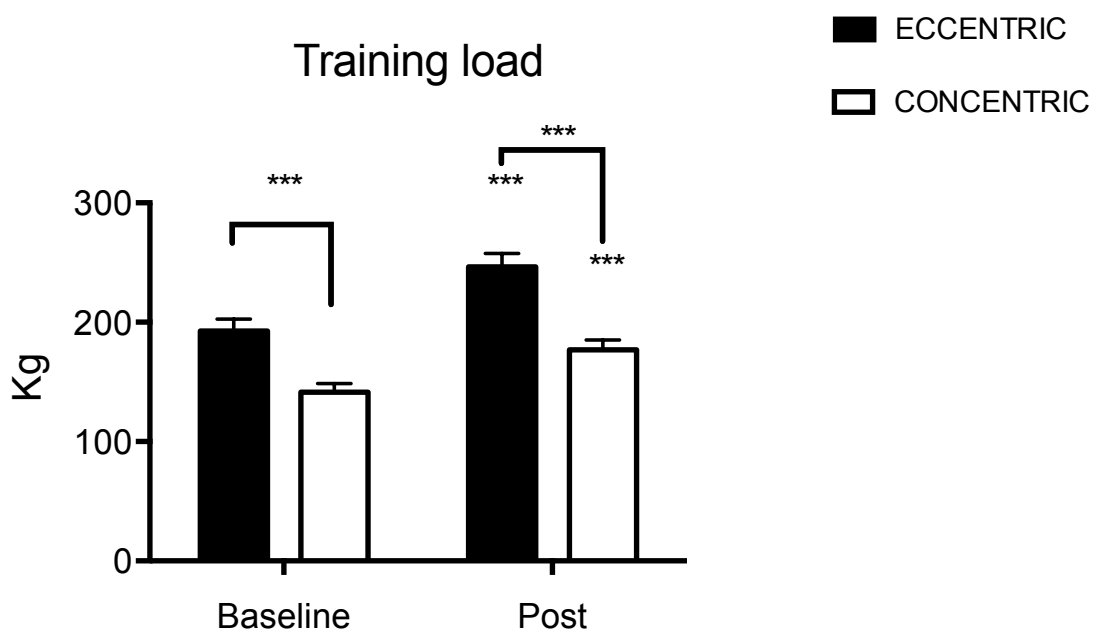
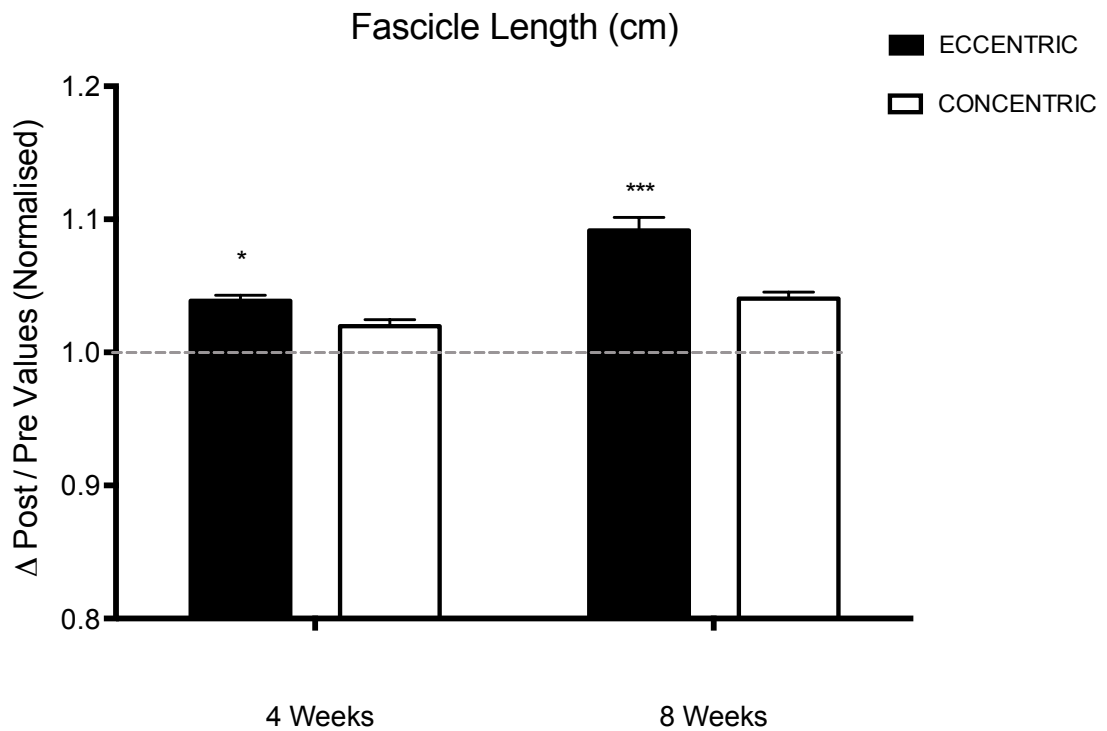
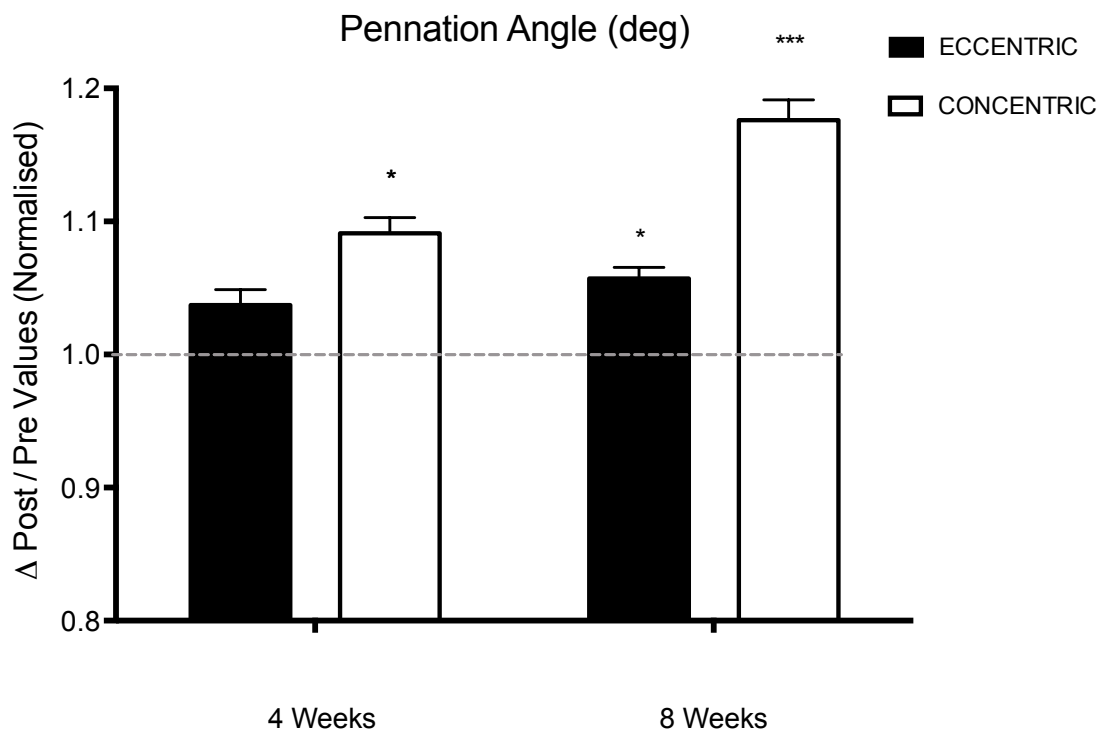


Fig 2. Training load used during leg-press exercise. Changes at baseline and 8 weeks for ECC and CON loading paradigms; \*\*\* =  $P < 0.0001$ . Values are presented as means  $\pm$  SEM.



*Fig 3. Post/Pre value ratio of fascicle length at 4 and 8 weeks of either ECC or CON training protocol. Y= 1 represent the baseline value. \* = P < 0.05, \*\*\* = P < 0.0001. Values are presented as means ± SEM.*



*Fig 4. Post/Pre value ratio of pennation angle at 4 and 8 weeks of either ECC or CON training protocol. Y= 1 represent the baseline value. \* = P < 0.05, \*\*\* = P < 0.0001. Values are presented as means ± SEM.*

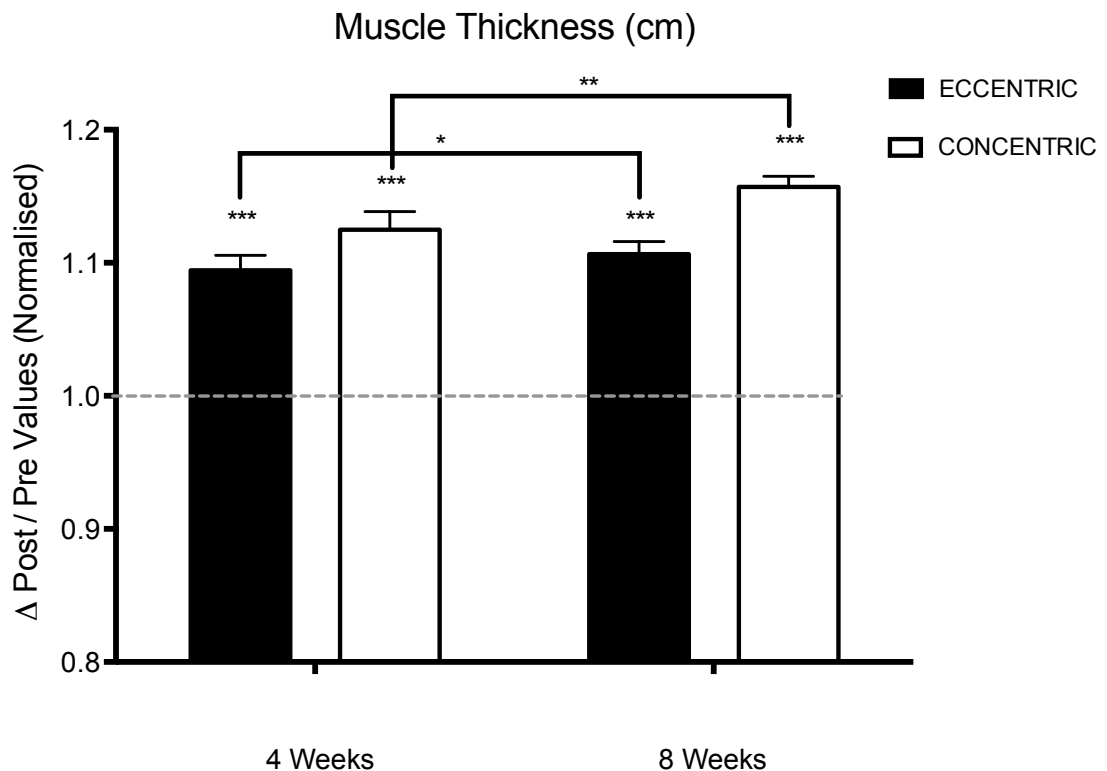


Fig 5. Post/Pre value ratio of muscle thickness at 4 and 8 weeks of either ECC or CON training protocol.  $Y=1$  represent the baseline value. \* =  $P < 0.05$ , \*\* =  $P < 0.001$ , \*\*\* =  $P < 0.0001$ . Values are presented as means  $\pm$  SEM.

#### *Muscle function and training load changes*

Changes in isometric maximum voluntary contraction (MVC) are shown in figure 6. Both ECC and CON trained limbs were observed to ( $P < 0.01$ ) isometric MVC at 4 weeks ( $12.5\% \pm 3.2$  vs.  $14\% \pm 3.3$ , respectively). After the whole training period (8 weeks) similar gains in MVC were found ( $P < 0.0001$ ) after ECC and CON interventions ( $20\% \pm 4.6$  vs.  $16\% \pm 4$ ). No significant differences were found between baseline values and between the two time points (4 and 8 weeks) for both loading modalities.

As expected, ECC training load was greater than CON one throughout all the study ( $P < 0.0001$ ): increments for both loading modes are shown in figure 1. ECC and CON interventions resulted in similar gains in training load ( $28.4 \pm 2.3\%$  vs.  $25.8 \pm 3.5$ , respectively) after 8 weeks of training ( $P < 0.0001$ ).

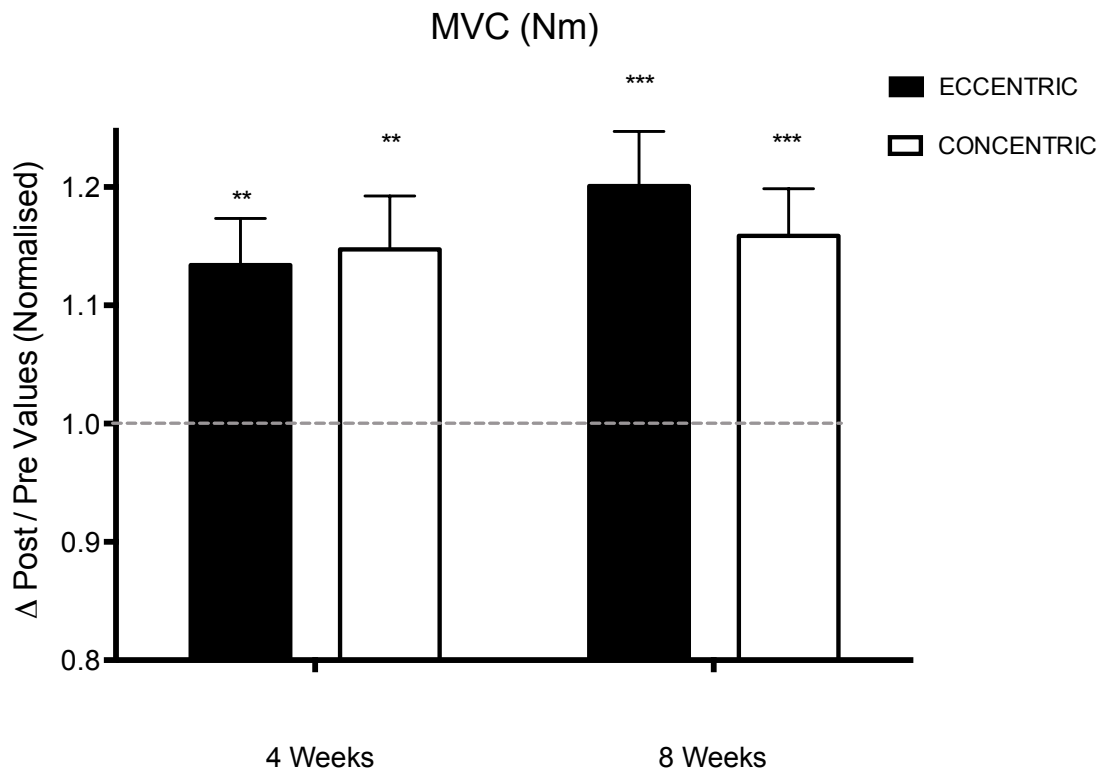


Fig 6. Post/Pre value ratio of isometric MVC at 4 and 8 weeks of either ECC or CON training protocol. Y= 1 represent the baseline value. \*\* =  $P < 0.001$ , \*\*\* =  $P < 0.0001$ . Values are presented as means  $\pm$  SEM.

*Muscle protein synthesis: incorporation of deuterium into protein bound alanine and myofibrillar fractional synthetic rate (FSR)*

Values of incorporation of D<sub>2</sub>O into protein bound alanine were assessed at 3 time points (baseline, 4 weeks and 8 weeks) in two different sites of VL muscle (middle portion, i.e. MID, and close to the myotendineous junction, i.e. MTJ) (Fig 7). After 4 weeks of RET, both ECC and CON trained limbs showed similar augmented D<sub>2</sub>O incorporation from bioptic samples taken either at MID (ECC = 689.1 vs. CON = 664.8,  $\delta^2\text{H}$  values) or MTJ (ECC = 676 vs. CON = 616,  $\delta^2\text{H}$  values) sites of VL muscle with no discrepancies between ( $P < 0.001$ ). Although a slight increase was observed for both

loading modes in both muscle sites after 8 weeks compared to 4 weeks time point (MID - ECC = 802 vs. CON = 788,  $\delta^2\text{H}$  values; MTJ – ECC = 762 vs. CON = 780,  $\delta^2\text{H}$  values), this was not statistically significant.

Figure 8 presents the daily myofibrillar fractional synthetic rates for ECC and CON training, evaluated between the start of RET and 4 and 8 weeks time points. FSRs were calculated from muscle biopsies collected from MID and MTJ VL sites. Again, no statistically significant differences were found between muscle sites and loading modalities at both time points. However, ECC MID and MTJ, together with CON MID (but not CON MTJ) site FSRs significantly decreased at 8 weeks, compared to the values at week 4 ( $P < 0.05$ ).

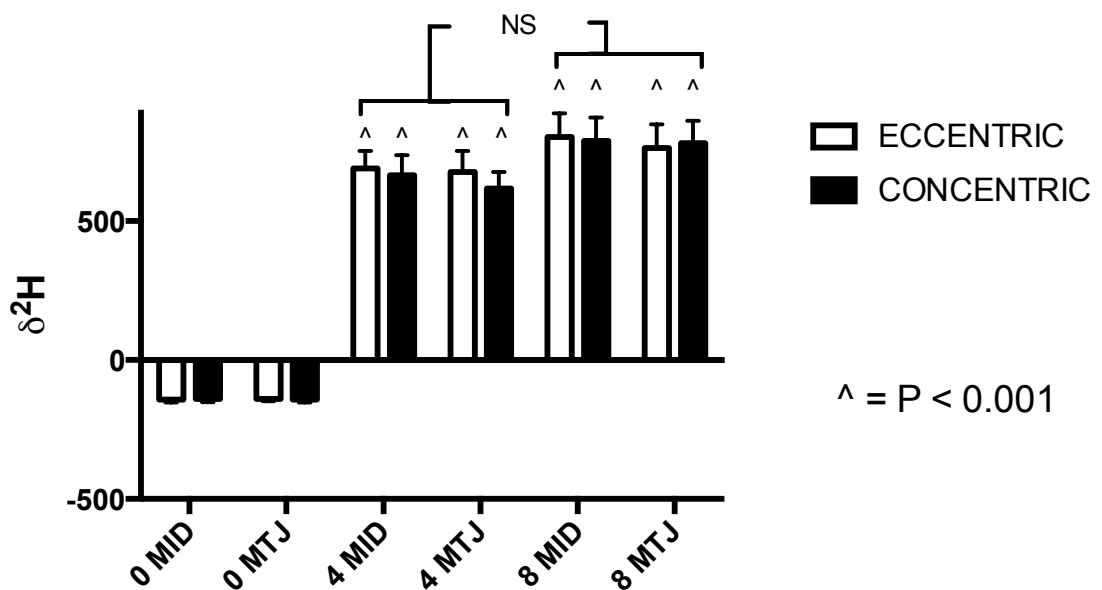


Fig 7. Incorporation of deuterium ( $\delta^2\text{H}$ ) into protein bound alanine over the training period and calculated from muscle biopsies collected at MID and MTJ sites of vastus lateralis muscle. ^ =  $P < 0.0001$  and NS = non significant. Values are presented as means  $\pm$  SEM.



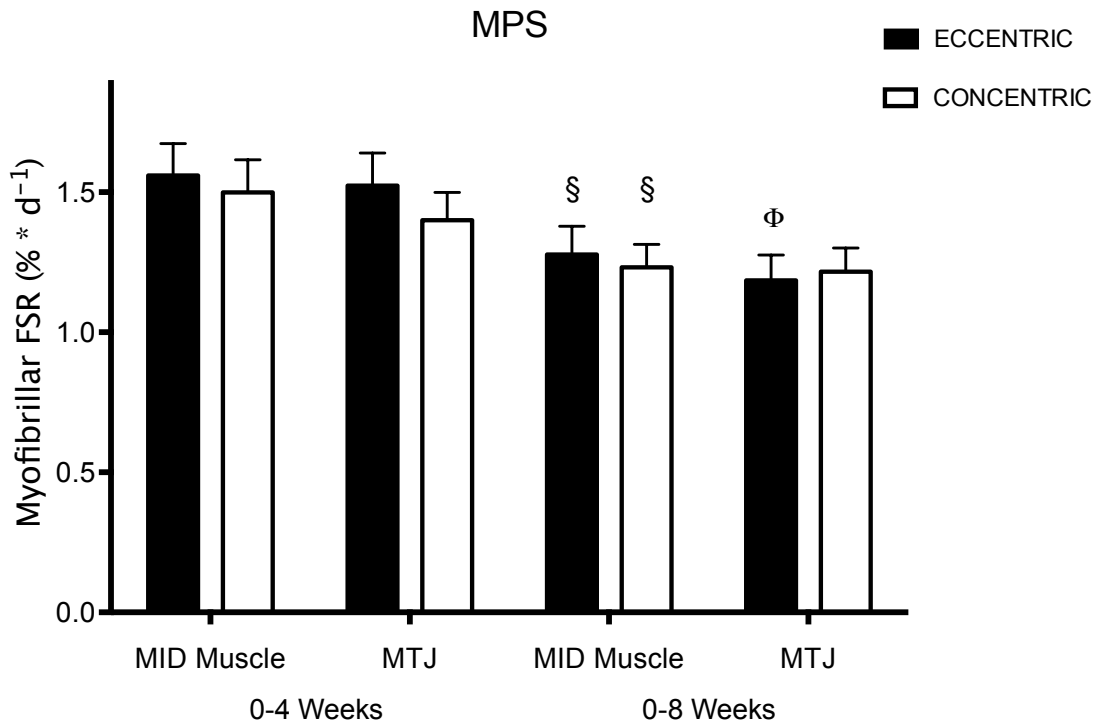


Fig 8. Myofibrillar protein synthesis rates for ECC and CON groups over the period of 4 and 8 weeks calculated from muscle biopsies collected at MID and MTJ sites of VL muscle. § =  $P < 0.001$  compared to 0-4weeks FSR value; Φ =  $P < 0.0001$  compared to 0-4 weeks FSR value. Values are presented as means  $\pm$  SEM.

## Discussion

The present study aimed to investigate the chronic metabolic adaptations (i.e. protein synthesis) to ECC and CON training and test the homogeneity (or heterogeneity) of these responses along the muscle belly of m. vastus lateralis. To the author's best knowledge, to date, this is the first study that not only tries to analyse chronic MPS changes (in terms of fractional synthetic rates) after pure ECC vs. CON training regimes, but also attempts to clarify if skeletal muscle responds homogeneously or heterogeneously to the exercise stimulus throughout its length.

In order to compare the effects of ECC and CON stimuli on skeletal muscle, volunteers were asked to train unilaterally with one leg performing just shortening contractions, while the other limb was trained just with

lengthening actions. The unilateral training model (i.e. within the subject design), in which each limb is the internal control of the other, is well established and has been adopted by several previous studies either for the knee extensors (Moore et al., 2005; Kim et al., 2005; Cuthberston et al., 2006; Kostek et al., 2007; Kumar et al., 2009) or for the elbow flexors (Moore et al., 2012; West et al., 2010). The advantages of such design are of minimising the variability of the training responses within groups (Hubal et al., 2005; Moore et al., 2012), and that the hypertrophic responses induced by RET are the result of localised muscular adaptations (West et al., 2010). Furthermore, the training protocols were matched for relative load (80% of either CON or ECC 1RM), following a design previously described by Franchi et al. (2014). Consequently, the training load (Fig 6) was greater for the ECC trained leg compared to that of the CON trained leg (i.e. for the ECC leg: ECC/CON load ratio =  $1.35 \pm 0.02$  and  $1.39 \pm 0.01$ , baseline and post-exercise values respectively). This loading protocol was chosen as to obtain a similar EMG activity in the two contraction modes, in an attempt to force muscle to operate along the same ECC-CON force-velocity curve as described by Reeves et al. (2009) and successively by Franchi et al. (2014). Notably, despite the greater loading, ECC and CON training regimes produced similar increases in muscle thickness (defined as “the distance between the superficial and deep aponeuroses of a muscle” – Narici & Maganaris, 2006). Indeed, similar changes in MT were observed after 4 weeks of either lengthening or shortening RET (8.0% vs. 10.5%, respectively); and after 8-weeks, where both ECC and CON regimes lead to similar MT increments ( $12.5\% \pm 15.7\%$ , respectively). Thus, the present

findings show no significant discrepancies in hypertrophic response between ECC and CON training when matched for relative load. Similar results in muscle CSA increase in response to the two loading modes have been reported by Blazevich et al. (Blazevich et al., 2007), and by Jones & Rutherford (Jones & Rutherford, 1987) respectively after 10 weeks of CON and ECC RET (matched for work using isokinetic contractions) and after 12 weeks of CON or ECC weightlifting program at 80% of the CON 1RM. In the present study, although the ECC leg trained with significantly higher loads compared to CON (but with similar motor unit recruitment), this lead neither to a larger increase in muscle size nor to a greater increase in muscle strength. In fact, the functional adaptations to ECC vs. CON modes revealed similar increases in isometric MVC both after 4 weeks (ECC 12%; CON 14%) and 8 weeks of RET (ECC 20%; CON 16%). These observations are also supported by previous findings of Moore et al (2012), showing a similar increase in biceps muscle CSA and isometric MVC in response to a 9-week protocol of either CON or ECC isokinetic contractions matched for work. Therefore, it appears that Wernbom's observations (2007) suggesting the substantial lack of evidence of the superiority of eccentric compared to concentric RET for achieving a greater muscle mass are confirmed either by the present findings and by the findings of Moore et al (2012).

Although the changes in muscle thickness produced by the two loading regimes were not different, the changes in fascicle length and pennation angle were highly loading-specific. As expected, both at 4 and 8 weeks of RET, the increase in Lf was significantly greater for ECC than for CON RET (4.5% vs. 2 % and 9% vs. 4 %, respectively): conversely, PA showed a very

large increase already after 4 weeks of CON (9%) compared to ECC exercise (3%). Although ECC exercise showed a statistically significant increase of pennation angle after 8 weeks of training compared to baseline values (5.7%), the changes was considerably lower than the one brought by CON RET (17.6 %). These values indicate once again that the architectural adaptations of human skeletal muscle are contraction-specific. Therefore, chronic use of lengthening vs. shortening contractions leads to a similar degree of hypertrophy over time, but through divergent remodelling patterns, as suggested by Franchi and colleagues (2014).

Since the classical studies by Goldspink (Goldspink, 1968) and Williams (Williams & Goldspink, 1971) on animal muscle, it is known that striated muscle fibres post-natal growth is achieved longitudinally by the addition of new serial sarcomeres. Successive experiments (Williams et al., 1988, Williams, 1990) showed how longitudinal growth of muscle fibres could be achieved with passive and/or intermittent stretch of the related muscle. From these findings, together with the observations of Newham and colleagues (1983) on the marked ultrastructural changes of human muscle after lengthening contractions, Morgan (D L Morgan, 1990) proposed the so-called “popping sarcomeres” hypothesis, which states that the weaker sarcomeres are over-stretched as result of lengthening contraction and inhomogeneous length of sarcomeres themselves (Gordon et al., 1966), leading to a shearing of myofibrils and subsequent exercise induced muscle damage (Newham et al., 1983). Therefore, skeletal muscle adapts to lengthening action by increasing the number of serial sarcomeres (Williams et al., 1988, and 1990; Proske & Morgan, 2001; Morgan & Proske, 2004). Moreover, Butterfield and

colleagues (Butterfield et al., 2005) have shown that serial sarcomeres addition is contraction type dependent in rats after uphill and downhill treadmill running: loss of serial sarcomere occurred after uphill concentric-biased contractions while downhill eccentric-biased contractions resulted in a greater gain of sarcomeres in series. Therefore, dynamic shortening contractions seem to not promote longitudinal growth as much as muscle action involving stretching.

The different architectural adaptations to CON as opposed to ECC training suggest that this contraction-specific differential growth of skeletal muscle may be controlled by different responses of muscle anabolism (differential stimulation of new contractile material synthesis, i.e. myofibrillarPS). It also appears legitimate to question whether these remodelling mechanisms could differ along the muscle belly.

Previous literature has successfully attempted to gain insight into the potentially different gain in muscle protein synthesis brought by lengthening and shortening contractions (Phillips et al., 1997; Moore et al., 2005).

The main findings suggest that muscle protein synthesis does not present significant differences in response to the two mechanical stimuli, although ECC might result in a different temporal stimulation of MPS compared to CON. Indeed, while Phillips and colleagues reported no significant discrepancies in mixed MPS between the loading modes at 3-24 and 48 hours after a single exercise bout, Moore's study showed an early noteworthy increase (4.5 hours time point) in myofibrillar FSR after ECC compared to CON performed on isokinetic dynamometer. However, no

significant differences were observed at 8.5 hours time point as myofibrillar PS was reported to increase similarly after both kind of exercise.

Nonetheless, the presence of acute-only MPS data sets lead us to focus on the long-term adaptations to ECC vs. CON. In order to perform such chronic measurements of muscle metabolism, we used deuterium oxide stable isotope: this technique has been recently utilised for evaluation of protein synthesis by gas-chromatography mass spectrometry (GC-MS) in humans after acute RET exercise (Gasier et al., 2012) and after 6 weeks of aerobic/endurance training (Robinson et al., 2011). Robinson and colleagues (2011) proved the appropriateness of the method to measure MPS over prolonged periods (i.e. 6 weeks) in free-living adults. In addition, Gasier et al., (2012) reported an increase of myofibrillar protein synthesis (MyoPS) but not mixed muscle protein synthesis at 24 h following a single RET bout, thus affirming once again the importance of delineating fraction specific differences.

Moreover, the use of D<sub>2</sub>O has been recently validated for assessment of MPS over an 8-day RET protocol by pyrolysis - isotope ratio mass spectrometry (IRMS, more sensitive than GC-MS for measuring tissue isotopic enrichment) (Wilkinson & Franchi et al. 2014). In the present study it is shown how the methodology is suitable for measuring of cumulative incorporation of deuterium into new muscle proteins (i.e. and therefore MPS) over long-term resistive-exercise training regimes.

Data on incorporation of D<sub>2</sub>O into protein bound alanine from myofibrillar fractions has been found significant for both contraction types after 4 and 8 weeks of training, but with similar values. Furthermore, there seems to be a

substantial homogeneity between muscle sites, as MID and MTJ incorporation values are very close for each differently trained leg.

Thus, no significant differences have been observed neither between loading types nor between mid-length vs. distal muscle sites through the whole training period. A few considerations arise from the latter findings. Firstly, changes in MID MPS could reflect the one in muscle thickness (measured at the same muscle site), suggesting even in a stronger manner that similar hypertrophy is reached by ECC and CON either when matched for work (Blazevich et al. 2007; Moore et al. 2012) or when matched for relative maximum load (Franchi et al., 2014).

Nonetheless, the chronic data here presented are in accordance with those reported in the acute phase after exercise by the aforementioned works (Phillips et al., 1997; Moore et al., 2005; Cuthberston et al., 2006) in which no substantial differences were observed by comparing the two loading modalities. Secondly, the incorporation data clearly suggest that hypertrophic adaptations start from the very first days (Wilkinson & Franchi et al. 2014) /weeks of resistive- exercise both at molecular (MPS) and morphological-architectural level, as considerable changes in Lf, PA and MT were observed already after 4 weeks of training (as previously reported by Seynnes et al. 2007).

Considering that ECC and CON have been shown to lead to different regional increase in ACSA (Seger et al., 1998; Franchi et al., 2014), the fact that MPS was unchanged between middle (MID) and distal (MTJ) muscle sites was unexpected. In Seger's study, young healthy males were trained unilaterally for 10 weeks eccentrically and concentrically and showed a

preferential increase of distal muscle area after ECC. As extensively discussed in the introduction section, Franchi and colleagues analogously reported a greater change in muscle CSA in the distal part of VL after ECC compared to CON RET, while CON promoted a larger increase in muscle volume towards the middle of VL length.

Thus, skeletal muscle seems to respond homogeneously to ECC vs. CON, resulting in very similar chronic changes in MPS as acute studies previously suggested.

Although the muscle showed homogeneous anabolic response at two different locations, it must be stated that the analyses of the present study were carried out only on myofibrillar fraction of muscle samples: therefore, this may suggest that differential adaptation throughout the muscle length could be brought by changes in synthesis of other fractions: for example Collagen proteins (i.e. greater addition of Extra Cellular Matrix –ECM). Collagen expression have been shown to increase in rats muscle much more after lengthening contractions compared to the one in shortening (Heinemeier et al., 2007), thus this could still be regarded as a possible adaptation occurred in the present study after training. Further investigation is needed to clarify such hypothesis.

However, what could cause differential adaptation in muscle architectural remodeling to ECC and CON exercise still remains cryptic.

If this contraction-dependent remodeling in muscle architecture is not matched by the changes in MPS, then something else must be the responsible, the on/off switch for such characteristic regulation of muscle growth strategies. Although Franchi's work (2014) showed an ECC but not



CON contraction-specific activation of some of the MAPK family (p38, ERK<sup>1/2</sup>, p90RSK) 30 minutes after an exercise bout in humans (with similar findings in Lf and PA changes), this does not seem to address the question at the core. It may be that the mechanisms underlying these changes are mechano-transductory driven. In particular, focal adhesion kinase (FAK) complex has been shown to modulate mechanical stimuli transducing them into chemical/intracellular responses (Flück et al., 1999); (Durieux et al., 2007). FAK activation is regulated in a load-dependent manner and has been object of many investigations on animals in which loading (Gordon et al., 2001) and un-loading (Flück et al., 2002) states were the main factors of augmentation or non-response (respectively) in FAK phosphorylation.

Because un-loading scenarios lead to loss in muscle mass (i.e thus loss of contractile material), a change in muscle architecture is expected to be the following consequence (Narici & Maffulli, 2010). On this topic, deBoer and colleagues (de Boer et al., 2007; de Boer et al., 2007) showed a very significant decrease of total content and activation of FAK in humans following 10 days of unloading of the knee extensor in humans, resulting in a significant loss of sarcomeres correlated with a decrease of muscle fascicle length. In the light of the fact that FAK might be involved in the response to unloading and change in muscle architecture, it seems justified to speculate whether there might be a link between different architectural adaptations brought by the chronic use of lengthening vs. shortening contractions and FAK activity during loading states, as one could think of a different modulation caused by complete opposite mechanical stimuli. Further research is needed in order to attempt to clarify these possibilities.

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## Epilogue

The work presented in this thesis focused on eccentric and concentric muscular contractions (lengthening vs. shortening actions), in order to gain novel insights into the morphological, functional, molecular and metabolic adaptations of human skeletal muscle in response to acute and chronic loading paradigms.

The thesis involved three novel studies investigating 1) the morphological, functional, and molecular adaptations to 10 weeks of pure ECC vs. CON loading in human skeletal muscle, 2) the study of changes in muscle metabolism (muscle protein synthesis) during RET using an innovative deuterium oxide stable isotope tracing technique for measuring periods in free-living humans, and 3) The evaluation of MPS at two different muscle locations (vastus lateralis mid-length vs. distal portion) in order to investigate whether heterogeneities in the distribution of muscle hypertrophy along the muscle belly could be ascribed to regional differences in MPS in response to RET.

The major findings and outcomes of the three studies are:

## **Study 1.**

### **Architectural, functional, and molecular responses to concentric and eccentric loading in human skeletal muscle**

In this study, the structural remodeling of human skeletal muscle was investigated in response to a 10-weeks pure unilateral CON and ECC training period where the two modalities were, for the first time, matched for relative load, in order to compare the different RET types for relative loading stimulus. While similar gains in muscle size were observed as result of the chronic use of either ECC or CON training, it is contended that muscular hypertrophy was achieved through two opposite architectural remodeling patterns. The adaptations in muscle architecture are in agreement with the analogous findings reported in chapter 3 and reflect the peculiar plasticity of human skeletal muscle, able to reach high degrees of muscle hypertrophy but with two different modalities: muscle growth can occur either through the addition of sarcomere in parallel, or in series, or as a result of both (Russell et al., 2000). Early studies by Williams and Goldspink (Williams & Goldspink, 1971) showed that longitudinal muscle growth (increase in Lf) can be achieved through the accumulation of new contractile material along the muscle fibre (serial sarcomeres addition, Goldspink, 1968), seen as a strategy for the muscle to preserve itself from possible muscular damage scenarios. As a matter of fact, eccentric actions have been shown to lead to a greater degree of muscle damage (Schoenfeld, 2012), caused by the shearing of myofibrils and “weaker” sarcomeres disruption when muscle are

forcibly lengthened: skeletal muscle, by increasing the number of sarcomeres placed in series and therefore Lf, is thought of preserving itself from undergoing significant sarcomeres damage again because of less strain of each sarcomere (Morgan & Proske, 2004).

The greater change in fascicle angle (pennation angle) observed after CON exercise is instead generally accepted as a reflection of the larger addition of sarcomeres in parallel: the muscle is thought of placing more contractile material in parallel along the deep aponeurosis of the muscle to increase its force-generating capacity (Gans, 1982).

Consequently, the first task of this study was that of investigating, using ultrasonography, how the two types of contractions were “mechanically” different during shortening and lengthening muscle contractions on a leg-press. As expected, Lf shortened during CON exercise while lengthened during ECC contractions and a rise in PA was only seen during the CON exercise. Thus, ECC and CON actions actually reflected the different mechanical stimuli applied to the muscle by the two loading modes. It was subsequently attempted to link the morpho-architectural changes to the molecular signaling pathways implicated in muscle remodeling (MAPKs). The results obtained by immune blotting showed a clear preferential activation of members of the MAP Kinases family only after ECC exercise bout but not after CON. Although in this study MAPKs activation was found to be contraction type-dependent (as in previous animal models) one could speculate that different mechanisms of muscle remodeling are operating even at the molecular level, it remains to be established, however, whether such “remodeling pathway” activation is the main cause of these differential

architectural adaptations. An explanation for the similar hypertrophy observed with ECC and CON RET may be afforded by the greater myofibrillar disruption known to be caused by ECC loading (Newham et al., 1983) , followed by a belated activation of the inflammatory pathways, likely antagonizing muscle hypertrophy.

Hence, the main findings of study 1 are that a mismatch was found between the changes in muscle volume and in muscle architecture in response to ECC and CON training. Whereas, the activation of distinct molecular pathways seem to be associated with the different architectural responses to ECC and CON training, the overall gain in muscle mass (volume) is similar between the two training modes, possibly pointing to the presence of muscle damage and inflammation as blunting factors to a greater muscle hypertrophy with ECC training.

Nevertheless, few questions were still unresolved at this point. First, regional differences in VL muscle hypertrophy were found in the present study (i.e. higher degree of hypertrophy in the distal part of the VL with ECC RET vis-à-vis a greater increase in volume specifically in the VL mid-length portion with CON RET). Thus, if such morphological, architectural and molecular adaptations occur, are these responses driven by different mechanisms of regulation of muscle mass in terms of activation of muscle protein synthesis? And if so, do the muscle show homogeneity or heterogeneity throughout its whole length regarding the responses to these stimuli?

New insights into the possibility of monitoring daily/week changes in muscle metabolism were required, as one muscle biopsy represents just a singular snapshot that can provide information on acute responses, but not on chronic

changes in MPS that could ultimately be related to the findings above mentioned.

## **Study 2.**

### **A validation of the application of D<sub>2</sub>O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein sub-fraction synthesis in free-living humans**

Study 1 attempted to describe the implication of possible biological mechanisms underlying the contraction-specific remodelling of muscle size and architecture, analysis of cellular signalling pathways provides valuable information about the regulation of transcriptional and translational processes after exercise but without providing an actual quantification of new protein synthesis occurred. Traditionally, calculation of MPS has been gauged using venous/arterial labeled AA infusion with multiple biopsy collections, but this technique is rather restrictive, as it allows measures of acute responses and just in controlled environment (laboratories). Instead, the tracer method (as deuterium oxide) used for this study, allows measures of cumulative tracer incorporation into proteins over a period of several weeks or months providing the actual quantification of muscular anabolic response to definite interventions.

The study demonstrated for the first time that it is possible, using the D<sub>2</sub>O tracing technique together with the use of pyrolysis - isotope ratio mass spectrometry (IRMS), to assess the temporal response of FSR within multiple

muscle protein sub-fractions over a short-period. Furthermore, these findings validate the method also for evaluation of MPS in response to a 8-day RET (temporal and cumulative anabolic responses to exercise). These results not only indicated that the unilateral training study design (i.e. with an internal control within the subject, as one leg was untrained and used as a control) is suitable for accurately monitoring changes MPS between the two limbs (and so that localised muscular responses provide reliable data for muscle metabolism), but also that the “first steps” of hypertrophy could be detected already after two days from the start of a resistance-training period (therefore supporting the validity of both previous and chapter 3 findings on muscular early hypertrophic changes to exercise).

Study 2 also lead the way to an exciting novel method for measuring MPS and muscle anabolism in extended ‘free-living’ situations, being therefore able to “fill the gap” between acute and chronic adaptations in muscle metabolism. The validation of this technique was vital for study 3, as we could then test some of the hypothesis formulated after discussing study 1 outcomes.

### **Study 3.**

**Chronic changes in muscle protein synthesis in response to eccentric and concentric loading measured at two different regions of the *vastus lateralis* muscle in humans**

The design and rationale of study 3 were inspired by the results obtained in Study 1 and the novel validation of D<sub>2</sub>O tracing technique discussed in Study 2. In this last investigation (Study 3) morphological adaptations induced by CON and ECC RET training were related to muscle metabolism (myofibrillar PS) measured using the technique validated in Study 2. To the best author's knowledge, this is the first study that investigated myofibrillar protein synthesis at two different locations of the muscle belly, collected at the same time points. Muscle hypertrophy was found to reach similar between ECC and CON values after 8-weeks, as indicated by results of increase in muscle thickness.

Moreover, a significant increase in MT was observed after just 4 weeks of CON and ECC loading confirming earlier reports of early muscle hypertrophy induced by high-intensity RET (Seynnes et al., 2007). The observation of the similar increase in muscle size between CON and ECC RET, is in agreement both with the findings on the incorporation of deuterium into new proteins and the myofibrillar fraction rates of synthesis. A considerably increased incorporation of D<sub>2</sub>O into protein-bound alanine was found both at 4 and 8 weeks; the same occurred for FSR measured between 0-4 weeks and 0-8 weeks. Hence these findings clearly show that the addition of new contractile material in response to RET is very rapid and that sizeable muscle growth can already be detected after four weeks of training,

Although the changes in muscle thickness and metabolisms were found to be closely related, the architectural adaptations clearly differed between the two loading regimes, in an analogous manner to the data presented in Study 1. Hence, fascicle length and pennation angle were once again observed to

change in a contraction-dependent fashion (Lf: ECC > CON, PA: ECC < CON).

Therefore, considering that, a) ECC and CON resulted in similar muscular hypertrophy (volume Study 1, muscle thickness Study 3) and that, b) ECC and CON have been shown to lead to different regional increases in muscle CSA in Study 1, the finding that MPS was unchanged between middle (MID) and distal (MTJ) muscle sites was unexpected.

Furthermore, the present data still do not clarify what factor(s) could be responsible for such characteristic remodeling pattern of muscle architecture. As Study 1 suggested the presence of contraction-dependent molecular responses in terms of preferential MAPK after lengthening contractions, it is speculated that maybe a likely involvement of mechano-transducing pathways could be the cause of these adaptations. In support to this hypothesis, focal adhesion kinase (FAK) has been found to be correlated in the change in muscle architecture after muscle overloading in humans (M V Narici et al., 2011). In fact, FAK activity and importance is well documented during loading states (Klossner et al., 2009), therefore it could be speculated that complete opposite mechanical stimuli may lead to different FAK modulation and thus this might be relevant for macro-architectural changes (differential addition of sarcomeres in series vs. in parallel).

## **Conclusions and future directions**

The present work attempted to clarify what are the mechanisms of remodeling of human skeletal muscle when trained with two completely different mechanical stimuli. Divergent changes in muscle architecture were



observed to be the main consequence of the training programs, but they did not influence on the overall increase in muscle size. From a purely theoretical point of view, the different architectural response would be expected to impact on the two fundamental mechanical properties of skeletal muscle: the length-force and the force-velocity relation. It would be interesting if this possibility were investigated by future studies.

Fundamentally, the present findings show that ECC and CON training lead to a similar hypertrophy but through different morphological adaptations. Although the existing literature presents controversial findings regarding the superiority of each loading type on increases in muscle mass (Wernbom et al., 2007; Roig et al., 2009), it is important to emphasise that in studies 1 & 3 of this thesis, the ECC loading was greater than the CON one. It follows that the mechanical stimulus applied to the muscle was greater during ECC, so one would think that training with this loading type would have resulted in larger hypertrophy compared to CON and yet, this was not the case. A possible explanation for this phenomenon could be found in the rise of inflammatory processes following eccentric contractions and also muscle damage, which could have likely blunted the anabolic response to training more than after concentric exercise. Support to this hypothesis seems provided by the study of Mitch & Goldberg et al (Mitch & Goldberg, 1996).

Cellular signaling pathways should provide information on what processes are activated or suppressed at molecular level after exercise, potentially describing what factors could have played a role in such similar morphological adaptations and in such different changes in muscle architecture. As the mechanical stimuli applied to the muscle were opposite,

we can speculate on the involvement of costameric proteins (mechanosensor sites of focal adhesion in the sarcolemma, Klossner et al., 2013) in these growth differentiation processes. Costameres provide connections between internal portion of muscle fibres and the extracellular matrix (ECM) outside the fibres, and they respond mechanical forces applied to the muscle fibres, transferring those into biochemical signalling, which can modulate cell remodeling and growth (Klossner et al., 2013). Specific proteins are involved in this mechano-transduction process, such as FAK (Flück et al., 1999, Klossner et al., 2009), SRF (serum response factor – Guerci & Sotiropoulos, 2012) and TSC (Tenascin-C, a protein found in the ECM and activated by mechanical stimuli, (Flück et al., 2008) and they are all regulators of skeletal muscle hypertrophy recruited through a load/mechanical-dependent process. Further investigation is needed to assess the activation and the role of these targets in response to different contractions type. Furthermore, different time points must be investigated after the application of those mechanical stimuli, as activation of mechanotransductory pathways could be happening at very early stages straight after contraction as Klossner and colleagues (2013) found significant increase of phosphorylated FAK just after 2 min after stretching was applied to cultured cells.

The importance of gauging muscular molecular/metabolic responses at earlier time points is explained in Fig 1 of this section. The data of 3 different studies that have recently been carried out in our laboratory (Wilkinson and Franchi et al. 2014; Brook et al. unpublished; Franchi et al. unpublished – *i.e. chapter 3 data*) have been plotted to investigate/clarify the response in myofibrillar protein synthesis to different exercise protocols over time

(measured by using the deuterium oxide tracing technique). The MPS data have been collected from the works by Wilkinson & Franchi (2014) and by Brook and colleagues (unpublished) on young men undergoing a unilateral Conventional exercise protocol (with one leg not being trained and functioning as control – REST line on the graph); therefore FSR data between 0-2, 0-4, 0-8 days (4sets x 8 -10 reps at 80% 1RM concentric, training any other day for 8 days) and 0-3 and 0-6 weeks (6 sets x 8 reps at 75% 1RM concentric, 3 x wk for 6 weeks) have been plotted for Conventional exercise. The ECC vs. CON data are the same as presented in chapter 3. Compared to the rest leg, myofibrillar FSR are much higher in the first days of Conventional training than the one calculated at 3 and 6 weeks for the same training modality and than the one calculated a 4 and 8 weeks for ECC and CON loading protocols. Nevertheless, following the robust MPS increase of the first days, the slope of decay of FSR values in the successive weeks is very similar regardless the typologies of RET adopted. These data strongly suggest that hypertrophic responses to unaccustomed exercise occur very early during RET periods, justifying early architectural adaptations discussed in chapter 3 and previously reported (Seynnes et al. 2007). Furthermore, the only differences reported between ECC and CON exercise on myofibrillar PS could be found in Moore and colleague's work (Moore et al., 2005), in which mPS increased more after lengthening contractions compared to shortening ones 4.5 hours after a single exercise bout. Thus, data acquisition using earlier time points should be included in future studies, in order to try to investigate the potential differences between loading types.

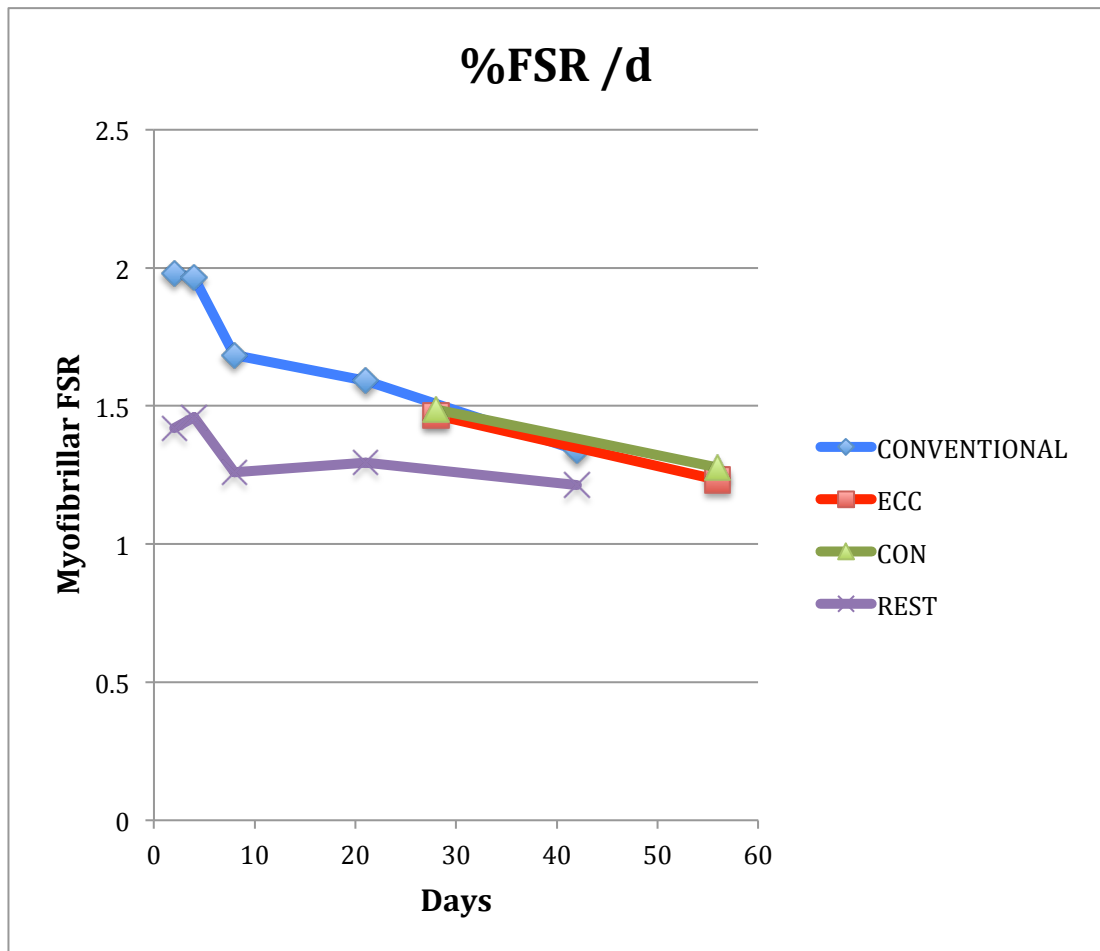


Fig 1. Myofibrillar FSR of different loading protocols overtime.

Last but not least, as sarcopenia (i.e. the loss of muscle mass with ageing, Narici & Maffulli, 2010) is achieved also with an architectural remodeling of muscle (decrease in muscle thickness, fascicle length and pennation angle) and thus with loss of sarcomeres in series and in parallel, understanding where a contraction mode leads to, is of great interest in order to assess what could be the best intervention with different populations. Moreover, eccentric exercise is usually performed with higher loads but at a lower energy cost compared to concentric one, hence it has been suggested as particularly suitable for older frail people (LaStayo et al., 2003); nevertheless, because of the high degree of inflammation caused by exercise-induced

muscle damage, eccentric exercise is not always seen as the best way of training. However, further research focusing on the profits of eccentric exercise is needed, as different intensities of exercise may result as beneficial in older age, without causing such damaging/inflammatory response. Moreover, this study has mainly focused on muscular responses to the two loading modes, but surely would be of interest investigating the adaptations of the muscle-tendon unit, as the newly introduced stable isotope technique using deuterium oxide could improve the knowledge on tendon metabolism/remodeling to ECC vs. CON exercise and in different populations (young vs. old). In addition, knowing how tendon metabolism works not only could be of interest to better understand muscle-tendon unit interaction during ECC vs. CON loading states (i.e. muscle architecture remodeling + tendon behaviour to such adaptations), but also might result useful in gaining new insights into rehabilitation programs after knee surgeries and injuries both in athletes, or young and old populations.

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## **Acknowledgements**

Well, this is it.

Siamo arrivati alla fine.

I personally think it is all quite surreal.

Just 5 years and a half ago I was on a plane. Destination: not just a new country, but also a whole new life. Although, when in few years I will look back at this lines, I might think that completing a PhD program was easy compared to other stuff, I would like to write a reminder to myself, as it totally wasn't. Do not get me wrong, it has been very enjoyable, a journey of learning and experiencing. But it has been hard too: I had to sacrifice many things, losing the grip of friendships and I had to let love go out of my life. That was the hardest thing.

But I had an objective in mind; I focused the last 5 years on it, first by completing the MRes program, then diving into this PhD adventure. Thanks to the following people, I finally achieved something that represents the ultimate reason of why I left home and everything else.

*In primis*, I would like to thank *mamma Maura*, *papa' Giovanni* and *Benedetta*, my beloved sister. Mum and Dad made me, and with Benny's help, they all shaped my soul: they are the primal reason of why I am a happy man.

**I love you from the deep of my heart.**

Thank you *Marco* for all you have done for me. You had the courage to believe in me, when I was just a guy with no conception of research

whatsoever. You have been an exceptional Director of Studies, but first of all you have been an amazing role model: an extraordinary scientist from who I learnt pretty much all I know now, with the exquisite touch of a kind real man. You took care of me like I was one of your kids, acting from time to time like a second father.

For these reasons, I will be eternally grateful. Grazie.

Thank you *Neil*, always so patient, always ready to sit down and to explain things to me. Never denied a smile, a good handshake, or words of encouragement. You are the living proof that a brilliant scientist does not necessarily have to be arrogant and aggressive. I am extremely grateful for your help and will never forget the way you proudly looked and smiled at me after my VIVA. Grazie.

Thank you *Phil*, your guidance has been much appreciated. You hosted me at your place the very first time I came to Derby, and although the many beers (eh eh), it was a lovely evening. It must have not been easy for you having me as student straight away, from one day to another, when I moved from Manchester. Your help and support in this PhD program have been so important, so I am thankful to you for believing in me. Grazie.

Some people have passed by, some other have gone already, few remained as constant presence in my life. The following people in some kind of way touched my soul, and even if maybe they will never read this, I want to thank them (nonetheless, it is my thesis).

*Styles*, no words needed. While reading this, you probably know already what I want to say. Always rocking other lives. I genuinely love you. Grazie, clone.

*Alex*, if there is a friend who still makes me believe that true love does exist is you. You and Emma are a superb couple and little Marco is just the coolest kid alive. So grateful I met you. God bless your family. Grazie.

*Aaron*, you probably don't know, but you have profoundly changed my life. You made me richer: culturally, introducing me to your habits, to your roots and to friends from many different places. You made me richer by trusting our friendship. All those evenings spent at the cornerhouse, sipping "dat devil juice", those were the best moments in my PhD frenetic weeks. You are one of the best guys I met in the last 5 years. Bless you. Grazie.

*Danny*, you are the first English friend I met. You are just a genuine and nice guy. Without you, my experience would have not been as good here in UK. So glad we are still close friends. In addition, you definitely are one of the greatest wingman in the history of mankind. Grazie.

*TMW*, you have been a fantastic flatmate and friend. We balanced our lives between PhD work and social life while living together, and I am proud to say you will always be the English part of my family. Grazie.

Sev, thanks for all the support in these years and the very nice friendship. You're one of us. You're a real rocker. I am so looking forward to collaborate with you in the future. Keep on rocking, dude. Grazie.

VDV, Ale R, il vostro supporto e' fondamentale. Keep going. Never back down. (Grazie).

*To all the UoN and MMU Crew who supported me.*

*Alla Professoressa Vago, senza la quale nulla sarebbe iniziato.*

*Al Professor Mondoni e al Professor Botton.*

*A tutti i Blues.*

*A Reyes, Mary, Rob & Stef.*

*Ad Andre, per avermi supportato e sopportato.*

*A Vegio, Uoffa, Kello, Gianfo, Vincent & Cris.*

*To Petra.*

*To Marie.*

Thank you.

It's a long way to the top, if you wanna rock n' roll.

[AC/DC]