Regional regulation of focal adhesion kinase after concentric and eccentric loading is related to remodeling of human skeletal muscle.

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Regional regulation of focal adhesion kinase after concentric and eccentric loading is related to remodeling of human skeletal muscle

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Running title: Focal adhesion kinase activation and muscle plasticity with eccentric and concentric training in humans

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Abstract

Aims

We assessed focal adhesion kinase (FAK) response to concentric (CON) versus eccentric (ECC) resistance training (RT) at two vastus lateralis (VL) sites, and the relationships between FAK, muscle protein synthesis (MPS), and morphological remodeling.

Methods

Six young males trained both legs unilaterally 3 times/week for 8 weeks; one leg performed CON RT, the contralateral performed ECC RT. Muscle biopsies were collected after training from VL midbelly (MID) and distal (Distal) sites at 0, 4, 8 weeks. FAK content and activation were evaluated by immunoblotting. MPS was assessed by deuterium-oxide tracer; morphological adaptations were evaluated by ultrasound and DXA.

Results

pY397-FAK 8 weeks levels were ~4-fold greater after ECC at the Distal site compared to CON ($p<0.05$); pY397FAK to total FAK ratio was greater in ECC versus CON at 4 (~2.2 fold, $p<0.05$) and 8 weeks (~9fold, $p<0.001$) at the Distal site. Meta-vinculin was found transiently increased at 4 weeks at the Distal site only after ECC RT. ECC presented greater fascicle length (Lf) increases (10.5% vs. 4%), whereas CON showed greater in pennation angle (PA) changes (12.3% vs. 2.1%). MPS did not differ between exercise types or muscle sites at all time points. Distal pY397-FAK and pY397-FAK/FAK values correlated to changes in Lf at 8weeks ($r=0.76$, $p<0.01$ and $r=0.66$, $p<0.05$, respectively).

Conclusion

FAK phosphorylation was greater at 8-wks after ECC RT and was muscle region-specific. FAK activity correlated to contraction-dependent architectural remodeling, suggesting a potential role of FAK in orienting muscle structural changes in response to distinct mechanical stimuli.

Keywords: Mechanotransduction, FAK, Muscle Architecture, Eccentric contractions, Muscle Remodelling

List of abbreviations

CON = Concentric

D_2O = Deuterium Oxide

DXA = Dual Absorption X-Ray Absorptiometry

ECC = Eccentric

FAK = Focal Adhesion Kinase

FSR = Fractional Synthetic Rate

Lf = Fascicle Length

MHCII = Myosin Heavy Chain type II

MID = Mid-belly Muscle Portion

MPS = Muscle Protein Synthesis

MT = Muscle Thickness

MVC = Maximum Voluntary Contraction

PA = Pennation Angle

RT = Resistance Training

VL = Vastus Lateralis

Introduction

The impact of mechanical loading on skeletal muscle is a crucial physiological stimulus of myofibrillogenesis [1–3]. This is illustrated by the pronounced anabolic response of skeletal muscle to the repeated impact of resistance exercise [4]. Resistance training (RT) can be carried out with different modalities of contraction, such as concentric (CON), eccentric (ECC) or isometric. The impact of different mechanical stimuli on skeletal muscle varies considerably, affecting the deposition of sarcomeres in parallel and in series, and thus muscle functional properties [5]. In this

regard, we have previously shown that CON vs. ECC RT (matched for relative maximum load and neural activation) produces similar hypertrophy of the vastus lateralis muscle through different architectural remodeling [6,7]. Whereas muscle growth with ECC RT exercise was largely reflected by an increase in the length of muscle fascicles, similar increase in muscle volume was reached with CON RT mainly through an increase in pennation angle. Although no differences in muscle protein synthesis was found after 4 weeks of ECC and CON RT [8], the molecular responses underlying muscle architectural remodeling involve a contraction-specific activation of the MAP Kinase family [7]. Yet, the possible link between structural remodeling and these molecular signaling pathways is still unclear [7,9–11].

Changes in muscle architecture reflect the longitudinal and radial growth of muscle fibers [6,12], brought by the incorporation of new sarcomeres, which are held in register by Z-disks. The integrin-based subsarcolemmal focal adhesion complexes, so called costameres, are integral part of the mechanism of sarcomerogenesis [13] because they provide an anchor for the cytoskeletal elements that hold Z-disk in register and ultimately controlling sarcomere assembly [14]. This is evidenced by changes in sarcomerogenesis with modulation of costamere components in cell cultures [15,16]. In human anti-gravity muscle, altered levels of costamere-associated proteins (FAK, vinculin isoforms, integrin) to be modulated by increased or reduced loading [17,18]. For gamma- and meta-vinculin, this response has been shown to differ between the first 4 weeks of an 8 weeks training program [18] and has been associated with level changes of slow and fast myosin heavy chain [18–20]. In this respect, the integrin-associated focal adhesion kinase (FAK) plays an important role as it organizes the turnover of focal adhesions and constitutes an upstream element of intracellular signal transduction with mechanical impact on integrin based adhesion receptor complexes [21]. In addition, the phosphorylation of Y397 within the N-terminal domain of FAK is functionally relevant because it liberates the kinase domain from a conformation-mediated inhibition [21].

In skeletal muscle, Y397 phosphorylation of FAK (pY397-FAK) usually increases within 15 - 30 minutes after RT [22]. The repeated impact of load-bearing stimuli on muscle has been found to further increase pY397-FAK in possible relation with an increase in FAK protein concentration [23–25]. This is instrumental for IGF-I-mediated growth of skeletal muscle cells, which, in rodents, has been shown to be associated with a positive regulation of protein synthesis and muscle hypertrophy [23–25]. It has been observed that not only pY397-FAK content is related to expressional regulation of costamere components by muscle loading [19], but also that alterations in FAK and pY397-FAK to

muscle loading are concomitant to changes in muscle mass and architecture, as supported by a correlation between FAK and ultrasound-derived muscle thickness [26].

Thus, the study hypothesis was that the concentration and Y397 phosphorylation of FAK in response to training would have differed between ECC and CON RT and between the mid-belly and distal regions, likely reflecting distinct contraction-specific structural adaptations.

We therefore investigated whether ECC or CON RT result in a larger content of pY397-FAK and of the costamere-associated proteins gamma and meta-vinculin.

It is of interest to highlight that sarcomere addition in rat muscle has been shown to be greater towards the fiber ends in response to stretch/lengthening loading [27,28]. In addition due to the intrinsic differences in muscle tension between ECC and CON contractions, and due to evidences for regional distribution of costamere components, whereby the highest levels have been observed towards the myotendinous junction [29], we also investigated whether regional alterations in pY397-FAK may be connected to the distinct architectural remodeling to 8 weeks of CON as opposed to ECC RT in humans.

Results

Muscle morphological adaptations– Fig 2 shows the changes in muscle mass (Thigh) and architecture (VL) in response to either ECC or CON RT. All data are presented as means ± S.D. Table 1 shows instead the baseline values for each leg of each volunteer (N.S. differences in any morphological parameter between legs at baseline). Muscle architecture features were assessed at 50% of the VL length (MID muscle portion).

As Fig 2 A shows, both ECC and CON training protocols resulted in a similar increase of thigh lean mass, at 4 and 8wks ($P<0.01$) time points: (ECC = 2.5±1.4%, $P<0.01$, vs. CON = 2.9±1.3%, $P<0.001$, after 4 weeks of RT; ECC = 4.1±2.3%, $P<0.001$, vs. CON = 3.9±2%, $P<0.001$, after 8 weeks).

Similarly, significant changes in VL muscle thickness (Fig 2 B) were observed at 4 and 8wks of both ECC and CON RT. After 4-weeks, MT increased by 6.8±4.6%, $P<0.01$, in the ECC leg and by 6.8±3.9%, $P<0.01$, in the CON leg; a further increase in MT was observed at 8-weeks (ECC = 11.1±5.5%, $P<0.001$, vs. CON = 11.8±4.7%, $P<0.001$, N.S. difference between loading types).

Lf (Fig 2 C) significantly increased in both CON and ECC legs, but the change was much greater after ECC RT compared to CON RT at 4 ($P<0.001$) and 8wks ($P<0.001$) time points (ECC = 4.9±1.9%, $P<0.001$, vs. CON = 1.9±1.4%, $P<0.05$, after 4 weeks of RT; ECC = 10.5±1.2%, $P<0.001$ vs. CON = 4±0.8%, $P<0.01$ after 8 weeks). Pennation angle (Fig 1 D) showed a significant increase after CON RT only, this being significantly different ($P<0.001$) to the almost negligible increase in PA brought by ECC RT at 4 and 8wks time points (ECC = 0.8±1%, $P>0.05$, vs. CON = 6.3±2.1%, $P<0.001$, after 4 weeks of RT; ECC = 2±1%, $P>0.05$, vs. CON = 12.3±3.8%, $P<0.001$, after 8 weeks).

Muscle function: MVC and training load– Table 2 presents the adaptations of muscle function and training load. Data are presented as means ± S.D. Isometric Maximum Voluntary Contraction (MVC) and training load increased in both ECC and CON legs at 4 and 8wks time points. Training load was always greater in ECC RT compared to CON RT resulting in ~1.4 times greater than CON load throughout the whole training period.

Muscle protein synthesis– Fig 3 shows the myofibrillar FSR values after ECC vs. CON RT and at the two distinct muscle sites (MID vs. Distal).

After 4 weeks of ECC or CON RT, myofibrillar FSR rates did not differ between loading types and muscle sites (ECC = 1.59±0.24%/day vs. CON = 1.52±0.21%/day at MID muscle site, ECC = 1.61±0.2%/day vs. CON = 1.58±0.2%/day at Distal muscle site). Myofibrillar FSR did not show any further increase at 8 weeks, with no differences between loading types and muscle sites (ECC = 1.21±0.19%/day vs. CON = 1.23±0.16%/day at the MID muscle site, ECC = 1.20±0.17%/day vs. CON = 1.31 ±0.14%/day at the Distal muscle site).

Activation status of FAK and its expression level after concentric and eccentric resistance training – Figure 4 shows the detected proteins in a comparison between ECC vs. CON RT at distinct muscle sites (MID and Distal portions).

Y397-FAK phosphorylation, 60 to 90 minutes after exercise, was not different at MID muscle portion between training modalities at any of the time points (Fig 4A).

Y397-FAK phosphorylation level at Distal muscle site was 4 times fold greater after ECC RT at 8 weeks time point compared to CON RT ($p<0.05$) (Fig 4B).

When muscle sites were compared within each contraction mode at both sites (i.e. MID vs. Distal comparison in either CON or ECC RT), no significant differences were reported for pY397-FAK.

FAK protein level per total protein (Fig 4C) after the first exercise bout showed a trend for CON RT at MID muscle site to be greater, but was not significantly different from the levels found after ECC RT ($p=0.12$). CON RT presented a successive decrease in FAK level at 4 and 8 weeks of training compared to week 0 time point. Nonetheless, a ~3.5-fold transient increase of FAK was observed at MID muscle portion after ECC RT only 4 weeks into the training period compared to the response after the first bout performed at week 0 ($P<0.05$). A successive return to 0-wks time point values was then found at 8 weeks time point ($p<0.05$ compared to 4 weeks level).

FAK protein level at Distal muscle site (Fig 4D) did not show any significant differences within and between ECC vs. CON groups at any of the time points.

When muscle sites were compared within the single intervention (i.e. MID vs. Distal comparison in either CON or ECC RT), FAK levels were found ~5-fold greater at 4 weeks after ECC RT in the MID portion compared to the Distal one ($P<0.05$).

The specific Y397-phosphorylation of FAK (i.e. the ratio of pY397FAK to total FAK) in *vastus lateralis* muscle was significanlty greater at the Distal site after ECC RT at 4 and 8 weeks time points. pY397FAK/FAK showed a ~2.2-fold difference ($P<0.05$) between ECC vs. CON RT at 4 weeks and a ~9-fold difference ($P<0.001$) at 8-weeks of RT, with a ~4.1-fold decrease observed after CON RT between 4 weeks and 8 weeks values.

When muscle sites were compared within the single intervention (i.e. MID vs. Distal comparison in either CON or ECC RT), pY397FAK/FAK levels were found ~2-fold greater ($P=0.05$) at 4 weeks after CON RT in the MID portion compared to the Distal one, whereas, at 4 weeks time point, after ECC RT, pY397FAK/FAK levels were ~4-fold greater in the Distal portion compared to the MID one ($P<0.01$).

Meta-vinculin, Gamma-vinculin, and fast myosin levels after concentric and eccentric resistance training – Fig 5 presents the levels of vinculin isoforms in a comparison between ECC vs. CON RT at distinct muscle sites (MID and Distal portions). Meta-vinculin presented a significant 2-fold increase ($P<0.05$) between 0 weeks and 4 weeks time points only at the distal site of vastus lateralis muscle. Gamma-vinculin did not present any significant changes by time and between loading modalities. Similarly, fast myosin, although showing a small trend to increase during exercise, did not show any significant changes (data are not shown in the manuscript, please refer to the supplemental material section).

Correlations– The significant linear relationships observed between FAK-related parameters and changes in muscle architecture are shown in Table 3. Distal muscle portion phosphorylation at the Y397 tyrosine site of focal adhesion kinase (pFAK) positively correlated with the delta of Lf between 8 and 0 weeks time points (i.e. 8weeks value – 0 weeks value, expressed in cm) ($r=0.67$, $p<0.05$), and with the % increase in Lf after 8weeks ($r=0.76$, $p<0.01$). Likewise, the specific activation of FAK (pFAK/FAK) presented a positive linear relationship with the delta of Lf between 8 and 0 weeks time points ($r=0.69$, $p<0.05$) and with the % increase in Lf after 8weeks ($r=0.66$, $p<0.05$). In addition, pFAK/FAK values were also found to be negatively correlated to the delta of PA between 8 and 0 weeks time points ($r=-0.65$, $p<0.05$).

The significant linear relationships found between meta-vinculin, gamma-vinculin, FAK-related parameters and fast myosin levels are presented in table 4. Meta-vinculin levels significantly correlated to MHCII at all the time points: however, at 4 weeks the linear relationship was much stronger ($r=0.90$, $p<0.0001$) compared to the one at week 0 ($r=0.53$, $p<0.05$). The correlation at 8 weeks remained significant ($r=0.74$, $p<0.001$). Meta-vinculin also correlated to the values of p-Y397FAK at 4 weeks ($r=0.40$, $p<0.01$) and 8 weeks ($r=0.53$ $p<0.01$), whereas no significant linear relationship was observed at week 0 ($r=0.23$, $p=0.26$).

Gamma-vinculin levels were observed to correlate to FAK content when all values at all time points were included in the calculation ($r=0.42$, $p<0.01$).

Discussion

Physiological changes of muscle mass are a result of mechano-regulated phenomena [35,36] that rely both on signaling and gene expression [37,38], leading to new synthesized proteins [4] and to the re-assembly of myofibrils [2]. Our data provide the novel information that post-exercise levels of pY397-FAK, FAK and its specific activation i.e., pY397-FAK/FAK, as well as meta-vinculin, are differently regulated by ECC and CON RT and that such processes are muscle region-specific. Similar MPS responses were found after ECC vs. CON RT and were consistent with similar changes in muscle mass and MT. MPS responses could not explain (at any of the time points) the distinct contraction-type dependent architectural remodeling observed. However, ECC RT resulted in greater activation of Y397-FAK and Y397-FAK/FAK at the distal VL portion compared to CON RT, a muscular site where serial sarcomere addition has been found to occur in animals [27]. Furthermore, a transient significant increase of meta-vinculin was observed at 4-weeks only after ECC RT and only at the distal muscle site. In the light of these findings and of that both levels pY397-FAK and pY397-FAK/FAK correlated with parameters of architectural remodeling, we propose that a mechano-regulated relationship between focal adhesion turnover and the pattern of muscle hypertrophy (longitudinal vs. radial growth) may exist.

VL morphological adaptations were in line with previous observations [6-8]: thigh lean mass showed further increase in both legs (i.e. after both loading protocols) at 8 weeks, however the changes were smaller in the second half of the training period (4-8 weeks) compared to the first 4 weeks (ECC RT +1.4% vs. CON RT 0.6%). Similar observations apply to MT changes (4-8 Weeks increase = ECC RT +4.4% vs. CON RT 4.1%). These results were expected, since previous studies have shown that, when properly matched for either work [39] or relative maximum load [7], both ECC and CON RT can lead to similar hypertrophy (for a review on this specific topic please see Franchi et al. 2017 [5]). The results in MPS (i.e., myofibrillar FSR) clearly support such contention; this is the first report of MPS responses over such duration (i.e., 0-8-weeks) to pure CON vs. pure ECC loading paradigms. Moreover, the observation that myofibrillar FSR resulted greater in the first 4 weeks of training is in line with recent work [31] and reflects the kinetics of the changes of muscle growth with morphological indexes (thigh lean mass and MT).

However, muscle architectural adaptations, in terms of Lf and PA, not only confirmed the contraction-specific pattern previously observed at 4 weeks time point but kept increasing with a similar rate in the following second half (4-8wks) of training period. This is interesting especially if

considering that, as presented above, after both loading types, MPS (at each muscle site) MT and Lean mass showed instead a slower rate of increase in the second half of the training period. These observations lead to few considerations: firstly, the MPS data confirmed the concept that RT-induced hypertrophy occurs rapidly, within the first days and weeks of a training protocol [8,32,33], subsequently slowing [31]. Secondly, considering that architectural adaptations of skeletal muscle may reflect an addition of sarcomeres either in series (i.e., longitudinal growth, preferentially occurring after ECC RT or in parallel (i.e. radial growth, preferentially occurring after CON RT) [7], the observation that changes in FSR were not region-specific suggests that the overall addition of sarcomeres, whether in series or in parallel, was uniform along the muscle length for both ECC and CON legs. This further indicates that distinct patterns of muscle remodeling cannot be fully explained by the quantity of newly synthesized proteins.

Our novel findings point to differences in the mechano-transductor FAK activation and meta-vinculin levels between ECC and CON RT. To the best of the authors' knowledge, this represents the first study on human muscle reporting different activation of FAK in response to acute bouts of lengthening vs. shortening (matched for relative maximum load) during a chronic application of such distinct mechanical stimuli over-time. Thus, the data of FAK activity of the present study are unprecedented in humans but are in agreement with previous observations (in muscle cells) on the role of mechanotransduction in regulating muscle growth [36]. Hornberger and colleagues [40] showed that C2C12 myotubes were able to respond differently to uniaxial compared to multiaxial stretch, with only the latter resulting in a significant activation of ribosomal S6 kinase: this supports the contention that different forces can trigger different mechano-sensitive pathways in muscle cells. FAK exerts a double role in mechano-regulation of skeletal muscle through its impact on turnover of integrin-based sarcolemmal adhesion sites, i.e. costameres [19,41] and the initiation of integrin-based signaling towards p70S6K-mediated protein synthesis [42,43].

Here we show that potentially, when different types of contractions (and thus distinct mechanical stimuli) are chronically provided, human skeletal muscle may be essentially able to sense and differentiate distinct mechanical forces applied overtime.

In humans, FAK and pY397-FAK levels have been shown to increase after chronic loading [18,44]. Our results show that the concentration of FAK at the distal muscle portion was not affected by concentric and/or eccentric training whereas FAK was transiently increased after ECC RT in the mid-

belly portion at 4 weeks. This finding supports previous observations of an increase in FAK protein concentration in humans VL muscle after downhill skiing [25]. Regarding FAK activation state, Gehlert and colleagues have previously shown that maximal ECC bout resulted in greater phosphorylation of Y397-FAK compared to a submaximal bout (75% of CON and ECC force) of conventional exercise (CON+ECC) matched for time under tension [22]. Thus, higher forces (i.e., and higher tension developed) appear to induce higher activation of this mechanosensitive pathway. These results are supported by the findings of Rahnert & Burkholder, which showed that the activation of FAK and p38 MAPK was related to the force-time integral, when modulated for the force compared to the total time of contraction [45]. In the present study, ECC and CON RT were matched for maximum relative load and neural drive [8], in order to provide the same relative intensity of training [6]. Nonetheless, because of the nature of lengthening contractions, ECC RT resulted in greater force developed during training than CON RT [46], consequent greater work and potentially, because of the higher mechanical stimulus provided, greater stress and strain applied onto the muscle fascicles. All these previous factors (i.e. higher tension and higher force-time integral) could explain the distinct activation of FAK between the loading paradigms.

Furthermore, the differences in levels of pY397-FAK and pY397-FAK /FAK (i.e. the specific ratio between activated and total FAK) appear to be region-specific in the VL, being most activated at the distal site. This is another unique finding that warrants further investigation. Previously, hypertrophy induced by eccentric loading in rodents has been shown to preferentially involve the addition of sarcomeres at the muscle ends [27,28,47]. Our results do not provide evidence for regional differences in myofibrillar FSR (p=0.629); however, activation of Y397-FAK was specifically enhanced by eccentric exercise at 8 weeks in the distal portion of VL. This is supported by Li and colleagues' findings, which shown an increase in pY397-FAK after 9 weeks of isoinertial training (i.e., with eccentric overload) [18]. Moreover, in the present study, the specific phosphorylation of Y397-FAK (pY397-FAK / total FAK) was greater after ECC RT compared to CON RT at 4 weeks and at 8 weeks time points in the distal portion of the muscle. As with other focal adhesion proteins, FAK has been shown to concentrate at the myotendinous junction of muscle fibers in laboratory animals [29,48]. This greater distal activation may relate to recent findings showing that sarcomeres elongation is not uniform across intact muscle, with the higher stretch occurring near the myotendineous junction [49]. In addition, the heterogeneity of collagen type composition of the human myotendineous junction [50] may have had an influence on the multiaxial forces acting through extra-cellular matrix at that particular site and during specific mechanical stimuli (ECC RT). Phosphorylation of Y397 site has been shown to release FAK into a confirmation, which favors activation of its kinase domain [21]. In addition, Y397FAK phosphorylation is a key signal event for costamerogenesis and focal adhesions turnover [18].

The proposed underlying mechanism may involve the role of focal adhesions as template for the assembly of sarcomeres [19] and/or the reinforcement of the integration between the sarcolemma and sarcomeres with fascicle length changes during contraction [51,52]. Furthermore, vinculin levels have been previously observed to be modulated by increased or reduced loading, in animal [19,20] and human muscle[18]. In the present study, meta-vinculin significantly increased only after ECC RT at 4 weeks and at the distal muscle site. Meta-vinculin changes during unloading have been previously associated to a shift of skeletal muscle to a fast phenotype [20]: This notion is supported by a strong linear relationship between meta-vinculin and fast myosin levels at all time points (although higher at 4 and 8 weeks compared to week 0 time point). Taking into account that meta-vinculin values also moderately correlated to pY397FAK levels at week 4 and week 8, we may be able to speculate also on a potential role of meta-vinculin involved in the addition/deposition of contractile material in human skeletal muscle. Thus, a potential role for FAK (and meta-vinculin) in the regulation of serial sarcomere addition towards the muscle ends in response to ECC RT is proposed.

This hypothesis is strengthened by the positive linear relationship found between both pY397-FAK and pY397-FAK / total FAK levels and fascicle length increase at 8weeks (Table 3.). Previous work from our lab reported a linear relationship between FAK content and increase in VL thickness after 12 weeks of downhill ski training [26]; the results from the present study further highlight the potential connection between contraction-dependent remodeling and mechano-sensitive pathways.

Higher activation of MAP Kinases family has been observed preferentially after lengthening contractions in rats [9] in terms of ERK 1/2 and p38 MAPK. This activation was shown to be proportionally related to tension [10,53] and to the force-time integral [45] together, in the case of the latter study, with FAK phosphorylation. ERK are rapidly activated following mechanical stimuli [32], as p38 [54,55], which phosphorylation is promoted by integrin signaling [56]; mechanical stretch in cardiac myocytes has been shown to modulate these MAPKs through both an independent and dependent FAK-mediated regulation [57]. Interestingly, ERK 1/2 expression/knockout in cardiac muscle have been shown to influence the direction of cardiac cells growth, modulating the increasing of length (ECC hypertrophy) vs. thickness (CON hypertrophy) of cardiac myocytes [11]. Because of previous results from our lab, which confirmed the findings of MAPKs being highly phosphorylated after lengthening contractions in humans [7], and the data of present study, we could hypothesize that both FAK and MAP kinases signaling pathways may play a role in the differentiation of structural remodeling of human skeletal muscle.

A number of limitations apply to our investigation. First, our findings derive from a small number of subjects. Moreover, we adopted a unilateral training desing, in which each leg was randomly assigned to a contraction type, thus one leg trained just concentrically (CON) while the contralateral performed eccentric (ECC) training only".

Nonetheless, while acknowledging the possibility of transfer effects on muscle strength adaptations to both training modalities, it must be said that the unilateral within subject design, is a well-established modality in RT and has been adopted by several studies either in the knee extensors [58–62] or in the elbow flexors [39,63]. The advantages of such a design is the minimising of variability in the training responses within groups [39,64], and that the hypertrophic responses to RET are the result of localised muscular adaptations [63].

In relation to the number of biopsies being taken, we chose not to collect a baseline biopsy to reduce the burden. This was also justified because FAK concentration in skeletal muscle is unchanged in the first hours after a muscle stimulus [19,22]. As well we identify that ultrasound snapshots were acquired only at VL mid length (50%) whilst no scans were performed at the distal myotendinous muscle portion. However, the distal biopsy site was identified by ultrasound at 4 cm above the actual myotendinous junction; because a 10cm probe was used for the snapshots acquired for muscle architecture analyses, we can conclude that portions of fascicle visualized in such greater field of view terminated in the distal site of VL. Thus, the changes in fascicle length presented could be representative of both bioptic sites. Lastly, it may be argued that a single point of measurement 60-90 post-exercise may under-estimate the possible maximal pY397 phosphorylation of FAK. Previous investigations have shown that changes in level of FAK are not manifested at 1hr after exercise [19] and that changes in level of p-Y397FAK are instead detectable from already 20 seconds after stretch [19] and are clearly visible 1hr after loading [42], even up to 6hrs after loading [42]. However, the clear differences between muscle sites and contraction types presented consistent trends over the training period.

Conclusion –

Summarizing, we reported that the specific activation of FAK at Y397 tyrosine site resulted to be greater after ECC RT at 4weeks and 8 weeks compared to CON RT. Such observations occurred specifically at the distal muscle portion, a site that is usually preferential for sarcomeres addition in longitudinal muscle growth. Similar muscle hypertrophy was reached in response to 8-weeks of ECC vs. CON RT, but through distinct architectural remodeling, and similar MPS was found overtime

between the two loading modalities. In addition, no differences were found in MPS between mid and distal muscle sites. The findings both on exercise-induced changes in the proxy of focal adhesion signaling, pY397-FAK, and on muscle remodeling, emphasize a potential role of focal adhesion-modulation in orienting the direction of fiber growth in response to a specific increase in muscle loading.

Materials and Methods

Design – Six young, healthy recreationally active males (age, 24 ± 1 year old; height, 183 ± 8.2 cm; weight, 81.1+ 12 kg; BMI, 24 + 2.7 Kg/m^2) were recruited from different University of Nottingham sites and entered an 8-weeks program of CON vs. ECC RT. Each exercise bout was carried out in the fasted state. Muscle anatomy and function was assessed prior to exercise before training, at 4 weeks and 8 weeks of a RT program (8 weeks representing the end of training period). At these time points, muscle biopsies were collected from the VL muscle 60 to 90 minutes after a single exercise session. Biopsies (30-40mg) were used for the analysis of the fractional synthetic rates (FSR) of myofibrillar protein, using the deuterium-oxide (D_2O) tracer technique and ~10-20mg tissue underwent analysis of FAK-related parameters. Volunteers were clinically screened by means of a medical questionnaire, to exclude sufferers of joint disease and metabolic, respiratory, or cardiovascular impairments. Written informed consent was obtained from every participant. The study was approved by The University of Nottingham Ethics Committee and was performed in accordance with the Helsinki Declaration.

Parts of anatomical, functional and protein synthetic responses after only 4 weeks of training have been published previously [8]. This manuscript presents data subsets (n=6) of anatomical, functional and protein synthesis adaptations (0-4wk) with an additional time point (8-wk, previously unpublished), whereas all the FAK, meta and gamma-vinculin and fast myosin analyses have not been previously published.

Resistance training - Training was performed on customized a leg-press machine (Technogym, Gambettola Italy) specifically adapted to perform either CON or ECC contractions, as described in three previous studies from our lab [6-8]. Volunteers trained both legs but in a unilateral manner: each leg was randomly assigned to a contraction type, thus one leg trained just concentrically (CON) while the contralateral performed eccentric (ECC) training only [8]. Each exercise bout consisted of 4 sets of

8-10 contractions at 80% load of the 1-repetition maximum (1RM) for each contraction type (i.e., one leg training concentrically at 80% of the concentric 1RM and the contralateral training at 80% of the eccentric 1RM).

Each contraction was performed over of the duration of 2-3 seconds (i.e. ~2 seconds for concentric actions and ~3 seconds for the eccentric ones) and 1 min of rest was allowed between sets. The exercise was repeated 3-times per week over 8 weeks. Training load was monitored and progressively increased ~ every 10 days accordingly to the change in ECC or CON 1RM.

Muscle function – Maximum voluntary isometric knee extensor torque was assessed unilaterally via isokinetic dynamometry (Isocom, Isokinetic Technologies, Eurokinetics, UK). Volunteers performed isometric contractions in a randomised fashion at different knee joint angles: 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 centre of rotation was aligned with the dynamometer fulcrum. Contractions lasted 4 s, with a rest period of 30 s between contractions, and 90 s between knee joint angle changes. Maximum isometric torque was assessed at 3 different time points (0, 4-wks and 8-Wks).

Muscle Architecture- Muscle architecture was determined at rest in supine position corresponding to full knee extension using B-mode ultrasonography (Mylab 70, Esaote Biomedica, Genova, Italy), with a 100 mm, 10–15 MHz, linear array probe as previously described [8]. Briefly, images were acquired from the middle of VL length, with the mid-point of the probe placed longitudinally exactly at 50% of VL length and on the mid-sagittal line of the muscle. The transducer was then aligned in the fascicle plane to capture an optimal portion of fascicles [6]. Images were collected and digitally analysed by the same un-blinded operator. 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 [30], were performed by using ImageJ 1.42q software (National Institutes of Health, USA). The visible portion of the fascicle length was directly assessed, then, when fascicles extended off the ultrasound window, an estimation of the non-visible part was performed using a linear extrapolation of fibres and aponeuroses [7].

In addition thigh lean mass was measured by Dual absorption X-Ray absorptiometry, determined from the lowest point of the ischium to knee space as previously described [31].

Muscle Biopsies – Biopsies (~100mg tissue) were collected with conchotome forceps from two distinct regions of vastus lateralis muscle (from a "DISTAL" site, close to the myotendinous junction and from a "MID" at the 50% of muscle length), washed in ice-cold saline solution, then frozen in liquid nitrogen and stored in air-tight cryotubes at -80°C. The biopsies collected at the myotendineous junction (MTJ) were ultrasound guided, in order to avoid the collection of any tendineous tissue. Basal biopsies were taken on the midsagittal line of the muscle in the mid-belly (MID) and at 40 mm from the myotendinous junction (DISTAL), at 50% of the distance between lateral and medial borders of vastus lateralis muscle. Subsequent biopsies at week 4 were performed 30-mm posterior to the first basal distal biopsy and 30-mm proximal to the basal mid-belly biopsy site, while biopsies at week 8 were performed 30-mm anterior the first basal distal biopsy and 30-mm distal the first basal mid-belly biopsy site. All biopsies were performed under local anaesthesia (1% Lignocaine, B. Braun Melsungen, Germany) using an aseptic technique. Ultrasound scanning with Phillips iU22 and 40 mm Phillips L9-6 linear array transducer (Phillips Healtcare, Reigate, United Kingdom) permitted determination of MTJ and muscle boundaries. Smaller muscle CSA in the DISTAL region compared to the MID muscle portion necessitated US guidance throughout infiltration of local anaesthesia and execution of conchotomy. Biopsies were collected at 0, 4 and 8-Wks time points, in a window between 60 and 90 minutes after the single exercise bout.

Deuterium oxide tracing technique: body water enrichment and deuterated alanine incorporation into myofibrillar protein-

These procedures have been thoroughly described for a recent previous study from our lab [8]. Briefly, immediately post biopsy on day 0, participants provided a saliva sample and then ingested a single 150 ml oral bolus of D_2O (70 Atoms%, Sigma Aldrich, Poole, UK), labelling the body water pool to ~0.2%. We monitor body water enrichment throughout the study by asking each participant 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). Body water enrichment was assessed by direct liquid injection of the 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) (as explained in a previous study [32]. The myofibrillar fraction was isolated from ~30-40 mg of muscle using our standard approach [32,33]. 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) [31,32].

Fractional synthesis rate of myofibrillar protein– The fractional synthetic rate (FSR) of myofibrillar (MyoPS) protein synthesis was determined (as previously established [32] and reported in detail for this population [8]) from the incorporation of deuterium labelled 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 (i.e. between 0-4 weeks and 0-8 weeks RT).

In brief, the standard equation:

$$FSR\ (\%\ d^{-1}) = -Ln[-1[(APE_{Ala})/(APE_{P})]/\ t]$$

This equation is where, APE_{Ala} = deuterium enrichment of protein bound alanine, APE_{P} = precursor enrichment and *t* represents the duration of time between biopsies, as described by Brook and colleagues [27, 29].

Protein biochemistry – FAK, vinculin isoforms and fast type myosin levels per total protein was estimated by immunodetection of total protein in homogenate as described [19]. The levels of Y397-phosphorylation of FAK (i.e. pY397-FAK) per total protein, and specific Y397-phosphorylation of FAK (i.e. Y397-FAK per FAK), were estimated using immunoprecipitation as described [19]. In a total 72 samples were assessed arising from 2 interventions (concentric, eccentric), 3 time points (0, 4 and 8 weeks) and 2 locations (mid-belly biopsy and the biopsy collected close to distal myotendinous junction) (n= 6 subjects). The 12 sample points for one subject were analyzed in one assay and expressed respective to a control sample to reduce variability between blots. For the detection of costamere proteins 20 micrograms of total protein was loaded per lane, except for one sample, where 10 micrograms were loaded due to insufficient protein amount. For the detection of FAK phosphorylation, soluble protein of 1 mg protein homogenate was incubated with pY397-FAK antibody followed by protein A-sepharose. The resulting immunoprecipitate was subjected to

separation by SDS-PAGE immunoblotting with FAK antibody. Protein levels were expressed per total protein being subjected to the immunoprecipitation. Specific Y397-phosphorylation of FAK was calculated as the ratio between pY397-FAK levels and total FAK levels. FAK-pY397 and total FAK were detected using a home-made polyclonal rabbit a- FAK serum (1:1,000) as described previously [34], gamma- and meta-vinculin were detected using a mouse a-vinculin serum (gift of Dr. M. A. Glukhova, Paris, France; 1:100), whereas fast myosin was detected using a mouse a-skeletal myosin (fast) (M4276, Sigma–Aldrich), as previously described [18]. Then, the Goat a-mouse antibody #A9917 (Sigma–Aldrich) secondary antibody (1:20000) was applied on the respective membranes.

Statistics – Data throughout the manuscript are presented as means ± S.D. Statistical analyses of training-induced changes were carried out with Graph Pad Prism. A two-way ANOVA was performed between 0, 4 weeks and 8 weeks time points. Effects were localized with the post-hoc test of Bonferroni. Effects were determined as significant at a p-value set at p=<0.05. Linear relationships were tested by using the Pearson's product moment correlation coefficient (*r*). The level of significance was set at $p < 0.05$.

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Authors' Contribution

MVF, PJA, KS, MVN: *Conceived the study design*; MVF, MVN, MF: *Conceived the FAK and vinculin-related experiments*; KWM, MVF: *performed the muscle biopsy collection*; SR, MVF, MF: *performed the data acquisition, analysis and interpretation*; MVF, MF: *drafted the manuscript*; MVF, SR, PV *prepared the manuscript figures*; MVF, KS, MF *edited the manuscript*; MVF, PV, MF: *performed additional analyses and revisions*.

Conflict of Interest

I and we authors do not have any conflict of interest to declare.

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Table 1: Subject characteristics and muscle morphology: Values represent mean + SD.

Age (years)	24.1±5.8
Height (cm)	183.1±8.2
Weight (Kg)	81.1±12.1
BMI (Kg/m^2)	24.1±2.7

		Baseline
Thigh lean mass [g]	***CON leg:***	5980.3 ± 1279.9
	ECC leg:	5994.7 ± 1281.4
	p-value CON vs. ECC	*0.676*
VL thickness [cm]	***CON leg:***	2.56 ± 0.15
	ECC leg:	2.50 ± 0.18
	p-value CON vs. ECC	*0.541*
Fascicle length [cm]	***CON leg:***	8.84 ± 1.53
	ECC leg:	8.90 ± 1.52
	p-value CON vs. ECC	*0.81*
Pennation angle [°]	***CON leg:***	17.2 ± 2.2
	ECC leg:	17.4 ± 1.1
	p-value CON vs. ECC	*0.953*

Table 2: MVC and Training Loads: Values represent mean + SD.

		Baseline	***4 Weeks***	***8 Weeks***
Torque [Nm]	***CON leg:***	261.8 ± 49.1	288.3 ± 44.3	296.7 ± 52.0
	p-value vs. 0w		*<0.01*	*<0.05*
	ECC leg:	261.3 ± 59.2	283.2 ± 53	308.4 ± 62.2
	p-value vs. 0w		*<0.01*	*<0.01*
	p-value CON vs. ECC	*0.987*	*0.86*	*0.569*
Training loads [kg]	***CON leg:***	158.3 ± 33.6	181.8± 34.6	197.5 ± 32.2
	p-value vs. 0w		*<0.001*	*<0.001*
	ECC leg:	218.3 ± 46.7	250.9± 45.7	275.8 ± 47.9
	p-value vs. 0w		*<0.001*	*<0.001*
	p-value CON vs. ECC	*<0.001*	*<0.001*	*<0.001*

Table 3: ***Pearsons' correlations between FAK-related parameters and changes in muscle architecture in response to ECC and CON RT***

FAK-parameters	*Muscle Architecture*	*r value*	*p value*
p-Y397FAK-8wks	delta Lf 8-0Wks	0.67	<0.05
p-Y397FAK-8wks	Lf % increase 8-0Wks	0.76	<0.01
p-Y397FAK/FAK-8wks	delta Lf 8-0Wks	0.69	<0.05
p-Y397FAK/FAK-8wks	Lf % increase 8-0Wks	0.66	<0.05
p-Y397FAK/FAK-8wks	delta PA 8-0Wks	-0.65	<0.05

Table 4: ***Pearsons' correlations (interrelations) between FAK-related parameters, Gamma-Vinculin, Meta-Vinculin and MHCII content in response to ECC and CON RT***

Parameter 1	*Parameter 2*	*r value*	*p value*
Meta-Vinculin-0wks	MHCII-0wks	0.53	<0.05
Meta-Vinculin-4wks	MHCII-4wks	0.90	<0.0001
Meta-Vinculin-8wks	MHCII-8wks	0.74	<0.001
Meta-Vinculin-0wks	p-Y397FAK-0wks	0.23	>0.05
Meta-Vinculin-4wks	p-Y397FAK-4wks	0.40	<0.01
Meta-Vinculin-8wks	p-Y397FAK-8wks	0.56	<0.01
Meta-Vinculin-8wks	p-Y397FAK/FAK-8wks	0.52	<0.01
Gamma-Vinculin-0,4,8wks	FAK-0,4,8wks	0.42	<0.01

Figures

Fig 1. ***Study Design Scheme***.

Fig 2. ***Changes in muscle mass and architecture***. Post/Pre value ratios of A) thigh lean mass, B) muscle thickness, C) fascicle length and D) pennation angle after 4weeks and 8 weeks of ECC and CON RT. Y=1 represent the baseline values. Values are presented as means±S.D.

Fig 3. ***Changes in muscle protein synthesis***. Myofibrillar protein synthesis rates for ECC and CON legs over 4 weeks and 8 weeks training period calculated from muscle biopsies collected at MID and DISTAL muscle sites of VL. Values are presented as means±S.D.

Fig 4. ***Focal adhesion kinase activity and content after ECC vs. CON single session over the RT period***. A) FAK phosphorylation at the Y397 tyrosine site, FAK total content and B) their ratio (pFAK/FAK) at MID and DISTAL sites of VL muscle at 0weeks, 4 weeks and 8weeks time points. Values are presented as means±S.D. The respective representative blots are shown under the related graphs. The vertical black lines in the p-Y397FAK blot images indicate that the successive series of 3 bands was obtained from a different subject.

Fig 5. ***Meta-vinculin and gamma-vinculin content after ECC vs. CON single session over the RT period***. Meta-and gamma-vinculin at MID and DISTAL sites of VL muscle at 0weeks, 4 weeks and 8weeks time points. Values are presented as means±S.D. The respective representative blots are shown under the related graphs.