

ORIGINAL ARTICLE

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

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Abstract

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

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 mid-belly (MID) and distal (distal) sites at 0, 4, 8 weeks. Focal adhesion kinase 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 ($P < .05$); pY397FAK to total FAK ratio was greater in ECC vs CON at 4 (~2.2-fold, $P < .05$) and 8 weeks (~9-fold, $P < .001$) 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 8 weeks ($r = .76$, $P < .01$ and $r = .66$, $P < .05$ respectively).

Conclusion: Focal adhesion kinase phosphorylation was greater at 8 weeks after ECC RT and was muscle region-specific. FAK activity correlated to contraction-dependent architectural remodelling, suggesting a potential role of FAK in orienting muscle structural changes in response to distinct mechanical stimuli.

KEYWORDS

eccentric contractions, focal adhesion kinase, mechanotransduction, muscle architecture, muscle remodelling

1 | 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 remodelling.^{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 were found after 4 weeks of ECC and CON RT,⁸ the molecular responses underlying muscle architectural remodelling involve a contraction-specific activation of the MAP Kinase family.⁷ Yet, the possible link between structural remodelling and these molecular signalling pathways is still unclear.^{7,9–11}

Changes in muscle architecture reflect the longitudinal and radial growth of muscle fibres,^{6,12} brought by the incorporation of new sarcomeres, which are held in register by Z-discs. The integrin-based subsarcolemmal focal adhesion complexes, so-called costameres, are integral part of the mechanism of sarcomerogenesis¹³ because they provide an anchor for the cytoskeletal elements that hold Z-disc 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-week training programme¹⁸ 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, have 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 results 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 fibre 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 remodelling to 8 weeks of CON as opposed to ECC RT in humans.

2 | RESULTS

2.1 | Muscle morphological adaptations

Figure 1 shows the changes in muscle mass (Thigh) and architecture (VL) in response to either ECC or CON RT. All data are presented as means \pm SD. 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 Figure 1A shows, both ECC and CON training protocols resulted in a similar increase of thigh lean mass, at 4- and 8-weeks ($P < .01$) time points: (ECC = $2.5 \pm 1.4\%$, $P < .01$, vs CON = $2.9 \pm 1.3\%$, $P < .001$, after 4 weeks of RT; ECC = $4.1 \pm 2.3\%$, $P < .001$, vs CON = $3.9 \pm 2\%$, $P < .001$, after 8 weeks).

Similarly, significant changes in VL muscle thickness (Figure 1B) were observed at 4 and 8 weeks of both ECC and CON RT. After 4 weeks, MT increased by $6.8 \pm 4.6\%$, $P < .01$, in the ECC leg and by $6.8 \pm 3.9\%$, $P < .01$, in the CON leg; a further increase in MT was observed at 8 weeks (ECC = $11.1 \pm 5.5\%$, $P < .001$, vs

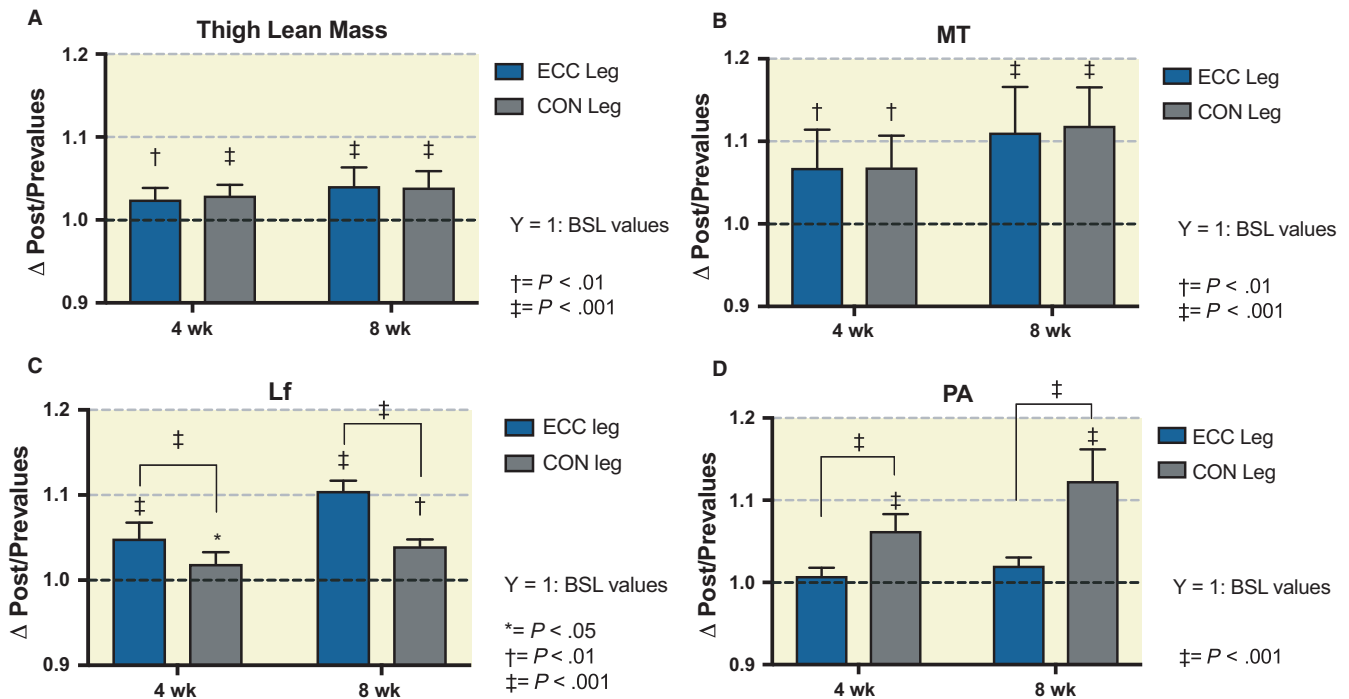


FIGURE 1 Changes in muscle mass and architecture. Post/Prevalue ratios of (A) thigh lean mass, (B) muscle thickness, (C) fascicle length and (D) pennation angle after 4 weeks and 8 weeks of ECC and CON RT. Y = 1 represent the baseline values. Values are presented as means \pm SD

TABLE 1 Subject characteristics and muscle morphology: values represent mean \pm SD

Age (years)	24.1 \pm 5.8
Height (cm)	183.1 \pm 8.2
Weight (Kg)	81.1 \pm 12.1
BMI (Kg/m ²)	24.1 \pm 2.7
Baseline	
Thigh lean mass (g)	
CON leg	5980.3 \pm 1279.9
ECC leg	5994.7 \pm 1281.4
P-value CON vs ECC	.676
VL thickness (cm)	
CON leg	2.56 \pm 0.15
ECC leg	2.50 \pm 0.18
P-value CON vs ECC	.541
Fascicle length (cm)	
CON leg	8.84 \pm 1.53
ECC leg	8.90 \pm 1.52
P-value CON vs ECC	.81
Pennation angle (°)	
CON leg	17.2 \pm 2.2
ECC leg	17.4 \pm 1.1
P-value CON vs ECC	.953

CON = 11.8 \pm 4.7%, $P < .001$, N.S. difference between loading types).

Fascicle length (Figure 1C) significantly increased in both CON and ECC legs, but the change was much greater after ECC RT compared with CON RT at 4- ($P < .001$) and 8-weeks ($P < .001$) time points (ECC = 4.9 \pm 1.9%, $P < .001$, vs CON = 1.9 \pm 1.4%, $P < .05$, after 4 weeks of RT; ECC = 10.5 \pm 1.2%, $P < .001$ vs CON = 4 \pm 0.8%, $P < .01$ after 8 weeks). Pennation angle (Figure 2D) showed a significant increase after CON RT only, this being significantly different ($P < .001$) to the almost negligible increase in PA brought by ECC RT at 4- and 8-weeks time points (ECC = 0.8 \pm 1%, $P > 0.05$, vs CON = 6.3 \pm 2.1%, $P < .001$, after 4 weeks of RT; ECC = 2 \pm 1%, $P > 0.05$, vs CON = 12.3 \pm 3.8%, $P < .001$, after 8 weeks).

2.2 | Muscle function: MVC and training load

Table 2 presents the adaptations of muscle function and training load. Data are presented as means \pm SD. Isometric maximum voluntary contraction (MVC) and training load increased in both ECC and CON legs at 4- and 8-weeks time points. Training load was always greater in ECC RT compared with CON RT resulting in \sim 1.4 times greater than CON load throughout the whole training period.

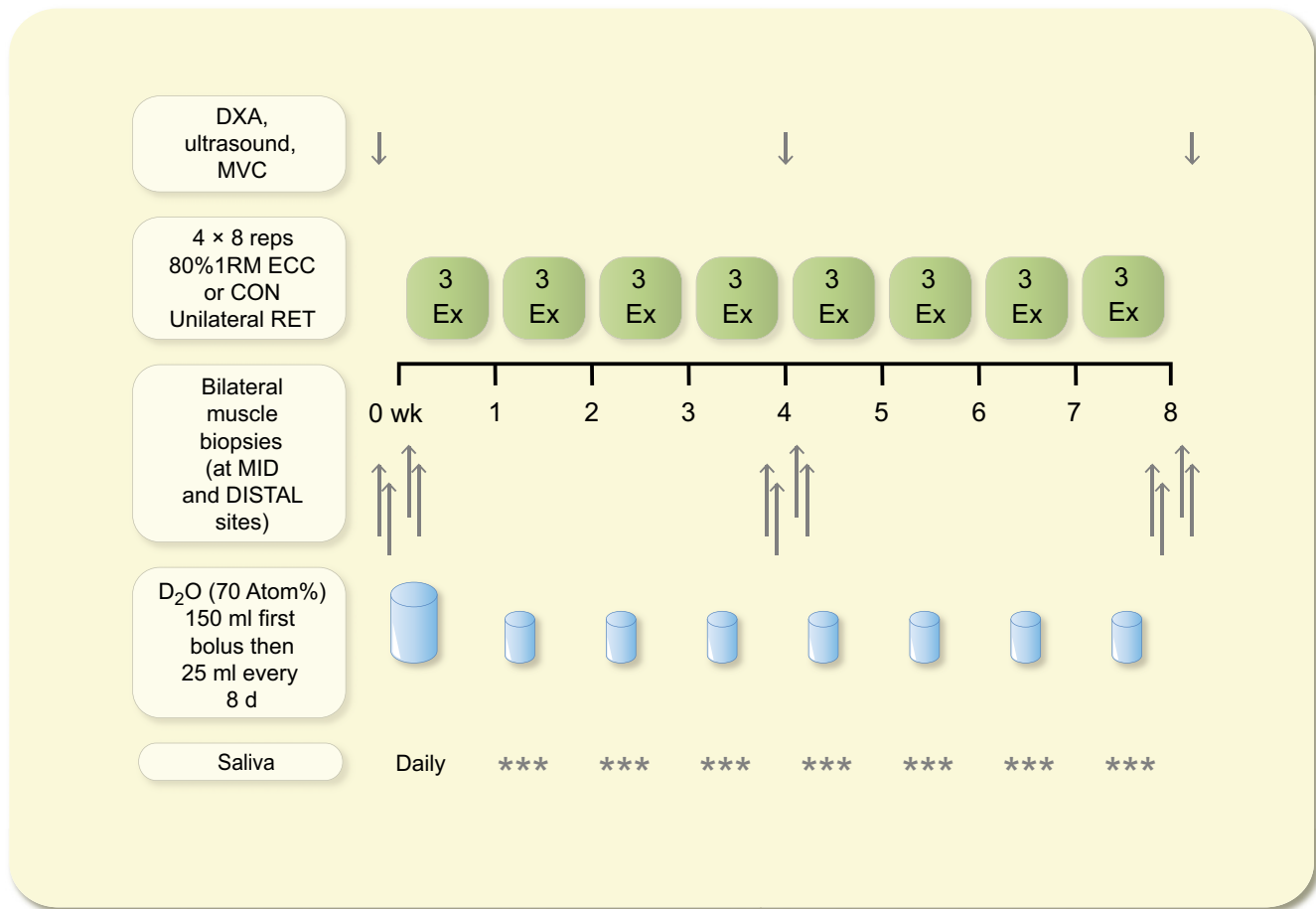


FIGURE 2 Study Design Scheme

2.3 | Muscle protein synthesis

Figure 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 = $1.59 \pm 0.24\%/day$ vs CON = $1.52 \pm 0.21\%/day$ at MID muscle site, ECC = $1.61 \pm 0.2\%/day$ vs CON = $1.58 \pm 0.2\%/day$ 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 = $1.21 \pm 0.19\%/day$ vs CON = $1.23 \pm 0.16\%/day$ at the MID muscle site, ECC = $1.20 \pm 0.17\%/day$ vs CON = $1.31 \pm 0.14\%/day$ at the distal muscle site).

2.4 | 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).

TABLE 2 Maximum voluntary contraction and training loads: values represent mean + SD

	Baseline	4 Wks	8 Wks
Torque (Nm)			
CON leg	261.8 ± 49.1	288.3 ± 44.3	296.7 ± 52.0
<i>P</i> -value vs 0 wk		<.01	<.05
ECC leg	261.3 ± 59.2	283.2 ± 53	308.4 ± 62.2
<i>P</i> -value vs 0 wk		<.01	<.01
<i>P</i> -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
<i>P</i> -value vs 0 wk		<.001	<.001
ECC leg	218.3 ± 46.7	250.9 ± 45.7	275.8 ± 47.9
<i>P</i> -value vs 0 wk		<.001	<.001
<i>P</i> -value CON vs ECC	<.001	<.001	<.001

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

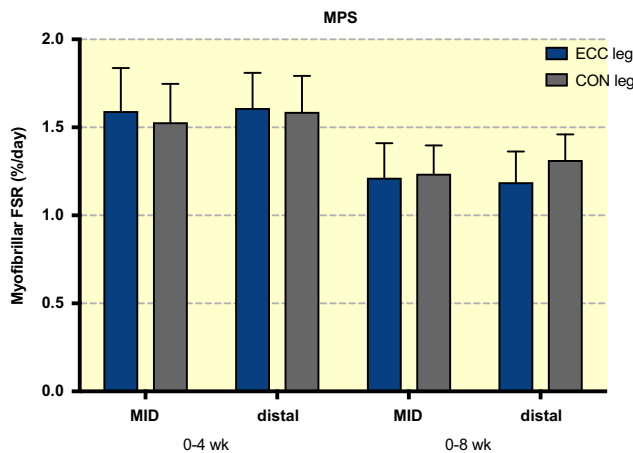


FIGURE 3 Changes in muscle protein synthesis. Myofibrillar protein synthesis rates for ECC and CON legs over 4 weeks and 8 weeks training period calculated from muscle biopsies collected at MID and DISTAL muscle sites of VL. Values are presented as means \pm SD

Y397-FAK phosphorylation level at distal muscle site was 4 times greater after ECC RT at 8-week time point compared with CON RT ($P < .05$) (Figure 4A).

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

Focal adhesion kinase protein level per total protein (Figure 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 ($P = .12$). CON RT presented a successive decrease in FAK level at 4 and 8 weeks of training compared with 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 with the response after the first bout performed at week 0 ($P < .05$). A successive return to 0-week time point values was then found at 8-week time point ($P < .05$ compared to 4-week level).

Focal adhesion kinase protein level at distal muscle site (Figure 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 (ie, 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 with the distal one ($P < .05$).

The specific Y397-phosphorylation of FAK (ie, the ratio of pY397FAK to total FAK) in *vastus lateralis* muscle was significantly greater at the distal site after ECC RT at 4- and 8-week time points. pY397FAK/FAK showed a ~ 2.2 -

fold difference ($P < .05$) between ECC vs CON RT at 4 weeks and a ~ 9 -fold difference ($P < .001$) at 8 weeks of RT, with a ~ 4.1 -fold decrease observed after CON RT between 4-week and 8-week values.

When muscle sites were compared within the single intervention (ie, MID vs distal comparison in either CON or ECC RT), pY397FAK/FAK levels were found ~ 2 -fold greater ($P = .05$) at 4 weeks after CON RT in the MID portion compared with the distal one, whereas, at 4-week time point, after ECC RT, pY397FAK/FAK levels were ~ 4 -fold greater in the distal portion compared with the MID one ($P < .01$).

2.5 | Meta-vinculin, Gamma-vinculin and fast myosin levels after concentric and eccentric resistance training

Figure 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 twofold increase ($P < .05$) between 0-week and 4-week 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).

2.6 | 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 fascicle length (Lf) between 8- and 0-week time points (ie, 8-week value—0-week value, expressed in cm) ($r = .67$, $P < .05$), and with the % increase in Lf after 8 weeks ($r = .76$, $P < .01$). Likewise, the specific activation of FAK (pFAK/FAK) presented a positive linear relationship with the delta of Lf between 8- and 0-week time points ($r = .69$, $P < .05$) and with the % increase in Lf after 8 weeks ($r = .66$, $P < .05$). In addition, pFAK/FAK values were also found to be negatively correlated with the delta of PA between 8- and 0-week time points ($r = -.65$, $P < .05$).

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 ($r = .90$, $P < .0001$) compared with the one

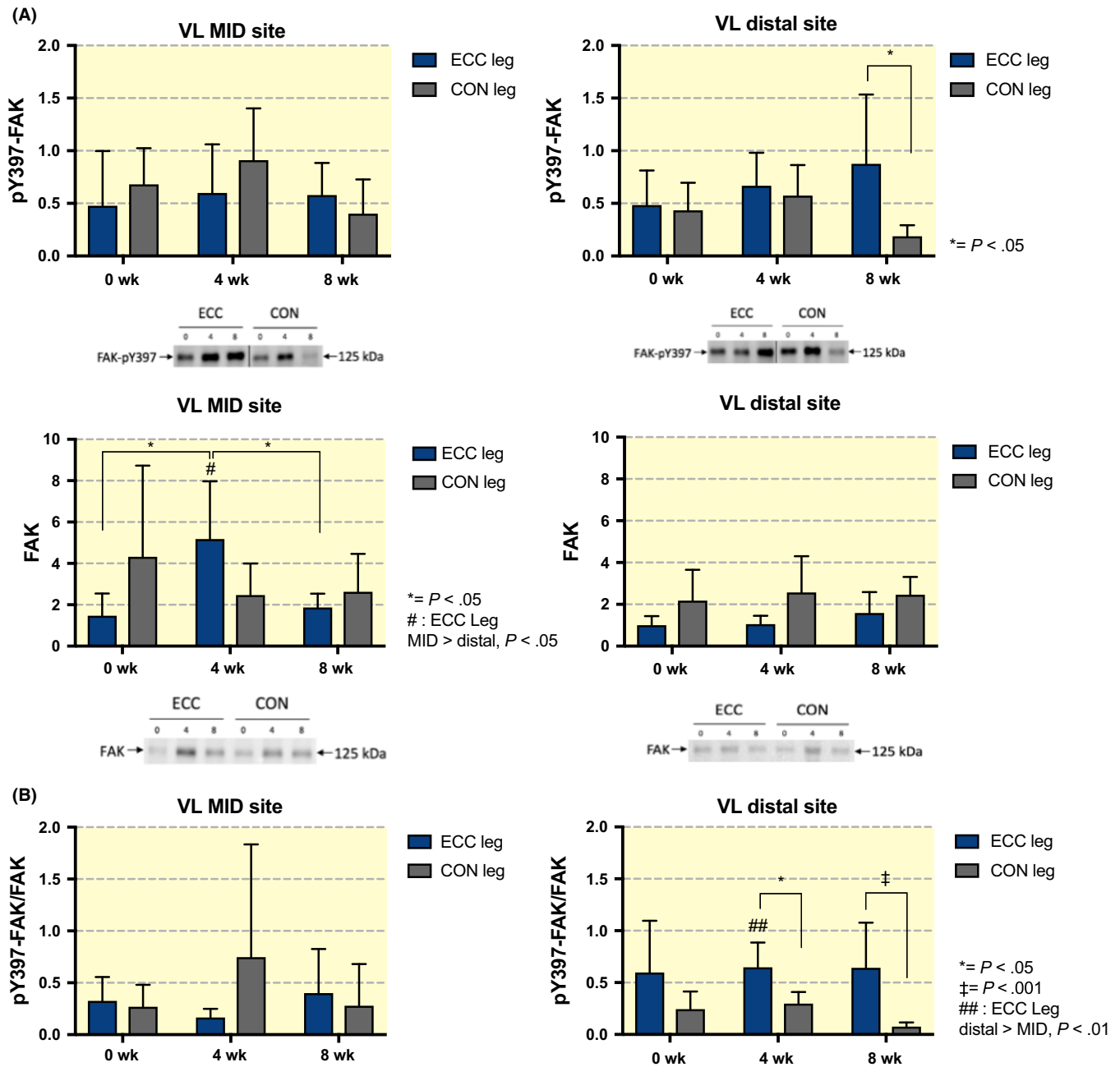


FIGURE 4 Focal adhesion kinase activity and content after ECC vs CON single session over the RT period. (A) FAK phosphorylation at the Y397 tyrosine site, FAK total content and (B) their ratio (pFAK/FAK) at MID and DISTAL sites of VL muscle at 0-week, 4-week and 8-week time points. Values are presented as means \pm SD. 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

at week 0 ($r = .53$, $P < .05$). The correlation at 8 weeks remained significant ($r = .74$, $P < .001$). Meta-vinculin also correlated to the values of p-Y397FAK at 4 weeks ($r = .40$, $P < .01$) and 8 weeks ($r = .53$, $P < .01$), whereas no significant linear relationship was observed at week 0 ($r = .23$, $P = .26$).

Gamma-vinculin levels were observed to correlate with FAK content when all values at all time points were included in the calculation ($r = .42$, $P < .01$).

3 | DISCUSSION

Physiological changes of muscle mass are a result of mechano-regulated phenomena^{30,31} that rely both on signalling and gene expression,^{32,33} leading to newly synthesized proteins⁴ 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, that is, pY397-FAK/FAK, as well as meta-vinculin, are differently

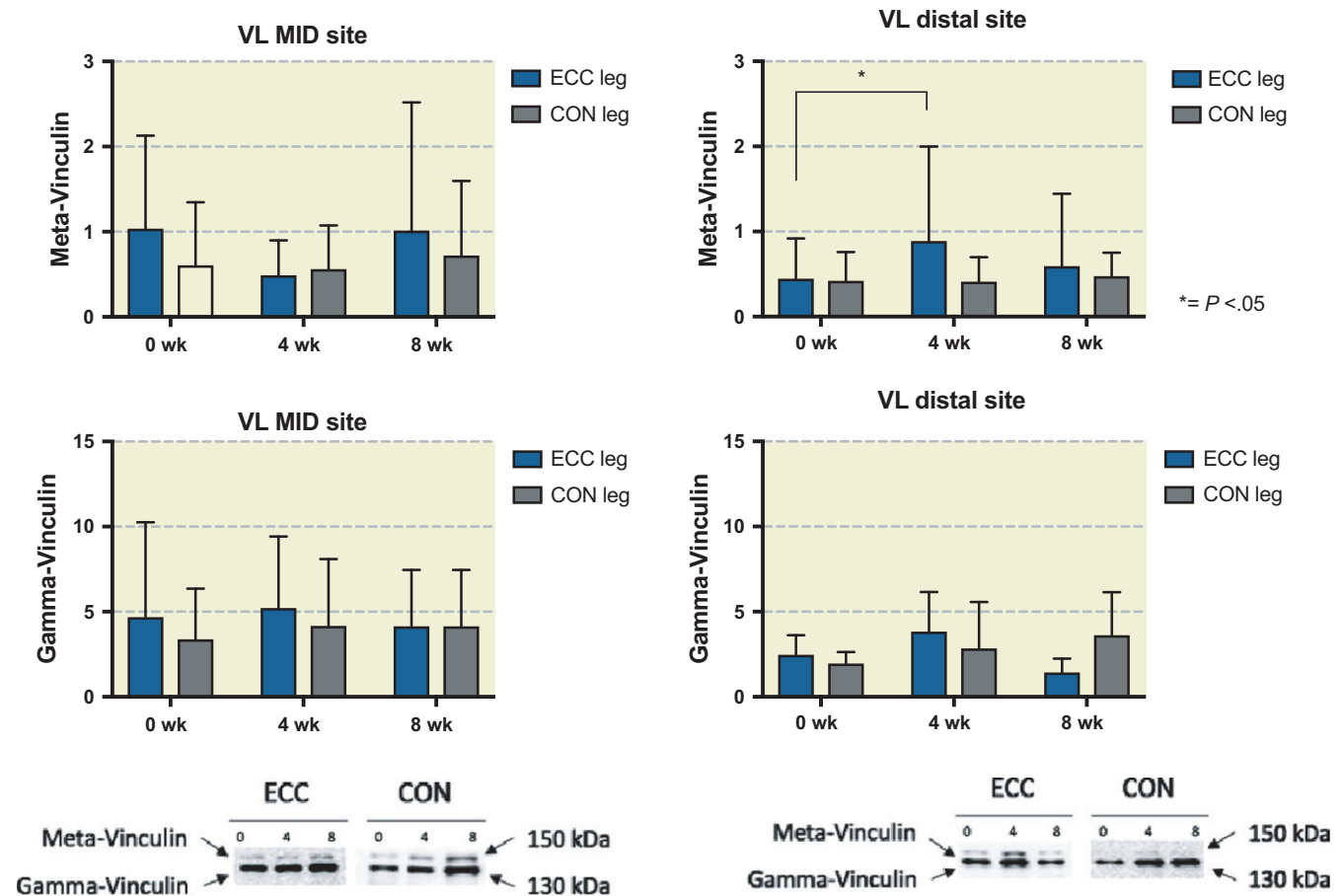


FIGURE 5 Meta-vinculin and gamma-vinculin content after ECC vs CON single session over the RT period. Meta- and gamma-vinculin at MID and DISTAL sites of VL muscle at 0-week, 4-week and 8-week time points. Values are presented as means \pm SD. The respective representative blots are shown under the related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

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

FAK-parameters	Muscle Architecture	<i>r</i> value	<i>P</i> value
p-Y397FAK-8 wks	delta Lf 8-0 wks	.67	<.05
p-Y397FAK-8 wks	Lf % increase 8-0 wks	.76	<.01
p-Y397FAK/FAK-8 wks	delta Lf 8-0Wks	.69	<.05
p-Y397FAK/FAK-8 wks	Lf % increase 8-0 wks	.66	<.05
p-Y397FAK/FAK-8 wks	delta PA 8-0 wks	-.65	<.05

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 remodelling observed. However, ECC RT resulted in greater activation of Y397-FAK and Y397-FAK/FAK at the distal VL portion compared with CON RT, a muscular site where serial sarcomere addition has been found to occur in animals.²⁷

TABLE 4 Pearson's correlations (interrelations) between FAK-related parameters, Gamma-Vinculin, Meta-Vinculin and MHCII content in response to ECC and CON RT

Parameter 1	Parameter 2	<i>r</i> value	<i>P</i> value
Meta-Vinculin-0 wks	MHCII-0 wks	.53	<.05
Meta-Vinculin-4 wks	MHCII-4 wks	.90	<.0001
Meta-Vinculin-8 wks	MHCII-8 wks	.74	<.001
Meta-Vinculin-0 wks	p-Y397FAK-0 wks	.23	>.05
Meta-Vinculin-4 wks	p-Y397FAK-4 wks	.40	<.01
Meta-Vinculin-8 wks	p-Y397FAK-8 wks	.56	<.01
Meta-Vinculin-8 wks	p-Y397FAK/FAK-8 wks	.52	<.01
Gamma-Vinculin-0,4,8 wks	FAK-0,4,8 wks	.42	<.01

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 remodelling, we propose that a mechano-regulated relationship between focal adhesion turnover and the pattern of muscle hypertrophy (longitudinal vs radial growth) may exist.

Vastus lateralis morphological adaptations were in line with previous observations^{6–8}: thigh lean mass showed further increase in both legs (ie, after both loading protocols) at 8 weeks; however, the changes were smaller in the second half of the training period (4–8 weeks) compared with the first 4 weeks (ECC RT +1.4% vs CON RT 0.6%). Similar observations apply to MT changes (4- to 8-week 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 work³⁴ 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 al⁵). The results in MPS (ie, myofibrillar FSR) clearly support such contention; this is the first report of MPS responses over such duration (ie, 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 work³⁵ 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-week time point but kept increasing with a similar rate in the following second half (4–8 weeks) 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,^{8,36,37} subsequently slowing.³⁵ Secondly, considering that architectural adaptations of skeletal muscle may reflect an addition of sarcomeres either in series (ie, longitudinal growth, preferentially occurring after ECC RT or in parallel (ie, 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 remodelling cannot be fully explained by the quantity of newly synthesized proteins.

Our novel findings point to differences in the mechano-transducer 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 overtime. 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.³¹ Hornberger et al³⁸ showed that C2C12 myotubes were able to respond differently to uniaxial compared with 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 mechanosensitive 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, that is costameres^{19,39} and the initiation of integrin-based signalling towards p70S6K-mediated protein synthesis.^{40,41}

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.^{18,42} 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 with a submaximal bout (75% of CON and ECC force) of conventional exercise (CON + ECC) matched for time under tension.²² Thus, higher forces (ie, 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.⁴³ In the present study, ECC and CON RT were matched for maximum relative load and neural drive,⁸ 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,⁴⁴ 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 (ie, 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 (ie, 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.^{27,28,45} Our results do not provide evidence for regional differences in myofibrillar FSR ($P = .629$); 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 (ie, 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 with CON RT at 4-week and at 8-week 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 fibres in laboratory animals.^{29,46} 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 myotendinous junction.⁴⁷ In addition, the heterogeneity of collagen type composition of the human myotendinous junction⁴⁸ may have had an influence on the multiaxial forces acting through extracellular 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 favours activation of its kinase domain.²¹ In addition, Y397FAK phosphorylation is a key signal event for costamereogenesis and focal adhesions turnover.¹⁸ The proposed underlying mechanism may involve the role of focal adhesions as template for the assembly of sarcomeres¹⁹ and/or the reinforcement of the integration between the sarcolemma and sarcomeres with fascicle length changes during contraction.^{49,50} 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 with a shift of skeletal muscle to a fast phenotype²⁰: 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 8 weeks (Table 3). Previous work from our laboratory reported a linear relationship between FAK content and increase in VL thickness after 12 weeks of downhill ski training²⁶; the results from the present study further highlight the potential connection between contraction-dependent remodelling and mechanosensitive pathways.

Higher activation of MAP Kinases family has been observed preferentially after lengthening contractions in rats⁹ in terms of ERK 1/2 and p38 MAPK. This activation was shown to be proportionally related to tension^{10,51} and to the force-time integral⁴³ together, in the case of the latter study, with FAK phosphorylation. ERK is rapidly activated following mechanical stimuli,³⁶ as p38,^{52,53} which phosphorylation is promoted by integrin signalling⁵⁴; mechanical stretch in cardiac myocytes has been shown to modulate these MAPKs through both an independent and dependent FAK-mediated regulation.⁵⁵ 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 laboratory, 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 signalling pathways may play a role in the differentiation of structural remodelling 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 design, 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 extensors⁵⁶⁻⁶⁰ or in the elbow flexors.^{34,61} The advantages of such a design are the minimizing of variability in the training responses within groups,^{34,62} and that the hypertrophic responses to RET are the result of localized muscular adaptations.⁶¹

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 10 cm 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 underestimate the possible maximal pY397 phosphorylation of FAK. Previous investigations have shown that changes in level of FAK are not manifested at 1 hour after exercise¹⁹ and that changes in level of p-Y397FAK are instead detectable from already 20 seconds after stretch¹⁹ and are clearly visible 1 hour after loading,⁴⁰ even up to 6 hours after loading.⁴⁰ However, the clear differences between muscle sites and contraction types presented consistent trends over the training period.

4 | CONCLUSION

Summarizing, we reported that the specific activation of FAK at Y397 tyrosine site resulted to be greater after ECC RT at 4 weeks and 8 weeks compared with 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 remodelling, 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 signalling, pY397-FAK and on muscle remodelling emphasize a potential role of focal adhesion-modulation in orienting the direction of fibre growth in response to a specific increase in muscle loading.

5 | MATERIALS AND METHODS

5.1 | 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/m²) were recruited from different University of Nottingham sites and entered an 8-week programme of CON vs ECC RT. Each exercise bout was carried out in the fasted state. Muscle anatomy and function were assessed prior to exercise before training, at 4 weeks and 8 weeks of a RT programme (8 weeks representing the end of training period). At these time points,

muscle biopsies were collected from the VL muscle 60–90 minutes after a single exercise session. Biopsies (30–40 mg) were used for the analysis of the fractional synthetic rates (FSR) of myofibrillar protein, using the deuterium oxide (D₂O) tracer technique and ~10–20 mg 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 (0–4 weeks) with an additional time point (8-weeks, previously unpublished), whereas all the FAK, meta and gamma-vinculin and fast myosin analyses have not been previously published.

5.2 | 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 laboratory.^{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 (ie, 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 for the duration of 2–3 seconds (ie, ~2 seconds for concentric actions and ~3 seconds for the eccentric ones), and 1 minutes 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.

5.3 | 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 randomized 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 seconds, with a rest period of 30 seconds between contractions, and 90 seconds between knee joint angle changes. Maximum isometric torque was assessed at 3 different time points (0, 4-weeks and 8-weeks).

5.4 | 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 unblinded operator. Quantification of muscle architectural adaptations, as assessment of 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,^{6,3} were performed 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.³⁵

5.5 | Muscle biopsies

Biopsies (~100 mg 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 myotendinous junction (MTJ) were ultrasound guided, to avoid the collection of any tendinous tissue. Basal biopsies were taken on the mid-sagittal 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 Healthcare, 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-weeks time points, in a window between 60 and 90 minutes after the single exercise bout.

5.6 | 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 laboratory.⁸ Briefly, immediately postbiopsy on day 0, participants provided a saliva sample and then ingested a single 150-mL oral bolus of D₂O (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 ie, 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.³⁶ The myofibrillar fraction was isolated from ~30 to 40 mg of muscle using our standard approach.^{36,37} 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).^{35,36}

5.7 | Fractional synthesis rate of myofibrillar protein

The fractional synthetic rate (FSR) of myofibrillar (MyoPS) protein synthesis was determined (as previously established³⁶ and reported in detail for this population⁸) 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 (ie, between 0- and 4-week and 0- to 8-week RT).

In brief, the standard equation:

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

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 et al^{35,37}

5.8 | Protein biochemistry

Focal adhesion kinase, vinculin isoforms and fast type myosin levels per total protein were estimated by immunodetection of total protein in homogenate as described.¹⁹ The levels of Y397-phosphorylation of FAK (ie, pY397-FAK) per total protein, and specific Y397-phosphorylation of FAK (ie, 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 analysed in one assay and expressed relative to a control sample to reduce variability between blots. For the detection of costamere proteins, 20 μg of total protein was loaded per lane, except for one sample, where 10 μg was 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 α -FAK serum (1:1,000) as described previously,⁶⁴ γ - and meta-vinculin were detected using a mouse α -vinculin serum (gift of Dr. M. A. Glukhova, Paris, France; 1:100), whereas fast myosin was detected using a mouse α -skeletal myosin (fast) (M4276, Sigma-Aldrich), as previously described.¹⁸ Then, the Goat α -mouse antibody #A9917 (Sigma-Aldrich) secondary antibody (1:20 000) was applied on the respective membranes.

5.9 | Statistics

Data throughout the manuscript are presented as means \pm SD. Statistical analyses of training-induced changes were carried out with GraphPad Prism. A two-way

ANOVA was performed between 0-, 4-week and 8-week time points. Effects were localized with the post hoc test of Bonferroni. Effects were determined as significant at a P -value set at $P \leq .05$. Linear relationships were tested using the Pearson's product-moment correlation coefficient (r). The level of significance was set at $P < .05$.

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CONFLICT OF INTEREST

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

AUTHORS' CONTRIBUTION

MVF, PJA, KS, MVN conceived the study design. MVF, MVN, MF conceived the FAK and vinculin-related experiments. KWM and 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.

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