OGT and OGA expression in postmenopausal skeletal muscle associates with hormone replacement therapy and muscle cross-sectional area

Minna H. M. Toivonen[1] Eija Pöllänen[1], Maarit Ahtiainen[1], Harri Suominen[1], Dennis R. Taaffe[2], Sulin Cheng[1], Timo Takala[3], Urho M. Kujala[1], Markku I. Tammi[4], Sarianna Sipilä[1], Vuokko Kovanen[1]

[1]Department of Health Sciences and Gerontology Research Centre, University of Jyväskylä, P. O. Box 35, 40014 University of Jyväskylä, Finland
[2]School of Environmental and Life Sciences, University of Newcastle, 10 Chittaway Road, Ourimbah NSW 2258, Australia
[3]Department of Sports and Exercise Medicine, Oulu Deaconess Institute, Uusikatu 46, P. O. Box 365, 90101 Oulu, Finland, Department of Health Sciences, University of Oulu, Aapistie 5 A, P. O. Box 5000, 90014 University of Oulu, Finland
[4]Institute of Biomedicine, University of Eastern Finland, Yliopistonranta 1, P. O. Box 1627 70211 Kuopio, Finland

Corresponding author:
Minna H. M. Toivonen
Department of Health Sciences
and Gerontology Research Centre
University of Jyväskylä
P. O. Box 35
FI - 40014 University of Jyväskylä
Tel. +358 (0) 40 8053597
E-mail: minna.h.m.toivonen@jyu.fi

Abstract

Protein glycosylation via O-linked N-acetylglucosaminylation (O-GlcNAcylation) is an important post-translational regulatory mechanism, mediated by O-GlcNAc transferase (OGT) and responsive to nutrients and stress. OGT attaches an O-GlcNAc moiety to proteins, while O-GlcNAcase (OGA) catalyzes O-GlcNAc removal. In skeletal muscle of experimental animals, prolonged increase in O-GlcNAcylation associates with age and muscle atrophy, but no published data exist on humans. Here we examined the effects of hormone replacement therapy (HRT) and power training (PT) on muscle OGT and OGA gene expression in postmenopausal women generally prone to age-related muscle weakness. The associations of OGT and OGA gene expressions with muscle phenotype were analyzed as well. Twenty-seven 50−57-year-old women participated in a yearlong randomized placebo-controlled trial: HRT (n=10), PT (n=8) and control (n=9). OGT and OGA mRNA levels were measured from muscle samples obtained at baseline and after one year. Knee extensor muscle cross-sectional area (CSA), knee extension force, running speed and vertical jumping height were measured. During the yearlong intervention, HRT suppressed the aging-associated upregulation of OGT mRNA that occurred in the controls. The effects of PT were similar but weaker. HRT also tended to increase the OGA mRNA level compared to the controls. The change in the ratio of OGT to OGA gene expressions correlated negatively with the change in muscle CSA. Our results suggest that OGT and OGA gene expressions are associated with muscle size, during the critical postmenopausal period. HRT and PT influence muscle OGT and OGA gene expression, which may be one of the mechanisms by which HRT and PT prevent aging-related loss of muscle mass.

Keywords: Muscle atrophy, aging, postmenopausal hormone replacement therapy, plyometric power training, estrogen, muscle cross-sectional area

1. Introduction

Protein glycosylation via O-linked N-acetylglucosaminylation (O-GlcNAcylation) is an important post-translational regulatory mechanism, mediated by O-GlcNAc transferase (OGT) (Ozcan et al., 2010). OGT catalyzes the addition of a single N-acetylglucosamine (GlcNAc) moiety to serine and threonine amino acid residues in nuclear, cytoplasmic and mitochondrial proteins, providing reversible modification comparable to protein phosphorylation. Unlike phosphorylation, O-GlcNAcylation is controlled by only two enzymes, OGT and O-GlcNAcase (OGA), the latter being an enzyme that releases O-GlcNAc moiety from proteins. O-GlcNAcylation has an important role in insulin signaling since O-GlcNAcylation of insulin signal pathway proteins by O-GlcNAc attenuates the insulin signal transduction (Yang et al., 2008).

Prolonged increase in O-GlcNAcylation of muscle proteins causes insulin resistance in rat skeletal muscle (Arias et al., 2004), and it has been hypothesized that sustainably increased protein O-GlcNAcylation might be involved in the muscular pathology (i.e. muscle atrophy, fiber type changes and metabolic disturbance) associated with diabetes (Cieniewski-Bernard et al., 2009). In addition, inactive splice variant of OGA has been shown to induce muscle atrophy, concomitantly with an increase in proapoptotic proteins (Huang et al., 2011). Furthermore, recent studies with mice showed that in cardiac muscle, exercise-induced hypertrophy is associated with an improvement in contractile function and decrease in protein O-GlcNAcylation, independent of blood glucose and fatty acid levels (Belke, 2011; Bennett et al., 2012). In rats, ageing increases O-GlcNAcylation levels in many tissues including skeletal muscle (Fulop et al., 2008). This may have an important role in age-related impairments like muscle weakness and loss of muscle mass. However, the knowledge of effects of ageing on human muscle protein O-GlcNAcylation is currently lacking.

The decline in muscle mass and strength with advancing age is well established and has several health concerns. Women are especially prone to this phenomenon after menopause when estrogen levels decrease dramatically (Maltais et al., 2009). We used a yearlong randomized controlled trial, the Exercise and Hormone Replacement Therapy-study (Ex/HRT), to investigate the effects of plyometric power training (PT) and HRT on muscle mass and performance in postmenopausal women (Sipilä et al., 2001; Taaffe et al., 2005). We showed that HRT induced on an average 6% increase in knee extensor muscle cross-sectional area while the change in PT and control groups

was minimal. Moreover, muscle power production increased by 6% with PT and 7% with HRT, while in those not undergoing either treatment it was reduced by 5%. In addition, running speed increased by 4% both after HRT and PT and was reduced by 2% without treatments. There were no significant changes in knee extension strength.

The transcriptome-wide study conducted with muscle samples of the Ex/HRT participants revealed that the expression of many genes related to energy metabolism and its regulation are affected by HRT and PT (Pöllänen et al., 2010). OGT was one of the genes upregulated in the muscles of postmenopausal women in the control group in comparison to those receiving HRT or PT. In the present study we investigated the associations of OGT and OGA gene expression with muscle phenotype and improvements obtained due to HRT and PT. Additionally, myotubes in culture were exposed to estradiol to find its possible immediate effects on muscle cells.

2. Material and methods

2.1. Study design

The original yearlong randomized placebo-controlled Ex/HRT-trial (ISRCTN49902272) has been previously described in detail by Sipilä et al. (2001). Briefly, 80 early postmenopausal women (50-57-years) were randomly assigned to one of the four groups: PT (n=20), HRT (n=20), PT+HRT (n=20) and control (CO, n=20). HRT (2 mg estradiol, 1 mg noretisterone acetate, Kliogest, Novo Nordisk) was administered double-blinded and continuously one tablet per day. Women not receiving HRT took placebo one tablet per day. PT participants performed progressive plyometric power training under supervision twice a week and a series of exercises at home four days per week. Women who were not in the exercise groups were advised to maintain their daily routines and physical activity level. In the present study we used muscle phenotype and microarray data from participants, from whom baseline and 12 month muscle samples were available. These included eight PT, ten HRT and nine CO women. The PT+HRT group was excluded from gene expression analysis due to the small number of muscle samples available. The study was carried out in accordance with the Declaration of Helsinki (1989) of the World Medical Association and was approved by the ethics committee of the Central Finland Health Care District. Written informed consent was obtained from all subjects.

2.2. Microarray analyses for muscle gene expression

Muscle biopsy sampling has been previously described in detail (Pöllänen et al., 2007). Briefly, needle muscle biopsies were obtained from the mid-part of *vastus lateralis* (midpoint between the greater trochanter and the lateral joint line of the knee) on the side of dominant hand. All the visible fat and connective tissue were removed and samples were snap-frozen in liquid nitrogen. Samples were stored at −80 °C until analyses. Total RNA was isolated according to manufacturer's instructions with Trizol-reagent (Invitrogen, Carlsbad, CA, USA). The whole genome gene expressions arrays (HumanRef-8 v1.0 or HumanWG-6 v1.0 BeadChips; Illumina Inc., San Diego, CA) were used to measure gene expression in baseline and follow-up muscle samples as described by Pöllänen et al. (2010). The expression of OGT and OGA genes were picked up from the arrays.

2.3. Muscle cell culture experiments

The primary human muscle cells were derived from a quadriceps muscle biopsy of a 5-day-old female infant (Edom et al., 1994). Non-differentiated mononuclear myoblasts were allowed to proliferate until they reached 80 % confluence by cultivating them in 4:1 DMEM: medium 199, supplemented with 20 % FBS, 100 U/mL penicillin, 100 µg/mL streptomycin and 1 % sodium pyruvate. Myoblasts were induced to differentiate by changing the proliferation medium to phenol red-free DMEM supplemented with 10 U/mL penicillin, 10 µg/mL streptomycin, 1 % sodium pyruvate, 10 µg/mL insulin and 100 µg/mL apotransferrin. After five days in differentiation medium the multinucleated myotubes were treated with 10 nM estradiol or vehicle. Hormone-treated and control cells were collected at five time points: 0 h, 6 h, 24 h and 72 h for RNA and protein analysis. All of the culture reagents were purchased from Invitrogen except insulin and apotransferrin, which were purchased from Sigma Aldrich (St. Louis, USA).

Total RNA was extracted from the cells with Total RNA Purification Kit (Norgen, Thorold, Canada) according to the manufacturer's instructions. RNA concentration and purity were measured with NanoDrop (Thermo Fisher Scientific Inc., Wilmington, DE, USA) and the samples were treated with RNase-Free DNase I (Norgen) according to the manufacturer's instructions. For protein analyses cells were homogenized with a 18 G needle in ice-cold RIPA buffer (Pierce, Thermo Scientific, Rockford, IL USA) containing Halt Protease and Phosphatase Inhibitor Cocktail (Pierce) and 10 µM pepstatin (Sigma Aldrich), and centrifuged (16 000 g, 15 min, 4 °C). Total protein concentration of the supernatant was assessed using BCA Protein Assay Kit (Pierce).

2.4. Real time RT-PCR

RT-PCR analyses were done for the cell culture samples by using commercial TaqMan gene expression assays Hs00269228_m1 and Hs00201970_m1 (Applied Biosystems, Foster city, CA) for OGT and OGA genes, respectively. The RNA was transcribed into cDNA and used in real time RT-PCR according to the manufacturer's (Applied Biosystems) instructions. The Applied Biosystems ABI 7300 unit was used to perform the assays using standard PCR conditions recommended by the manufacturer. Each sample was run in duplicate and each gene was run in a separate plate. The same reference sample (a mixture of several muscle cell culture samples) was included in each plate to control run-to-run variation. Dilution series of the reference sample were used to determine the amplification efficiency (E) for each gene. GAPDH (Hs99999905_m1) was used as an endogenous control transcript and relative quantity of each gene was determined as follows: $RQ = E^{(Cq(\text{reference sample}) - Cq(\text{gene of interest}))}$ and normalized with RQ of GAPDH.

2.5. Western blotting

The protein samples (20 µg protein/sample) from muscle cell culture were solubilized in 4 x Protein Loading Buffer (LI-COR Biosciences, Lincoln, NE, USA) and heat-denatured 5 min in 95 ° C. Protein samples were separated on 4−20 % gradient gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to nitrocellulose membrane (Amersham™ Hybond™-ECL, GE Healthcare UK Ltd, Little Chalfont, England). The blots were blocked with 50 % Odyssey® Blocking Buffer (LI-COR Biosciences) in PBS, washed with PBS and 0.1 % Tween 20 and incubated with the primary antibody against GAPDH (1:40 000, Sigma Aldrich) and O-GlcNAc (CTD110.6, 1:5000, Pierce). After washing the blots were incubated in IRDye 800-conjugated anti-rabbit (1:40 000, LI-COR Biosciences) and DyLight 680-conjugated anti-mouse (1:5000, Pierce) fluorescent secondary antibodies. The blots were visualized and quantified with Odyssey® CLx imaging system (LI-COR Biosciences). The total amount of O-GlcNAc-proteins was normalized with the amount of GAPDH proteins.

2.6. Data analysis

Data analyses were carried out with PASW Statistics 18 (SPSS, Inc., Somers, NY, USA). Since the gene expression data did not fulfill the criteria for parametric testing, the differences in percent

changes between groups were analyzed by the Kruskal-Wallis test followed by the post-hoc Mann Whitney U test. Percent change was calculated as (12 month value – baseline value)/baseline value x 100. The Pearson correlation coefficient was used to determine the relationship between OGT and OGA gene expression, muscle CSA and knee extension force. Although according Shapiro-Wilk test the gene expression data was not normally distributed, it was considered acceptable based on skewness and kurtosis, and therefore parametric tests were employed for all correlations. The cell culture data was analyzed by the Mann-Whitney U test by comparing the treated cells to untreated cells at each time point. An α level of 0.05 was required for significance in all statistical analyses.

3. Results

3.1. Gene expressions of OGT and OGA enzymes

The increase in OGT gene expression was significantly greater in CO group (22.1 %) than in HRT group (6.2 %, $p = 0.01$) (Fig 1a). Also in PT group the increase tended to be smaller (14.3 %), but it did not differ significantly from the increase in CO group (Fig 1a).

The changes in the OGA gene expression did not differ significantly between the groups, although in HRT group the OGA gene expression tended to increase (13.8 %, −0.6 % and 2.5 % in HRT, PT and CO group respectively) (Fig 1b).

The ratio of OGT to OGA gene expression, an indirect indicator for protein O-GlcNAcylation capacity, decreased significantly in HRT group (−3.9 %) compared to CO group (20.0 %, $p = 0.004$) (Fig 1c). There were no significant difference in the OGT/OGA ratio change between PT (15.2 %) and CO groups.

3.2. Associations of OGT and OGA gene expressions with muscle mass and performance

There was a trend for the change in OGT gene expression to be negatively associated with change in knee extensor muscle CSA, while the change in the OGA gene expression tended to correlate positively with the change in muscle CSA (Table 1). Consequently, the change in OGT/OGA ratio i.e. capacity for O-GlcNAcylation was inversely correlated with the change in muscle CSA ($r = −0.61$, $p = 0.001$, Fig 2), but no significant correlation was found with the change in knee extension force, running speed or vertical jumping height (Table 1).

3.3. Cell culture experiments

The exposure of myotubes to estradiol up to 72 hours, beginning at day five in the differentiation medium, did not induce any significant effect ($p > 0.05$) on the gene expression of OGT and OGA when compared to the unexposed myotubes. In addition, the overall protein O-GlcNAcylation level did not differ between the estradiol exposed and unexposed myotubes (Fig 3).

4. Discussion

The current study focused on the gene expression of two enzymes, OGT and OGA, responsible for the level of protein O-GlcNAcylation, an important post-translational regulatory mechanism for protein function. The target tissue was skeletal muscle of early postmenopausal women, who went through a one-year intervention with HRT, PT or treatment with placebo (CO group). The results indicate that during this critical period in a woman's life the capacity for protein O-GlcNAcylation (OGT/OGA ratio) increases in skeletal muscle tissue, which can be regarded as an aging effect in connection to the menopausal period. In addition, this increase in O-GlcNAcylation capacity was associated with a decrease in muscle mass. The use of HRT counteracted this phenomenon, possibly, by slowing down the increase in OGT gene expression, by tending to increase OGA gene expression and, consequently, by decreasing the ratio of OGT to OGA in favour of diminished protein O-GlcNAcylation.

The smaller effect of PT on OGT and OGA gene expression is in line with the relatively small effect of PT on muscle CSA compared to the effects of HRT. The impact type of physical training utilized in the current study was not designed to increase muscle CSA, but had a greater effect on muscle composition and performance (Sipilä et al., 2001). Although the OGT/OGA ratio correlated negatively with muscle CSA, no significant correlations were found with knee extension force, running speed or vertical jumping height. Possibly, the extent of protein O-GlcNAcylation associated with the homeostasis of muscle protein metabolism is not necessarily sufficient to increase muscle performance, in which other factors like neuronal input have more significant effects. However, the current study design opens a new perspective to search the regulation of muscle health via protein O-GlcNAcylation with advancing age, especially in early postmenopausal women, who are more prone to muscle wasting than men of the same age.

Relatively little is known about the effects of aging on protein O-GlcNAcylation, aberrant O-GlcNAcylation seems to be related to several age-related diseases (Chou and Hart, 2001; Dias and Hart, 2007). A study whit 5- and 24-month old male rats revealed that senescence increased the total level of O-GlcNAc modified proteins in several tissues, including skeletal muscle (Fulop et al., 2008). However, the increase was not associated with a significant increase in the level of OGT protein or mRNA, suggesting that increase in the extent of protein O-GlcNAcylation involves other regulatory factors. In our study, gene expression of OGT increased in all study groups during the one year intervention, although the increase was significantly smaller in the HRT groups compared to CO and PT, suggesting that aging enhances OGT expression and HRT is able to partially attenuate the aging affect. The gene expression of OGA was less affected in PT and CO groups, but in the HRT group it tended to increase. Altogether, these results suggest that menopause-related hormonal changes affect the gene expression of muscle OGT and OGA. However, we could not confirm an immediate, direct effect of estradiol on myotube cultures, suggesting more complex pathways of action (e.g. involment of systemic effects) for estradiol regulation of OGT and OGA gene expression and protein O-GlcNAcylation in skeletal muscle.

In conclusion, our results show that the ratio of OGT to OGA, an indicator for tissue's O-GlcNAcylation capacity, is associated with muscle size in women during the early menopausal years. We suggest that a proper balance between these two enzymes is important for maintaining muscle mass and that an increase in O-GlcNAcylation capacity (OGT/OGA ratio) is associated with decreased muscle mass. HRT and perhaps also PT attenuate the increase in O-GlcNAcylation capacity associated with advancing age and postmenopausal state. This could be one of the mechanisms via which HRT and physical activity prevent menopause-related muscle loss in women. However, the present study focused mostly on gene transcripts, which necessitates further O-GlcNAc analyses of specific proteins as well as analyses of OGT and OGA enzymes.

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Fig. 1. Gene expressions of OGT and OGA. Individual changes in the gene expression of OGT (A), OGA (B) and the OGT/OGA ratio (C) in the different study groups. Horizontal bars indicate group mean values. *p = 0.01, **p = 0.004.

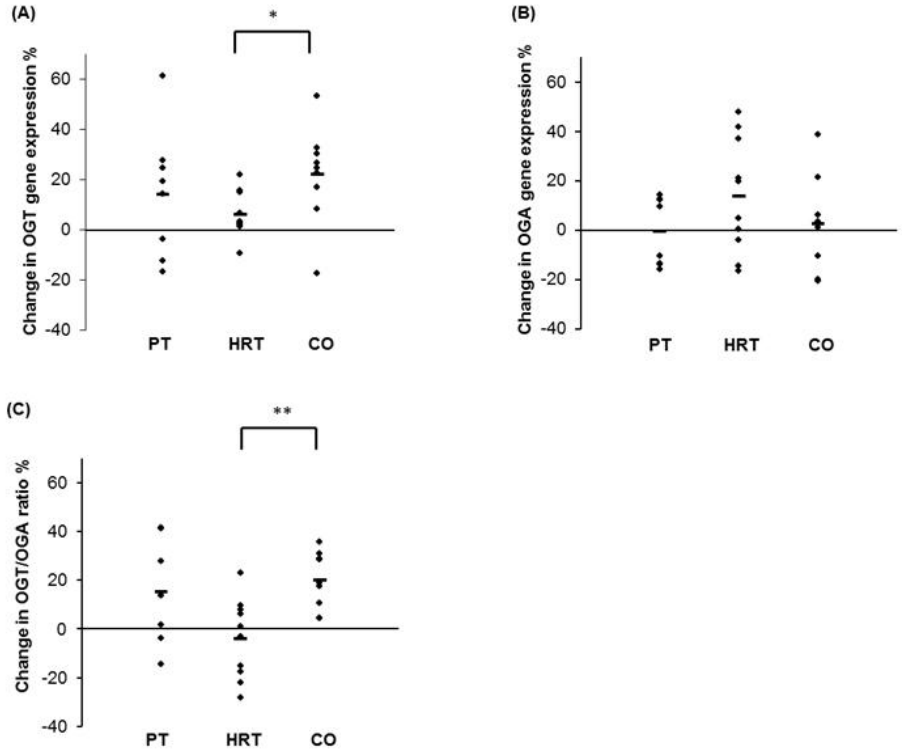

Fig. 2. Association of OGT/OGA ratio with knee extensor muscle CSA.

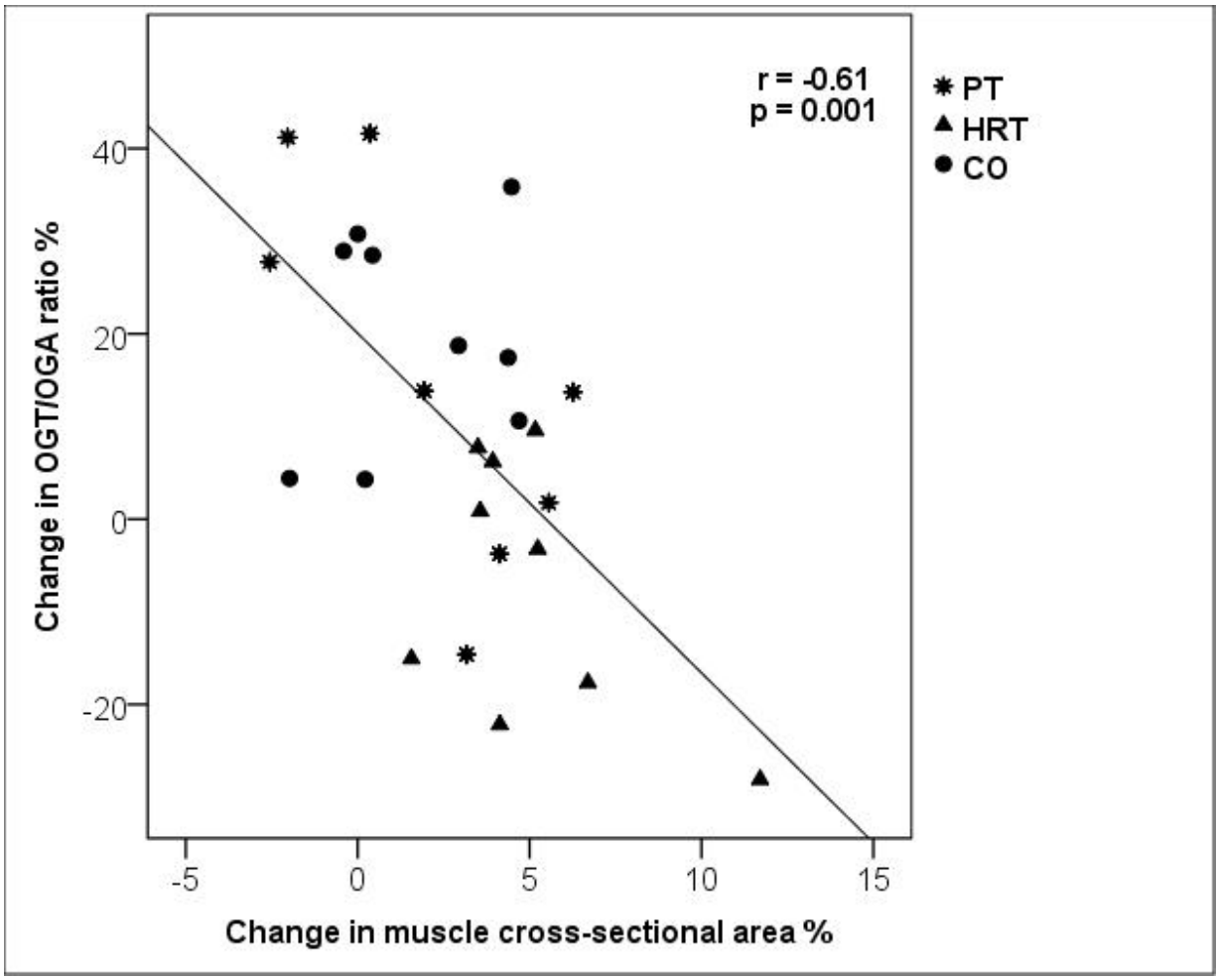

Table 1

Pearson correlations between expressions of OGT and OGA genes, OGT/OGA ratio, and muscle phenotype

	Change in OGT expression %	Change in OGA expression %	Change in OGT/OGA ratio %
Change in muscle CSA %	r = −0.37 p = 0.07	r = 0.39 p = 0.06	**r = −0.61** **p = 0.001**
Change in knee extension force %	r = 0.06 p = 0.78	r = 0.15 p = 0.46	r = −0.06 p = 0.79
Change in running speed %	r = 0.13 p = 0.54	r = 0.30 p = 0.13	r = −0.21 p = 0.31
Change in vertical jumping height %	r = 0.07 p = 0.71	r = 0.22 p = 0.27	r = −0.11 p = 0.57

Significant values are in bold. CSA: cross-sectional area, OGT: O-GlcNAc transferase, OGA: O-GlcNAcase

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