Original article

TITLE: Stiffness of Hip Adductor Myofibrils is decreased in Children with Spastic Cerebral Palsy.

Timothy R. Leonard, [1] Jason J. Howard, [2] Kelly Larkin-Kaiser, [1] Venus Joumaa, [1] Karl Logan, [3] Benjamin Orlik, [3] Ron El-Hawary, [3] Luke Gauthier, [3] and Walter Herzog*[1].

[1]Faculty of Kinesiology, University of Calgary, Calgary, AB, Canada; [2]Weill Cornell Medicine, Sidra Medicine, Doha, Qatar, [3]IWK Health Centre, Halifax, NS, Canada.

***Corresponding author**

Dr. W. Herzog

Faculty of Kinesiology

University of Calgary

2500 University Drive N.W.

Calgary, Alberta, Canada, T2N 1N4

Tel (403)220-8525

Fax (403)284-3553

wherzog@ucalgary.ca

https://doi-org.ezproxy.lib.ucalgary.ca/10.1016/j.jbiomech.2019.02.023

ABSTRACT

Cerebral palsy (CP) is the result of a static brain lesion which causes spasticity and muscle contracture. The source of the increased passive stiffness in patients is not understood and while whole muscle down to single muscle fibres have been investigated, the smallest functional unit of muscle (the sarcomere) has not been. Muscle biopsies (adductor longus and gracilis) from pediatric patients were obtained (CP n=9 and control n=2) and analyzed for mechanical stiffness, in-vivo sarcomere length and titin isoforms. Adductor longus muscle was the focus of this study and the results for sarcomere length showed a significant increase in length for CP (3.6μm) compared to controls (2.6μm). Passive stress at the same sarcomere length for CP compared to control was significantly lower in CP and the elastic modulus for the physiological range of muscle was lower in CP compared to control (98.2kPa and 166.1kPa, respectively). Our results show that CP muscle at its most reduced level (the myofibril) is more compliant compared to normal , which is completely opposite to what is observed at higher structural levels (single fibres, muscle fibre bundles and whole muscle). It is noteworthy that at the *in vivo* sarcomere length in CP, the passive forces are greater than normal, purely as a functional of these more compliant sarcomeres operating at long lengths. Titin isoforms were not different between CP and non-CP adductor longus but titin:nebulin was reduced in CP muscle, which may be due to titin loss or an over-expression of nebulin in CP muscles.

Keywords: Cerebral palsy, passive stress, myofibrils, titin, muscle stiffness.

INTRODUCTION

Cerebral palsy (CP) is the most common cause of physical disability in children (Oskoui et al., 2013). The clinical manifestations are progressive with growth (Kerr Graham and Selber, 2003) and the spastic motor type is most commonly found in children with CP (Howard et al., 2005), first manifesting with a velocity-dependent increase in muscle stiffness, and progressing to a fixed increase in muscle stiffness over time. It is generally accepted that spastic CP muscle is stiffer than normal muscle, but there is little agreement on the mechanisms behind this observation. Previous research has shown that the sarcomere, the basic contractile unit of skeletal muscle, is overstretched in spastic muscle tissue compared to normal, and operates at long sarcomere lengths (Lieber and Fridén, 2002; Mathewson et al., 2015; Mathewson and Lieber, 2015; Smith et al., 2011). At these increased lengths, the overstretched sarcomeres would have low active force-generating capacity (Gordon et al., 1966) and high passive forces, which agrees with the clinical situation whereby muscles are not only tight but also weak. Despite the increased sarcomere length, the muscle portion of the muscle-tendon unit has been found to be shorter in CP muscle as compared to normal (Matthiasdottir et al., 2014; Wren et al., 2010), and has been associated with the development of static contracture .

The primary aim of these experiments was to compare passive stress generation under stretch (i.e. stiffness) between myofibril samples acquired from the adductor longus of children with CP and those from typically developing children. Given that the isolated myofibril is devoid of passive structural elements outside of the sarcomere, such as the extracellular matrix, this analysis provides crucial insight into the mechanics of sarcomeres and titin in CP. Our

hypothesis was that the stresses generated by CP myofibrils under stretch are higher than for typically developing children, in accordance with results reported for single muscle fibre and fibre bundle preparations (Fridén and Lieber, 2003; Smith et al., 2011), because of a smaller (and stiffer) titin isoform in CP muscle.

A secondary aim was to compare passive stress under stretch between the adductor longus and gracilis from children with CP to determine if muscles in the hip adductor group have similar pathomechanics at the myofibrillar level.

METHODS

Study Design and Participant Selection

Patients with spastic quadriplegic CP (GMFCS III to V) who subsequently underwent tendon release (typically adductor longus, gracilis, and iliopsoas) for the treatment of progressive hip displacement were enrolled in this study. Biopsies of the adductor longus (CP-AL) and gracilis (CP- gracilis) were obtained during the course of a soft tissue release procedure for the treatment of hip displacement. The control group were patients with developmental dysplasia of the hip (DDH) who underwent an open hip adductor lengthening during operative reduction of hip dislocation (non-CP-AL).

Following ethics approval from our institutional review board, written informed consent from the parents of all patients included in our study was obtained.

Pre-operative Measures: CP Participants

All clinical and radiographic measures were recorded pre-operatively and included: Gross Motor Function Classification (GMFCS) level, hip range of motion (goniometry), spasticity

measures (Modified Tardieu Scale), and radiographic measures (Reimer's hip migration percentage).

On the morning of the surgery, each participant underwent a physical examination with the participant in the supine position, the maximum hip abduction was measured by goniometer for two positions: with the hip and knee at 90° flexion (adductor longus) and with the hip and knee at 0° flexion (gracilis). To determine the presence of dynamic and/or static contractures, measurements were taken using the modified Tardieu scale, whereby the angle of maximum hip abduction was taken at the point of 'spastic catch' after the joint was moved in a quick stretch (R1) and after the joint was moved slowly to its end point (R2), respectively.

Muscle Biopsy Procedure

Under general anesthesia, biopsies of operated muscles were excised by the treating orthopaedic surgeon (3mm length, 3mm diameter), and held at the *in vivo* length using a specially designed single-use polypropylene biopsy clamp (Howard et al., 2014). Sarcomere lengths (SLs) obtained using similar biopsy clamps compared to the results obtained from *in vivo* laser measurements show good agreement (Ward et al., 2009). With the hip in maximum (but not forced) abduction, the adductor longus and gracilis were biopsied with the hip and knee at 90° flexion and 0° flexion, respectively. Samples of muscle from the unclamped remnant ends of the excised muscle were collected and either frozen in liquid nitrogen and then stored at -80 ºC for later titin gel electrophoresis or were placed in a special rigor solution for generation of myofibrils at a later date.

Although both the AL and gracilis were biopsied in CP participants, only the AL muscle was biopsied and analyzed in the control group, as gracilis was not the surgical focus for the non-CP participants.

Measurement of *In Vivo* Sarcomere Length

The muscle for SL determination from all participants remained clamped and were stored in a 10% buffered formalin solution for 30 days. The methods for preparation are the same as used previously (Fleeter et al., 1985; Koh and Herzog, 1998). Briefly, the samples were processed in 30% nitric acid then transferred to glycerol. Fascicles were isolated and laser diffraction was used to estimate a mean SL and then randomly checked against results using optical microscopy. Twenty individual muscle fascicles were isolated from each muscle biopsy, mounted onto a glass slide and scanned for mean SL at 5 regions along the length of each fascicle.

Passive Stress Measurement

Samples were stored in a rigor/glycerol solution containing protease inhibitors at -20ºC for 14 days. Protease inhibitors to minimize titin and nebulin degradation (Complete ®, Roche Diagnostics Inc., Montreal, Canada) were placed in all storage and processing solutions used for generating myofibrils. Solutions and the myofibril testing apparatus used are described elsewhere (Fauver et al., 1998; Herzog et al., 2014; Joumaa et al., 2008, 2007) and a representative myofibril image is shown in Figure 1. The protocol was a ramp-hold-return design and every myofibril was lengthened from slack length (<2.0μm) to a target mean SL of 2.4, 2.8, 3.2, 3.6 and 4.0μm, sequentially. Target SL was not always achieved due to the cantilever compliance. The differences between target SL and actual mean SL were small and actual SL was always

used for analysis purposes. Lengthening was performed at a speed of 0.1µm/sarcomere/second) to minimize visco-eleastic effects on the peak stress at the end of the lengthening. At each target length, the myofibril was held for 1 minute to allow for stress-relaxation to reach steady-state. Steady-state stress and SLs were measured at the end of the 1-minute hold. The sample was then returned to slack length for 10 minutes before continuing the test to the next target SL; 10 minutes at slack length has been shown to allow full recovery of the passive force prior to the next test ramp (Herzog et al., 2014). For the elastic modulus tests aimed at a SL of 4.0µm, the actual SL ranged from 3.7µm to 3.9µm, because of the small compliance of the force cantilevers, as substantial passive force was generated at these SLs. Peak stress at mean SL 3.8µm (values used from 3.7µm to 3.9µm) was measured immediately once the stretch was completed and prior to the 1-minute hold.

Samples from the AL of both CP and non-CP participants were tested. For gracilis, only biopsies from CP participants were tested as no control tissue was available. Stresses were reported for comparison of myofibrils with different cross-sectional areas. For comparisons of passive stress between the groups at matched SL, the results were stratified into groups according to SL: 2.25-2.60, 2.61-2.85, 2.86-3.00, 3.01-3.25, 3.26-3.50, 3.51-3.75, 3.76-4.0, 4.01-4.25 µm.

The primary outcome measure was passive stress at pre-determined average SL. Repeated measures ANOVA (α=0.05) was performed to compare stress at matched SLs between CP-AL and non-CP-AL.

Stress relaxation reported as a percentage decrease was calculated for CP-AL and non-CP-AL samples. This calculation was performed for tests where the mean SL of 3.8µm was

observed. This SL was chosen because forces are sufficiently high that stress-relaxation is easily measured over the 1-minute hold. A comparison of the percentage stress-relaxation between CP-AL and non-CP-AL was done using the Mann-Whitney test (α=0.05).

The average elastic modulus was calculated for each AL myofibril for SL range of 2.4 to ~3.8µm. A comparison of the elastic modulus between CP-AL and non-CP-AL was done using the Mann-Whitney test (α=0.05).

Average elastic modulus = $(F/A)/(\Delta L/L_O)$

F= Change in force at steady state after a stretch from a SL of 2.4 µm to ~3.8 µm (nN)

A= Cross sectional area of the myofibril (μm^2)

ΔL= Change in length of the myofibril (µm)

L_O = Initial length of the myofibril (µm)

Peak stress at SL ~3.8µm was analyzed using the same statistical approach. The Mann-Whitney test (α=0.05) was used for titin molecular weight comparisons as well as for titin-nebulin content comparisons between CP and non-CP results.

Titin Gel Electrophoresis

Frozen biopsy sub-samples were processed using a standard protocol (Neagoe et al., 2003; Tatsumi and Hattori, 1995). Protease inhibitors were added to the homogenization buffer to minimize proteolytic degradation. The gels were stained with Coomassie Blue and scanned using a Bio-Rad GS-800 densitometer. To estimate the molecular weight of titin, each well of the gel was loaded with a muscle sample in conjunction with rabbit psoas muscle (2 isoforms expressed, 3416 and 3295kDa), rat heart (3000kDa) and rabbit soleus (3600kDa) (Neagoe et al.,

2003; Prado et al., 2005). Measurements of titin molecular weight were done using ImageJ software. Mean values (±SD) of titin molecular weights were calculated from three to six observations per muscle sample. The ratio of the optical density of titin to nebulin was calculated and reported, mean value (±SD). Nebulin is a large protein found in association with the thin filament; a single nebulin molecule spanning each thin filament, thus the titin:nebulin ratio hints at the abundance of titin relative to the contractile thick and thin filaments in a sarcomere (Ottenheijm et al., 2009).

RESULTS

Nine children with CP (CP Group; mean age 102±47 months), and 2 subjects with DDH (Control Group: mean age 38±23 months) met the study inclusion criteria. The mean age for all study participants was 8.8 ± 4.2 years (range, 2-12 years).

The mean pre-operative hip abduction (with knee and hip at 0 degrees flexion) was 11º ± 9º and 22º ± 15º for R1 and R2, respectively. The mean intraoperative hip abduction (with knee and hip at 0 degrees flexion) was 28º ± 12º. The mean pre-operative MP was 54 ± 21 % (range, 14-86 %).

In Vivo Sarcomere Length

The mean SL *in vivo* for CP-AL and CP-gracilis was 3.6±0.3μm and 3.5±0.1μm, respectively. Due to technical reasons, the mean SL could not be determined with confidence from the single AL muscle biopsy taken from each of the 2 non-CP participants. Fortunately, a great deal of literature exists which accurately describes the *in vivo* SL of muscles from typically

developing children (Mathewson et al., 2015), and so values from the literature are reported here (2.6μm) and used for comparative purposes to our CP SL measurements.

Myofibril Passive Force Measurements

For the CP participants, 46 AL myofibrils and 40 gracilis myofibrils were isolated and analyzed. For the controls, 8 AL myofibrils were isolated and analyzed. The total number of stress-SL observations for CP-AL, CP-gracilis, and non-CP-AL was 255, 208, and 43, respectively. There was no difference between the diameters of CP-AL (1.2±0.2μm) and non-CP-AL myofibrils (1.1±0.2μm) (p=0.47). At all ranges of matched SL, passive stress was significantly lower in CP-AL compared to non-CP-AL myofibrils except for the longest length grouping (Figure 2). Stresses at matched SL were not different for CP-AL versus CP-gracilis (Figure 3). Peak stress at SL 3.8μm (3.81±0.07μm) was 64±18 $nN/\mu m^2$ for CP-AL, and 129±30 $nN/\mu m^2$ for non-CP-AL (p=0.0002). Stress-relaxation (percentage) for CP-AL was 18±6% and for non-CP-AL was 19±6% (p=0.92). The elastic modulus for CP-AL was 98±45kPa and for non-CP-AL was 166±22kPa (p=0.0005).

Titin Molecular Weight and Content

Titin molecular weight for CP-AL and non-CP-AL were the same (3611±41kDa and 3615±32kDa respectively, p = 0.76; Figure 4). Titin molecular weight for CP-gracilis was 3588±26kDa, not different from the CP-AL group (p=0.08). The ratio of titin:nebulin content for CP-AL was 1.47±0.37 and was different from that for non-CP-AL, 3.26±0.16 (p=0.004). The titin:nebulin ratio for non-CP-AL was the same as that measured for normal rabbit psoas muscle (3.69±0.50; p=0.92).

DISCUSSION

CP group muscles had significantly decreased stress levels at matched SLs compared to non-CP muscles. This was evident at all SLs except for measurements at SL greater than 4μm. The reason for the lack of significance for SLs over 4μm was attributable to fewer data points and large variations at these long SLs. Paradoxically, and at odds with our hypothesis, these decreased stresses and lower average elastic modulus values for CP muscle suggest that CP myofibrils are less stiff than those of typically developing children. There was no difference between the adductor longus and gracilis muscles for children with CP. Thus, the myofibrils were found to be less stiff for both of the CP muscles targeted in this study, suggesting that this observation may be generalized, at least for muscles within the same functional group. Further research is necessary to confirm this supposition.

The etiology of pathological increases in passive joint stiffness in CP has been reported to derive from contributions at several structural levels: originating both from non-myogenic (i.e. joint capsule and extracellular matrix) and myogenic sources (i.e. muscle fibres and myofibrils) (Barber et al., 2011a; Lieber et al., 2003; Mirbagheri et al., 2001; Sinkjaer and Magnussen, 1994). Friden & Lieber (2003) proposed that the increased stiffness in CP muscle arose from two major sources: collagen and titin. Increased collagen content of the extracellular matrix (ECM) in CP muscle has been implicated as a primary cause of increased muscle stiffness (Booth et al., 2001; Smith et al., 2011). Increased collagen deposition has also been observed in denervated (Virtanen et al., 1992) and immobilized muscles (Williams and Goldspink, 1984), suggesting that collagen content within the ECM may be linked to neuronal activity.

The molecular spring titin is an important structural element within the sarcomere, tethering the thick filament (myosin) to the Z-discs . This protein centers the thick filament within the sarcomere and is thought to account for the majority of passive force generated when myofibrils are stretched (Bartoo et al., 1997; Granzier and Labeit, 2002; Linke and Fernandez, 2002; Tskhovrebova and Trinick, 2002; Wang et al., 1991). Accordingly, previous work has shown that changes in passive stiffness of myocytes are accompanied by changes in titin size (Nagueh et al., 2004). Furthermore, titin has been suggested to possess a 'mechano-sensing' capability, with isoform expression (and thus molecular size) being shown to change with mechanical stimuli (Granzier and Labeit, 2006; Ottenheijm et al., 2011). Indeed, it has been surmised that titin could be responsible for modulating myofibrillar stiffness in CP muscle (Foran et al., 2005).

To investigate titin mechanics in isolation from other contributors to spastic muscle stiffness, we used single myofibrils since this muscular element is simply an in-series arrangement of single sarcomeres. No extracellular matrix or basement membrane proteins are present, therefore, a single myofibril is thought to generate passive tension only through titin (Linke et al., 1994). Despite our findings of decreased passive force generation for the CP myofibrils, we found no difference in titin isoform masses between adductor longus in the non-CP and CP groups, nor between the adductor longus and gracilis within the CP groups, which is in agreement with previous reports, specifically gracilis-normal and gracilis-CP: 3588±18kDa and 3667±22kDa, respectively (Smith et al., 2011). The resolution of our gel system is ~50kDa, similar to that reported by Prado et al. (2005). We acknowledge that estimating the true size of high molecular weight proteins is not an easy task and therefore we added known weight markers, such as titin from the rat heart and rabbit psoas within the same lane as our

experimental samples. It is possible that while the molecular mass of titin for CP and non-CP samples were not different, the composition of titin, and thus its stiffness, could be different. However, there is no precedence for cases of titin with similar molecular weights having vastly different mechanical properties.

We report here the novel finding of a large decrease in the titin:nebulin ratio in CP compared to non-CP muscles suggesting a loss of titin content. Loss of titin may be an adaptive response resulting in a more compliant myofibril to partially offset the high passive stresses experienced at long *in vivo* SLs of CP patients. Loss of titin content has been observed previously in response to disuse atrophy and in response to cardiac myopathy (Makarenko et al., 2004; Udaka et al., 2008). However, the reduction in the titin:nebulin ratio does not necessarily mean a loss of titin in the sarcomeres. Degradation of titin and/or nebulin due to proteolytic activity is a concern. Steps were taken to minimize this degradation, which could be observed in the T2 band on the electrophoresis gels. The T2 bands in our Figure 4 were faint, suggesting that titin degradation was minimal. Our homogenization buffer contained leupeptin which has been shown to dramatically reduce proteolysis and subsequent T1 degradation (Helmes et al., 1996). Furthermore, the normal rabbit and the non-CP patient titin:nebulin ratios were the same, while the titin:nebulin ratio for the CP patients was significantly reduced when compared to the non-CP muscle. Since all muscle samples for gel electrophoresis were handled in the same fashion, it is unlikely that the non-CP samples were uniquely mishandled so as to degrade the nebulin and subsequently skew the results. A reduction in the titin:nebulin ratio in CP muscles could be the result of an over-expression of nebulin in CP muscles. However, as far as we know, over-expression of nebulin in CP muscles has not been shown. Also, since nebulin has no effect on passive force in single myofibrils, but titin is responsible for 95-100% of the passive force (Linke

et al., 1994), it seems reasonable to conclude that the dramatic decrease in passive forces in CP myofibrils compared to controls, was indeed caused by a loss of titin rather than an overexpression of nebulin.

In CP participants, we found that *in vivo* SL in the AL was significantly longer than reported values from typically developing muscle. This finding is in agreement with other researchers for muscles including the gracilis, semitendinosus and flexor carpi ulnaris (Lieber and Fridén, 2002; Mathewson et al., 2015; Smith et al., 2011). In particular, for gracilis, the only CP muscle in our study that has a previously reported SL in the medical literature, our observation of mean SL= 3.5μm is in agreement with the SL reported (gracilis SL=3.54μm) (Smith et al., 2011).

Control tissue was not obtained from the gracilis, as this muscle was not the subject of a pre-determined surgical intervention for the typically developing children. As such, the CP-gracilis myofibrils were compared to the other hip adductor muscle harvested (CP-AL) and were found to have SLs that were not different from SL in CP-AL. Titin isoform mass was not different between the two CP muscles, and there were no differences in passive stress generation between the CP hip adductor group muscles.

Muscles in typically developing children are believed to operate effectively at SLs of approximately 2.5 to 2.7μm (Herzog et al., 2010; Walker and Schrodt, 1974). The mean *in vivo* SL for the 2 muscles investigated here in CP children (approximately 3.56μm) is on the descending limb of the force-length curve (Herzog et al., 1992; Walker and Schrodt, 1974) where actin-myosin filament overlap would be reduced (Mathewson et al., 2015) and these

sarcomeres would have a decreased active force generation capacity (Carmick, 1993; Kerr et al., 2004; Rose and McGill, 2005). This is evident clinically, with CP muscle weakness being a predominant feature (Kerr Graham and Selber, 2003; Palisano et al., 1997).

Decreased passive stress observed in CP myofibrils compared to controls at the same SL, is complicated by the typical *in vivo* SL of the CP-AL and non-CP-AL which are very different (3.6μm and 2.6μm, respectively). This *in vivo* SL mismatch plays a dominating role in passive force production in the sarcomere and for the muscle as a whole. CP tissue is under substantially increased passive stress *in vivo* as compared to typically developing children (Figure 5).

STUDY LIMITATIONS

1. The lack of directly measured SL for the AL in the non-CP group is a limitation and needs to be followed-up in future work. Assuming the normal operating length for normal skeletal muscle to be approximately 2.5 to 2.7μm (Walker and Schrodt, 1974) and in the absence of direct measurements, using this range for modeling normal SL has been done previously (Smith et al., 2011). Measured and then corrected SL values for typically developing children have been reported at 2.17μm (Mathewson et al., 2015) and an estimate of normal SL based on 44 previously published values results in a mean of 2.6μm with a 99.99% CI of 2.43–2.91 (Mathewson et al., 2015). Our directly measured SL for CP-AL (3.6±0.3μm) is well beyond the upper confidence value for these previously reported normal values (2.91μm) and agrees with previously published direct measurements of CP muscle SL.

2. The discrepancy in ages between the CP and non-CP children was primarily a function of the clinical features of the control group diagnosis versus the CP group. Subjects for our control group - DDH - typically present within the first 2 years of life and surgical intervention, including adductor longus tenotomy, is often required at that time. Neurogenic hip displacement in CP, however, typically requires surgical intervention at an older age; typically between ages 4 to 10 years. With a prevalence of approximately 1 in 1000, DDH is the pediatric diagnosis with the highest frequency that requires adductor longus surgery. Hence, though we agree the age discrepancy is a limitation, we chose the diagnosis with the highest prevalence for our control group which we feel was the most appropriate choice. The only measurement affected by age was the sarcomere length since our biopsy clamp was too big for effective fixing of muscle samples.
3. Protein degradation is a concern when protein masses and ratios of protein content are estimated. Rapidly freezing and proper storage of muscle samples is essential. When assessing the mechanical behaviour of proteins, especially a large protein like titin, we were careful that protease inhibitors were used consistently and the timing of experiments were (e.g. 14 days after harvest) consistent.
4. The quantity of nebulin was variable and the contents of the band were assumed to be nebulin even though no Western Blot analysis was performed to confirm that the ~800 kDa band was nebulin and nebulin alone. Work by others (Witt et al. (2006) where a nebulin knock-out mouse was evaluated using Western blot analysis, the ~800 kDa band observed in skeletal muscle is nebulin alone (Their Figure 2A). Nevertheless, it would be useful to confirm this assumption independently in human biopsy samples using Western blot analysis.

Conclusion

Contrary to our hypothesis, at matched SLs, passive stresses and elastic moduli were much lower in CP myofibrils compared to typically developing control myofibrils. This finding is in contrast to findings in single fibres, fascicles and muscles, and as such, and might be an adaptation to reduce an already excessive passive force in spastic muscles. Despite the lower stresses in CP compared to control myofibrils, passive stress at *in vivo* sarcomere lengths is greater in CP than in typically developing children. A ~50% reduction in the titin:nebulin ratio seems to explain well the ~50% reduction in passive stress and elastic modulus of CP sarcomeres, and might be an adaptive response to partially offset the high passive stresses experienced at long *in vivo* sarcomere lengths of CP patients.

Conflict of Interest Statement

No conflict of interest to disclose.

Acknowledgments

Canadian Institutes of Health Research, the Canada Research Chair program, the Killam Foundation. The force cantilevers were fabricated at the Cornell NanoScale Facility. Thanks to Steve Van Iderstine for coordinating this research and Dr. Ellen Wood for determination of motor type and GMFCS level and Prof. H. Kerr Graham (Royal Children's Hospital, Melbourne, Australia) for reviewing the manuscript and providing comments.

REFERENCES

366 Barber, L., Barrett, R., Lichtwark, G., 2011. Passive muscle mechanical properties of the medial
367 gastrocnemius in young adults with spastic cerebral palsy. J. Biomech. 44, 2496–2500.
368 https://doi.org/10.1016/j.jbiomech.2011.06.008
369 Bartoo, M.L., Linke, W.A., Pollack, G.H., 1997. Basis of passive tension and stiffness in isolated
370 rabbit myofibrils. Am. J. Physiol. 273, C266-276.
371 Booth, C.M., Cortina-Borja, M.J., Theologis, T.N., 2001. Collagen accumulation in muscles of
372 children with cerebral palsy and correlation with severity of spasticity. Dev. Med. Child
373 Neurol. 43, 314–320.
374 Carmick, J., 1993. Clinical use of neuromuscular electrical stimulation for children with cerebral
375 palsy, Part 2: Upper extremity. Phys. Ther. 73, 514–22; discussion 523-7.
376 Fauver, M.E., Dunaway, D.L., Lilienfeld, D.H., Craighead, H.G., Pollack, G.H., 1998.
377 Microfabricated cantilevers for measurement of subcellular and molecular forces. IEEE
378 Trans. Biomed. Eng. 45, 891–898. https://doi.org/10.1109/10.686797
379 Fleeter, T.B., Adams, J.P., Brenner, B., Podolsky, R.J., 1985. A laser diffraction method for
380 measuring muscle sarcomere length in vivo for application to tendon transfers. J. Hand
381 Surg. 10, 542–546. https://doi.org/10.1016/S0363-5023(85)80080-0
382 Foran, J.R.H., Steinman, S., Barash, I., Chambers, H.G., Lieber, R.L., 2005. Structural and
383 mechanical alterations in spastic skeletal muscle. Dev. Med. Child Neurol. 47, 713–717.
384 https://doi.org/10.1017/S0012162205001465
385 Fridén, J., Lieber, R.L., 2003. Spastic muscle cells are shorter and stiffer than normal cells.
386 Muscle Nerve 27, 157–164. https://doi.org/10.1002/mus.10247
387 Gordon, A.M., Huxley, A.F., Julian, F.J., 1966. The variation in isometric tension with
388 sarcomere length in vertebrate muscle fibres. J. Physiol. 184, 170–192.

389 Granzier, H., Labeit, S., 2002. Cardiac titin: an adjustable multi-functional spring. J. Physiol.
390 541, 335–342.
391 Granzier, H.L., Labeit, S., 2006. The giant muscle protein titin is an adjustable molecular spring.
392 Exerc. Sport Sci. Rev. 34, 50–53.
393 Helmes, M., Trombitás, K., Granzier, H., 1996. Titin develops restoring force in rat cardiac
394 myocytes. Circ. Res. 79, 619–626.
395 Herzog, J.A., Leonard, T.R., Jinha, A., Herzog, W., 2014. Titin (visco-) elasticity in skeletal
396 muscle myofibrils. Mol. Cell. Biomech. MCB 11, 1–17.
397 Herzog, W., Joumaa, V., Leonard, T.R., 2010. The force-length relationship of mechanically
398 isolated sarcomeres. Adv. Exp. Med. Biol. 682, 141–61. https://doi.org/10.1007/978-1-
399 4419-6366-6_8
400 Herzog, W., Kamal, S., Clarke, H.D., 1992. Myofilament lengths of cat skeletal muscle:
401 Theoretical considerations and functional implications. J. Biomech. 25, 945–948.
402 Howard, J., Leonard, T., Kaiser, K., Herzog, J., Gauthier, L., Logan, K., Orlik, B., El-hawary, R.,
403 Herzog, W., 2014. High passive stresses in spastic muscle are not generated from
404 myofibrils for children with cerebral palsy, in: Developmental Medicine & Child
405 Neurology, S5. Presented at the AACPDM 68th Annual Meeting, San Diego, CA, p. 62.
406 Howard, J., Soo, B., Graham, H.K., Boyd, R.N., Reid, S., Lanigan, A., Wolfe, R., Reddihough,
407 D.S., 2005. Cerebral palsy in Victoria: motor types, topography and gross motor function.
408 J. Paediatr. Child Health 41, 479–483. https://doi.org/10.1111/j.1440-1754.2005.00687.x
409 Joumaa, V., Leonard, T.R., Herzog, W., 2008. Residual force enhancement in myofibrils and
410 sarcomeres. Proc. Biol. Sci. 275, 1411–1419. https://doi.org/10.1098/rspb.2008.0142

411 Joumaa, V., Rassier, D.E., Leonard, T.R., Herzog, W., 2007. Passive force enhancement in
412 single myofibrils. Pflugers Arch. 455, 367–371. https://doi.org/10.1007/s00424-007-
413 0287-2
414 Kerr, C., McDowell, B., McDonough, S., 2004. Electrical stimulation in cerebral palsy: a review
415 of effects on strength and motor function. Dev. Med. Child Neurol. 46, 205–13.
416 Kerr Graham, H., Selber, P., 2003. Musculoskeletal aspects of cerebral palsy. J. Bone Joint Surg.
417 Br. 85, 157–166.
418 Koh, T.J., Herzog, W., 1998. Excursion is important in regulating sarcomere number in the
419 growing rabbit tibialis anterior. J. Physiol. 508 (Pt 1), 267–280.
420 Lieber, R.L., Fridén, J., 2002. Spasticity causes a fundamental rearrangement of muscle-joint
421 interaction. Muscle Nerve 25, 265–270.
422 Lieber, R.L., Runesson, E., Einarsson, F., Friden, J., 2003. Inferior mechanical properties of
423 spastic muscle bundles due to hypertrophic but compromised extracellular matrix
424 material. Muscle Nerve 28, 464–471.
425 Linke, W.A., Fernandez, J.M., 2002. Cardiac titin: molecular basis of elasticity and cellular
426 contribution to elastic and viscous stiffness components in myocardium. J. Muscle Res.
427 Cell Motil. 23, 483–497.
428 Linke, W.A., Popov, V.I., Pollack, G.H., 1994. Passive and active tension in single cardiac
429 myofibrils. Biophys. J. 67, 782–792. https://doi.org/10.1016/S0006-3495(94)80538-7
430 Makarenko, I., Opitz, C.A., Leake, M.C., Neagoe, C., Kulke, M., Gwathmey, J.K., del Monte, F.,
431 Hajjar, R.J., Linke, W.A., 2004. Passive stiffness changes caused by upregulation of
432 compliant titin isoforms in human dilated cardiomyopathy hearts. Circ. Res. 95, 708–716.
433 https://doi.org/10.1161/01.RES.0000143901.37063.2f

Mathewson, M.A., Lieber, R.L., 2015. Pathophysiology of muscle contractures in cerebral palsy. Phys. Med. Rehabil. Clin. N. Am. 26, 57–67. https://doi.org/10.1016/j.pmr.2014.09.005

Mathewson, M.A., Ward, S.R., Chambers, H.G., Lieber, R.L., 2015. High resolution muscle measurements provide insights into equinus contractures in patients with cerebral palsy. J. Orthop. Res. Off. Publ. Orthop. Res. Soc. 33, 33–39. https://doi.org/10.1002/jor.22728

Matthiasdottir, S., Hahn, M., Yaraskavitch, M., Herzog, W., 2014. Muscle and fascicle excursion in children with cerebral palsy. Clin. Biomech. 29, 458–462.

Mirbagheri, M.M., Barbeau, H., Ladouceur, M., Kearney, R.E., 2001. Intrinsic and reflex stiffness in normal and spastic, spinal cord injured subjects. Exp. Brain Res. 141, 446–459. https://doi.org/10.1007/s00221-001-0901-z

Nagueh, S.F., Shah, G., Wu, Y., Torre-Amione, G., King, N.M.P., Lahmers, S., Witt, C.C., Becker, K., Labeit, S., Granzier, H.L., 2004. Altered titin expression, myocardial stiffness, and left ventricular function in patients with dilated cardiomyopathy. Circulation 110, 155–62. https://doi.org/10.1161/01.CIR.0000135591.37759.AF

Neagoe, C., Opitz, C.A., Makarenko, I., Linke, W.A., 2003. Gigantic variety: expression patterns of titin isoforms in striated muscles and consequences for myofibrillar passive stiffness. J. Muscle Res. Cell Motil. 24, 175–189. https://doi.org/10.1023/A:1026053530766

Oskoui, M., Coutinho, F., Dykeman, J., Jetté, N., Pringsheim, T., 2013. An update on the prevalence of cerebral palsy: a systematic review and meta-analysis. Dev. Med. Child Neurol. 62. https://doi.org/10.1111/dmcn.12080

Ottenheijm, C.A.C., van Hees, H.W.H., Heunks, L.M.A., Granzier, H., 2011. Titin-based mechanosensing and signaling: role in diaphragm atrophy during unloading? Am. J.

456 Physiol. Lung Cell. Mol. Physiol. 300, L161-166.
457 https://doi.org/10.1152/ajplung.00288.2010
458 Ottenheijm, C.A.C., Witt, C.C., Stienen, G.J., Labeit, S., Beggs, A.H., Granzier, H., 2009. Thin
459 filament length dysregulation contributes to muscle weakness in nemaline myopathy
460 patients with nebulin deficiency. Hum. Mol. Genet. 18, 2359–2369.
461 https://doi.org/10.1093/hmg/ddp168
462 Palisano, R., Rosenbaum, P., Walter, S., Russell, D., Wood, E., Galuppi, B., 1997. Development
463 and reliability of a system to classify gross motor function in children with cerebral palsy.
464 Dev. Med. Child Neurol. 39, 214–23.
465 Prado, L.G., Makarenko, I., Andresen, C., Krüger, M., Opitz, C.A., Linke, W.A., 2005. Isoform
466 diversity of giant proteins in relation to passive and active contractile properties of rabbit
467 skeletal muscles. J. Gen. Physiol. 126, 461–480. https://doi.org/10.1085/jgp.200509364
468 Rose, J., McGill, K.C., 2005. Neuromuscular activation and motor-unit firing characteristics in
469 cerebral palsy. Dev. Med. Child Neurol. 47, 329–36.
470 Sinkjaer, T., Magnussen, I., 1994. Passive, intrinsic and reflex-mediated stiffness in the ankle
471 extensors of hemiparetic patients. Brain J. Neurol. 117 (Pt 2), 355–363.
472 Smith, L.R., Lee, K.S., Ward, S.R., Chambers, H.G., Lieber, R.L., 2011. Hamstring contractures
473 in children with spastic cerebral palsy result from a stiffer extracellular matrix and
474 increased in vivo sarcomere length. J. Physiol. 589, 2625–2639.
475 https://doi.org/10.1113/jphysiol.2010.203364
476 Tatsumi, R., Hattori, A., 1995. Detection of Giant Myofibrillar Proteins Connectin and Nebulin
477 by Electrophoresis in 2% Polyacrylamide Slab Gels Strengthened with Agarose. Anal.
478 Biochem. 224, 28–31. https://doi.org/10.1006/abio.1995.1004

Tskhovrebova, L., Trinick, J., 2002. Role of titin in vertebrate striated muscle. Philos. Trans. R. Soc. B Biol. Sci. 357, 199–206. https://doi.org/10.1098/rstb.2001.1028

Udaka, J., Ohmori, S., Terui, T., Ohtsuki, I., Ishiwata, S., Kurihara, S., Fukuda, N., 2008. Disuse-induced preferential loss of the giant protein titin depresses muscle performance via abnormal sarcomeric organization. J. Gen. Physiol. 131, 33–41. https://doi.org/10.1085/jgp.200709888

Virtanen, P., Tolonen, U., Savolainen, J., Takala, T.E., 1992. Effect of reinnervation on collagen synthesis in rat skeletal muscle. J. Appl. Physiol. Bethesda Md 1985 72, 2069–2074.

Walker, S.M., Schrodt, G.R., 1974. I segment lengths and thin filament periods in skeletal muscle fibers of the Rhesus monkey and the human. Anat. Rec. 178, 63–81. https://doi.org/10.1002/ar.1091780107

Wang, K., McCarter, R., Wright, J., Beverly, J., Ramirez-Mitchell, R., 1991. Regulation of skeletal muscle stiffness and elasticity by titin isoforms: a test of the segmental extension model of resting tension. Proc. Natl. Acad. Sci. U. S. A. 88, 7101–7105.

Ward, S.R., Takahashi, M., Winters, T.M., Kwan, A., Lieber, R.L., 2009. A novel muscle biopsy clamp yields accurate in vivo sarcomere length values. J. Biomech. 42, 193–6. https://doi.org/10.1016/j.jbiomech.2008.10.004

Williams, P.E., Goldspink, G., 1984. Connective tissue changes in immobilised muscle. J. Anat. 138 (Pt 2), 343–350.

Wren, T.A.L., Cheatwood, A.P., Rethlefsen, S.A., Hara, R., Perez, F.J., Kay, R.M., 2010. Achilles Tendon Length and Medial Gastrocnemius Architecture in Children With Cerebral Palsy and Equinus Gait. J. Pediatr. Orthop. 30, 479. https://doi.org/10.1097/BPO.0b013e3181e00c80

502

503

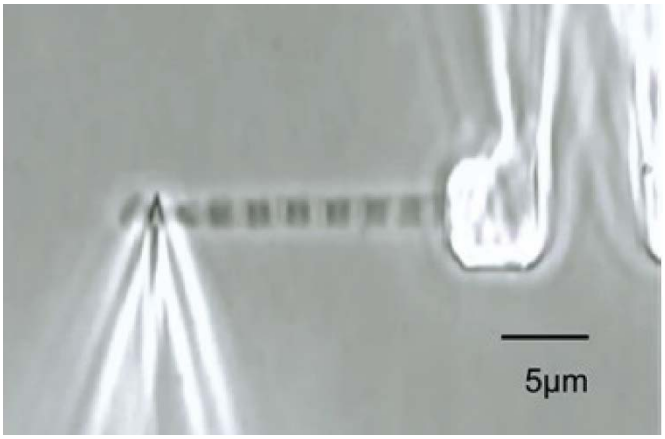


Figure 1

Figure 1: Testing apparatus for myofibril passive stress measurements. Here, an individual myofibril is attached at one end to a glass pipette which is connected to a motor used for specimen length control, and the other end to one of a cantilever pair used to measure force. The myofibril can be seen in the center of the image as a striated line delineating 7 sarcomeres in series.

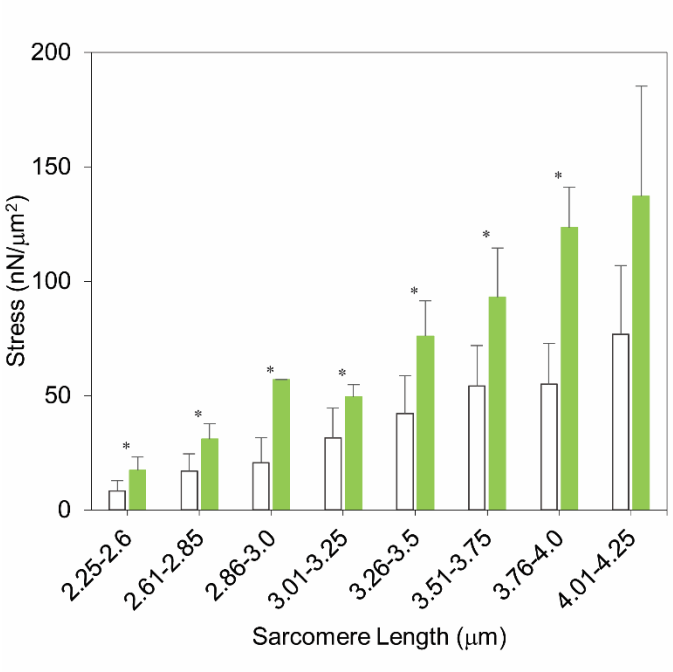


Figure 2

Figure 2: Passive stress generation versus sarcomere length for all data for CP and non-CP adductor longus (AL). Mean stress ± SD for CP-AL (white) and non-CP-AL (solid green) are significantly different at all sarcomere lengths tested, except for the longest range (>4μm). (*$p<0.05$). The non-significant result at the longest sarcomere lengths is explained by the reduced number of observations at this length compared to the other lengths.

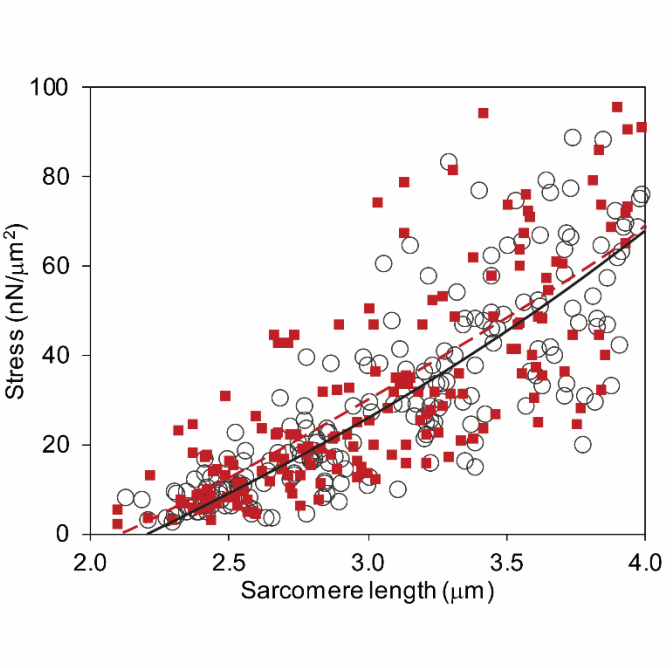
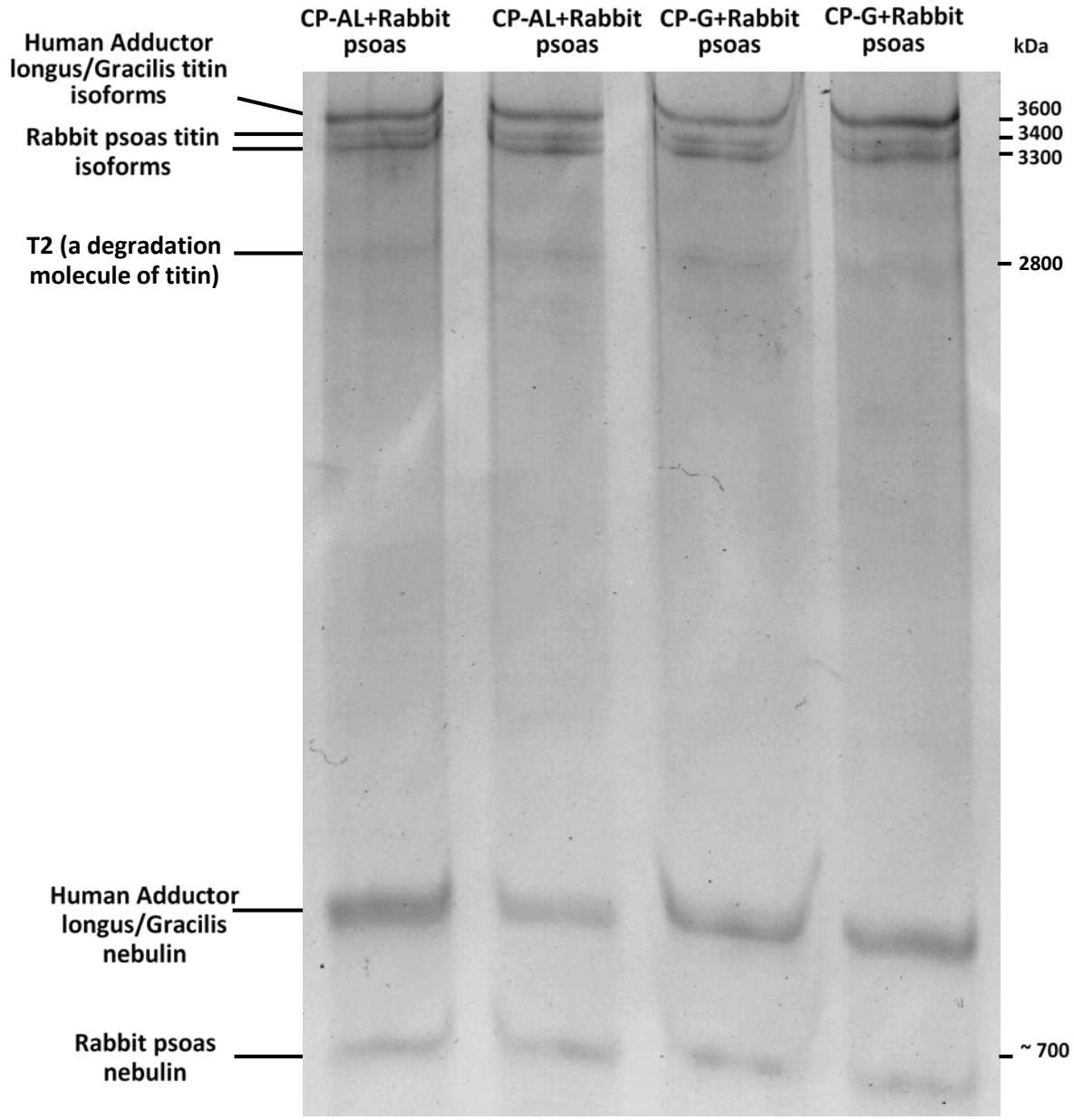


Figure 3

Figure 3: Stress versus Sarcomere Length for CP adductor longus and CP gracilis muscles. No significant difference was found when comparing the two CP muscles, CP-AL open white circles and black best-fit line, CP-gracilis solid red squares and red dashed best-fit line.

a)


CP-AL+Rabbit psoas
CP-AL+Rabbit psoas
CP-G+Rabbit psoas
CP-G+Rabbit psoas
kDa
Human Adductor longus/Gracilis titin isoforms
Rabbit psoas titin isoforms
T2 (a degradation molecule of titin)
Human Adductor longus/Gracilis nebulin
Rabbit psoas nebulin
3600
3400
3300
2800
~ 700

528

529

b)

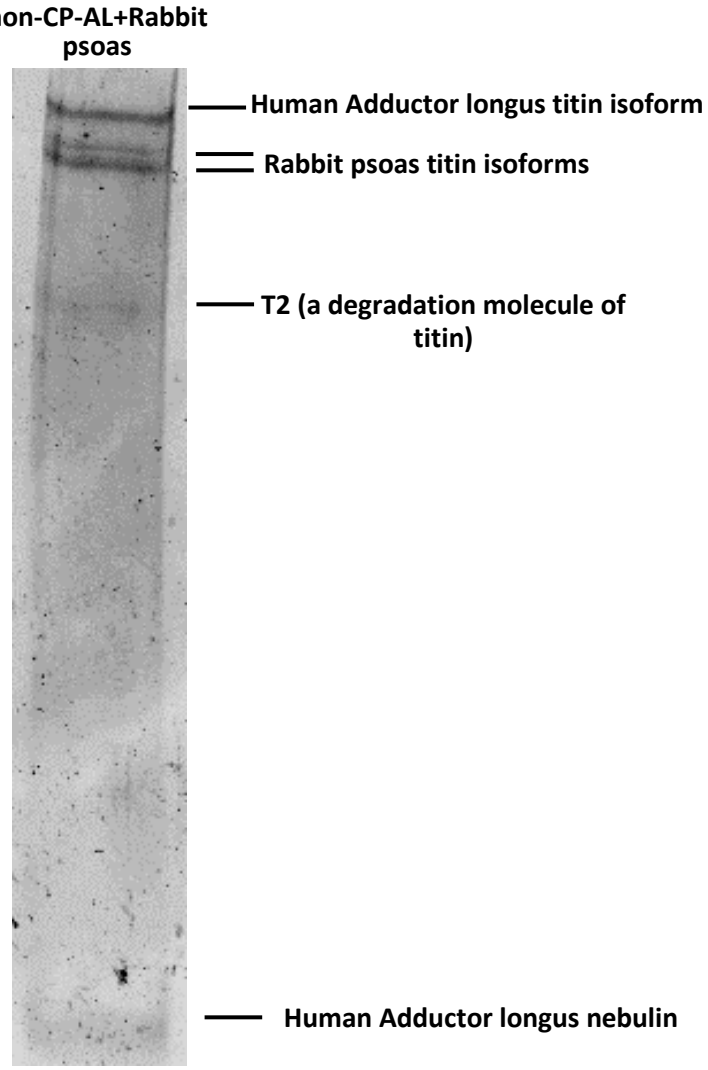


Figure 4

Figure 4: a) Agarose-strengthened 2% acrylamide gel. Extracts from rabbit psoas muscle were loaded in the same well with extracts from CP adductor longus and gracilis muscles in order to obtain an internal titin size standard. Titin size values were 3611±41 kDa, 3615±32 kDa and 3588±26 kDa, for CP-AL, non-CP-AL and CP-gracilis muscles respectively.

b) Control non-CP-AL with rabbit psoas. Faint T2 degradation band is present and human nebulin band is at the bottom of the gel. Rabbit nebulin was presumably present but is smaller and was likely pushed off the gel.

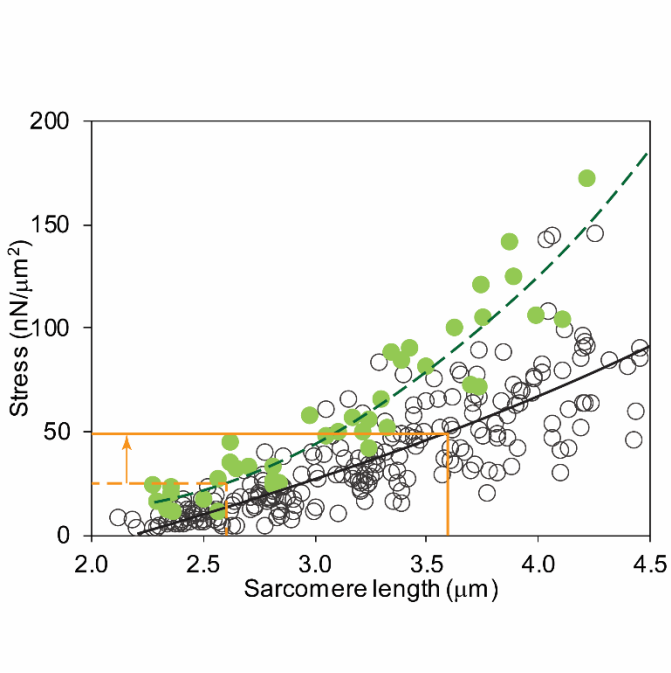


Figure 5

Figure 5: Adductor longus CP and non-CP myofibril Stress versus Sarcomere Length. CP myofibrils are under increased stress at *in vivo* sarcomere lengths (3.6 μm) compared to stress for typically developing children (SL about 2.6 μm). Best fit curves representing passive stress generation with all data points versus sarcomere length for CP-AL (black line, open white circles) and non-CP-AL (dashed green line, solid green circles) are displayed. While the CP-AL stresses were found to be lower for matched sarcomere lengths, at *in vivo* sarcomere lengths (CP=3.6μm: solid orange line) and (typically developing control =2.6μm: dashed orange line), the CP stresses are significantly higher (orange arrow) than for typically developing controls.