UNIVERSITY OF CALIFORNIA, SAN DIEGO

Multiscale Structure-Function Deficits in Muscle from Children with Cerebral Palsy

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy

in

Bioengineering

by

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2014

The Dissertation of Margie Ann Mathewson is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2014

DEDICATION

To my fantastic family,

for being unendingly supportive and encouraging my love of science, even when that meant having a cage of six inch long green caterpillars in the living room.

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LIST OF ABBREVIATIONS

CP	Cerebral palsy
ECM	Extracellular Matrix
Lf	Fiber length
GAST	Gastrocnemius
GR	Gracilis
GMFCS	Gross Motor Function Classification Score
Ls	Sarcomere length
Myf5	Myogenic factor 5
MyoD	Myogenic differentiation 1
MyoG	Myogenin
Rac1	Ras-related C3 botulinum toxin substrate 1
ST	Semitendinosus
SOL	Soleus
TD	Typically Developing
VCL	Vinculin

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The content of Chapter 1 is being prepared for submission to Physical Medicine and Rehabilitation Clinics of North America. The dissertation author was the primary author of this paper. The title and authors of the manuscript are as follows: Mathewson MA and Lieber RL. Skeletal Muscle Adaptations to Cerebral Palsy.

Chapter 2 has been submitted for publication to the Journal of Bone and Joint Surgery. The dissertation author was the primary author of this paper. The title and authors of the manuscript are as follows: Mathewson MA, Ward SR, Chambers HG, and Lieber RL. High Resolution Muscle Measurements Provide Insights into Equinus Contractures in Patients with Cerebral Palsy.

Chapter 3 has been submitted for publication to the Journal of Orthopedic Research. The dissertation author was the primary author of this paper. The title and authors of the manuscript are as follows: Mathewson MA, Chambers HG, Girard PJ, Tenenhaus M, Schwartz AK, and Lieber RL. Stiff Muscle Fibers in Calf Muscles of Patients with Cerebral Palsy Lead to High Passive Muscle Stiffness.

The material in Chapter 4 in part is being prepared for submission for publication by authors Mathewson MA, Meyer GA, Chambers HG, Engler A, and Lieber RL. The dissertation author was the primary investigator and author of this paper.

None of this would have been possible without the love and support of the author's family. They have been a source of encouragement from the very beginning. The author would also like to thank Joe Phaneuf for putting up with hearing about the highs and lows of graduate school for years and years. His calm presence and complete confidence in her abilities helped keep everything in perspective.

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Peer-Reviewed Research Articles

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ABSTRACT OF THE DISSERTATION

Multiscale Structure-Function Deficits in Muscle from Children with Cerebral Palsy

by

Margie Ann Mathewson

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Cerebral palsy (CP) is a heterogeneous disorder caused by an upper motor neuron lesion in the developing brain, leading to significant muscle impairments including muscle contractures, which limit range of motion around a joint. A deeper understanding of muscle adaptations in CP could lead to dramatic therapeutic improvements. The aim of this work, therefore, was to identify architectural and cellular components of muscle dysfunction in CP.

Chapter 1 introduces basic muscle physiology and gives an overview of previously reported muscular changes that occur in CP. **Chapter 2** presents the first simultaneous study of muscle fascicle and sarcomere length. In the soleus muscle, fascicle lengths appeared similar between patients with CP and typically developing (TD) individuals only because their sarcomeres were highly stretched. The combination of macro and microscopic data provide a clearer picture of the muscle in CP and point to reduced force production potential due to long *in vivo* sarcomere lengths.

To determine the implications of long sarcomeres on the *in vivo* passive mechanical environment in CP, **Chapter 3** measured passive fiber and fiber+extracellular matrix bundle stiffness in the soleus and gastrocnemius of patients with CP. At measured *in vivo* sarcomere lengths, both bundles and fibers appeared much stiffer in CP muscle. The similar stiffness of fibers and bundles at *in vivo* lengths indicated that the increase in stiffness was driven by stiffer fibers rather than by increase extracellular matrix stiffness.

To probe possible culprits for fiber alterations such as long sarcomeres and increased stiffness seen in Chapters 2 and 3, **Chapter 4** explored characteristics of satellite cells, muscle stem cells critical for growth and repair. These cells must be able to proliferate, move to sites of injury and growth, and differentiate to form mature fibers. Reduced CP satellite cell populations have been previously reported, so proliferation deficits were predicted. When proliferation, motility, and fusion were measured, however, no difference was seen in either proliferation or motility. Interestingly, differentiation and fusion capability was reduced in CP cells. This work paves the way for further studies into satellite cell function in CP, which could lead to completely new therapeutic directions.

Chapter 1

Introduction

1.1 Introduction to the Dissertation

Cerebral palsy, caused by an injury to the developing brain, leads to significant impairment of muscle function. Understanding changes at the level of the muscle in patients with CP is the focus of this research.

Chapter 2, which has been submitted for publication in the Journal of Bone and Joint Surgery, explores the architecture of the soleus muscle in patients with cerebral palsy. Compared to typically developing children, the soleus of patients with cerebral palsy has macroscopically similar muscle fibers. Deeper study reveals highly stretched sarcomeres and correspondingly fewer sarcomeres in series, leading to altered predicted force production and muscle excursion capability.

Chapter 3, which has been submitted for publication in the Journal of Orthopedic Research, extends the architectural information presented in Chapter 2 by considering passive mechanical characteristics of muscles involved in equinus contracture. This work showed that gastrocnemius and soleus fibers are passively stiffer in patients with CP, both at matched and *in vivo* sarcomere lengths. Stiff fibers appear to drive an overall increase in bundle stiffness at the long *in vivo* sarcomere lengths found in these muscles.

Chapter 4, which is being prepared for publication, characterizes the behavior of muscle stem cells, called satellite cells, which have been singled out as potential contributed to altered muscle fiber development and function. While proliferation and motility abilities of satellite cells isolated from patients with cerebral palsy appeared normal, differentiation and fusion capability was decreased in cells from these patients.

Chapter 5 summarizes the major results of this research, placing them in the broader context of clinical knowledge and research surrounding cerebral palsy.

1.2 Cerebral Palsy Causes and Characteristics

Cerebral palsy (CP) is a motor disorder caused by a nonprogressive injury to the developing brain (Rosenbaum *et al.*, 2007). The injury occurs perinatally, and while causes are rarely known (Bax *et al.*, 2005; Sankar & Mundkur, 2005), CP is more common in infants born preterm with small birth weights (Johnson, 2002). CP occurs in 2-3 of every 1000 live births (Arneson *et al.*, 2009) and has heterogeneous symptoms, anatomical involvement, and functional impairment, including lifelong changes in motor function (Bax *et al.*, 2005; Rosenbaum *et al.*, 2007). These alterations stem from both changes in the neural drive to muscles (Rose & McGill, 2005) and changes to muscles themselves. Spastic CP, which involves injury to the pyramidal system, is the most common form of CP, making up nearly 75% of all cases (Sankar & Mundkur, 2005). Spasticity has been defined as a "velocity dependent resistance to stretch" (Tilton, 2003). Limb involvement varies, with patients showing symptoms in either all four limbs (tetraplegia or quadriplegia), primarily on one side of the body including one upper and lower extremity (hemiplegia), or primarily in the lower extremities (diplegia) (Gorter *et al.*, 2004). Patient functional mobility can be classified using several rating scales, including the Gross Motor Function Classification System (GMFCS), which rates patient mobility on a scale of 1-5 from high to low function, respectively (Sankar & Mundkur, 2005; Rosenbaum *et al.*, 2007). While the injury associated with CP initially occurs in the developing brain, symptoms are commonly treated at the muscle level. Because the population affected with CP is large and heterogeneous, a better understanding, especially among clinicians and therapists, of muscular adaptations in CP may lead to improvements in treatment or even development of completely novel therapeutic strategies.

To understand the adaptations that occur in muscle from CP patients, it is important to first review how typically developing muscle functions.

1.3 Healthy Skeletal Muscle Structure and Function

1.3.1 Muscle Structure

The fundamental unit of muscle force production is the sarcomere. Sarcomeres produce force by the interaction of two proteins, actin and myosin. Force production is affected both by muscle velocity and by the amount of overlap between these two proteins, or sarcomere length. The sarcomere length-tension relationship has been characterized in the Blix curve (Blix, 1894). Sarcomeres are joined end to end in series to form myofibrils. Bundles of myofibrils form myofibers, or multinucleated muscle cells. These muscle fibers are joined into muscle fiber bundles, or fascicles (Fig. 1).

At each increasing size scale, extracellular matrix (ECM), the surrounding connective tissue, encapsulates muscle structures. Endomysium surrounds individual myofibers (Trotter & Purslow, 1992), perimysium surrounds muscle fascicles (Rowe, 1981), and epimysium surrounds the whole muscle (Järvinen *et al.*, 2002; Gao *et al.*, 2008) (Fig. 1). The composition and arrangement of these structures is important to muscle function and can vary in muscle disorders.

The extensive growth and regeneration capacity seen in muscle is due to its intrinsic stem cell population. The majority of these stem cells are called satellite cells (Mauro, 1961) and are found below the basal lamina of myofibers and are normally quiescent except when activated during times of muscle disease or injury (Schultz *et al.*, 1978). Satellite cell number and viability, rather than being constant throughout life, decreases with age or diseases that are characterized by extensive regeneration (Renault *et al.*, 2002). Conditions such as muscular dystrophy, which require constant regeneration of muscle fibers, are believed to eventually lead to exhaustion of the satellite cell population (Heslop *et al.*, 2000) and the concomitant loss in a muscle's ability to adapt to the new functional demand.

1.3.2 Plasticity

Muscle has strong regenerative capacity and can respond and change based on functional demands. For example, muscle fibers atrophy (leading to a decrease in muscle size) when subject to decreased use, aging, and some diseases. Serial sarcomere number can also change in response to growth (Williams & Goldspink, 1971), as well as limb immobilization with the muscle in a shortened or lengthened position. This serial sarcomere number change due to chronic muscle length change was shown in several classic studies in both mouse (Goldspink, 1968) and cat (Tabary *et al.*, 1972). These muscles, immobilized in a shortened position, rapidly adjust their sarcomere number to restore sarcomere length to previous values. A similar response was reported in a human case study of distraction osteogenesis in which a leg length discrepancy was corrected as a patients' bone was gradually lengthened over time (Boakes *et al.*, 2007). Sarcomere lengths were measured and sarcomere number calculated over the course of the treatment. As stretching of the bone and muscle occurred, sarcomere number rapidly increased and sarcomere length nearly returned to the pre-treatment value (Boakes *et al.*, 2007). Serial sarcomere number from patients with CP appears to be altered compared to typically developing muscles, however, suggesting that the plasticity seen in typically developing muscles may not be present to the same extent in CP.

1.4 Muscle Architecture and Function Adaptations in Cerebral Palsy

1.4.1 Force Production and Muscle Function

Alterations in gait, balance, and force production have been reported for patients with CP (Abel *et al.*, 1999; Moreau *et al.*, 2009; Ballaz *et al.*, 2010; Wren *et al.*, 2010; Moreau *et al.*, 2012). For example, knee extensor force decreases with CP, which can have serious implications for mobility (Moreau *et al.*, 2012). Overall voluntary force production is also decreased, as shown by many investigators (Ross & Engsberg, 2007; Tammik *et al.*, 2008;

Barber *et al.*, 2012). There is also evidence that greater cocontraction, or simultaneous activation of a muscle and its antagonist, occurs in CP (Damiano *et al.*, 2000). Compounding the problem of decreased force production, ankle stiffness was also shown to be 51% higher in CP, indicating increased resistance to passive ankle flexion (Barber *et al.*, 2011a).

1.4.2 Muscle Architecture

Muscles in spastic cerebral palsy often develop contractures, in which joint range of motion is limited and muscles appear functionally "short" (James, 2001). Many researchers have measured changes in muscle properties such as muscle belly size, muscle length, fascicle length, and sarcomere length all of which may help to explain this observation. Ultrasound is probably the most common tool used to describe basic muscle structural changes, such as fiber length and tissue thickness (Legerlotz *et al.*, 2010) (Fig. 2). Previous architecture studies focused primarily on the gastrocnemius muscle, an ankle plantarflexor and knee flexor that is commonly implicated in ankle equinus contractures (Mohagheghi *et al.*, 2007) and which also plays a role in knee flexion contractures. Ultrasound measurements show that gastrocnemius muscle volume is smaller in patients with CP. When affected and unaffected limbs were compared in hemiplegic patients, muscle volume was decreased by 28% in the affected gastrocnemius, and by nearly 50% than muscles of typically developing children (Malaiya *et al.*, 2007). Muscle belly lengths have also been shown to decrease in CP. One 3D ultrasound study of the medial gastrocnemius showed decreased muscle belly lengths, but no change in fascicle length when normalized to tibia length (Malaiya *et al.*, 2007). Based on the description of muscle structure presented above, it is clear that ultrasound alone cannot make functional predictions based on gross tissue dimensions since the composite sarcomeres cannot be detected using this modality.

In contrast with what might be expected for contractures, which are often considered

permanently contracted or shortened muscles, previous fascicle length measurements in patients with CP have been inconclusive. While some studies report shorter muscle fascicles in CP (Mohagheghi *et al.*, 2007, 2008), others report no difference between typically developing and CP fascicle lengths (Malaiya *et al.*, 2007; Barber *et al.*, 2011b). In contrast to the variety of results reported regarding for fascicle length, sarcomere length, the best predictor of active muscle force, have been consistently shown to be longer in CP patients (Fig. 3). While one study questioned the functional significance of sarcomere lengthening based on force measurements across the joint range of motion (Smeulders *et al.*, 2004), previous direct studies of sarcomere length show long sarcomeres in CP in both upper (Lieber & Fridén, 2002) and lower extremity flexors (Smith *et al.*, 2011). It therefore appears that sarcomere length regulation does not occur similarly in patients with typical development and those with CP, as CP sarcomeres do not maintain a relatively constant length as in normal development.

1.5 Tissue Properties

1.5.1 Mechanical Properties

While CP contractures are often thought to be stiff due to muscle overactivation, there are also critical contributions to stiffness that simply result from increased intrinsic passive stiffness of the tissue. A recent study explored the passive mechanical properties of two lower extremity muscles, the semitendinosus and gracilis (Smith *et al.*, 2011). Fibers, whose passive mechanical properties are thought to depend mainly on the giant structural protein titin, showed similar stiffness in typically developing and CP tissue of both muscles. Titin length was no different between the two groups, as would be expected from the similar fiber stiffness. Gracilis bundles were much stiffer than either typically developing bundles or bundles from the CP semitendinosus. CP semitendinosus bundles were also stiffer than their

typically developing controls (Fig. 4). This difference in stiffness is likely due to the contributions of the extracellular matrix at the bundle level. The change in bundle stiffness between typically developing and CP tissue suggests that collagen content has increased or that there is some type of abnormal organization in the muscle extracellular matrix. While increased collagen was observed in these muscles (Smith *et al.*, 2011), there are currently no tools available to quantify muscle extracellular structures accurately. Interestingly, earlier studies in the upper extremity yielded very different results, reporting stiffer fibers (Fridén & Lieber, 2003) but more compliant bundles in patients with CP (Lieber *et al.*, 2003) even though the ECM in CP muscle bundles tested in this study occupied more space than the ECM of typically developing controls (Lieber *et al.*, 2003). The differences in stiffness among muscles, potentially due to differences in ECM quality and arrangement, highlight the importance of studying muscles individually rather than making generalizations, especially in the case of highly heterogeneous disorders such as CP. In addition, it is important to develop new tools that can allow accurate measurement of muscle properties that have the greatest clinical relevance.

1.5.2 Histology

The histological profile of muscle from patients with CP varies (Fig. 5A). While the shape of individual muscle fibers may not change as drastically as it does in some other muscular disorders, most studies report a decrease in fiber size (Rose *et al.*, 1994; Marbini *et al.*, 2002) and an increase in fiber size variability (Rose *et al.*, 1994; Booth *et al.*, 2001). Moderate rounding of muscle fibers has also been reported (Booth *et al.*, 2001; Marbini *et al.*, 2002). In one study, capillary density was 30% lower in patients with CP (Pontén & Stal, 2007). Lipid content has been shown to increase in some cases (Rose *et al.*, 1994; Marbini *et al.*, 2002). Collagen content also appears increased (Booth *et al.*, 2001; Smith *et al.*, 2011) and

this has been correlated with increased muscle fiber bundle stiffness (Smith *et al.*, 2011). Some investigators have even reported a significant correlation between collagen increases and patient function, using the Modified Ashworth Scale and patient balance measurements (Booth *et al.*, 2001).

Alterations in myosin heavy chain, the major muscle contractile protein that determines fiber type, also changes in patients with CP, although the direction of these changes varies among muscles studied and techniques used (Fig. 5B). One study, using NADH and ATPase staining which reflect muscle oxidative capacity and contractile speed respectively, reported increased fiber size variability in patients with CP and also found that patients with CP were more likely to have a strong predominance of one fiber type over the other (either type 1 or type 2). These differences were often greater than 40%, while typically developing patients showed no predominance (Rose *et al.*, 1994). Another study using similar histochemical techniques in gastrocnemius biopsies reported increases in type 1 fiber percentage and decreases in type 2 fiber percentage, especially histochemically-defined 2B fibers (Ito *et al.*, 1996). In the adductor longus and triceps surea muscles, increased type 1 fiber percentage was reported with ATPase staining as well (Marbini *et al.*, 2002). A study of hamstring muscle in which myosin isoforms were electrophoretically separated demonstrated a 30% increase in type 1 myosin heavy chain percentage in patients with CP. When monoclonal antibodies were used to label different myosin heavy chain isoforms in the biceps brachii, a dramatic increase in type 2 fiber percentage was reported, however, with type 2x myosin heavy chain fibers making up 30% of CP muscle compared to 4% of TD (Pontén & Stal, 2007). Clearly, variation exists among muscles and further studies are needed to understand the impact of cerebral palsy on muscle fiber type predominance. From a

physiological perspective, however, fiber type percentage is not likely to cause dramatic functional impairment but rather, reflects an altered use pattern of the muscle.

1.5.3 Gene Expression

Another technique used to understand muscle at the cellular level is gene expression analysis. Using microarrays, it is possible to compare thousands of genes simultaneously. Two recent studies quantified muscle genome wide expression. The first, which compared muscles of the forearm, found distinct transcriptional differences between patients with and without CP (Smith *et al.*, 2009). Alterations were seen in multiple pathways, with important differences in ECM-related genes, a myosin heavy chain fiber-type shift toward faster myosin, oxidative metabolism decrease, and altered genes that allow excitation-contraction coupling (Smith *et al.*, 2009). The second study, which measured gene expression in hamstrings, found similar results including a dramatic increase in ECM production-related gene expression (which matched the collagen content measured from the same samples) and decreased oxidative metabolism gene expression (Smith *et al.*, 2012). These results agree with physiological observations in patients with CP. These transcriptional data appear to reveal a "confusion" in muscle cells that exist in the CP environment.

1.5.4 Stem Cells

In a very novel approach to understanding CP muscle, a recent publication highlighted the possible importance of certain muscle stem cells, called satellite cells, in patients with CP (Fig. 6). In one study, flow cytometry, a method used to count and isolate tagged cells of different types, was used to count cells in human biopsies (Smith *et al.*, 2013). While there was no change in either hematopoietic or endothelial cell numbers between groups, patients with CP had fewer than half as many satellite cells compared to typically developing control

subjects (Fig. 7A). This reveals decreased intrinsic satellite cell number or satellite cell depletion in CP and may indicate that this change contributes to abnormal sarcomere length, changes in material properties and contracture formation. The question as to the cellular mechanism that could explain these changes remain unanswered. However, there is the exciting possibility that, should this mechanism be determined, a new therapeutic approach to curing skeletal muscle contractures might be developed.

1.6 Summary

While cerebral palsy is caused by a brain injury, critical symptoms manifest in the muscle. Contractures and spasticity seen clinically correspond to changes in muscle sarcomere length, fiber type, ECM concentration, fiber and fiber bundle stiffness, and even stem cell numbers. Better understanding of muscular changes and development of new treatments that focus on these aspects might lead to new avenues for improving function in patients with cerebral palsy.

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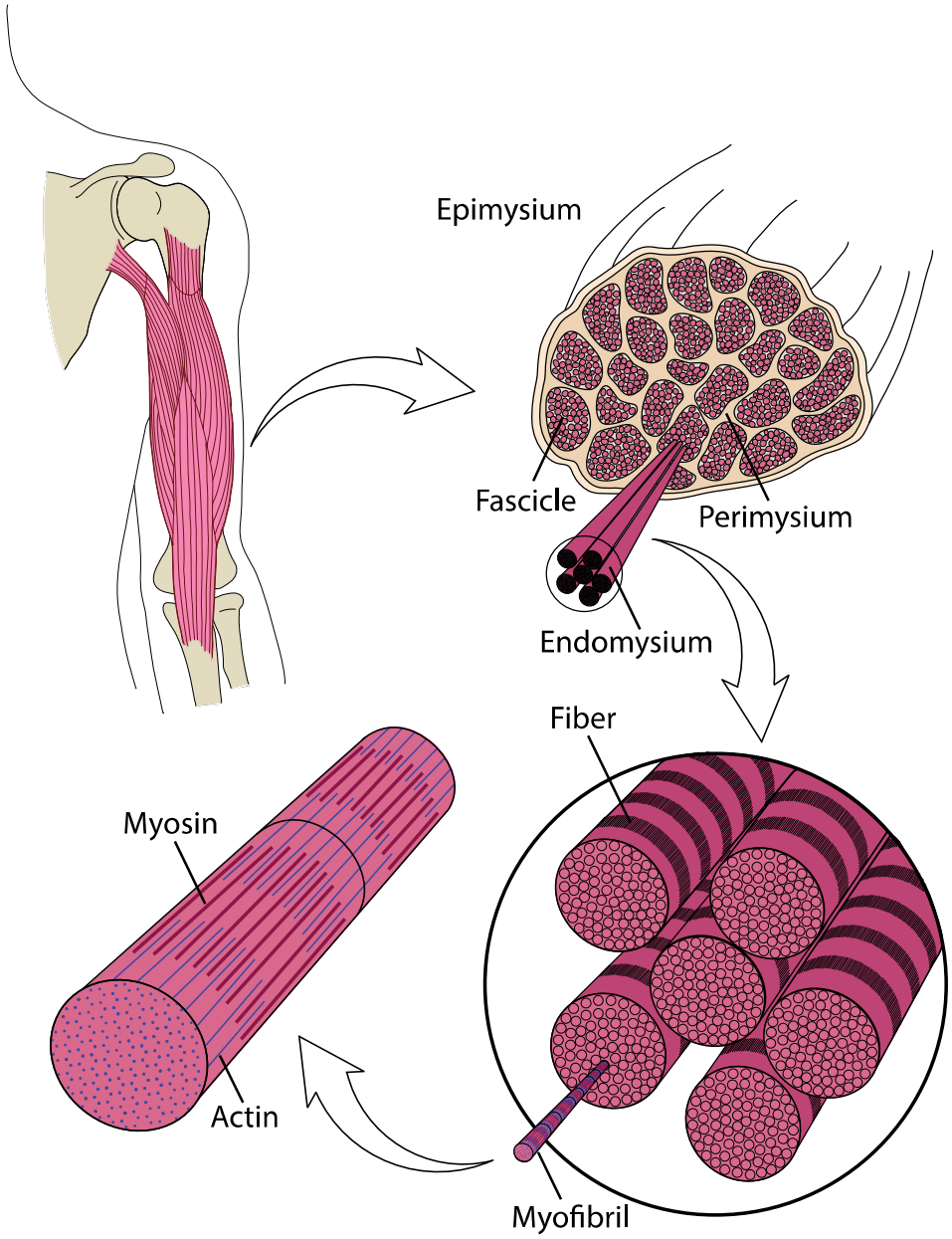

Figure 1.1. Structural hierarchy of skeletal muscle. Skeletal muscle is composed of bundles of muscle fibers called fascicles. Individual fibers in these fascicles consist of myofibrils, which are composed of the contractile proteins actin and myosin. Connective tissue, which surrounds the muscle at many levels, is organized into epimysium, surrounding the whole muscle, perimysium, surrounding fascicles, and endomysium, surrounding muscle fibers.

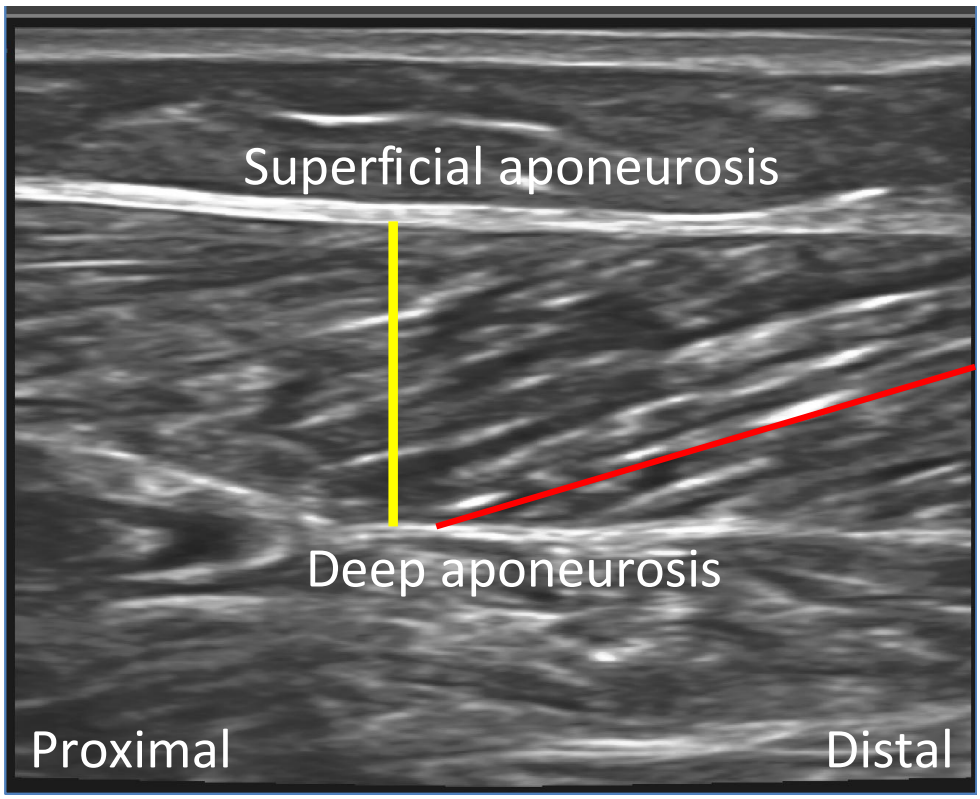

Figure 1.2. Typical ultrasound image of a human soleus muscle. Muscle appears dark in ultrasound and connective tissue light. Superficial and deep aponeuroses surround the muscle on either side. Red line indicates the path of a muscle fascicle, and yellow line represents the muscle belly thickness.

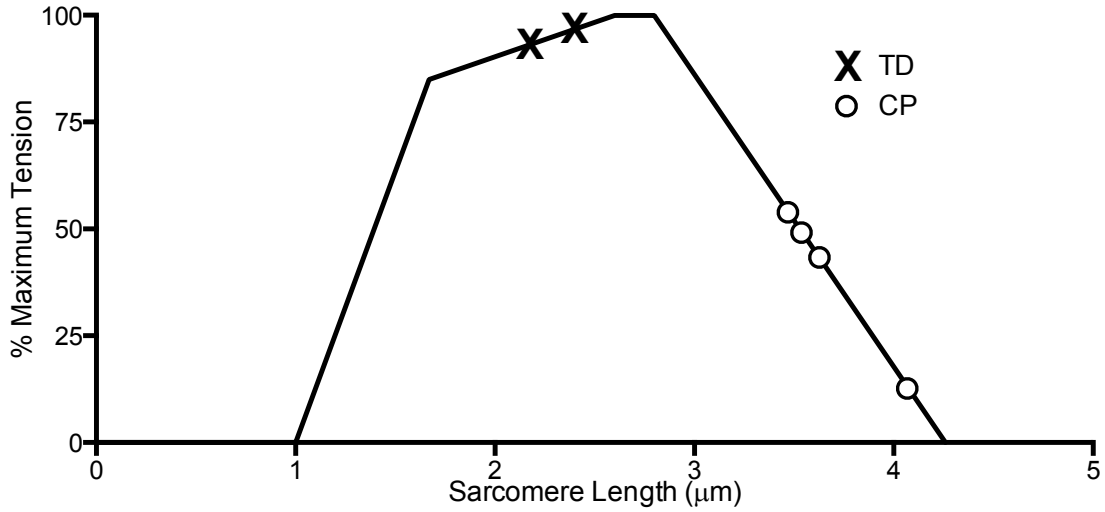

Figure 1.3. Schematic length-tension curve of skeletal muscle. When sarcomere length data for CP (O symbols) and TD muscles (X symbols) are plotted on this curve, sarcomeres clearly act in fundamentally different region of the curve for CP compared to TD subjects. For example, if TD sarcomeres are stretched, maximal force production will increase, while the opposite will happen for CP sarcomeres. Muscles represented include gracilis, semitendinosus, soleus, and flexor carpi ulnaris (Data replotted from previous studies Mathewson *et al*, submitted, and (Lieber & Fridén, 2002; Smith *et al.*, 2011))

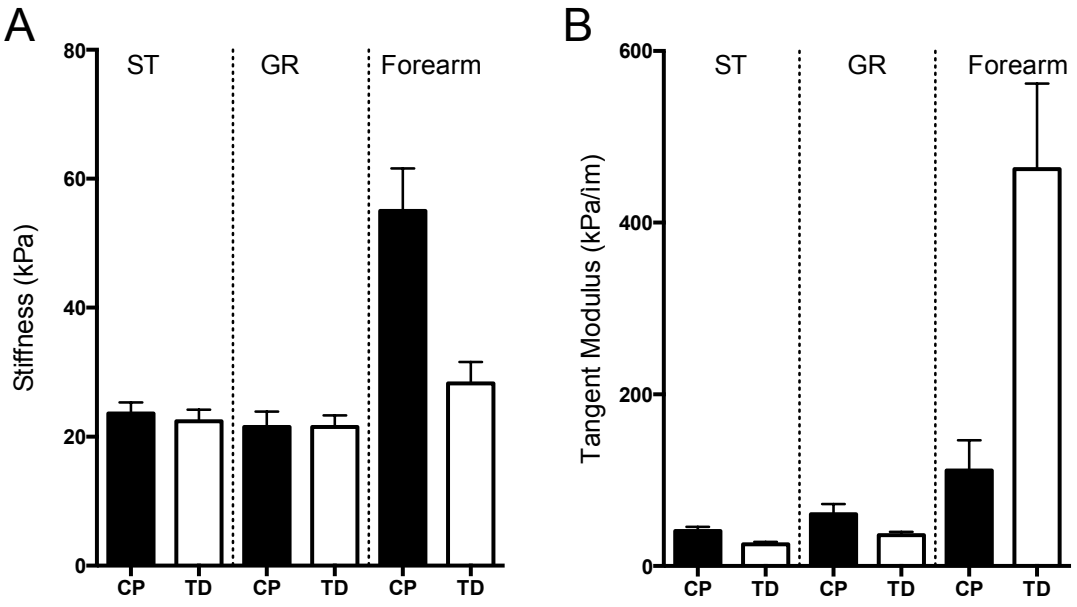

Figure 1.4. Comparison between fiber and bundle stiffness in CP and TD children. Data replotted from three previous studies (Fridén & Lieber, 2003; Lieber *et al.*, 2003; Smith *et al.*, 2011) Note that axes are not the same for A and B. **(A)** While fibers between CP and TD individuals are similar in stiffness in the gracilis (GR) and semitendinosus (ST), they are stiffer in the forearm muscles of individuals with CP. **(B)** Bundles of muscle fibers and their surrounding ECM were stiffer in CP in the GR and ST, but less stiff in the forearm.

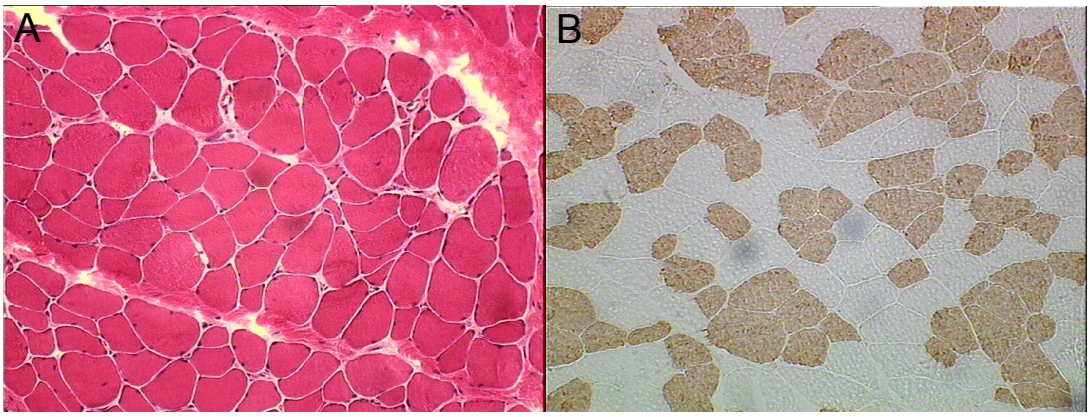

Figure 1.5. Light micrographs of muscle from a patient with CP. **(A)** Hematoxylin and Eosin permits evaluation of basic muscle tissue morphology. **(B)** ATPase histochemical staining permits determination of muscle fiber type.

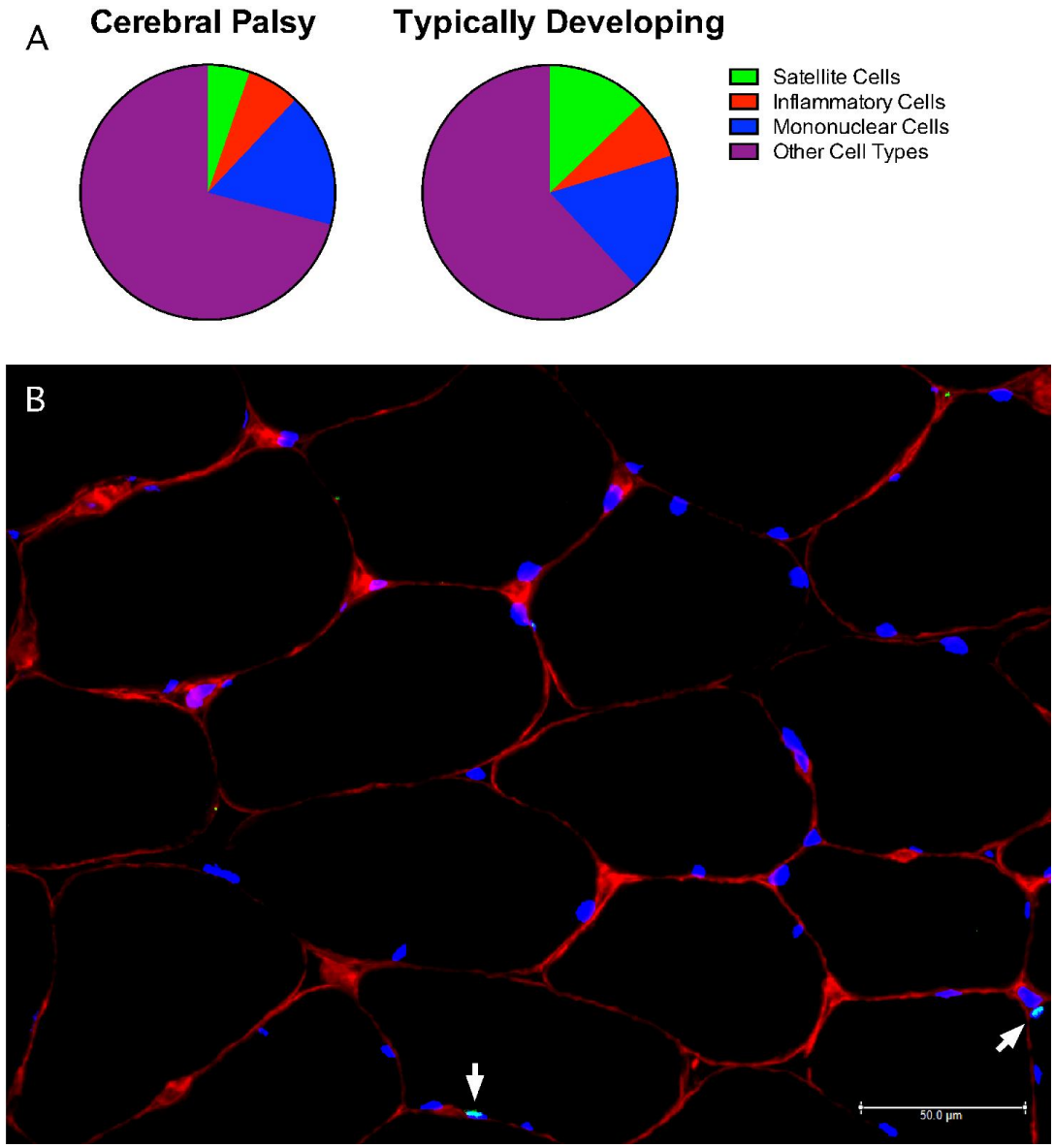

Figure 1.6. Fluorescence activated cell sorting (FACS) of several muscle mononuclear populations. **(A)** While the percentage of other cell types such as mononuclear cell and inflammatory cells are unchanged in patients with CP, the fraction of satellite cells in CP muscle is half of what is found in TD muscle. (data modified from Smith *et al. DMCN* 2012 **55**:3(Smith *et al.*, 2013)) **(B)** Immunohistochemical image of a satellite cell is shown in its native environment. Satellite cells are identified by looking for Pax7 positive staining (light green) on top of a cell nucleus (DAPI positive-blue) that is located under the basal lamina (laminin staining-red). Satellite cells are indicated with white arrowheads. (Image courtesy of the National Skeletal Muscle Research Center, www.muscle)

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Chapter 2

High Resolution Muscle Measurements Provide Insights into Equinus Contracture in Patients with Cerebral Palsy

2.1 Abstract

Muscle contractures that occur after upper motor neuron lesion are often surgically released or lengthened. However, surgical manipulation of muscle length changes a muscle's sarcomere length (L_s), which can affect force production. To predict the effects of surgery, both macro- (fascicle length (L_f)) and micro- (L_s) level structural measurements are needed. Therefore, the purpose of this study was to quantify both L_s and L_f in patients with cerebral palsy (CP) as well as typically developing (TD) children.

Soleus ultrasound images were obtained from children with CP (n=20, age=12.1±5.3 years) and TD children (n=21, 12.4±3.4 years). L_f was calculated from fascicle angle and distance between fascial planes. With the joint in the same position, CP biopsies were obtained, formalin fixed and L_s measured by laser diffraction. Since soleus L_s values were not measurable in TD children, TD L_s values were obtained using three independent methods. Myofilament length was also measured in age-matched TD and CP hamstring muscles.

While average L_f did not differ between groups (CP=3.6±1.2 cm, TD=3.5±0.9 cm; $p>0.6$), L_s was dramatically longer in children with CP (4.07±0.45 μm vs. TD=2.17±0.24 μm; $p<0.0001$) indicating that gross tissue dimensions were similar only because CP contractures were highly stretched internally.

While L_f values were similar between children with CP and TD children, this was due to highly stretched sarcomeres within the soleus muscle. Surgical manipulation of muscle-tendon unit length will thus alter muscle sarcomere length and change force generating capacity of the muscle. At constant neuromuscular activation, surgical lengthening of a muscle-tendon unit will result in either muscle strengthening or weakening, depending on pre-surgical L_s. In a muscle with sarcomeres highly stretched due to contracture, muscle-tendon unit lengthening could allow sarcomeres to shorten up the length-tension curve, producing

increased force. In the future, surgical outcomes could be improved by considering how procedures will specifically change sarcomere length, and therefore muscle force production, in individual patients.

2.2 Introduction

Surgical manipulation of muscle length is common in orthopedic surgery procedures, including tendon lengthening, total hip arthroplasty, and tendon transfer. Muscle length changes caused by surgery have functional consequences, since muscles are very length sensitive. The muscle length-tension curve, or "Blix" curve (Blix, 1894), defines the fraction of maximum muscle force produced as a function of length. Specifically, the sarcomere length-tension curve defines active muscle force as a function of *sarcomere* length (L_s) (Fig. 2.1). Sarcomeres are microscopic length-sensitive force generators in muscle whose properties are well known (Gordon *et al.*, 1966). While direct measurement of muscle length intraoperatively and indirect calculation of fascicle length (L_f) using ultrasound are fairly easy, neither of these methods provides insights into a muscle's L_s. Knowledge of L_s requires use of specialized instrumentation (Lieber *et al.*, 1984).

Because of the length-sensitivity of sarcomeres, muscles will either become stronger or weaker after surgery, depending on how their length is changed relative to their pre-surgical position on the length-tension curve. Specifically, for a muscle at a constant level of neural drive, if L_s is longer than optimal, increasing its length during surgery will *decrease* force production. Conversely, if L_s is shorter than optimal, increasing its length will *increase* force production. For this reason, intraoperative surgical decisions can make a muscle either weaker or stronger depending on starting conditions. Not only does L_s impact muscle function, but the number of sarcomeres in series also has an effect. If two muscles differ only in serial sarcomere number, the muscle with more sarcomeres in series will have greater excursion and

will be in a different position on the length-tension curve at a given joint angle compared to the other muscle (Loren *et al.*, 1996). Thus, for a surgeon to make a knowledgeable decision about muscle function due to surgery both L_s and serial sarcomere number must be known. While some investigators discuss L_f as it if were a surrogate for L_s (Lichtwark & Wilson, 2008), it is not. Long fascicles may be associated with short sarcomeres and *vice versa*, leading to different numbers of sarcomeres in series and different excursion and force production characteristics. Therefore, unambiguous evaluation of a muscle's condition requires simultaneous evaluation of both its L_f and L_s. Since sarcomeric force producing proteins actin and myosin dictate the dimensions of the length tension curve (Gokhin *et al.*, 2009), defining the length of these protein filaments is also important to define functional capacity.

One intraoperative situation in which a complete understanding of a muscle's condition may be especially useful is when performing surgery on patients with contractures due to cerebral palsy (CP). Muscle lengthenings are common in CP, as in surgical correction of equinus contracture (Etnyre *et al.*, 1993; Dreher *et al.*, 2012; Svehlik *et al.*, 2012), and, while this surgery is believed to improve patient function, there is evidence that overcorrection may be a complication (Segal *et al.*, 1989). While it is generally believed that fascicles in patients with CP are shorter compared to typically developing (TD) children, the literature is ambiguous (Barrett & Lichtwark, 2010). We suggest, based on the discussion above, that this is because L_f data have been interpreted in the absence of any L_s data. Therefore, the purpose of this study was to measure both L_f and L_s in patients with equinus contractures secondary to CP to more appropriately compare fiber length between children with and without CP.

2.3 Materials and Methods

2.3.1 Study Participants

All participants were recruited from the pediatric hospital's orthopedic department. The Institutional Review Board approved this study, and all patients and patient guardians provided informed consent or assent. Patients with CP were recruited from those undergoing tendon lengthening for ankle equinus contracture (n=20, age=12.1±5.3 years, mean±standard deviation) while TD patients were attending a clinic for injuries unrelated to the imaged leg (n=21, age=12.4±3.4 years). Participants with CP included all GMFCS levels and affected limbs (GMFCS I=3, II=11, III=2, IV=3, V=1; diplegic=10, hemiplegic=4, quadriplegic=6) and both genders (CP = 7 female, 13 male; TD = 11 female, 10 male).

2.3.2 Ultrasound Measurements of Fascicle Length

Ultrasound images of the medial aspect of the soleus were obtained with a t3200 Terason portable ultrasound system (Teratech Corporation, Burlington, MA, USA) in B-mode using a 15L4 Linear Array probe (variable frequency 4-15MHz) with constant settings for all patients. Participants sat in one chair and placed their foot on another (Fig. 2.2A). Before imaging, tibial length was measured as the distance between the medial malleolus and tibial plateau. For TD patients, ultrasound images were taken in two ankle orientations: 0° and 30° of plantarflexion. Ultrasound images of patients with CP were obtained in maximum passive dorsiflexion, ranging from 0° to 45° of plantarflexion (32.8°±7.9°, n=20), depending on contracture severity. Images were converted to TIFF format in OsiriX software (Rosset *et al.*, 2004) and quantified using ImageJ (Schneider *et al.*, 2012).

Soleus superficial and deep fascial planes were identified and the distance between them (d) was measured perpendicular to the aponeuroses (Fig. 2.2B). Fascicle angle (θ) corresponding to each distance measurement was also measured using the ImageJ angle

calculation tool (Fig. 2.2C). Five to ten points along the soleus with clearly identifiable fascicles were measured for each patient. L_f was calculated as the hypotenuse of the triangle corresponding to the angle (θ) and line between fascial planes (d) as previously described by others (Shortland *et al.*, 2002) using trigonometry. Values were averaged to yield a single L_f for each patient. Measurements were reproducible, with intraclass correlation coefficient 0.94 (n=4 CP, n=4 TD) for measurements made on separate days, similar to those reported elsewhere (Legerlotz *et al.*, 2010).

2.3.3 Muscle Sarcomere Length Measurements

In children with CP, intraoperative soleus biopsies from the region previously imaged were obtained with custom-made biopsy clamps (Ward *et al.*, 2009b). Ankles were set, with a goniometer, to the same angle as during ultrasound measurement (maximum dorsiflexion). A small section of tissue was isolated by blunt dissection and clamped. Care was taken not to stretch the tissue or disturb its *in vivo* position. Biopsies were fixed for three days in 10% buffered formalin, rinsed in phosphate buffered saline (PBS), and stored in fresh PBS until L_s determination. Thin tissue bundles were dissected from the biopsy at 10-15 sites and mounted on a glass slide using Permount mounting media (Fisher Scientific, Waltham, MA, USC) and then L_s was measured by laser diffraction as described previously (Lieber *et al.*, 1984) (Fig. 2.3A). At least 10 measurements were obtained per biopsy and average L_s was calculated for each patient. Because the soleus is almost never exposed during surgery in young TD patients, reported TD L_s are from a previous study (Ward *et al.*, 2009a) (n=19) in older adults (however, see below for other comparable values used). Plantarflexion in the previous study was slightly greater from in this study (TD= 49.0°±13.8°, CP=32.8°±7.9°), therefore, the L_s value from TD patients was mathematically adjusted (from 2.12 μm at 49.0° to 2.17 μm at 32.8°) using parameters from the previous work (Ward *et al.*, 2009a).

We acknowledge that the use of elderly cadaveric data for comparison to children with CP is not optimal but have waited over 2 years for informed consent from the parents of a TD child and yet failed to obtain it. Thus, we used three other independent approaches to estimate TD L_s values:

1) To establish "normal" human L_s values, we performed a complete meta-analysis of all published human muscle sarcomere length to calculate the confidence interval for human sarcomere length with the joint in a neutral position. Forty-four L_s values were obtained from six papers published between 1988 and 2011 (Cutts, 1988; Lieber *et al.*, 1994; Lieber & Fridén, 1997; Lieber & Fridén, 2002; Ward *et al.*, 2009a; Regev *et al.*, 2011). Values were screened for normality and then the mean and 99.99% confidence interval were calculated.
2) To determine the effect of age on L_s, we compared previously measured sarcomere lengths from younger and older subjects for several matched muscles. Specifically, we compared sarcomere lengths between 5 middle aged (50±6 years, n=14 for deltoid, n=20 for all other muscles) and elderly (89±12 years, n=12) rotator cuff muscles (Ward *et al.*, 2006; Altobelli, 2008; Altobelli *et al.*, submitted), young (39.8±11.73 years, n=5) and elderly (85.2±17.42 years, n=10) pelvic floor muscles ((Tuttle *et al.*, 2014) and personal communication), and 28 middle aged (49.7±5.7, n=13) and elderly (82.5±9.4, n=19-20) lower limb muscles ((Ward *et al.*, 2009a) and personal communication).
3) We derived normal soleus L_s from the most popular musculoskeletal modeling software, which contains muscle architectural data, moment arm values and explicitly includes tendon compliance. Using OpenSim (Delp *et al.*, 2007; Arnold *et al.*, 2010), we determined soleus L_f change over the joint range 30°

plantarflexion to 30° dorsiflexion and multiplied by the L_s used for normalization (2.7 μm) to determine the corresponding L_s over the range.

For our study, serial sarcomere number (i.e., number of sarcomeres in series within a fascicle) was calculated by combining ultrasound and L_s data as previously described (Boakes *et al.*, 2007) using the following equation:

$$N_s = \frac{L_f}{L_S}$$

where N_s is the serial sarcomere number, L_f is fascicle length, and L_s is sarcomere length. Both theoretical and experimental data demonstrate that serial sarcomere number is the best predictor of excursion capability of a muscle (Lieber & Fridén, 2000; Winters *et al.*, 2011).

2.3.4 Actin Filament Measurement

The two protein filaments critical for active muscle force production are composed of actin and myosin. While the myosin filament is consistent in length across muscles and species, actin filament length varies significantly (Page & Huxley, 1963; Granzier *et al.*, 1991; Burkholder & Lieber, 2001; Littlefield & Fowler, 2008). To determine whether systematic differences were present in myofilament length from patients with CP (which would lead to differences in predicted force production capability) we explicitly measured actin filament length in a separate cohort of patients from both groups. To obtain age-matched muscle samples for comparison, which were not available for the soleus, we measured filament lengths from the gracilis (GR) and semitendinosus (ST) muscles, both of which can be obtained in TD and CP subjects (Smith *et al.*, 2011),. Because Ls differences were also found between TD and CP tissue in these muscles, we believed they would be appropriate for thin filament length comparison. Tissue was collected from patients undergoing hamstring lengthening for CP-related contracture (n= 6 GR, n= 6 ST) or anterior cruciate ligament repair

with hamstring autograft (n= 4 GR, n= 4 ST). These samples were prepared using a modification of the protocol described previously (Gokhin *et al.*, 2012) and analyzed using distributed deconvolution software (Littlefield & Fowler, 2002). Briefly, samples were stretched, tied to stick, and immersed in relaxing solution (100mM KCl, 5mM EGTA, 5mM $MgCl_2$, 10mM imidazole, pH 7.0) overnight. They were then fixed in 4% Formalin for 24 hours, removed from the stick, and immersed in 15% sucrose followed by 30% sucrose in relaxing solution for 8-12 hours per solution. Samples were imbedded in OCT, frozen, and cut into 12 μm sections, then immunostained for actin (rhodamine-phalloidin, 1:100, Invitrogen, Carlsbad, CA), actin capping protein tropomodulin (affinity-purified rabbit polyclonal anti-human Tmod1 [R1749bl3c]), and a central sarcomeric protein α-actinin (EA53, 1:100; Sigma-Aldrich, St. Louis, MO) in order to mark the key regions of the thin filament and enable imaging (see Fig. 2.6A for measurement schematic). Slides were imaged with a Nikon TE2000-U microscope using a ×100/1.4 numerical aperture-oil objective lens at zoom 3. Actin lengths were quantified for both actin and tropomodulin stained images using distributed deconvolution software (Littlefield & Fowler, 2002) and average calculated for each patient group and muscle.

2.3.5 Data Analysis

Based on experimentally measured L_f variability (σ) of 1.22 cm for CP and 0.90 cm for TD patients, this study was powered at the 80% level to detect a 0.96 cm difference (~25%) in L_f. L_f, L_s, and serial sarcomere number. Statistical comparisons between groups were made using the Student's t-test. Significance level (α) was selected as 0.05. Data are represented as mean±standard deviation.

2.4 Results

2.4.1 Fascicle Length Comparison

Average soleus L_f was similar between patients with CP (3.6±1.2 cm) and TD individuals (3.5±0.9 cm) (Fig. 2.2D, p=0.64). It has been stated that L_f must be normalized to bone length to permit comparison between groups (Shortland *et al.*, 2004), but normalization of soleus L_f to tibial length neither changed the significance of the results (p=0.24) nor the resulting statistical power calculation.

2.4.2 Sarcomere Length Comparison

In contrast to the similar L_f observed between groups, L_s for patients with CP was 4.07±0.45 μm, which was dramatically longer (p<0.0001) compared to TD patients, 2.17±0.24 μm (Fig. 2.3B). Based on the length-tension properties of human sarcomeres, sarcomeres at 4.07 μm would produce much less force compared to sarcomeres at 2.17 μm, (Fig 3C). At a L_s of 4.07 μm, decreasing L_s by tendon lengthening will allow the muscle to generate greater force while, at a L_s of 2.17 μm, decreasing L_s by tendon lengthening will result in decreased force. This logic makes it obvious that tendon lengthening could make TD sarcomeres weaker but sarcomeres from patients with CP stronger, which may represent a fundamental difference between groups.

Independent sarcomere length comparisons supported the use of the elderly TD L_s discussed above. Meta-analysis of 44 previously measured sarcomere lengths had a mean of 2.67 μm and 99.99% CI=2.43-2.91 (Fig. 2.4). Based on these measurements, the CP sarcomere length was highly significantly different from previously measured sarcomere lengths (p<0.0001). None of the 36 comparisons between younger and elderly sarcomere lengths in the rotator cuff, pelvic floor, and lower limb were significantly different between age groups. Paired values were highly similar for all muscles, with a mean difference between

young and old of 0.036 μm. Based on this average young-old difference, the difference between young CP and elderly TD samples, nearly 2 μm, is more than 14 standard deviations from the mean and extremely unlikely to be due to age. Using OpenSim, soleus sarcomere length at 30° of plantarflexion is predicted to be 1.17 μm, a value that is clearly much smaller than realistic *in vivo* values. Approximations made during model construction may have lead to overly short estimates of sarcomere length in the soleus. Since modeling results were much shorter than any experimentally measured sarcomere length, we believe cadaveric values are more likely to provide an accurate comparison than the model. Values predicted by OpenSim range from 1.17 μm at 30° of plantarflexion to 3.60 μm at 30° of dorsiflexion. Thus, using every comparative method that we can envision, children with CP have dramatically longer sarcomeres compared to our estimates of normal soleus L_s or normal L_s in other muscles (Fig. 2.4).

2.4.3 Serial Sarcomere Number

Combining the L_f and L_s data yielded a value for serial sarcomere number that was 40% lower ($p<0.0001$) in patients with CP (9,190±3,810) compared to serial sarcomere number (using previous L_s data) in TD individuals (16,040±4,160) (Fig. 2.5). Since serial sarcomere number is an indicator of a muscle's excursion ability, and patients in both groups have similar bony dimensions, patients with CP have fascicles with a greatly decreased capacity for joint rotation. This may represent a fundamental change that limits range of motion in patients with contractures.

2.4.4 Actin Filament Length Measurement

Measurements of actin filament length using either phalloidin (Fig. 2.6B) or tropomodulin (Fig. 2.6C) labeling showed no difference between TD patients and those with

CP in either muscle (using phalloidin , GR: CP=1.04±0.12 μm, TD=1.16±0.07 μm; ST: CP=1.08±0.12 μm, TD=1.03±0.19 μm; using tropomodulin, GR: CP=1.18±0.18 μm, TD=1.01±0.17 μm; ST: CP=1.05±0.15 μm, TD=0.90±0.14 μm). These data demonstrate that the basic contractile protein dimensions in the gracilis and semitendinosus are similar between patients with CP and TD, indicating that dimensions of their length-tension curves are comparable.

2.5 Discussion

The purpose of this study was to integrate macroscopic muscle properties (fascicle length) with its microscopic properties (L_s) in order to understand structural changes in muscle contractures that can provide insights into surgical decision-making. Considering only L_f, which is the norm in the literature, muscle function would be predicted to be similar between patients with CP and TD patients since this value was similar between groups (Fig. 2C). However, sarcomeres are much longer in CP (Fig. 2.3B), which suggests these muscles would actually have a significant force production deficit (Fig. 2.3C). Using both L_f and L_s values, we calculated that there are fewer serial sarcomeres in patients with CP, which would lead to a significant deficit in muscle excursion. This study clearly demonstrates that, without considering L_s, imaging modalities measuring only macroscopic muscle features may provide incomplete or even misleading information.

Muscle weakness is a well-known feature of CP (Damiano *et al.*, 1995; Wiley & Damiano, 1998; Damiano *et al.*, 2001; Elder *et al.*, 2003; Stackhouse *et al.*, 2005), but previous studies have not been able to explain weakness based on measurements of muscle size or volume (Elder *et al.*, 2003; Moreau *et al.*, 2012). L_f alone in patients with CP also offers no explanation for muscle weakness (Moreau *et al.*, 2012) and fiber area is only modestly decreased secondary to CP (Fridén & Lieber, 2003; Smith *et al.*, 2011). For these

reasons, weakness is often primarily attributed to neuromuscular control, which is clearly altered in patients with CP (Rose & McGill, 2005). Although we agree that neuromuscular control is impaired in this patient population, this study demonstrates for the first time a clear candidate for muscle weakness—reduced serial sarcomere number and increased L_s. While L_f is similar between these two groups, it is only because sarcomeres in the patients with CP are highly stretched.

Previous studies of serial sarcomere number development in mice (Goldspink, 1968; Williams & Goldspink, 1971), rats (Koh & Tidball, 1999; Shrager *et al.*, 2002), rabbits (Fridén *et al.*, 2000; Takahashi *et al.*, 2010), cats (Tabary *et al.*, 1972), and goats (Lindsey *et al.*, 2002) demonstrated that, when a muscle is stretched, as during bone growth, serial sarcomeres are added to maintain original L_s. Sarcomere number adaptation has even been shown in a human muscle case of adolescent distraction osteogenesis (Boakes *et al.*, 2007). As such, the idea that sarcomere number adapts to surgical muscle length change has permeated the surgical literature. Data collected in this study, however, suggest that L_s regulation in patients with CP may differ from that of TD children. Strain from bone growth, which would normally cause an increase in serial sarcomere number, does not appear cause the appropriate sarcomere number increase in patients with CP, leading to a muscle with normal L_f but highly stretched sarcomeres. These findings have profound clinical implications for patients with CP. If surgeons assume that bone growth is accompanied by corresponding L_f changes (due to the addition of serial sarcomeres), this will be incorrect. Previous models of force production after surgical intervention, even when taking into account reduced serial sarcomere number, have assumed TD L_s in patients with CP (Delp *et al.*, 1995). This assumption leads to inaccurate estimates of force production changes with surgery.

Data from this study suggest that muscle-tendon lengthening of contractures, along with improving joint range of motion, could theoretically lead to improved muscle force production. If a simple muscle-tendon lengthening leads to muscle fiber shortening, sarcomeres would correspondingly shorten and be placed on a more favorable portion of the length-tension curve. This would lead to an increase in the intrinsic ability of the muscle to generate force.

While improved force production with surgical lengthening has been shown in two long-term studies of muscle-tendon lengthening for equinus contracture, in which persistent force production improvements were seen (Orendurff *et al.*, 2002; Dreher *et al.*, 2012), other sources indicate that deficits in force production and function can result from surgeries (Borton *et al.*, 2001; Dietz *et al.*, 2006). These conflicting results highlight the importance of considering the specifics of each patient and each surgical procedure when making predictions and point to the need for a fully integrated study of children with CP in which muscle properties and motor control are both appropriately quantified to determine the physiological basis of weakness.

The current data also make it clear that, depending on the surgery, sarcomeres might shorten or lengthen, putting them on very different force producing regions of the length tension curve. Differences in muscle-tendon lengthening methods (such as lengthening of gastrocnemius aponeurosis instead of tendon) could lead to different muscle architecture outcomes. For example, if a muscle-tendon unit is lengthened at the level of the muscle, sarcomeres might be stretched in some regions rather than allowed to shorten, which could lead to decreased force production. In simple cases that allow muscle fiber shortening, muscle-tendon lengthenings in CP might reduce disability associated with contracture by placing sarcomeres in a more favorable region of the sarcomere length-tension curve. However, the

surgeon also relies on passive tension of the muscle to correct joint contracture, and the patient relies on correct passive tension for function, so further muscle-tendon unit lengthening may result in a muscle that is too passively "slack." Thus, these L_s data must be used in conjunction with intraoperative decision-making regarding joint position and muscle passive tension as well.

2.6 Limitations

The main limitation of this study is the lack of age-matched soleus L_s data from TD children. (Note that, while we obtained IRB approval to obtain soleus biopsies from young TD patients, over a 2-year period we never had the opportunity to ask for even a single consent.) The primary comparative data for TD soleus L_s were obtained from cadaveric studies of elderly individuals (Ward *et al.*, 2009a). While using age-matched control soleus data would be ideal, the difference in L_s between groups is so great that we believe the overall effect is not in question, only the magnitude. However, acknowledging this limitation, we implemented three other independent methods to determine the degree to which soleus L_s values measured were abnormal. First, we compiled 44 human sarcomere lengths from 6 previously published reports (see Methods; Fig. 2.4). Our average L_s value of 4.07 μm exceeds the 99.99% confidence interval by 1.16 μm. In fact, given the normal distribution of sarcomere lengths generated by this data set, the probability that a 4.07 μm L_s would be considered normal is only 0.016%. Second, we have performed studies of sarcomere length in cadaveric tissues in older and younger subjects for 5 rotator cuff muscles (Ward *et al.*, 2006; Altobelli, 2008; Altobelli *et al.*, submitted), 3 pelvic floor muscles (Tuttle *et al.*, 2014), and 28 lower limb muscles (Ward *et al.*, 2009a). For all 36 muscles explicitly studied, sarcomere lengths were universally relatively short [mean = 2.74±0.33 μm; n=72 groups (1 younger and 1 elderly per muscle)] and were never significantly different in paired muscles as a function of age. These

data agree with previous studies in mammals, in which increasing muscle L_f during development was shown to occur mostly by sarcomere number increase (Goldspink, 1968; Williams & Goldspink, 1971; Yamashita *et al.*, 2007). Therefore, we do not expect a dramatic L_s difference between children and adults, but instead L_f differences. Finally, using musculoskeletal modeling software, predicted soleus L_s values ranged from only 1.17 μm in full plantarflexion to 3.60 μm in full dorsiflexion (dotted line, Fig. 2.4), still very different from the 4.07 μm measured here. Therefore, given the exceedingly low probability that a sarcomere length of 4.07 μm could be obtained from a normal human muscle, the lack of an age effect, the short sarcomere lengths measured from cadaveric specimens, and the very short sarcomere lengths derived from modeling software, we are confident that our conclusion that soleus L_s in CP is very long is justified.

Another limitation of the study was that actin filament measurements were made in the semitendinosus and gracilis, rather than in the soleus. Sarcomere length increases seen in the soleus, however, are similar to those in the gracilis and semitendinosus of children with CP. Therefore, we suggest that actin filament differences are unlikely to be the driver of sarcomere length differences in the soleus, since they do not appear to play a role in the gracilis or semitendinosus.

2.7 Conclusions

This is not the first study to report long sarcomeres in patients with CP, which was demonstrated in forearm (Lieber & Fridén, 2002; Pontén *et al.*, 2007) and hamstring muscles (Smith *et al.*, 2011). It is, however, the first study to measure both L_s and L_f in the same muscle of the same patient. Without knowing both values, it is impossible to determine how many sarcomeres the muscle has in series, and this serial sarcomere number is what actually determines the excursion capability of a muscle. Our results clearly demonstrate the

importance of knowing both values for proper surgical decision-making. Patents with CP have highly stretched fascicles that appear normal by ultrasound but have highly stretched sarcomeres at the microscopic level. Because L_s regulation appears impaired in these patients, therapies designed under the assumption of normal sarcomere number addition may not have the expected effect. Stretching, for example, might increase muscle weakness if it makes already-stretched sarcomeres even longer. Taken together, these data highlight the importance of a comprehensive understanding of a muscle's macroscopic and microscopic properties for appropriate surgical decision-making. Future studies must be performed to determine the biological basis for these muscular abnormalities with the hope that novel therapies could be developed to resolve contractures using state-of-the-art biological approaches.

2.8 Acknowledgements

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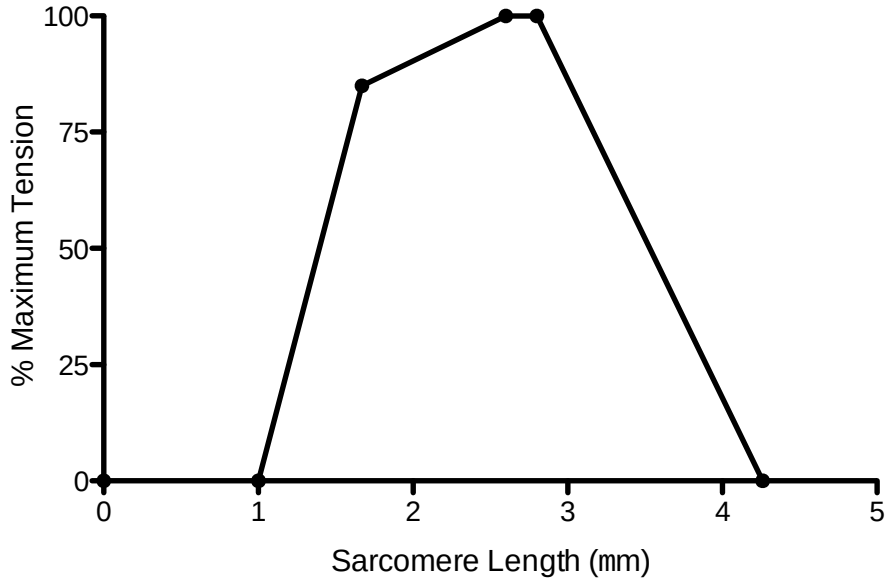

Figure 2.1. Muscle length-tension curve derived for human muscle. This curve was created using filament length data from a previous report (Lieber *et al.*, 1994) based on the sliding filament theory elucidated by Gordon et al. (Gordon *et al.*, 1966).

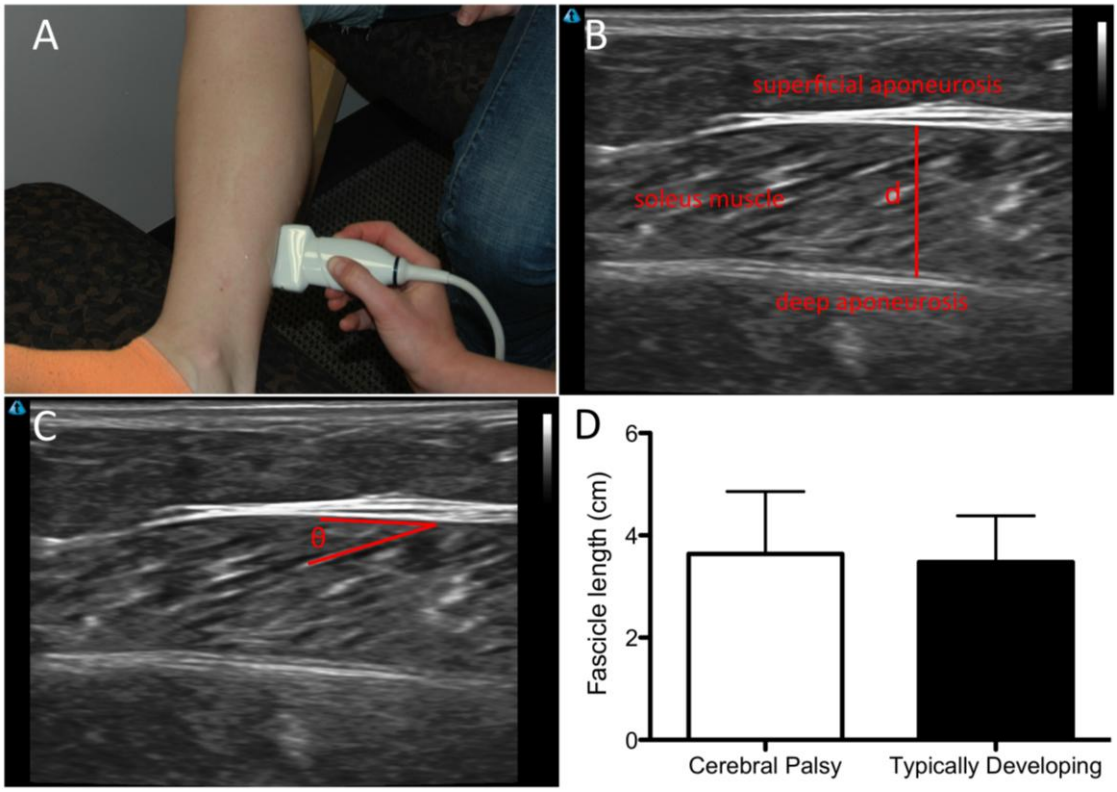

Figure 2.2. Method for fascicle length measurement in human subjects. A) Example of ultrasound leg positioning. B) Distance measurement from superior to inferior soleus fascial planes on an ultrasound image from a patient with CP. Red line one the image indicates the location of a measurement of d. C) Fascicle angle measurement with respect to the upper fascia plane on an ultrasound image. D) Ultrasound soleus fascicle length measurements of patients with CP and patients with typical development demonstrate that fascicle lengths were not significantly different between the two groups (p=0.64).

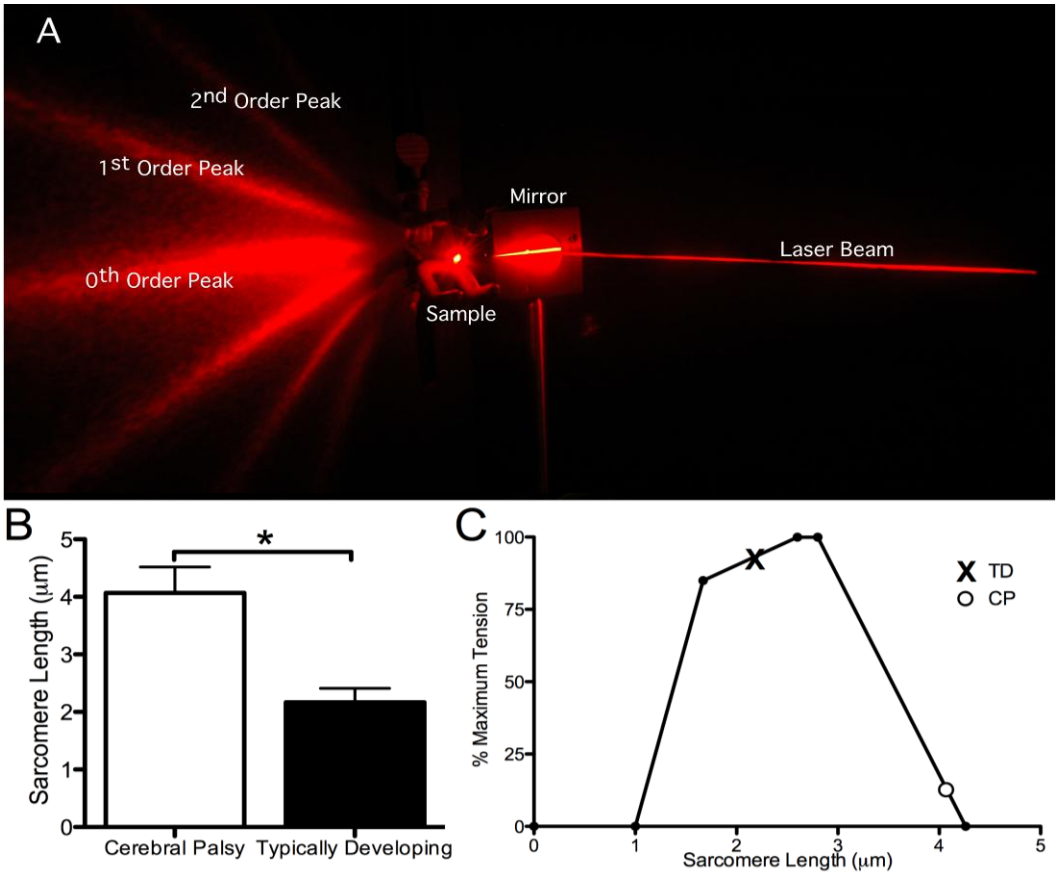

Figure 2.3. A) Photograph of the laser diffraction method for measuring muscle sarcomere length. Zeroth, first, and second order diffraction lines are highlighted in the image. B) Soleus sarcomere length from patients with CP and elderly TD patients were significantly different ($p<0.0001$). Soleus sarcomere length for patients with CP were measured in the group of patients whose fascicle lengths were measured by ultrasound, while TD values were taken from a previous report from our laboratory which used cadaveric specimens (Ward *et al.*, 2009a). (However, see Discussion for other comparisons.) C) Comparison of theoretical force production for a muscle from a patient with CP (represented by an open circle) vs. TD (represented by an "X") shows that muscles from patents with CP compared to TD patients are located on very different positions of the sarcomere length-tension curve.

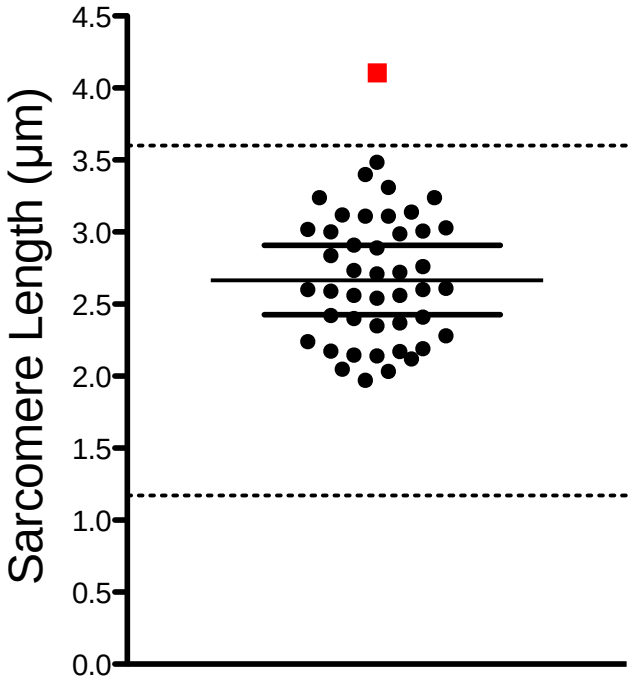

Figure 2.4. Comparison of forty-four previously published sarcomere length values from across the body (Cutts, 1988; Lieber *et al.*, 1994; Lieber & Fridén, 1997; Lieber & Fridén, 2002; Ward *et al.*, 2009a; Regev *et al.*, 2011) (black circles) to measured CP soleus sarcomere length (red square). Black bars represent the mean ± 99.99% confidence interval. Dashed lines indicate OpenSim L_s range predictions from 30° of plantarflexion to 30° of dorsiflexion.

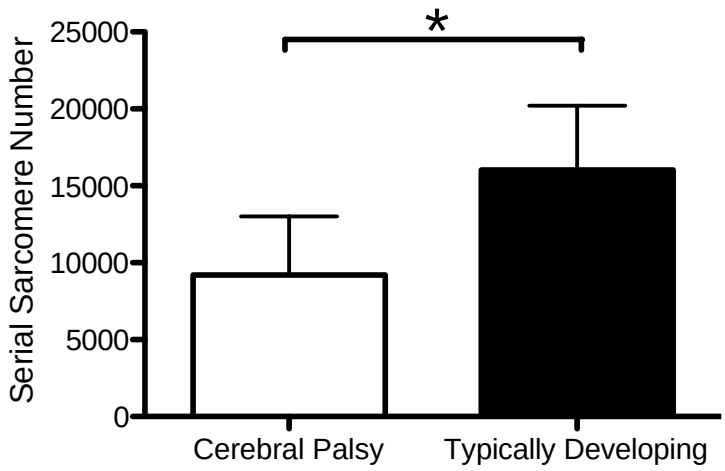

Figure 2.5. Calculated serial sarcomere number in the soleus of CP patients ($p<0.0001$). Patients with CP had nearly half the number of serial sarcomeres as patients with TD, which predicts highly reduced excursion.

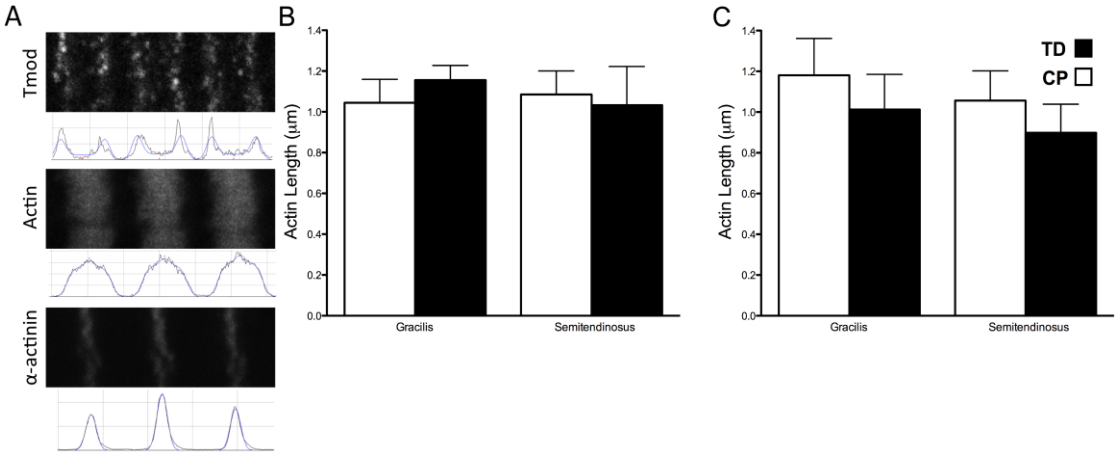

Figure 2.6. A) Schematic showing immunolabeled sarcomere components and their deconvolution program profiles that enable explicit filament length measurements. Labels are, from top to bottom, tropomodulin, Phalloidin, and α-actinin. **B)** Phalloidin and **C**) tropomodulin measurements of actin filament length show no significant difference ($p>0.1$) between TD patients and those with CP.

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Chapter 3

Stiff Muscle Fibers in Calf Muscles of Patients with Cerebral Palsy Lead to High Passive Muscle Stiffness

3.1 Abstract

Cerebral palsy (CP), caused by an injury to the developing brain, can lead to alterations in muscle function. Subsequently, increased muscle stiffness and decreased joint range of motion are often seen in patients with CP. We examined mechanical and biochemical properties of the gastrocnemius and soleus muscles, which are involved in equinus muscle contracture. Passive mechanical testing of single muscle fibers and fiber bundles from gastrocnemius and soleus muscle of patients with CP showed a significant increase in fiber stiffness ($p<0.01$), while bundles showed no stiffness difference ($p=0.28$). When *in vivo* sarcomere lengths were measured and fiber and bundle stiffness compared at these lengths, both fibers and bundles of patients with CP were predicted to be much stiffer *in vivo* compared to typically developing (TD) individuals. Interestingly, differences in fiber and bundle stiffness were not explained by typical biochemical measures such as titin molecular weight, (a giant protein thought to impact fiber stiffness), or collagen content, (a proxy for extracellular matrix amount). We suggest that the passive mechanical properties of fibers and bundles are thus poorly understood.

3.2 Introduction

Cerebral palsy (CP) is caused by an injury to the brain during fetal development (Rosenbaum *et al.*, 2007). Altered muscle function commonly occurs in patients with CP. These patients frequently form muscle contractures, in which joint range of motion is reduced (James, 2001; Rosenbaum *et al.*, 2007). While the root cause of these contractures is the perinatal brain injury, the way in which muscular changes lead to contracture is still not fully understood. Several classifications systems for CP exist, including the Gross Motor Function Classification System (GMFCS), where level I includes patients with almost no dysfunction and levels IV and V include non-ambulators. Current treatments for muscle dysfunction in CP

exist (Tilton, 2003), and include physical and occupational therapy, bracing, surgical lengthening, transfer or release of tendons, and pharmacological interventions such as botulinum toxin and baclofen. To date, despite these therapeutic interventions, no cure exists and little can be done to alter the natural history of the disorder. It is a goal to improve understanding of muscle adaptations to contracture in patients with CP that could lead to new, improved, and even less invasive treatments.

In patients with CP, muscle is significantly altered compared to typically developing (TD) individuals, yet cellular and molecular mechanisms that cause the differences are unknown. Previous reports focused on the two main mechanical load-bearing structures in muscle—the connective tissue extracellular matrix (ECM) and the individual muscle fibers. One mechanism by which muscle could differ between CP and TD is through connective tissue changes, either in amount or orientation of collagen and other ECM components. In previous reports of hamstrings and upper extremity muscles, extracellular matrix (ECM) content was higher in CP, measured both histologically and biochemically (Booth *et al.*, 2001; Smith *et al.*, 2011). Consistent with these findings is the fact that ECM related gene expression is significantly altered in both wrist (Smith *et al.*, 2009) and knee flexors (Smith *et al.*, 2012) in CP.

Previous studies also characterized changes in individual muscle fibers from patients with CP. Surprisingly, titin, the giant protein believed to be responsible for fiber stiffness, appears unchanged between CP and TD individuals (Smith *et al.*, 2011). It was suggested that systematic differences in muscle fibers, such as altered fiber type, could be responsible for changes in fiber mechanics. Indeed, several studies showed changes in the composition of myosin heavy chain protein in CP. Studies using ATPase staining reported fast-to-slow transitions in CP and type I fiber predominance (Rose *et al.*, 1994; Ito *et al.*, 1996; Marbini *et*

al., 2002). More recent reports eletrophoretically measuring myosin heavy chain isoform composition found the opposite trend, with muscles either increasing in Type II myosin or decreasing in Type I myosin (Pontén & Stal, 2007; Smith *et al.*, 2011; Gantelius *et al.*, 2012). These differences may indicate differences in myosin measurement technique, but they also may reflect the tremendous heterogeneity of patients with CP, both between patients and among different muscle of the same patient.

A general summary of previous reports clearly indicates that muscles throughout the body differ, both in TD individuals and in those with CP. There is a difference in function of muscles that are biarticular versus those that are monoarticular. Hamstring muscles are biarticular in comparison to a monoarticular muscle such as the brachioradialis of the arm or the soleus of the leg. Dramatic differences in activation pattern have been recorded between muscles that span one and two joints in nonhuman models, and these variations may hold true for humans as well (Carroll *et al.*, 2008). It is suggested that biarticular muscles such as the biceps brachii, psoas, and gastrocnemius function as modulators of activity while monarticular muscles are more involved in movement and strength generation (Gage *et al.*, 1995). A recent study even showed substantial systematic biochemical variation among muscles in different body regions (Tirrell *et al.*, 2012). It is therefore problematic to assume that results from one muscle or group hold true for other muscles or groups.

Because little is known about how the differences described above affect muscle adaptation to CP and because of the high clinical prevalence of equinus contractures, this study focuses on the soleus (SOL) and gastrocnemius (GAST) muscles of the calf. These muscles are both innervated by branches of the tibial nerve (Loh *et al.*, 2003) but have different architectural properties (Ward *et al.*, 2009a) and cross different numbers of joints; the SOL crosses only the ankle while the GAST crosses both the ankle and knee. Given the ability

to make these physiological and anatomical comparisons, the purpose of this study was to measure the passive mechanical properties of fibers and fiber bundles in these two muscles in an effort to improve our understanding of the passive mechanical properties of muscle in the context of the changes observed in CP.

3.3 Methods

3.3.1 Participants

Children with CP undergoing muscle-tendon unit lengthening for equinus contracture were recruited in a local children's hospital (age = 11.1±5.1 yrs., n=24), and TD adults undergoing surgery for tibial plateau fracture or wound repair with SOL or GAST muscle flaps were recruited from a university hospital (age = 47.7±15.3 yrs., n=13) (Table 1). All studies were approved by the University of California Institutional Review Board (IRB). Written informed consent was obtained from all adult subjects and parents of pediatric subjects, and written assent was obtained from subjects under 18 years of age.

3.3.2 Biopsy Collection

During surgery, muscle biopsies of the SOL and GAST were collected using a custom biopsy clamp (Ward *et al.*, 2009b). Care was taken to maintain muscles in their *in vivo* position to prevent sarcomere stretch or deformation. Biopsies were split into three pieces. One was frozen in liquid nitrogen and stored at -80 ° C for biochemical analysis. The second was placed in a glycerinated muscle relaxing solution containing (mM): imidazole (59.4), KCH_4O_3S (86.0), $Ca(KCH_4O_3S)_2$ (0.13), $Mg(KCH_4O_3S)_2$ (10.8), K_3EGTA (5.5), KH_2PO_4 (1.0), Na_2ATP (5.1), and 50.0 μM leupeptin and stored at -20 ° C for mechanical testing. The third portion of the biopsy was left in the clamps at their *in situ* length and fixed in 10%

buffered formalin, followed by storage in phosphate buffered saline, as described previously for sarcomere length determination (Smith *et al.*, 2011).

3.3.3 Sarcomere Length Measurements

Small muscle bundles (2-10 fibers) were teased from the fixed samples and mounted on glass slides using Permount mounting media (Thermo Fisher Scientific, Pittsburgh, PA, USA). Sarcomere lengths of 10-15 muscle bundles were measured using laser diffraction (Lieber *et al.*, 1984) and average biopsy sarcomere length was calculated using the grating equation.

3.3.4 Passive Mechanical Testing

Passive mechanical testing was carried out on unfixed muscle fibers and fiber bundles stored in glycerinated muscle relaxing solution as previously described (Fridén & Lieber, 2003; Smith *et al.*, 2011). Briefly, single fibers or bundles of 10-20 muscle fibers and their surrounding connective tissue were dissected from biopsies in muscle relaxing solution at pH 7.1 consisting of (mM): imidazole (59.4), KCH_4O_3S (86.0), $Ca(KCH_4O_3S)_2$ (0.13), $Mg(KCH_4O_3S)_2$ (10.8), K_3EGTA (5.5), KH_2PO_4 (1.0), Na_2ATP (5.1), and 50.0 μM leupeptin. Samples were placed in a chamber filled with chilled relaxing solution and secured to two pins with 10-O suture, one attached to a motor arm (Newport MT-RS; Irvine, CA, USA) and the other to a force transducer (Model 405A, sensitivity 10 V g−1, Aurora Scientific, Ontario, Canada). Fibers or bundles were stretched in increments of 0.25 μm/sarcomere/stretch, with a 3 min stress-relaxation period between stretches. Three minutes relaxation was chosen since fibers and bundles reach an asymptotic force by this point. Force was recorded and sarcomere length was measured by laser diffraction after each stretch. Measured force was converted to stress using fiber or bundle size. A custom designed LabView (National Instruments, Austin,

TX, USA) program was used for data analysis. Tangent stiffness was calculated at each sarcomere length.

3.3.5 Biochemical Analysis - Titin

Titin molecular weight was quantified using SDS-VAGE (Warren *et al.*, 2003) as previously described (Tirrell *et al.*, 2012). Individual muscle fibers used for stiffness measurements were saved (n=10 from each muscle per group) and loaded into 1% SeaKem Gold agarose gels (Lonza, Basel, Switzerland) held in place within the gel apparatus with a small 12.8% acrylamide plug. Human SOL titin (3700 kDa) and rat cardiac titin (2992 kDa) were used as molecular mass standards. Gels were run at 15 mA for 5 h at 4 °C. Gels were stained following the BioRad Silver Stain Plus protocol and bands were identified and quantified using densitometry (Bio-Rad, Hercules, CA, USA).

3.3.6 Biochemical Analysis – Myosin Heavy Chain (MHC)

Myosin heavy chain composition was measured to determine muscle fiber type as previously described (Talmadge & Roy, 1993). Samples (n=10 of each muscle per group) were weighed, homogenized in a bullet blender, and suspended in sample buffer with protease cocktail at 125 μg/mL. They were then boiled and run at 4°C on SDS-PAGE gels for 1h at 10 mA followed by 20 hours at 275 V. Gels were composed of a 4% acrylamide stacking region and an 8% acrylamide resolving region. Gels were trimmed and stained using the BioRad Silver Stain Plus staining kit (Bio-Rad, Hercules, CA, USA) and bands were quantified using densitometry.

3.3.7 Biochemical Analysis – Collagen

A modified hydroxyproline assay was used to measure collagen content in muscle samples (Stegemann & Stalder, 1967). Ten samples from each group were weighed, then

hydrolyzed overnight at 110°C in 6N HCl. Tubes were cooled to room temperature and samples were placed in a 96 well plate and desiccated in a vacuum desiccator for 1 hour. Chloramine T solution was added to wells and plates were left to react for 20 min. Isopropanol and 60% perchloric acid were used to dissolve p-Dimethylaminobenzaldehyde, which was added to wells. Samples were then covered and incubated at 60°C for 30 minutes. Absorbances were then read at 550 nm. A standard curve was used to determine hydroxyproline content, and collagen concentration was found using a hydroxyproline to collagen mass conversion factor of 7.46.

3.3.8 Statistical Analysis

Statistical analysis of data was performed using Prism software (GraphPad Software, La Jolla, CA, USA). All results are given as mean±standard deviation unless stated otherwise. To compare CP and TD values, two-tailed Student's *t*-tests were performed with $P < 0.05$ set as the significance level. Linear regression was used to compare stress-sarcomere length curves.

3.4 Results

3.4.1 Sarcomere Length

GAST sarcomere lengths in CP were 3.71±0.44 μm and SOL sarcomeres lengths were 4.07±0.45 μm, consistent with those previously reported (Mathewson *et al.,* in review). Both average sarcomere lengths were dramatically longer ($p<0.0001$) than corresponding values (SOL=2.2 μm, GAST=2.6 μm) we previously reported for adults (Ward *et al.*, 2009a) (Fig. 3.1).

3.4.2 Passive Mechanics

At sarcomere lengths from 2.5-4 μm, both GAST and SOL fiber bundle stress-sarcomere length curves were similar between CP and TD muscles (Figs. 2A and 2B). For single fibers, however, measured passive stress curves were significantly higher in patients with CP for both muscles ($p<0.01$) ((Figs. 2C and 2D.). Tangent stiffness did not vary significantly among patients with different involved limbs (hemiplegic, diplegic, or quadriplegic) or across the clinical functional scores (as indicated by the Global Mean Functional Classification System (GMFCS) (Palisano *et al.*, 1997)) except in the case of GAST fibers, which were significantly stiffer ($p<0.01$) in patients with classifications of GMFCS III-V compared to patients with lower GMFCS scores. While fibers tended to be slightly smaller on average in patients with CP, the overall fiber size distribution was similar (Figs. 2E and 2F). At the *in vivo* sarcomere length (CP SOL=4.1 μm, TD SOL=2.2 μm; CP GAST=3.7 μm, TD GAST=2.6 μm; red asterisks, Fig. 3.2), both fibers and bundles from patients with CP were significantly stiffer ($p<0.0001$) (Fig. 3.3). Dramatic differences of at least an order of magnitude were found between CP and TD pairs for both muscles studied. Thus, both increased muscle fiber tangent stiffness in children with CP and altered sarcomere length of both fibers and bundled contributed to this tremendous difference in tangent stiffness. To estimate fiber and connective tissue contribution to bundle tangent stiffness, we calculated ECM mechanical properties by subtracting the fiber contribution (assuming 80% of CP and 90% of TD bundle area is fibers (Lieber *et al.*, 2003)) from the bundle properties. This resultant curve demonstrated that ECM contribution would dominate bundle tangent stiffness at relatively long sarcomere lengths (longer than measured *in vivo* values) (Figs. 3E and 3F).

3.4.3 Titin

Surprisingly, in spite of the fact that fiber tangent stiffness varied significantly

between patients with CP compared to TD individuals, for both muscles, titin mass was actually slightly *greater* in patients with CP (SOL: CP=3770±51, TD=3710±51; GAST: CP=3750±34, TD=3670±31) (Fig. 3.4A). Typically, increased stiffness is associated with smaller titin mass (Prado *et al.*, 2005). Also, unlike previous studies performed at the myofibrillar level suggesting a causal relationship between titin mass and fiber stiffness (Linke *et al.*, 1996; Ottenheijm *et al.*, 2009), no significant correlation was found between titin molecular weight and passive tangent stiffness for either muscle studied (Fig. 3.4B). It was also clear, viewing individual subgroups, that no correlation was apparent for any of the individual subgroups. Titin size was also not significantly correlated with age, gender, range of motion of either the knee or ankle, or GMFCS classification for either the GAST or SOL.

3.4.4 Myosin Heavy Chain

As observed in recent studies of myosin heavy chain composition (Pontén & Stal, 2007; Smith *et al.*, 2011; Gantelius *et al.*, 2012), muscle homogenate from patients with CP showed significantly more type IIx MHC in both SOL and GAST and significantly less MHC type I in the GAST (Table 2). No difference was seen in MHC type IIa content for either group.

3.4.5 Collagen

In contrast to previous reports from children with CP (Booth *et al.*, 2001; Smith *et al.*, 2011) there was no significant difference in collagen content between CP and TD muscle samples for either GAST or SOL (GAST: CP=6.59±5.27 µg/mg, TD=11.98±11.05 µg/mg; SOL: CP=6.14±3.26 µg/mg, TD=10.94±16.08 µg/mg) (Fig 5A). Sample variation was high, especially in TD individuals. While TD muscles showed a trend toward higher collagen, this might have been due to the increased average age of the subset of patients tested in this group

(SOL: CP=12.2±6.5, TD=50.8±16.8; GAST: CP=10.4±2.8, TD=50.7±14.8). Collagen content was not significantly correlated with the tangent stiffness of bundles in either group or muscle (Fig. 3.5B).

3.5 Discussion

The purpose of this paper was to measure muscle fiber and muscle fiber bundle stiffness in the GAST and SOL muscles of patients with CP and to compare them to TD individuals. While previous studies reported increased stiffness at the fiber and/or bundle level, it was not clear to what extent these studies would generalize to the triceps surae. Specifically, no consensus has been reached on how muscle use pattern, architecture, innervation, or joint crossings affect muscle adaptation and no current mechanism explains the passive mechanical properties of fibers or bundles. By comparing the GAST and SOL, which have similar location and innervation but different architecture and function, our goal was to parse out some of these differences. Surprisingly, both muscles were very were similar in all parameters studied. This result suggests that use pattern, location, and possibly innervation are important determinants of muscle adaptation to CP but that number of joints crossed and architecture are less important.

In terms of intrinsic mechanical properties, the only significant change observed was that fibers from patients with CP were significantly stiffer compared to TD fibers (Fig. 3.3). In many other studies increased fiber stiffness is associated with changes in the giant protein titin, which connects myosin to the Z-disk. In our study, however, titin size did not correlate with fiber tangent stiffness. In fact, while we would expect to see longer titin in more compliant fibers, we actually observed the opposite result, leading to us to question titin's function in fibers and how titin size correlates with tangent stiffness. We were also unable to account for differences between groups based on any visual appearance of residual connective

tissue adhering to fibers.

Fiber bundles and fibers actually had similar tangent stiffnesses within each muscle in patients with CP, in contrast to previous work, which showed bundle tangent stiffness was significantly higher than that of fibers (Smith *et al.*, 2011). Bundles from TD patients, however, were stiffer than fibers (Fig. 3.3). The fact that bundles, which include ECM, do not show increased tangent stiffness in CP, suggests there may be differences in ECM of muscles in patients with and without CP. Previous work has shown a stiffer ECM in hamstring muscles of patients with CP (Smith *et al.*, 2011), unlike what we observed in this study. One possible explanation of our current results is that ECM of calf muscles becomes less organized and less able to resist force at the lengths measured in this study even though the totally amount of collagen is the same. Clearly, muscle adaptations to altered use vary throughout the body. Interpreting data is hampered by lack of any structural model for load bearing in the extracellular space.

To interpret material property changes in fibers and fibred bundles in terms of *in vivo* function, we "calibrated" mechanical properties of these specimens in the context of their *in vivo* length, measured using muscle biopsy clamps (Fig. 3.1). The tangent stiffness increases in both fibers and bundles at *in vivo* lengths that was observed (Figs. 3C and 3D) was clearly due to the long lengths at which sarcomeres existed within each muscle of patients with CP. Fundamental differences in ECM stiffness (measured from fiber bundles) was not observed, although fibers from patients with CP were stiffer than TD fibers at the same sarcomere length. When an ECM stiffness curve was calculated, it was found that little ECM contribution was present at *in vivo* lengths but ECM was predicted to dominate bundle tangent stiffness only at longer sarcomere lengths. Inspection of this relationship reveals that mechanical properties of the ECM essentially shift relative to *in vivo* sarcomere length. This

may indicate that ECM mechanical properties follow *in vivo* sarcomere length. These results also demonstrate that stiffness seen in calf muscles of patients with CP is due to their highly stretched position and stiffer fibers rather than changes to ECM such as alterations in collagen content.

3.6 Limitations

The main limitation of this study is the lack of age-matched control tissue for passive mechanical or biochemical testing. Because TD children rarely if ever have their SOL and GAST exposed during surgery, we were only able to measure the properties of adult muscle biopsies. Indeed, while we had received IRB approval for GAST and SOL tissue collection from TD children, no case in which collection was possible occurred over the three year period of sample collection. We believe that matching muscles is critical for this study, which is why we opted for adult muscles rather than younger muscles from a different anatomical location. Despite the age difference between the TD and CP samples, we believe our tangent stiffness results hold true. If any difference would be expected, older adults, who have been shown to have increased collagen (Alnaqeeb *et al.*, 1984), would be expected to be stiffer than younger patients. The fact that our younger patients with CP showed much higher tangent stiffness at *in vivo* lengths suggests that increased tangent stiffness truly exists, and may be, if anything, under reported in this study. Changes in biochemical parameters such as collagen content and MHC isoform distribution, however, could be affected by age. We measured no difference between collagen concentrations in our two groups, which could have been due to the difference in age (~35 yrs.) between the children with CP and TD adults. Previous work in other muscles found increases in collagen in patients with CP (Booth *et al.*, 2001; Smith *et al.*, 2011), but these changes may have been blunted by age differences in our study, as older adults are expected to have increased collagen due to aging (Alnaqeeb *et al.*, 1984). Previous

work has also suggested a slight fast to slow myosin transition (increase in type I MHC) with aging (Short *et al.*, 2005), which may have impacted the slow to fast transition in patients with CP we see here. Samples from patients undergoing tibial plateau surgery were harvested from viable, healthy muscle away from the zone of injury. The samples were all obtained under direct supervision by one of the authors, thereby minimizing differences in harvesting technique.

3.7 Conclusions

Clearly, the highly stretched sarcomeres found in SOL and GAST muscles of patients with CP lead to muscles that are more passively stiff than those of TD individuals, although our results suggest that this may vary by anatomical location. High passive stiffness at *in vivo* sarcomere lengths may contribute to contracture formation in children with CP. Therapies that decrease sarcomere length or allow the muscle to shorten, therefore, could lead to functional improvements in these patients. For example, surgical lengthening that causes muscle shortening will therefore allow sarcomeres to shorten, likely leading to lower passive muscle stiffness. Different muscles, however, may respond differently to therapies. Our results point to innervation or anatomical location predicting muscle response rather than architecture or joint crossings. The question regarding the long-term effects on due to therapies such as serial casting, stretching, and surgery remain unanswered. Further research is necessary to understand how muscles at different locations in the body of patients with CP respond to therapies. However, these studies are hampered by our lack of understanding of the structure and function of the extracellular matrix and muscle fiber passive load bearing structures. These important physiological properties clearly have clinical significance in limiting patient range of motion and thus, future therapies aimed at altering ECM properties would have high clinical impact.

3.8 Acknowledgements

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Table 3.1. Characteristics of study participants

Patient	Sex	Age	Limbs	Ankle Angle	Popliteal Angle	GMFCS
CP	F	4.6				
CP	M	13.0	D		110	1
CP	M	11.0	T	0	120	3
CP	M	11.3	Q	-40	90	4
CP	M	4.8	Q	-45	180	5
CP	F	24.1	Q	-30		2
CP	M	11.4	D	5	150	2
CP	M	6.7	D	-30	150	2
CP	M	9.6	Q	0	170	5
CP	F	14.3	D	-20	170	1
CP	F	13.6	H	-10	140	2
CP	F	21.3	D	-15	120	2
CP	F	9.2	D	-20	95	3
CP	F	14.9	D	-20	150	2
CP	M	12.9	H	-15	145	2
CP	F	6.3	H	-30	160	1
CP	M	12.6	D	-40	150	1
CP	M	4.2	H	-50	150	2
CP	F	11.5	Q	-30	170	4
CP	M	7.8	D	-15	150	2
CP	F	5.1	Q	-30	120	2
CP	M	11.2	Di	-20	120	2
CP	M	13.2	Q		110	4
CP	M	22.7	H	-20	120	2
TD	F	59.2				
TD	F	38.1				
TD	F	42.2				
TD	M	50.6				
TD	F	52.3				
TD	M	42.7				
TD	F	70.7				
TD	F	76.3				
TD	M	50.5				
TD	M	25.9				
TD	F	57.1				
TD	M	40.1				
TD	M	41.2				

Table 3.2. Myosin heavy chain measurements. Values are mean±SEM. * show significant differences between pairs. Because values are averages of percentages, they do not add to exactly 100%.

	CP Soleus	**TD Soleus**	**CP Gastroc**	**TD Gastroc**
MHC Type I	51.4±5.8%	56.8±6.5%	26.9±2.5% *	51.5±1.2% *
MHC Type IIa	18.1±4.0%	18.1±3.5%	27.6±2.05%	23.5±5.1%
MHC Type IIx	30.5±4.0% *	27.1±5.6% *	44.3±3.0% *	24.9±3.7% *

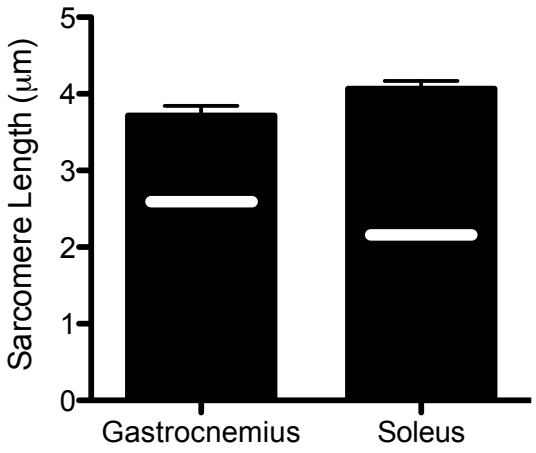


Figure 3.1. Sarcomere lengths from patients with CP are significantly longer compared to those of TD individuals. TD values, shown with a white bar, were obtained from a previous study (Ward *et al.*, 2009a), and CP SOL sarcomere lengths were previously reported (Mathewson *et al.*, in review).

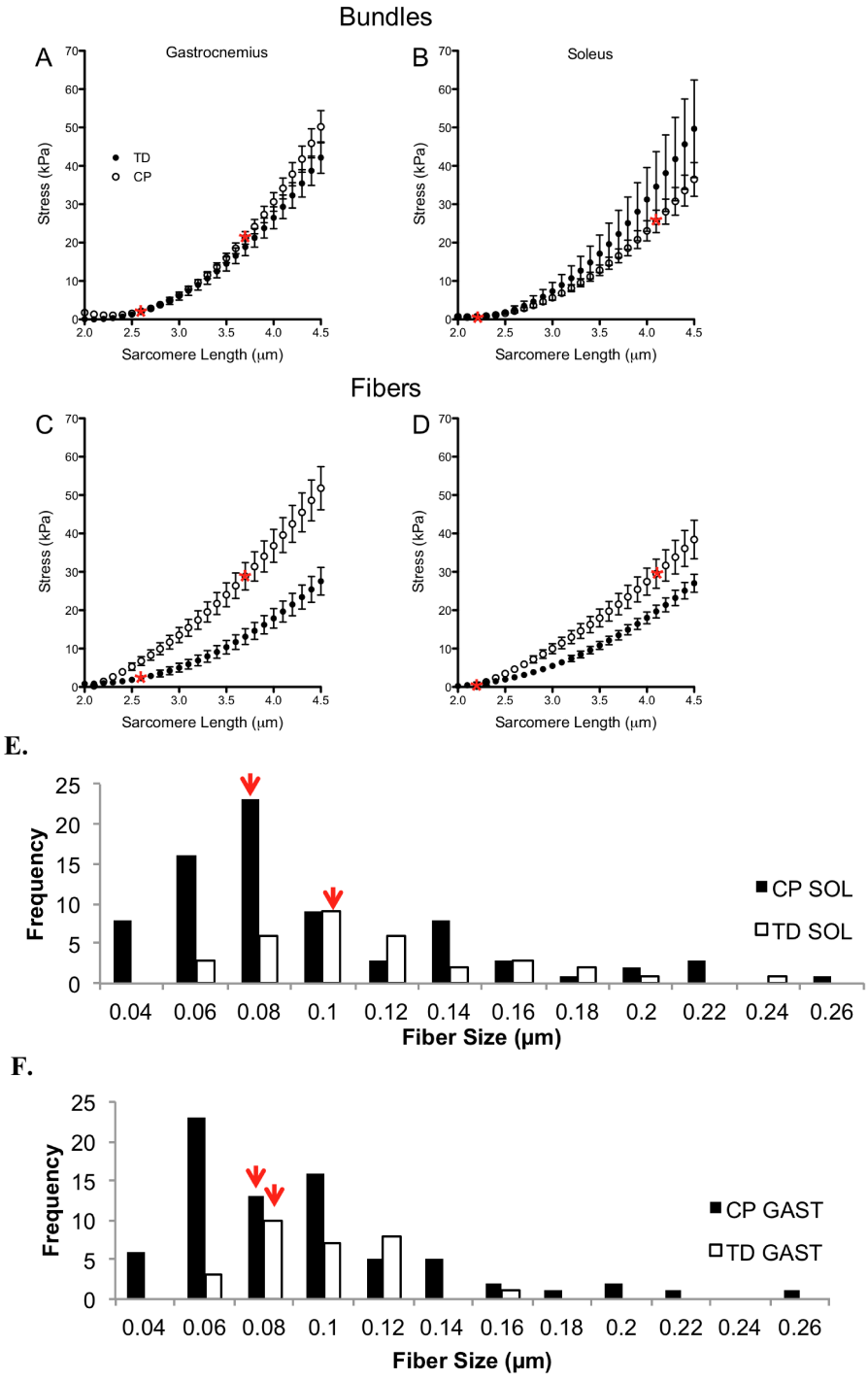


Figure 3.2. Sarcomere length vs. measured stress curves for bundles **(A-B)** and fibers **(C-D)** from each muscle. Data are shown as mean±SEM. Red asterisks show *in vivo* sarcomere lengths measured by laser diffraction (see Methods). Group fiber size distributions are presented as histograms of SOL **(E)** and GAST **(F),** with red arrows showing bins containing group averages.

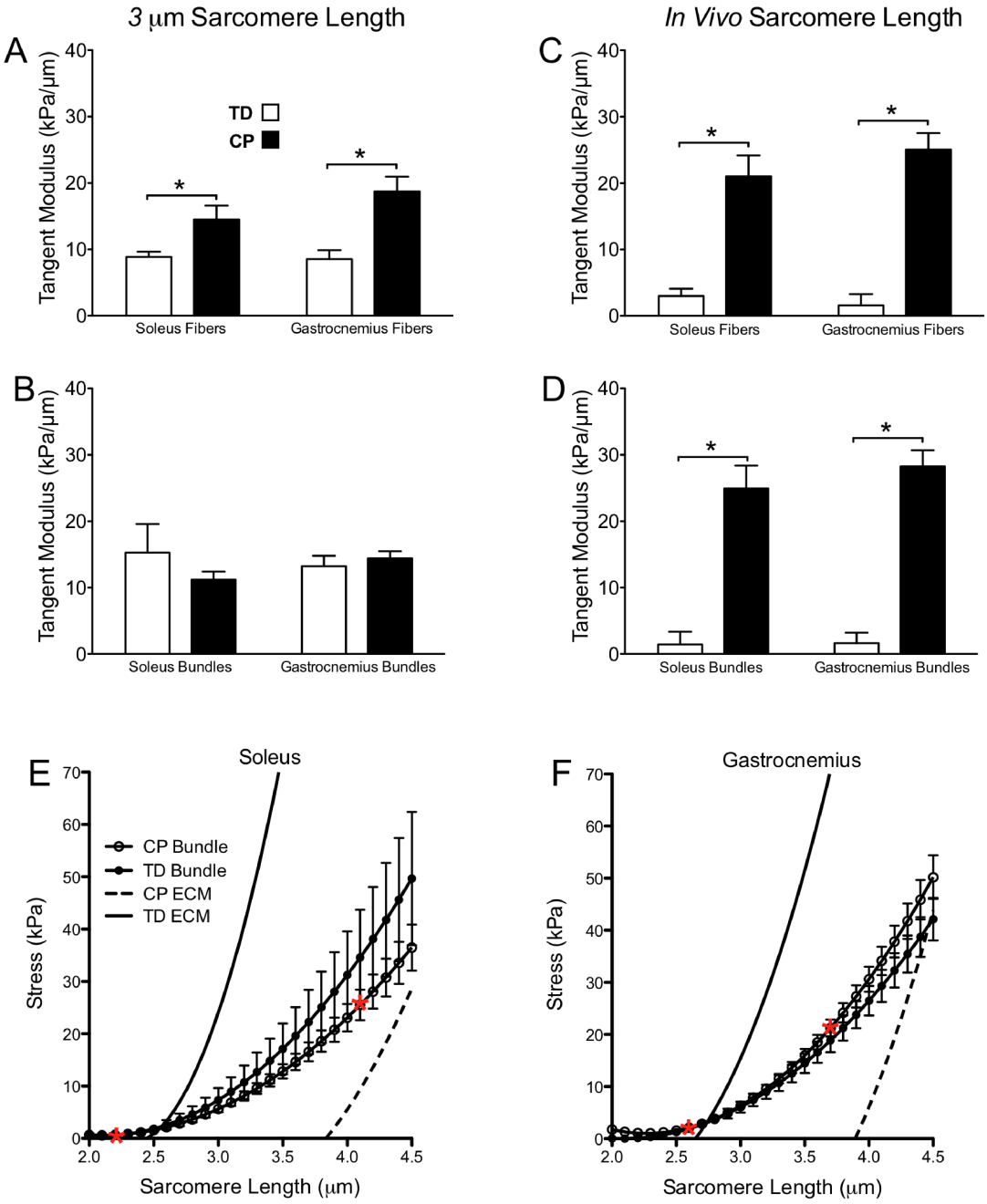
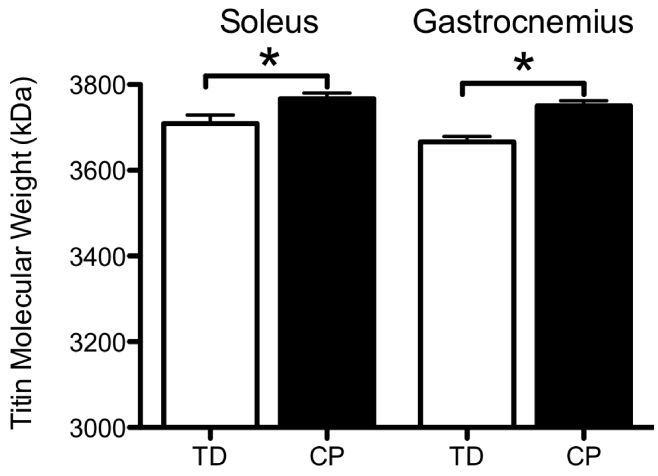


Figure 3.3. Tangent stiffness of fibers and bundles. **(A)** At an intermediate sarcomere length of 3 μm, fibers **(A)** had a significantly higher calculated tangent stiffness in both muscles in patients with CP than in their TD counterparts. Bundles **(B)** were not significantly different. When compared at their average *in vivo* sarcomere length (c.f. Fig. 3.1), however, both fibers **(C)** and bundles **(D)** had significantly higher calculated tangent stiffness in muscles from patients with CP compared to those of TD individuals. **(E)** ECM mechanical properties calculated as described in Methods for the SOL and GAST **(F).** ECM stiffness contributes most to bundle tangent stiffness near *in vivo* sarcomere length.

A.



B.

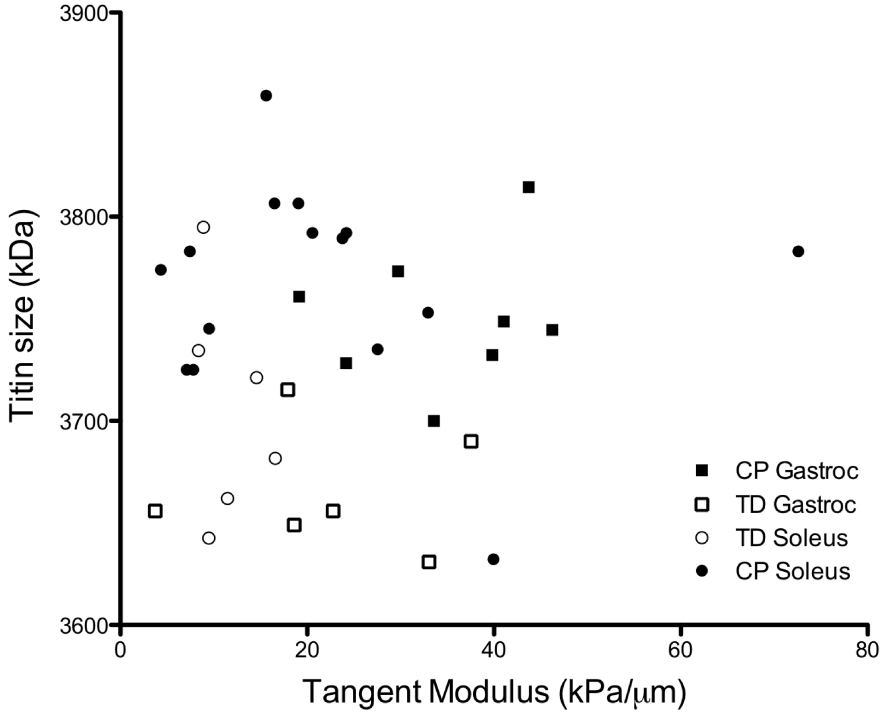
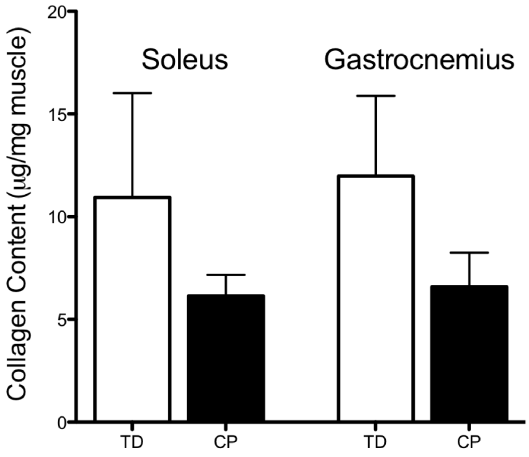


Figure 3.4. A slight increase in titin molecular mass was seen in patients with CP (note that y-axis only shows the range 3000-3800 kDa to highlight differences) **(B)** Titin size did not correlate with measured fiber tangent stiffness.

A.



B.

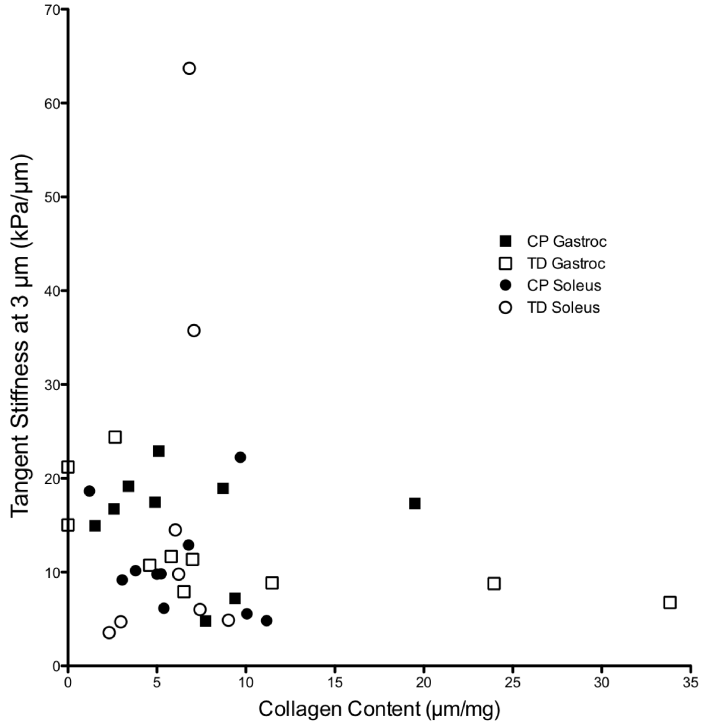


Figure 3.5. (A) Collagen content was not significantly different between TD individuals and those with CP. **(B)** Collagen content was not significantly correlated with bundle tangent stiffness at any length for either muscle or group.

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Chapter 4

Satellite Cells from Patients with Cerebral Palsy Show *In Vitro* Differentiation and Fusion Deficits But No Changes In Proliferation or Motility

4.1 Abstract

Patients with cerebral palsy (CP) often develop muscular symptoms such as contractures, which limit joint range of motion. Possible culprits for muscle deficits in CP are stem cells, called satellite cells, whose population is decreased in muscle from patients with CP compared to typically developing (TD) individuals. In this study, we set out to characterize properties of satellite cells that might impact muscle growth and repair in CP: their capacity for proliferation and population renewal, their ability to translocate, which is critical for reaching sites of growth and repair, and their potential to differentiate and fuse to regenerate muscle fibers. Satellite cells from CP muscle showed no deficits in proliferation or motility compared to satellite cells from TD muscle, but their differentiation and fusion capability was reduced, as shown by a significant decrease in myogenic regulatory factor 5 and a trend toward lower myogenin ($p=0.07$) and myosin heavy chain expression ($p=0.06$) and dramatically lower nuclei/myotube ratio and total myotube area ($p=0.02$). No difference in global DNA methylation was seen between CP and TD samples, but this result does not rule out differences in specific methylation sites. Decreased ability for satellite cell differentiation and fusion could negatively impact both muscle growth and repair in patients with CP, so further research into the mechanism of these changes has the potential to lead to exciting new CP muscle therapies.

4.2 Introduction

Cerebral palsy (CP) is a heterogeneous disorder caused by a perinatal injury to the developing brain (Bax, 1964; Bax *et al.*, 2005; Sankar & Mundkur, 2005; Rosenbaum *et al.*, 2007). While the primary insult in CP is this nonprogressive brain injury, muscles function can be dramatically altered in patients with CP. Symptoms can include spasticity, contracture, altered muscle control, and generalized muscle weakness (Bax, 1964; Rose *et al.*, 1994;

Tilton, 2003; Bax *et al.*, 2005; Rose & McGill, 2005; Sankar & Mundkur, 2005; Rosenbaum *et al.*, 2007). For patients with muscle contractures, joint range of motion in the extremities can be severely reduced. While treatments for CP, including surgery and pharmacologic agents such as botulinum toxin and baclofen (Tilton, 2003), can provide temporary relief from muscle symptoms, current treatments do not repair the muscle environment or prevent further detrimental alterations in muscle tissue.

Research on muscle of patents with CP has shown several changes. Overall muscle belly size has been shown to be reduced in CP (Mohagheghi *et al.*, 2007; Moreau *et al.*, 2009; Barber *et al.*, 2011) as well as the diameter of individual muscle fibers (Marbini *et al.*, 2002; Smith *et al.*, 2011). Other changes include alterations in slow and fast muscle fiber type predominance (Rose *et al.*, 1994; Ito *et al.*, 1996; Marbini *et al.*, 2002; Pontén & Stal, 2007; Smith *et al.*, 2011) as well as changes in the muscle extracellular matrix (Booth *et al.*, 2001; Lieber *et al.*, 2003; Smith *et al.*, 2011). Interestingly, muscle fibers are highly stretched (Lieber *et al.*, 1994; Smith *et al.*, 2011), with sarcomeres that are sometimes as much as twice as long as those of TD individuals (Mathewson, *et al.*, submitted). Studies of passive muscle mechanics have shown variability across the body (Fridén & Lieber, 2003; Smith *et al.*, 2011) (Mathewson, *et al.*, submitted), but several have found that muscle fibers are intrinsically stiffer in patients with CP (Fridén & Lieber, 2003) (Mathewson, *et al.*, submitted). The common theme in many of these studies appears to be changes at the muscle fiber level, with apparent reductions in both longitudinal and radial fiber growth in patients with CP.

When considering alterations at the fiber level, possible culprits for changes in muscle development and function are muscle stem cells, called satellite cells (Mauro, 1961). Satellite cells, found between the sarcolemma and basal lamina of muscle fibers, are quiescent in normal, healthy muscle (Schultz *et al.*, 1978). During times of growth or injury, however,

satellite cells can become activated to contribute to both longitudinal and radial fiber growth (Moss & Leblond, 1971), as well as injury repair (Snow, 1978; Collins *et al.*, 2005). There is evidence, however, the regenerative capacity of satellite cells may be limited. In cases of aging or disease, the satellite cell pool can become exhausted, with decreased ability to repair muscle after damage (Schultz & Lipton, 1982; Heslop *et al.*, 2000; Renault *et al.*, 2002). Patients with Duchenne Muscular Dystrophy, in which constant muscle regeneration occurs, appear to have upregulated satellite cell proliferation, leading to early exhaustion of the satellite cell pool (Wakayama, 1976; Kottlors & Kirschner, 2010). Satellite cell changes seen in aging or diseases such as muscular dystrophy occur through repeated cycles of proliferation and muscle repair, which is not seen in patients with CP. Current evidence, however, does suggest that satellite cells may be altered in patients with CP.

Decreased satellite cell numbers in muscle from patients with CP have been observed, both by fluorescence activated cell sorting and by immunohistochemical analysis (Smith *et al.*, 2013). While some evidence indicates that reduced satellite cell population leads to reduced regenerative capability in muscle (Shefer *et al.*, 2006; Day *et al.*, 2010), the implications for growing and developing muscle in patients with CP are currently unknown. Both the causes and effects of lower muscle stem cell numbers in CP are open questions, so we set out to characterize the basic properties of satellite cells in individuals with CP and TD. For proper muscle development and repair, satellite cells must be able to proliferate and replenish themselves, move within the muscle to the site of growth or injury, and differentiate and fuse with existing muscle fibers. We therefore evaluated *in vitro* proliferation, motility, and differentiation ability of cells from patients with CP as well as from TD children. We hypothesized that satellite cell proliferation would be reduced in patients with CP, consistent

with reduced satellite cell numbers seen previously, but that motility and differentiation capabilities would be unchanged.

4.3 Materials and Methods

4.3.1 Biopsy Collection

Biopsies were collected from the semitendinosus and gracilis muscles of the hamstrings from patients undergoing hamstring lengthening for CP or ACL repair with hamstring autograft. All procedures were approved by the local Institutional Review Board and assent was obtained from patients and consent from parents in accordance with the IRB. Samples were collected from n=8 subjects from each group (CP: age=9.8±5.7 yrs, 1 female; TD: age=16.2±2.1 yrs, 4 female). Table 4.1 shows patient characteristics.

4.3.2 Cell Isolation

Cells were isolated by fluorescent activated cell sorting (FACS) using a modification of a previously described procedure (Smith *et al.*, 2013). Briefly, biopsies were digested in a collagenase-dispase solution, mechanically broken down with forceps, filtered twice, spun at 600g for 10 min, and resuspended in FACS buffer (pH 7.4 1mM Ethylenediaminetetraacetic acid (EDTA) and 2.5% normal goat serum in phosphate buffered saline (PBS)). Cells were stained with anti-CD56 (neural cell adhesion molecule, NCAM) (Abcam, Cambridge, MA, USA) which has previously been shown to reliably identify muscle satellite cells (Illa *et al.*, 1992; Capkovic *et al.*, 2008), anti CD31 (Abcam, Cambridge, MA, USA), which is a marker of endothelial cells, and anti CD45 (Abcam, Cambridge, MA, USA), which is a marker of leukocytes. CD31 and CD45 were combined into a dump channel and remaining NCAM only positive cells were collected using a FACSAria II (BD Biosciences, San Jose, CA, USA). Gating was set very stringently to include only clearly NCAM positive cells and exclude

double positive cells. Many possible satellite cells were likely discarded, but the population collected was very pure and appeared homogeneous in culture.

4.3.3 Cell Culture

After sorting, cells were plated and grown in myoblast growth media containing 20% fetal bovine serum, a 50/50 mix of Ham's F10 and DMEM (Dulbecco's Modified Eagle Medium), 50 U/ml penicillin and 50 mg/ml streptomycin, and basic fibroblast growth factor at 1/1000. Primary isolated cells (P0) were passaged once and frozen at P1.

4.3.4 Proliferation Studies

In order to measure growth rates in CP and TD satellite cells (n=6 subjects/group), divided nuclei were labeled and the numbers of labeled nuclei were measured at 12 and 18 hour time points. Cells were thawed and plated on clean glass coverslips coated with fibronectin crosslinked to Sulfo-SANPAH (Thermo Scientific, Rockford, IL, USA). Replicating DNA was labeled with EdU (5-ethynyl-2'-deoxyuridine), a thymidine analogue, using the Click-it EdU kit (Life Technologies, Grand Island, NY, USA), which allowed identification of cells that had divided in the time period of interest. Coverslips were imaged and total nuclei (Fig. 4.1A) and EdU positive nuclei (Fig. 4.1B) were counted with a custom Matlab (Mathworks, Natick, MA, USA) program.

4.3.5 Motility Measurements

Cells were plated in 12 well plates at 1000 cells/well (n=8 subjects/group). They were allowed to attach for 24 hours before the assay begin. After attachment, cell plates were placed in a temperature and CO_2 controlled chamber and imaged for a 24-hour period. Five locations in each well were imaged once every 10 minutes (Fig. 4.2A) and images were combined into

stacks in ImageJ (Schneider *et al.*, 2012). Three randomly selected cells per image were tracked using the ImageJ Manual Track plugin for a total of 15 cells tracked per sample.

4.3.6 Differentiation

Cells were plated at 50,000 cells/well in 6 well plates and at 20,000 cells/well in 12 well plates and allowed to grow to confluence (n=7-8 subjects/group). Once confluent, media was changed from growth media to low serum differentiation media (10% horse serum in DMEM with insulin at 10 μg/mL and 50 U/ml penicillin and 50 mg/ml streptomycin) and cells were grown for 4 more days. Cells in 6 well plates were collected and frozen in TRIzol (Life Technologies, Grand Island, NY, USA) for PCR. Custom designed primers (Integrated DNA Technologies, Coralville, IA, USA) were created for early stage differentiation marker myogenic differentiation 1 (MYOD1), myogenic factor 5 (Myf5), myogenin (MyoG) and late stage marker Myosin Heavy Chain 3 (MHC3), as well as for vinculin (VCL), a focal adhesion protein, and Ras-Related C3 Botulinum Toxin Substrate 1 (Rac1), involved in myoblast proliferation and differentiation, with the following sequences: (MYOD1) F: ACTTTGCTATCTACAGCCGGG, R: TAGAAGTCGTCCGTTGTGGC; (Myf5) F: ATGCACAGATAAAAACTCCTT, R: AATCAATGGCCCCCTATCAGAAA; (MyoG) F: GCGGGCGGCCACACTGA , R: GGGGGCTCGCAAGGATG; (MHC3) F: TTGCTGTCTTCTGCTCTCATCC, R:GGAGCAGCTATGCCGAACAC; (VCL) F:TGAAGCTCGCAAATGGTCCAGCAAG, R:ACCTCATCTGAGGCCTTGGCGATGT; (Rac1) F:ATGCAGGCCATCAAGTGTGTGGTG, R:TTACAACAGCAGGCATTTTCTCTTCC. Expression values were measured by RT-PCR using the following protocol. RNA was isolated with TRIzol (Life Technologies, Grand Island, NY, USA) and cDNA was synthesized using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen by Life Technologies, Grand Island, NY, USA). Samples

were run in duplicate on a CFX96 Real-Time System (BioRad, Hercules, CA, USA) using the following protocol: heating to 50 °C for 2 min followed by heating to 95 °C for 10 min, then 40 cycles of heating to 95 °C for 15 sec and to 60 °C for 1 min. Values were normalized to GAPDH expression, which was similar for all samples.

A second set of cells, grown in 12 well plates, was stained to measure myotube number and morphology. Samples were fixed for 10 minutes in ice-cold methanol. Cells were then permeabilized for 15 minutes in 1% Triton-X in PBS, washed in PBS, and incubated in primary MHC antibody (MF-20-s, Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA) at 1:30 in 2% bovine serum albumin (BSA) for one hour. Wells were then washed three times in PBS and incubated with a secondary antibody (Alexa Fluor 488) at 1:400 in 2% BSA for 20 minutes. Wells were washed three times in PBS, Hoechst dye was added at 1:1000 in water for 2 minutes, and cells were washed and left in PBS for imaging at 10x.

4.3.7 Methylation Measurements

Frozen cells were thawed and DNA was collected using the MagMAX DNA isolation kit (Life Technologies, Grand Island, NY, USA) (n=8 subjects/group). Using the Imprint Methylated DNA Quantification Kit (Sigma Aldrich, St Louis, MO, USA), global methylation was measured at 450 nm with a Synergy HT Microplate Reader (BioTek Winooski, VT, USA). Methylation averages were calculated and normalized to the TD average.

4.3.8 Statistical Analysis

Data analysis was completed with Prism software (GraphPad Software, La Jolla, CA, USA) using Student's T-test. Unless otherwise stated, results are presented as mean±SEM.

4.4 Results

4.4.1 Proliferation Studies

At both 12 and 18 hours of proliferation, no difference was seen between CP and TD satellite cells (12 hrs: CP=69.83±3.24%, TD=71.95 ± 3.07%, $p>0.64$; 18 hrs: CP=83.10±5.64%, TD=86.24 ± 2.26%, $p>0.61$) (Fig. 4.1C and D). Based on sample size and variability, this experiment was powered at 80% to detect a 12% difference between the groups.

4.4.2 Motility

We calculated the total path length satellite cells traveled over 24 hours. There was no difference between groups in total movement (CP=680.8 ± 73.1 μm, TD=642.4 ± 61.7 μm, $p>0.69$) (Fig. 4.2B). To see if one group showed more directed movement than the other (traveled a greater distance in any one direction), we calculated the maximum distance traveled from the cells point of origin (Fig. 4.2C). Again, there was no difference between groups (CP= 161.6 ± 15.7 μm, TD= 149.4 ± 13.8 μm, $p>0.56$). This study was powered at 80% to detect a 30% difference between groups. Means, however, were nearly identical for the two groups.

4.4.3 Differentiation

qPCR was carried to measure two markers of myoblast differentiation, the early commitment marker MyoD and the late stage marker MHC. Expression of both transcripts was normalized to GAPDH. GAPDH expression was similar between groups, as was expected due to their similar confluence and cell numbers. The early marker MyoD showed no expression difference between groups (CP=1.41 ± 0.08, TD= 1.50 ± 0.07 $p>0.46$), but Myf5 was significantly reduced in CP (CP= 1.49 ± 0.05, TD=1.63 ± 0.01, $p=0.01$). Myogenin and

late-stage commitment marker MHC also showed a strong trend toward being decreased in cells from patients with CP (MyoG: CP=1.30 ± 0.05, TD=1.39 ± 0.02, p=0.07; MHC: CP=1.81 ± 0.11, TD= 2.05 ± 0.04, p=0.06). Vinculin, a focal adhesion protein, and Rac1, involved in myoblast proliferation and differentiation, were both unchanged in CP (VCL: CP=1.53 ± 0.03, TD=1.56 ± 0.01, p>0.3; Rac1: CP=1.23 ± 0.05, TD= 1.22 ± 0.05, p>0.9) (Fig. 4.3).

When myotube morphology was quantified, the decrease in differentiation in CP satellite cells was more pronounced (Representative images Fig. 4.4A). Nuclei/myotube or fusion index, a measure of how many myoblasts came together to form each myotube, was significantly decreased in CP, at almost half the value of TD cells (CP=4.42 ± 0.61, TD=7.46 ± 0.94, p=0.02) (Fig. 4.4B). MHC positive myotubes as a fraction of total well area was also significantly decreased in CP cells (CP=17.38 ± 3.80, TD= 30.03 ± 2.96, p=0.02) (Fig. 4.4C). MHC positive myotubes from patients with CP were long and thin, covering much less surface area than those from TD subjects (Fig 4.4A).

4.4.4 Methylation Measurements

Methylation measurements showed high variance for both groups. While individual methylation values varied within groups, there was no systematic difference between groups (Fig. 4.5). When data was normalized to the average of TD measurements, CP=1.15 ± 0.11, TD= 1.00 ± 0.42, p>0.43. Because of very high sample variability, this experiment was powered at 80% to detect a difference on the normalized scale of 0.39 (a difference of around 40% from control).

4.5 Discussion

While previous measurements showing decreased satellite cell number in patients with CP (Smith *et al.*, 2013) led us to predict a decrease in proliferative capability in these cells, no difference was seen in proliferation at 12 or 18 hours between CP and TD satellite cells. Movement was also similar between CP and TD satellite cells. Interestingly, it was differentiation capability that appeared altered in patients with CP. When qPCR was used to measure expression of markers of myogenic differentiation, no difference was seen for the early marker MyoD. Myf5, however, was significantly decreased in CP satellite cells. Myogenin and a later marker of differentiation, MHC, also showed a strong trend toward being decreased in cells from patients with CP. Taken together, these PCR date suggest a decrease in the differentiation program. To get a full picture of differences between the myotubes, however, morphological data was necessary. Cultures from the two groups were dramatically different, with fewer thin, spindly myotubes forming in CP cultures compared to the many thick and highly multinucleated tubes in TD cultures. From morphological observations, it appears possible that problems with myoblast fusion may be present in patients with CP.

If satellite cell differentiation and particularly fusion capability is reduced in the satellite cell population of patients with CP, this could have dramatic consequences during muscle growth and repair. The relationship between sarcomere length and satellite cell activation and fusion is currently unknown. It is possible, however, that fewer cells successfully fuse with fibers and participate in muscle formation as patients with CP are growing, leading to the “stretched” muscle fibers with long sarcomeres seen previously (Lieber *et al.*, 1994; Smith *et al.*, 2011). Reduced differentiation and fusion capacity might also explain previous histological observations of CP muscle. Multiple researchers have found

high fiber size heterogeneity in CP (Castle *et al.*, 1979; Rose *et al.*, 1994), which is consistent with an altered ability of myoblast to fuse with existing muscle fibers during growth.

Reduced differentiation capacity might also explain overall reductions in satellite cell number in patients with CP. If, for example, myoblast fusion capability with existing myotubes is limited, more satellite cells may be required to provide the number of fusion events needed for muscle repair and growth. In a population thus shifted toward differentiation, the increased number of satellite cells needed to provide differentiating myotubes might lead to an eventual exhaustion of the overall pool even without overt cycles of rapid regeneration seen in diseases such as Duchenne Muscular Dystrophy.

4.6 Limitations

A main limitation of this study is the *in vitro,* tissue culture plate environment in which the cells were studied. Extrapolating from isolated cell behavior to predict how cells will function in the body obviously excludes many important parameters. The goal of this first exploration of satellite cells from CP muscle was to determine if intrinsic differences in the cells existed outside of the CP muscle environment. Ideally, future cell studies will be able to better recapitulate the environment experienced by these cells by matching factors such as oxygen tension, substrate stiffness, and extracellular matrix composition to values measured in CP. Currently, these values are not known, but considering them would provide a better view into what might actually occur *in vivo*.

Another limitation was imperfect matching of TD and CP subjects. The average age was 6 years older for TD participants. We would expect this age difference to cause minimal differences in satellite cells as all study participants were young (under 20 years of age). Previous studies of stem cells from subjects at a variety of ages shows similar proliferative ability for patients in the age range considered in this study (Renault *et al.*, 2000). Finally, no

correlation was seen between age or gender for either group in any of the parameters studied, suggesting that these factors did not systematically alter the results.

4.7 Conclusions

The results presented here open the door to many more questions about the behavior and function of satellite cells in CP. These *in vitro* data suggest decreases in satellite cell number seen previously in patients with CP do not appear to be due to an intrinsic difference in proliferative capability of the cells. Another possible culprit, decreased myoblast differentiation and fusion capability, may contribute to altered muscle fiber structure and function. When interpreting these data, however, it is important to keep in mind the significance of the altered muscle environment in CP. The complex interaction between satellite cells and their environment must be better understood in order to make accurate predictions of satellite cell behavior in CP and to understand the basis for their decreased number.

4.8 Acknowledgements

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Table 4.1. Characteristics of study participants

Patient	Sex	Age	Limbs	Popliteal Angle	GMFCS
CP	M	7.5	D	100	2
CP	M	2.7	Q	110	4
CP	M	8.7	Q	110	5
CP	M	7.4	D	100	2
CP	F	18.4	Q	100	5
CP	M	5.33	Q	90	5
CP	M	10.4	Q	135	4
CP	M	18.0	Q	90	4
TD	F	18.6			
TD	F	14.5			
TD	M	15.8			
TD	F	17.5			
TD	M	15.3			
TD	M	14.7			
TD	F	13.8			
TD	M	19.4			

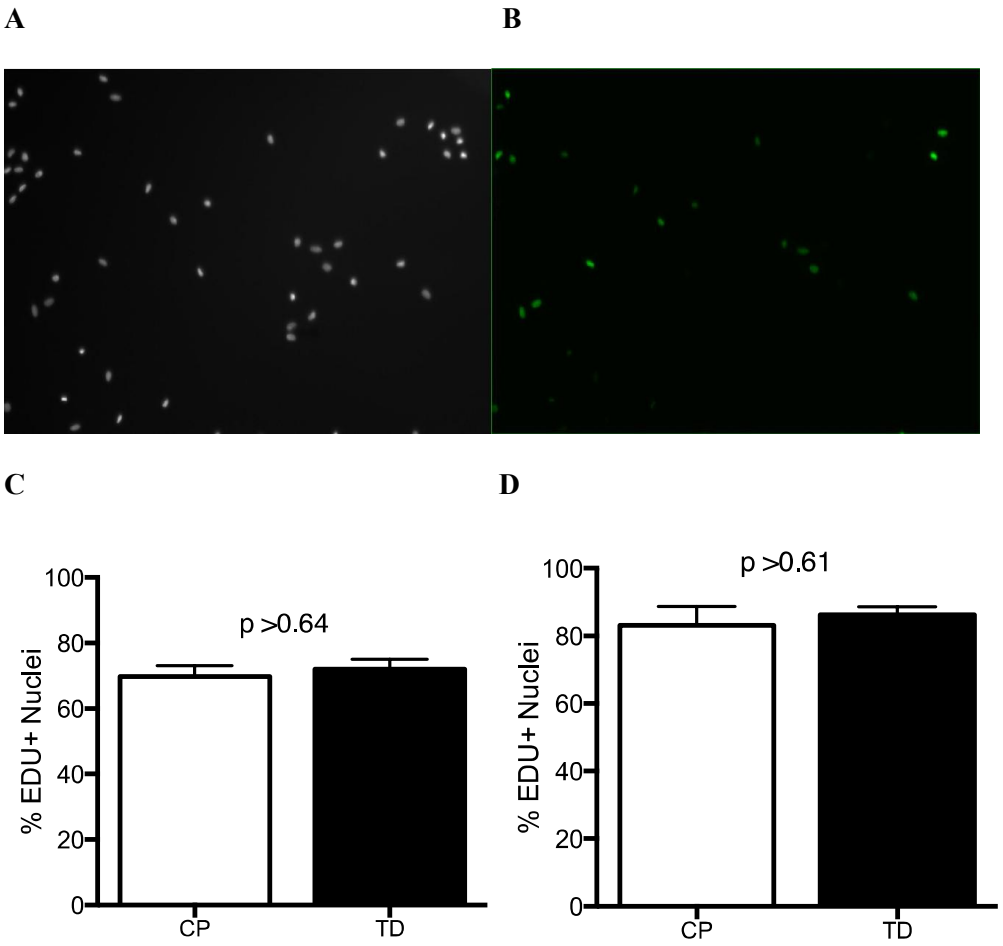
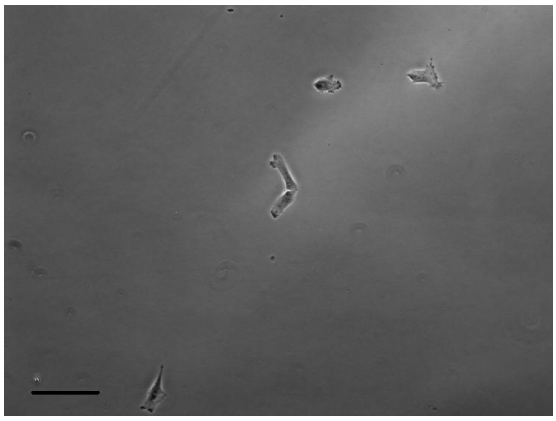
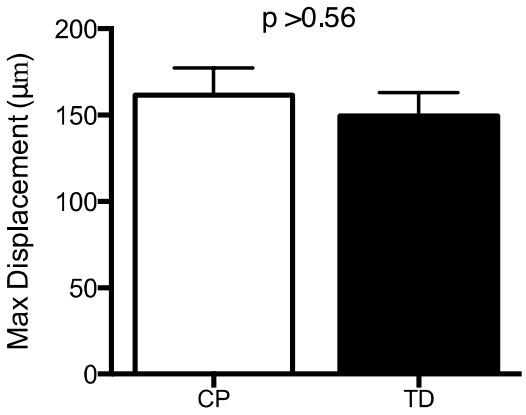


Figure 4.1. Proliferation measurements using EdU incorporation taken at 10x magnification **A)** Example image of total nuclei **B)** Example image of EdU positive nuclei **C)** The same fraction of nuclei were EdU positive at 12 and **D)** 18 hours, indicating that similar percentages of cells from each group had divided during those time periods.

A



B



C

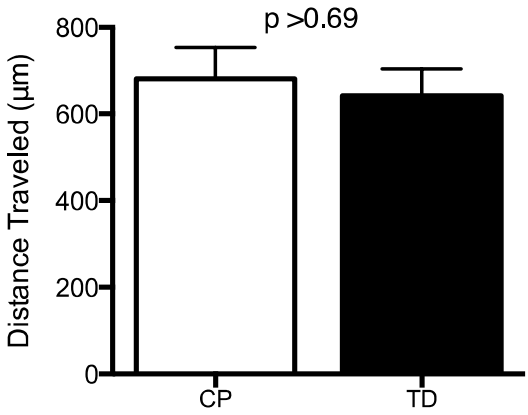


Figure 4.2. A) Brightfield image of satellite cells from a patient with CP. Scale bar indicates 100 **µ**m. **B)** There was no difference in the maximum distance cells traveled from their origin (directional motility) **C)** or in the total path length between CP and TD satellite cells.

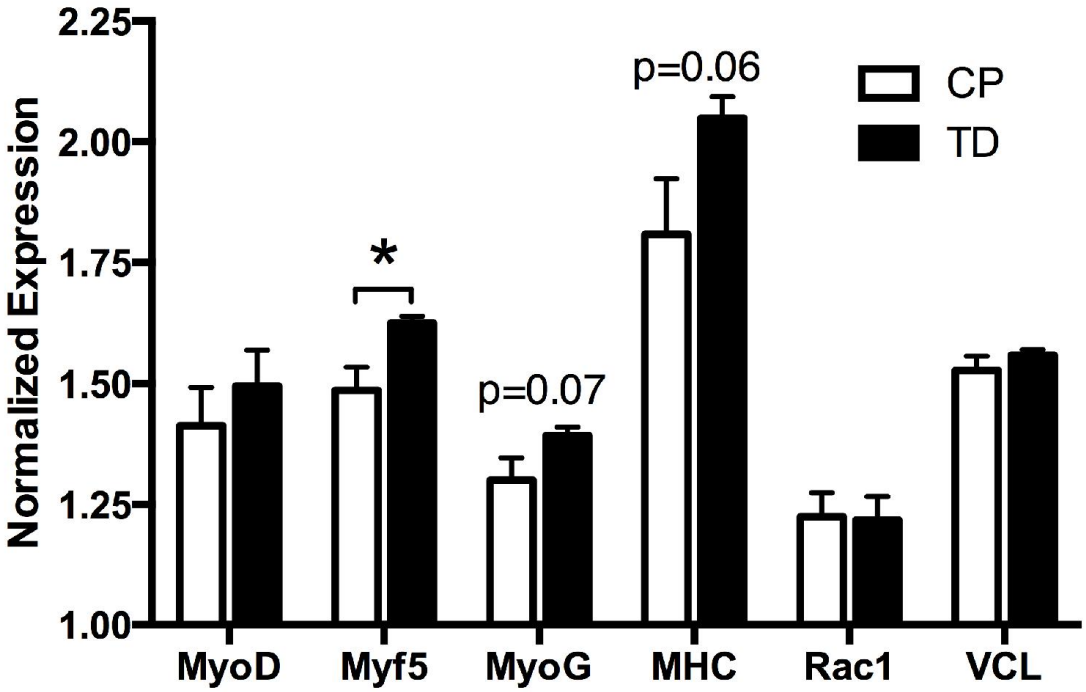
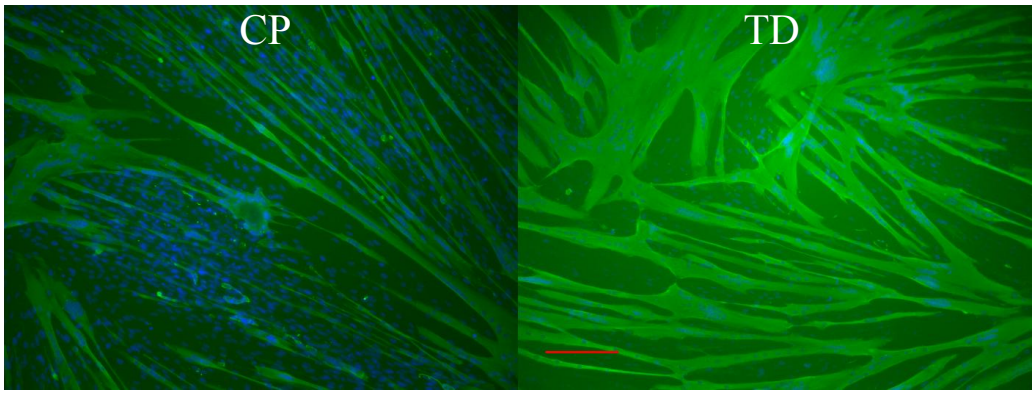
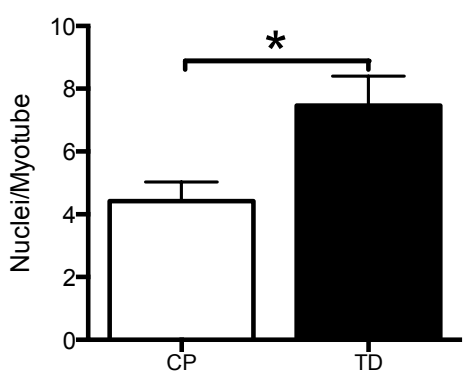


Figure 4.3. Differentiation and fusion PCR results. While there was no difference in early myogenic differentiation marker MyoD, Myf5 expression was significantly reduced in CP and MyoG and MHC levels showed a strong trend toward being decreased in CP satellite cells. Rac1, involved in myoblast proliferation and differentiation, and vinculin, involved in cell-cell adhesions, were both similar in CP and TD cells.

A



B



C

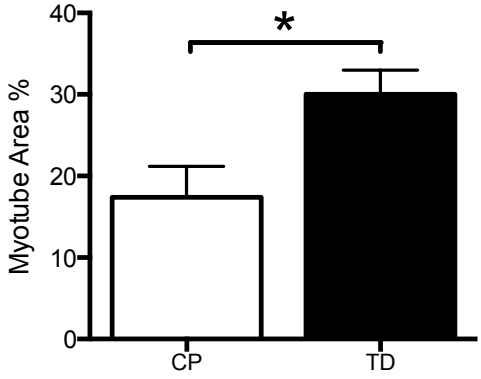


Figure 4.4. A) Representative images of CP and TD satellite cells. CP satellite produced long, thin myotubes with fewer nuclei than TD myotubes. Scale bar represents 200 μm. **B)** The average number of nuclei per myotube was lower in CP samples as was **C)** the total fraction of the image field covered by MHC positive staining.

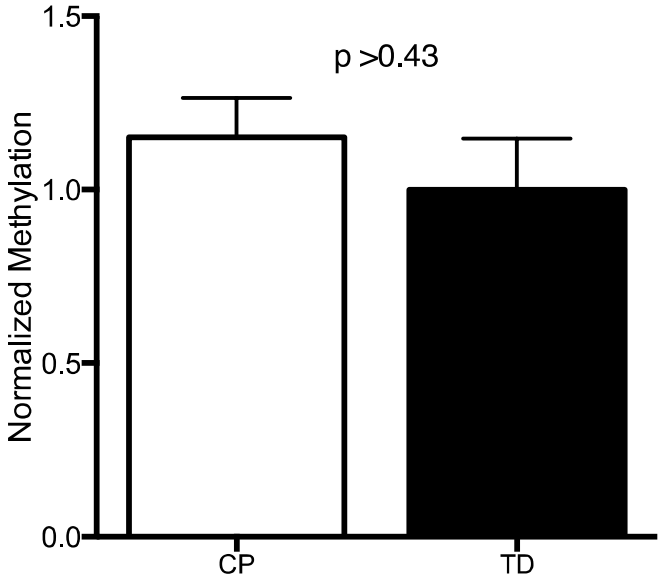


Figure 4.5. Global methylated DNA amounts did not differ systematically between CP and TD satellite cells.

4.9 References

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Chapter 5

Summary and Significance

5.1 Summary of Findings

While previous research has explored some aspects of muscle alteration in patients with CP, many questions remain about within-patient heterogeneity between muscles as well as underlying drivers of muscle change. The goal of this work was to further explore properties previously reported in other muscles and to begin to determine possible biological mechanisms for changes observed.

In Chapter 2 we presented the first example of paired fascicle length and sarcomere length measurements in patients with CP. Previous studies had made assertions about the state of the muscle using only fascicle length, which provided an incomplete picture of actual muscle architecture. Our results showed similar fascicle lengths for both groups but highly stretched sarcomeres in individuals with CP. Using the sarcomere length-tension curve, patients with CP would be predicted to have dramatically reduced force production based on their long sarcomere lengths. Longer sarcomeres in a fascicle of the same length also suggest that patients with CP have many fewer sarcomeres in series. The number of end-on sarcomeres in a muscle fascicle determines its excursion capability. With fewer serial sarcomeres, individuals with CP would be predicted to have less muscle excursion than their TD counterparts.

Chapter 3 expanded on the long sarcomere lengths found in Chapter 2 by measuring passive mechanical properties of the soleus and gastrocnemius muscles of the calf in patients with CP. When matched sarcomere lengths (eg. 3 μm) were considered, fibers were significantly stiffer in patients with CP but bundles were not different from TD individuals. At *in vivo* sarcomere lengths, however, which present a more *in vivo* like stiffness, both bundles and fibers were much stiffer in patients with CP for both muscles. Interestingly, the stiffness of CP fibers and bundles was not significantly different at *in vivo* lengths, suggesting that

increases in bundle stiffness seen in CP were due to increased fiber stiffness rather than changes to the ECM.

Changes to fibers seen in previous chapters, both at the level of stiffness and of sarcomere length, led us to focus on satellite cells, the building blocks of muscle, in Chapter 4. Satellite cells must be able to proliferate, move to the site of growth or injury, and differentiate and fuse with existing muscle fibers to promote normal muscle development and repair. While previous studies showing decreased satellite cell numbers in CP led us to expect decreases in proliferative ability, satellite cells from CP muscle had no deficits in proliferation or motility. They did, however, show a decreased ability to differentiate and fuse to form myotubes. While global methylation was found to be unchanged in CP, specific changes in methylation pattern might lead to differentiation deficits seen in CP satellite cells.

5.2 Significance of Findings

These results highlight the complexity of muscle alterations in CP. While long sarcomeres found in Chapter 2 agree with sarcomere lengths from the literature (Lieber *et al.*, 1994; Smith *et al.*, 2011), previous passive mechanical studies of muscle in CP vary by anatomical location (Fridén & Lieber, 2003; Lieber *et al.*, 2003; Smith *et al.*, 2011). The results found in Chapter 3, stiffer fibers contributing to increased bundle stiffness, differ from both the forearm, which showed increased fiber stiffness and decreased bundle stiffness (Fridén & Lieber, 2003; Lieber *et al.*, 2003), and the hamstring muscles, which had equally stiff fibers but stiffer bundles in CP (Smith *et al.*, 2011). Differences in use patterns, innervations, joint crossings, and many other factors may play into the inconsistencies in the literature. Heterogeneity within and among patients is a hallmark of CP, however, so the more areas that are studied, the more complete picture doctors and researchers can form about muscle in CP.

Satellite cell studies presented in Chapter 4 provide a truly novel view of contractures in CP. While one previous study had noted decreased satellite cell number in patients with CP (Smith *et al.*, 2013), satellite cell characteristics were completely unexplored. Because of the underlying brain injury, muscle adaptations were often assumed to be at the level of innervation rather than developmental adaptations to the muscles themselves. This evidence that satellite cells, fundamentally important for muscle growth and development, are different in patients with CP, suggests that the current understanding of CP contractures is missing a critical muscle biological component. The early *in vitro* data presented here will pave the way for future studies of satellite cells, both at the level of growth and behavior as well as the epigenetic mechanisms the drive changes in differentiation capability. Future therapies for CP may eventually be able to focus on restoring muscle function by providing "healthier" satellite cells or epigenetically altering existing satellite cells to behave more like those from TD individuals.

5.3 Future Directions

5.3.1 Epigenetics of Satellite Cell Alterations

The current understanding of CP suggests that genetic mutations are not a major cause of the disorder (Naeye *et al.*, 1989; Bax *et al.*, 2005; Rosenbaum *et al.*, 2007; Fairhurst, 2011). Genetic differences in satellite cells, therefore, are not expected to explain alterations in differentiation seen in the study presented here. Epigenetics is a growing field that provides a non genetic explanation for heritable changes seen in a variety of cell types (Riddihough & Zahn, 2010). While environmental effects on satellite cells are a strong possibility, the fact that satellite cell differences persist outside their native environment suggests epigenetic changes as a likely culprit for decreased differentiation ability in these cells. By probing methylation and histone modifications in regions specific to differentiation and myoblast

fusion, it would be possible to pinpoint alterations in CP satellite cells. So far, no deep biological mechanism of this sort has been proven to produce CP muscle changes, so an understanding of epigenetic alterations in satellite cells could lead to novel therapies.

5.3.2 *In Vivo* Satellite Cell Niche Characterization

While the information gained through culture of isolated satellite cells on tissue culture plastic gives basic information about CP and TD satellite cell differences, it cannot provide a picture of how satellite cells actually behave *in vivo*. Many factors of the satellite cell environment, including fiber stiffness (Fridén & Lieber, 2003), capillary density and therefore oxygen tension (Pontén & Stal, 2007), and extracellular matrix quantity and composition (Lieber *et al.*, 2003; Smith *et al.*, 2011), among others, differ between CP and TD muscles. These environmental factors likely play a critical role in how satellite cells behave and interact with their surroundings in patients with CP. A detailed investigation into the satellite cell niche could provide the basis for dramatically improved cell culture systems. Atomic force microscopy, combined with a critical consideration of what forces satellite cells actually experience (shear vs. tension vs. compression) could give information about substrate stiffness. Careful histology would offer information about capillary density alteration and provide the basis for calculations of oxygenation within the muscle. Western blotting and Elisa assays combined with high-resolution microscopy could be used to determine ECM environment surrounding the satellite cell. Using data from studies such as these, a dramatically more *in vivo*-like cell culture system could be created, allowing us to probe satellite cell behavior in an environment closer to what is seen in CP.

5.3.3 Other Mononuclear Cells

A critical component of muscle growth, repair, and regeneration is the host of cell types that provide growth cues and support for satellite cells. The work presented here does not consider the contributions of these many important cell types to muscle development and repair in CP. Fibroblasts, for example have been shown to secrete collagen IV, which regulates satellite cell proliferation and renewal ability (Urciuolo *et al.*, 2013). Other studies have found fibroblasts critical for other satellite cell functions such as muscle growth and repair (Murphy *et al.*, 2011). Several techniques exist that could allow experiments to determine the impact and characteristics of these cells types. Fluorescence activated cell sorting, which was used to collect satellite cells for culture, still lacks reliable, documented antibodies for cell types such as fibroblasts. While fibroblast antibody examples exist in the literature (Strutz *et al.*, 1995; Camelliti *et al.*, 2005; Goodpaster *et al.*, 2008), none reliably labeled fibroblasts in our preliminary studies. Identification of a robust fibroblast marker would allow coculture or transwell culture of satellite cells with these important support cells and might give a much more accurate picture of satellite cell behavior. Until a marker is found, preplating techniques could be used to collect fibroblast-like cells. A complete picture of how satellite cells, other mononuclear cells, and their environment interact could point to more mechanistic explanations of how satellite cell, and subsequently muscle, alterations occur in CP and could pave the way for satellite cell-based therapies in the future.

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