EVALUATING MUSCLE DEFICITS AND CENTRAL ADIPOSITY IN CHILDREN WITH CEREBRAL PALSY

by

CHUAN ZHANG

(Under the Direction of Christopher Modlesky)

ABSTRACT

The purpose of this dissertation was to evaluate the skeletal muscle deficits and the level of central adiposity in children with cerebral palsy (CP) with four studies. The aim of the first study was to determine whether fat-free soft tissue mass (FFST), a marker of skeletal muscle mass, from dual-energy X-ray absorptiometry (DXA) is more compromised at the appendicular than non-appendicular sites in children with CP. The main finding was that children with CP have a remarkable FFST deficit that is more pronounced in the appendicular than the non-appendicular regions and more pronounced in the lower than the upper appendages. The aim of the second study was to determine whether DXA accurately estimates midleg muscle mass in ambulatory children with spastic CP. The main finding was that DXA-based statistical models accurately estimate midleg muscle mass in children with CP when the models are composed using data from children with CP rather than typically developing children. The aim of the third study was to determine whether serum levels of myostatin, follistatin and IGF-1 are consistent with the muscle deficit in children with CP. The main finding was that circulating myostatin,

follistatin and IGF-1 are not different between ambulatory children with CP and typically developing children. However, FFST per unit IGF-1 was lower in children with CP, suggesting that IGF-1 may play an important role in their muscle underdevelopment. The aim of the fourth study was to determine whether the level of central adiposity in children with CP is related to their motor function and physical activity. The main finding was that the level of central adiposity was greater in children with CP than in typically developing children, but it was not related to the level of gross motor function or physical activity except for subcutaneous fat, which was negatively related to gross motor function.

In conclusion, children with CP have much lower skeletal muscle mass and higher central adiposity than their typically developing peers. Future studies should identify intervention strategies that effectively attenuate the muscle deficits and reduce the level of central adiposity in children with CP.

INDEX WORDS: Cerebral palsy; skeletal muscle; visceral fat; physical activity; dual-energy X-ray absorptiometry; magnetic resonance imaging

EVALUATING MUSCLE DEFICITS AND CENTRAL ADIPOSITY IN CHILDREN WITH CEREBRAL PALSY

by

CHUAN ZHANG

B.Ed, Beijing Sport University, China, 2013

MA, Teachers College of Columbia University, 2015

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSPHY

ATHENS, GEORGIA

2019

EVALUATING MUSCLE DEFICITS AND CENTRAL ADIPOSITY IN CHILDREN WITH CEREBRAL PALSY

by

CHUAN ZHANG

Major Professor:	Christopher Modlesky
Committee:	Richard Lewis
	Ellen Evans

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 201

ACKNOWLEDGEMENTS

I thank Dr. Christopher Modlesky for serving as my major professor, and for the excellent guidance and support he has given me throughout the four years of my doctoral study. I thank Dr. Ellen Evans and Dr. Richard Lewis for serving as my committee members, taking time to meet with me and providing great feedback to help me make my dissertation better. I thank Dr. Kevin McCully for being my postdoctoral mentor, and I look forward to making great things happen. I thank Dr. Harshvardhan Singh, Dr. Daniel G Whitney, Benjamin Connor, Nicholas Laganeli, Cara Esposito, Rachel Campo, Ewan Williams, Albino Schifino, Katherine Collins, Kathleen Ollendick, Joel Licea, Suzanne Volger, Sydni Wilhoite and Owais Khan for being great undergraduate, graduate and professional coworkers, and making this path easier. I thank all of the faculty members, staff, graduate students and undergraduate students who have provided assistance during my doctoral studies and during the completion of this dissertation. I thank the National Institutes of Health and the United Cerebral Palsy Foundation for their generous grant support for our studies. I am very thankful to all our research participants and their families for taking part in our studies, and I do hope they enjoyed the time that they spent with me. I also want to thank Eric Magrum for weight training with me to help me stay active, and I thank the Chinese app Tiktok for helping me pass through some difficult times. Very importantly, I thank my cousin, Yingying Xu and my parents, Yingzhou Zhang and Baozhi Qiao, for standing by my side whenever I needed, and for giving me courage to walk through this adventure. Lastly, I also want to thank myself, for it was a long and hard journey, and I am glad I am able to stand at the finish line.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION

Cerebral palsy (CP), a disorder of movement and posture, is the most common childhood disability. It was estimated that the prevalence of CP is about 3.1 to 3.6 per 1000 new births [1], affecting nearly about 1 million individuals in the United States [2]. Children with CP exhibit a significantly lower level of physical activity compared to their typically developing peers without neurological disorders, with ambulatory children with CP shown 40 % reduction [3] and nonambulatory children with CP shown 70 % reduction [4]. This is important because childhood is viewed as a crucial period for musculoskeletal growth and development [5], and a lack of mechanical stimulation can be detrimental to such accretion. On the other hand, the lack of physical activity could lead to global and regional body fat profile changes, which haven't been thoroughly investigated in children with CP.

A more pronounced deficit in fat-free soft tissue (FFST) mass, a surrogate marker for skeletal muscle mass, at the appendages than at the trunk has been observed in other mobility related disorders, such as spinal cord injury [6,7]. However, it is unclear whether a similar deficit pattern is present in children with CP. Gaining this knowledge is essential because it will assist scientists in understanding the level of the FFST deficit across the body of children with CP. It has also been demonstrated that in addition to having lower muscle volume [3,8-10], the amount of muscle for a given amount of FFST is lower in children with CP than typically developing children [11]. This leads to erroneous estimates of muscle mass when it is assessed using indirect measures, such as FFST from dual-energy X-ray absorptiometry [11]. Therefore, prediction models

that can accurately estimate muscle mass from FFST in children with CP are needed. Further, while the cause of the musculature deficit in children with CP is generally attributed to the decreased levels of physical activity and unloading [12], there may be other contributing factors. There is evidence that the availability of bioactive agents such as myostatin, follistatin and insulin-like growth factor 1 (IGF-1) could affect skeletal muscle development, therefore may play a role in the underdeveloped musculature in different musculoskeletal disorders. However, this is poorly studied in individuals with CP. Lastly, while it has been reported that ambulatory children with CP have elevated central adiposity depots compared to their typically developing peers [13], a site where fat distribution can highly impact cardiovascular health [14], no studies have evaluated the level of central adiposity across all levels of gross motor function in children with CP.

Therefore, the purposes of this dissertation were four fold: 1) to determine whether FFST is more compromised at the appendicular sites than at non in children with CP, 2) to determine whether DXA can be used to estimate DXA to estimate mid-leg muscle mass in ambulatory children with CP, 3) to determine whether serum bioactive agents myostatin, follistatin and insulin-like growth factor (IGF-1) levels are consistent with the muscle underdevelopment observed in children with CP, and 4) to determine whether a lower level of gross motor function and a lower level of physical activity are associated with a higher level of central adiposity in children with CP.

CHAPTER 2

LITERATURE REVIEW

2.1 Cerebral Palsy (CP)

CP is an umbrella term that refers to a group of non-progressive disorders caused by a disturbance to a fetal or infant brain [15]. It is a heterogeneous disorder with multiple ways to define the different subtypes of CP. If classified based on motor types, CP can be divided into hypertonic (spastic), dyskinetic, hypotonic, mixed motor types and ataxic CP [16]. Spastic CP is the predominant subtype, with more than 80 % of individuals with CP have spasticity [17].

2.2 Motor Function

It is well recognized that children with CP have problems with motor control. The corticospinal tract, which is responsible for volitional motor control, is usually disrupted in children with CP due to an injury to the brain. As a result, children with CP exhibit a wide range of gross and fine motor deficiencies that impair the ability to perform the most fundamental of activities, such as walking and running, as well as more sophisticated activities, such as bimanual motor tasks [18].

2.2.1 Gross Motor Function in Children with CP

The gross motor function is usually measured according to the gross motor function classification system (GMFCS). This is a five-point scale, with higher numbers indicate more advanced motor function deficit [19]. Specifically, GMFCS I indicates that the child has a the ability to walk indoors or outdoors and climb stairs without limitations, but can only run or jump

at a reduced speed compared to typically developing children. GMFCS II indicates that the child can walk indoors and outdoors, but may face difficulties walking on uneven surfaces, and need the help of a rail when climbing upstairs. A limited ability to run and jump is also observed in children with CP who had GMFCS II. GMFCS III indicates that the child has the ability to walk indoors and outdoors with the help of assistive devices, such as a cane, and may need a wheelchair to assist long-distance movement. GMFCS IV indicates that the child needs a wheelchair to move around but still preserves certain mobility. GMFCS V indicates that the child completely lacks the ability for independent mobility and all types of motor function are limited. The validity and reliability of using GMFCS to assess gross motor function in children with CP is established [20].

Depending on their ambulatory status, children with CP are sometimes divided into ambulatory group and nonambulatory subgroups. By definition, children with GMFCS I and II are considered "ambulatory", however the classification for those with GMFCS III has been somewhat conflicting, with some studies considering them ambulatory [21,22] and some considering them nonambulatory [23]. The distinction may be whether the child can ambulate independently. For studies focused on bone and muscle, where independent ambulation is very important, it may be better to classify children with CP who have GMFCS III as nonambulatory.

2.2.2 Fine Motor Function in Children with CP

The Manual Ability Classification System (MACS) was developed specifically to classify the upper extremity fine motor function in children with CP [24]. Similar to GMFCS, MACS is a five-point scale system with higher numbers indicating more fine motor deficiency. MACS I

indicates that achild has the ability to handle objects easily and successfully. MACS II indicates that a child can handle most objects in a reduced speed or quality. MACS III indicates that a child handles things with difficulty and may need external help. MACS IV indicates that a child can only handle a limited selection of things that can be easily managed. MACS V indicates that a child cannot handle things and has a very limited ability to perform simple actions. It is important to notice that this scale was developed to assess the coordination of both hands rather than the ability differences between different hands. Therefore, it is going to be reflective of one's ability perform fine motor skills that are closely related to daily life, like eating, brushing teeth, putting clothes on, etc.

The validity and reliability of the MACS scale has been evaluated and deemed to be excellent [24,25]. In addition, there is a growing interest in identifying the relationship between GMFCS and MACS in children with CP. While discrepancies do occur [26], in general, a moderate to high level of correlation has been observed between gross and fine motor classification scales [24,27,28] (*r* ranges from 0.62~0.79).

2.3 Typical Muscle Growth and Development

During embryonic development, the middle portion of the embryo, which is referred to as the mesoderm, condenses into somites. The dorsal portion of the somites become dermomyotome which give rise to skeletal muscle progenitor cells (also named myoblast). The myoblasts eventually fuse together to form multinucleated muscle fibers, a process that is controlled by a multitude of factors [29]. Myoblasts that do not differentiate into muscle fibers become satellite cells, which are a group of cells with a single nucleus generated around myofibers that are mitotically quiescent but important for later mature skeletal muscle hypertrophy[30,31].

In the event of external stimulation, such as exercise or mechanical loading, satellite cells become activated from quiescent status to provide additional myonuclei for muscle fibers and fuse with them to achieve muscle hypertrophy. A crucial mediator of this process is insulin-like growth factor 1 (IGF-1) [31].

2.4 Muscle Spasticity and Related Muscle Changes

A classic, and perhaps the most cited, definition for spasticity was created by Lance who defined spasticity as "a motor disorder characterized by a velocity-dependent increase in tonic stretch reflexes with exaggerated tendon jerks, resulting from hyperexcitability of the stretch reflex, as a component of the upper motor neuron syndrome" [32]. However, there is still no consensus on whether this is the most accurate definition for spasticity. Other definitions for spasticity have been proposed [33-35], with some people questioning whether those definitions truly capture the spasticity phenomena [36]. The lack of agreement among researchers on how to definie such an impairment may, at least partly, lie in the heterogeneous nature of spastcity [37]. Nevertheless, it's clear that clinically, spasticity is characterized by increased involuntary hypertonia and is associated with compromised movement ability.

2.4.1 Muscle Stiffness in Children with CP

It has come to the attention of clinicians and researchers that children with CP also have increased muscle stiffness, which may contribute to their abnormal gait and diminished force generation capacity [38]. The exact causes of such phenomena is uncertain, but was suggested to be more than just the abnormal muscle-tendon unit length [38].

Muscle stiffness can be quantified using elastrography, which can be achieved along with MRI or some specialized ultrasound devices. The basic idea behind elastrography is to introduce

a local vibration to a specific tissue area and quantify the velocity of the vibration propagation using imaging techniques was first applied to diagnose liver fibrosis [39].

Studies that have used ultrasound elastography to assess muscle stiffness in children with CP suggest that children with CP have stiffer muscles than their typically developing peers [40,41]. A study by Kwon et al. found that medial gastrocnemius muscles are stiffer in children with CP (n = 15) than in children without CP (n = 13) [40]. Similarly, using ultrasonic elstography, Brandenburg et al. showed that compared to their age and sex matched typically developing peers (n = 13), children with CP (n = 13) have a higher level of muscle stiffness as indicated by greater shear modulus in lateral gastrocnemius muscle at both neutral and plantarflexion ankle positions [41]. Some argue that the stiffness seen in muscle in children with CP is mainly due to alterations in collagen in the extracellular matrix but not due to changes in individual muscle fibers [42].

2.4.2 Muscle Strength in Children with CP

It is well established that children with spastic CP have compromised muscle strength. Using a hand-held dynamometer, Wiley et al. compared the lower extremity strength profile in 15 ambulatory children with spastic hemiplegic CP, 15 ambulatory children with spastic diplegic CP and 16 typically developing children who had similar ages to those with CP [43]. Not surprisingly, children with spastic CP compared to typically developing children showed significantly lower strength in all muscle groups tested, with hip extensors, ankle dorsiflexors and plantarflexors being the weakest. Interestingly, this study also demonstrated that, in addition to having a strength deficit on the non-dominant side, children with spastic hemiplegic CP also showed compromised strength in the dominant side compared to their typically developing peers.

The finding suggests that there may be a generalstrength deficiency in children with spastic CP that tends to be more prevalent on one side of the body, such as the right upper and lower extremity, or in one region of the body, such as the lower extremities vs. the upper extremities. .

In addition, to demonstrate that children with spastic CP have diminished lower-extremity strength in general, Elder et al. went a step further to try to identify the potential factors that contribute to muscle weakness in indviduals with CP [44]. In their study of 28 children with spastic CP (14 with hemiplegia and 14 with diplegia) and 18 typically developing children, they used magnetic resonance imaging (MRI) to quantify muscle cross-sectional area (CSA) of plantarflexors (PF) and dorsiflexors (DF). They also used electromyography (EMG) to assess muscle activity of the soleus, medial gastrocnemius (MG) and tibialis anterior during voluntary contraction. Aside from demonstrating that children with CP had lower maximal torque, they also showed for the first time, that this population has reduced specific tension, that is, PF or DF torque normalized by their respective anatomical CSA. The study is of particular importance because it showed that the lower extremity strength in children with spastic CP is compromised even after accounting for their smaller muscles. Based on EMG results during maximal contraction, they discovered that other than the apparent muscle architectural changes, the inability to activate the PF is the major reason for the plantarflexion weakness. Whereas, the combined effects of incomplete activation and PF co-activation are contributing to DF weakness.

There are other studies that also looked at the strength profiles in children with spastic CP [45,46], all of which clearly demonstrated a compromised ability for maximal contraction at the lower extremities in this population. Fortunately, this pattern is not irreversible, as multiple studies have suggested an increase in muscle strength after strength/resistance training in children with spastic CP [47-50]. Other training paradigms like neuromuscular electrical stimulation

[51] (NMES) or whole body vibration training [52] have also showed promising results to improve strength in this population. The later ones seem to be drawing more attention, as they are passive training paradigms and do not inflict too much perceived fatigue on the children. Therefore. children with CP are more likely to adhere to whole body vibration training.

2.4.3 Gross Muscle Morphology Changes in Children with CP

Gross muscle architecture in children with spastic CP is generally assessed using either ultrasound or MRI. While computed tomography (CT) has the ability to assess muscle architecture as well, it is seldom used in muscle studies in children with CP.

2.4.3.1 Muscle Fascicle Length

One of the first studies to assess fascicle length in children with CP was done by Shortland et al., who used ultrasound to examine MG gross muscle architecture in ambulatory children with spastic CP [53] (n = 7). Compared to typically developing children (n = 5) and normal adults (n = 5), children with CP had no difference in muscle fascicle length, as well as fascicle length normalized by leg length. The importance of this study was that it suggested the shortness seen in MG muscle belly length was not due to reduced fascicle length. The finding was later supported by Malaiya et al. [54].

However, this conclusion was later challenged by Mohaghehi et al. who performed ultrasound scans on 18 children with spastic CP and 50 typically developing children. They concluded that both the absolute and normalized fascicle lengths were shorter in the gastrocnemius muscles in the CP group [55]. The same group also conducted another study which reported 18 % lower gastrocnemus fascicle length in the paretic leg compared to non-paretic leg

in children with hemiplegic CP [56]. Although it is currently unclear why such discrepancies exist, contributing factors may be the different characteristics of the research participants (i.e. diplegic only vs. mixed types of CP), the position where the ultrasound probe was placed, the joint angle at which measurements were taken, and the range of severity of CP for children involved in the study. More recent studies continue to present conflicting results on this matter [57-60], with some suggesting a lack of between groups difference [57], and others reporting higher [60] and lower [58,59] fascicle lengths in children with CP. Therefore, studies with better designs are needed before a definitive conclusion can be drawn.

2.4.3.2 Pennation/Fascicle Angle

Interestingly, although fascicle length is usually calculated based on muscle thickness and fascicle angle, not all studies that obtained fascicle length in children with CP also reported fascicle angle [60]. Whether children with spastic CP have altered muscle fascicle (or pennation) angle remains uncertain.

Shortland et al. [53] and Malaiya et al. [54] reported no differences in fascicle angle in the MG of children with CP compared to tyically developing children when the tested limb was at rest, while a reduced fascicle angle was found at when the limb was placed at 30^{o} of plantarflexion. Similarly, Moreau et al. reported no difference in fascicle angle in the biceps femoris of children with CP compared to typically developing children when rested in the supine position [58]. However, the same study also reported a 3^{o} lower fascicle angle for the vastus lateralis muscle in children with CP. On the other hand, Gao et al. found a slightly larger, but non-significant, fascicle angle in the MG of those with CP compared to typically developing children at the neutral plantarflexion and dorsiflexion positions [59].

Consistent with other studies, Barber et al. [57] reported no difference in the fascicle angle of the MG in a group of young children with CP (n = 15; 2 – 5 y) compared to typically developing children (n = 20) when the ankle was at the resting angle. However, children with CP had a smaller MG fascicle angle when the ankle was at the maximal plantarflexion position [57]. The results suggest that if fascicle alterations occur in children with CP, they may occur at at a very early stage of life.

To sum up, similar to muscle fascicle length, there is conflicting evidence about the fascicle/pennation angle alterations in children with CP, with some studies suggesting no significant changes, while others indicate either higher or lower in this population compare to their typically developing peers. Therefore, a definitive conclusion cannot be made on this matter until studies with better designs and quality control are conducted.

2.4.3.3 Muscle Thickness and CSA

Unlike muscle fascicle length and fascicle angle, studies have consistently demonstrated lower muscle thickness and CSA in children with spastic CP compared to typically developing children and has been linked to their muscle weakness. Muscle CSA can be expressed as anatomical CSA, which is the absolute area for a muscle at the transverse plane. Muscle CSA can also be expressed as physiological CSA, which also takes the fascicle angle into consideration [61]. Therefore, physiological CSA is bigger than anatomical CSA for a pennate muscle. Multiple studies have confirmed that physiological CSA is closely related to force generation capacity, perhaps even closer than anatomical CSA in human [62,63]. However, the precise calculation for physiologic.l CSA usually requires multiple instruments, such as MRI and

ultrasound, to assess it. Thus, little information is available for the physiological CSA in children with spastic CP.

Compared to their typically developing peers, children with CP were found to have smaller muscle thickness for the MG [55], lateral gastrocnemius [55], vastus lateralis [58] and rectus femoris [58] as measured by ultrasound. Mohagheghi et al. also found that the paretic limb vs. the non-paretic limb of children with spastic hemiplegic CP has20 % lower muscle thickness in the MG and lateral gastrocnemius when measured at the resting ankle position [56]. Given that muscle thickness is significantly correlated with mobility and gross motor function in children and adolescents with CP [64], and the fact that this measurement is easy to obtain with ultrasonography, clinicians may consider including muscle thickness as part of the routine assessment in those with CP.

Muscle anatomical CSA can be measured with either MRI or ultrasound, however, MRI is considered the "gold standard". using MRI, a lower muscle CSA was found for both the anterior and posterior compartments of the leg in children with hemiplegic CP (n = 8) compared to those without CP (n = 18), and the CSA was lower in their affected side than their non-affected side [44]. Using MRI, Bandholm et al. found that compared to typically developing children (n = 6), children with CP (n = 9) had lower anatomical CSA. Also, in a subgroup of children with hemiplegic CP (n = 8), a 32 % side-to-side difference was present [65]. Several other studies that utilized ultrasonography also confirmed a reduced muscle anatomical CSA for the biceps femoris and vastus lateralis [58,66] in children and adolescents with CP. Only a limited number of studies have assessed the physiological CSA in children with CP. In a group of very young children with CP (n = 15, age 2-5 y), the MG physiological CSA was found to be lower

when compared to their typically developing peers [57]. Similar findings have been reported for young adults with CP [67].

Taken together, it is clear that the muscle thickness and CSA are compromised when compared to typically developing children, and for children with hemiplegic CP, the paretic limb muscle thickness and CSA are more deficient relative to the non-paretic limb.

2.4.3.4 Muscle Volume

Similar to muscle thickness and CSA, muscle volume has consistently been shown to be lower in children with CP. Specifically, MG volume was shown to be much lower in children with hemiplegic CP [54] and diplegic CP [68], and rectus femoris and vastus lateralis were found to have lower volumes in children and adolescents with CP when compared to their typically developing peers [58]. In addition, dorsiflexor and plantarflexor muscles groups have lower volumes in children with CP compared to typcally developing controls [44]. Research have also demonstrated a collective lower extremity muscle volume reduction for this population [3,4,8]. Importantly, recent studies [69,70] and unpublished observation from our laboratory suggest that the volume for individual muscles at the leg in children with CP was significantly compromised as measured by MRI , even for ambulatory children with CP when compared to their age, sex and ethnicity matched controls. The level of deficit present in those studies ranges from 10 % to ~60 %. Interestingly, some studies also demonstrated a more compromised muscle volume distally (leg) than proximally (thigh) [10,70]. Moreover, a noticeable side-to-side muscle volume difference was present in children with hemiplegic CP [44,54].

2.4.4 Muscle Fat Profile Changes in Children with CP

Only a handful of studies have quantified the adipose tissue infiltration of muscles in children with CP. Johnson et al. [4] found a 2.3 fold higher mean intermuscular fat CSA and 1.7 fold higher mean subfascial fat CSA for non-ambulatory children with quadriplegic CP at the mid-thigh when compared to control matched to children with CP for age, sex and pubertal development. Interestingly, no differences in subcutaneous fat CSA was observed. This is consistent with another study which reported no group difference in subcutaneous fat volume at the mid-third compartment of the lower leg in ambulatory children with spastic CP compared to typically developing children [3]. However, there was a 3.3 fold higher subfascial fat and a 3.7 fold higher intermuscular fat volume in the children with CP. Importantly, the same study, along with another recent study [8], pointed out that the intramuscular fat infiltration is elevated by ~ 50 % in ambulatory children with CP, as measured by MRI, when compared to their typically developing peers. To sum up, children with CP have elevated intermuscular and intramuscular fat, but not subcutaneous fat when compared to typically developing children.

2.4.5 Bioactive Agents That May Contribute to the Muscle Changes in Children with CP

It is generally believed that the underdevelopment of muscles observed in children with spastic CP is mainly due to a lack of physical activity whch causes a lack of mechanical stimulation, disuse and a failure to reach their full genetic potential [58,64]. However, endocrine factors may influence this process as well [12]. It is currently uncertain whether some of the bioactive agents that are commonly known to affect muscle growth and development play a role in the muscle architectural and volumetric changes in children with CP.

2.4.5.1 Growth Hormone (GH)

GH releasing hormone (GHRH) secreted by the hypothalamus located within the brain, along with somatostatin, also known as GH inhibiting hormone (GHIH), work together to control the GH release from the somatotropic cells located at the anterior pituitary gland. GH acts directly on a majority of the internal organs like bone, muscle and adipocyte to promote anabolic effects, but also on the liver to stimulate the release of IGF-1, which induces growth and development. The status of an individual's growth will, in turn, affect the release of GHRH and GHIH within a feedback loop. This whole process is also known as the GH axis. Importantly, it was pointed out that the anabolic effects of GH are more related to the increased rate of protein synthesis rather than slowed protein degradation rate [71].

GH deficiency is known to affect human muscle development. For example, thigh muscle CSA, muscle force and body weight were significantly lower in a group of adults with long standing GH deficiency (n = 24) compared to controls (n = 41) [72]. For men who have confirmed diagnoses of GH deficiency (n = 42), total body muscle mass was significantly smaller when compared to their age- and sex- matched controls, despite all of them having received GH treatment during their childhood [73]. On the other hand, a number of studieis have shown that GH injection and supplementation could improve muscle quality in those with GH deficiency or in populations characterized by diminished muscle mass and strength [74-76]. Similar anabolic effects were also observed in healthy adults [77].

Exercise/physical activity has been recognized as an influential factor for GH secretion during childhood. In a study by Keenen et al. [78] involving 25 typically developing children, a two-fold higher level of serum GH was observed immediately after moderate exercise when compared to resting status, and remained at the same elevated level for 20 minutes after the

exercise stopped. Similar observations were also reported by Cappa et al. [79], who found a significant serum GH increase after either completing the Bruce walking protocol or a bicycle ergometry test. Importantly, the same study also found that children can have a second GH spike when the Bruce protocol was carried out again, two hours later. Whereas, no such GH increase was observed in adults [79], which indicated more endocrinological GH secretion flexibility in children compared to adults. Interestingly, in the early days, exercise was suggested to be a screening tool for children with GH deficiency, and the post-exercise GH level was set as the initial exclusion criterion [80]. However, the lack of mobility leads to a significantly lower physical activity level and exercise capacity in children with CP, which may potentially influence their GH availability.

Although direct assessment of the relationship between GH deficiency and skeletal muscle underdevelopment in children with CP are lacking, it was proposed that the prevalence of such phenomena in this population may account for part of their slowed growth velocity [81-85], and that GH therapy may be needed for them achieve normal stature. Given the clear link shown between GH and muscle growth in animal and human studies, it is suspected that GH is low in children with CP and may play a role in their compromised musculature.

2.4.5.2 IGF-1

IGF-1 has been suggested to play a crucial role in muscle hypertrophy. During postnatal muscle growth, myonuclei located within mature muscle fibers cease to divide. However, for the purpose of muscle growth, additional myonuclei are needed. Satellite cells, a group of quiescent cells located between the basal lamina and sarcolemma of myofibers, can be activated and go through proliferation to provide more myonuclei, and eventually fuse with existing myofibers to

achieve muscle hypertrophy. It has been shown that IGF-1 is an important regulator of this process [31,86].

IGF-1 is an anabolic peptide that's mainly produced by the liver and also locally by the skeletal muscle. The majority of the circulating IGF-1 binds with insulin-like growth factor binding protein 3 (IGFBP-3), until it reaches the targeted tissue to bind with IGF-1 receptors to initialize a series of signaling cascades [87]. Importantly, other than activating satellite cells, IGF-1 has also been shown to increase gene transcription and protein synthesis [88]. As a result, both proliferation and differentiation of satellite cells are being promoted, although the pathways through which those processes are accomplished are very different [89].

IGF-1 has been shown to mediate muscle loss. In animal models, localized IGF-1 gene expression has been shown to attenuate skeletal muscle wasting in mice with weight loss and muscle wasting as a result of infusing angiotensin II, a condition mimicking advanced stage of congestive heart failure [90]. In mice with induced muscle injury, localized overexpression of IGF-1 leads to down-regulated proinflammatory cytokines and rapid muscle regeneration [91]. In human studies, IGF-1 with IGFBP-3 supplementation given to children who suffer a severe burn, a condition that's commonly associated with severe muscle wasting, leads to significant improvement in muscle protein synthesis [92].

Some studies have suggested that children with CP have lower circulatory levels of IGF-1 [93,94]. However, the studies are poorly designed. For example, in one study that reported lower serum IGF-1 levels in children with CP compared to their typically developing peers, there was a much wider variation in the levles of the children with CP (mean ± SD: 67 ± 45 vs. 74 ± 6 ng/ml) [93]. The considerable variation may be the result of including children with CP across the full range of GMFCS (I-V). Despite the significant between group difference detected, the effect

size was small (Cohen's d = 0.2) and the investigators used an independent t test, which assume equal variation in IGF-1 in the two groups. Therefore, studies with better quality control with regard to this matter are needed before definitive conclusions can be drawn.

2.4.5.3 Myostatin

Myostatin, also known as growth and differentiate factor-8 (GDF-8), is a protein that belongs to the transforming growth factor (TGF –β) superfamily and is a potent muscle negative regulator which acts primarily in an autocrine manner. It was first found in 1997 when McPherron et al. [95] discovered that mice with a disrupted GDF-8 gene have muscles that weighed 2-3 times more than wild type mice. Other studies also suggested a substantial muscle mass increase in bovine with either mutated or deleted GDF-8 [96-98]. A similar phenomena was also found in other animals, such as sheep [99] and dogs [100], with myostatin knock out species showing significantly enhanced muscularity.

Ever since that initial study by McPherron, there has been a growing interest in myostatin as a mediator for muscle mass regulation. Myostatin knock-out mice consistently show increased muscle mass in various studies [101,102]. It's important to notice that such hypertrophy was due to both increased muscle fiber diameter expansion as well as an increased number of muscle fibers [95]. Pharmacologically suppressed myostatin led to increased skeletal muscle mass and strength in adult mice, which indicated that myostatin could be a potential therapeutic target for muscle wasting [103]. On the other hand, it was demonstrated that systematically injected myostatin could lead to cachexia [104].

The mechanism through which myostatin exerts its negative effect on muscle has been studied extensively and can be complicated. In short, it is believed that myostatin, when activated, binds with activin receptor ActRIIB, causing the assembly of Smad2/Smad3 with

Smad4 (Smad is a protein family responsible for transferring TGF –β from the cell surface into the cell). The heterodimer will then be translocated to the nucleus, leading to the downregulation of MyoD, Pax3, myf5, myogenin and other genes known to affect muscle development and repair. Recently, studies have shown that myostatin may also regulate muscle mass by activating mitogen-activated protein kinases p38 and ERK1/2. The former has been shown to inhibit myogenesis-related gene expression. The role of latter kinase is controversial and may be mediated by myostatin levels and the presence of pathological conditions [105].

The strongest evidence to support the influential role of myostatin on muscle mass regulation in humans is the report of a child who had a myostatin gene mutation [106]. The child showed extraordinary musculature in his thighs and upper arms right after birth. At the age of 4.5 y, his quadriceps muscle CSA was 7.2 SD higher than his age- and sex-matched controls, as examined by ultrasound. Conversely, in populations which are characterized by diminished muscle volumes, such as aging people [107] and patients with chronic obstructive pulmonary disease [108], an elevated circulating myostatin level was found, and was inversely correlated with muscle wasting. On the other hand, it was reported that in patients with cancer cachexia, serum myostatin level was reduced compared to cancer patients without cachexia, and was positively correlated with their muscle index [109]. Similar results were found in heart failure patients who presented with muscle wasting. Furihata et al. [110] discovered decreased serum myostatin levels in patients with chronic heart failure, which were associated with lower muscle wasting. The exact mechanisms behind such differences is currently uncertain. It could be that the contribution of circulating myostatin to muscle dystrophy varies depending on the subjects' status. The elevated serum myostatin level seen in some patients may simply be a compensatory response to

their existing conditions [110]. In some disease status, myostatin may not affect muscle mass at all [109].

Myostatin may also be regulated by physical activity. In a study which involved subjects who were accustomed to exercise training, prolonged post-exercise suppression of myostatin gene-expression was found, which may contribute to the myogenesis process [111]. Other studies also suggested that exercise intervention can reduce myostatin gene expression [112-114]. No literature seems to have specifically examined the effects of sedentary lifestyle on myostatin gene expression or its circulating levels, but it can be inferred from the aforementioned studies that if a relationship exists, those who are physically inactive are more likely to have an increased level of this protein secretion.

To the best of the author's knowledge, only one prior study has looked at myostatin in children with CP. Using surgically removed muscle tissues, Smith et al. described an increased myostatin gene expression in children with spastic CP [115]. However, the sample size was very small with only 6 children with CP and 2 control children. Furthermore, the average age of the children with CP was considerably higher (12.8 ± 1.5 vs. 8.5 ± 2.1 y). Therefore, whether serum myostatin level is altered in children with spastic CP, a population known to have weak, underdeveloped muscles and a low level of physical activity, hasn't been determined and warrants further investigation.

2.4.5.4 Follistatin

Follistatin plays a role in muscle metabolic regulation as well. It acts as an antagonist of the transforming growth factor (TGF –β) superfamily by directly binding with it to ihibit its activity and thus promote muscle growth [116]. Using a mice model, administering follistatin

showed promising results in both reducing disease-induced sarcopenia [117] and facilitating skeletal muscle healing after injury [118]. Interestingly, in one study which looked at the effects of follistatin overexpression on mice muscle, greater muscle mass accumulation was observed in myostatin-null mice compared to wild type mice, suggesting that the effects of follistatin on muscle is not entirely through inhibiting myostatin activity, but also other potential signaling pathways [119], which has yet to be elucidated. Nevertheless, few studies have directly evaluated the association of circulating follistatin levels and muscle development in human, and the serum follistatin profile in children with CP remains to be explored.

2.5 Ectopic Fat Depots in Children with CP

Other than increased fat infiltration in and around skeletal muscle, children with CP also have increased bone marrow fat infiltration, as pointed out by Whitney et al [3]. This is of particular importance, because bone marrow contains mesenchymal stem cells, with the potential to promote both osteogenesis and adipogenesis. However it has been suggested that adipocytes are generated at the expense of osteoblast [120]. The increased bone marrow fat accumulation would mean that less osteoblasts are available for bone repair and formation, a process that may partly account for the decreased bone volume and quality in children with CP [121-123]. Increased bone marrow fat may also affect new red blood cell formation in this population, as the long bone marrow is a major site for hematopoietic stem cells depot after birth [124]. The increased intramuscular fat implies there is a higher intramyocellular lipids (IMCL) content, which has been tied to higher insulin resistance [125].

2.6 Central Adiposity in Children with CP

Fat distribution, especially fat depots at the abdominal regions of the body has been suggested to have a great influence on cardiovascular risk [126-128]. Visceral and subcutaneous

adipose tissue seem to play rather different roles in the physiological system. Visceral adipose tissue tends to be more metabolically active and more inclined to respond to stimuli due to the better vasculature and more inflammatory cells, and prone to generate free fatty acids. In contrast, subcutaneous adipose tissue is less metabolically active, and prone to absorb free fatty acids and thus serving as a "buffer" site for excessive energy intake [129]. Importantly, some of the recent reviews and studies have suggested that visceral fat may release free fatty acids and cytokines to the liver directly via the portal vein, a process that can substantially compromise the insulin sensitivity at the liver [130]. Therefore, an alteration in the abdominal fat profile, especially fat mass accumulation at the visceral cavity, may lead to the increased metabolic risks [129,131,132].

Plenty of studies have demonstrated a link between visceral fat accumulation and insulin resistance [133-136], however, such studies are lacking in children with CP. There is evidence that ambulatory children with CP have greater central adiposity when compare to their age, sex and race matched typically developing children [13]. However, no studies have evaluated the level of central adiposity across all levels of GMFCS in children with CP. Therefore, whether the level of central adiposity is related to GMFCS is currently unknown. In addition, very low level of physical activity is present in children with CP [3,4,137], and studies have suggested a clear link between physical activity and central adiposity accumulation in other clinical populations [138,139]. Yet, the relationship between physical activity and central adiposity hasn't been evaluated in children with CP and warrants further investigation.

2.7 Cardiometabolic Risks in Children with CP

As previously discussed, children with CP exhibit a wide range of ectopic fat accumulation. Locations includes, but are not limited to, within and between skeletal muscles, bone marrow, whole body and abdominal sites, all of which have been shown to be linked to

increased cardiometabolic risk. It has been reported that in 435 adults with CP between the age of 40 and 60, 252 (57.8 %) were found to have multimorbidity, including 137 unique multiple combinations of multimorbidities [140]. However, epidemiological studies examining whether similar trends can be observed in younger populations with CP are lacking.

As mentioned before, the increased intramuscular fat accumulation observed in children with CP could indicate a higher skeletal muscle IMCL content, although more research is needed to confirm that. Skeletal muscle is a major site for glucose uptake and thus help keep circulating glucose at an acceptable level. However, other than serving as a competitive fuel source may decrease the needs for muscle to uptake glucose, high level of IMCL accumulation may also activate a series of signaling cascades that eventually affect the insulin-induced glucose uptake via impairing GLUT4 transporter, and activate intracellular pro-inflammatory pathways which will both contribute to insulin resistance [141]. Quantifying IMCL used to be difficult, but the emerging magnetic resonance spectroscopy (MRS) technique has made the *in vivo* assessment of IMCL possible. A number of studies showed a significant correlation between insulin resistance and IMCL content in skeletal muscle in healthy and glucose uptake impaired population [142-146]. However, to date, whether children with CP have elevated insulin resistance when compared to their typically developing peers, and whether there is an association between IMCL or intramuscular fat and insulin resistance in ambulatory children with CP, is unknown.

Whole body and central adiposity have long been recognized as risk factors for insulin resistance, especially for visceral fat in children [147,148]. The mechanisms for fat accumulation to induce insulin resistance is very complicated and remains to be elucidated, functional defects and impaired insulin signaling pathways have been identified [149]. Weiss et al. demonstrated that for obese children with impaired glucose tolerance (n = 14), elevated abdominal subcutaneous and

visceral fat was observed when compared to those with similar age, sex and degree of obesity, but with normal glucose tolerance (n = 14). Moreover, visceral fat was inversely related to glucose disposal and metabolism [150]. In another study which involved 32 Hispanic children between the ages of 8 and 13 and with a family history of type 2 diabetes, visceral fat content showed the strongest correlation with measurements of insulin, including fasting insulin, acute insulin response and insulin sensitivity [151], total body fat mass was related to fasting insulin and insulin resistance. However, the results in another study which involved both white (n = 68) and African American children (n = 51) suggested that whole body fat mass is actually the predominant factor to influence insulin sensitivity, while visceral fat content may contribute additionally to fasting insulin [152]. It should also be noted that due to the high demand of time and costs, very few studies of this nature with the focus on children have used the glucose clamp technique, the "gold standard" measurement for insulin resistance. Therefore, the small discrepancies seen in those studies may be attributed to measurement error. Nevertheless, it is clear that whole body and central fat accumulation can affect insulin action. However, the relationship between total body, central adiposity and insulin resistance in children with CP hasn't been evaluated. Importantly, an epidemiological study suggested that physical activity level is associated with insulin resistance even after adjusting for total or central adiposity in Portuguese children [153]. Considering that children with CP have a physical disability, decreased physical activity level and increased adiposity accumulation, it wouldn't be surprising that this population also develops higher insulin resistance than their typically developing peers.

In vivo studies of bone marrow fat and metabolic risks are scarce, potentially due to the difficulty of evaluating bone marrow fat content. Nevertheless, a few available studies have focused primarily on vertebral bone, and pointed out a positive correlation between bone marrow

fat accumulation and increased metabolic risks. It was demonstrated that human bone marrow fat at the vertebral, femur and tibia were correlated with serum lipids level [154]. In a study involving 13 postmenopausal women with type 2 diabetes, vertebral bone marrow fat content correlated significantly with serum HbA1c level, a blood marker known to reflect long term blood glucose control [155]. The mechanisms for bone marrow fat to influence human metabolism is yet to be explored, but the potential lack of hematopoietic stem cells in individuals with increased marrow fat content may play a role, as they use glucose (glycolysis) as energy resources [156].

2.8 Specific Aims

My long-term goal is to attenuate the compromised musculature, muscle quality and ectopic fat deposition in children with CP. The overall objective of this project is to evaluate the level of skeletal muscle deficits, the role for some of the bioactive agents in those changes, and to determine the level of central adiposity across all levels of gross motor function in children with CP. My central hypotheses are 1) FFST is more compromised in the appendicular than the non-appendicular regions in children with CP that can be estimated using simple physical characteristics data, 2) bioactive agents like myostatin, follistatin and IGF-1 play a role in the muscle development compromise in children with CP and 3) the level of central adiposity is related to the level of gross motor function in children with CP. The rationale for this project is that it will allow us to better understand the level of muscle deficit of children with CP, elucidating the validity of using dual-energy X-ray absorptiometry to assess these deficits, and gain knowledge about the their long-term cardiometabolic health via understanding their regional fat distributions.

I plan to test my central hypothesis by using the following specific aims:

Specific Aim 1. To determine whether FFST, a marker of skeletal muscle mass, is more compromised at the appendicular than non-appendicular sites in children with CP, and whether simple physical characteristics can be used to accurately estimate appendicular FFST (AFFST).

Hypothesis 1.1. AFFST and non-appendicular FFST will be significantly lower in children with CP compared to their typically developing peers, but the extent of the deficit will be more pronounced at the appendicular regions.

Hypothesis 1.2. AFFST and its indexes (AFFST/height and AFFST/height^2) will be overestimated by models developed using data from typically developing children, but will be accurately estimated using data from children with CP.

Specific Aim 2. To determine whether DXA accurately estimates midleg muscle mass in ambulatory children with spastic CP.

Hypothesis 2.1. Midleg muscle mass in children with CP will be overestimated by a DXA-based statistical model developed using data from typically developing children.

Hypothesis 2.2. Midleg muscle mass will be accurately estimated by a DXA-based model developed using data from children with CP.

Specific Aim 3. To determine whether serum levels of the bioactive agents myostatin, follistatin and IGF-1 are consistent with the muscle deficit in children with CP.

Hypothesis 3.1. Compared to typically developing children, children with CP will have higher levels of myostatin and lower levels of follistatin and IGF-1 in the serum.

Specific Aim 4. To determine if the level of central adiposity in children with CP is positively related to their motor function and negatively related to their physical activity.

Hypothesis 4.1. Nonambulatory children with CP will have greater central adiposity when compared to their typically developing peers and ambulatory children with CP, and the level of central adiposity in children with CP will be positively related to their gross motor function.

Hypothesis 4.2. The level of central adiposity will be significantly and inversely related to physical activity in children with CP.

Findings in this project will not only allow us to gain a better understanding of the underlying mechanisms that underly the compromised musculature in children with CP, but also elucidate how those changes may affect our ability to evaluate their muscle quantity. In addition, findings about the level of central adiposity may help explain the greater cardiometabolic events seen in individuals with CP at the middle stages of their lives.

CHAPTER 3

PREFERENTIAL DEFICIT OF FAT-FREE SOFT TISSUE IN THE APPENDICULAR REGION OF CHILDREN WITH CEREBRAL PALSY AND PROPOSED STATISTICAL MODELS TO CAPTURE THE DEFICIT

Zhang, C., Colquitt, G., Miller, F., Shen,Y., Modlesky, C.M. Submitted to Clinical Nutrition, 4/11/2019

3.1 Abstract

Background: Cerebral palsy (CP) is a neurological disorder characterized by a profound skeletal muscle deficit. However, whether there is a regional-specific skeletal muscle deficit in children with CP is unknown.

Objective: The purpose of this study was to determine whether fat-free soft tissue mass (FFST), a commonly used surrogate for skeletal muscle mass, is more compromised at the appendages than at the trunk in children with CP. A second purpose was to determine whether physical characteristics can be used to accurately estimate appendicular FFST (AFFST) in children with CP.

Methods: Forty-two children with CP (4 – 13 y) and 42 typically developing children matched to children with CP for sex, age and race were studied. Whole body FFST ($FFST_{whole}$), FFST in the upper extremities ($FFST_{upper}$), FFST in the lower extremities ($FFST_{lower}$), the ratio of AFFST to height (AFFST/ht), the ratio of AFFST to height^2 (AFFST/ht^2) and non-appendicular FFST were estimated from dual-energy X-ray absorptiometry. Statistical models were developed to estimate AFFST, AFFST/ht and AFFST/ht^2 in both groups of children, and the leave-one-out method was used to validate the models.

Results: Children with CP had 21 % lower $FFST_{whole}$, 30 % lower AFFST, 34 % lower $FFST_{lower}$, 14 % lower non-appendicular FFST, 23 % lower AFFST/ht, 19 % lower AFFST/ht^2 and 9 % lower AFFST/$FFST_{whole}$ (all $p < 0.05$). Statistical models developed using data from typically developing children overestimated AFFST, AFFST/ht and AFFST/ht^2 by 35 %, 30 % and 21 % (all $p < 0.05$), respectively in children with CP. Separate models developed using data from children with CP yielded better accuracy, with the estimated results highly correlated (r^2 = 0.78, 0.66 and 0.50, respectively; all $p < 0.001$) and not different from calculated AFFST,

AFFST/ht and AFFST/ht^2 (all $p > 0.99$). However, when the difference in estimated values and measured values of AFFST, AFFST/ht and AFFST/ht^2 were plotted against measured values, there was an inverse relationship (r = -0.38, -0.47 and -0.61, respectively, all $p < 0.05$).

Conclusion: Children with CP have a remarkable deficit in FFST that is more pronounced in appendicular than the non-appendicular regions and more pronounced in the lower than the upper appendages. Preliminary models developed using data from children with CP can provide reasonable estimates of AFFST and indexes of AFFST relative to height, but further development of the models may be needed.

Keywords: cerebral palsy; appendicular fat-free soft tissue; dual-energy X-ray absorptiometry; statistical models; muscle mass

3.2 Introduction

Cerebral palsy (CP) is a neurological disorder of movement and posture that onsets before, during or shortly after birth and lasts across the lifespan. It currently affects about 1 million people in the United States [2]. It is well established that children with CP have skeletal muscle deficits compared to their typically developing peers. The scope of such compromise includes, but is not limited to, decreased muscle size [3,4,53,54], decreased force generation capacity [43,157,158], and increased inter- and intramuscular fat infiltration [3,4]. However, the evaluation of skeletal muscle deficits in children with CP has primarily focused on the lower extremities and studies that have evaluated the muscle development in all limbs in children with CP are lacking due to technical difficulties.

Appendicular fat-free soft tissue (AFFST), as measured by dual-energy X-ray absorptiometry (DXA), has traditionally been used as an indicator of sarcopenia in the aging population [159], and cachexia in clinical populations [160]. In healthy adults, the upper and lower limbs contain the largest portion of total body skeletal muscle mass [161]. The AFFST is calculated by summing the FFST in the upper appendages ($FFST_{upper}$) and the lower appendages ($FFST_{lower}$), to provide a summative index that serves as a surrogate for skeletal muscle mass in the extremities. Because muscles at the appendicular sites are frequently involved in daily movement and physical activity, deficits in AFFST may severely impair one's mobility and affect quality of life.

To our knowledge, the degree and distribution of AFFST deficit among children with CP have not been elucidated. It has been reported that the lean tissue compromise in individuals with spinal cord injury was more pronounced in the arms and legs than in the trunk when compared to their sex-, age-, height-, and weight-matched controls [6,7]. Spinal cord injury is a motor condition that results in low level of mobility due to the injury to the central nervous system. Because of the similar etiology, spinal cord injury is sometimes used to infer the long-term consequences of having CP [162,163]. However, whether there is a regional preference for FFST deficit in children with CP (i.e., whether the FFST deficit is more profound at the appendicular regions than at the trunk in children with CP) is unknown.

The purpose of this study was to determine whether FFST is more compromised at the appendicular sites than at the trunk in children with CP, and whether we can use simple physical characteristics data to accurately estimate AFFST and its derived indexes that account for the lower heights of children with CP. It is hypothesized that 1) AFFST and non-appendicular (trunk) FFST would be lower in children with CP compared to their typically developing peers,

but the extent of the deficit would be more pronounced at the appendicular regions, and 2) AFFST and AFFST indexes of children with CP would be overestimated when derived from models developed using data from typically developing children, but would be more accurately estimated using models based on data from children with CP.

3.3 Methods

3.3.1 Participants

Forty-two children with spastic CP, between the ages of 4-13 were recruited from CP programs at local hospitals to participate in this study. Forty two typically developing children who had no known neurological disorders, had height, body mass and BMI between the 5th and 95th percentile for respective age and matched to children with CP for age (± 1.5 y), sex and race were also invited to participate. This study was approved by the Institutional Review Board. Prior to any testing, consent and assent were obtained from the legal guardian and the child participant, respectively.

3.3.2 Anthropometrics

Height and body mass for all children were assessed with minimal clothing on and without shoes or braces. Height was assessed using a stadiometer (Seca 217; Seca GmbH & Co. KG., Hamburg, Germany) in typically developing children and ambulatory children with CP to the nearest 0.1 cm. For nonambulatory children with CP , height was estimated using forearm length and methods described by Miller et al [164]. Body mass for all children was assessed using a digital weight scale to the nearest 0.2 kg (Detecto, 6550, Cardinal Scale, Webb City, MO). BMI

was calculated based on the acquired height and body mass values. Height, body mass and BMI percentiles were determined based on the growth charts published by the Center for Disease Control and Prevention [165].

3.3.3 Gross motor function

Gross motor function in children with CP was assessed by a physician or physician assistant according to the Gross Motor Function Classification System (GMFCS). GMFCS is a 5 point system with a larger number indicating a higher level of functional deficiency [19]. Specifically, level I indicates the ability to walk and run but at a reduced speed while level two indicates limited walking ability and minimal ability for running independently. Children with GMFCS levels of I and II were considered ambulatory. Level III indicates the ability to walk only with an assistive device or the help of others. Level IV and V reflect minimal or lack of independent motor function and the need for a wheelchair. Children with GMFCS levels III –V were considered nonambulatory because they were unable to ambulate independently.

3.3.4 Body composition

Whole body DXA scans for all participants were acquired using a Delphi-W (Hologic, Bedford, MA) DXA densitometer. Children with CP were secured from the waist down using a modified version of the BodyFix (Medical Intelligence, Inc., Schwabműnchen, GER) during scanning acquisition to reduce potential involuntary contractions. The procedure has been shown to have no effect on body composition measurements [166]. Fat-free soft tissue mass ($FFST_{whole}$) and fat mass for the whole body were first determined (excluding the head). Upper and lower extremity regions of the whole body scan were isolated, and the FFST in each limb

were added together to calculate AFFST. In addition, FFST in each upper extremity were added together to calculate upper body FFST ($FFST_{upper}$), and FFST in each lower extremity were added together to calculate lower body FFST ($FFST_{lower}$). The remaining FFST (excluding the head) was considered non-appendicular FFST. To account for the shorter statue that children with CP usually exhibit, height-adjusted indexes were calculated as follows:

$$AFFST/ht = AFFST\ (kg)\ /height\ (m)$$

$$AFFST/ht^2 = AFFST\ (kg)\ /height^2\ (m^2)$$

3.3.5 Statistical analysis

Data were analyzed using SPSS version 24.0 (IBM Corp, Armonk, NY). All data were checked for normality first, and mean group differences between CP and typically developing children were compared using independent t-test or Mann-Whitney U test accordingly. To compare data from nonambulatory children with CP, ambulatory children with CP and typically developing children, subgroup analyses were performed using ANOVA for physical characteristics and body composition measurements. For body composition measurements, age was used as a covariate because the nonanbulatory children with CP were slightly older, though the difference was not significantly different, and a Bonferroni adjustment was applied for post-hoc comparisons to control for type I error. Effect size was estimated using Cohen's d (*d*), with 0.2, 0.5 and 0.8 indicating small, medium and large effect size, respectively [167] when applicable. An alpha level of 0.05 was used for all significance tests.

Multiple regression analyses were first performed in typically developing children to determine whether simple physical characteristics data (i.e., age, sex, height, body mass and

BMI) could be used to accurately estimate AFFST, AFFST/ht and AFFST/ht^2. All independent predictors were examined for interactions and if they were significant contributors to the model. Models either included BMI of body mass and height depending on which explained the most variance in the dependent variable. Models did not include all three because inclusion of all three did not improve any of the models. The final models were cross-validated using the leave-one-out technique [168]. Models were also applied to children with CP to determine whether accurate estimates could be obtained. Separate models using data from children with CP were also created and checked for validity. Paired t-tests were used to determine if AFFST, AFFST/ht and AFFST/ht^2 estimated from predictive models were significantly different from their respective values measured using DXA. The validity of the models was also assessed using Bland-Altman plots [169] and scatter plots.

3.4 Results

3.4.1 Physical characteristics

Eighteen children with CP were classified as nonambulatory and 24 were classified as ambulatory. Physical characteristics for all participants were summarized in **Table 1**. No significant differences were detected for age, BMI or BMI percentile between typically developing children and children with CP. However, children with CP had lower height and height percentile (d = 0.690 and 1.358, respectively, both $p < 0.05$), and lower body mass and body mass percentile (d = 0.413 and 0.842, respectively, both $p < 0.05$) as compared to the typically developing children.

When children with CP were separated based on ambulatory status, nonambulatory children with CP had lower height percentile and body mass percentile (d = 1.705 and 0.976, respectively, both $p < 0.05$) compared to typically developing children. Ambulatory children with CP had lower height, height percentile and body mass percentile (d = 0.732, 1.091 and 0.707, respectively, all $p < 0.05$) compared to the typically developing children.

Table 1. Physical characteristics of children with cerebral palsy (CP) and typically developing children (Con).

	CP (n = 42)	NACP (n =18)	ACP (n =24)	Con (n = 42)
Age (y)	9.1 ± 2.5	9.9 ± 2.1	8.5 ± 2.6	9.2 ± 2.3
Height (m)	1.26 ± 0.15[a]	1.27 ± 0.15	1.25 ± 0.16[a]	1.36 ± 0.14
Height (%)	21 ± 26[a]	15 ± 22[a]	27 ± 28[a]	57 ± 27
Body mass (kg)	28.1 ± 11.2[a]	28.7 ± 12.2	27.6 ± 10.7	32.6 ± 10.6
Body mass (%)	31 ± 33[a]	27 ± 33[a]	35 ± 33[a]	56 ± 26
BMI (kg/m^2)	17.2 ± 4.3	17.3 ± 4.9	17.1 ± 3.9	17.3 ± 2.9
BMI (%)	47 ± 36	44 ± 39	50 ± 34	53 ± 30
GMFCS (I/II/III/IV/V)	14/10/7/1/10	0/0/7/1/10	14/10/0/0/0	_

Values are mean ± SD. NACP = nonambulatory CP; ACP = ambulatory CP; BMI = body mass index. % for height, body mass and BMI reflects the percentile relative to age- and sex- based norms; GMFCS = gross motor function classification system. [a]Different from Con, $p < 0.05$. [b]Different from ACP, $p < 0.05$.

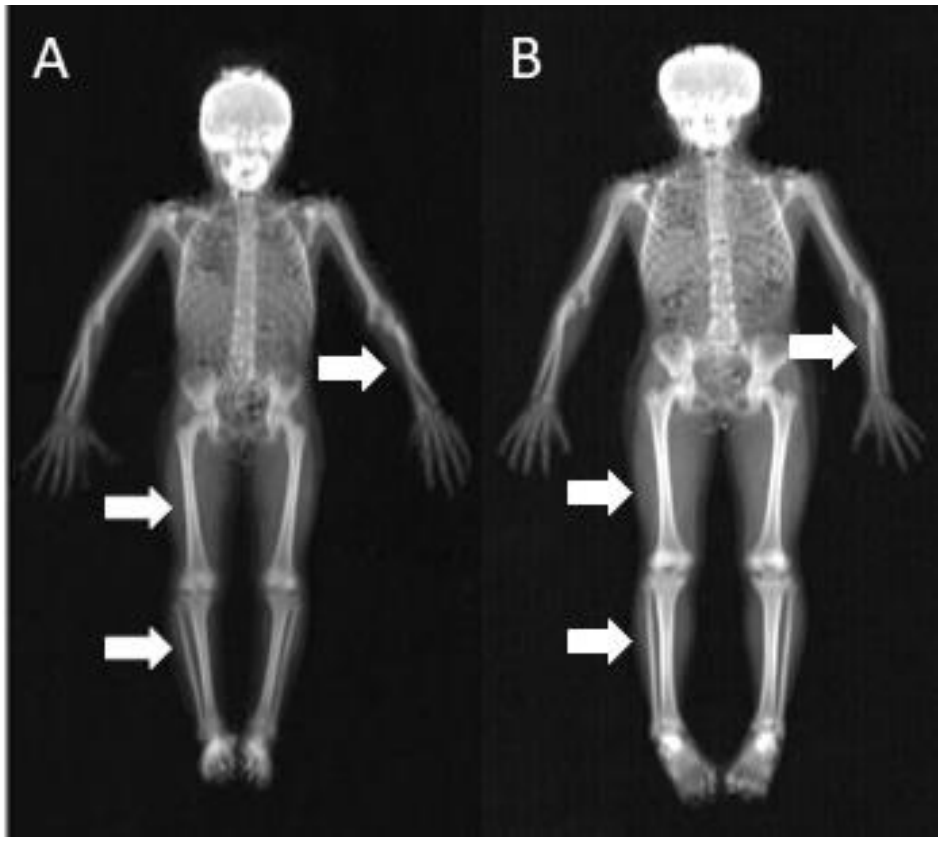


Figure 1. Whole body dual-energy X-ray Absorptiometry (DXA) scan for a 9.4 year old child with cerebral palsy (A; CP) at GMFCS level II, and a typically developing child (B; Con) with the same age, sex and race. The white arrows point to the soft tissue, a combination of fat mass and fat free soft tissue at the appendicular regions. Children with CP have much less soft tissue compared to Con.

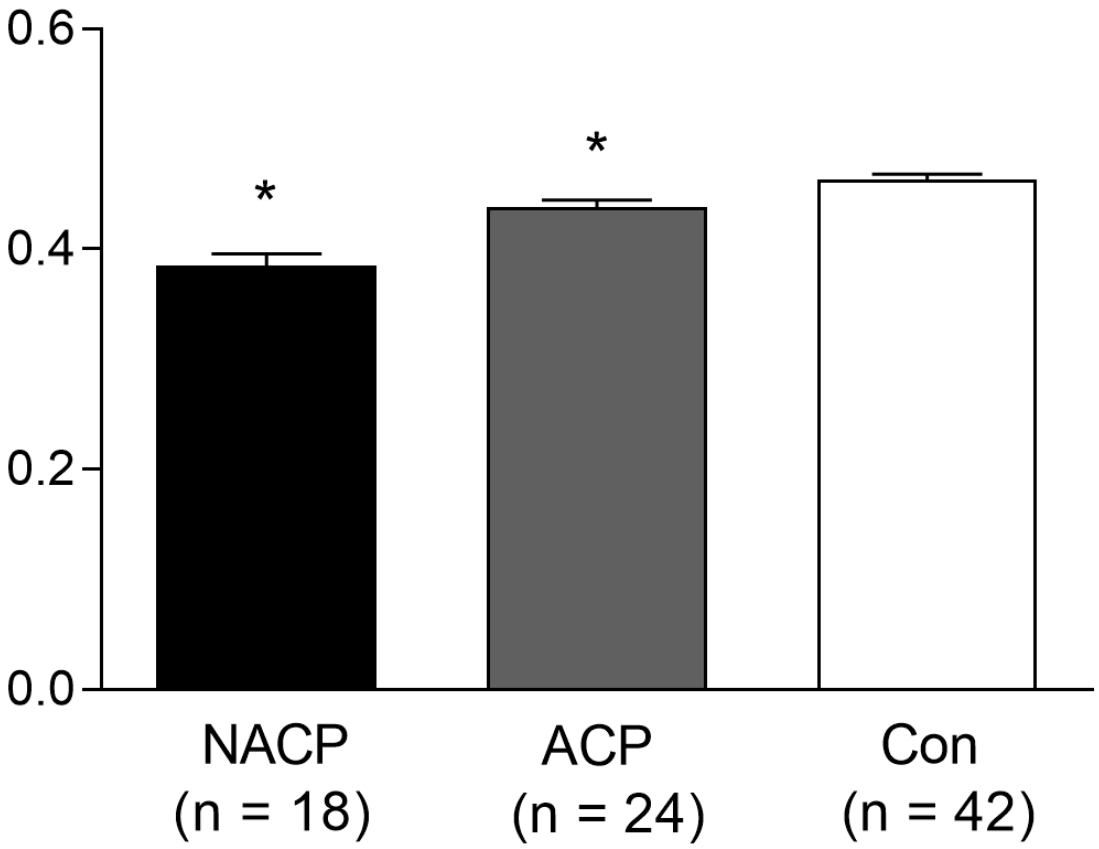


Figure 2. Appendicular fat-free soft tissue mass (AFFST) to whole body fat-free soft tissue mass ($FFST_{whole}$) ratios for all participants. NACP = non-ambulatory children with cerebral palsy; ACP = ambulatory children with cerebral palsy; Con = typically developing children. *Different from typically developing children, $p < 0.05$.

3.4.2 Body composition

Body composition estimates from DXA are summarized in **Table 2** and representative DXA scans from a child with CP and a typically developing child are presented in **Figure 1**. There was no group difference in fat mass, but compared to controls, children with CP had 21% lower $FFST_{whole}$ ($d = 0.661$, $p = 0.002$). AFFST and non-appendicular FFST were both lower in children with CP than in controls, but the degree of the difference was greater for AFFST (30 % lower, $d = 0.909$, $p < 0.001$) than non-appendicular FFST (14 % lower, $d = 0.444$, $p = 0.019$).

Compared to controls, children with CP also had 23% lower AFFST/ht, 19% lower AFFST/ht^2, 9% lower AFFST/$FFST_{whole,}$ 34 % lower $FFST_{lower}$, and 31% higher $FFST_{upper}/FFST_{lower}$ (d = 0.909, 0.994, 0.907, 1.034, and 1.403, respectively, all $p < 0.05$).

When children with CP were separated based on ambulatory status, all measures of FFST were lower in nonambulatory children with CP compared to typically developing children (d range = 0.403 to 1.980, all $p < 0.05$), except $FFST_{upper}/FFST_{lower}$ which was higher compared to typically developing children (d = 1.663, $p < 0.001$). The ambulatory children with CP had lower $FFST_{whole}$ compared to typically developing children, although it was marginally insignificant (d = 0.576, p = 0.056). The ambulatory children with CP also had lower AFFST, AFFST/ht, AFFST/$FFST_{whole}$ and $FFST_{lower}$, compared to the typically developing children (d = 0.708, 0.647, 0.667 and 0.830, respectively, all $p < 0.05$). In addition, the ambulatory children with CP had higher $FFST_{upper}/FFST_{lower}$ compared to typically developing children (d = 1.229, $p < 0.001$), but there was no group difference in non-appendicular FFST or $FFST_{upper}$ (d = 0.468 and 0.286, respectively, both $p > 0.05$). The lower AFFST/$FFST_{whole}$ in the nonambulatory and ambulatory children with CP compared to the typically developing children is demonstrated in **Figure 2**.

Table 2. Body composition estimates of children with cerebral palsy (CP) and typically developing children (Con) using dual-energy X-ray absorptiometry.

	CP (n = 42)	NACP (n = 18)	ACP (n = 24)	Con (n = 42)
Fat mass (kg)	8.4 ± 5.7	9.4 ± 7.1	7.6 ± 4.5	7.4 ± 4.3
$FFST_{whole}$ (kg)	15.3 ± 5.8[a]	14.8 ± 5.2[a]	15.7 ± 6.2	19.3 ± 6.3
AFFST (kg)	6.4 ± 2.6[a]	5.7 ± 2.2[a]	6.9 ± 2.9[a]	9.1 ± 3.3
non-appendicular FFST (kg)	8.9 ± 3.3[a]	9.1 ± 3.2[a]	8.8 ± 3.4	10.3 ± 3.0
AFFST/ht (kg/m)	5.0 ± 1.6[a]	4.4 ± 1.2[a]	5.4 ± 1.7[a]	6.5 ± 1.7
$AFFST/ht^2$ (kg/m^2)	3.9 ± 1.0[a]	3.4 ± 0.7[a]	4.2 ± 1.0	4.8 ± 0.8
$AFFST/FFST_{whole}$	0.42 ± 0.05[a]	0.39 ± 0.04[a]	0.44 ± 0.03[a]	0.46 ± 0.03
$FFST_{upper}$ (kg)	1.7 ± 0.7	1.6 ± 0.6[a]	1.8 ± 0.7	2.0 ± 0.7
$FFST_{lower}$ (kg)	4.7 ± 2.0[a]	4.1 ± 1.6[a]	5.1 ± 2.2[a]	7.1 ± 2.6
$FFST_{upper}/FFST_{lower}$	0.37 ± 0.08[a]	0.40 ± 0.09[a]	0.36 ± 0.07	0.29 ± 0.04

Values are mean ± SD. NACP = nonambulatory CP (Gross Motor Function Classification (GMFCS) level I and II); ACP = ambulatory CP (GMFCS level III,IV and V); FFST = fat-free soft tissue mass; $FFST_{whole}$ = whole body FFST; AFFST = appendicular FFST in the upper and lower appendages; AFFST/ht = ratio of AFFST to height; $AFFST/ht^2$ = ratio of AFFST to $height^2$; $FFST_{upper}$ = FFST in the upper extremities; $FFST_{lower}$ = FFST in the lower extremities. [a]Different from Con, $p < 0.05$.

3.4.3 Statistical models developed in typically developing children

Multiple regression analysis was performed using physical characteristics data from typically developing children to estimate AFFST, AFFST/ht and $AFFST/ht^2$. The resulting models yielded good estimates in typically developing children, as indicated by the high amount of variance explained (R^2 = 0.92, 0.88 and 0.73, respectively, all $p < 0.001$; **Table 3**). When the models were cross-validated using the leave-one-out technique, the estimates were strongly

related and not different from measured AFFST, AFFST/ht and AFFST/ht^2 (r^2 = 0.89, 0.84 and 0.65, respectively; all $p > 0.91$; **Figure 3**). However, when the difference in estimated values and measured values of AFFST, AFFST/ht and AFFST/ht^2 were plotted against measured values, there was an inverse relationship (r = -0.31, -0.39 and -0.56, respectively, all $p < 0.05$; **Figure 5D-F**). This indicated a trend for an overestimation of AFFST, AFFST/ht and AFFST/ht^2 for typically developing children with lower values and an underestimation for children with higher values.

Although the models developed using data from typically developing children yielded estimates of AFFST, AFFST/ht and AFFST/ht^2 that were moderately-to-strongly related to the measured values during the cross-validation in children with CP, they overestimated AFFST, AFFST/ht and AFFST/ht^2 by 11, 14 and 15 %, respectively (all $p < 0.01$; **Figure 3)**. The overestimation was demonstrated by most data points and the regression line residing below the lines of identity in the scatter plots (**Figure 3A-C**), and by most data points and mean difference lines above the no difference line in the Bland-Altman plots (**Figure 3D-F**). When the difference in estimated values and measured values of AFFST, AFFST/ht and AFFST/ht^2 were plotted against measured values, there was a significant inverse relationship for AFFST/ht^2 (r = -0.60, $p < 0.001$; **Figure 5F**). This indicated a trend for a greater overestimation AFFST/ht^2 for children with CP with lower values. The differences between the estimated values and the measured values of AFFST, AFFST/ht and AFFST/ht^2 were significantly correlated with the low AFFST/FFST$_{whole}$ in children with CP (r = -0.52, -0.66 and -0.74, respectively; all $p < 0.001$), as shown in **Figure 4**.

Table 3. Statistical models developed to estimate appendicular fat-free soft tissue (AFFST) mass and AFFST indexes in typically developing children.

Model	Outcome measure	Coefficients	β	*t*-Value	SE	*p*-Value	Model R^2	Model adjusted R^2
1	AFFST (kg)						0.917	0.908
		Intercept	-14.106	-4.685	3.011	0.000		
		Sex	-0.416	-1.306	0.318	0.199		
		Age (year)	-0.048	-0.273	0.177	0.786		
		Height (m)	14.485	4.112	3.523	0.000		
		Body mass (kg)	0.128	4.512	0.028	0.000		
2	AFFST/ht (kg/m)						0.877	0.864
		Intercept	-3.565	-1.875	1.901	0.069		
		Sex	-0.411	-2.044	0.201	0.048		
		Age (year)	0.527	0.219	0.112	0.828		
		Height (m)	5.487	2.468	2.224	0.018		
		Body mass (kg)	0.08	4.479	0.018	0.000		
3	AFFST/ht^2 (kg/m^2)						0.732	0.703
		Intercept	2.238	1.672	1.339	0.103		
		Sex	-0.352	-2.488	0.142	0.017		
		Age (year)	0.048	0.611	0.079	0.545		
		Height (m)	0.386	0.246	1.566	0.807		
		Body mass (kg)	0.052	4.148	0.013	0.000		

AFFST/ht = ratio of AFFST to height; AFFST/ht^2 = ratio of AFFST to height^2; male = 0 and female = 1 for sex; SEE = Standard error of estimation. All models $p < 0.001$.

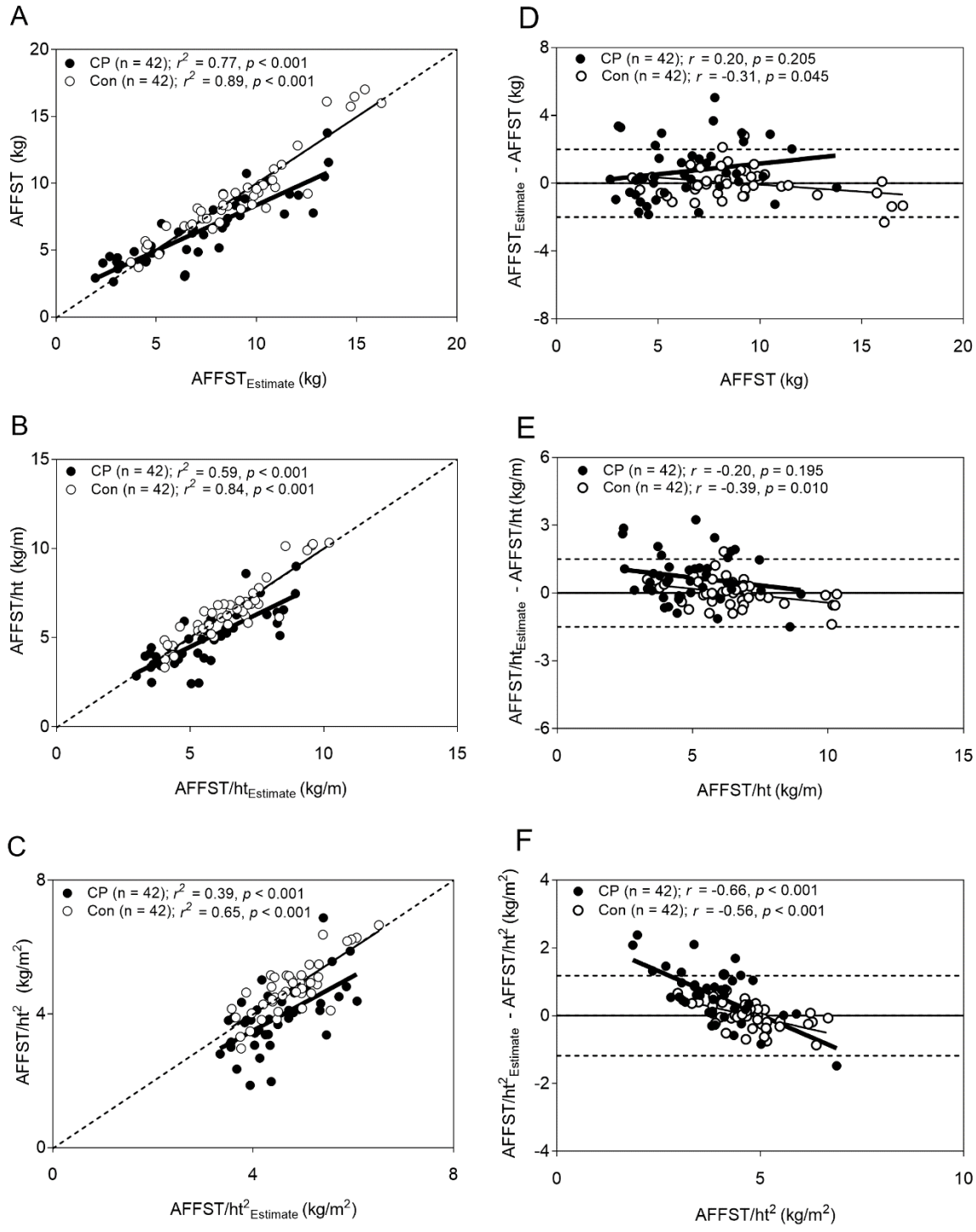


Figure 3. Scatter plots demonstrating the cross-validation of models for appendicular fat-free soft tissue mass from dual-energy X-ray absorptiometry (AFFST), the ratio of AFFST to height

(AFFST/ht), and the ratio of AFFST to height squared (AFFST/ht^2) developed using physical characteristics data from typically developing children (A-C). The models were cross-validated using the leave-one out method and data from the typically developing children. The models were also cross-validated using data from children with CP. Estimated values are on the x-axis. Measured values are on the y-axis. The dotted lines represent the lines of identity. The cross-validation was also evaluated using Bland-Altman plots (D-F). The dotted lines represent AFFST estimated minus AFFST measured ± SD for the typically developing children. The thick solid lines represent the regression lines for children with CP, and the thin solid lines represent the regression lines for typically developing children.

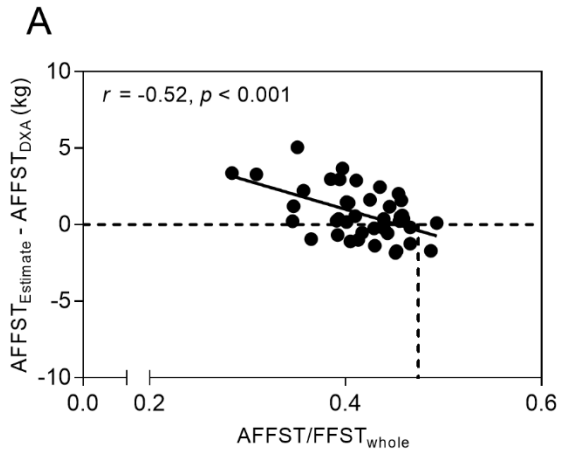
3.4.4 Statistical models developed in children with CP

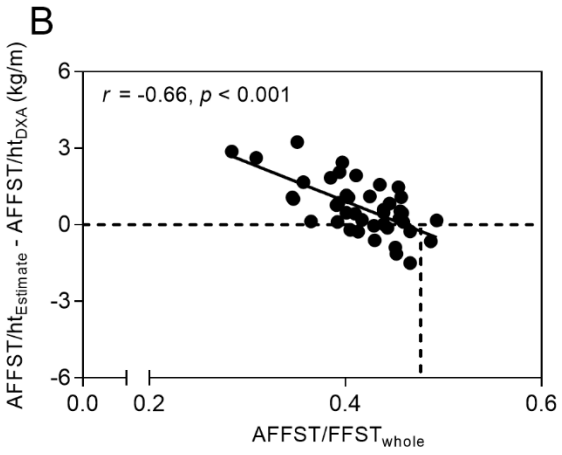
Multiple regression analysis was performed using physical characteristics data from children with CP to estimate AFFST, AFFST/ht and AFFST/ht^2. The resulting models yielded good estimates, as indicated by the high amount of variance explained. (R^2 = 0.85, 0.77 and 0.64, respectively, all $p < 0.001$; **Table 4**). When the models were cross-validated using the leave-one-out method, the results were strongly related to (r^2 = 0.78, 0.66 and 0.50, respectively, all $p < 0.001$; **Figure 5**) and not different from measured AFFST, AFFST/ht and AFFST/ht^2 (all $p > 0.95$). Excellent estimation is indicated visually by most data points and the regression lines near the lines of identity in the scatter plots (**Figure 5A-C)**, and by most data points and the mean difference lines near the no difference lines in the Bland-Altman plots (**Figure 5D-F**). However, when the difference in estimated values and measured values of AFFST, AFFST/ht and AFFST/ht^2 were plotted against measured values, there was an inverse relationship (r = -0.38, -0.47 and -0.61, respectively, all $p < 0.05$; **Figure 5D-F**). This indicated a trend for an overestimation of AFFST, AFFST/ht and AFFST/ht^2 for children with CP with lower values and an underestimation for children with higher values.

Table 4. Statistical models developed to estimate appendicular fat-free soft tissue mass (AFFST) and AFFST indexes in children with cerebral palsy.

Model	Outcome measure	Coefficients	β	*t*-Value	SE	*p*-Value	Model R^2	Model adjusted R^2
1	AFFST (kg)						0.854	0.833
		Intercept	-3.512	-1.529	2.296	0.135		
		Sex	-0.595	-1.602	0.371	0.118		
		Age (year)	0.220	1.422	0.155	0.164		
		Height (m)	4.342	1.493	2.908	0.144		
		Body mass (kg)	0.118	4.721	0.025	0.000		
		Ambulatory status	-1.484	-3.696	0.402	0.001		
2	AFFST/ht (kg/m)						0.770	0.738
		Intercept	2.277	1.323	1.721	0.194		
		Sex	-0.449	-1.612	0.278	0.116		
		Age (year)	0.179	1.547	0.116	0.131		
		Height (m)	-0.666	-0.306	2.179	0.762		
		Body mass (kg)	0.091	4.853	0.019	0.000		
		Ambulatory status	-1.134	-3.769	0.301	0.001		
3	AFFST/ht^2 (kg/m^2)						0.637	0.597
		Intercept	1.360	2.744	0.496	0.009		
		Sex	-0.334	-1.561	0.214	0.127		
		Age (year)	0.106	2.500	0.042	0.017		
		BMI (kg/m^2)	0.120	5.062	0.024	0.000		
		Ambulatory status	-0.839	-3.914	0.214	0.000		

AFFST/ht= ratio of AFFST to height; AFFST/ht^2 = ratio of AFFST to $height^2$; male = 0 and female = 1 for sex; ambulatory = 0, and nonambulatory = 1 for ambulatory status; SEE = Standard error of estimation. All models $p < 0.001$.


A
r = -0.52, p < 0.001
AFFST_Estimate - AFFST_DXA (kg)
AFFST/FFST_whole
10
5
0
-5
-10
0.0
0.2
0.4
0.6


B
r = -0.66, p < 0.001
AFFST/ht_Estimate - AFFST/ht_DXA (kg/m)
AFFST/FFST_whole
6
3
0
-3
-6
0.0
0.2
0.4
0.6

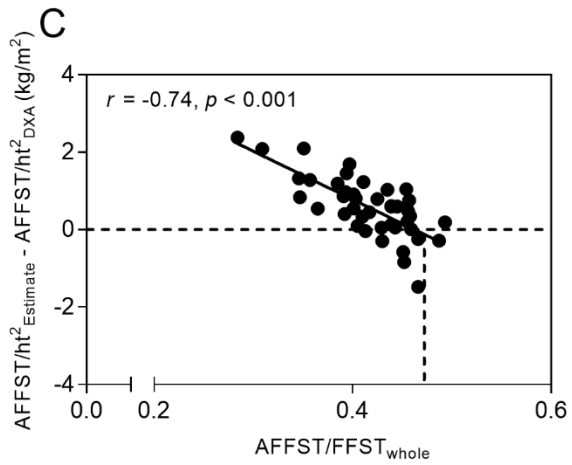

C
r = -0.74, p < 0.001
AFFST/ht²_Estimate - AFFST/ht²_DXA (kg/m²)
AFFST/FFST_whole
4
2
0
-2
-4
0.0
0.2
0.4
0.6

Figure 4. Scatter plots of the ratio of appendicular fat-free soft tissue mass to whole body fat-free soft tissue mass ($AFFST/FFST_{whole}$) compared to (A) the difference in appendicular fat-free soft tissue mass (AFFST), (B) the ratio of AFFST to height (AFFST/ht), and (C) the ratio of AFFST to height^2 ($AFFST/ht^2$) and their estimates in children with cerebral palsy (CP) by statistical models developed using data from typically developing children. $AFFST_{Estimate}$, $AFFST/ht_{Estimate}$ and $AFFST/ht^2_{Estimate}$ represent the estimates by the models developed using data from typically developing children. The horizontal dotted line represents the point where the difference between $AFFST_{Estimate}$ and AFFST measured is zero. The vertical dotted line represents the average $AFFST/FFST_{whole}$ for typically developing children. The solid lines represent the regression lines for children with CP.

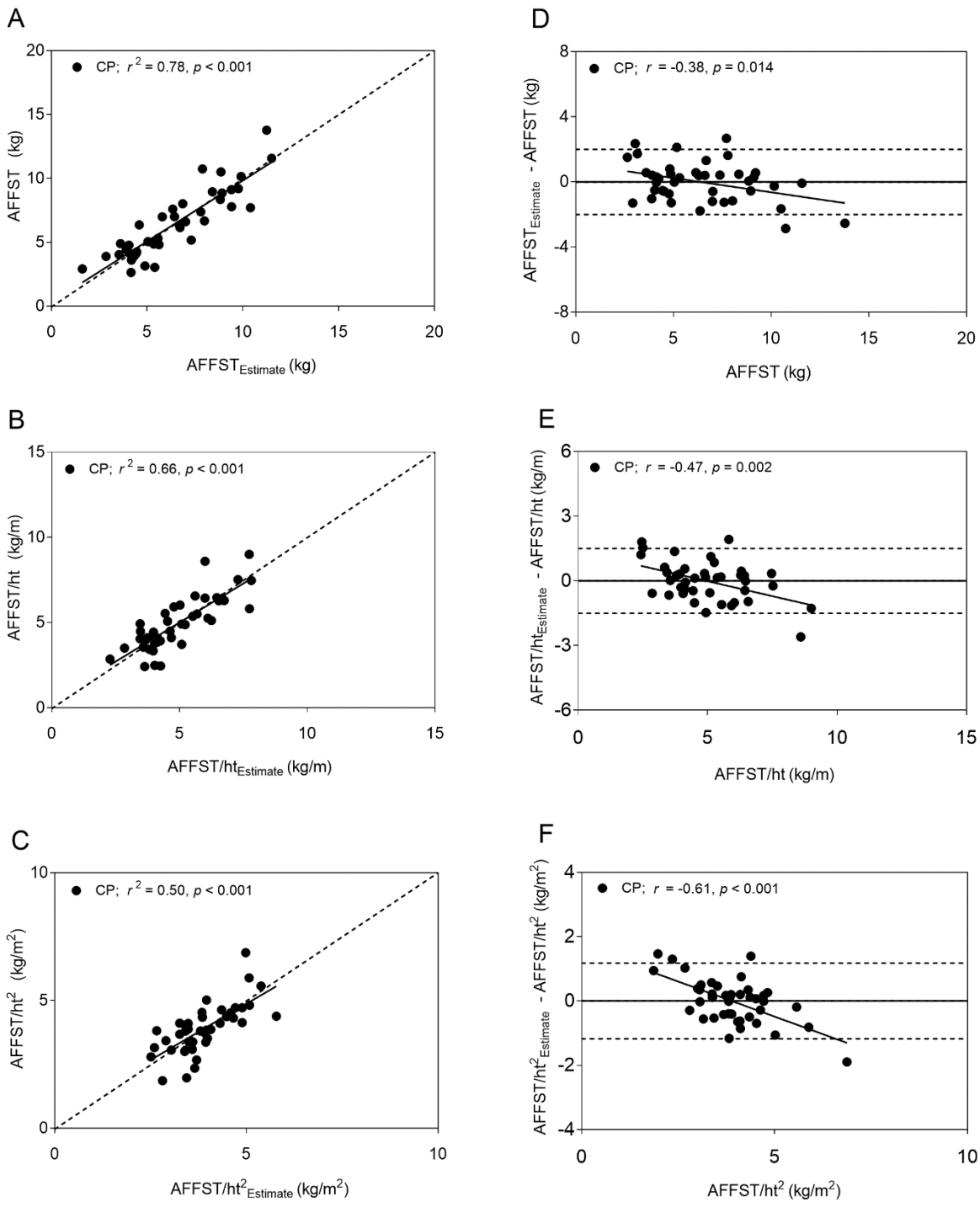

A
CP; r^2 = 0.78, p < 0.001
AFFST (kg)
AFFST$_{Estimate}$ (kg)
D
CP; r = -0.38, p = 0.014
AFFST$_{Estimate}$ - AFFST (kg)
AFFST (kg)
B
CP; r^2 = 0.66, p < 0.001
AFFST/ht (kg/m)
AFFST/ht$_{Estimate}$ (kg/m)
E
CP; r = -0.47, p = 0.002
AFFST/ht$_{Estimate}$ - AFFST/ht (kg/m)
AFFST/ht (kg/m)
C
CP; r^2 = 0.50, p < 0.001
AFFST/ht^2 (kg/m^2)
AFFST/ht$^2_{Estimate}$ (kg/m^2)
F
CP; r = -0.61, p < 0.001
AFFST/ht$^2_{Estimate}$ - AFFST/ht^2 (kg/m^2)
AFFST/ht^2 (kg/m^2)

Figure 5. Scatter plots demonstrating the cross-validation of models for appendicular fat-free soft tissue mass from dual-energy X-ray absorptiometry (AFFST), the ratio of AFFST to height (AFFST/ht), and the ratio of AFFST to height squared (AFFST/ht^2) developed using physical characteristics data from children with cerebral palsy (CP; A-C). The models were also cross-validated using data from children with CP and the leave-one-out method. Estimated values are on the x-axis. Measured values are on the y-axis. The dotted lines represent the lines of identity. The cross-validation was also evaluated using Bland-Altman plots (D-F). The dotted lines represent AFFST estimated minus AFFST measured ± SD. The solid lines represent the regression lines for children with CP.

3.5 Discussion

To our knowledge, this is the first study to demonstrate that the FFST deficit in children with CP is greater at the appendicular regions than at the non-appendicular region. In addition, we demonstrated that AFFST, along with its height-adjusted indexes (AFFST/ht and AFFST/ht^2), can be estimated with reasonable accuracy in children with CP using models based on physical characteristics. However, the models must be generated using data from children with CP. Although models developed using data from typically developing children yield reasonably accurate estimates of AFFST and its indexes in typically developing children, significant overestimates result when the models are applied to children with CP. Low AFFST is a concern because it has been linked to multiple comorbidities in adults and older people, including functional impairment [170], physical disabilities [170], impaired bone structure [171,172], impaired balance [171] and cardiovascular risks [173,174]. The low AFFST in children with CP may be an early indicator of these complications, which are all present at an accelerated rate in individuals with CP [3,122,140,175-177].

In the present study, AFFST and non-appendicular FFST were both lower in children with CP, but there was a greater compromise in AFFST as demonstrated by the greater percentage difference (30 % vs 14 %) and greater effect size (d = 0.909 vs. d = 0.444) when

compared to typically developing children. These deficits are also reflected by the lower $AFFST/FFST_{whole}$ in children with CP. The observation that the deficit in FFST is more pronounced in the appendicular than the non-appendicular regions is consistent with previous studies that show a proportionately smaller FFST in the arms and legs than in the trunk of individuals with spinal cord injury [6,7], a group that experiences exceptional loss of muscle and physical activity after injury. The preferential deficit of FFST in the appendages versus the trunk may be attributed to the larger proportion of muscle in the FFST of the appendages and the larger proportion of non-muscular tissue (e.g. internal organs) in the FFST of the trunk [161]. It is likely that skeletal muscle compared to non-muscle is more affected by mobility-related issues.

The reason for the diminished AFFST in children with CP compared to their typically developing peers is likely multi-factorial. Physical activity levels in children with CP are very low, with some reports of 70 to 80 % lower levels in nonambulatory children with CP [4,178] and ~40% reduction in ambulatory children with CP [3]. Such a low level of physical activity may contribute to a muscular deficit simply by disuse; specifically, there is insufficient stimulus for muscle growth. This problem may be exacerbated as children with CP grow older because there is evidence that gait function declines as children with CP age, which may contribute to further sedentary behavior in this population [179]. Another potential contributing factor is malnutrition, which is common in children with CP [180]. It was reported that the prevalence of malnutrition is much higher in women with sarcopenia, as defined by $AFFST/ht^2$, than in those without sarcopenia [181]. Whether a similar phenomenon is present in children with CP is yet to be determined. Catabolic hormones like myostatin may also play a role in this process. There is some evidence that myostatin is upregulated in children with CP [115]. It was reported that myostatin RNA expression tends to be higher (29%; $p = 0.09$) in older adults with sarcopenia

than in those without sarcopenia [182]. However, to our knowledge, the relationship between myostatin and AFFST has not been examined in children with CP. More research is required to identify specific mechanisms underlying the lower AFFST in children with CP compared to typically developing children.

Another novel finding in the current study was the disproportionate FFST compromise in the lower limbs compared to the upper limbs in children with CP. Although FFST was lower in the upper and lower limbs of the nonambulatory children with CP when compared to typically developing children, the deficit was smaller in the upper limbs. This was confirmed by the higher $FFST_{upper}/FFST_{lower}$ in the nonambulatory children with CP compared to typically developing children. In the ambulatory children with CP, a detectable compromise in FFST was found in the lower limbs, but not in the upper limbs. The detectable FFST deficit in the upper limbs of nonambulatory children, but not the ambulatory children with CP is probably due to a much lower usage of the arms by the nonambulatory children. Most of the nonambulatory children with CP (14 out of 18) were quadriplegic, where all four limbs are affected. On the other hand, all of the ambulatory children with CP were either diplegic or hemiplegic, where one or two lower limbs are affected and the upper limbs are usually unaffected. It is likely that a deficit in FFST is present in children with milder forms of CP, such as those who are ambulatory, but larger samples and/or more sensitive measures that specifically assess muscle are needed.

The finding that models developed using data from typically developing children and simple characteristics, such as sex, age, height, body mass, BMI and ambulatory status, overestimate AFFST and indexes of AFFST when applied to children with CP is consistent with previous studies. For example, DXA-based models developed using data from typically

developing children overestimated midthigh muscle mass by 12-15 % [11] and midleg muscle mass by 13-22 % [8] in children with CP. The finding that AFFST and AFFST indexes can be estimated in children with CP by models developed using data from children with CP is encouraging. However, the models tended to overestimate AFFST in children with CP with lower values and underestimate AFFST children with higher values. Therefore, further efforts are needed to develop models to estimate AFFST and associated indexes.

In addition to the novel findings already reviewed, there are strengths of this study that should be discussed. First, children with CP and typically developing children were matched for age, sex and race. Moreover, the height, body mass and BMI for the typically developing children were not different from the 50^{th} sex- and age-based percentiles. Therefore, the differences in body composition observed in the current study likely reflect the population difference between children with CP and their typically developing peers. Second, FFST at appendicular sites was expressed not only as an absolute value, but also as indexes that corrected for the shorter statue of the children with CP (i.e., AFFST/ht and AFFST/ht^2). A marked reduction was still present with the relative measures of AFFST indicating that the level of compromise in children with CP is striking.

The limitations of this study must also be addressed. First, although commonly used to represent skeletal muscle mass at the appendicular regions, AFFST is not a perfect surrogate for skeletal muscle mass. Apart from skeletal muscle, FFST also contains tendons, ligaments, vessels and other connective tissues. Therefore, the actual appendicular skeletal muscle mass is only a portion of the AFFST measured by DXA. In addition, children with CP have a lower proportion of muscle in the FFST as reflected by a lower ratio of skeletal muscle mass to FFST [8,11]. Therefore, the actual deficit in muscle is probably even greater in this population than is

reflected by AFFST. Second, regression models were developed in the present study using limited physical characteristic data, such as age, sex and height. In a previous study of adults, it was found that AFFST is accurately estimated by models that include sex, height and weight, as well as other predictors, such as hip circumference and grip strength [159]. Whether adding other anthropometric measures and muscle strength can increase predictability for models specific to children with CP is unknown and needs further investigation. Despite the limitation, the models developed in the present study provided moderate-to-strong estimates of AFFST and indexes of AFFST in children with CP. Lastly, it is unknown if the statistical models developed in the present study can be used to monitor changes in children with CP due to growth, maturation, surgery or alterations in physical activity, nutrition or rehabilitation. Future studies are needed to assess the usefulness of these models for scientists studying and clinicians monitoring the muscle development and health in children with CP.

3.6 Conclusion

Children with CP have a remarkable deficit in FFST that is more pronounced in appendicular than non-appendicular (trunk) regions, with greater deficits noted in the lower extremities than in the upper extremities. Although the unique FFST profile is more pronounced in nonambulatory children, it is also present in ambulatory children with CP. Preliminary models developed in the current study using data from children with CP can provide good estimates of AFFST, but further development of the models to estimate AFFST indexes may be needed.

3.7 Acknowledgement

We thank all the participants and their families for their support. CMM and FM designed the study; CMM, CZ and FM conducted the data collection; CZ, CMM, GC and YS analyzed the data; CZ, CMM and GC wrote the manuscript. All authors edited and approved the final manuscript. The authors declare that they have no conflicts of interest. This study was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development and the National Center for Medical Rehabilitation Research (grant numbers HD050530, HD071397 and HD090126), the United Cerebral Palsy Research and Educational Foundation and the University of Georgia Athletic Association.

CHAPTER 4

STATISTICAL MODELS TO ASSESS LEG MUSCLE MASS IN AMBULATORY CHILDREN WITH SPASTIC CEREBRAL PALSY USING DUAL-ENERGY X-RAY ABSORPTIOMETRY

Zhang, C., Whitney, D.G., Singh, F., Slade, J.M., Miller, F., Modlesky, C.M. Accepted by Journal of Clinical Densitometry. Reprinted here with permission of publisher.

4.1 Abstract

Background: Cerebral palsy (CP) is a movement disorder associated with small and weak muscles. However, methods that accurately assess muscle mass in children with CP are scarce.

Objective: The objective was to determine whether dual-energy X-ray absorptiometry (DXA) accurately estimates of midleg muscle mass in ambulatory children with spastic CP.

Methods: Ambulatory children with spastic CP and typically developing children 5-11 y were studied (n = 15/group). Fat-free soft tissue mass (FFST) and fat mass at the middle third of the tibia (i.e., midleg) were estimated using DXA. Muscle mass ($muscle_{MRI}$) and muscle mass corrected for intramuscular fat ($muscle_{MRIfc}$) in the midleg were estimated using magnetic resonance imaging (MRI). Statistical models were created to predict $muscle_{MRI}$ and $muscle_{MRIfc}$ using DXA.

Results: Children with CP compared to typically developing children had lower FFST (38 %), $muscle_{MRI}$ (40 %) and $muscle_{MRIfc}$ (47 %) (all $p < 0.05$) and a lower ratio of $muscle_{MRIfc}$ to FFST (17 %, $p < 0.05$). DXA-based models developed using data from typically developing children explained 90 % of the variance in $muscle_{MRI}$ and 83 % of the variance in $muscle_{MRIfc}$ in children with CP (both $p < 0.05$); however, the models overestimated $muscle_{MRI}$ (13 %) and $muscle_{MRIfc}$ (22 %) (both $p < 0.05$). The overestimation was inversely related to the ratio of $muscle_{MRI}$ to FFST ($r = -0.79$, $p < 0.001$) and $muscle_{MRIfc}$ to FFST ($r = -0.95$, $p < 0.001$). DXA-based models developed using data from children with CP explained 91 % of the variance in $muscle_{MRI}$ and 90 % of the variance in $muscle_{MRIfc}$ in children with CP (both $p < 0.05$). Moreover, the estimates were not different from $muscle_{MRI}$ and $muscle_{MRIfc}$ (both $p > 0.99$).

***Conclusion*:** DXA-based statistical models accurately estimate midleg muscle mass in children with CP when the models are composed using data from children with CP rather than typically developing children.

Keywords: muscle mass; cerebral palsy; dual-energy X-ray absorptiometry; magnetic resonance imaging; intramuscular fat; statistical models

4.2 Introduction

As the largest tissue component of the lean body mass in humans, skeletal muscle plays an important role in daily life. Apart from force-generating capacity [183], which is crucial for movement and balance [171], it is also involved in numerous physiological processes that help the body achieve homeostasis [184]. Altered skeletal muscle quantity and quality have been associated with multiple morbidities, such as insulin resistance [185], functional impairment and disability [170], obesity [146] and osteoporosis [186]. Therefore, obtaining information about human skeletal muscle development and accretion is necessary for disease prevention and treatment.

Magnetic resonance imaging (MRI) is considered the "gold standard" for non-invasive skeletal muscle mass assessment [187]. However, some limitations associated with MRI include high cost, low availability, and time-consuming acquisition and processing procedures. An alternative to MRI is dual-energy X-ray absorptiometry (DXA) because of its lower cost, and shorter scanning and processing time. Studies have shown that fat-free soft tissue (FFST) from DXA can be used to estimate skeletal muscle mass [11,188-190]. However, few studies have focused on children [11,188,190] and fewer have focused on children with arrested muscle accretion, such as children with cerebral palsy (CP) [11]. Muscle assessment in children with CP is particularly important because CP is the most common childhood disability and it is associated with muscles

that are small [4,10,44], weak [44] and highly infiltrated with fat [3]. The muscle size deficit in children with CP is profound in the lower extremities, especially the legs [10]. Moreover, the concentration of intramuscular fat in the legs is ~ 50 % higher in ambulatory children with CP than in typically developing children [3].

There is evidence that a DXA-based statistical model developed for typically developing children overestimates skeletal muscle mass in non-ambulatory children with CP, which has been linked to their lower proportion of muscle in the FFST [11]. Whether a similar problem is present and correctable in ambulatory children with spastic CP is unknown. In addition, most MRI structural images used to assess muscle volume cannot separate intramuscular fat from muscle tissue. Therefore, the proportion of muscle in the FFST may be even lower and the overestimation of muscle mass by DXA-based models higher in children with CP than previously demonstrated when the high degree of intramuscular fat is considered.

The objective of this study was to determine whether DXA can be used to estimate midleg muscle mass in ambulatory children with spastic CP. It was hypothesized that midleg muscle mass in children with CP would be overestimated by a DXA-based statistical model developed using data from typically developing children while a model developed using data from children with CP would yield accurate estimation.

4.3 Methods

4.3.1 Participants

Sixteen ambulatory children with spastic CP (n = 5 girls, n = 12 Caucasian, n = 2 African American and n =2 Hispanic) and between 5 and 11 years old were recruited from AI duPont Hospital for Children (Wilmington, DE) CP clinic and other pediatric clinics from the greater

Atlantic area. Fifteen typically developing children (n = 4 girls, n = 11 Caucasian, n = 2 African American, n = 1 Hispanic and n =1 Asian) without known neurological disorders and had similar ages to children with CP also participated. This study received approval from the Institutional Review Board. Written consent and assent were obtained from the participant and the parent, respectively.

4.3.2 Anthropometrics

Height was assessed using a stadiometer (Seca 217; Seca GmbH & Co. KG., Hamburg, GER). Body mass was assessed using a digital weight scale (Detecto 6550, Cardinal Scale, Webb City, MO). Both measurements were taken while children wore minimal clothing. Body mass index (BMI) was subsequently calculated. Height, body mass and BMI percentiles were determined using growth charts [191].

4.3.3 Sexual Maturity

Sexual maturity was assessed using the Tanner staging technique [192]. This is a five-point scale with I indicating no development and V indicating full development. There are two parts to this assessment. Pubic hair development in both boys and girls, and testicular/penile development in boys and breast development in girls were assessed by a physician assistant.

4.3.4 Gross Motor Function

Gross motor function in children with CP was classified according to the gross motor function classification system (GMFCS) [19]. GMFCS is a five-point scale ranging from I to V

with a higher number indicating lower gross motor function. Participants with CP in this study had GMFCS I or II.

4.3.5 DXA

FFST and fat mass at the level of the middle-third (i.e., midleg) of the non-dominant side in typically developing children and the more affected side in children with CP were assessed using DXA (Delphi W, version 11.2; Whole Body Analysis; Hologic Inc) and a whole body scan. The BodyFIX (Medical Intelligence, Inc., Schwabműnchen, GER) was used to limit motion in children with CP [11]. After completion of the scan, a region of interest (ROI) box was placed at the middle-third of the tibia to represent the midleg and fat mass and FFST were determined. The coefficient of variation (CV) for repeat measures of FFST and fat mass in the midleg were 0.6 % and 0.7 %, respectively [11].

4.3.6 MRI

Midleg muscle mass and muscle mass corrected for intramuscular fat were determined using MRI (GE, 1.5 T, Milwaukee, WI) in the same leg tested using DXA. To help children remain still during the MRI testing, they were secured from the waist down using the BodyFix to limit motion [3]. They also watched a movie of their choice using an MRI-compatible goggle system (CinemaVision, Resonance Technology Inc, Northridge, CA). All scans with visible motion in the images were redone. Axial images (0.5 cm slice thickness and 0.5 cm spacing) were collected from the tibia plateau to the malleolar surface using a semiflex long bone array coil (ScanMed, Omaha, NE) and two scans. The first scan (TR = 650, TE = 14, NEX = 3, Bandwidth = 15.63, frequency 512 and phase 256, field of view 12 cm) yielded T-1 weighted

images. The second scan (IDEAL: fast-spin-echo, TR = 600, TE = min full, NEX = 2, Bandwidth = 31.25, frequency 320 and phase 224) yielded fat and water images.

Images were processed with a program developed using Interactive Data Language (Research Systems, Inc., Boulder, CO). T1-weighted images at the level of the midleg were automatically identified and processed. Skeletal muscle area was separated from other tissues [193] with minor manual adjustments applied as needed. The identified area for the first and last images were multiplied by a correction factor (< 1), and the rest of the images were multiplied by 1 to account for the slice thickness and spacing, and sum of all results was defined as mid-third tibia muscle volume (MV). The mid-third tibia MV was then multiplied by 1.04 g/cm^3, the assumed density of muscle [161] to calculate muscle mass ($muscle_{MRI}$). The reliability in our laboratory is excellent as indicated by intraclass correlation coefficients > 0.99 and CVs of 0.5 % [11].

To correct muscle mass for intramuscular fat concentration ($muscle_{MRIfc}$), areas that were assigned to muscle in the T1-weighted images were used to identify muscle areas in the corresponding fat and water images. The signal intensity (SI) were used to calculate fat concentration using the following equation: Fat concentration = SI from fat images / (SI from fat images + SI from water images) * 100 [154]. Skeletal MV corrected for intramuscular fat concentration was calculated using the following equation: Corrected MV = MV – (MV x fat concentration). The corrected MV was then multiplied by the assumed muscle density [161] to yield $muscle_{MRIfc}$.

4.3.7 Statistical Analysis

Data analyses were conducted using SPSS (version 24.0; SPSS, Chicago, IL). Physical characteristics and data from DXA and MRI were checked for normality first, and group comparisons were made using independent t-tests or Mann-Whitney test accordingly. The magnitude of the effects were determined using Cohen's d (*d*), with 0.2, 0.5 and 0.8 indicating small, medium and large effects, respectively [167].

A simple linear regression analysis was performed using FFST as the independent variable to estimate $muscle_{MRI}$ and $muscle_{MRIfc}$ in typically developing children and in children with CP. Age, height, body mass, BMI and fat mass were added to the model as predictors one at a time to determine whether those variables could significantly improve model predictability. The final models were cross-validated using the leave-one-out technique [194]. Paired t-tests were performed to determine whether model estimated muscle mass was different from $muscle_{MRI}$ and $muscle_{MRIfc}$.

4.4 Results

One Caucasian boy with CP did not complete the second MRI scan (IDEAL) and was excluded from the analyses. Physical characteristics for the remaining participants are summarized in **Table 5**. Eight children with CP had GMFCS I and 7 had GMFCS II. No group differences in age, body mass, BMI, BMI percentile, sexual maturity or fat mass were detected (all $p > 0.05$). Children with CP had a lower body mass percentile, height and height percentile (all $p < 0.05$). Children with CP also had 38 % lower FFST, 40 % lower $muscle_{MRI}$ and 47 % lower $muscle_{MRIfc}$ (all $p < 0.05$). Intramuscular fat concentration was higher in children with CP (24 % vs 15 %; $p = 0.001$). Children with CP had a 17 % lower ratio of $muscle_{MRIFC}$ to FFST

(0.62 ± 0.13 vs. 0.75 ± 0.06; d = 1.205, p = 0.004; **Figure. 6B**). Although children with CP also had a lower $muscle_{MRI}$ to FFST ratio, the difference was not statistically significant (0.82 ± 0.13 vs. 0.88 ± 0.06; d = 0.564, p = 0.138; **Figure. 6A**). A visual depiction of the muscle and fat discrepancies between a boy with CP and an age-matched typically developing boy is shown in **Figure 7**.

Table 5. Physical characteristics, DXA measurements and MRI skeletal muscle mass in children with cerebral palsy (CP) and typically developing children (Con).

	CP (n = 15)	Con (n = 15)	*d*	*p*
Ages (years)	8.0 ± 2.4	8.3 ± 2.1	0.133	0.700
Tanner stage (I/II/III)				
Pubic hair	11/3/1	14/1/0	0.550	0.345
Testicular-penile/breast	11/4/0	13/1/1	0.137	0.595
Height (m)	1.20 ± 0.12	1.30 ± 0.11	0.869	0.022
Height (%)	17 ± 28	58 ± 30	1.413	< 0.001
Body mass (kg)	24.7 ± 9.3	28.9 ± 7.0	0.510	0.067
Body mass (%)	32 ± 31	60 ± 26	0.979	0.012
BMI (kg/m^2)	16.9 ± 3.4	16.8 ± 2.4	0.034	0.461
BMI (%)	50 ± 36	55 ± 28	0.155	0.694
GMFCS (I/II)	8/7	-		
FFST (g)	163 ± 59	263 ± 77	1.447	< 0.001
Fat mass (g)	125 ± 78	148 ± 61	0.321	0.161
$Muscle_{MRI}$ (g)	136 ± 61	228 ± 65	1.485	< 0.001
Intramuscular fat concentration (%)	24 ± 8	15 ± 3	1.490	0.001
$Muscle_{MRIfc}$ (g)	103 ± 48	194 ± 54	1.786	< 0.001

Values are mean ± SD. BMI = body mass index. % for height, body mass and BMI reflects the percentile relative to age- and sex- based norms; % for intramuscular fat concentration reflects the percent fat within the muscle; DXA= dual-energy X-ray absorptiometry; MRI = magnetic resonance imaging; GMFCS = gross motor function classification system; FFST = fat free soft tissue; $Muscle_{MRI}$, muscle mass assessed by MRI; $Muscle_{MRIfc}$, muscle mass assessed by MRI and corrected for fat concentration. *d* = Cohen's d.

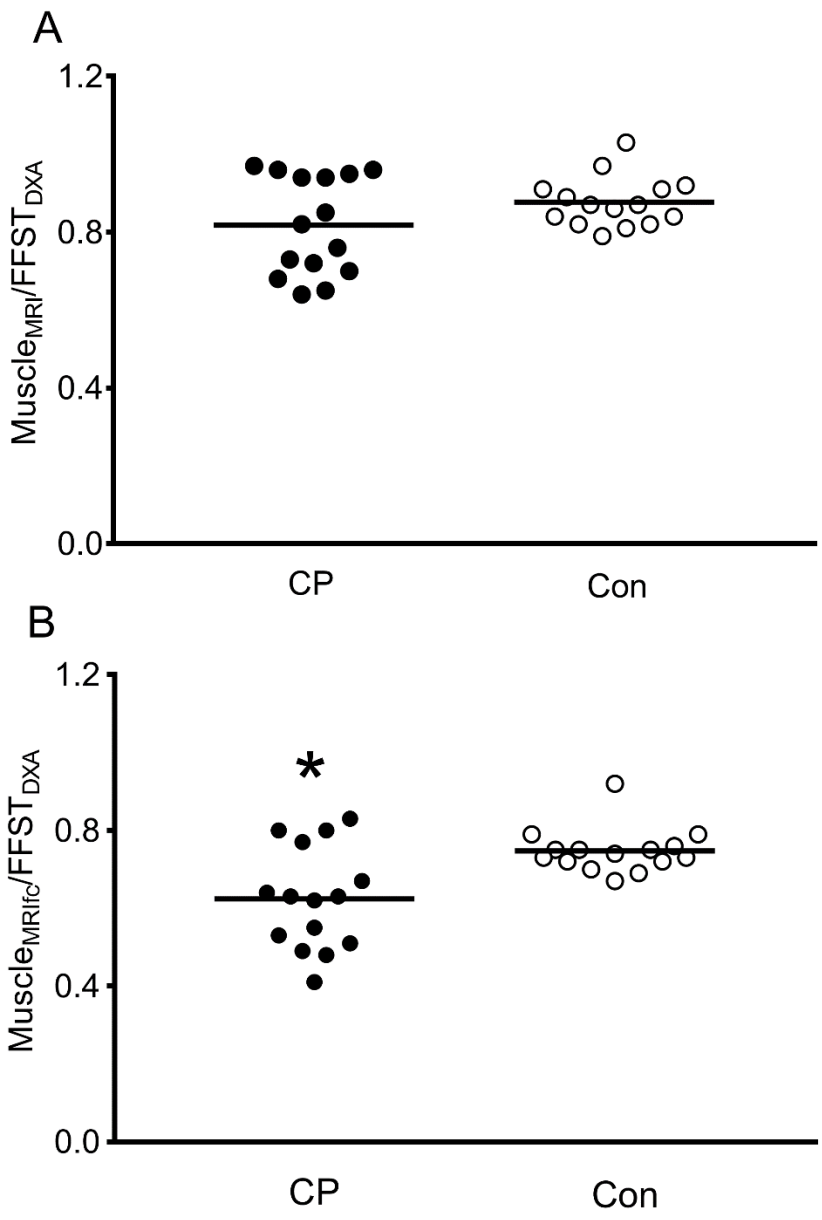


Figure. 6. Ratio of muscle mass from MRI ($Muscle_{MRI}$) to fat free soft tissue from DXA ($FFST_{DXA}$; A) and muscle mass from MRI corrected for fat concentration ($Muscle_{MRIfc}$) to $FFST_{DXA}$ (B) in the midleg of children with cerebral palsy (CP; n = 15) and typically developing children (Con; n = 15). *Group difference, $p < 0.05$.

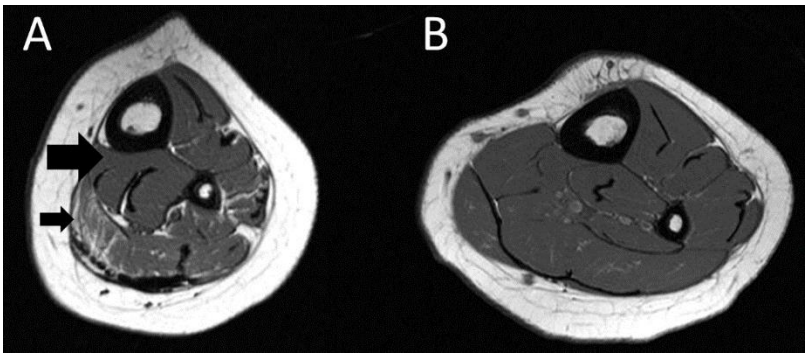


Figure 7. Magnetic resonance images from a boy with cerebral palsy (CP; A) and a typically developing boy (B) both 8.5 years of age at the level of the midtibia. The boy with CP had much lower muscle mass (large black arrow in A) and much higher intramuscular fat (smaller black arrow in A) compared to his typically developing peer.

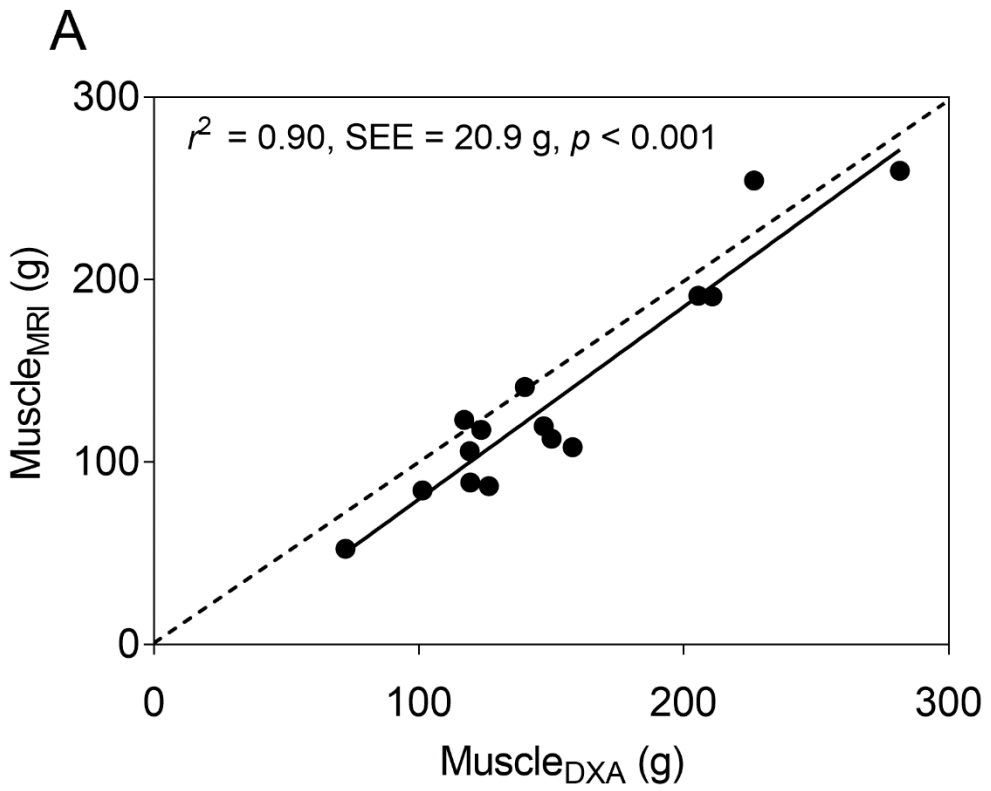
4.4.1 Statistical model developed using typically developing children

Simple linear regression using FFST as the predictor explained 95 % of the variance in $muscle_{MRI}$ in typically developing children (**Table 6**, model 1). Adding age or height didn't significantly improve the model (both $p > 0.05$). However, adding body mass, BMI or fat mass individually significantly improved the model (all $p < 0.05$). Adding fat mass yielded the highest R^2 value explaining 98% of the variance in $muscle_{MRI}$ (**Table 6**, model 2). When the model with FFST and fat mass was cross-validated using the leave-one-out method, the estimated muscle mass ($muscle_{DXA}$) was strongly related ($r^2 = 0.97$, SEE = 11.8 g, $p < 0.001$) and not different from $muscle_{MRI}$ ($p = 0.770$). When the model was cross-validated in children with CP, $muscle_{DXA}$ was strongly related to $muscle_{MRI}$, as shown in the scatter plot in **Figure. 8A** ($r^2 = 0.90$, SEE = 20.0 g, $p < 0.001$). However, the model overestimated $muscle_{MRI}$ by 13 % (17.3 g, $p = 0.004$), as shown by most data points and the regression line residing mainly below the line of identity in **Figure. 8A** and by most data points and mean difference line above the no difference line in the Bland-Altman plot in **Figure. 8B**.

Table 6. Statistical models developed to estimate midleg muscle mass from MRI using data from typically developing children (Con) and children with cerebral palsy (CP).

Group	Model	R^2	SEE (g)
Con	1. FFST (g) * 0.816 + 13.953	0.96	14.1
	2. FFST (g) * 0.704 + fat mass (g) * 0.221 + 10.924	0.98	9.5
CP	3. FFST (g) * 0.986 – 24.577	0.91	19.3

All models are statistically significant, $p < 0.001$; MRI = magnetic resonance imaging; FFST = fat free soft tissue; n = 15/group.


A
r^2 = 0.90, SEE = 20.9 g, p < 0.001
$Muscle_{MRI}$ (g)
$Muscle_{DXA}$ (g)
0
100
200
300

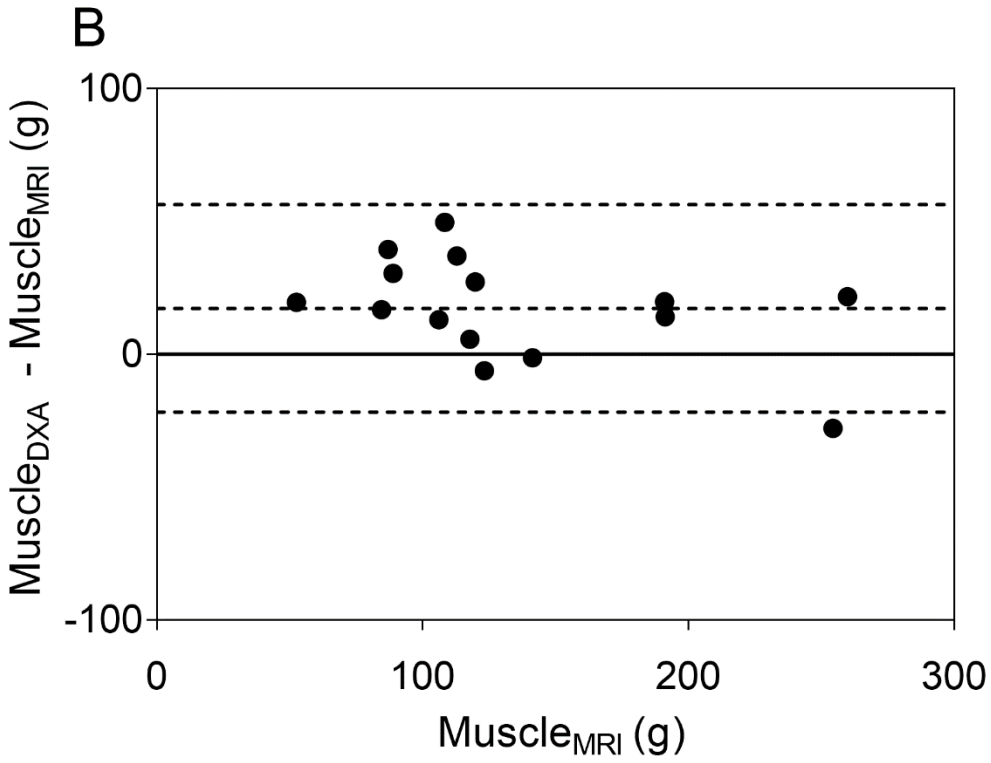

B
$Muscle_{DXA}$ - $Muscle_{MRI}$ (g)
$Muscle_{MRI}$ (g)
100
0
-100
0
100
200
300

Figure 8. A) The scatter plot shows midleg muscle mass ($Muscle_{MRI}$) in children with cerebral palsy (CP; n = 15) estimated using a dual-energy X-ray absorptiometry (DXA) -based model ($Muscle_{DXA}$) developed using data from typically developing children. $Muscle_{DXA}$ was estimated using fat-free soft tissue mass (FFST) from DXA. The dotted line represents the line of identity. The solid line represents the regression line. B) The Bland-Altman plot shows the level of agreement between $Muscle_{DXA}$ and $Muscle_{MRI}$. The dotted lines indicate the mean difference ± 2 SD between actual muscle mass from MRI and the estimated muscle mass by the DXA-based model in children with CP. The solid line indicates no difference between actual and estimated muscle mass.

Simple linear regression using FFST as the predictor explained 96 % of the variance in $muscle_{MRIfc}$ in typically developing children (**Table 7**, model 1). None of the additional predictors significantly improved the model (all $p > 0.05$). When the final model with only FFST as the predictor was cross-validated using the leave-one-out method, the estimated muscle mass was also strongly related ($r^2 = 0.95$, SEE = 12.7 g, $p < 0.001$) and not different from $muscle_{MRIfc}$ ($p = 0.858$). When the same model was cross-validated in children with CP, the estimated muscle mass ($muscle_{DXAfc}$) was strongly related to $muscle_{MRIfc}$, as shown in **Figure. 9A** ($r^2 = 0.83$, SEE = 17.6 g, $p < 0.001$). However, the model overestimated $muscle_{MRIfc}$ by 22 % (22.3 g, $p = 0.001$), as shown by most data points and the regression line residing below the line of identity in **Figure. 9A** and by most data points and the mean difference line above the no difference line in the Bland-Altman plot in **Figure. 9B**.

Table 7. Statistical models developed to estimate midleg muscle mass from MRI corrected for fat concentration using data from typically developing children (Con) and children with cerebral palsy (CP).

Group	Model	R^2	SEE (g)
Con	1. FFST (g) * 0.684 + 14.350	0.96	10.7
CP	2. FFST (g) * 0.740 – 17.086	0.83	20.9
	3. FFST (g) * 0.993 – body mass (kg) * 2.123 – 5.766	0.90	16.8

All models are statistically significant, $p < 0.001$; MRI = magnetic resonance imaging; FFST = fat free soft tissue; n = 15/group.

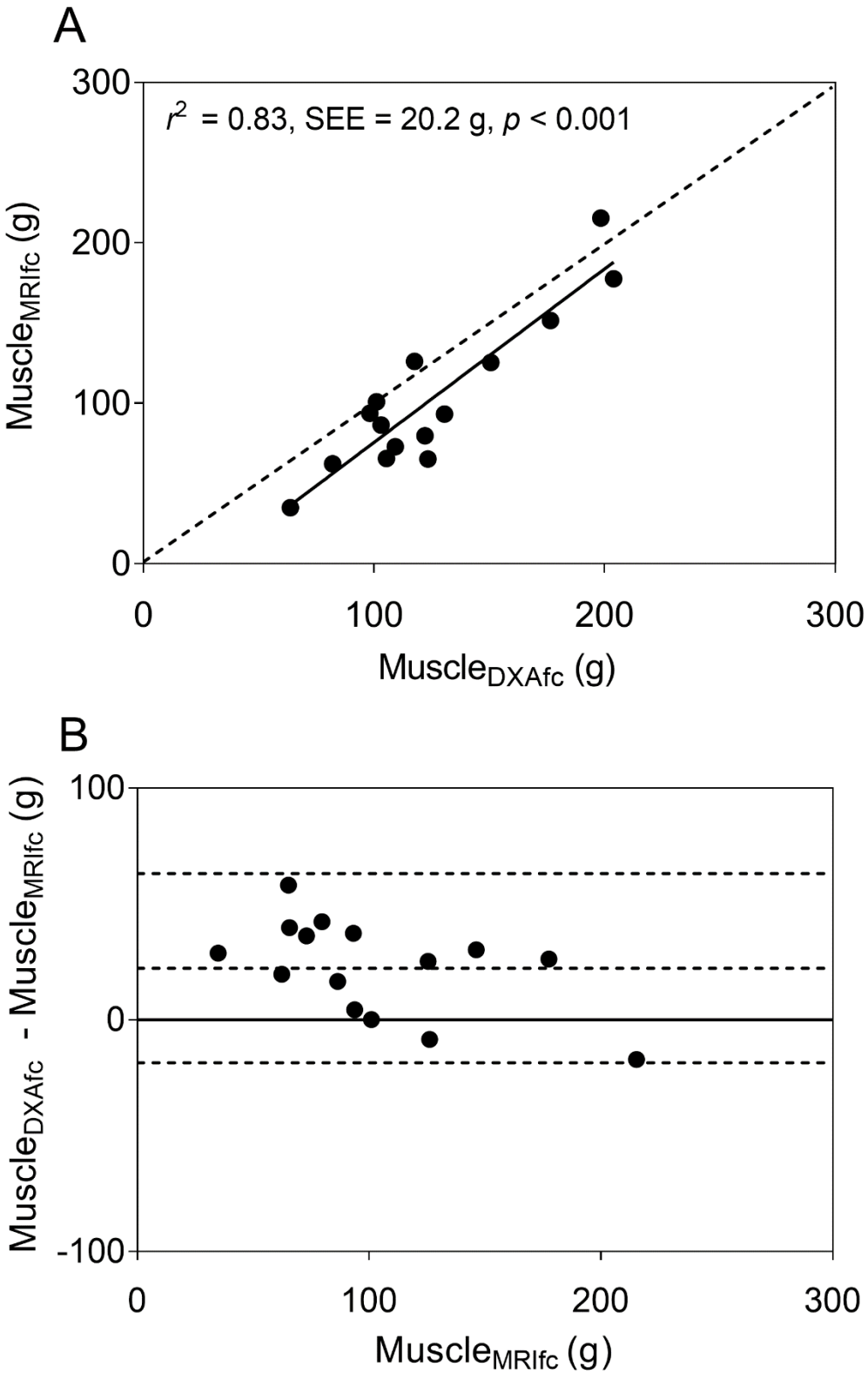
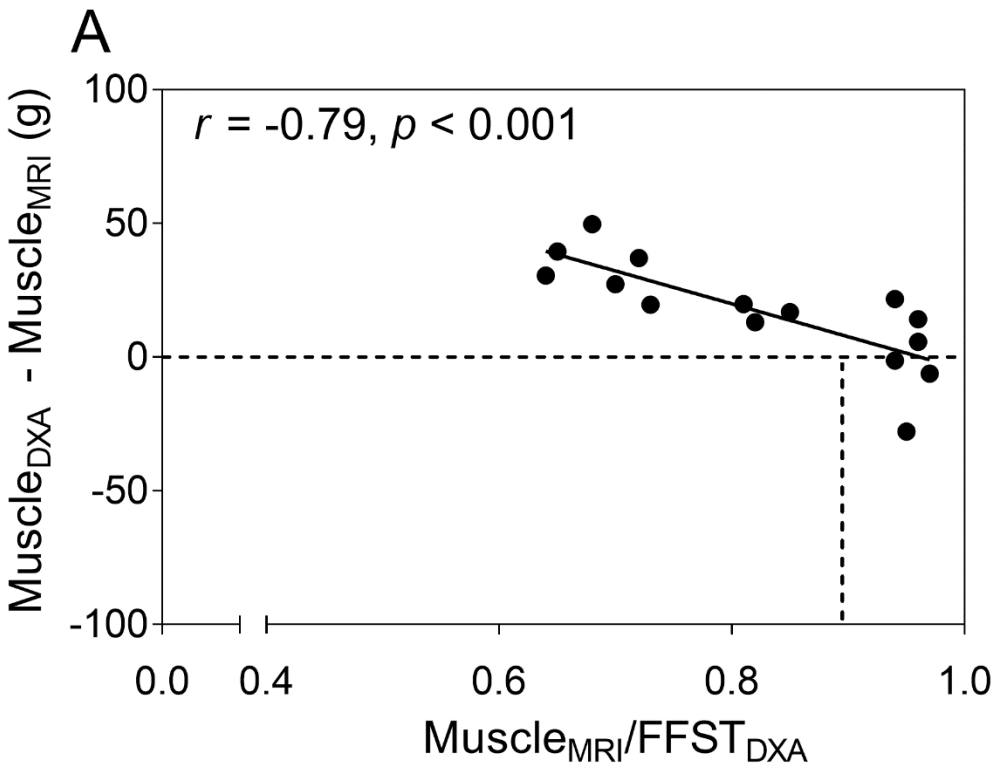

A
r^2 = 0.83, SEE = 20.2 g, p < 0.001
$Muscle_{MRIfc}$ (g)
$Muscle_{DXAfc}$ (g)
0
100
200
300
B
$Muscle_{DXAfc}$ - $Muscle_{MRIfc}$ (g)
$Muscle_{MRIfc}$ (g)
-100

Figure 9. A) The scatter plot shows midleg muscle mass adjusted for intramuscular fat concentration ($Muscle_{MRIfc}$; A) in children with cerebral palsy (CP; n = 15) estimated using a dual-energy X-ray absorptiometry (DXA) -based model ($Muscle_{DXAfc}$) developed using data from typically developing children. $Muscle_{DXAfc}$ was estimated using fat-free soft tissue mass (FFST) from DXA. The dotted line represents the line of identity. The solid line represents the regression line. B) The Bland-Altman plot shows the level of agreement between $Muscle_{DXAfc}$ and $Muscle_{MRIfc}$. The dotted lines indicate the mean difference ± 2 SD between actual muscle mass from MRI and the estimated muscle mass by the DXA-based model in children with CP. The solid line indicates no difference between actual and estimated muscle mass.

The difference between $muscle_{DXA}$ and $muscle_{MRI}$ in children with CP was inversely related to the ratio of muscle mass to FFST (r = -0.79, $p < 0.001$), as shown in **Figure. 10A**. The positive values for the difference between $muscle_{DXA}$ and $muscle_{MRI}$ indicate an overestimation of muscle mass by the DXA-based model, which was greatest in children with lowest ratio of $muscle_{MRI}$ to FFST. The overestimation declined as the average ratio of muscle to FFST in typically developing children (0.88) was approached. A similar inverse relationship was demonstrated between the difference in predicted muscle mass and $muscle_{MRIfc}$ and the ratio of $muscle_{MRIfc}$ to FFST (r = -0.95, $p < 0.001$; **Figure. 10B**). The overestimation was greatest in children with lowest ratio of muscle to FFST and became smaller as the average ratio of muscle to FFST in typically developing children (0.75) was approached.


A
r = -0.79, p < 0.001
100
50
0
-50
-100
0.0
0.4
0.6
0.8
1.0
MuscleDXA - MuscleMRI (g)
MuscleMRI/FFSTDXA

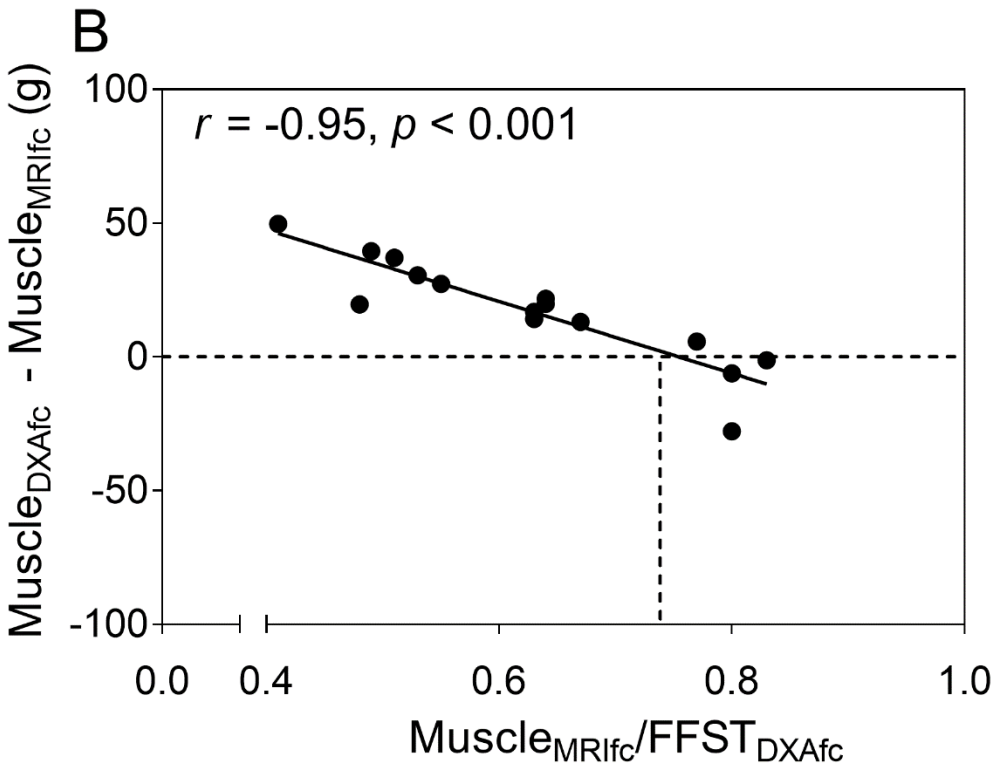

B
r = -0.95, p < 0.001
100
50
0
-50
-100
0.0
0.4
0.6
0.8
1.0
MuscleDXAfc - MuscleMRIfc (g)
MuscleMRIfc/FFSTDXAfc

Figure 10. A) Scatter plot of the ratio of muscle mass from MRI ($Muscle_{MRI}$) to fat-free soft tissue mass from DXA ($FFST_{DXA}$) in the midleg compared to the difference between muscle mass estimated by the DXA-based model using data from controls ($Muscle_{DXA}$) and $Muscle_{MRI}$ in children with cerebral palsy (CP; n = 15). B) Scatter plot of the ratio of $muscle_{MRI}$ corrected for intramuscular fat concentration ($Muscle_{MRIfc}$) to $FFST_{DXA}$ in the midleg compared to the difference between $Muscle_{DXA}$ corrected for intramuscular fat concentration ($Muscle_{DXAfc}$) and muscle mass $Muscle_{MRIfc}$. The dashed horizontal lines represent the points where the difference between muscle mass estimated from DXA and MRI are zero. The dashed vertical lines represents the mean ratios of muscle mass from MRI to $FFST_{DXA}$ (0.88 for $Muscle_{MRI}/FFST_{DXA}$ and 0.75 for $Muscle_{MRIfc}/FFST_{DXA}$) for typically developing children.

4.4.2 Statistical model developed using children with CP

A simple regression model using FFST as the predictor explained 91% of the variance in $muscle_{MRI}$ in children with CP (**Table 6**, model 3). Adding age, height, body mass, BMI or fat mass as independent variables didn't significantly improve the model (all $p > 0.05$). Estimated muscle mass using the model with FFST as the predictor was not different from $muscle_{MRI}$ ($p > 0.990$). The validity of estimation was excellent as shown by most data points and the regression line residing near the line of identity in **Figure. 11A** and by most data points and the mean difference line near the no difference line in the Bland-Altman plot in **Figure. 11B**. When the model was cross-validated using the leave-one out method, the estimated muscle mass was strongly related ($r^2 = 0.87$, SEE = 21.8 g, $p < 0.001$) and not different from $muscle_{MRI}$ ($p = 0.893$).

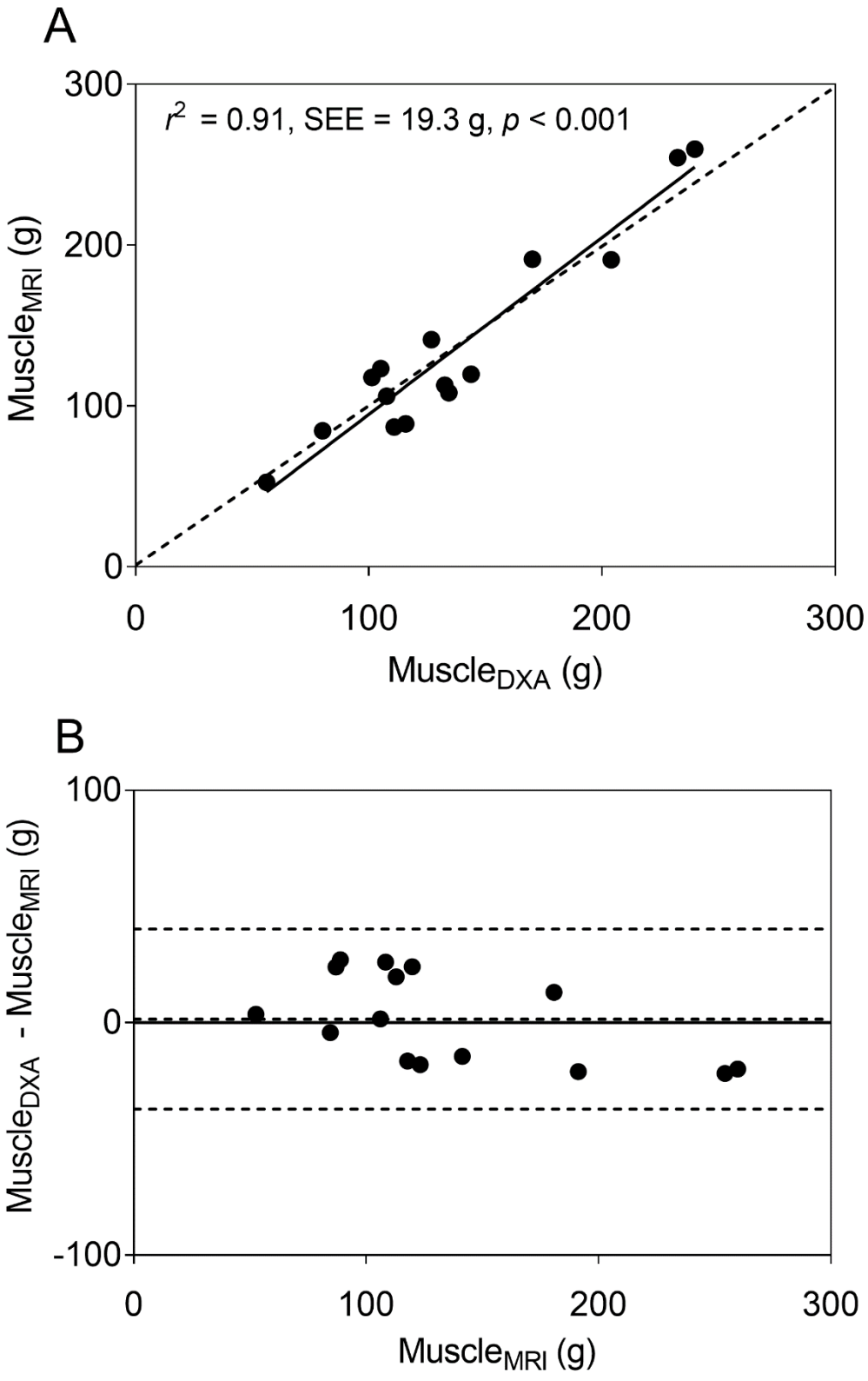

A
r^2 = 0.91, SEE = 19.3 g, p < 0.001
Muscle$_{MRI}$ (g)
Muscle$_{DXA}$ (g)
0
100
200
300
B
Muscle$_{DXA}$ - Muscle$_{MRI}$ (g)
Muscle$_{MRI}$ (g)
-100

Figure 11. A) The scatter plot shows midleg muscle mass ($Muscle_{MRI}$) in children with cerebral palsy (CP; n = 15) estimated using a dual-energy X-ray absorptiometry (DXA) -based model ($Muscle_{DXA}$) developed using data from children with CP. $Muscle_{DXA}$ was estimated using fat-free soft tissue mass (FFST) from DXA. The dotted line represents the line of identity. The solid line represents the regression line. B) The Bland-Altman plot shows the level of agreement between $Muscle_{DXA}$ and $Muscle_{MRI}$. The dotted lines indicate the mean difference ± 2 SD between actual muscle mass from MRI and the estimated muscle mass by the DXA-based model. The solid line indicates no difference between actual and estimated muscle mass.

A second regression model using FFST as the predictor explained 83 % of the variance in $muscle_{MRIfc}$ in children with CP (**Table 7,** model 2). Adding age, height, BMI or fat mass didn't significantly improve the model (all $p > 0.05$). However, when body mass was added to the model, significant improvement was observed with 90 % of the variance in $muscle_{MRIfc}$ explained (**Table 7**, model 3). Estimated corrected muscle mass using this model was not different from $muscle_{MRIfc}$ ($p = 0.990$). The validity of estimation was excellent as shown by most data points and the regression line residing near the line of identity in **Figure. 12A** and by most data points and the mean difference line near the no-difference line in the Bland-Altman plot in **Figure. 12B**. When the model was cross-validated using the leave-one out method, the estimated corrected muscle mass was strongly related ($r^2 = 0.83$, SEE = 20.5 g, $p < 0.001$) and not different from $muscle_{MRIfc}$ ($p = 0.785$).

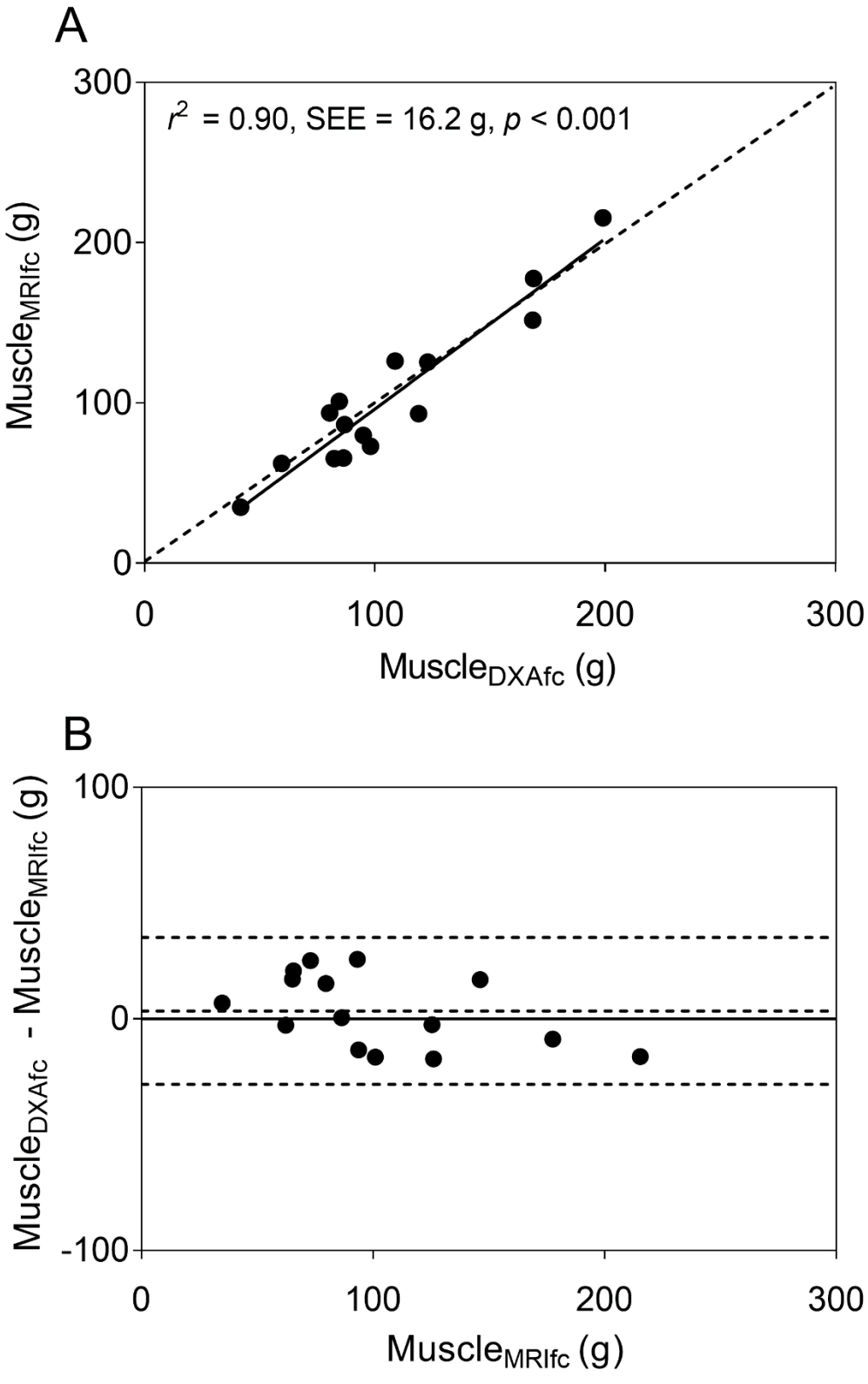

A
300
200
100
0
r^2 = 0.90, SEE = 16.2 g, p < 0.001
$Muscle_{MRIfc}$ (g)
0
100
200
300
$Muscle_{DXAfc}$ (g)
B
100
0
-100
$Muscle_{DXAfc}$ - $Muscle_{MRIfc}$ (g)
0
100
200
300
$Muscle_{MRIfc}$ (g)

Figure 12. A) The scatter plot shows midleg muscle mass adjusted for intramuscular fat concentration ($Muscle_{MRIfc}$; A) in children with cerebral palsy (CP; n = 15) estimated using a dual-energy X-ray absorptiometry (DXA) -based model ($Muscle_{DXAfc}$) developed using data from children with CP. $Muscle_{DXAfc}$ was estimated using fat-free soft tissue mass (FFST) from DXA. The dotted line represents the line of identity. The solid line represents the regression line. B) The Bland-Altman plot shows the level of agreement between $Muscle_{DXAfc}$ and $Muscle_{MRIfc}$. The dotted lines indicate the mean difference ± 2 SD between actual muscle mass from MRI and the estimated muscle mass by the DXA-based model in children with CP. The solid line indicates no difference between actual and estimated muscle mass.

4.5 Discussion

To our knowledge, this is the first study to show that a DXA-based statistical model can provide accurate estimates of leg muscle mass in ambulatory children with spastic CP. Although models produced using typically developing children provided accurate estimates of muscle mass in typically developing children, they overestimated muscle mass by 13 % and 22 %, respectively, before and after correction for intramuscular fat in children with CP. The findings are important because children with CP have very low muscle mass in the leg and methods that provide accurate estimates of muscle mass are needed.

The finding that statistical models developed for typically developing children do not provide accurate estimates of muscle mass in children with CP is consistent with a previous study. In a small group of non-ambulatory children with CP, midthigh muscle mass was overestimated by 12-15 % ($p < 0.05$) when a DXA-based model developed using typically developing children was used. The overestimation was attributed to the lower ratio of muscle to FFST in children with CP. An inverse relationship between this ratio and the degree of muscle mass overestimation supports the strong tie between the relative amount of muscle in the FFST and the accuracy of muscle mass estimated by DXA [11]. Similarly, in the present study, muscle mass was overestimated by 13 % when a model developed using typically developing children

was employed, and the ratio of muscle to FFST was lower in the children with CP. Moreover, there was a strong inverse relationship between this ratio and the degree of muscle mass overestimation by a DXA-based models produced for typically developing children. Other than skeletal muscle, FFST also contains skin, vessels and other connective tissues. Assuming the non-skeletal muscle portion of FFST is not altered in children with CP, the compromised muscle mass will lead to a lower ratio of $muscle_{MRI}$ to FFST and $muscle_{MRIfc}$ to FFST in this population. It can be inferred that if children with CP had similar ratios of muscle mass to FFST as typically developing children, there would be no, or minimal overestimation, as indicated in **Figure. 10**.

Interestingly, in typically developing children, when fat mass was included as a predictor with FFST, the model improved significantly. However, the same improvement was not found in children with CP. Although both groups had a similar amount of fat mass as measured by DXA, the fat mass in children with CP actually represented a larger proportion of the leg soft tissue, as children with CP had 38 % smaller FFST. Similarly, a study that assessed thigh skeletal muscle mass in men with spinal cord injury found a stronger relation between the difference in skeletal muscle and FFST when adipose tissue was accounted for in controls than that in people with spinal cord injury [195]. The reason fat mass contributes to DXA-derived estimates of skeletal muscle mass in a healthy population but not in clinical populations who manifest reduced muscle mass is currently unknown but warrants further investigation.

To our knowledge, this is the first study to assess the accuracy of DXA estimates of muscle mass adjusted for intramuscular fat. This is important because intramuscular fat does not contribute to the force production of muscle and has been shown to be associated with decreased physical function [196]. Compared to typically developing children, children with CP have a higher intramuscular fat concentration. Therefore, the disparity in muscle mass was even greater when

intramuscular fat was subtracted from muscle mass (47 % vs. 40 % lower muscle mass in children with CP). As a result, DXA overestimated muscle mass corrected for fat concentration to a greater degree (22 % vs. 13 %, respectively). A DXA-based model developed for children with CP yielded much better accuracy, as indicated by the high amount of variance (90 %) explained by the model and no difference in DXA and MRI estimates of muscle mass.

Small muscles have been linked to poor physical function and low participation in physical activity [4,197], and thus may increase risk of chronic disease [198,199]. The risk is likely to rise as individuals with CP age [176,200]. This idea is supported by the much higher cardiovascular [175] and osteoporosis [176] risks in adults with CP compared to general population . Therefore, the muscle deficit in children with CP is an important therapeutic target for interventions aimed at improving physical function and physical activity and reducing long-term risk of developing chronic diseases. The present study ascertains the validity of using DXA to predict leg muscle mass, a region that has been found to have the largest muscle deficit in individuals with CP [10]. The findings suggest that the DXA-based models developed in the present study provide an accessible, relatively cheap and accurate method to assess the skeletal muscle status in ambulatory children with spastic CP. However, studies are needed to determine whether the models can accurately capture the muscle changes in children with CP due to natural growth or treatment, such as resistance training [49], vibration treatment [201] and botulinum toxin [202].

There are imitations associated with this study. First, because of the relatively small number of subjects in each group, the results should be interpreted with caution. However, no differences in age or sexual maturity were observed and the composition of boys and girls was similar. Second, to estimate skeletal muscle mass, muscle volume was multiplied by 1.04 g/cm^3, which is the assumed muscle density of muscle determined from adult cadavers [161]. It's possible

that for children that are still growing, their true muscle density may not be the same as adults. It is also reasonable to infer that for children with CP whose muscle volume and quality are compromised, their muscle density may be lower than their typically developing peers. Therefore, the true muscle deficit in children with CP may be at a greater magnitude than what was presented in the current study. However, this potential discrepancy has been partly mediated by correcting for intramuscular fat concentration. The assumed density of fat [203] is lower than muscle, so the density of muscle for children with CP after correcting for fat concentration would be closer to that of typically developing children. Third, the findings from the present study can't be extrapolated to other regions of the body.

4.6 Conclusion

In conclusion, DXA-based statistical models can accurately estimate midleg muscle mass in ambulatory children with spastic CP when the models are developed using data from children with CP. Models developed using data from typically developing children overestimate leg muscle mass in children with CP due to the lower proportion of muscle in the FFST of children with CP. Future studies are needed to determine whether the models developed in the present study are valid in children with more severe forms of CP and whether the models accurately estimate change that occurs with growth or intervention. Future studies are also needed to develop models to estimate muscle mass at other body regions in children with CP.

4.7 Acknowledgement

We thank all the participants and their families. We thank Patricia Groves for assisting with data collection, and Nancy Lennon for assisting with recruitment. This study was supported

by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant numbers HD071397 and HD090126).

CHAPTER 5

ROLE OF CIRCULATING MYOSTATIN, FOLLISTATIN AND IGF-1 IN THE MUSCLE UNDERDEVELOPMENT IN AMBULATORY CHILDREN WITH SPASTIC CEREBRAL PALSY

Zhang, C., Miller, F., Modlesky, C.M. To be submitted to Muscle & Nerve.

5.1 Abstract

Background: Cerebral palsy (CP) is a neurological disorder characterized by the decreased muscle volume compared to their typically developing peers. The mechanism for the muscle underdevelopment hasn't been thoroughly examined.

Objective: To determine whether serum bioactive agents myostatin, follistatin and insulin-like growth factor (IGF-1) levels are consistent with the muscle underdevelopment observed in children with CP.

Methods: 13 children with CP (4 – 11 y) and 13 typically developing children matched with children with CP for sex, age and race were studied. Muscle volume in the middle third of the leg (midleg) were estimated using magnetic resonance imaging (MRI), and whole body (excluding the head) fat-free soft tissue mass (FFST) and FFST index (FFST/height^2; FFSTI) were estimated using dual-energy X-ray absorptiometry (DXA). Serum myostatin, follistatin and IGF-1 levels were determined using ELISA.

Results: Children with CP had 35% lower muscle volume, 21% lower FFST and 11% lower FFSTI when compared to their typically developing children (all $p < 0.05$). No between group differences were found for myostatin, follistatin or IGF-1 (all $p > 0.05$). In addition, none of the serum markers were related to any of the muscle measurements (all $p > 0.05$). No between group differences were found for FFST per unit myostatin or follistatin (both $p > 0.05$), but FFST per unit IGF-1 was significantly lower in children with CP ($p = 0.047$).

Conclusion: Current study suggest that circulating myostatin, follistatin and IGF-1 are not different between ambulatory children with CP and their typically developing peers. However, FFST per unit IGF-1 was lower in children with CP, suggesting that IGF-1 may play an important role in the muscle underdevelopment in children with CP.

Keywords: Cerebral palsy; myostatin; follistatin; insulin-like growth factor; muscle.

5.2 Introduction

Cerebral palsy (CP) is a neurological disorder caused by the damage to the brain that happened around the time of birth. Children with CP usually exhibit a series of mobility issues including muscle weakness, which is partly characterized by the decreased muscle volume compared to their typically developing peers [8,9,54,157]. However, despite the well-established growth hormone deficiency prevalent in this population [81], the role of other bioactive agents in the muscle compromise in children with CP has not been elucidated.

Myostatin is a potent regulator of muscle. It is mainly secreted by muscle and acts in an autocrine manner to decrease myogenesis activity, [204] thus inhibiting muscle mass accretion. Myostatin-deficient mammals exhibit great increases in muscle mass [97,101,106,205,206]. Similar patterns have been reported in those injected with myostatin-inhibitors [207], with increases in muscle fiber number and cross-section also observed [208]. Such improvement in muscle mass was attributed to multiple factors, including, but not limited to, elevated myogenesis and decreased adipogenesis [101]. In addition, systematically injected myostatin has been shown to induce cachexia in mice [104]. In humans, serum myostatin level was linked to skeletal muscle loss in those who are aging[107], patients with chronic obstructive pulmonary disease [108] and patients with heart failure [110]. On the other hand, follistatin acts as an antagonist of the transforming growth factor (TGF –β) superfamily, in which myostatin belongs to, by directly binding with TGF –β to inhibit its activity and thus promote muscle growth [116]. In mice, administering follistatin may reduce disease-induced sarcopenia [117] and facilitate skeletal muscle healing after injury [118]. In humans, follistatin gene therapy has been shown to be a promising treatment for Becker

muscular dystrophy [209]. However, despite the strong influence of myostatin and follistatin on muscle development, few studies have examined their roles in the underdevelopment of muscle in children with CP.

Insulin-like growth factor 1 (IGF-1) is also well-recognized as a mediator for muscle mass. Mice that lack IGF-1 receptors demonstrate considerable hypoplasia while those that exhibit IGF-1 gene overexpression alleviates the negative effects associated with various muscle wasting conditions [210-212]. IGF-1 also stimulates satellite cell differentiation into new muscle cells to prepare for muscle regeneration after damage [31]. Thus, IGF-1 is important in both muscle growth, as well as later development and hypertrophy. However, well-designed studies aimed at determining the status of IGF-1 in children with CP are lacking.

Therefore, the purpose of the present study was to determine the fasting serum myostatin, follistatin and IGF-1 profiles in children with CP, and to determine whether the circulating levels of these bioactive agents are associated with the curbed muscle development observed in this population. We hypothesized that children with CP have higher serum myostatin and lower serum follistatin and IGF-1 levels compared to typically developing children.

5.3 Methods

5.3.1 Participants

Thirteen ambulatory children with spastic CP were recruited from local hospitals CP programs. Thirteen typical developing children without known neurological disorders, with height, body mass and BMI between the 5th and 95th percentile and matched to the children with

CP for age (± 1.5 years), sex and race were also recruited via word of mouth and flyers. This study was approved by the Institutional Review Board.

5.3.2 Anthropometrics

Height was measured to the nearest 0.1 cm using a stadiometer (Seca 217; Seca GmbH & Co. KG., Hamburg, Germany), and weight was measured to the nearest 0.2 kg using a weight scales (Detecto D1130; Detecto, Webb City, MO). Both height and weight were measured with the child have minimal clothing on. BMI was determined using the following equation: BMI=Height (m)/weight^2 (kg). Height, weight and BMI percentile were subsequently determined using the growth chart recommended by the Center for Disease Control and Prevention [165].

5.3.3 Sexual maturity

Sexual maturity was assessed by a physician assistant using the Tanner Staging technique. This is a 5-point score system with I indicating no development and V representing full development. For both boys and girls, pubic hair development was assessed. In addition, penis/testicles development in boys and breast development in girls were assessed. All assessments were performed by the same physician assistant to ensure consistency.

5.3.4 Gross motor function classification system (GMFCS)

Gross motor function for children with CP was assessed by a physician assistant using GMFCS [20]. This is a 5 point system with higher numbers indicating greater functional limitations. Specifically, level I indicates the ability to walk and run but at the reduced speed while level two indicates limited walking ability and minimal ability for running independently.

All assessments were performed by the same physician assistant. All participants had GMFCS I and II.

5.3.5 Dual-energy X-ray absorptiometry (DXA)

Whole body DXA scans were acquired using a Delphi-W (Hologic, Bedford, MA) densitometer. Children with CP were secured from waist down during the procedure using a BodyFix system (Medical Intelligence, Inc., Schwabműnchen, GER) and a modified version of the procedure to reduce potential involuntary contractures. The modified BodyFIX procedure does not influence body composition estimates [166]. Whole body fat-free soft tissue (FFST; excluding the head) of each participant was determined from the whole body scan, and FFST index (FFSTI) was calculated using the following equation [213]:

$$\text{FFSTI} = \frac{\text{FFST}}{\text{Height}^2}\left(\frac{\text{g}}{\text{m}^2}\right)$$

In addition, we also calculated the amount of FFST per unit biomarker (FFST/myostatin, 10^9 L; FFST/follistatin, 10^9 L; FFST/IGF-1, 10^6L).

5.3.6 Magnetic resonance imaging (MRI)

A GE MRI scanner (1.5 T, Milwaukee, WI) was used to assess the leg musculature at the more-affected side in children with CP and at the non-dominant side in typical developing children. All images were acquired with the child secured from waist down using the same BodyFix system during the DXA scan acquisition, as previously described [214]. A three plane localizer was used to identify the region of interest. A semiflex long bone array coil (ScanMed, Omaha, NE) was used to collect axial images from the tibia plateau to the malleolar articular surface (0.5 cm thick separated by 0.5 cm of spacing). The employed imaging sequence (fast

spin echo, TR = 650, TE = 14, FOV = 12, NEX = 3, BW = 15.63) yielded T1-weighted images with a field of view of 12 cm, and a reconstructed image matrix of 512×512.

All image collection was overseen by the senior author (CMM). Images at the level of mid-third tibia, which was identified automatically with custom software created with Interactive Data Language (Research Systems, Boulder, Colorado), were analyzed. The muscle cross-sectional area of each image slice at the level of mid-third tibia was segmented using a fuzzy clustering algorism [193] with manual adjustment applied as needed, and the respective volumes were calculated by multiplying the spacing and thickness (1cm) between adjacent slices. The test-retest reliability for muscle volume measurements was performed by testing a subsample of children twice on different days or on the same day after repositioning, which yielded intraclass correlation coefficients > 0.99 and coefficients of variation (CV) 2.6% as indication of excellent reliability.

5.3.7 Biochemical sample collection and processing

Subjects were instructed not to eat or drink anything except for water after 10:00 pm the day before sample collection. Blood sample was taken by a certified pediatric phlebotomist between 8:00 am and 9:00 am in the morning to ensure at least ten hours of overnight fasting. All samples were prepared and stored at − 80 °C freezer until processing. Myostatin, follistatin and IGF-1 levels were measured using ELISA kits (R&D Systems, Minneapolis, MN).

5.3.8 Statistics

All data were processed using SPSS (version 24.0; SPSS, Chicago, IL). All datasets were checked for normality first. For between group comparisons, if data were normally distributed,

two sample t-tests were performed; otherwise Mann-Whitney tests were performed. One sample t-tests were used to determine whether typically developing children and children with CP had height, body mass and BMI percentiles different from the sex- and age-based 50th percentile. Cohen'd (*d*) was calculated whenever applicable, with 0.2, 0.5 and 0.8 indicating small, medium and large effect size, respectively [167]. To determine the relationship between myostatin, follistatin, IGF-1and muscle, FFST mass measurements, Pearson correlation analyses were performed. Alpha level was set at 0.05.

5.4 Results

Physical characteristics for subjects were summarized in **Table 8**. Six children with CP were classified as GMFCS I and 7 were classified as GMFCS II. Typically developing children had height, body mass and BMI not different from the 50th percentile (all $p > 0.05$). No between group differences were detected for age, Tanner Staging, height, body mass, BMI or BMI percentile (all $p > 0.05$). On the other hand, height percentile was lower than the 50th percentile in children with CP ($p < 0.001$). Children with CP also had lower height percentile and body mass percentile compared to typically developing children (both $p < 0.05$).

Table 8. Physical characteristics in children with cerebral palsy (CP) and controls (Con).

	CP (n = 13)	Con (n = 13)	*d*	*p*
Age(y)	8.3 ± 2.5	8.5 ± 2.2	0.085	0.845
Tanner stage (I/II/III)				
Pubic hair	9/3/1	12/1/0	_	0.311
Tests/breast	8/5/0	11/1/1	_	0.390
Height (m)	1.22 ± 0.14	1.30 ± 0.11	0.635	0.103
Height (%)	15.7 ± 16.3	53.3 ± 28.4	1.633	0.001*
Body mass (kg)	25.9 ± 9.8	29.7 ± 8.1	0.423	0.292
Body mass (%)	33.2 ± 32.9	58.5 ± 26.7	0.844	0.041*
BMI (kg/m^2)	16.9 ± 3.6	17.3 ± 3.0	0.121	0.511
BMI (%)	52.5 ± 36.6	57.0 ± 30.1	0.134	0.736
GMFCS (I/II)	6/7	_		

Values are means ± SD. % reflects the percentile relative to sex- and age-based norms. GMFCS = gross motor function classification system.
* $p < 0.05$. d = Cohen's d.

Muscle related measurements were summarized in **Figure 13**. Compared to controls, children with CP had significantly lower FFST ($d = 0.824$; $p = 0.046$). While marginally insignificant, children with CP also have lower FFSTI ($d = 0.845$; $p = 0.058$). Compared to controls, children with CP had a 35% lower leg muscle volume ($d = 1.347$; $p = 0.003$).

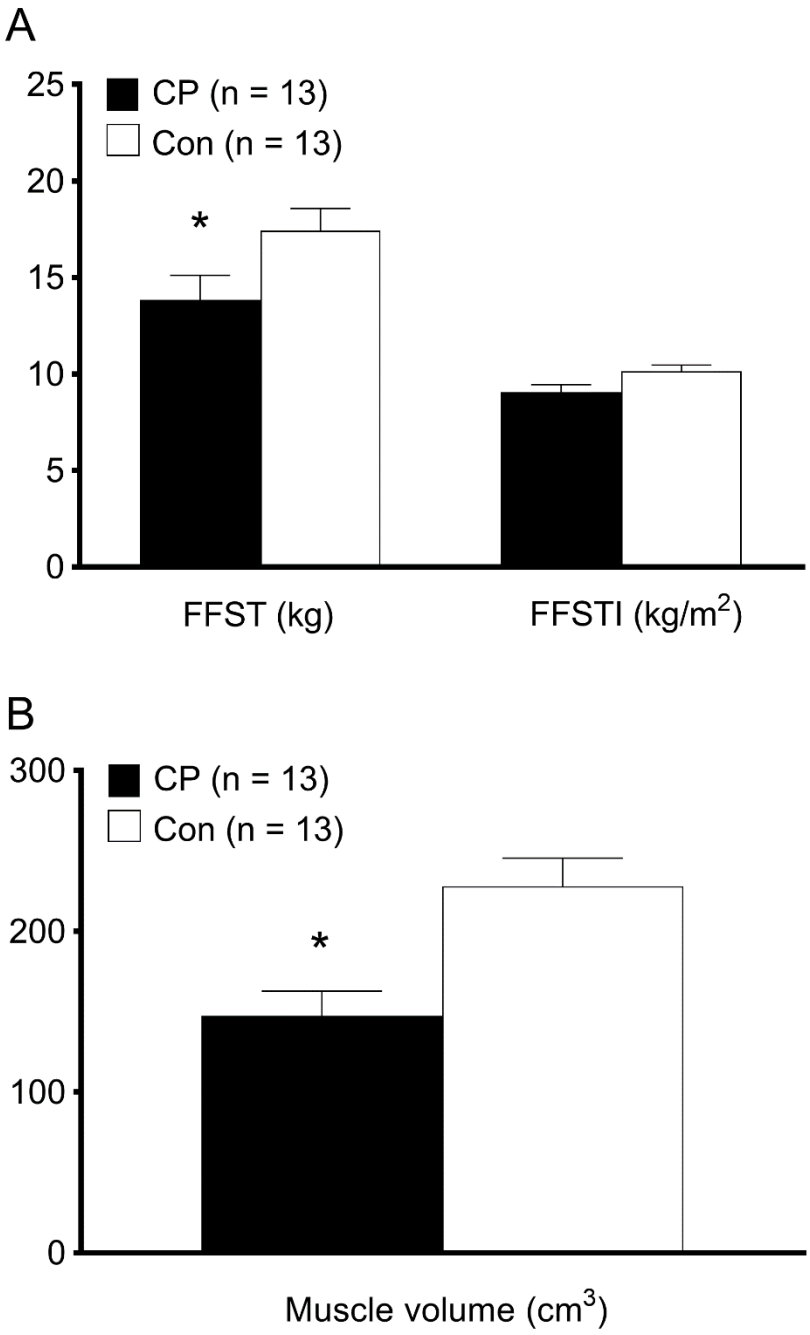


Figure 13. Bar graphs showing the comparisons for fat-free soft tissue (FFST) and FFST index (FFSTI; A) of the total body and muscle volume at the level of midthird tibia (B) between typically developing children (Con) and children with cerebral palsy (CP). *Significant between group difference.

Although slightly higher in children with CP, serum myostatin, follistatin and IGF-1 levels were not different compared to typically developing children (d = 0.217, 0.368 and 0.350, respectively; all $p > 0.05$; **Figure 14A**). There were no group differences in FFST/myostatin and FFST/follistatin (d =0.348 and 0.440, respectively; both $p > 0.05$; **Figure 14B**). However, FFST/IGF-1 was lower in children with CP compared to controls (d = 0.821; p = 0.047; **Figure 14B**). In addition, no association between serum biomarkers and muscle related measurements was detected (all $p > 0.05$).

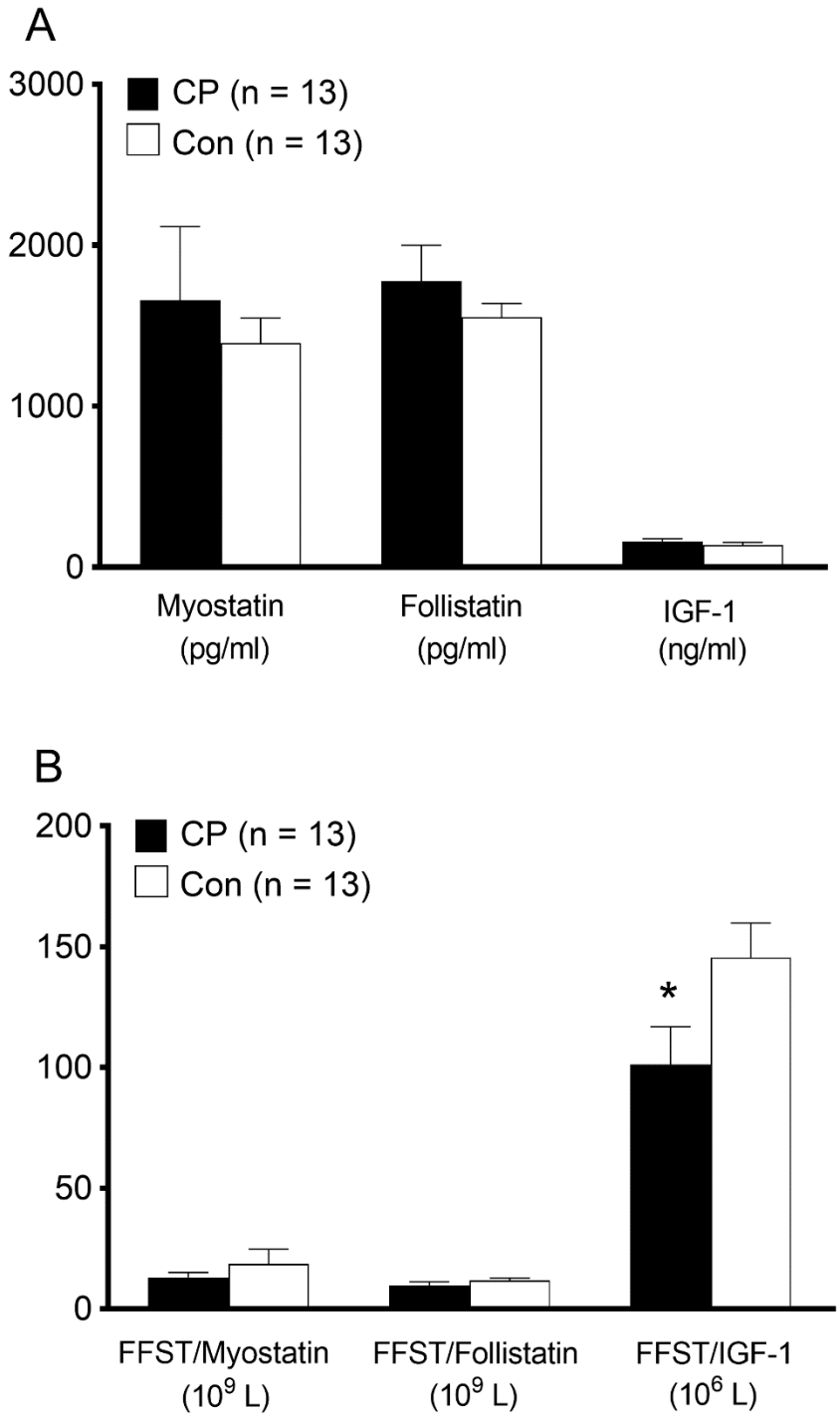


Figure 14. Bar graphs showing the comparisons for myostatin, follistatin and insulin-like growth factor 1 (IGF-1) between children with cerebral palsy (CP) and typically developing children (Con). Serum myostatin, follistatin and IGF-1 were not different in children with CP compared to Con (A). Fat-free soft tissue (FFST) per unit myostatin and follistatin were not different between children with CP and Con, however, FFST per unit IGF-1 was lower in children with CP (B).

5.5 Discussion

Only one prior study that we are aware of has examined the potential role of myostatin in the muscle compromise in children with CP. Using surgically removed spastic wrist muscle samples, Smith et al. found increased myostatin gene expression in children with spastic CP compared to controls [115]. However, it is imperative that we interpret the results with caution, as their sample size was very limited (6 children with CP vs 2 controls) and there was a considerable age difference between groups (12.8 ± 1.5 years for children with CP vs 8.5 ± 2.1 for controls). It is important to recognize that children with CP and typically developing children in the Smith study were probably at different stages of development, which could significantly confound the results. Despite the limitations, this study was the first attempt at examining the potential role of myostatin in the muscle compromise observed in children with CP.

The results from the current study suggest a minimal role of serum myostatin in the curbed muscle development in children with CP. This finding is inconsistent with the studies which found significant associations between serum myostatin and muscle loss/cachexia/sarcopenia in other clinical populations [107,108,110]. The discrepancies may be due to several factors. First, the subjects in the previous studies were generally in more advanced ages with varies clinical manifestations. It is possible that the effect of serum myostatin on muscle loss could be more critical in aging clinical population, but is limited in children who haven't reached their full genetic potential on muscular development. Second, only children with mild CP (i.e., GMFCS I-II) were included in the current study. Compared to those with more involved forms of CP (GMFCS higher than II), their muscle compromises were less pronounced, as indicated by the 35% reduction observed in the current study versus 51% observed in the latter group[4]. It is possible the effect of myostatin on muscle underdevelopment in children with a mild

form of CP is minimal; however it can become more pronounced in more advanced stages of CP, which is a deduction that warrants further investigation. In addition, as a bioactive agent that is secreted in an autocrine/paracrine manner, its effects may be localized in nature and thus not reflected in the serum level.

Follistatin was first identified in the human reproductive system. The pathways through which follistatin regulates muscle growth have been studied extensively, with some studies suggesting that the regulation occurs by inhibiting the action of the TGF –β superfamily[215], while others studies suggest that it occurs independent of myostatin[216]. Despite the conflicting viewpoints on the underlying mechanism, it is clear that higher follistatin level is associated with increased muscle mass, regardless of whether it was achieved via external administering [117] or genetic alteration [118]. For this, increasing systematic follistatin level is viewed as a potential therapeutic strategy for sarcopenia [217] and other muscle loss [209]. However, we found no association between serum follistatin levels and any of the muscle measurements in children with CP.

Reduced IGF-1 signaling is thought to play a central role in the degeneration process of aging muscles [218]. In addition, circulating IGF-1 was found to be significantly lower than controls in various models/disease conditions of muscle wasting or underdevelopment [219,220]. On the other hand, IGF-1 administration/overexpression has been shown to attenuate muscle wasting/loss [92,210,211,221]. Studies examining the status of IGF-1 secretion in children with CP have been somewhat inconsistent, with some suggesting a lower circulatory level when compared to typically developing children [93] or established norms [94], while one study actually indicated increased IGF-1 gene expression in those with spastic CP [115]. The discrepancies may be due, in part, to poor study designs. The study which suggested a reduced IGF-1 level in

children with CP when compared to norms lacked a control group. While the study that compared the serum IGF-1 level between children with CP and typically developing children did include a control group, there were many more children with CP compared to controls (n = 58 vs n = 19). In addition, although IGF-1 levels were statistically significantly different between groups using t-test, there was a tremendous amount of variation in the CP group (mean ± SD: 66.74 ± 44.78 ng/ml), which led to a very low effect size (0.22 as calculated by Cohen's d). As for the study by Smith et al., which suggested a greaterIGF-1 transcriptional profile in children with CP than controls, it included a very limited number of participants (6 CP vs. 2 controls). More importantly, CP children were significantly older than the two typically developing children (12.8 vs 8.5). The study of age and puberty on IGF-1 gene expression is scarce, but it has been suggested that serum IGF-1 level increases as children sexually mature [222]. It is only reasonable to assume that IGF-1 gene expression increases through puberty. Therefore, the results observed in the Smith et al. study may be confounded by age and sexual maturity differences between the two groups.

The present study addressed the abovementioned study design issues by: 1) matching children with CP and typically developing controls for age, sex and race, and 2) assuring that the typically developing children did not have extreme physical characteristics (i.e., height, body mass and BMI < 5^{th} and > 95^{th} percentile for age and sex). The lack of group differences in serum IGF-1 in the current study may be attributed to the same factors speculated for myostatin. Specifically, IGF-1 may play a more important role in the muscle compromise in the more advanced stage of CP, a population characterized by a greater level of skeletal muscle deficit, and aging clinical population, but may have limited effects for ambulatory children with CP. In addition, it was demonstrated that systematic IGF-1 injection in mice didn't attenuate muscle

wasting induced by angiotensin II infusion, but localized IGF-1 gene overexpression prevented muscle wasting. Therefore, perhaps for muscle development, IGF-1 has an autocrine role that may not be reflected in the circulating level [90,223]. However, a significantly lower FFST per unit IGF-1 was observed in children with CP compared to controls. This is an interesting finding because one of the primary mechanisms for IGF-1 to influence skeletal muscle development is by activating satellite cells for new muscle fiber formation [31]. It was suggested that children with spastic CP have lower amount of satellite cells compared to typically developing children [224]. Taken together, it seems that children with CP have a lower capacity to generate new muscle fibers, which may partially explain their compromised muscle development. Nonetheless, more research is needed to confirm this deduction.

There are strengths of the study that should be mentioned. First and foremost, the control group and CP group were matched for sex, age and race. As a result of careful subject selection, no differences were detected in sex, age, sexual maturity or any other physical characteristics, except for height and body mass percentage, between two groups. This helped minimize the potential variance caused solely by differences in developmental stage. The second strength is the technique used to acquire muscle related measurements. Lower leg muscle volume was estimated at the level of the middle third of the tibia using MRI, while FFST and FFSTI were derived from DXA. MRI is considered the "gold standard" for estimating soft tissue content *in vivo* [187], while DXA-derived FFST measurements have been validated [225]. Therefore, the imaging modalities employed in the current study ensured the accuracy of muscle estimation.

Limitations of this study should also be addressed. First, the number of participants included in the current study is relatively small (n = 13/group), so the results should be interpreted with caution. However, as part of the effort to overcome such an issue, all CP

participants were matched to a typical developing child for sex, age and race to reduce variation. Therefore, the between group comparisons are more likely to reflect the true population variations. Second, only serum markers were analyzed. As mentioned previously, some of the biomarkers have a strong tendency to act in an autocrine manner, so a close examination of the muscle tissue gene expression and protein content may better indicate their roles in muscle development in children with CP. Yet such samples are difficult to acquire under non-surgical situations for children.

5.6 Conclusion

The current study suggests that circulating myostatin, follistatin and IGF-1 are not different between ambulatory children with CP and their typically developing peers. However, FFST per unit IGF-1 is lower in children with CP, suggesting that IGF-1 may play an important role in the poor muscle development of children with CP.

5.7 Acknowledgement

We thank all participants and their families. This study was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant numbers HD071397 and HD090126) and the University of Georgia Athletic Association.

CHAPTER 6

ELEVATED VISCERAL FAT IN CHILDREN WITH CEREBRAL PALSY IS NOT RELATED TO GROSS MOTOR FUNCTION OR PHYSICAL ACTIVITY

Zhang, C., Licea, J., Miller, F., Modlesky, C.M. To be submitted to Pediatric Obesity.

6.1 Abstract

Background: Ambulatory children with cerebral palsy (ACP) compared to typically developing children have a greater level of visceral fat. However, no studies have evaluated the level of central adiposity across all levels of gross motor function in children with CP.

Objective: The purpose of this study was to determine if a lower level of gross motor function, as assessed using the gross motor function classification system (GMFCS), and a lower level of physical activity, as assessed using a physical activity monitor worn on the hip, are associated with a higher level of central adiposity in children with CP.

Methods: Thirty-five children with CP (age 5-12 years; 20 NACP, GMFCS III-V; 15 ACP, GMFCS I-II) and 35 typically developing children were studied. Fat mass and fat mass index (FMI; fat mass/height^2) at the abdominal, subcutaneous and visceral regions were determined using dual-energy X-ray absorptiometry.

Results: Both NACP and ACP had higher visceral FMI than typically developing children (both $p < 0.05$). ACP also had higher abdominal FMI than typically developing children ($p = 0.021$). No differences in central adiposity measures were detected between NACP and ACP (all $p > 0.05$). Moreover, abdominal and visceral fat measurements were not significantly related to GMFCS levels or physical activity in children with CP (all $p > 0.05$). However, subcutaneous fat mass and FMI were inversely related to GMFCS ($p = 0.059$ and 0.048, respectively). Measures of central adiposity were inversely related to physical activity in typically developing children (all $p < 0.05$).

Conclusion: The level of central adiposity was greater in children with CP than in their typically developing peers, but it was not related to the level of gross motor function or physical activity except for subcutaneous fat, which was negatively related to GMFCS.

Keywords: cerebral palsy; visceral fat; dual energy X-ray absorptometry; fat mass index

6.2 Introduction

Cerebral palsy (CP) is the most common childhood physical disability [226]. Numerous studies have suggested that children with CP have altered body composition, including a lower total body fat-free mass and higher total body fat mass [227,228] for a given body mass index. Less is known about the regional body composition alterations, especially fat distribution changes at the abdominal region in children with CP. This is important because compared to other fat depots in the abdomen, visceral adipose tissue is metabolically very active in that it has a greater capacity to produce free fatty acids, it is more prone to react to adrenergic stimulation, and it produces many hormones and bioactive molecules that can affect homeostasis [129,229]. On the other hand, subcutaneous adipose tissue serves as a physiological buffer for excessive energy intake and is more prone to absorb free fatty acids and triglycerides [129]. Therefore, the functional and metabolic differences between subcutaneous and visceral adipose tissue at the abdominal region suggest that changes reported in abdominal fat distribution may be associated with metabolic profile changes, as has been pointed out in obese adolescents [230], premenopausal women [231] and older adults [232].

There is evidence that ambulatory children with CP (ACP) have greater central adiposity when compared to age-, sex- and race-matched typically developing counterparts after accounting for their shorter stature [13]. However, whether the level of central adiposity in children with CP is related to their level of gross motor function and more prominent in nonambulatory children with CP (NACP), a group of children with greater functional limitations, is unknown. In addition, it has been suggested that a key moderator for central adiposity distribution is

physical activity [138,233], which is significantly lower in children with CP when compared to their typically developing peers [3,4]. Therefore, the relationship between physical activity and central adiposity distribution in children with CP warrants further investigation.

The purpose of this study was to determine whether the level central adiposity in children with CP is related to: 1) their level of gross motor function and ambulatory status, and 2) their level of physical activity. We hypothesized that: 1) NACP would have greater central adiposity when compared to their typically developing peers and ACP, and 2) greater central adiposity would be more pronounced in NACP than ACP and typically developing children and would be associated with a lower level of gross motor function and a lower level of physical activity in children with CP.

6.3 Methods

6.3.1 Participants

Thirty-five children with CP and between the ages of 5 and 12 and 35 typically developing children without known neurological disorders, had height, body mass and BMI between the 5th and 95th percentile and matched to children with CP for age (± 1.5 years), sex and race were also recruited locally to serve as controls. Some of these children participated in previous studies using the same recruitment and testing procedures [11,166]. This study was approved by the Institutional Review Board. Informed consent was obtained from the parent or the legal guardian, and assent was obtained from the participant, if able, before any data collection was carried out.

6.3.2 Anthropometrics

Height and body mass were assessed while the children were wearing minimal clothing. For typically developing children and children with CP who can independently stand, height was assessed using a stadiometer (Seca 217; Seca GmbH & Co. KG., Hamburg, Germany) to the nearest 0.1 cm. For children with CP who were unable to stand independently, height was assessed using forearm length and a method described by Miller et al. [164]. For all children, body mass was assessed with a digital weight scale to the nearest 0.2 kg (Detecto, 6550; Cardinal Scale, Webb City, MO). Height, body mass and BMI percentile were subsequently determined according to the growth chart published by the Center for Disease Control and Prevention [191].

6.3.3 Sexual Maturity

Sexual maturity was assessed by a physician assistant using the Tanner staging technique [234]. This is a five-point scale ranging from I to V with higher numbers indicating greater sexual maturation. Pubic hair development was assessed in all children. In addition, breast development was assessed in girls, and testicular/penile development was assessed in boys.

6.3.4 Gross Motor Function

Gross motor function in children with CP was assessed by a physician assistant using the GMFCS [19]. This is a scale ranging from I to V with higher number indicating lower motor function. Individuals who have GMFCS I and II still have the ability to walk and run, but at a reduced speed. Individuals who have GMFCS III have the ability to walk only with assistive devices or other people's help. Individuals with GMFCS IV and V will have minimal or lack of independent motor function, and cannot move around without a wheelchair. For the purpose of

this study, participants with GMFCS level I and II were classified ACP, as they can independently ambulate without an assistive device. Participants with GMFCS level III to V were classified as NACP.

6.3.5 Dual-energy X-ray absorptiometry (DXA)

A DXA densitometer (Delphi-W, Hologic; Bedford, MA) and standard imaging and positioning protocols were used to acquire whole body composition measurements. For children with CP, the BodyFix system (Medical Intelligence Inc, Schwabmunchen, Germany) and a modified version of the standard protocol was used to secure them from the waist down to help prevent potential involuntary contractions. The procedure has been shown to have no effect on body composition measurements [166]. Whole body and trunk fat mass and fat free mass (excluding the head) were first determined from the scan. Details about how to determine central adiposity was described elsewhere [13]. Briefly, central adiposity was determined within the abdominal region and based on the manufacturer's instructions. Visceral fat mass was estimated by manually place the region of interest line to include the visceral cavity. Subcutaneous fat region was determined by manually segmenting the abdominal wall muscles and visceral cavity regions out from the abdominal region, and subcutaneous fat mass was subsequently determined. Excellent agreement between DXA and CT measured visceral fat content has been demonstrated in literature [235,236].

To account for the shorter statue that children with CP usually exhibit [227], fat mass index (FMI) and fat-free mass index (FFMI) were calculated for whole body, trunk and FMI for abdominal, subcutaneous and visceral regions using the following equations:

FMI = fat mass (kg) / height^2 (m)

FFMI = fat-free mass (kg) / height^2 (m)

6.3.6 Physical activity

Physical activity was assessed using an accelerometer-based physical activity monitor (Actical; MiniMitter, Sunriver, Oregon) worn around the waist over the hip of the non-dominant hip in typically developing children and of the more affected side in children with CP. The monitors were worn continuously for four full days (day and night; 3 weekdays and 1 weekend day). Four-day recording time was chosen because it provides reliable physical activity data [237]. The averaged physical activity total counts over the four-day period was reported.

6.3.7 Statistical analysis

Data were analyzed using SPSS version 24.0 (IBM Corp, Armonk, NY). Between group differences were determined using an independent t-test if data were normally distributed and a Mann-Whitney test if data were not normally distributed. Subgroup analysis was also carried out to determine whether there is a group difference for physical characteristics, body composition and central adiposity measurements among typically developing children, NACP and ACP using analysis of covariance if data were normally distributed and the Kruskal-Wallis test if data were not normally distributed. A one sample t-test was used to determine whether typically developing controls and children with CP had height, body mass and BMI percentiles different from the 50th percentile. Spearman correlation analysis was performed to determine if there is a relationship between GMFCS and central adiposity measurements in children with CP and Spearman rho values (r_s) are reported. Pearson correlation analysis was performed to determine the relationship between central adiposity measurements and physical activity in typically developing children

and children with CP and correlation coefficient values (*r*) are reported. Cook's D and standardized DfBeta were used to check for data with undue influence on the relationships. If especially influential data points were detected (Cook's D and standardized DfBeta >1), appropriate alternate analyses were planned. Alpha level was set as 0.05. Data were presented as mean ± SD in tables and mean ± SE in figures. To assess the magnitude of effects, Cohen's d (*d*) was calculated whenever applicable, with 0.2, 0.5 and 0.8 representing small, medium and large effect size, respectively [167].

6.4 Results

Physical characteristics data were summarized in **Table 9**. For typically developing children, height, body mass and BMI percentiles were not different from the 50th percentile. For children with CP, height and body mass percentiles were significantly lower from the 50th percentile (both $p < 0.001$). Children with CP had lower height, height percentile and body mass percentile compared to typically developing children ($d = 0.644$, 1.118 and 0.586, respectively; all $p < 0.05$). When children with CP were separated based on ambulatory status, NACP had lower height percentile and body mass percentile compared to typically developing children ($d =$ 1.389 and 0.747, respectively; both $p < 0.05$). ACP had lower height and height percentile compared to typically developing children ($d = 0.755$ and 0.935; both $p < 0.05$). No other differences in physical characteristics between children with CP and typically developing children were detected (all $p > 0.05$). No differences in physical characteristics between ACP and NACP groups were detected (all $p > 0.05$).

Table 9. Physical characteristics of children with cerebral palsy (CP) and typically developing children (Con).

	CP (n = 35)	NACP (n = 15)	ACP (n = 20)	Con (n = 35)
Age (y)	9.5 ± 2.5	10.4 ± 1.8	8.7 ± 2.7	9.5 ± 2.1
Tanner stage (I/II/III/IV/V)				
Pubic hair	23/9/2/0/1	9/4/1/0/1	14/5/1/0/0	26/6/2/1/0
Testicular-penile/breast	25/7/2/1/0	10/4/0/1/0	15/3/2/0/0	19/7/3/1/0
Height (m)	1.28 ± 0.15[a]	1.30 ± 0.14	1.26 ± 0.15[a]	1.37 ± 0.12
Height (%)	23 ± 27[a]	17 ± 24[a]	27 ± 29[a]	54 ± 29
Body mass (kg)	29.4 ± 11.5	30.4 ± 12.4	28.7 ± 11.0	31.1 ± 8.5
Body mass (%)	33 ± 34[a]	28 ± 34[a]	37 ± 34	51 ± 27
BMI (kg/m²)	17.5 ± 4.7	17.5 ± 5.2	17.6 ± 4.4	16.7 ± 2.0
BMI (%)	48 ± 36	44 ± 40	51 ± 34	48 ± 30
GMFCS (I/II/III/IV/V)	10/10/6/1/8	0/0/6/1/8	10/10/0/0/0	_

Values are mean ± SD. BMI = body mass index. % for height, body mass and BMI reflects the percentile relative to age- and sex- based norms; CP = all children with cerebral palsy; NACP = nonambulatory children with cerebral palsy; ACP = ambulatory children with cerebral palsy; Con = typically developing controls; GMFCS = gross motor function classification system
[a]Significant difference compared to typically developing children.

Measures of total and trunk body composition are summarized in **Table 10**. Children with CP had lower total body fat-free mass, higher FMI and lower FFMI compared to typically developing children (d = 0.676, 0.676 and 0.927, respectively; all $p < 0.05$). When separated

based on ambulatory status, NACP had lower total body fat-free mass, higher FMI and lower FFMI compared to typically developing children (d = 0.740, 0.695 and 1.165, respectively; both $p < 0.05$). ACP also had lower total body fat-free mass, higher FMI and lower FFMI compared to typically developing children (d = 0.620, 0.655 and 0.764; all $p < 0.05$). There was no difference in body composition between NACP and ACP (all $p > 0.05$).

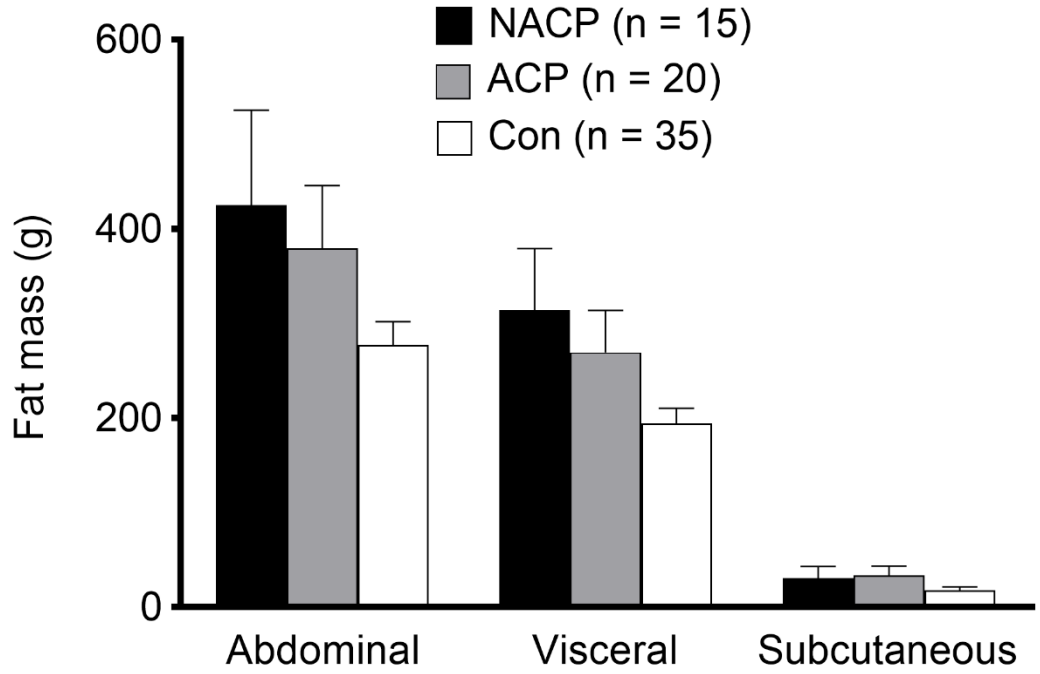
Table 10. Body composition in children with cerebral palsy (CP) and typically developing children (Con).

	CP (n = 35)	NACP (n = 15)	ACP (n = 20)	Con (n = 35)
Mass				
Total body fat (kg)	9.2 ± 5.7	10.1 ± 6.8	8.6 ± 4.8	7.4 ± 4.8
Total body fat-free (kg)	16.5 ± 6.0[a]	16.1 ± 5.9[a]	16.7 ± 6.2[a]	20.4 ± 5.5
Trunk fat (kg)	4.1 ± 3.0	4.7 ± 3.7	3.6 ± 2.3	3.1 ± 1.7
Trunk fat-free (kg)	9.3 ± 3.3	9.7 ± 3.4	9.1 ± 3.2	10.7 ± 2.7
Mass Index				
Total body FMI (kg/m^2)	5.5 ± 2.9[a]	5.7 ± 3.4[a]	5.3 ± 2.5[a]	3.9 ± 1.5
Total body FFMI (kg/m^2)	9.8 ± 2.0[a]	9.3 ± 1.8[a]	10.2 ± 2.0[a]	12.3 ± 3.2
Trunk FMI (kg/m^2)	2.4 ± 1.5	2.7 ± 1.9	2.2 ± 1.3	1.8 ± 1.0
Trunk FFMI (kg/m^2)	5.5 ± 1.0	5.6 ± 1.0	5.5 ± 1.0	5.7 ± 0.6

CP = all children with cerebral palsy; NACP = nonambulatory children with cerebral palsy; ACP = ambulatory children with cerebral palsy; Con = typically developing controls; FMI = fat mass index; FFMI = fat-free mass index.
[a]Significant difference compared to typically developing children. No difference for any of the measurements were found between NACP and ACP.

Children with CP had higher FMI at the abdominal and visceral sites compared to typically developing children (d = 0.641 and 0.754, respectively; both $p < 0.05$). NACP had higher visceral FMI compared to typically developing children (d = 0.776; p = 0.019; **Figure 15B**). ACP had greater abdominal and visceral FMI compared to typically developing children (d = 0.722 and 0.652 respectively; both $p < 0.05$; **Figure 15B**). No differences in any of the central adiposity measurements were detected between NACP and ACP (all $p > 0.05$).

A


NACP (n = 15)
ACP (n = 20)
Con (n = 35)
Fat mass (g)
600
400
200
0
Abdominal
Visceral
Subcutaneous

B

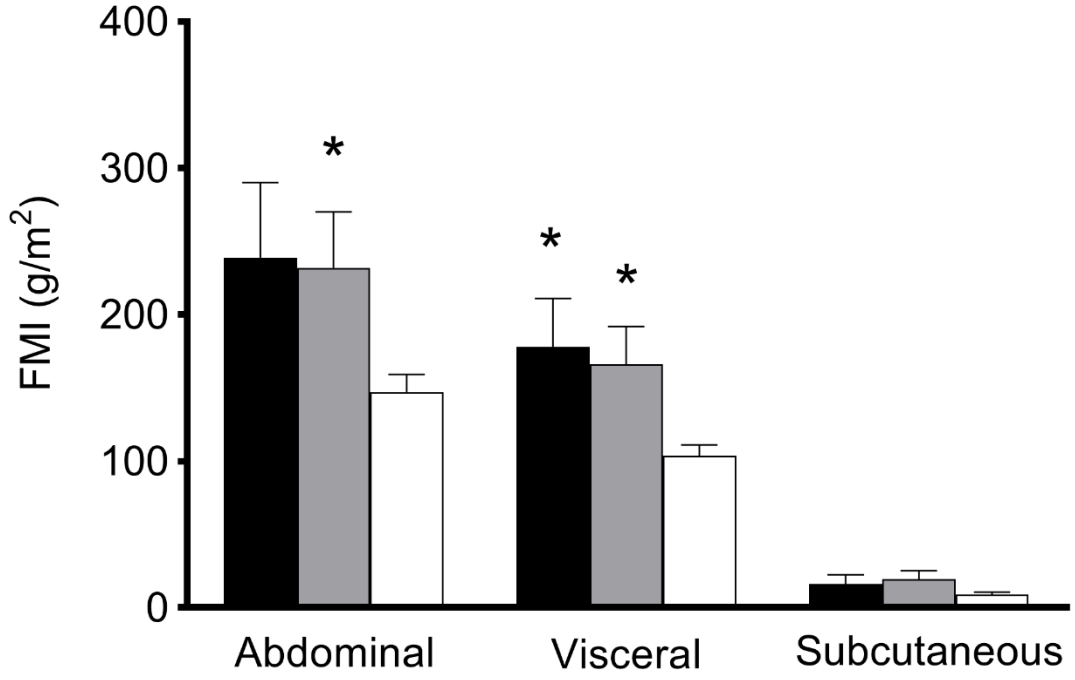

FMI (g/m2)
400
300
200
100
0
*
*
*
Abdominal
Visceral
Subcutaneous

Figure 15. Central adiposity measurements in all children. NACP = nonambulatory children with cerebral palsy; ACP = ambulatory children with cerebral palsy; Con = typically developing children; FMI, fat mass index.
*Significant difference compared to Con. No difference for any of the measurements were found between NACP and ACP. Data are presented as means ± SE

Scatter plots of GMFCS versus abdominal, visceral and subcutaneous fat depots are reported in **Figure 16**. Spearman correlation analysis showed that abdominal and visceral fat mass and indexes were not related to GMFCS (all $p > 0.05$; **Figure 16**) in children with CP. However, subcutaneous FMI was negatively correlated with GMFCS ($r_s = -0.366$, $p = 0.048$; **Figure 16F**). In addition, there was a trend for subcutaneous fat mass to be negatively correlated with GMFCS ($r_s = -0.322$, $p = 0.059$; **Figure 16C**), although it was marginally insignificant.

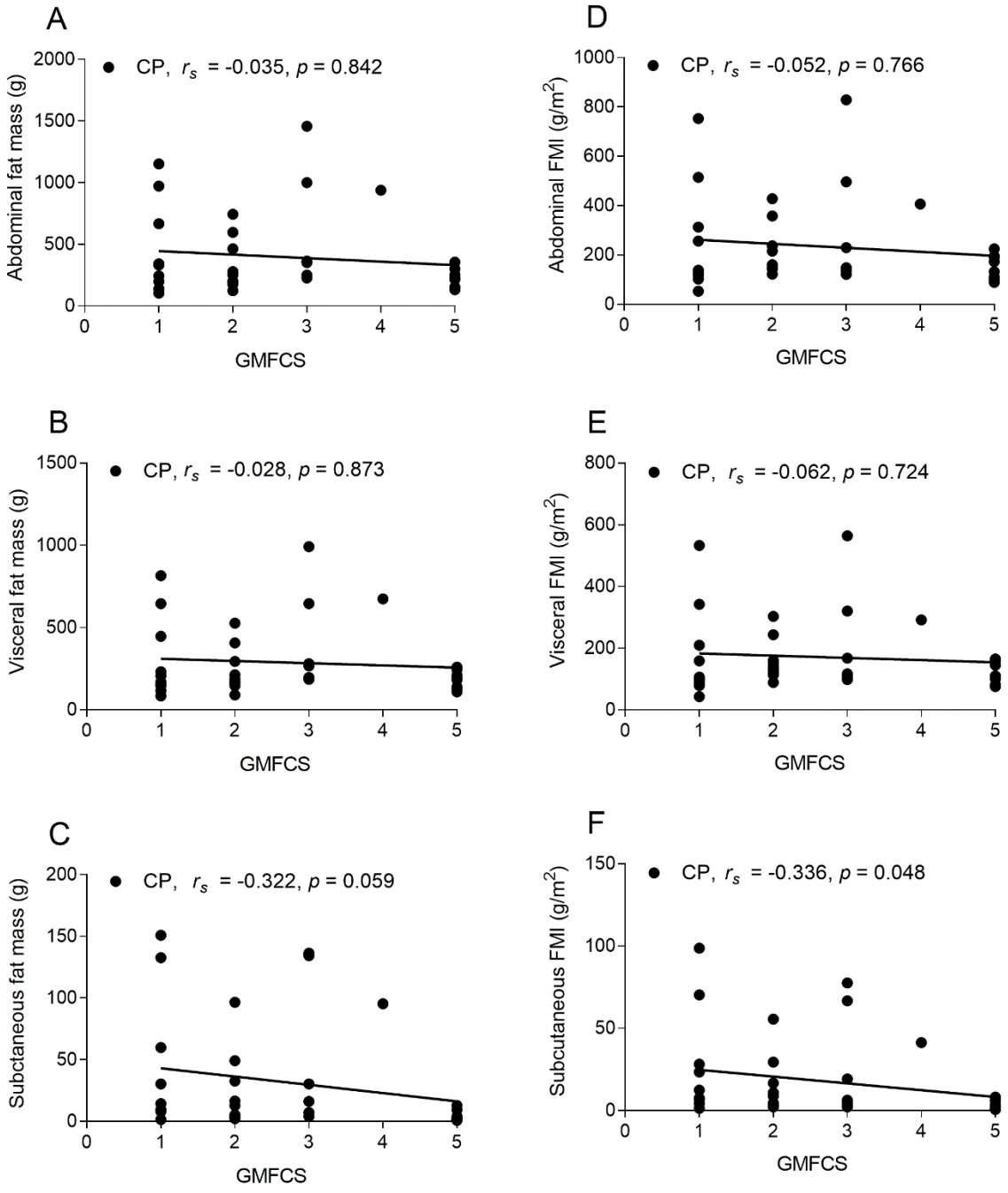


Figure 16. Scatter plots demonstrating a lack of association between central adiposity and gross motor function classification system (GMFCS) in children with cerebral palsy (CP) using Spearman's correlation (r_s). FMI = fat mass index.

Children with CP had 51 % lower physical activity compared to their typically developing peers (d = 1.404; p < 0.001). When separated based on ambulatory status, NACP had 71 % lower and ACP had 36 % lower physical activity than typically developing children (d = 2.038 and 1.057, respectively; both p < 0.05; **Figure 17**). In addition, NACP also had lower physical activity than ACP (d = 1.010; p = 0.017; **Figure 17**). Correlation analysis showed inverse and significant relationships between central adiposity measurements and physical activity (r ranges from - 0.352 to -0.379, all p < 0.05) in typically developing children. However, no significant relationships were observed for children with CP (r ranges from -0.092 to 0.246, all p > 0.05; **Figure 18**).

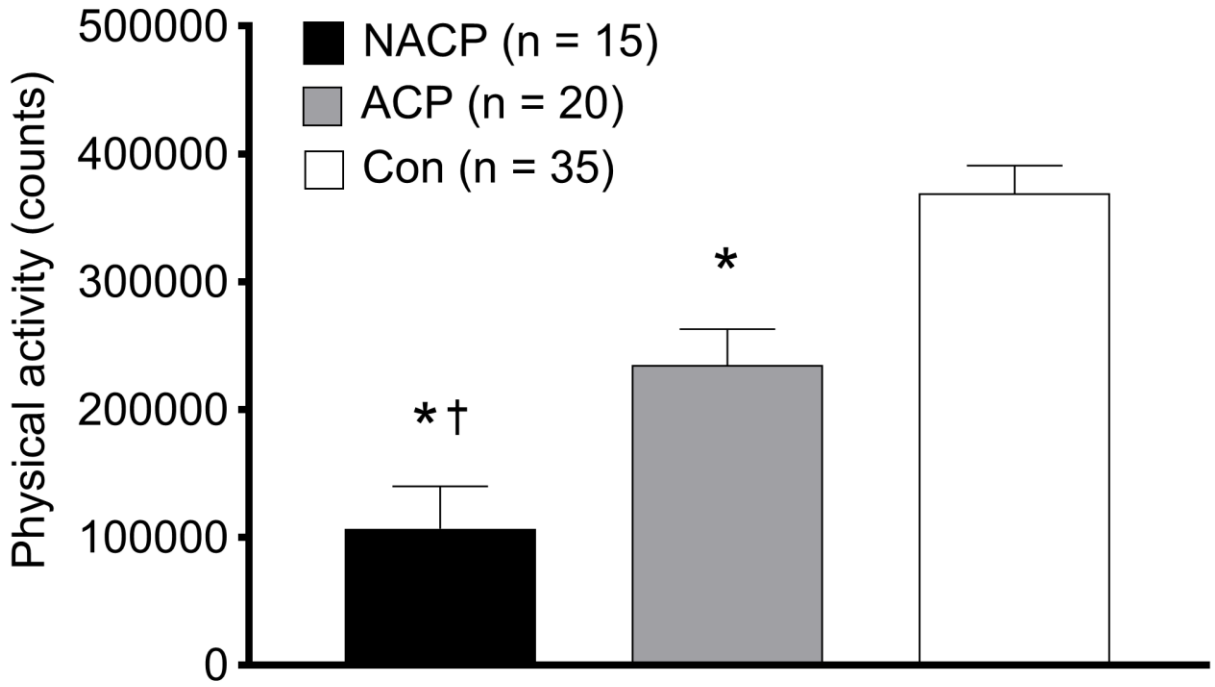


Figure 17. Physical activity measurements in all children. NACP, nonambulatory children with cerebral palsy; ACP, ambulatory children with cerebral palsy; controls, typically developing children.
*Significant difference compared to typically developing children. †Significant difference compared to ACP. Data are presented as mean ± SE.

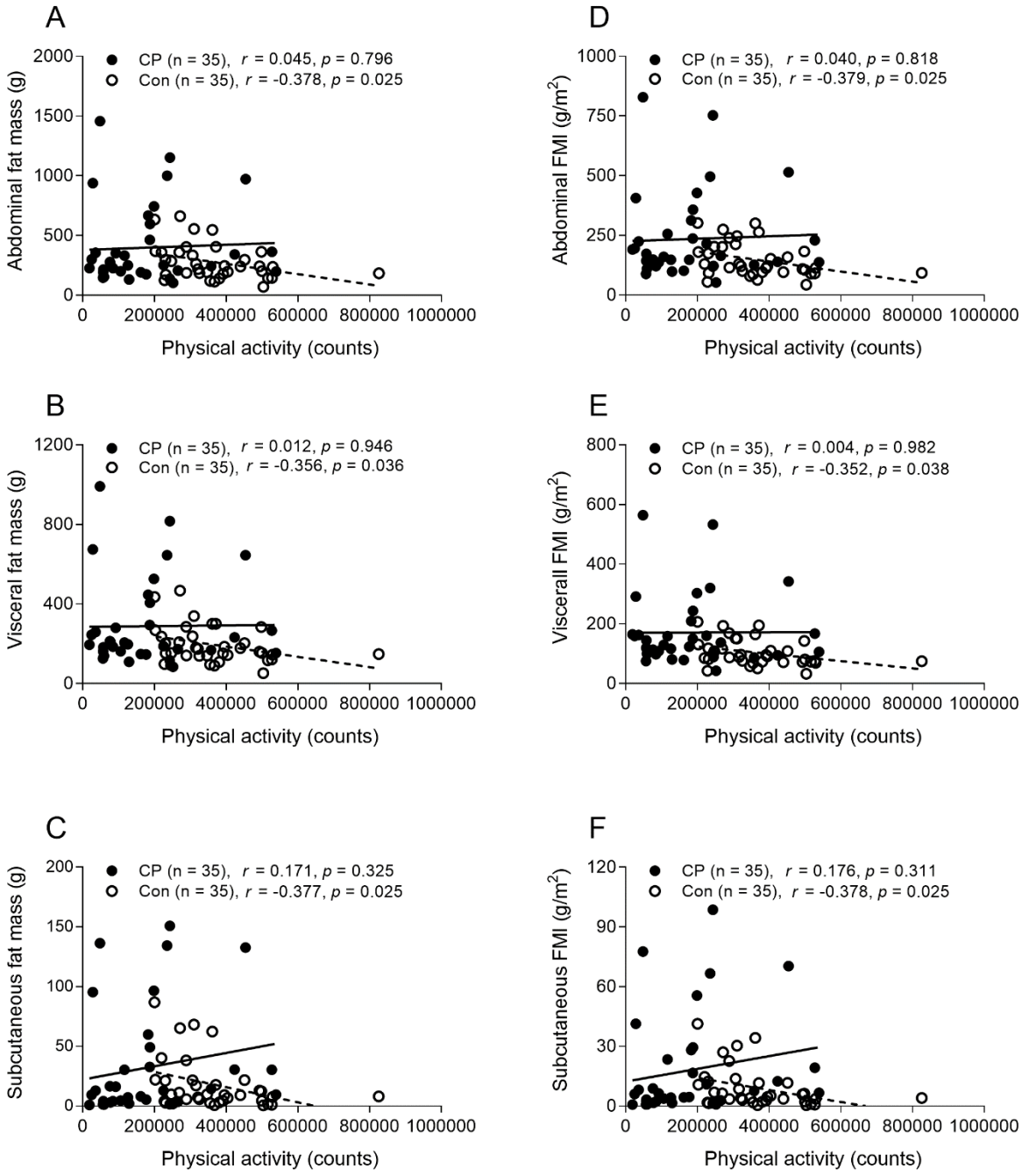


Figure 18. Scatterplots demonstration the relationship between central adiposity measurements and physical activity counts in children with cerebral palsy (CP) and in typically developing children (Con). FMI, fat mass index.
The thick solid lines represent the regression lines for children with CP, and the thin solid lines represent the regression lines for Con.

6.5 Discussion

To our knowledge, this is the first study to assess the level of central adiposity across all levels of gross motor function in children with CP. We found that NACP and ACP had greater visceral fat when compared to their typically developing peers, but the degree of elevation was not different in the two groups. The finding may be clinically important because excess visceral fat is associated with an increased risk of cardiometabolic disease [238-240] and individuals with CP have a higher risk of cardiometabolic disease than the general population [140,175]. We also found that GMFCS and physical activity were not related to abdominal or visceral fat in children with CP; whereas, GMFCS was inversely related to measures of subcutaneous fat. This unique finding suggests that different factors may drive the level of fat in the different depots in children with CP.

The finding that NACP had a greater degree of adiposity in the visceral region when compared to typically developing children is not surprising and is consistent with a recent study that found greater fat accumulation in the abdominal and visceral regions in ACP than in typically developing children [13]. It is also consistent with studies that found elevated abdominal fat in adults with CP [241,242]. However, the finding that the degree of central adiposity was not greater in NACP than ACP is somewhat surprising. It was hypothesized that NACP would have a higher level of visceral adiposity due to their lower functional level and lower participation in physical activity [4] than observed in ACP [3]. Although the expected greater deficit in physical activity in NACP than in ACP was confirmed in the present study (i.e., ~50 % lower), a significant relationship between physical activity and fat in the abdominal and visceral depots was not observed in children with CP. A significant relationship between GMFCS and fat in the abdominal and visceral depots was also not observed. These findings are inconsistent with a

previous study that reported an inverse relationship between disease progression and central adiposity in individuals with chronic obstructive pulmonary disease [243]. It is also inconsistent with another study that reported a negative relationship between physical activity and visceral fat in children at risk of obesity [244]. An inverse relationship between physical activity and intermuscular adipose tissue in the thigh has also been observed in children with NACP [4].

Although GMFCS and physical activity were not significantly related to any measure of abdominal or visceral fat, we found a trend for an inverse relationship between GMFCS and subcutaneous fat in children with CP. This implies that there is less subcutaneous fat accumulation in children with CP who are less functional. The reason for this unusual finding is not clearly understood; however, it may be attributed to the high prevalence of feeding problems in children with more involved forms of CP. In a group of children with CP who were mostly severe and accompanied by a feeding problem, gastrostomy tube feeding intervention was shown to increase subcutaneous fat deposition at various sites [245]. Hence, the lower fat mass at subcutaneous sites in NACP may indicate a need for greater energy intake. However, future studies are needed to draw a definitive conclusion.

It was important to study the level of visceral fat in children with CP because it is relate to cardiometabolic risk. Considerable evidence supports the notion of a greater relationship between cardiometabolic risk and fat in the visceral than in other depots. For example, in a study of 161 Caucasian men with wide range of adiposity, it was found that visceral fat was an independent predictor of metabolic risk factors while no such relationship was discovered for subcutaneous fat [246]. Similarly, visceral fat was reported to be associated with cardiovascular risk factors in Chinese with type 2 diabetes [247]. Importantly, the level of visceral fat deposition seems to affect insulin sensitivity, as pointed out by a study in which obese insulin resistant youth

showed much higher visceral fat mass than those who were obese and insulin sensitive [248]. Therefore, the greater visceral fat seen in children with CP across all levels of gross motor function in the current study implies a greater cardiovascular risk and insulin resistance in children with CP than in the general population of children. This notion is consistent with recent studies showing a much higher multimorbidity prevalence in middle-aged adults with CP [140,175] than in those without CP.

There are some strengths to this study that are worth reviewing. First, the degree of adiposity in children with CP was assessed while adjusting for stature. Children with CP, especially in those with the most functional limitations, are shorter than typically developing children [249]. If the smaller size is not taken into account, the true level of fat and fat-free mass may not be captured and their obesity status misclassified [227]. Second, the inclusion of children with CP across all levels of gross motor function allowed for the study of central adiposity in children across all levels of functionally. Third, considerable effort went into identifying a representative sample of typically developing children for the study. Typically developing children matched children with CP for sex-, age- and race. Moreover, the typically developing children enrolled in this study had height, weight and BMI not different from 50th percentile. Therefore, the group differences that were presented in the current study likely provided an accurate representation of the level of central adiposity in children with CP.

There are limitations to this study also worth reviewing. First, no biochemical markers were measured, so whether the greater central adiposity observed in children with CP will lead to greater cardiovascular disease risks in uncertain and warrants further investigation. Second, physical activity was assessed for four days using an accelerometer-based physical activity monitor. While this method has been shown to be reliable in assessing physical activity [237], it

does not give us information about the physical activity history of the participants, which may be more important for central adiposity accumulation. Future studies should address the effects of habitual physical activity level on central adiposity in children with CP.

6.6 Conclusion

In conclusion, the study findings suggest that NACP and ACP both have high levels of visceral fat when compared to their typically developing peers, but the degree of the elevated visceral fat is not different in the two groups. The finding that gross motor function and physical activity were not related to abdominal and visceral fat in children with CP suggest that other factors contribute to the accumulation of fat in these depots; however, a more comprehensive, longer-term assessment of physical activity is warranted in future studies. The novel finding that subcutaneous fat is inversely related to gross motor function in children with CP also warrants further study.

6.7 Acknowledgement

We thank all the participants and their families. This study was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development (grant numbers HD050530, HD071397 and HD090126) and the University of Georgia Athletic Association. CMM and FM designed the study; CMM and CZ collected and analyzed the data; CZ, CMM and JL wrote the manuscript; all authors read and approved the final version of this manuscript.

CHAPTER 7

CONCLUDING SUMMARY

The overall objective of this dissertation was to evaluate skeletal muscle deficits, the roles of circulatory factors that may potentially contribute to such deficits, as well as the level of central adiposity in children with CP. A brief summary for all the specific aims are below.

Summary of specific aim 1: Using DXA, it was demonstrated that children with CP have a remarkable deficit in FFST that is more pronounced in appendicular than the non-appendicular regions and more pronounced in the lower extremities than the upper extremities. Preliminary models developed in the current study using data from children with CP can provide good estimates of appendicular FFST. However, there is a trend for greater overestimation for children with CP with lower values and an underestimation for children with higher values. Therefore further development of the models to estimate appendicular FFST indexes may be needed.

Future directions: This study only applied simple physical characteristics measurements as predictors to estimate appendicular FFST and indexes. Future studies should look into whether adding other physical characteristics data such as hip circumference, and kinetics data such as grip strength or leg strength will improve the accuracy of model predictability.

Summary of specific aim 2: It was demonstrated that DXA-based statistical models can accurately estimate midleg muscle mass in ambulatory children with spastic CP when the models are developed using data from children with CP. Models developed using data from typically

developing children overestimate leg muscle mass in children with CP due to the lower proportion of muscle in the FFST of children with CP.

Future directions: Future studies are needed to determine whether the models developed in the present study are valid in children with more severe forms of CP and whether the models accurately estimate change that occurs with growth or intervention. Future studies are also needed to develop models to estimate muscle mass at other body regions in children with CP.

Summary of specific aim 3: It was suggested that circulating myostatin, follistatin and IGF-1 are not different between ambulatory children with CP and their typically developing peers, nor were any of the serum markers associated with the curbed muscle development in ambulatory children with CP.

Future directions: This study only assessed the levels of circulatory bioactive agents myostatin, follistatin and IGF-1 in children with CP. Studies examining the role of other bioactive agents in the muscle compromise in children with CP is needed. In addition, more well-designed studies looking at the level of gene-expression for the bioactive agents should be explored.

Summary of specific aim 4: Using DXA, we found that the level of central adiposity was greater in children with CP than in typically developing peers, at both the abdominal and visceral sites, but they were not related to the level of gross motor function. In addition, we found that the level of central adiposity is inversely related to physical activity in typically developing children but not in children with CP.

Future directions: Studies to determine how the elevated central adiposity influences the cardiometabolic health in children with CP are needed. Studies of this nature should also incorporate the assessment of nutritional intake. Visceral fat has also shown to be associated with

inferior bone development in children and young adults, how it contributes to the skeletal fragility in children with CP warrants further investigation,

In conclusion, children with CP have much lower skeletal muscle mass and higher central adiposity compare to their typically developing peers. Strategies that can attenuate the skeletal muscle deficits and reduce the level of central adiposity in children with CP are needed.

References

1. Christensen D, Van Naarden Braun K, Doernberg NS, et al. Prevalence of cerebral palsy, co-occurring autism spectrum disorders, and motor functioning - Autism and Developmental Disabilities Monitoring Network, USA, 2008. *Dev Med Child Neurol.* 2014;56(1):59-65.
2. CDC. Economic costs associated with mental retardation, cerebral palsy, hearing loss, and vision impairment--United States, 2003. *MMWR Morb Mortal Wkly Rep.* 2004;53(3):57-59.
3. Whitney DG, Singh H, Miller F, et al. Cortical bone deficit and fat infiltration of bone marrow and skeletal muscle in ambulatory children with mild spastic cerebral palsy. *Bone.* 2017;94:90-97.
4. Johnson DL, Miller F, Subramanian P, Modlesky CM. Adipose tissue infiltration of skeletal muscle in children with cerebral palsy. *J Pediatr.* 2009;154(5):715-720.
5. Modlesky CM, Lewis RD. Does exercise during growth have a long-term effect on bone health? *Exerc Sport Sci Rev.* 2002;30(4):171-176.
6. Bauman W, Spungen A. Body composition in aging: adverse changes in able-bodied persons and in those with spinal cord injury. *Top Spinal Cord Inj Rehabil.* 2001;6(3):22-36.
7. Spungen A, Adkins R, Bauman W, Kemp B, Waters R. The effect of level and com pleteness of lesion on body composition in subjects with spinal cord injury. *J Spinal Cord Med.* 1999;22:43.

8. Zhang C, Whitney DG, Singh H, et al. Statistical models to assess leg muscle mass in ambulatory children with spastic cerebral palsy using dual-energy X-ray absorptiometry. *J Clin Densitom.* 2018.

9. Noble JJ, Fry NR, Lewis AP, Keevil SF, Gough M, Shortland AP. Lower limb muscle volumes in bilateral spastic cerebral palsy. *Brain Dev-Jpn.* 2014;36(4):294-300.

10. Lampe R, Grassl S, Mitternacht J, Gerdesmeyer L, Gradinger R. MRT-measurements of muscle volumes of the lower extremities of youths with spastic hemiplegia caused by cerebral palsy. *Brain Dev.* 2006;28(8):500-506.

11. Modlesky CM, Cavaiola ML, Smith JJ, Rowe DA, Johnson DL, Miller F. A DXA-based mathematical model predicts midthigh muscle mass from magnetic resonance imaging in typically developing children but not in those with quadriplegic cerebral palsy. *J Nutr.* 2010;140(12):2260-2265.

12. Gough M, Shortland AP. Could muscle deformity in children with spastic cerebral palsy be related to an impairment of muscle growth and altered adaptation? *Dev Med Child Neurol.* 2012;54(6):495-499.

13. Whitney DG, Singh H, Zhang C, Miller F, Modlesky CM. Greater visceral fat but no difference in measures of total body fat in ambulatory children with spastic cerebral palsy compared to typically developing children. *J Clin Densitom.* 2018:In press.

14. Mahabadi AA, Massaro JM, Rosito GA, et al. Association of pericardial fat, intrathoracic fat, and visceral abdominal fat with cardiovascular disease burden: the Framingham Heart Study. *Eur Heart J.* 2009;30(7):850-856.

15. Bax M, Goldstein M, Rosenbaum P, Leviton A, Paneth N. Proposed definition and classification of cerebral palsy, April 2005. *Dev Med Child Neurol.* 2005;47(8):571-576.

16. Howard J, Soo B, Graham HK, et al. Cerebral palsy in Victoria: Motor types, topography and gross motor function. *J Paediatr Child H.* 2005;41(9-10):479-483.

17. Hollung SJ, Vik T, Wiik R, Bakken IJ, Andersen GL. Completeness and correctness of cerebral palsy diagnoses in two health registers: implications for estimating prevalence. *Dev Med Child Neurol.* 2017;59(4):402-406.

18. Himmelmann K, Beckung E, Hagberg G, Uvebrant P. Gross and fine motor function and accompanying impairments in cerebral palsy. *Dev Med Child Neurol.* 2006;48(6):417-423.

19. Wood E, Rosenbaum P. The gross motor function classification system for cerebral palsy: a study of reliability and stability over time. *Dev Med Child Neurol.* 2000;42(5):292-296.

20. Bodkin AW, Robinson C, Perales FP. Reliability and validity of the gross motor function classification system for cerebral palsy. *Pediatr Phys Ther.* 2003;15(4):247-252.

21. Rogozinski BM, Davids JR, Davis RB, et al. Prevalence of obesity in ambulatory children with cerebral palsy. *J Bone Joint Surg Am.* 2007;89a(11):2421-2426.

22. Sullivan E, Barnes D, Linton JL, et al. Relationships among functional outcome measures used for assessing children with ambulatory CP. *Dev Med Child Neurol.* 2007;49(5):338-344.

23. Whitney D, Miller F, Pohlig R, Modlesky C. BMI does not capture the high fat mass index and low fat-free mass index in children with cerebral palsy and proposed statistical models that improve this accuracy. *Int J Obesity.* 2018;In press.

24. Eliasson AC, Krumlinde-Sundholm L, Rosblad B, et al. The Manual Ability Classification System (MACS) for children with cerebral palsy: scale development and evidence of validity and reliability. *Dev Med Child Neurol.* 2006;48(7):549-554.

25. Morris C, Kurinczuk JJ, Fitzpatrick R, Rosenbaum PL. Reliability of the manual ability classification system for children with cerebral palsy. *Dev Med Child Neurol.* 2006;48(12):950-953.

26. Carnahan KD, Arner M, Hagglund G. Association between gross motor function (GMFCS) and manual ability (MACS) in children with cerebral palsy. A population-based study of 359 children. *BMC Musculoskelet Disord.* 2007;8.

27. Gunel MK, Mutlu A, Tarsuslu T, Livanelioglu A. Relationship among the Manual Ability Classification System (MACS), the Gross Motor Function Classification System (GMFCS), and the functional status (WeeFIM) in children with spastic cerebral palsy. *Eur J Pediatr.* 2009;168(4):477-485.

28. Morris C, Kurinczuk JJ, Fitzpatrick R, Rosenbaum PL. Do the abilities of children with cerebral palsy explain their activities and participation? *Dev Med Child Neurol.* 2006;48(12):954-961.

29. Bentzinger CF, Wang YX, Rudnicki MA. Building Muscle: Molecular Regulation of Myogenesis. *Csh Perspect Biol.* 2012;4(2).

30. Le Grand F, Rudnicki MA. Skeletal muscle satellite cells and adult myogenesis. *Curr Opin Cell Biol.* 2007;19(6):628-633.

31. Machida S, Booth FW. Insulin-like growth factor 1 and muscle growth: implication for satellite cell proliferation. *P Nutr Soc.* 2004;63(2):337-340.

32. Lance JW. Symposium synopsis. *Spasticity: disordered motor control.* 1980:487-489.

33. Young RR. Spasticity - a Review. *Neurology.* 1994;44(11):12-20.

34. Sanger TD, Delgado MR, Gaebler-Spira D, Hallett M, Mink JW, Disorde TFCM. Classification and definition of disorders causing hypertonia in childhood. *Pediatrics.* 2003;111(1).

35. Pandyan AD, Gregoric M, Barnes MP, et al. Spasticity: Clinical perceptions, neurological realities and meaningful measurement. *Disabil Rehabil.* 2005;27(1-2):2-6.

36. Malhotra S, Pandyan AD, Day CR, Jones PW, Hermens H. Spasticity, an impairment that is poorly defined and poorly measured. *Clin Rehabil.* 2009;23(7):651-658.

37. Wissel J, Verrier M, Simpson DM, et al. Post-stroke Spasticity: Predictors of Early Development and Considerations for Therapeutic Intervention. *Pm&R.* 2015;7(1):60-67.

38. Delp SL. What causes increased muscle stiffness in cerebral palsy? *Muscle & Nerve.* 2003;27(2):131-132.

39. Castera L, Forns X, Alberti A. Non-invasive evaluation of liver fibrosis using transient elastography. *J Hepatol.* 2008;48(5):835-847.

40. Kwon DR, Park GY, Lee SU, Chung I. Spastic cerebral palsy in children: dynamic sonoelastographic findings of medial gastrocnemius. *Radiology.* 2012;263(3):794-801.

41. Brandenburg JE, Eby SF, Song PF, et al. Quantifying passive muscle stiffness in children with and without cerebral palsy using ultrasound shear wave elastography. *Dev Med Child Neurol.* 2016;58(12):1288-1294.

42. Smith LR, Lee KS, Ward SR, Chambers HG, Lieber RL. Hamstring contractures in children with spastic cerebral palsy result from a stiffer extracellular matrix and increased in vivo sarcomere length. *J Physiol.* 2011;589(Pt 10):2625-2639.

43. Wiley ME, Damiano DL. Lower-extremity strength profiles in spastic cerebral palsy. *Dev Med Child Neurol.* 1998;40(2):100-107.

44. Elder GCB, Kirk J, Stewart G, et al. Contributing factors to muscle weakness in children with cerebral palsy. *Dev Med Child Neurol.* 2003;45(8):542-550.

45. Brouwer B, Wheeldon RK, Stradiotto-Parker N, Allum J. Reflex excitability and isometric force production in cerebral palsy: the effect of serial casting. *Dev Med Child Neurol.* 1998;40(3):168-175.

46. Engsberg JR, Ross SA, Olree KS, Park TS. Ankle spasticity and strength in children with spastic diplegic cerebral palsy. *Dev Med Child Neurol.* 2000;42(1):42-47.

47. Damiano DL, Abel MF. Functional outcomes of strength training in spastic cerebral palsy. *Arch Phys Med Rehabil.* 1998;79(2):119-125.

48. Dodd KJ, Taylor NF, Damiano DL. A systematic review of the effectiveness of strength-training programs for people with cerebral palsy. *Arch Phys Med Rehabil.* 2002;83(8):1157-1164.

49. McNee AE, Gough M, Morrissey MC, Shortland AP. Increases in muscle volume after plantarflexor strength training in children with spastic cerebral palsy. *Dev Med Child Neurol.* 2009;51(6):429-435.

50. Eek MN, Tranberg R, Zugner R, Alkema K, Beckung E. Muscle strength training to improve gait function in children with cerebral palsy. *Dev Med Child Neurol.* 2008;50(10):759-764.

51. Stackhouse SK, Binder-Macleod SA, Stackhouse CA, McCarthy JJ, Prosser LA, Lee SCK. Neuromuscular electrical stimulation versus volitional isometric strength training in

children with spastic Diplegic cerebral palsy: A preliminary study. *Neurorehab Neural Re.* 2007;21(6):475-485.

52. El-Shamy SM. Effect of whole-body vibration on muscle strength and balance in diplegic cerebral palsy: a randomized controlled trial. *Am J Phys Med Rehabil.* 2014;93(2):114-121.

53. Shortland AP, Harris CA, Gough M, Robinson RO. Architecture of the medial gastrocnemius in children with spastic diplegia. *Dev Med Child Neurol.* 2002;44(3):158-163.

54. Malaiya R, McNee AE, Fry NR, Eve LC, Gough M, Shortland AP. The morphology of the medial gastrocnemius in typically developing children and children with spastic hemiplegic cerebral palsy. *J Electromyogr Kinesiol.* 2007;17(6):657-663.

55. Mohagheghi AA, Khan T, Meadows TH, Giannikas K, Baltzopoulos V, Maganaris CN. In vivo gastrocnemius muscle fascicle length in children with and without diplegic cerebral palsy. *Dev Med Child Neurol.* 2008;50(1):44-50.

56. Mohagheghi AA, Khan T, Meadows TH, Giannikas K, Baltzopoulos V, Maganaris CN. Differences in gastrocnemius muscle architecture between the paretic and non-paretic legs in children with hemiplegic cerebral palsy. *Clin Biomech.* 2007;22(6):718-724.

57. Barber L, Hastings-Ison T, Baker R, Barrett R, Lichtwark G. Medial gastrocnemius muscle volume and fascicle length in children aged 2 to 5 years with cerebral palsy. *Dev Med Child Neurol.* 2011;53(6):543-548.

58. Moreau NG, Teefey SA, Damiano DL. In vivo muscle architecture and size of the rectus femoris and vastus lateralis in children and adolescents with cerebral palsy. *Dev Med Child Neurol.* 2009;51(10):800-806.

59. Gao F, Zhao H, Gaebler-Spira D, Zhang LQ. In Vivo Evaluations of Morphologic Changes of Gastrocnemius Muscle Fascicles and Achilles Tendon in Children with Cerebral Palsy. *Am J Phys Med Rehab.* 2011;90(5):364-371.

60. Mathewson MA, Ward SR, Chambers HG, Lieber RL. High Resolution Muscle Measurements Provide Insights into Equinus Contractures in Patients with Cerebral Palsy. *J Orthop Res.* 2015;33(1):33-39.

61. Fukunaga T, Roy RR, Shellock FG, et al. Physiological cross-sectional area of human leg muscles based on magnetic resonance imaging. *J Orthop Res.* 1992;10(6):928-934.

62. Fukunaga T, Roy RR, Shellock FG, Hodgson JA, Edgerton VR. Specific tension of human plantar flexors and dorsiflexors. *J Appl Physiol.* 1996;80(1):158-165.

63. Fukunaga T, Miyatani M, Tachi M, Kouzaki M, Kawakami Y, Kanehisa H. Muscle volume is a major determinant of joint torque in humans. *Acta Physiol Scand.* 2001;172(4):249-255.

64. Ohata K, Tsuboyama T, Haruta T, Ichihashi N, Kato T, Nakamura T. Relation between muscle thickness, spasticity, and activity limitations in children and adolescents with cerebral palsy. *Dev Med Child Neurol.* 2008;50(2):152-156.

65. Bandholm T, Magnusson P, Jensen BR, Sonne-Holm S. Dorsiflexor muscle-group thickness in children with cerebral palsy: Relation to cross-sectional area. *Neurorehabilitation.* 2009;24(4):299-306.

66. Moreau NG, Falvo MJ, Damiano DL. Rapid force generation is impaired in cerebral palsy and is related to decreased muscle size and functional mobility. *Gait Posture.* 2012;35(1):154-158.

67. Barber L, Barrett R, Lichtwark G. Passive muscle mechanical properties of the medial gastrocnemius in young adults with spastic cerebral palsy. *J Biomech.* 2011;44(13):2496-2500.

68. Fry NR, Gough M, McNee AE, Shortland AP. Changes in the Volume and Length of the Medial Gastrocnemius After Surgical Recession in Children With Spastic Diplegic Cerebral Palsy. *J Pediatr Orthoped.* 2007;27(7):769-774.

69. Oberhofer K, Stott NS, Mithraratne K, Anderson IA. Subject-specific modelling of lower limb muscles in children with cerebral palsy. *Clin Biomech.* 2010;25(1):88-94.

70. Pitcher CA, Elliott CM, Valentine JP, et al. Muscle morphology of the lower leg in ambulant children with spastic cerebral palsy. *Muscle & Nerve.* 2018;58(6):818-823.

71. Florini JR, Ewton DZ, Coolican SA. Growth hormone and the insulin-like growth factor system in myogenesis. *Endocr Rev.* 1996;17(5):481-517.

72. Cuneo RC, Salomon F, Wiles CM, Sonksen PH. Skeletal-Muscle Performance in Adults with Growth-Hormone Deficiency. *Horm Res.* 1990;33:55-60.

73. Deboer H, Blok GJ, Voerman HJ, Devries PMJM, Vanderveen EA. Body-Composition in Adult Growth Hormone-Deficient Men, Assessed by Anthropometry and Bioimpedance Analysis. *J Clin Endocr Metab.* 1992;75(3):833-837.

74. Welle S TC, Statt MA, McHenry B. Growth hormone increases muscle mass and strength but does not rejuvenate myofibrillar protein synthesis in healthy subjects over 60 years old. *J Clin Endocrinol Metab.* 1996;81(9):3239-3243.

75. Cuneo RC, Salomon F, Wiles CM, Hesp R, Sonksen PH. Growth-Hormone Treatment in Growth Hormone-Deficient Adults .1. Effects on Muscle Mass and Strength. *J Appl Physiol.* 1991;70(2):688-694.

76. Garibotto G, Barreca A, Russo R, et al. Effects of recombinant human growth hormone on muscle protein turnover in malnourished hemodialysis patients. *J Clin Invest.* 1997;99(1):97-105.

77. Lundeberg S, Belfrage M, Wernerman J, Vonderdecken A, Thunell S, Vinnars E. Growth-Hormone Improves Muscle Protein-Metabolism and Whole-Body Nitrogen Economy in Man during a Hyponitrogenous Diet. *Metabolism.* 1991;40(3):315-322.

78. Keenan BS, Killmer LB, Sode J. Growth-Hormone Response to Exercise - Test of Pituitary Function in Children. *Pediatrics.* 1972;50(5):760-&.

79. Cappa M, Bizzarri C, Martinez C, et al. Neuroregulation of growth hormone during exercise in children. *Int J Sports Med.* 2000;21:S125-S128.

80. Lacey KA, Hewison A, Parkin JM. Exercise as a Screening-Test for Growth-Hormone Deficiency in Children. *Arch Dis Child.* 1973;48(7):508-512.

81. Coniglio SJ, Stevenson RD, Rogol AD. Apparent growth hormone deficiency in children with cerebral palsy. *Dev Med Child Neurol.* 1996;38(9):797-804.

82. Coniglio SJ, Stevenson RD. Growth hormone deficiency in two children with cerebral palsy. *Dev Med Child Neurol.* 1995;37(11):1013-1015.

83. Devesa J, Casteleiro N, Rodicio C, Lopez N, Reimunde P. Growth hormone deficiency and cerebral palsy. *Ther Clin Risk Manag.* 2010;6:413-418.

84. Hamza RT, Ismail MA, Hamed AI. Growth hormone deficiency in children and adolescents with cerebral palsy: relation to gross motor function and degree of spasticity. *Pak J Biol Sci.* 2011;14(7):433-440.

85. Kuperminc MN, Gurka MJ, Houlihan CM, et al. Puberty, statural growth, and growth hormone release in children with cerebral palsy. *J Pediatr Rehabil Med.* 2009;2(2):131-141.

86. Schultz E, Mccormick KM. Skeletal-Muscle Satellite Cells. *Rev Physiol Bioch P.* 1994;123:213-257.

87. Singleton JR, Feldman EL. Insulin-like growth factor-I in muscle metabolism and myotherapies. *Neurobiol Dis.* 2001;8(4):541-554.

88. Philippou A, Maridaki M, Halapas A, Koutsilieris M. The role of the insulin-like growth factor 1 (IGF-1) in skeletal muscle physiology. *In Vivo.* 2007;21(1):45-54.

89. Coolican SA, Samuel DS, Ewton DZ, McWade FJ, Florini JR. The mitogenic and myogenic actions of insulin-like growth factors utilize distinct signaling pathways. *J Biol Chem.* 1997;272(10):6653-6662.

90. Song YH, Li YX, Du J, Mitch WE, Rosenthal N, Delafontaine P. Muscle-specific expression of IGF-1 blocks angiotensin II-induced skeletal muscle wasting. *J Clin Invest.* 2005;115(2):451-458.

91. Pelosi L, Giacinti C, Nardis C, et al. Local expression of IGF-1 accelerates muscle regeneration by rapidly modulating inflammatory cytokines and chemokines. *Faseb J.* 2007;21(7):1393-1402.

92. Herndon DN, Ramzy PI, DebRoy MA, et al. Muscle protein catabolism after severe burn: Effects of IGF-1/IGFBP-3 treatment. *Ann Surg.* 1999;229(5):713-722.

93. Nazif H, Shatla R, Elsayed R, et al. Bone mineral density and insulin-like growth factor-1 in children with spastic cerebral palsy. *Child Nerv Syst.* 2017;33(4):625-630.

94. Ali O, Shim M, Fowler E, Cohen P, Oppenheim W. Spinal bone mineral density, IGF-1 and IGFBP-3 in children with cerebral palsy. *Horm Res.* 2007;68(6):316-320.

95. McPherron AC, Lawler AM, Lee SJ. Regulation of skeletal muscle mass in mice by a new TGF-beta superfamily member. *Nature.* 1997;387(6628):83-90.

96. McPherron AC, Lee SJ. Double muscling in cattle due to mutations in the myostatin gene. *P Natl Acad Sci USA.* 1997;94(23):12457-12461.

97. Grobet L, Martin LJR, Poncelet D, et al. A deletion in the bovine myostatin gene causes the double-muscled phenotype in cattle. *Nat Genet.* 1997;17(1):71-74.

98. Kambadur R, Sharma M, Smith TPL, Bass JJ. Mutations in myostatin (GDF8) in double-muscled Belgian blue and Piedmontese cattle. *Genome Res.* 1997;7(9):910-916.

99. Hu SW, Ni W, Sai WJF, et al. Knockdown of Myostatin Expression by RNAi Enhances Muscle Growth in Transgenic Sheep. *Plos One.* 2013;8(3).

100. Mosher DS, Quignon P, Bustamante CD, et al. A mutation in the myostatin gene increases muscle mass and enhances racing performance in heterozygote dogs. *Plos Genet.* 2007;3(5):779-786.

101. Lin J, Arnold HB, Della-Fera MA, Azain MJ, Hartzell DL, Baile CA. Myostatin knockout in mice increases myogenesis and decreases adipogenesis. *Biochem Bioph Res Co.* 2002;291(3):701-706.

102. Hamrick MW. Increased bone mineral density in the femora of GDF8 knockout mice. *Anat Rec Part A.* 2003;272a(1):388-391.

103. Whittemore LA, Song KN, Li XP, et al. Inhibition of myostatin in adult mice increases skeletal muscle mass and strength. *Biochem Bioph Res Co.* 2003;300(4):965-971.

104. Zimmers TA, Davies MV, Koniaris LG, et al. Induction of cachexia in mice by systemically administered myostatin. *Science.* 2002;296(5572):1486-1488.

105. Elkina Y, von Haehling S, Anker SD, Springer J. The role of myostatin in muscle wasting: an overview. *J Cachexia Sarcopenia Muscle.* 2011;2(3):143-151.

106. Uhlenberg B, Lucke B, Schuelke M. Myostatin mutation associated with gross muscle hypertrophy in a child - Reply. *New Engl J Med.* 2004;351(10):1030-1031.

107. Yarasheski KE, Bhasin S, Sinha-Hikim I, Pak-Loduca J, Gonzalez-Cadavid NF. Serum myostatin-immunoreactive protein is increased in 60-92 year old women and men with muscle wasting. *J Nutr Health Aging.* 2002;6(5):343-348.

108. Ju CR, Chen RC. Serum myostatin levels and skeletal muscle wasting in chronic obstructive pulmonary disease. *Resp Med.* 2012;106(1):102-108.

109. Loumaye A, de Barsy M, Nachit M, et al. Role of Activin A and Myostatin in Human Cancer Cachexia. *J Clin Endocr Metab.* 2015;100(5):2030-2038.

110. Furihata T, Kinugawa S, Fukushima A, et al. Serum myostatin levels are independently associated with skeletal muscle wasting in patients with heart failure. *Int J Cardiol.* 2016;220:483-487.

111. Louis E, Raue U, Yang YF, Jemiolo B, Trappe S. Time course of proteolytic, cytokine, and myostatin gene expression after acute exercise in human skeletal muscle. *J Appl Physiol.* 2007;103(5):1744-1751.

112. Hjorth M, Pourteymour S, Gorgens SW, et al. Myostatin in relation to physical activity and dysglycaemia and its effect on energy metabolism in human skeletal muscle cells. *Acta Physiol.* 2016;217(1):45-60.

113. Lenk K, Erbs S, Hollriegel R, et al. Exercise training leads to a reduction of elevated myostatin levels in patients with chronic heart failure. *Eur J Prev Cardiol.* 2012;19(3):404-411.

114. Hittel DS, Axelson M, Sarna N, Shearer J, Huffman KM, Kraus WE. Myostatin Decreases with Aerobic Exercise and Associates with Insulin Resistance. *Med Sci Sport Exer.* 2010;42(11):2023-2029.

115. Smith LR, Ponten E, Hedstrom Y, et al. Novel transcriptional profile in wrist muscles from cerebral palsy patients. *Bmc Med Genomics.* 2009;2(44).

116. Lee SJ, Lee YS, Zimmers TA, et al. Regulation of Muscle Mass by Follistatin and Activins. *Mol Endocrinol.* 2010;24(10):1998-2008.

117. Rose FF, Mattis VB, Rindt H, Lorson CL. Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy. *Hum Mol Genet.* 2009;18(6):997-1005.

118. Zhu JH, Li Y, Lu AP, et al. Follistatin Improves Skeletal Muscle Healing after Injury and Disease through an Interaction with Muscle Regeneration, Angiogenesis, and Fibrosis. *Am J Pathol.* 2011;179(2):915-930.

119. Lee SJ. Quadrupling Muscle Mass in Mice by Targeting TGF-beta Signaling Pathways. *Plos One.* 2007;2(8).

120. David V, Martin A, Lafage-Proust MH, et al. Mechanical loading down-regulates peroxisome proliferator-activated receptor gamma in bone marrow stromal cells and favors osteoblastogenesis at the expense of adipogenesis. *Endocrinology.* 2007;148(5):2553-2562.

121. Modlesky CM, Subramanian P, Miller F. Underdeveloped trabecular bone microarchitecture is detected in children with cerebral palsy using high-resolution magnetic resonance imaging. *Osteoporosis Int.* 2008;19(2):169-176.

122. Modlesky CM, Whitney DG, Singh H, Barbe MF, Kirby JT, Miller F. Underdevelopment of trabecular bone microarchitecture in the distal femur of nonambulatory children with cerebral palsy becomes more pronounced with distance from the growth plate. *Osteoporosis Int.* 2015;26(2):505-512.

123. Henderson RC, Lark RK, Gurka MJ, et al. Bone density and metabolism in children and adolescents with moderate to severe cerebral palsy. *Pediatrics.* 2002;110(1).

124. Gunsilius E, Gastl G, Petzer AL. Hematopoietic stem cells. *Biomed Pharmacother.* 2001;55(4):186-194.

125. Samuel VT, Shulman GI. Mechanisms for Insulin Resistance: Common Threads and Missing Links. *Cell.* 2012;148(5):852-871.

126. Hsieh SD, Yoshinaga H, Muto T. Waist-to-height ratio, a simple and practical index for assessing central fat distribution and metabolic risk in Japanese men and women. *Int J Obesity.* 2003;27(5):610-616.

127. Goodpaster BH, Krishnaswami S, Harris TB, et al. Obesity, regional body fat distribution, and the metabolic syndrome in older men and women. *Archives of Internal Medicine.* 2005;165(7):777-783.

128. Botton J, Heude B, Kettaneh A, et al. Cardiovascular risk factor levels and their relationships with overweight and fat distribution in children: The Fleurbaix Laventie Ville Sante II study. *Metabolism.* 2007;56(5):614-622.

129. Ibrahim MM. Subcutaneous and visceral adipose tissue: structural and functional differences. *Obesity Reviews.* 2010;11(1):11-18.

130. Item F, Konrad D. Visceral fat and metabolic inflammation: the portal theory revisited. *Obesity Reviews.* 2012;13:30-39.

131. Ebbert JO, Jensen MD. Fat Depots, Free Fatty Acids, and Dyslipidemia. *Nutrients.* 2013;5(2):498-508.

132. Bergman RN, Kim SP, Catalano KJ, et al. Why visceral fat is bad: Mechanisms of the metabolic syndrome. *Obesity.* 2006;14:16s-19s.

133. Banerji MA, Faridi N, Atluri R, Chaiken RL, Lebovitz HE. Body composition, visceral fat, leptin, and insulin resistance in Asian Indian men. *J Clin Endocr Metab.* 1999;84(1):137-144.

134. Gastaldelli A, Cusi K, Pettiti M, et al. Relationship between hepatic/visceral fat and hepatic insulin resistance in nondiabetic and type 2 diabetic subjects. *Gastroenterology.* 2007;133(2):496-506.

135. Hayashi T, Boyko EJ, McNeely MJ, Leonetti DL, Kahn SE, Fujimoto WY. Visceral adiposity, not abdominal subcutaneous fat area, predicts increased future insulin resistance in Japanese Americans. *Diabetes.* 2006;55:A69-A70.

136. Lord J, Thomas R, Fox B, Acharya U, Wilkin T. The central issue? Visceral fat mass is a good marker of insulin resistance and metabolic disturbance in women with polycystic ovary syndrome. *Bjog-Int J Obstet Gy.* 2006;113(10):1203-1209.

137. Bjornson KF, Belza B, Kartin D, Logsdon R, McLaughlin JF. Ambulatory physical activity performance in youth with cerebral palsy and youth who are developing typically. *Phys Ther.* 2007;87(3):248-257.

138. Seidell JC, Cigolini M, Deslypere JP, Charzewska J, Ellsinger BM, Cruz A. Body-Fat Distribution in Relation to Physical-Activity and Smoking-Habits in 38-Year-Old European Men - the European Fat Distribution Study. *Am J Epidemiol.* 1991;133(3):257-265.

139. Troisi RJ, Heinold JW, Vokonas PS, Weiss ST. Cigarette-Smoking, Dietary-Intake, and Physical-Activity - Effects on Body-Fat Distribution - the Normative Aging Study. *Am J Clin Nutr.* 1991;53(5):1104-1111.

140. Cremer N, Hurvitz EA, Peterson MD. Multimorbidity in Middle-Aged Adults with Cerebral Palsy. *Am J Med.* 2017;130(6).

141. Eckardt K, Taube A, Eckel J. Obesity-associated insulin resistance in skeletal muscle: Role of lipid accumulation and physical inactivity. *Rev Endocr Metab Dis.* 2011;12(3):163-172.

142. Jacob S, Machann J, Rett K, et al. Association of increased intramyocellular lipid content with insulin resistance in lean nondiabetic offspring of type 2 diabetic subjects. *Diabetes.* 1999;48(5):1113-1119.

143. Forouhi NG, Jenkinson G, Thomas EL, et al. Relation of triglyceride stores in skeletal muscle cells to central obesity and insulin sensitivity in European and South Asian men. *Diabetologia.* 1999;42(8):932-935.

144. Krssak M, Falk Petersen K, Dresner A, et al. Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia.* 1999;42(1):113-116.

145. Perseghin G, Lattuada G, Danna M, et al. Insulin resistance, intramyocellular lipid content, and plasma adiponectin in patients with type 1 diabetes. *Am J Physiol-Endoc M.* 2003;285(6):E1174-E1181.

146. Sinha R, Dufour S, Petersen KF, et al. Assessment of skeletal muscle triglyceride content by (1)H nuclear magnetic resonance spectroscopy in lean and obese adolescents: relationships to insulin sensitivity, total body fat, and central adiposity. *Diabetes.* 2002;51(4):1022-1027.

147. Frayn KN. Visceral fat and insulin resistance--causative or correlative? *Br J Nutr.* 2000;83 Suppl 1:S71-77.

148. Ross R, Aru J, Freeman J, Hudson R, Janssen I. Abdominal adiposity and insulin resistance in obese men. *Am J Physiol-Endoc M.* 2002;282(3):E657-E663.

149. Kahn BB, Flier JS. Obesity and insulin resistance. *J Clin Invest.* 2000;106(4):473-481.

150. Weiss R, Dufour S, Taksali SE, et al. Prediabetes in obese youth: a syndrome of impaired glucose tolerance, severe insulin resistance, and altered myocellular and abdominal fat partitioning. *Lancet.* 2003;362(9388):951-957.

151. Cruz ML, Bergman RN, Goran MI. Unique effect of visceral fat on insulin sensitivity in obese hispanic children with a family history of type 2 diabetes. *Diabetes Care.* 2002;25(9):1631-1636.

152. Goran MI, Bergman RN, Gower BA. Influence of total vs. visceral fat on insulin action and secretion in African American and white children. *Obes Res.* 2001;9(8):423-431.

153. Sardinha LB, Andersen LB, Anderssen SA, et al. Objectively measured time spent sedentary is associated with insulin resistance independent of overall and central body fat in 9-to 10-year-old Portuguese children. *Diabetes Care.* 2008;31(3):569-575.

154. Slade JM, Coe LM, Meyer RA, McCabe LR. Human bone marrow adiposity is linked with serum lipid levels not T1-diabetes. *J Diabetes Complications.* 2012;26(1):1-9.

155. Baum T, Yap SP, Karampinos DC, et al. Does vertebral bone marrow fat content correlate with abdominal adipose tissue, lumbar spine bone mineral density, and blood biomarkers in women with type 2 diabetes mellitus? *J Magn Reson Imaging.* 2012;35(1):117-124.

156. Takubo K, Nagamatsu G, Kobayashi CI, et al. Regulation of Glycolysis by Pdk Functions as a Metabolic Checkpoint for Cell Cycle Quiescence in Hematopoietic Stem Cells. *Cell Stem Cell.* 2013;12(1):49-61.

157. Elder GC, Kirk J, Stewart G, et al. Contributing factors to muscle weakness in children with cerebral palsy. *Dev Med Child Neurol.* 2003;45(8):542-550.

158. Stackhouse SK, Binder-Macleod SA, Lee SCK. Voluntary muscle activation, contractile properties, and fatigability in children with and without cerebral palsy. *Muscle & Nerve.* 2005;31(5):594-601.

159. Baumgartner RN, Koehler KM, Gallagher D, et al. Epidemiology of sarcopenia among the elderly in New Mexico. *Am J Epidemiol.* 1998;147(8):755-763.

160. Dodson S, Baracos VE, Jatoi A, et al. Muscle Wasting in Cancer Cachexia: Clinical Implications, Diagnosis, and Emerging Treatment Strategies. *Annu Rev Med.* 2011;62:265-279.

161. Snyder WS, Cook MJ, Nasset ES, Karhauserr LR, Howells GP, Tipton IH. Report of the task group on reference man. Paper presented at: International Comission on Radiological Protection1975; Oxford.

162. Bauman WA. The potential metabolic consequences of cerebral palsy: inferences from the general population and persons with spinal cord injury. *Dev Med Child Neurol.* 2009;51:64-78.

163. Barber L, Barrett R, Lichtwark G. Medial gastrocnemius muscle fascicle active torque-length and Achilles tendon properties in young adults with spastic cerebral palsy. *J Biomech.* 2012;45(15):2526-2530.

164. Miller F, Koreska J. Height measurement of patients with neuromuscular disease and contractures. *Dev Med Child Neurol.* 1992;34(1):55-60.

165. CDC. CDC Growth Charts: United States. In. *National Center for Health Statistics in collaboration with the National Center for Chronic Disease Prevention and Health Promotion.*2000.

166. Rawal R, Miller F, Modlesky CM. Effect of a novel procedure for limiting motion on body composition and bone estimates by dual-energy X-ray absorptiometry in children. *J Pediatr.* 2011;159(4):691-694.

167. Cohen J. *Statistical Power for the Behavioral Sciences.* 2nd ed. Hillsdale, NJ: Lawrence Erlbaum Associates; 1988.

168. Hawkins DM, Basak SC, Mills D. Assessing model fit by cross-validation. *J Chem Inf Comp Sci.* 2003;43(2):579-586.

169. Bland JM, Altman DG. Statistical Methods for Assessing Agreement between Two Methods of Clinical Measurement. *Lancet.* 1986;1(8476):307-310.

170. Janssen I, Heymsfield SB, Ross R. Low relative skeletal muscle mass (sarcopenia) in older persons is associated with functional impairment and physical disability. *J Am Geriatr Soc.* 2002;50(5):889-896.

171. Szulc P, Beck TJ, Marchand F, Delmas PD. Low skeletal muscle mass is associated with poor structural parameters of bone and impaired balance in elderly men--the MINOS study. *J Bone Miner Res.* 2005;20(5):721-729.

172. Blain H, Jaussent A, Thomas E, et al. Appendicular skeletal muscle mass is the strongest independent factor associated with femoral neck bone mineral density in adult and older men. *Exp Gerontol.* 2010;45(9):679-684.

173. c Visser M, Pahor M, Taaffe DR, et al. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: the Health ABC Study. *J Gerontol A Biol Sci Med Sci.* 2002;57(5):M326-332.

174. Pedersen M, Bruunsgaard H, Weis N, et al. Circulating levels of TNF-alpha and IL-6-relation to truncal fat mass and muscle mass in healthy elderly individuals and in patients with type-2 diabetes. *Mech Ageing Dev.* 2003;124(4):495-502.

175. Whitney DG, Hurvitz EA, Ryan JM, et al. Noncommunicable disease and multimorbidity in young adults with cerebral palsy. *Clin Epidemiol.* 2018;10:511-519.

176. Whitney DG, Hurvitz EA, Devlin MJ, et al. Age trajectories of musculoskeletal morbidities in adults with cerebral palsy. *Bone.* 2018;114:285-291.

177. Rose J, Wolff DR, Jones VK, Bloch DA, Oehlert JW, Gamble JG. Postural balance in children with cerebral palsy. *Dev Med Child Neurol.* 2002;44(1):58-63.

178. Modlesky CM, Kanoff SA, Johnson DL, Subramanian P, Miller F. Evaluation of the femoral midshaft in children with cerebral palsy using magnetic resonance imaging. *Osteoporosis Int.* 2009;20(4):609-615.

179. Morgan P, McGinley J. Gait function and decline in adults with cerebral palsy: a systematic review. *Disabil Rehabil.* 2014;36(1):1-9.

180. Perenc L, Przysada G, Trzeciak J. Cerebral Palsy in Children as a Risk Factor for Malnutrition. *Ann Nutr Metab.* 2015;66(4):224-232.

181. Velazquez Alva MdC, Irigoyen Camacho ME, Delgadillo Velazquez J, Lazarevich I. The relationship between sarcopenia, undernutrition, physical mobility and basic activities of daily living in a group of elderly women of Mexico City. *Nutricion hospitalaria.* 2013;28(2).

182. Ryan A, Serra M, Addison O. The role of skeletal muscle myostatin in sarcopenia in older adults. *Innovation in aging.* 2017;1(Suppl 1):361.

183. Sandow A. Fundamental mechanics of skeletal muscle contraction. *Am J Phys Med.* 1952;31(2):103-125.

184. Pedersen BK, Febbraio MA. Muscles, exercise and obesity: skeletal muscle as a secretory organ. *Nat Rev Endocrinol.* 2012;8(8):457-465.

185. Albu JB, Kovera AJ, Allen L, et al. Independent association of insulin resistance with larger amounts of intermuscular adipose tissue and a greater acute insulin response to glucose in African American than in white nondiabetic women. *Am J Clin Nutr.* 2005;82(6):1210-1217.

186. Kaji H. Linkage between muscle and bone: common catabolic signals resulting in osteoporosis and sarcopenia. *Curr Opin Clin Nutr Metab Care.* 2013;16(3):272-277.

187. Mitsiopoulos N, Baumgartner RN, Heymsfield SB, Lyons W, Gallagher D, Ross R. Cadaver validation of skeletal muscle measurement by magnetic resonance imaging and computerized tomography. *J Appl Physiol (1985).* 1998;85(1):115-122.

188. Kim J, Shen W, Gallagher D, et al. Total-body skeletal muscle mass: estimation by dual-energy X-ray absorptiometry in children and adolescents. *Am J Clin Nutr.* 2006;84(5):1014-1020.

189. Kim J, Wang Z, Heymsfield SB, Baumgartner RN, Gallagher D. Total-body skeletal muscle mass: estimation by a new dual-energy X-ray absorptiometry method. *Am J Clin Nutr.* 2002;76(2):378-383.

190. Midorikawa T, Ohta M, Hikihara Y, Torii S, Sakamoto S. Predicting skeletal muscle mass from dual-energy X-ray absorptiometry in Japanese prepubertal children. *Eur J Clin Nutr.* 2017;71(10):1218-1222.

191. Kuczmarski RJ, Ogden CL, Grummer-Strawn LM, et al. CDC growth charts: United States. *Adv Data.* 2000;314(314):1-27.

192. Tanner J. *Growth and Adolescence.* 2nd ed. Oxford: Blackwell Scientific Publications; 1962.

193. Suckling J, Sigmundsson T, Greenwood K, Bullmore ET. A modified fuzzy clustering algorithm for operator independent brain tissue classification of dual echo MR images. *Magn Reson Imaging.* 1999;17(7):1065-1076.

194. Hawkins DM, Basak SC, Mills D. Assessing model fit by cross-validation. *J Chem Inf Comput Sci.* 2003;43(2):579-586.

195. Modlesky CM, Bickel CS, Slade JM, Meyer RA, Cureton KJ, Dudley GA. Assessment of skeletal muscle mass in men with spinal cord injury using dual-energy X-ray absorptiometry and magnetic resonance imaging. *J Appl Physiol (1985).* 2004;96(2):561-565.

196. Therkelsen KE, Pedley A, Hoffmann U, Fox CS, Murabito JM. Intramuscular fat and physical performance at the Framingham Heart Study. *Age.* 2016;38(2).

197. Visser M, Goodpaster BH, Kritchevsky SB, et al. Muscle mass, muscle strength, and muscle fat infiltration as predictors of incident mobility limitations in well-functioning older persons. *J Gerontol a-Biol.* 2005;60(3):324-333.

198. Visser M, Pahor M, Taaffe DR, et al. Relationship of interleukin-6 and tumor necrosis factor-alpha with muscle mass and muscle strength in elderly men and women: The health ABC study. *J Gerontol a-Biol.* 2002;57(5):M326-M332.

199. Kim S, Won CW, Kim BS, Choi HR, Moon MY. The Association between the Low Muscle Mass and Osteoporosis in Elderly Korean People. *J Korean Med Sci.* 2014;29(7):995-1000.

200. Peterson MD, Gordon PM, Hurvitz EA, Burant CF. Secondary muscle pathology and metabolic dysregulation in adults with cerebral palsy. *Am J Physiol Endocrinol Metab.* 2012;303(9):E1085-1093.

201. Gusso S, Munns CF, Colle P, et al. Effects of whole-body vibration training on physical function, bone and muscle mass in adolescents and young adults with cerebral palsy. *Sci Rep-Uk.* 2016;6.

202. Williams SA, Reid S, Elliott C, Shipman P, Valentine J. Muscle volume alterations in spastic muscles immediately following botulinum toxin type-A treatment in children with cerebral palsy. *Dev Med Child Neurol.* 2013;55(9):813-820.

203. Fidanza F, Keys A, Anderson JT. Density of body fat in man and other mammals. *J Appl Physiol.* 1953;6(4):252-256.

204. Guo BS, Zhang ZK, Liang C, et al. Molecular Communication from Skeletal Muscle to Bone: A Review for Muscle-Derived Myokines Regulating Bone Metabolism. *Calcified Tissue Int.* 2017;100(2):184-192.

205. Welle S, Bhatt K, Pinkert CA, Tawil R, Thornton CA. Muscle growth after postdevelopmental myostatin gene knockout. *Am J Physiol-Endoc M.* 2007;292(4):E985-E991.

206. Clop A, Marcq F, Takeda H, et al. A mutation creating a potential illegitimate microRNA target site in the myostatin gene affects muscularity in sheep. *Nat Genet.* 2006;38(7):813-818.

207. Haidet AM, Rizo L, Handy C, et al. Long-term enhancement of skeletal muscle mass and strength by single gene administration of myostatin inhibitors. *P Natl Acad Sci USA.* 2008;105(11):4318-4322.

208. Lee SJ, McPherron AC. Regulation of myostatin activity and muscle growth. *P Natl Acad Sci USA.* 2001;98(16):9306-9311.

209. Mendell JR, Sahenk Z, Malik V, et al. A Phase 1/2a Follistatin Gene Therapy Trial for Becker Muscular Dystrophy. *Mol Ther.* 2015;23(1):192-201.

210. Kaspar BK, Llado J, Sherkat N, Rothstein JD, Gage FH. Retrograde viral delivery of IGF-1 prolongs survival in a mouse ALS model. *Science.* 2003;301(5634):839-842.

211. Palazzolo I, Stack C, Kong LL, et al. Overexpression of IGF-1 in Muscle Attenuates Disease in a Mouse Model of Spinal and Bulbar Muscular Atrophy. *Neuron.* 2009;63(3):316-328.

212. Schulze PC, Fang J, Kassik KA, et al. Transgenic overexpression of locally acting insulin-like growth factor-1 inhibits ubiquitin-mediated muscle atrophy in chronic left-ventricular dysfunction. *Circ Res.* 2005;97(5):418-426.

213. Schutz Y, Kyle UUG, Pichard C. Fat-free mass index and fat mass index percentiles in Caucasians aged 18-98y. *Int J Obesity.* 2002;26(7):953-960.

214. Modlesky CM, Subramanian P, Miller F. Underdeveloped trabecular bone microarchitecture is detected in children with cerebral palsy using high-resolution magnetic resonance imaging. *Osteoporos Int.* 2008;19(2):169-176.

215. Winbanks CE, Weeks KL, Thomson RE, et al. Follistatin-mediated skeletal muscle hypertrophy is regulated by Smad3 and mTOR independently of myostatin. *J Cell Biol.* 2012;197(7):997-1008.

216. Gilson H, Schakman O, Kalista S, Lause P, Tsuchida K, Thissen JP. Follistatin induces muscle hypertrophy through satellite cell proliferation and inhibition of both myostatin and activin. *Am J Physiol-Endoc M.* 2009;297(1):E157-E164.

217. Rodino-Klapac LR, Haidet AM, Kota J, Handy C, Kaspar BK, Mendell JR. Inhibition of Myostatin with Emphasis on Follistatin as a Therapy for Muscle Disease. *Muscle & Nerve.* 2009;39(3):283-296.

218. Grounds MD. Reasons for the degeneration of ageing skeletal muscle: a central role for IGF-1 signalling. *Biogerontology.* 2002;3(1-2):19-24.

219. Costelli P, Muscaritoli M, Bossola M, et al. IGF-1 is downregulated in experimental cancer cachexia. *Am J Physiol-Reg I.* 2006;291(3):R674-R683.

220. de Waal WJ, Hokken-Koelega AC, Stijnen T, de Muinck Keizer-Schrama SM, Drop SL. Endogenous and stimulated GH secretion, urinary GH excretion, and plasma IGF-I and

IGF-II levels in prepubertal children with short stature after intrauterine growth retardation. The Dutch Working Group on Growth Hormone. *Clin Endocrinol (Oxf).* 1994;41(5):621-630.

221. Sato K, Li Y, Foster W, et al. Improvement of muscle healing through enhancement of muscle regeneration and prevention of fibrosis. *Muscle & Nerve.* 2003;28(3):365-372.

222. Juul A, Dalgaard P, Blum WF, et al. Serum Levels of Insulin-Like Growth-Factor (Igf)-Binding Protein-3 (Igfbp-3) in Healthy Infants, Children, and Adolescents - the Relation to Igf-I, Igf-Ii, Igfbp-1, Igfbp-2, Age, Sex, Body-Mass Index, and Pubertal Maturation. *J Clin Endocr Metab.* 1995;80(8):2534-2542.

223. Brink M, Price SR, Chrast J, et al. Angiotensin II induces skeletal muscle wasting through enhanced protein degradation and down-regulates autocrine insulin-like growth factor I. *Endocrinology.* 2001;142(4):1489-1496.

224. Smith LR, Chambers HG, Lieber RL. Reduced satellite cell population may lead to contractures in children with cerebral palsy. *Dev Med Child Neurol.* 2013;55(3):264-270.

225. Bredella MA, Ghomi RH, Thomas BJ, et al. Comparison of DXA and CT in the Assessment of Body Composition in Premenopausal Women With Obesity and Anorexia Nervosa. *Obesity.* 2010;18(11):2227-2233.

226. Koman LA, Smith BP, Shilt JS. Cerebral palsy. *Lancet.* 2004;363(9421):1619-1631.

227. Whitney DG, Miller F, Pohlig RT, Modlesky CM. BMI does not capture the high fat mass index and low fat-free mass index in children with cerebral palsy and proposed statistical models that improve this accuracy. *Int J Obesity.* 2019;43(1):82-90.

228. Stallings VA, Cronk CE, Zemel BS, Charney EB. Body composition in children with spastic quadriplegic cerebral palsy. *J Pediatr.* 1995;126(5):833-839.

229. Shuster A, Patlas M, Pinthus JH, Mourtzakis M. The clinical importance of visceral adiposity: a critical review of methods for visceral adipose tissue analysis. *Brit J Radiol.* 2012;85(1009):1-10.

230. Taksali SE, Caprio S, Dziura J, et al. High visceral and low abdominal subcutaneous fat stores in the obese adolescent - A determinant of an adverse metabolic phenotype. *Diabetes.* 2008;57(2):367-371.

231. Pascot A, Lemieux S, Lemieux I, et al. Age-related increase in visceral adipose tissue and body fat and the metabolic risk profile of premenopausal women. *Diabetes Care.* 1999;22(9):1471-1478.

232. Koster A, Stenholm S, Alley DE, et al. Body Fat Distribution and Inflammation Among Obese Older Adults With and Without Metabolic Syndrome. *Obesity.* 2010;18(12):2354-2361.

233. Kanaley JA, Sames C, Swisher L, et al. Abdominal fat distribution in pre- and postmenopausal women: The impact of physical activity, age, and menopausal status. *Metabolism.* 2001;50(8):976-982.

234. Tanner J. Growth and Adolescence. Oxford: Blackwell Scientific Publications. 1962.

235. Micklesfield LK, Goedecke JH, Punyanitya M, Wilson KE, Kelly TL. Dual-Energy X-Ray Performs as Well as Clinical Computed Tomography for the Measurement of Visceral Fat. *Obesity.* 2012;20(5):1109-1114.

236. Kaul S, Rothney MP, Peters DM, et al. Dual-Energy X-Ray Absorptiometry for Quantification of Visceral Fat. *Obesity.* 2012;20(6):1313-1318.

237. Trost SG, Pate RR, Freedson PS, Sallis JF, Taylor WC. Using objective physical activity measures with youth: how many days of monitoring are needed? *Med Sci Sports Exerc.* 2000;32(2):426-431.

238. Sironi AM, Petz R, De Marchi D, et al. Impact of increased visceral and cardiac fat on cardiometabolic risk and disease. *Diabetic Med.* 2012;29(5):622-627.

239. Rothney MP, Catapano AL, Xia J, et al. Abdominal Visceral Fat Measurement Using Dual-Energy X-ray: Association with Cardiometabolic Risk Factors. *Obesity.* 2013;21(9):1798-1802.

240. Elffers TW, de Mutsert R, Lamb HJ, et al. Body fat distribution, in particular visceral fat, is associated with cardiometabolic risk factors in obese women. *Plos One.* 2017;12(9).

241. Peterson MD, Zhang P, Haapala HJ, Wang SC, Hurvitz EA. Greater Adipose Tissue Distribution and Diminished Spinal Musculoskeletal Density in Adults With Cerebral Palsy. *Arch Phys Med Rehabil.* 2015;96(10):1828-1833.

242. Ryan JM, Crowley VE, Hensey O, McGahey A, Gormley J. Waist circumference provides an indication of numerous cardiometabolic risk factors in adults with cerebral palsy. *Arch Phys Med Rehabil.* 2014;95(8):1540-1546.

243. Furutate R, Ishii T, Wakabayashi R, et al. Excessive visceral fat accumulation in advanced chronic obstructive pulmonary disease. *Int J Chron Obstruct Pulmon Dis.* 2011;6:423-430.

244. Saelens BE, Seeley RJ, Van Schaick K, Donnelly LF, O'Brien KJ. Visceral abdominal fat is correlated with whole-body fat and physical activity among 8-y-old children at risk of obesity. *Am J Clin Nutr.* 2007;85(1):46-53.

245. Sullivan PB, Juszczak E, Bachlet AM, et al. Gastrostomy tube feeding in children with cerebral palsy: a prospective, longitudinal study. *Dev Med Child Neurol.* 2005;47(2):77-85.

246. Nguyen-Duy TB, Nichaman MZ, Church TS, Blair SN, Ross R. Visceral fat and liver fat are independent predictors of metabolic risk factors in men. *Am J Physiol-Endoc M.* 2003;284(6):E1065-E1071.

247. Anderson PJ, Chan JCN, Chan YL, et al. Visceral fat and cardiovascular risk factors in Chinese NIDDM patients. *Diabetes Care.* 1997;20(12):1854-1858.

248. Weiss R, Taksali SE, Dufour S, et al. The "obese insulin-sensitive" adolescent: Importance of adiponectin and lipid partitioning. *J Clin Endocr Metab.* 2005;90(6):3731-3737.

249. Day SM, Strauss DJ, Vachon PJ, Rosenbloom L, Shavelle RM, Wu YW. Growth patterns in a population of children and adolescents with cerebral palsy. *Dev Med Child Neurol.* 2007;49(3):167-171.

Appendix A

Institutional Review Board Approval Letter

UNIVERSITY OF GEORGIA

Tucker Hall, Room 212
310 E. Campus Rd.
Athens, Georgia 30602
TEL 706-542-3199 | FAX 706-542-5638
IRB@uga.edu
http://research.uga.edu/hso/irb/

Office of Research
Institutional Review Board

APPROVAL OF PROTOCOL

August 11, 2017

Dear Christopher Modlesky:

On May 31, 2017, the IRB reviewed the following submission:

Type of Review:	Initial Study
Title of Study:	Effect of a high-frequency, low-magnitude vibration on muscle properties, physical activity and balance in children with cerebral palsy
Investigator:	Christopher Modlesky
IRB ID:	STUDY00004873
Funding:	NATIONAL INSTITUTES OF HEALTH;
Documents Reviewed:	Protocol, Recruitment Materials, Consent Documents, Sponsor Protocol, Site Authorization
Review Category:	Full Board

This study meets the criteria for permissible clinical research with children as set forth at 45 CFR 46.404. One parent or guardians' signed permission is sufficient to enroll minor subjects. Parent or guardian permission must be obtained. Assent from some of the children should be obtained based on their capacity to understand as per 45 CFR 46.408.

The IRB approved the protocol from 8/11/2017 to 5/30/2018 inclusive. Before or within 30 days of study closure, whichever is earlier, you are to submit a continuing review with required explanations. You can submit a continuing review by navigating to the active study and clicking Create Modification / CR.

If continuing review approval is not granted before the expiration date of to 5/30/2018 inclusive. Before 5/30/2018 or within 30 days of study closure, whichever is earlier, you are

to submit a continuing review with required explanations. You can submit a continuing review by navigating to the active study and clicking Create Modification / CR.
If continuing review approval is not granted before the expiration date of 5/30/2018, approval of this study expires on that date approval of this study expires on that date.

If consent will be documented, use the consent documents that were approved and stamped by the IRB. Go to the Documents tab to download them.

Please close this study when all human subject research activities and data analysis of identifiable information is complete.

In conducting this study, you are required to follow the requirements listed in the Investigator Manual (HRP-103).

Sincerely,
Gerald Crites, M.D.
Institutional Review Board Chairperson
University of Georgia